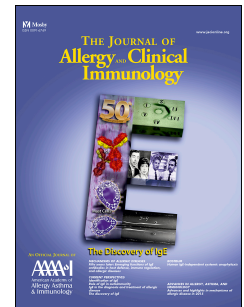


Accepted Manuscript

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PII: S0091-6749(17)30570-5

DOI: [10.1016/j.jaci.2017.03.017](https://doi.org/10.1016/j.jaci.2017.03.017)

Reference: YMAI 12732

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 1 July 2016

Revised Date: 22 February 2017

Accepted Date: 1 March 2017

Please cite this article as: Jahreis S, Trump S, Bauer M, Bauer T, Thürmann L, Feltens R, Wang Q, Gu L, Grützmann K, Röder S, Averbeck M, Weichenhan D, Plass C, Sack U, Borte M, Dubourg V, Schüürmann G, Simon JC, von Bergen M, Hackermüller J, Eils R, Lehmann I, Polte T, Maternal Phthalate Exposure Promotes Allergic Airway Inflammation over Two Generations Via Epigenetic Modifications, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.03.017.

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Maternal phthalate exposure promotes allergic airway inflammation over two generations via epigenetic modifications

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Supported by the Helmholtz Association grant VG-NG-337, the LIFE - Leipzig Research Center for Civilization Diseases (LIFE-007-D7), and the Helmholtz Initiative for Personalized Medicine (iMed).

ABSTRACT

Background: Prenatal and early postnatal exposures to environmental factors are considered responsible for the increasing prevalence of allergic diseases. Although there is some evidence for allergy-promoting effects in children due to exposure to plasticizers like phthalates, findings of previous studies are inconsistent and lack mechanistic information.

Objective: We investigated the effect of maternal phthalate exposure on asthma development in the subsequent generations and their underlying mechanisms including epigenetic alterations.

Methods: Phthalate metabolites were measured within the prospective mother-child cohort LINA and correlated with asthma development in the children. A murine trans-generational asthma model was used to identify involved pathways.

Results: In LINA maternal urinary concentrations of mono-n-butyl phthalate, a metabolite of butyl benzyl phthalate (BBP), were associated with an increased asthma risk in the children. Using a murine trans-generational asthma model, we demonstrate a direct effect of BBP on asthma severity in the offspring with a persistently increased airway inflammation up to the F2 generation. This disease-promoting effect was mediated by a BBP-induced global DNA hypermethylation in CD4⁺ T cells of the offspring as treatment with a DNA demethylating agent alleviated exacerbation of allergic airway inflammation. 13 transcriptionally down-regulated genes linked to promoter or enhancer hypermethylation were identified. Among these, the GATA-3 repressor Zfp111 emerged as a potential mediator of the enhanced susceptibility for Th2-driven allergic asthma.

Conclusion: These data provide strong evidence that maternal BBP exposure increases the risk for allergic airway inflammation in the offspring by modulating the expression of genes involved in Th2 differentiation via epigenetic alterations.

Key messages: - maternal MnBP levels associate with an increased asthma risk in children; - in mice, maternal exposure to BBP enhances airway inflammation in the F1 and F2 generation via epigenetic alterations

Capsule summary: The study demonstrates that maternal phthalate exposure is a risk factor for asthma development in subsequent generations. These disease-promoting effects are mediated by epigenetic changes resulting in an altered expression of genes important for Th2-driven immune regulation.

Keywords: airway inflammation, asthma, phthalates, epigenetics, T cells

Abbreviation used:

AHR - airway hyperreactivity

BAL - bronchoalveolar lavage

BBP - butyl benzyl phthalate

DEG - differentially expressed gene

DMR – differentially methylated region

MnBP - mono-n-butyl phthalate

OVA – ovalbumin

RL – lung resistance

Th2 cells – T helper 2 cells

WBS - whole genome bisulfite sequencing

INTRODUCTION

Allergic asthma is caused by a Th2 cell-mediated immune response to common environmental allergens and is characterized by airway inflammation with pulmonary eosinophilia, airway hyperreactivity (AHR), and increased serum immunoglobulin E (IgE) levels.¹ This inflammatory airway disease has a strong genetic background but also a multitude of environmental trigger factors have been described.^{2, 3} Epidemiological and experimental studies demonstrated that exposure to environmental pollutants like diesel exhaust particles, environmental tobacco smoke, or chemicals such as volatile organic compounds can lead to an exacerbation of respiratory symptoms and asthma.⁴⁻⁷ In particular, the prenatal and early postnatal period appear critical to environmental exposures, probably interfering with the developmental programming of the immune system and thereby altering the disease risk in later life.⁸ Epigenetic changes like modified DNA methylation have been described as mediators between exposure and disease development.^{8, 9} This has been shown for allergy-preventing effects induced by upbringing in a farming environment or exposure to microbial components as well as for asthma-promoting influences like maternal exposure to tobacco smoke during pregnancy.⁹⁻¹¹ These findings emphasize that the developing immune system is especially susceptible to perturbations by external factors.

Here, we focused on phthalates, a group of chemicals commonly used as plasticizers in large quantities worldwide. These chemicals are present in a wide range of consumer products like cosmetics, plastics, floor coverings, building materials, toys, and cleaning products.^{12, 13} Humans are exposed to phthalates mainly through ingestion or inhalation throughout their entire life starting *in utero*.^{14, 15} Although, there is some evidence of allergy-promoting effects in children by maternal phthalate exposure, findings of previous studies are conflicting regarding the effect direction,

the investigated exposure conditions and their assessment.¹⁶⁻¹⁸ Furthermore, little is known about the mechanisms of maternal phthalate exposure leading to an altered disease risk in children's later life.

In the present study, we report a significant association between maternal urinary concentrations of the phthalate metabolite mono-n-butyl phthalate (MnBP) and an increased asthma risk in children of our prospective mother-child cohort LINA.⁷ Using a murine trans-generational asthma model, we demonstrate that maternal exposure to butyl benzyl phthalate (BBP), a parent compound of MnBP, has a direct effect on severity of allergic airway inflammation in the offspring, observing an enhanced airway inflammation even in the F2 generation. This asthma-promoting effect was mediated by a BBP-induced DNA hypermethylation, as shown by diminished BBP effects after treating mice with a hypomethylation-inducing compound, and a subsequently reduced expression of genes involved in the differentiation of Th2 cells like the GATA-3 repressor *Zfp1* offering an explanation for the increased allergic immune response.

METHODS

For more detailed information, please see the Methods section in this article's Online Repository at www.jacionline.org.

LINA study design and sample collection

The LINA cohort study (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) recruited 629 mother-child-pairs between May 2006 and December 2008 in Leipzig, Germany, to investigate how environmental factors in the

pre- and postnatal period influence immune system development and resulting disease risks.¹⁹⁻²¹

Urine Samples and Sample Preparation

Phthalate metabolite quantification was carried out for 540 early morning maternal urine samples as described previously.²²

Exposure to BBP and OVA immunization

BALB/c mice were exposed to BBP (3 µg/ml) exclusively via drinking water (custom-built glass bottles) for different time periods (Figure 2) as described in detail in the Online Repository.

Measurement of airway responsiveness

Lung resistance (R_L) was measured by invasive plethysmography in response to inhaled methacholine as described previously²³⁻²⁵ and in more detail in the Online Repository.

Whole genome bisulfite sequencing, DMR calling and annotation

WGBS library construction and sequencing was performed as previously described²⁶ applying an tagmentation based, ultra-low input library preparation protocol with a quantity of 20 ng of input DNA. Sequencing and alignment (mm10) were carried out as described before.²⁷ DMR calling was performed as described previously.²⁷

RNA-sequencing

Total RNA was isolated and RNA-sequencing was performed as described previously.²⁸⁻³⁰

Mapping of DMRs to target genes

DMRs were mapped on gene bodies, promoters and putative enhancers of the differentially expressed genes (DEGs) as described in detail in the Online Repository.

MassARRAY methylation analysis

MassARRAY analysis was performed as described previously.²⁷

Statistical analysis

Equal parameter distribution was tested using Chi-square. To assess the contribution of phthalate exposure on risk increase for asthma development or elevated IgE levels in the children logistic regression models were implemented adjusting for known confounding factors of lung disease or atopy in early childhood.

Non-parametric tests were applied for all not normally distributed parameters (e.g. Mann-Whitney-U, Spearman correlations). All calculations were performed in STATISTICA for Windows, Version 10 (Statsoft Inc., Tulsa, OK, USA). Data were expressed as mean $\pm$ SEM and p-values < 0.05 were considered significant.

RESULTS

Association between maternal phthalate metabolites and the development of allergic asthma in children (LINA).

For the LINA mother-child study 629 mother-child pairs were recruited between 2006 and 2008. The six-year follow-up investigation included 420 (66.8%) of the families.

Regarding asthma development only physician diagnosed asthma was considered for this study. For 24 of the LINA children an asthma diagnosis was reported until the age of six years. General characteristics of the study participants are shown in Table E1. Apart from a slightly lower number of tobacco smoke exposed children participating until the age of six, there were no differences between the analyzed sub-cohort (n=371) and the total LINA cohort (n=629).

The concentration of 10 phthalate metabolites was determined in a total of 540 maternal urine samples of the 34th week of gestation. However, only measurements of the 371 children who participated in the study until the age of six years were considered for our analysis (Table E2). Interestingly, an increased risk to develop asthma symptoms until the age of 6 was observed only in children from mothers with elevated urinary mono-n-butyl phthalate (MnBP) levels (Figure 1A, B), while all other metabolites showed no significant association (Table E3 and E5). In addition to the lung phenotype, high maternal MnBP concentrations were also associated with a higher risk for allergic sensitization against inhalant allergens in the children (Figure 1C, D and Table E4 and E5).

Pre- and perinatal exposure to BBP increased allergic airway inflammation in a murine asthma model.

MnBP is next to mono benzyl phthalate (MBzP) the major metabolite of butyl benzyl phthalate (BBP).³¹ To further investigate whether the observed increase in asthma risk in the human study cohort might be directly caused by maternal exposure to BBP, we employed a well-established trans-generational ovalbumin (OVA)-induced asthma mouse model.^{10, 23, 32, 33} During the exposure period Balb/c mice received 3 µg/ml BBP via drinking water, a concentration relevant for the human exposure situation. MnBP levels measured in the urine of BBP-exposed dams were

comparable to those detected in highly exposed LINA mothers (Table E6 compared to 12.47 – 6894.87 ng/ml maternal urine). To analyse the effect of maternal BBP exposure on allergic airway inflammation, dams were exposed to BBP during pregnancy (prenatal) or during pregnancy and breastfeeding (perinatal). Grown-up offspring was then subjected to antigen sensitization without being further exposed to BBP (Figure 2A). In another treatment regimen adult mice were exposed to BBP starting one week before OVA sensitization until the end of the asthma protocol (Figure 2B). While exposure of adult mice to BBP had no effect on the asthma-like phenotype, prenatal and perinatal BBP exposure significantly increased the number of eosinophils within the BAL fluid compared to the offspring from un-exposed dams (Figure 3A). Maternal BBP exposure led to substantially more inflammatory infiltrates in the airways as demonstrated by histological examination of H&E-stained lung sections (Figure 3B), confirmed by software-based image analysis (Figure 3C). Accordingly, airway hyperreactivity (AHR) measured as lung resistance (R_L) was enhanced in the offspring from BBP-exposed dams (Figure 3D). Furthermore, OVA-specific IgE levels were elevated in pre- and perinatally-exposed offspring compared to control mice (Figure 4A). Moreover, OVA-re-stimulated spleen and lymph node cells from offspring of BBP-exposed dams produced significantly higher amounts of the Th2 cytokines IL-5 and IL-13, while there was no significant effect on the Th1 cytokine IFN- γ (Figure 4B, C).

To evaluate, which BBP metabolite promotes the occurrence of an asthma-like phenotype we perinatally exposed mice to MnBP or MBzP and subsequently characterized the asthma phenotype in the offspring. Interestingly, neither MnBP nor MBzP significantly increased the allergic airway inflammation (Figure E1A-D).

Maternal BBP exposure induces global hypermethylation mediating the asthma-promoting effect.

Early environmental influences have been shown to perturb epigenetic changes such as DNA methylation that might contribute to programming of disease risks.⁸ To evaluate whether maternal (perinatal) BBP exposure alters the global DNA methylation pattern in the F1 generation, we subjected splenic CD4⁺ T cells isolated from dams and their 3-week old offspring to WGBS. Differentially methylated regions (DMRs) were called with $\Delta\text{meth} > 5\%$ and a $p\text{-value} < 0.05$ between exposed groups (F0 or F1) vs. non-exposed control mice of the same generation ($n = 3$, median-FDR F0: 0.056, F1:0.036).

Our data show that maternal BBP exposure induced a global DNA hypermethylation in the F0 generation (63% hypermethylated regions among identified DMRs) and to an even higher extent also in the offspring (83%) (Figure 5A, Data Set 1). Similar to the F0 generation hypermethylation in F1 was not restricted to any particular genomic region but occurred genome-wide (Figure 5B).

To evaluate whether the BBP-induced hypermethylation is linked to the severe allergic airway inflammation observed in the offspring, one-week old pups from BBP-exposed dams were treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza) for two weeks until weaning.³⁴ Treatment of the offspring with Aza reversed enhanced airway inflammation caused by maternal BBP exposure as demonstrated by the reduced number of eosinophilic granulocytes in the BAL fluid (Figure 5C), diminished lung inflammation (Figure 5D) and attenuated OVA-specific lung resistance (Figure 5E), IgE levels (Figure 5F), and IL-5/IL-13 in splenocytes and IL-4/IL-5 levels in lymphnode cells (Figure 5G). Aza treatment of pups from unexposed dams had no effect on the asthma-like phenotype in the offspring.

BBP-induced hypermethylation leads to down-regulation of certain target genes.

To characterize the functional consequences of the BBP-induced hypermethylation on transcription we assessed gene expression by RNA sequencing in CD4⁺ T cells from 3-week old offspring alongside with our WGBS analysis. At an FDR < 0.01 we detected 1138 differential expressed genes (DEG) in the offspring of BBP-exposed dams in comparison to non-exposed mice. Treated *versus* non-treated mice clustered with respect to their genome-wide RNA expression by unsupervised clustering (Figure 6A, Data Set 2). 242 of the obtained DEGs could be linked to 346 DMRs (Figure 6B). Those target genes most likely mediating the BBP-related asthma-promotion should fulfill certain criteria (Figure E2 and Data Set 3). As already mentioned BBP-induced hypermethylation was correlated to an increased airway inflammation, which was alleviated by treatment with the DNA demethylating agent Aza. Therefore, in a first step we concentrated on those DEGs related to hypermethylated DMRs (201 DEGs) located in functionally translating regulatory elements (promoter/enhancer; 33 DEGs remaining). Since DNA hypermethylation is a key mechanism to gene silencing we focused only on those transcriptionally repressed genes leaving us with 13 potential candidates (Data Set 3). Among those, three genes were previously described to play a role in Th2-driven allergic immune diseases and were subsequently evaluated in more detail.

Fatty acid desaturase 1 (*Fads1*) is an enzyme that regulates desaturation of fatty acids and is involved in the synthesis of long-chain polyunsaturated fatty acid (LC-PUFA). It has been shown that down regulation of *Fads* genes is associated with lower polyunsaturated fatty acid levels and atopic eczema.³⁵ The second gene, fanconi anaemia complementation group A (*Fanca*), regulates the activity of regulatory T cells. Deletion of *Fanca* leads to a decreased efficiency to suppress T effector cell function.³⁶ Third, we focused on zinc finger protein 1 (*Zfp1*), also

known as friend of GATA protein 1 (Fog1), a gene that acts as a repressor of GATA-3-mediated Th2 cell development.³⁷⁻³⁹ Down-regulation of *Zfp1* could therefore promote a Th2-driven immune response. Validation of our RNA sequencing results by real time PCR confirmed a reduced expression of all 3 genes in CD4⁺ T cells from 3-week old offspring of BBP-exposed dams compared to control animals (Figure 6C). In contrast, in BBP-exposed F0 mice expression of *Fads1*, *Fanca* and *Zfp1* was not altered (Figure 6C). Furthermore, the down-regulation of *Fanca* and *Zfp1* was also seen in full-grown offspring of BBP-exposed dams (8 to 12 weeks old), while the effect of maternal BBP-exposure on *Fads1* expression in adult mice was not significant (Figure 6C). Aza treatment of BBP-treated pups not only alleviated the asthma-promoting effect as described earlier but also abrogated down-regulation of *Fanca* and *Zfp1* in the mature F1 generation (Figure 6D).

To evaluate whether the potential mediators of phenotype development identified in our mouse model may also play a role in humans we performed real time PCR analysis in whole blood samples of 4-year-old children of the LINA cohort. Interestingly, high urinary maternal MnBP concentrations (i.e. MnBP concentrations above the median) were associated with a significant decrease in *ZFPM1* expression in the children (MWU: p-value = 0.025, Figure E3A) in accordance with our observations in the mouse model, while no significant decrease of *FADS1* or *FANCA* expression in prenatally MnBP exposed children was observed (data not shown). Concomitantly with a decrease in expression of *ZFPM1* asthmatic children showed a significant increase in methylation at a CpG site (chr16:88564360, R=-0.87, p=0.001) located in an intronic human enhancer region corresponding to the region observed in mice, which also in humans appears to regulate its host gene *ZFPM1* (Figure E3B, C).

Altered function of CD4⁺ T cells in the offspring from BBP-exposed mice.

Fanca and Zfp116 both are described to play a crucial role in T cell differentiation and function.^{36, 37} To investigate a possible impact of maternal BBP exposure on these two endpoints in the next generation we isolated splenic CD4⁺ effector T cells from 8-week old offspring and stimulated the cells with anti-CD3/CD28 antibodies. While proliferation of T cells was not affected (data not shown), CD4⁺ T cells from BBP-exposed offspring appeared more activatable than T cells from non-exposed control mice as shown by the expression of the activation marker CD25 (Figure 7A). Interestingly, we detected significantly more IL-4-producing CD4⁺ T cells in the F1 generation from exposed mice, while the increased number of IL-13-producing cells reached borderline significance and the number of IFN- γ -producing cells was unaffected (Figure 7B). Furthermore, we found a reduced number of Foxp3-positive regulatory T cells in stimulated CD4⁺CD25⁺T cells from BBP-exposed offspring compared to cells derived from control mice (Figure 7C). However, the described differences did not reach statistical significance. Furthermore, the frequency of Foxp3-positive regulatory T cells in the blood and in unstimulated splenocytes of BBP-exposed offspring was not affected (Figure E4). Accordingly, the efficiency of CD4⁺CD25⁺ regulatory T cells to suppress responder T cells was slightly but not significantly impaired using cells from BBP-exposed mice compared to regulatory T cells from control animals (Figure 7D).

Maternal BBP exposure affects airway inflammation in the F2 generation.

To investigate whether the asthma-promoting effect of perinatal BBP exposure persists up to the F2 generation we mated perinatally exposed females with non-exposed males and induced an asthma-like phenotype in the F2 generation (Figure 2C). Surprisingly, exposure to BBP during pregnancy and breastfeeding enhanced

the eosinophilic airway inflammation (Figure 8A, B) and the AHR (Figure 8C) but did not alter OVA-specific IgE levels in the F2 generation (Figure 8D). Since treatment of F2 pups with Aza reduced their increased airway inflammation in relation to BBP exposure of their grandmother an involvement of epigenetic mechanisms including DNA hypermethylation in promoting this immune response is highly probable (Figure 8A, C). However, we did neither observe changes in DNA methylation related to BBP-exposure in F2 pups in *Fads1*-, *Fanca*- (both promoters) or *Zfp1-DMRs* (enhancer) identified in F1, nor were these genes significantly differentially expressed (Figure E5A, B).

DISCUSSION

The role of phthalate exposure in the rising prevalence of atopic diseases has been a topic of great interest in recent years. However, from current studies no clear conclusion can be drawn about the role of phthalates in asthma development or about the potential mechanisms involved.¹⁸ In the present study, we showed that higher concentrations of MnBP in maternal urine during pregnancy were associated with an increased risk of asthma in children up to the age of 6, while all other phthalate metabolites analyzed were not associated with this disease outcome. These results are in line with findings of a recent study by Whyatt *et al.*,⁴⁰ who found a positive association between the maternal urine phthalate metabolites MnBP and MBzP and the asthma risk in children between age 5 and 11 years,⁴⁰ while other studies only found such a correlation for MBzP but not for MnBP.⁴¹ These discrepancies might partially be due to differences in study size but might also be related to regional differences in exposure pattern to the different members of the phthalate family. Interestingly, beyond the increased risk of asthma development by

MnBP we also observed a link between maternal MnBP concentrations and the risk for sensitization against inhalant allergens in the children suggesting the involvement of an atopic mechanism.

MBzP and MnBP are both described as breakdown products of BBP⁴⁰ with MnBP being the major metabolite of BBP with larger quantities in urine compared to MBzP.^{31, 42} Thus, we investigated the effect of maternal BBP exposure on the asthma-like phenotype in our murine trans-generational model.

Applied BBP exposure concentrations were based on the tolerable daily intake of BBP in humans of 0.5 mg/kg body weight/day,⁴³ which corresponds to an estimated uptake of 0.48 – 0.6 mg BBP/kg body weight/day in our mice experiments (assuming 4 – 5 ml drinking water intake/day containing 3 µg/ml BBP). The resulting urinary MnBP concentrations in our murine model were similar to those observed in highly exposed mothers of the LINA cohort suggesting that BBP concentrations investigated here are representative for the real exposure situation in humans.

While pre- and perinatal exposure to BBP increased asthma severity in the offspring, exposure of adult mice did not affect the allergic immune response, further corroborating the notion that the most vulnerable time period for phthalate exposure is during pregnancy and shortly after birth.¹⁸

Another interesting finding was the inability of the BBP metabolites MnBP or MBzP to significantly increase the allergic airway inflammation in the offspring when applied perinatally to the dams. These data point to a direct effect of the parent compound BBP even without bio-transformation. However, further insight into the BBP-mediated initial molecular events in the dams or the fetus has to be explored in future studies.

There is growing evidence that pre- and perinatal exposure to diverse environmental chemicals may dysregulate the fetal or neonatal epigenome, with potential consequences for diseases manifesting in childhood.⁴⁴ Since the asthma-promoting

effects by BBP in the present study were only detectable in the offspring from exposed dams, we investigated the effects of BBP on the epigenetic landscape. Previous studies already showed an impact on DNA methylation by certain phthalates, but only based on targeted approaches restricted to pre-selected genes like glucose transporter genes (Glut4) or pro-inflammatory cytokines (TNF- α).⁴⁵⁻⁴⁷ In the current study, we subjected samples from our trans-generational mouse model to a genome-wide evaluation of DNA methylation changes related to BBP. This analysis was centered on the CD4⁺ T cell subset since these cells have a central role in initiation and maintenance of the allergic immune response.⁴⁸ Maternal BBP exposure led to a global deregulation in DNA methylation in the offspring dominated by a DNA hypermethylation. In contrast to our findings Wang *et al.* described a phthalate-induced DNA hypomethylation to be responsible for their observed increased asthma risk.⁴⁵ However, the study is based on methylation analysis of 21 selected genes measured in human blood samples compared to our genome-wide approach in a T cell subset. Nevertheless, we also observed phthalate-induced hypomethylation, e.g. in the promoter of IL-4 (chr11:53620267-53620300) similar to what has been described by Wang *et al.* in humans (Data Set 1). Counteracting the global DNA hypermethylation in our study by treating pups with the DNA methyltransferase inhibitor Aza prevented the BBP-related exacerbation of allergic airway inflammation suggesting a direct link between DNA hypermethylation and phenotype development.

Although, recent findings indicated a wide variety of relationships between DNA methylation and gene expression high promotor/enhancer methylation is classically associated with low levels of gene expression.^{49, 50} We therefore focused on significantly repressed genes with a promoter or enhancer hypermethylation to further elucidate the functional translation of the BBP associated hypermethylation. In

the offspring of BBP-exposed dams we identified 13 genes fulfilling these criteria including three interesting candidates involved in immune processes contributing to Th2 differentiation and therefore a higher susceptibility to develop allergic diseases.^{35, 36, 38} Considering the longitudinal stability of differential RNA expression induced by maternal BBP exposure from 3-week old pups to the adult offspring, *Fanca* and *Zfpm1* appeared to be the most promising genes. Characterising the CD4⁺ T cell function from offspring of BBP-exposed dams primarily revealed an increased susceptibility for a Th2-driven immune response as expected from down-regulation of the GATA-3 repressor *Zfpm1*.³⁷ In contrast, we could not show significant perturbations in Treg function, which would have been one possible consequence of a suppressed *Fanca* expression. Most interestingly, we could corroborate a possible mediating role of *Zfpm1* in the phthalate effect by transferring the results of the mouse model to our LINA cohort. *ZFPM1* mRNA expression was similarly decreased in children prenatally exposed to high levels of MnBP. This decrease in gene expression was correlated with an increase in *ZFPM1* methylation in the same region identified as a differentially methylated enhancer in our mouse model. The importance of a reduced expression of the GATA-3 repressor *Zfpm1* by maternal BBP exposure is supported by various studies therapeutically targeting GATA-3 to attenuate the Th2-regulated inflammatory response.^{51, 52}

To investigate whether the BBP-induced asthma-promoting effect functions also in the absence of direct environmental exposures via germline transmission of an altered epigenome⁵³ we characterized the asthma severity in the grandmotherly BBP exposed F2 generation. Although we observed an increased airway inflammation, the phenotype compared to the F1 generation was slightly different showing no effect on IgE serum levels. Furthermore, neither DNA methylation nor RNA expression of *Fads1*, *Fanca* and *Zfpm1* were affected in the F2 generation suggesting that the

underlying mechanisms for an exacerbated asthma-like phenotype in the F1 and the F2 generation are different.

In summary, the data from our study strongly suggest that early exposure to specific phthalates increases susceptibility to the development of allergic asthma in the offspring and even contributes to airway inflammation in the F2 generation. The asthma-promoting effect was mediated by epigenetic changes leading to an altered expression of genes, which play a crucial role in immune regulation. Therefore, chemical exposure of the developing immune system leads to an altered epigenetic prenatal programming contributing not only to asthma development in later life but potentially also to other immune-regulated diseases.

Acknowledgements

We thank Marita Reiprich, Michaela Loschinski, Beate Fink, Stephan Schreiber, Marion Bähr, Monika Helf, Oliver Mücke and Melanie Nowak for excellent technical assistance and all participants of the LINA study. We would like to thank the DKFZ Genomics and Proteomics Core Facility for the excellent technical support and expertise.

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Figure Legends

Figure 1. Maternal phthalate exposure and children's asthma and allergic sensitization. Maternal urine MnBP concentrations (34th gestational week) for children with asthma until the age-of-six (A) and sensitization against inhalant allergens (C) compared to controls (median and IQR). Frequency and risk (adjusted odds ratios (OR) per 100 ng/mg MnBP increase) for asthma (B) and sensitization against inhalant allergens (D) according to maternal MnBP concentrations (high=>median).

Figure 2. Phthalate exposure and induction of an asthma-like phenotype. Dams were exposed to BBP during pregnancy (prenatal) or until weaning when pups were 3 weeks old (perinatal exposure, A). Exposure of adult mice to BBP started one week before the first allergen injection and was continued until the end of the asthma induction protocol (B). Female F1 mice from exposed or control dams were mated with un-exposed males (C).

Figure 3. Pre- and perinatal exposure to BBP increases allergic airway inflammation in a murine asthma model. Total cell number in BAL fluid (A), airway inflammation examined by lung histology (B, H&E, x100), quantified by an investigator-independent computer-based analysis (C) and lung resistance (D) were examined in offspring from BBP-exposed dams and in BBP-exposed adult mice. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals/group in the offspring (from at least 5 dams) and adult mice from 4 independent experiments; * $P < 0.05$

Figure 4. Pre- and perinatal exposure to BBP enhances allergen-specific IgE levels and Th2 cytokine production. OVA-specific IgE serum levels (A), and

cytokine production of re-stimulated splenocytes (B) and lymph node cells (C) were analyzed in OVA-immunized offspring from BBP-exposed dams and in BBP-exposed adult mice. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals/group in the offspring (from at least 5 dams) and adult group from 4 independent experiments; $*P < 0.05$

Figure 5. Maternal BBP exposure induces global hypermethylation which mediates the asthma-promoting effect. Differentially methylated regions (DMRs) were called from dams and their offspring (A) between exposed (F0 or F1) vs. non-exposed groups. The numbers of DMRs in different DNA regions of the F1 generation (B) are displayed. Total cell number in BAL fluid (C), airway inflammation (D), lung resistance (E), OVA-specific IgE levels (F) and cytokine production in spleen and lymphnode cells (G) were examined in offspring from BBP-exposed dams compared to F1 mice treated with the DNA methyltransferase inhibitor Aza. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals/group in the offspring (from ≥ 6 dams) from 6 independent experiments; $*P < 0.05$ OVA vs. OVA + BBP; $^{\#}P < 0.05$ OVA + BBP vs. OVA + BBP + Aza.

Figure 6. BBP-induced hypermethylation leads to down-regulation of selected target genes.

The heatmap (A) displays differentially expressed genes from pups of BBP-exposed dams vs. control mice. 242 of 1338 differentially expressed genes (DEG) in the offspring from BBP-exposed dams can be linked to 346 DMRs (B). RNA expression of selected genes in dams ($n = 5$), pups or adult mice ($n \geq 6$ from at least 4 dams) was verified by qPCR (C). Mice from BBP-exposed dams were treated with Aza as described under Methods and RNA expression was measured in mature offspring (D;

n $\geq$ 6 from at least 2 dams). Data are expressed as mean $\pm$ SEM from at least two independent experiments, * P < 0.05 CON vs. BBP; # P < 0.05 BBP vs. BBP + Aza.

Figure 7. Altered function of CD4⁺ T cells in the offspring from BBP-exposed mice. The number of CD4⁺CD25⁺ T cells (A), the percentage of cytokine-producing T cells (B) and Foxp3⁺CD4⁺CD25⁺ T cells (C) are shown. The efficiency of CD4⁺CD25⁺ regulatory T cells to suppress proliferation of responder T cells is displayed in (D). Data are expressed as mean $\pm$ SEM, (n $\geq$ 5 from 3 independent experiments). * P < 0.05

Figure 8. Maternal BBP exposure affects airway inflammation in the F2 generation. Total cell number in BAL fluid (A), airway inflammation (B), lung resistance (C) and OVA-specific IgE levels (D) were examined in OVA-immunized F2 generation from prenatally BBP-exposed F1 dams compared to control mice. Data are expressed as mean $\pm$ SEM, n $\geq$ 9 animals/group in the F2 offspring (from $\geq$ 6 dams) from 6 independent experiments; * P < 0.05 OVA vs. OVA + BBP; # P < 0.05 OVA + BBP vs. OVA + BBP + Aza.

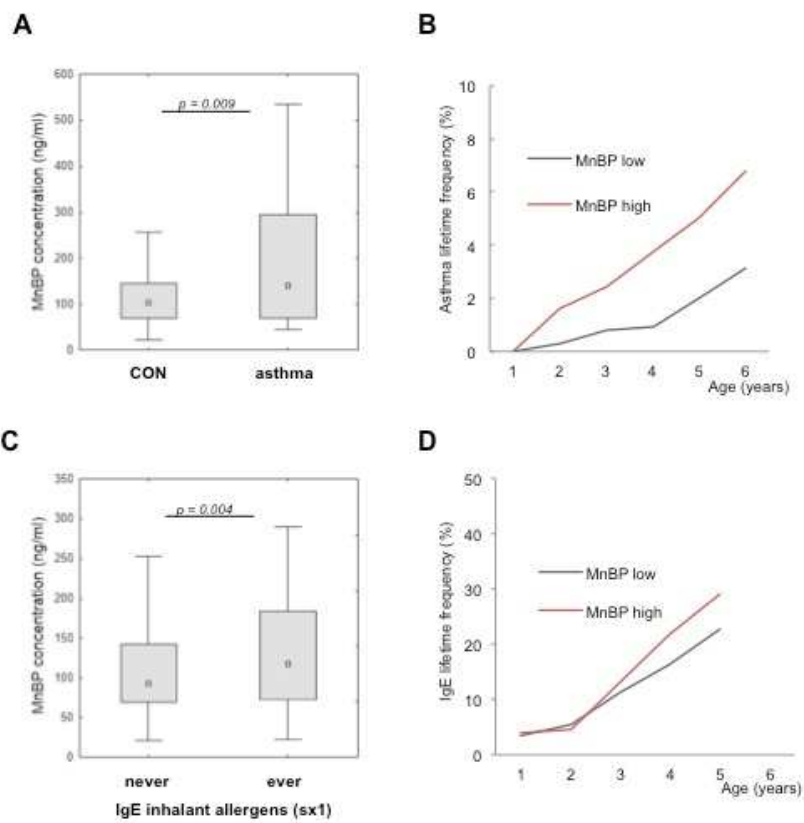
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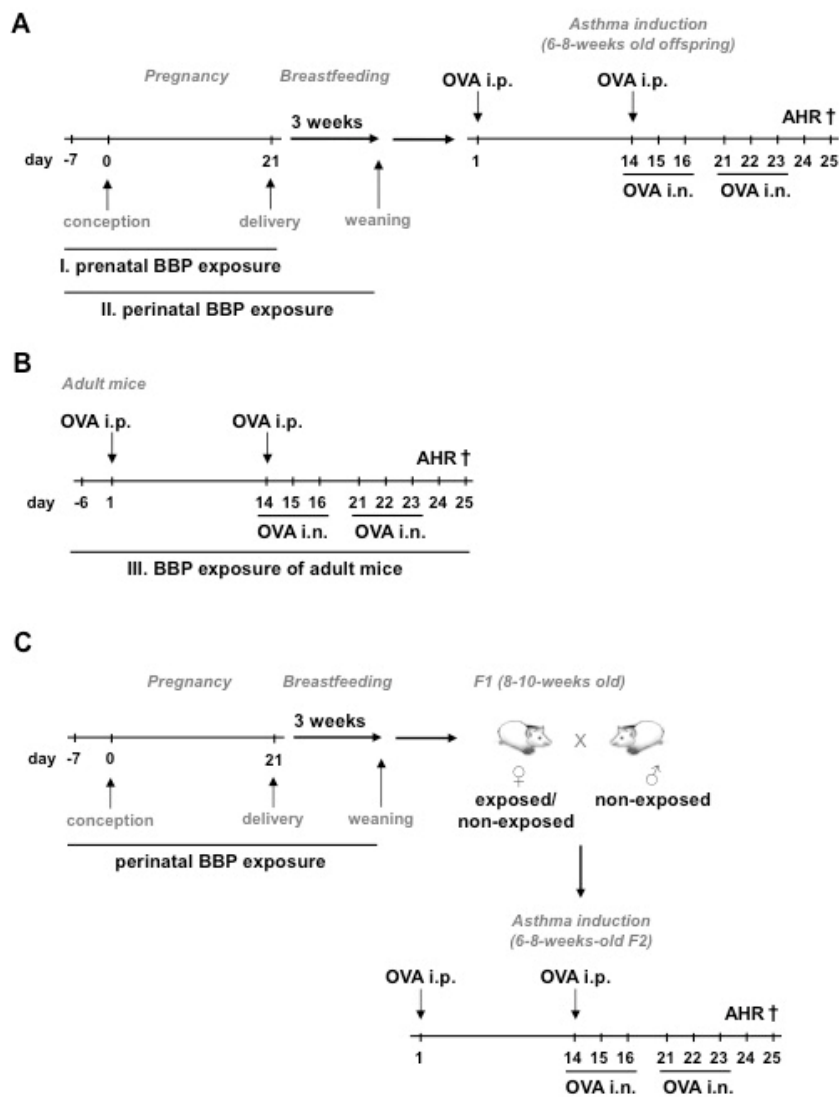


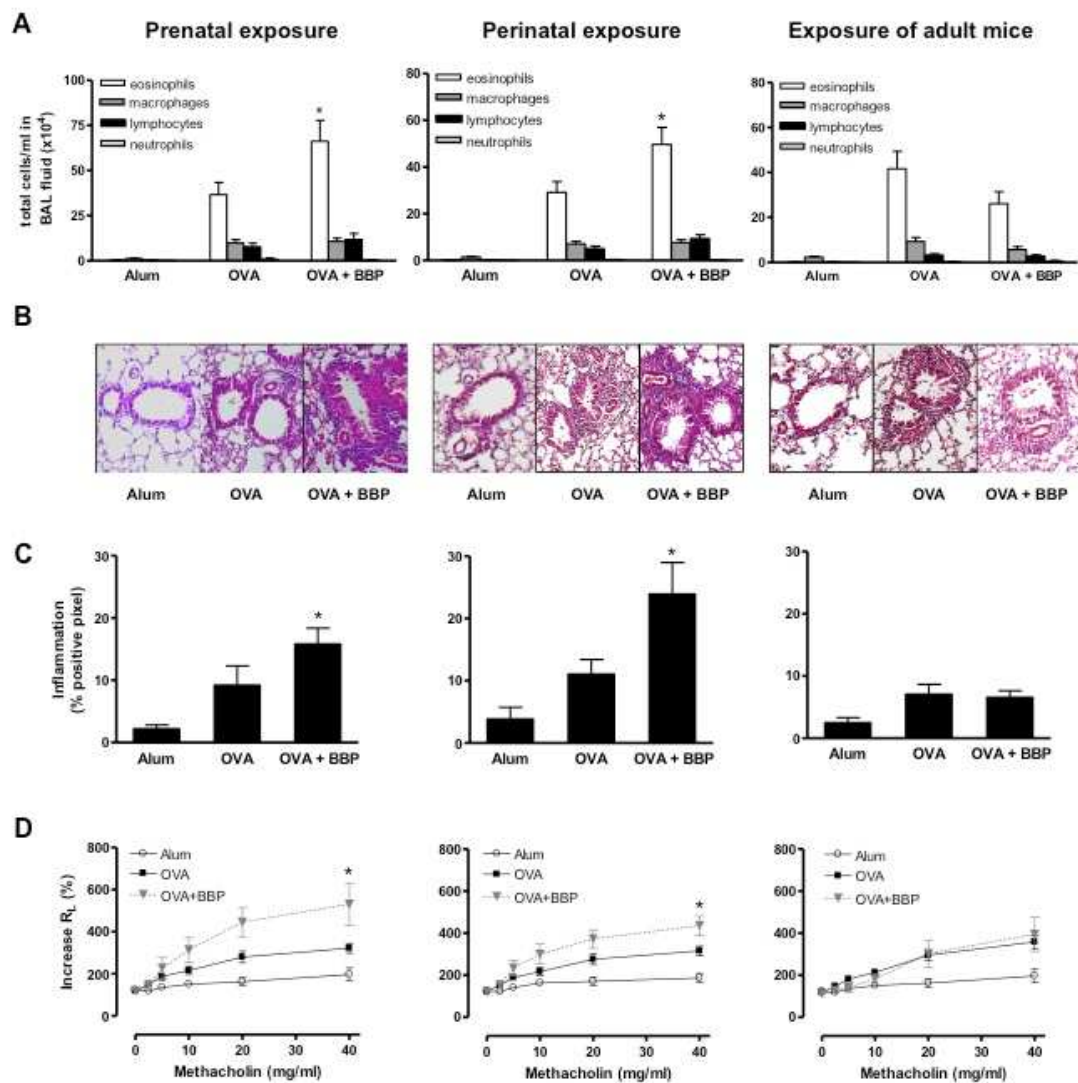
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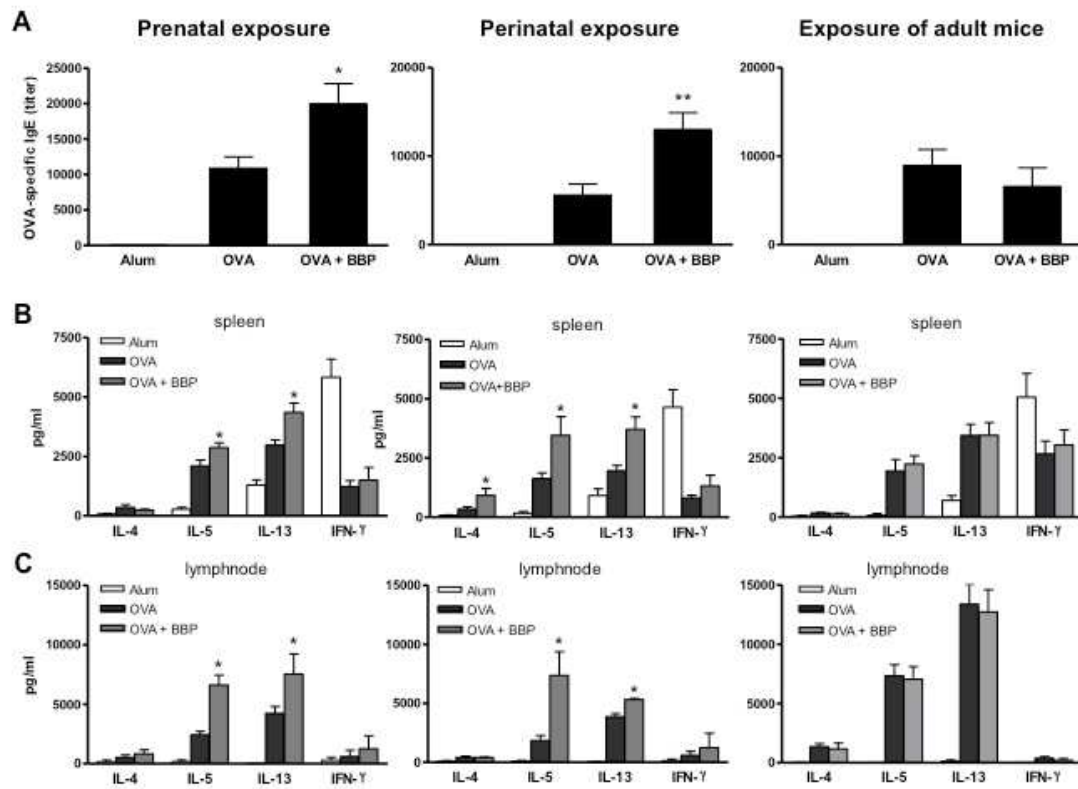
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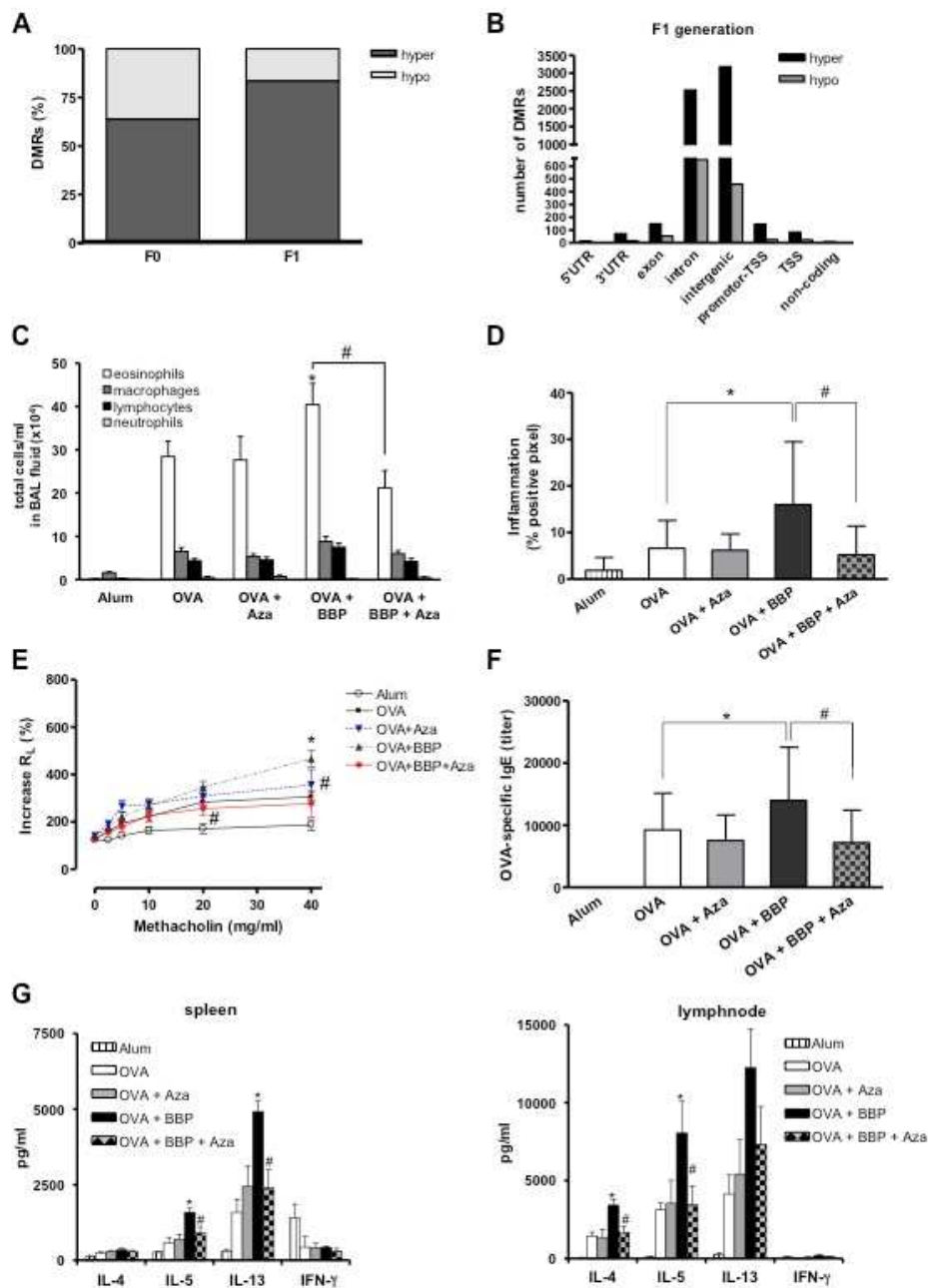


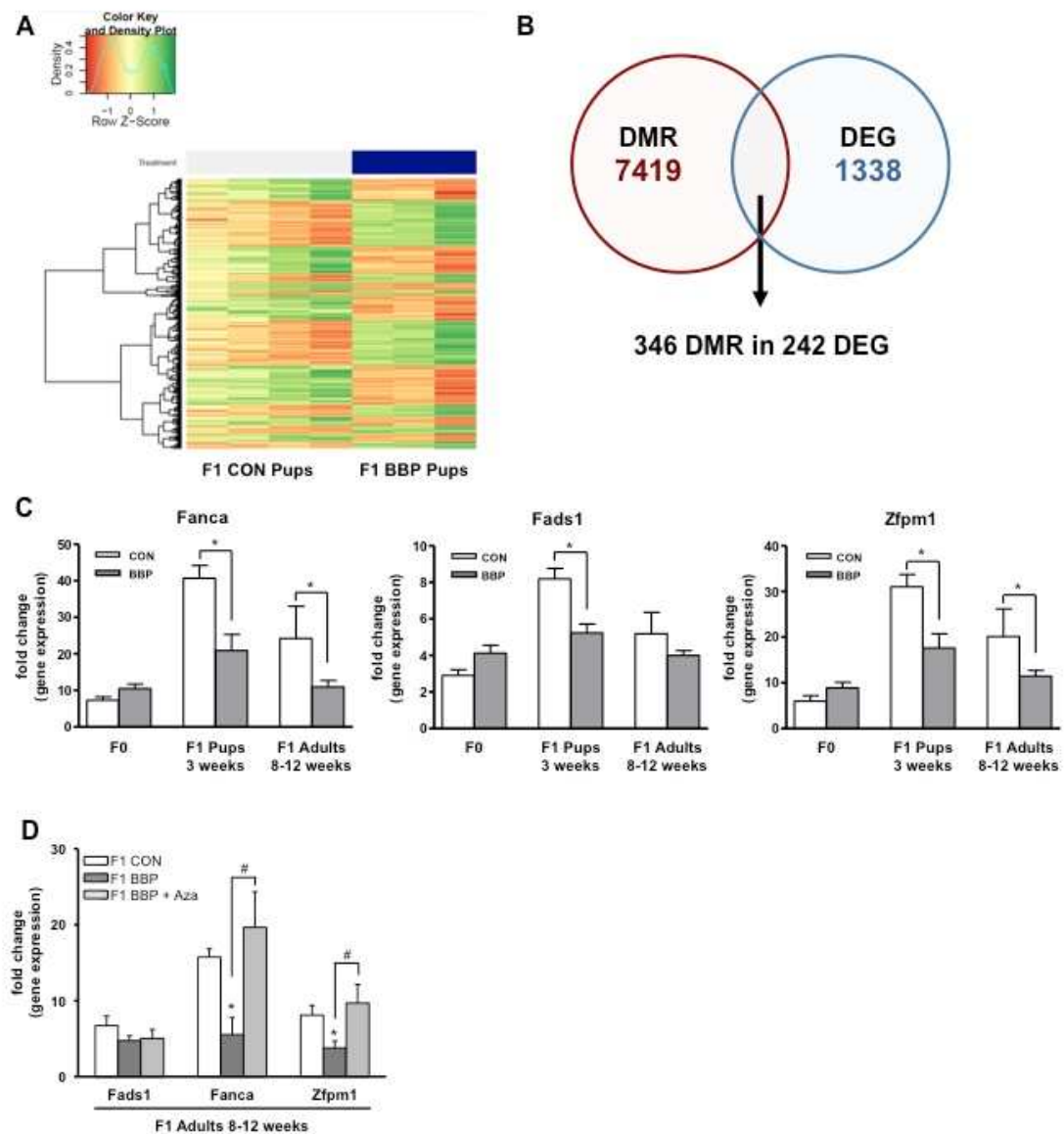
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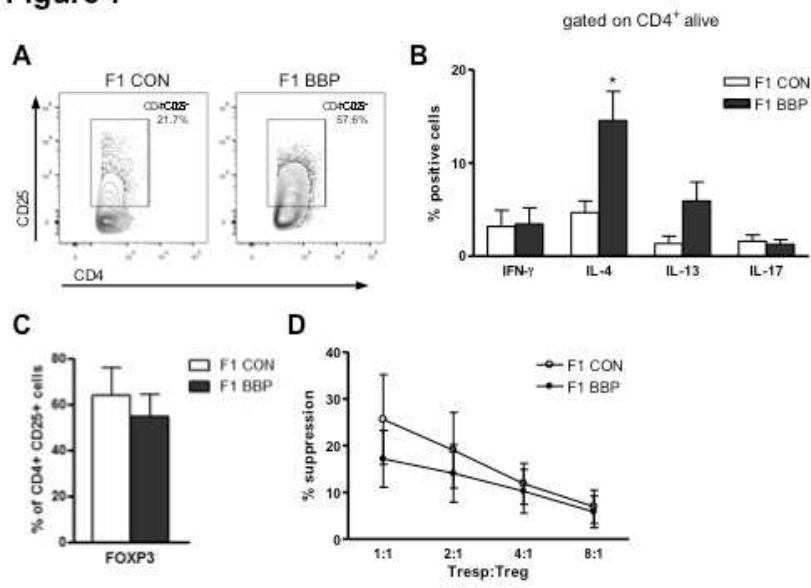
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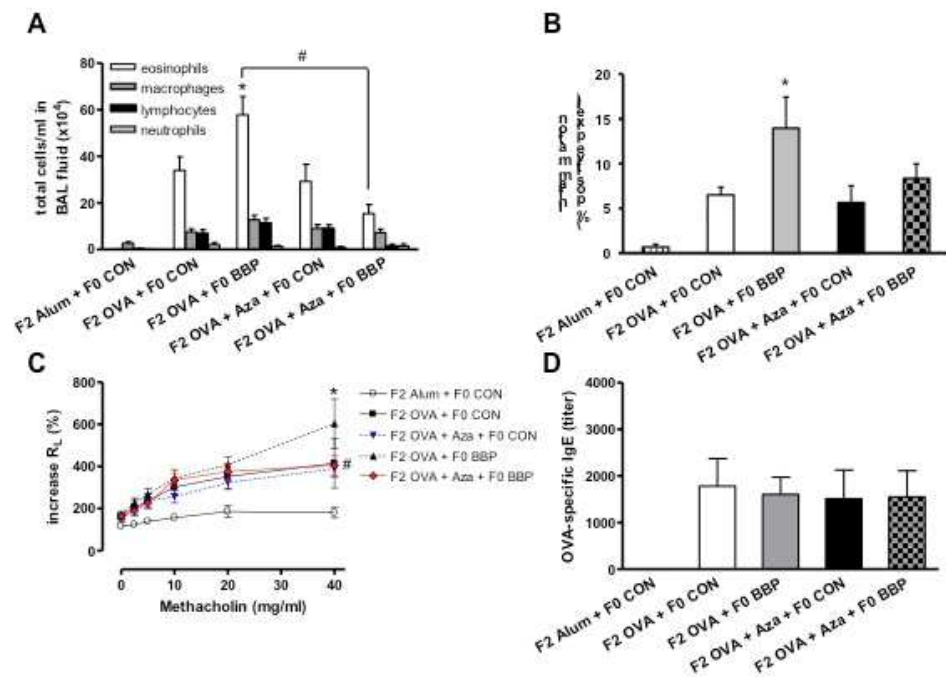
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Figure E1

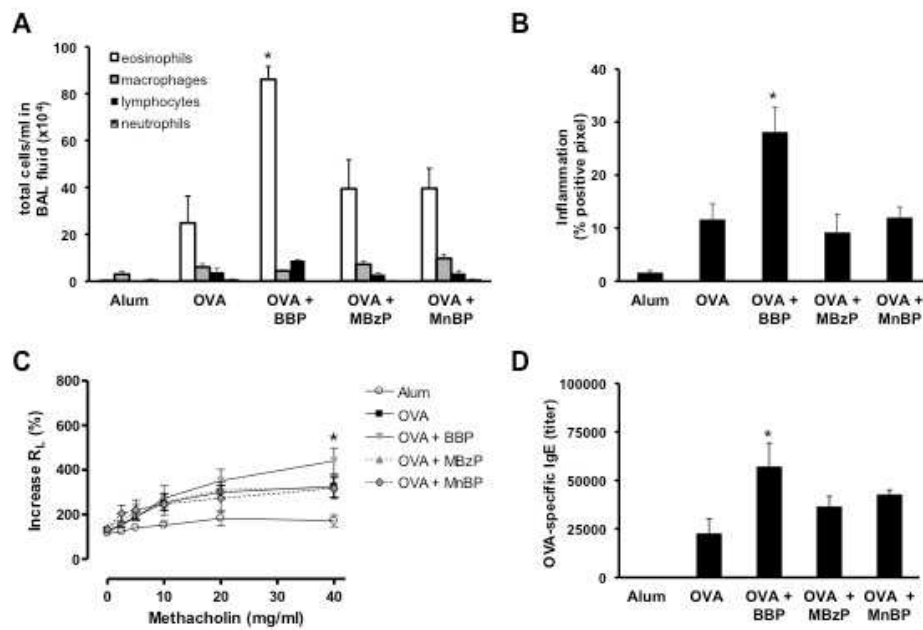


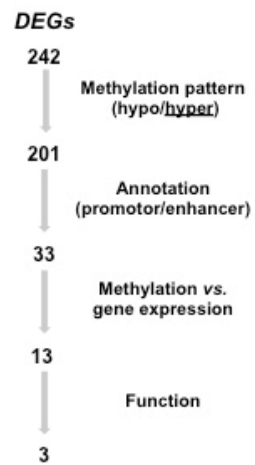
Figure E2

Figure E3

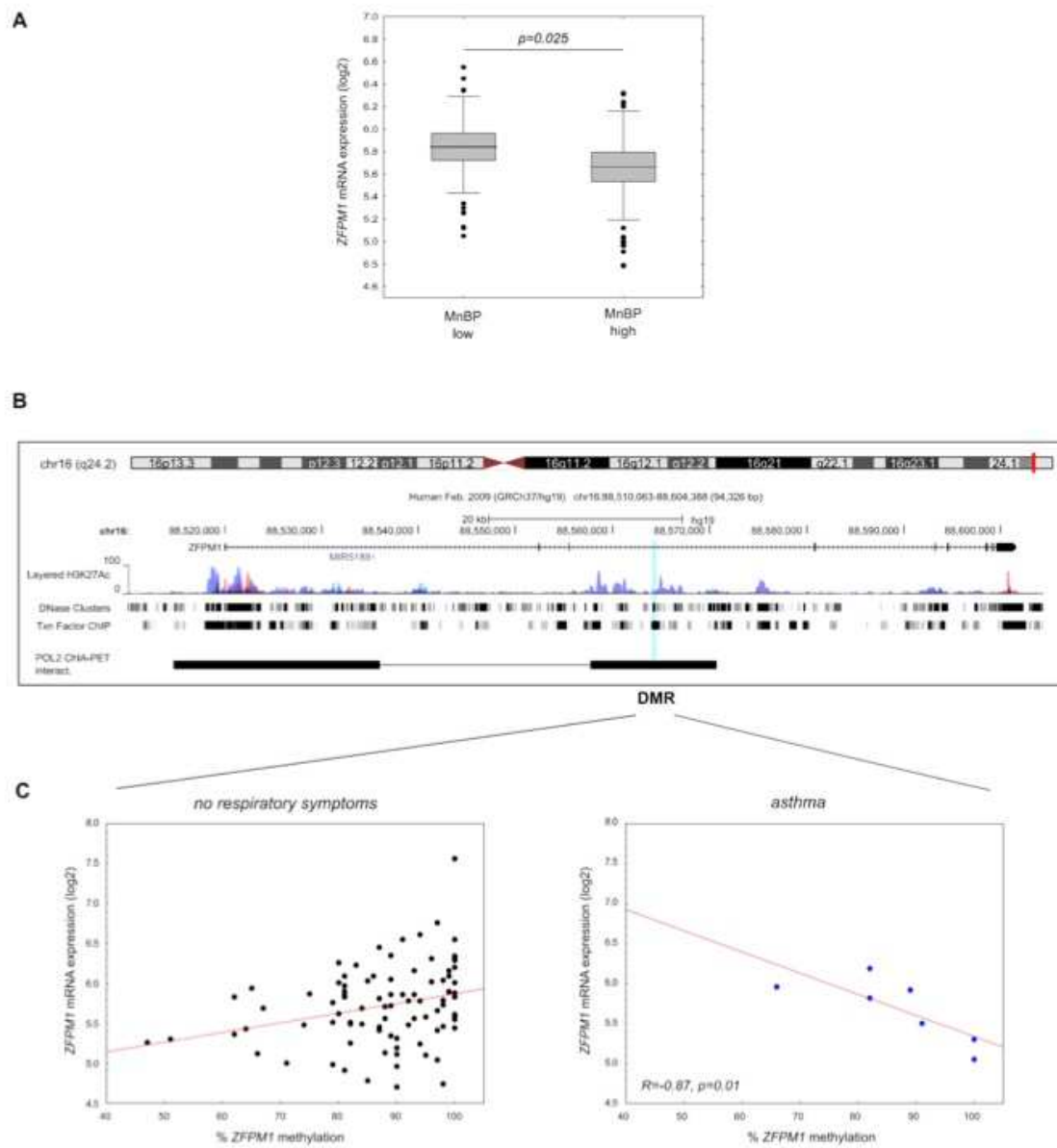


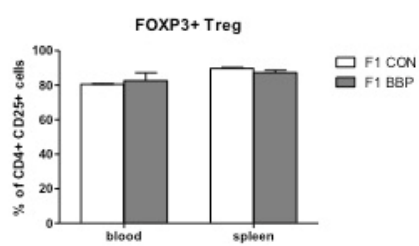
Figure E4

Figure E5