Pulmonary Exposure to Particles during Pregnancy Causes Increased Neonatal Asthma Susceptibility

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Maternal immune responses can promote allergy development in offspring, as shown in a model of increased susceptibility to asthma in babies of ovalbumin (OVA)-sensitized and -challenged mother mice. We investigated whether inflammatory responses to air pollution particles (diesel exhaust particles, DEP) or control "inert" titanium dioxide (TiO_2) particles are enhanced during pregnancy and whether exposure to particles can cause increased neonatal susceptibility to asthma. Pregnant BALB/c mice (or nonpregnant controls) received particle suspensions intranasally at Day 14 of pregnancy. Lung inflammatory responses were evaluated 48 hours after exposure. Offspring of particle- or buffer-treated mothers were sensitized and aerosolized with OVA, followed by assays of airway hyperresponsiveness (AHR) and allergic inflammation (AI). Nonpregnant females had the expected minimal response to "inert" TiO_2. In contrast, pregnant mice showed robust and persistent acute inflammation after both TiO_2 and DEP. Genomic profiling identified genes differentially expressed in pregnant lungs exposed to TiO_2. Neonates of mothers exposed to TiO_2 (and DEP, but not PBS) developed AHR and AI, indicating that pregnancy exposure to both "inert" TiO_2 and DEP caused increased asthma susceptibility in offspring. We conclude that (*1*) pregnancy enhances lung inflammatory responses to otherwise relatively innocuous inert particles; and (*2*) exposures of nonallergic pregnant females to inert or toxic environmental air particles can cause increased allergic susceptibility in offspring.

Keywords: maternal asthma; environmental particles; titanuim dioxide; diesel exhaust particles; susceptibility

The increased prevalence of asthma is a major public health problem (1–4). Asthma is a disease that primarily begins in early life, but can persist into adult life. One strong risk factor for asthma is maternal asthma (more so than paternal) (5, 6). Multiple mechanisms may contribute to the maternal effect, including genetic, environmental, and maternal immune system factors.

We have developed a murine model in which an identical genetic background allows experiments focused on maternal immunity and how it can affect susceptibility of offspring to allergy (7–9). In this model of maternal transmission of asthma risk, mother mice are sensitized and challenged with chicken ovalbumin (OVA) and their offspring are subjected to an "intentionally suboptimal" OVA sensitization and challenge protocol. An asthma-like phenotype of airway hyperresponsiveness (AHR) and allergic inflammation (AI) is seen only in offspring from asthmatic, but not normal, mothers.

CLINICAL RELEVANCE

A novel model allowing analysis of environmental exposures in pregnancy on offspring susceptibility to allergy identifies titanium dioxide particles as pro-inflammatory in pregnancy and pro-allergic for neonates.

Air pollution is well known to exacerbate existing asthma (10). The role of air pollution in the initiation of asthma is more controversial. Arguments against a link include epidemiologic data showing less asthma in highly polluted East Germany compared with West Germany (11) and the increase in asthma in Western countries where air pollution has in general been decreasing. On the other side of the controversy are epidemiologic data showing increased incidence of asthma in high-traffic areas (12, 13).

Some air pollutants—for example, diesel exhaust particles (DEP)—have been used extensively to address this question experimentally in people and in animal models. DEP can exacerbate established asthma in mice (14, 15) and nasal allergy outcomes in human studies (16). DEP can act as a strong adjuvant or co-factor in the initiation phase or sensitization to allergen in both mice and people (17, 18) and up-regulate production of pro-allergic cytokines (19, 20). Other air pollutants, like the titanium dioxide particles (TiO_2) or carbon black particles (CB) are known to be immunologically "inert" and typically used as control substances in immunotoxicity studies.

Since asthma begins in early life, we sought to determine if our model could be used to detect and analyze increased susceptibility arising from environmental exposure of pregnant mice. Our pilot studies indicated that a single intratracheal instillation of DEP into normal, nonallergic mother mice during pregnancy results in increased susceptibility to allergy in their offspring. We hypothesized that in pregnancy the response to particles is enhanced and that this may influence the offspring allergic susceptibility. In addition, we were interested in effects of immunologically "inert" particles (e.g., TiO_2) on both local pulmonary inflammation in the lungs of pregnant mice and on susceptibility of the offspring of exposed mothers to allergic sensitization.

MATERIALS AND METHODS

Animals

BALB/c mice were obtained from Charles River Laboratories (Cambridge, MA). All mice were housed in a clean barrier facility where animals are maintained at 22 to 24°C with a 12-hour dark/light cycle with an independent pressure-gradient–enabled ventilation system. Animal care complied with the Guide for the Care and Use of Laboratory Animals, and all experiments were approved by the Institutional Review Board.

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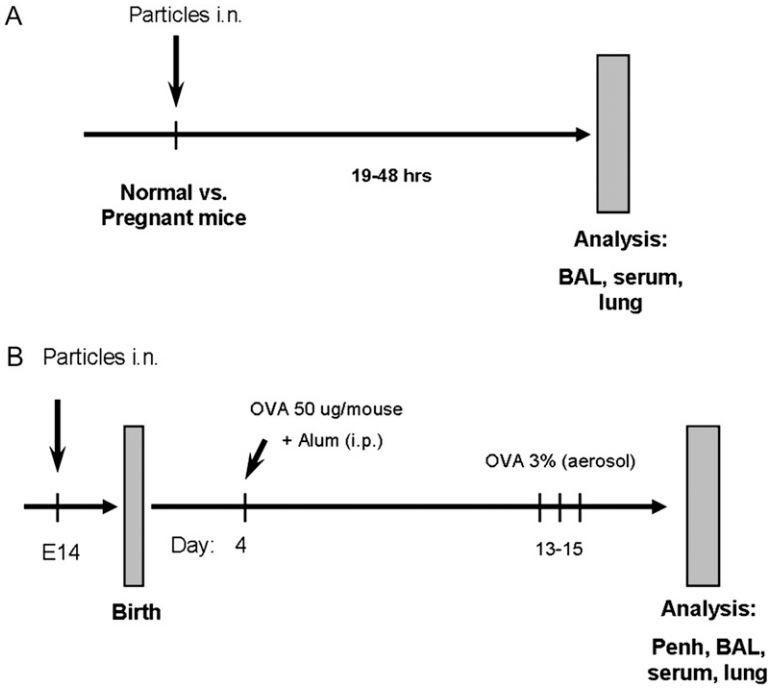

Figure 1. Experimental protocols. (*A*) Particles exposure protocol. Normal females or pregnant mice were treated with diesel exhaust particle (DEP) or titanium dioxide (TiO_2) particle suspensions (50 ug/mouse) and analyzed 19 or 48 hours later. (*B*) Maternal particles exposure + single intraperitoneal neonatal sensitization period. Pregnant mothers at Day 14 of pregnancy (E14) received 50 μg/mouse intranasally of DEP, carbon black (CB), or TiO_2 particle suspensions or PBS buffer (negative control). Offspring of these mothers were injected once with 0.1 ml of 50 μg/ml ovalbumin (OVA) + alum ("suboptimal") sensitization and challenged three times with 3% OVA aerosol.

Exposure to Environmental Particles

Respirable-size DEP, TiO_2, and CB particles were generously provided by Dr. Ian Gilmour (U.S. E.P.A.) and Dr. Joseph Brain (Harvard University). Particle samples were baked at 165°C for 3 hours to eliminate endotoxin, aliquoted and stored frozen at −80°C. Particle suspensions (50 μg in 50 μl for DEP and TiO_2, and 250 μg in 50 μl for CB) or PBS solution (vehicle) were administered by single intranasal insufflation of pregnant or normal BALB/c mice under light halothane anesthesia (21). We used two different protocols of particle exposure.

Protocol 1A: Comparison of innate immune response to particles in normal versus pregnant mice. To test whether pregnancy alters the normally minimal inflammatory response to "inert" particles, we administered TiO_2 and DEP suspensions (50 μg/mouse) or PBS solution by intranasal insufflation to normal or pregnant E14 mice (*see* Figure 1B). The mice were subjected to pathologic analysis 48 hours later.

Protocol 1B: Particle exposure during pregnancy and asthma susceptibility in offspring. The protocol is based on our

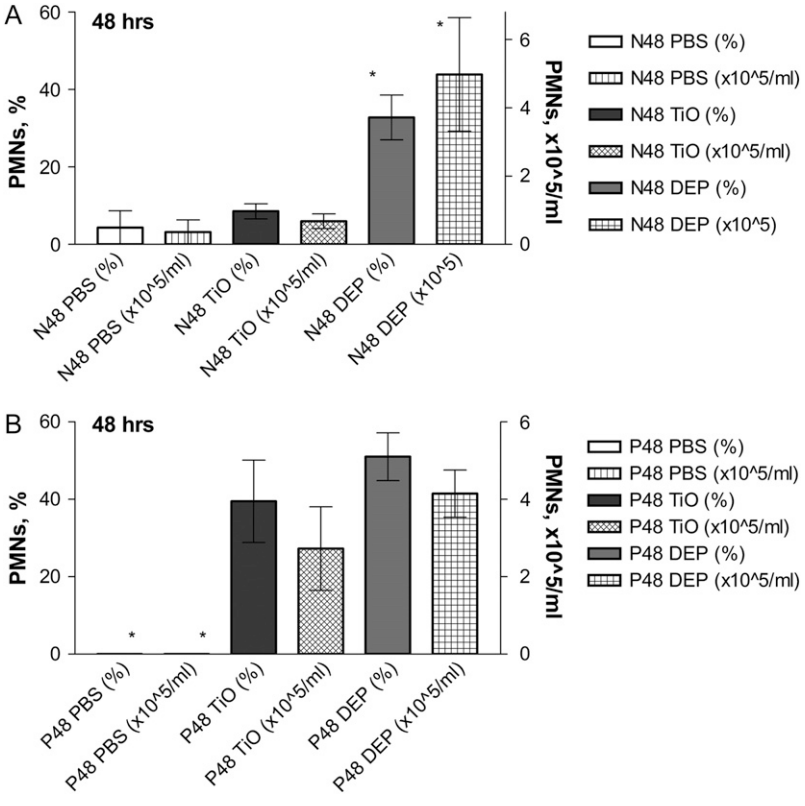

Figure 2. Direct analysis of bronchoalveolar lavage (BAL) responses in pregnant versus control females. Pregnant or normal mice were exposed to either DEP or TiO_2 particle suspension or PBS alone and BALs were obtained 48 hours later. Normal mice exposed to TiO_2 reveal minimal airway inflammation at 48 hours (*A*) after exposure. In contrast, pregnant mice reveal enhanced and prolonged inflammation seen even 48 hours after exposure to TiO_2 (*B*). Mean ± SEM ($n > 9$ each group). *$P < 0.05$.

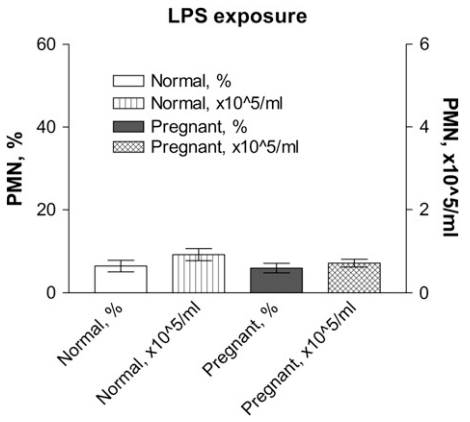

Figure 3. Inflammatory BAL response to LPS challenge. Pregnant mice or normal controls were exposed to LPS aerosol, and BALs were obtained 24 hours later. There is no significant difference in PMN counts in these groups. Mean ± SEM (n = 8).

prior studies showing that maternal immune events can influence the susceptibility of offspring's immune system to allergy (7). The model uses an "intentionally suboptimal" allergen (OVA, grade III; Sigma-Aldrich, St. Louis, MO) sensitization and challenge protocol in the newborn mice, as detailed in (7). Briefly, female mice received two intraperitoneal injections of 5 μg OVA with 1 mg alum in 0.1 ml PBS at 3 and 7 days of age, and after weaning are exposed to aerosols of allergen (3% OVA [wt/vol] in PBS [pH 7.4]) for 10 minutes on 3 consecutive days at 4, 8, and 12 weeks of age. These "asthmatic" and normal control mothers are mated with normal males and the offspring receive "suboptimal" protocol. In this study we replaced prior maternal sensitization with particle exposure (Figure 1A).

Offspring Allergen Sensitization and Challenge

On Day 4 after birth, newborns from particle-exposed and normal control mother mice received a single intraperitoneal injection of OVA with alum. On Days 12 to 14 of life, these baby mice were exposed to aerosolized 3% OVA within individual compartments of a mouse pie chamber (Braintree Scientific, Braintree, MA) using a Pari IS2 nebulizer (Sun Medical Supply, Kansas City, KS) connected to air compressor (PulmoAID; DeVilbiss, Somerset, PA). After this challenge, the mice were subjected to pulmonary function and pathologic analysis.

Pulmonary Function Testing

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco, Sharon, CT). Briefly, each mouse was placed in a chamber, and continuous measurements of box pressure/time wave were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation in BALB/c mice with the airway resistance examined by standard evaluation methods, was calculated from the box waveform. After measurement of baseline Penh, aerosolized PBS or methacholine (MCh, acetyl-methylcholine chloride; Sigma-Aldrich) in increasing concentrations (6, 12, 25, 50, and 100 mg/ml) was nebulized through an inlet of the chamber for 1 minute, and Penh measurements were taken for 9 minutes after each dose. Penh values for the first 2 and the last 2 minutes after each nebulization were discarded, and the values for 5 minutes in between were averaged and used to compare results. Increased Penh was interpreted as evidence of increased AHR.

Lipopolysaccharide Exposure

To test whether pregnancy alters inflammatory response to a non-specific agent, pregnant mice and normal controls were place in individually labeled compartments of a pie chamber and exposed to 2 μg/ml lipopolysaccharide (LPS) (serotype 055:B5, CAT:L2880, LOT:110K4046; Sigma-Aldrich) nebulized aerosol for 10 minutes. Bronchoalveolar lavage (BAL) samples were collected 24 hours later. We chose this time point based on abundant work from other labs (53) and our own prior experience in working with inhaled LPS exposure, showing optimal detection of peak BAL polymorphonuclear leukocytes (PMN) responses at this time point.

Pathologic Analysis

Animals were killed with sodium pentobarbital (Veterinary Laboratories, Lenexa, KS). The chest wall was opened and the animals were exsanguinated by cardiac puncture. The trachea was cannulated after blood collection. BAL was performed five times with 0.3 ml of sterile PBS instilled and harvested gently. Lavage fluid (recovery volume was ~ 90% of instilled) was collected and centrifuged at 1200 rpm (300 × g) for 10 minutes, and the cell pellet was resuspended in 0.1 ml PBS. Total cell yield was quantified by hemocytometer. BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation of samples at 800 rpm for 5 minutes (Cytospin 2; Shandon, Pittsburgh, PA). These slides were fixed in 95% methanol and stained with Diff-Quik (VWR, Boston, MA), a modified Wright-Giemsa stain, and a total of 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. After lavage, the lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with hematoxylin and eosin (H&E). For allergy responses, an index of pathologic changes in coded H&E slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 4–10 cells thick; 3, >10 cells thick) and overall prevalence (0, normal; 1, <25% of sample; 2, 25–50%; 3, 51–75%; 4, >75%). The index was calculated by multiplying severity by prevalence, with a maximum possible score of 9.

Cytokine Detection

Levels of cytokines in BAL fluid, serum or cell culture supernatants were measured via the multiplexed Luminex xMAP assay (Luminex, Austin, TX). LINCOplex kits were obtained from Linco Research (St. Charles, MI). The sensitivity of the kit varied between 0.3 to 20 pg/ml for serum/plasma samples depending on the cytokine. Samples were tested in duplicates.

Gene Chip Microarray and Data Analysis

Total lung RNA extraction and isolation was performed using a Qiagen RNAeasy Mini kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA purity and quality were analyzed by Agilent Bioanalyzer 2100 scan (Agilent, Santa Clara, CA). The hybridization was carried out at the Harvard Partners Genomic Center Microarray facility (Cambridge, MA) using the Affymetrix GeneChip platform and Affymetrix mouse 430 2.0 chips (Affymetrix, Santa Clara, CA). Signal intensities and detection calls were extracted using dChip (v. 2006). Chip images were evaluated for overall quality; PM/MM pairs were evaluated for outliers to judge on hybridization performance. Hybridization quality was found to be consistent with the manufacturer's requirements. Probesets were filtered based on detection call to exclude ones in which "P" call was not present in all four samples in any one group, this also excluded probesets with all "A" calls. The filtration resulted in about 24,000 probesets. RMA values for this list were extracted using RMAExpress (v. 0.4.1) with background correction, normalization, and log2 transformation and were analyzed using tMEV (v. 4.0). Resampling with bootstrapping using the Support Tree feature indicated appropriate sample clustering with 90 to 100% support level (not shown). High-level analysis was performed in tMEV 4.0 and included Significance Analysis for Microarrays (SAM) at false-discovery rate (FDR) of 0, ANOVA, and t test with Welch approximation. Fold change was calculated from corresponding natural, not log2 values. Meta-analysis was carried out using the Expression Analysis Systematic Explorer (EASE v. 2.0)

General Statistical Methods

Data are presented as mean ± SEM. Data analysis was performed using Microsoft Excel from Microsoft Office 2003 Pro (Microsoft

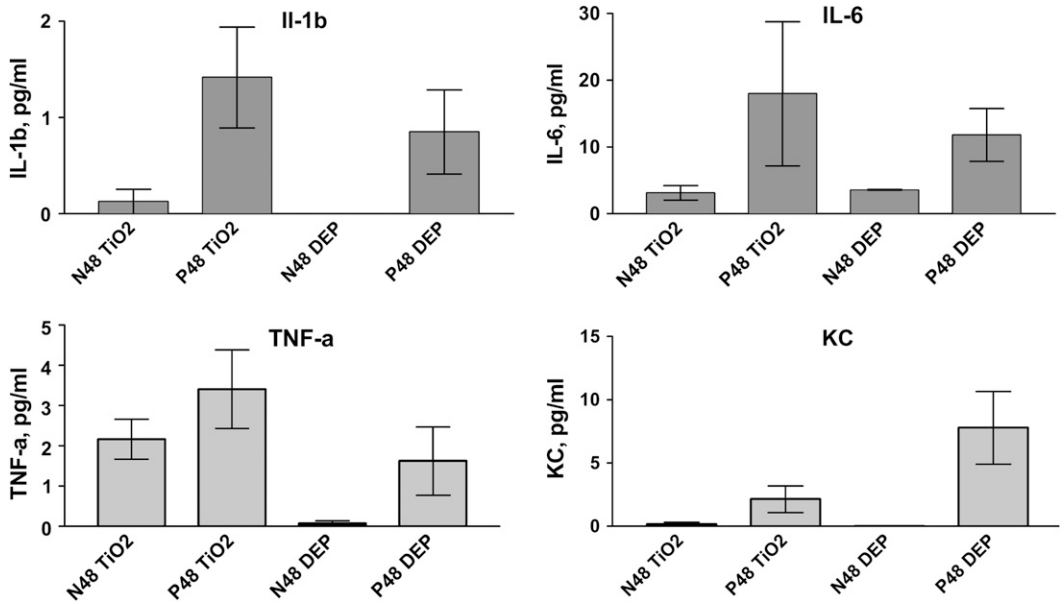

Figure 4. Serum cytokine levels after particle exposure. Pregnant (P) or normal (N) mice were exposed to either DEP or TiO_2 particle suspension and sera were obtained 48 hours later. Levels of proinflammatory cytokines are increased in pregnant mice compared with nonpregnant controls after both TiO_2 and DEP exposure (with $P < 0.05$). Mean ± SEM ($n = 9$ each group).

Corporation, Redmond, WA) and GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Statistical significance was accepted when $P < 0.05$. To estimate significance of differences between groups in multiple comparisons ANOVA with Tukey's Honest Significant Differences for unequal N *post hoc* test and Kruskal-Wallis test with Dunn's post-test were used, as appropriate. For pairwise comparisons nonparametric Mann-Whitney U test was used. For repeated measurements in the plethysmography procedure we used repeated-measures ANOVA.

RESULTS

Inflammatory Response to Inhaled Particles Is Enhanced in Pregnancy

To investigate whether pregnancy alters the inflammatory response to particles, we exposed pregnant and control normal female mice to particle suspensions of DEP or vehicle (PBS) (Figure 1, Protocol 1A). We initially analyzed TiO_2 as an "inert" negative control particle. In both normal and pregnant mice, BAL PMN counts were significantly increased at 48 hours after exposure to DEP, but not to PBS (Figure 2). Nonpregnant mice treated with TiO_2 displayed minimal increases in BAL PMN counts 48 hours after exposure (Figure 2A). In contrast, pregnant mice exhibited a robust acute neutrophilic inflammation (Figure 2B). No significant changes were noted in any other cell type (e.g., lymphocytes) (data not shown). The specificity of the enhanced inflammatory response to TiO_2 was tested by comparing responses to inhaled LPS. Both pregnant and nonpregnant females showed similar acute PMN influx into the lungs after exposure to aerosolized LPS (Figure 3). We used an exposure that causes mild inflammation in normal nonpregnant females (e.g., $\sim$ 10% PMNs in BAL samples) so as to allow sensitive detection of increased inflammation in pregnant mice. To investigate whether responses to particles in the lungs of pregnant mice were associated with systemic effects, serum samples from DEP- and TiO_2-treated pregnant and normal animals were analyzed for cytokine levels via a multiplex assay (Luminex). The data show that pregnant mice exposed to both DEP and TiO_2 had elevated levels of IL-1β, TNF-α, IL-6, and KC levels 48 hours after exposure, compared to nonpregnant controls (Figure 4).

Gene Expression Changes in Response to Inhaled Particles Are Different in Pregnant versus Normal Mice

To identify genes involved in the unexpected response of pregnant lungs to inert TiO_2 particles, we performed microarray analysis of mRNA gene expression patterns in pregnant and nonpregnant females treated with TiO_2 or PBS vehicle. Data analysis used significance analysis for microarrays (SAM). At false-discovery rate (FDR) of 0, SAM identified 130 probesets significantly different across the four groups (*see* Figure EA in the online supplement A). Pathway analysis indicated that most of these genes are involved in inflammatory response and immune regulation, cell proliferation/DNA metabolism, and metabolic processes (Table EA in online supplement B).

Using *t* test with Welch approximation and ANOVA, we identified a cluster of genes that were changed only upon exposure to TiO_2 in pregnant mice (were significantly different between Pregnant PBS and Pregnant TiO_2 groups) (Figure EB, *left*, in online supplement A). From this list we excluded genes that were significantly changed in normal mice upon TiO_2 exposure, or were changed in pregnant mice compared with normals. We also excluded noncoding sequences. Expression of these 80 genes (*see* Table 1) is changed (increased or decreased) only in response to TiO_2 on the background of pregnancy. We also identified genes that were changed upon exposure to TiO_2 in normal mice, but were not significantly different between pregnant mice exposed to PBS versus TiO_2 (Figure EB, *right*, in online supplement A; Table 2). Absence of change in these 108 genes in pregnant mice exposed to TiO_2 compared to PBS may also contribute to the studied phenomenon. Detailed pathway analysis with EASE (Expression Analysis Systematic Explorer, a functional enrichment analysis that identifies groups of genes based on their involvement in various processes) for the genes in Tables 1 and 2 is presented in online supplement B. Genomic data has been submitted to Gene Expression Omnibus (GEO) database and has been assigned Series Record # GSE7475.

Enhanced Response in Pregnancy Leads to Increased Allergic Susceptibility in Offspring

We investigated whether pregnancy-enhanced response to particles could influence the allergic susceptibility of the off-

TABLE 1. GENES POTENTIALLY INVOLVED IN TiO2 RESPONSE IN PREGNANT MICE

Probe Set ID	Representative Public ID	Gene Title	Gene Symbol	Process	Adjusted *P* Value	Fold Ti > PBS
1450920_at	AK013312	Cyclin B2	Ccnb2	Cell division and cell	0.0082	1.489921
1423774_a_at	BC005475	Protein regulator of cytokinesis 1	Prc1	cycle regulation,	0.0039	1.328305
1437716_x_at	BB251322	Kinesin family member 22	Kif22	cytokinesis, apoptosis	0.0079	1.299987
1449171_at	NM_009445	Ttk protein kinase	Ttk		0.0061	1.294891
1423775_s_at	BC005475	Protein regulator of cytokinesis 1	Prc1		0.0038	1.287928
1428104_at	AK011311	TPX2, microtubule-associated protein homolog	Tpx2		0.0052	1.257099
1460238_at	NM_018857	Mesothelin	Msln		0.0017	1.256257
1428480_at	AV307110	Cell division cycle associated 8	Cdca8		0.0059	1.248081
1417251_at	NM_023245	Palmdelphin	Palmd		0.0082	1.213763
1422498_at	AF319981	Melanoma antigen, family H, 1	Mageh1		0.0081	1.191047
1433543_at	BI690018	Anillin, actin binding protein (scraps homolog, Drosophila)	Anln		0.0086	1.179205
1449249_at	NM_018764	Protocadherin 7	Pcdh7		0.0059	1.154676
1439436_x_at	BB418702	Inner centromere protein	Incenp		0.0089	1.14459
1438951_x_at	BB168451	Nucleoporin 54	Nup54		0.0040	1.142115
1429594_at	BB030482	Solute carrier family 38, member 2	Slc38a2		0.0092	1.139607
1416114_at	NM_010097	SPARC-like 1 (mast9, hevin)	Sparcl1		0.0061	1.122001
1449445_x_at	BB436326	Microfibrillar-associated protein 1	Mfap1		0.0089	1.10335
1426129_at	BC003485	Breast cancer metastasis-suppressor 1	Brms1		0.0094	0.814778
1450060_at	NM_011082	Polymeric immunoglobulin receptor	Pigr	Immune response and regulation,	0.0084	1.75463
1427747_a_at	X14607	Lipocalin 2	Lcn2	complement cascade,	0.0075	1.736284
1438148_at	BB829808	Gene model 1960, (NCBI)	Gm1960	adhesion, proteolysis	0.0061	1.670798
1442187_at	AW490711	Bradykinin receptor, beta 2	Bdkrb2		0.0041	1.337381
1450652_at	NM_007802	Cathepsin K	Ctsk		0.0080	1.291786
1457664_x_at	AV227574	Complement component 2 (within H-2S)	C2		0.0094	1.280136
1417009_at	NM_023143	Complement component 1, r subcomponent	C1r		0.0012	1.260038
1416051_at	NM_013484	Complement component 2 (within H-2S)	C2		0.0046	1.214211
1456532_at	BB428671	Platelet-derived growth factor, D polypeptide	Pdgfd		0.0015	1.202271
1421812_at	AF043943	TAP binding protein	Tapbp		0.0091	1.113042
1455327_at	BI684973	SUMO/sentrin specific peptidase 2	Senp2		0.0033	0.889723
1435943_at	AI647687	Dipeptidase 1 (renal)	Dpep1		0.0090	0.840445
1435560_at	BI554446	Integrin alpha L (CD11a antigen)	Itgal		0.0036	0.757596
1421546_a_at	NM_012025	Rac GTPase-activating protein 1	Racgap1	Intracellular transport,	0.0074	1.447467
1438773_at	BB817972	Six transmembrane epithelial antigen of prostate 2	Steap2	cell metabolism	0.0062	1.360801
1449203_at	NM_130861	Solute carrier organic anion transporter family, member 1a5	Slco1a5		0.0046	1.319134
1417381_at	NM_007572	Complement component 1, q subcomponent, alpha polypeptide	C1qa		0.0046	1.300391
1447234_s_at	AU018928	Sorting nexin 6	Snx6		0.0059	1.113238
1417039_a_at	NM_025611	Cullin 7	Cul7		0.0075	1.104416
1421594_a_at	NM_031394	Synaptotagmin-like 2	Sytl2		0.0017	0.87377
1449227_at	NM_009890	Cholesterol 25-hydroxylase	Ch25h	Metabolism	0.0028	1.563081
1423256_a_at	BI154058	ATPase, H+ transporting, lysosomal V1 subunit G1 ///	Atp6v1g1		0.0031	1.115357
1455824_x_at	BB724781	STT3, subunit of the oligosaccharyltransferase complex, homolog A (S. cerevisiae)	Stt3a		0.0074	1.101699
1427128_at	BM195862	Protein tyrosine phosphatase, non-receptor type 23	Ptpn23		0.0096	1.093921
1422092_at	BC018418	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	Pfkfb2		0.0050	0.838748
1435893_at	BB127955	Very low density lipoprotein receptor	Vldlr		0.0068	0.81228
1438338_at	BB318769	Malate dehydrogenase 1, NAD (soluble)	Mdh1		0.0050	0.806002
1434437_x_at	AV301324	Ribonucleotide reductase M2	Rrm2	Transcription, DNA replication, metabolism and repair	0.0087	1.494858
1448535_at	NM_023876	Elongation protein 4 homolog (S. cerevisiae)	Elp4		0.0006	1.219804
1455490_at	AV027632	Upstream binding transcription factor, RNA polymerase I	Ubtf		0.0006	1.64884
1454694_a_at	BM211413	Topoisomerase (DNA) II alpha	Top2a		0.0080	1.535987
1424105_a_at	AF069051	Pituitary tumor-transforming 1	Pttg1		0.0096	1.302197
1418036_at	NM_008922	DNA primase, p58 subunit	Prim2		0.0054	1.177537
1416641_at	NM_010715	Ligase I, DNA, ATP-dependent	Lig1		0.0094	1.176809
1426484_at	AI788596	UBX domain containing 2	Ubxd2		0.0083	1.160992
1449295_at	NM_020483	SAP30 binding protein	Sap30bp		0.0052	1.123169
1435303_at	AV373814	TAF4B RNA polymerase II, TATA box binding protein (TBP)-associated factor	Taf4b		0.0051	1.078127
1455323_at	BB446066	RB-associated KRAB repressor	Rbak		0.0099	0.941894
1418397_at	BC019962	Zinc finger protein 275	Zfp275		0.0062	0.876814
1436360_at	BB811893	GLI-Kruppel family member HKR2	Hkr2		0.0087	0.859708

(*Continued*)

TABLE 1. (CONTINUED)

Probe Set ID	Representative Public ID	Gene Title	Gene Symbol	Process	Adjusted *P* Value	Fold Ti > PBS
1452617_at	BG073014	Single-stranded DNA binding protein 1	Ssbp1		0.0028	0.820666
1447198_at	AI853438	RecQ protein-like	Recql		0.0019	0.820491
1438766_at	AV001197	Proline-rich nuclear receptor coactivator 2	Pnrc2		0.0046	0.791521
1443867_at	BB320633	Ankyrin repeat domain 12	Ankrd12	Other	0.0087	1.512112
1416299_at	NM_011369	Shc SH2-domain binding protein 1	Shcbp1		0.0048	1.43836
1429411_a_at	AI595744	Enhancer of yellow 2 homolog (Drosophila)	Eny2		0.0072	1.372424
1422430_at	NM_021891	Fidgetin-like 1	Fignl1		0.0049	1.327348
1417926_at	NM_133762	Leucine zipper protein 5	Luzp5		0.0091	1.30666
1449015_at	NM_020509	Resistin like alpha	Retnla		0.0023	1.303933
1448894_at	NM_008012	Aldo-keto reductase family 1, member B8	Akr1b8		0.0086	1.252814
1454630_at	BB282890	Sterile alpha motif domain containing 14	Samd14		0.0097	1.191833
1429474_at	BE283373	Zinc binding alcohol dehydrogenase, domain containing 1	Zadh1		0.0030	1.188432
1424292_at	BC005799	DEP domain containing 1a	Depdc1a		0.0078	1.185658
1442059_at	BB385925	Fragile X mental retardation gene 1, autosomal homolog	Fxr1h		0.0070	1.140154
1452042_a_at	AV306255	Transmembrane protein 144	Tmem144		0.0011	1.125372
1416779_at	BE197945	Serum deprivation response	Sdpr		0.0084	1.115135
1424049_at	BC027203	Leucine rich repeat containing 42	Lrrc42		0.0082	1.100444
1417073_a_at	NM_021881	Quaking	Qk		0.0085	1.078538
1453848_s_at	AK002774	Zinc finger, BED domain containing 3	Zbed3		0.0052	1.075243
1425026_at	BC017549	SFT2 domain containing 2	Sft2d2		0.0099	1.055601
1454794_at	AV298495	Spastin	Spast		0.0053	0.940109
1420112_at	AI503516	Phosphofurin acidic cluster sorting protein 1	Pacs1		0.0015	0.763026

Meta-analysis on the list of genes significantly different in the group Preg TiO2 versus Preg PBS, with subtraction of genes significantly different in Norm TiO2 versus Norm PBS and of genes significantly different in all Preg versus all Norm. See heatmap in the online supplement, *left*.

spring. Offspring of mice exposed to DEP during pregnancy (Figure 1, Protocol 1B) showed increased AHR (Figure 5A) and allergic airway inflammation (AI) (Figures 5B–5D) compared with offspring of vehicle (PBS)-treated mice, indicating increased allergic susceptibility. We also analyzed offspring from pregnant mice treated with "inert" TiO_2 and CB particles. These offspring also showed increased susceptibility to allergy, manifesting as increased AHR and AI (Figure 5).

DISCUSSION

Our findings indicate that in pregnancy both local and systemic inflammatory responses to immunologically "inert" environmental particles are enhanced compared to the normal nonpregnant state. This phenomenon is associated with differential activation of multiple genes involved in immune response and regulation, cell metabolism and proliferation. An important biological effect is increased allergic susceptibility in offspring of mothers exposed during pregnancy.

TiO_2 (and CB) particles are a prototypical "inert" particle in pulmonary toxicology studies because of the minimal inflammatory response usually seen *in vivo* in animal models; they do not have soluble components. However, they are not completely innocuous. For example, specially coated TiO_2 particles were shown to cause pulmonary inflammation (31). Moreover, TiO_2 particles were shown to cause pulmonary inflammation with activation of antigen-presenting cells and production of certain chemokines (32, 33). They were also associated with increased production of IL-13 by mast cells (34) and, potentially germane to our study, were shown to cause increase IL-25 and IL-13 production by lung antigen-presenting cells (35). Similarly, there are a few studies showing that another generally "inert" particle type, CB particles may have also minor immune system effects (36).

Specific information on the subject of exposure to particles during pregnancy remains scarce. However, previous observations include findings that pulmonary immune response to certain environmental factors (e.g., ozone) (37, 38) can be enhanced in the already Th2-deviated milieu of pregnancy (39, 40). We compared the local pulmonary response of pregnant versus normal females to TiO_2 particles. Normal nonpregnant females showed minimal residual inflammation 48 hours after particle treatment, the expected finding with "inert" particles. In contrast, at the 48-hour analysis point, pregnant mice reveal persistence of enhanced inflammation, a finding not seen in nonpregnant females (Figure 2). These data indicate that "inert" particles are no longer innocuous and noninflammatory in the setting of pregnancy. At the same time exposure to nonparticulate inflammatory agent LPS did not cause enhanced responses in pregnancy as compared with nonpregnant mice (Figure 3), indicating that not all inflammatory responses are altered in pregnancy in our model. We are aware of a discordant finding of enhanced inflammation after a higher dose of LPS in pregnant rats (37), therefore this issue requires further study.

We speculate that several factors may be involved in the mechanism, including alteration of innate and adaptive immune responses under the influence of estrogen and progesterone, the essential hormones of pregnancy that are produced in increasing concentrations (41–43). These hormones induce a pro-Th2 skewing of immunity (as reviewed in Ref. 44). More interestingly, it was shown that estrogens and progesterone can alter function of macrophages (45, 46) and regulate macrophage cytokine production (47, 48). Similar data applies to DCs located in the reproductive organs (49, 50), and recent reports suggest that DCs have estrogen receptors and respond to estrogen stimulation (51). Other possible mechanisms include alteration of the placental milieu in an inflamed organism towards production of Th2-skewing products (52).

The postulate that innate immune responses to "inert" particles are altered predicts selective activation or deactivation of gene transcription. Indeed, genomic profiling of total lung RNA from normal and pregnant females exposed to either TiO_2 particles or PBS control identified several clusters of genes that may potentially be involved in the mechanism. We initially used a more stringent SAM analysis across all four groups and identified 130 sequences (mostly involved in inflammatory response and immune regulation, cell proliferation, DNA metabolism, and metabolic processes) to be differentially expressed

TABLE 2. GENES POTENTIALLY INVOLVED IN TiO2 RESPONSE IN NORMAL MICE

Probe Set ID	Representative Public ID	Gene Title	Gene Symbol	Process	Adjusted *P* Value	Fold Ti > PBS
1421394_a_at	BF137345	Baculoviral IAP repeat-containing 4	Birc4	Cell cycle regulation,	0.0048	1.709305
1426720_at	BG067463	Amyloid beta (A4) precursor protein-binding, family B, member 2	Apbb2	cell division and motility	0.0044	1.418741
1417086_at	BE688382	Platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	Pafah1b1		0.0029	1.26618
1450784_at	NM_016678	Reversion-inducing-cysteine-rich protein with kazal motifs	Reck		0.0099	1.178259
1434775_at	AW543460	par-3 (partitioning defective 3) homolog (C. elegans)	Pard3		0.0005	1.168492
1423663_at	BC025820	Folliculin	Flcn		0.0015	1.154846
1449491_at	NM_130859	Caspase recruitment domain family, member 10	Card10		0.0058	1.120665
1434000_at	BQ176608	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Kras		0.0082	1.118953
1423136_at	AI649186	Fibroblast growth factor 1	Fgf1		0.0012	1.113597
1426110_a_at	U48235	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Edg2	Immune response and regulaiton,	0.0043	1.51332
1419204_at	NM_007865	Delta-like 1 (Drosophila)	Dll1	cell adhesion, proteolysis	0.0041	1.491792
1421319_at	NM_011197	Prostaglandin F2 receptor negative regulator	Ptgfrn	and protein biosynthesis	0.0083	1.352902
1418634_at	NM_008714	Notch gene homolog 1 (Drosophila)	Notch1		0.0022	1.251013
1450817_at	NM_015830	Small optic lobes homolog (Drosophila)	Solh		0.0069	1.234057
1455137_at	AW536472	Rap guanine nucleotide exchange factor (GEF) 5	Rapgef5		0.0043	1.213749
1455251_at	AV071536	Integrin alpha 1	Itga1		0.0094	1.212642
1418901_at	NM_009883	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb		0.0090	1.178609
1423902_s_at	AF467766	Rho guanine nucleotide exchange factor (GEF) 12	Arhgef12		0.0048	1.15206
1437277_x_at	BB550124	Transglutaminase 2, C polypeptide	Tgm2		0.0026	1.115566
1418674_at	AB015978	Oncostatin M receptor	Osmr		0.0070	1.115232
1448590_at	NM_009933	Procollagen, type VI, alpha 1	Col6a1		0.0011	1.108705
1428622_at	AK014624	DEP domain containing 6	Depdc6		0.0043	1.091745
1452143_at	BQ174069	Spectrin beta 2	Spnb2		0.0093	1.060853
1453728_a_at	AK003008	Mitochondrial ribosomal protein S17	Mrps17		0.0085	0.909486
1423254_x_at	BB836796	Ribosomal protein S27-like	Rps27l		0.0069	0.884305
1434159_at	BG069810	Serine/threonine kinase 4	Stk4		0.0042	0.843541
1450925_a_at	BB836796	Ribosomal protein S27-like /// similar to 40S ribosomal protein S27-like protein	Rps27l /// LOC667571		0.0064	0.83938
1433825_at	BM245880	Neurotrophic tyrosine kinase, receptor, type 3	Ntrk3		0.0061	0.83153
1426898_at	AK009321	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	Map3k7ip1		0.0099	0.830648
1418642_at	BC006948	Lymphocyte cytosolic protein 2	Lcp2		0.0063	0.809947
1425294_at	BC024587	SLAM family member 8	Slamf8		0.0004	0.782785
1420657_at	AF053352	Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3		0.0061	0.766881
1420659_at	NM_030710	SLAM family member 6	Slamf6		0.0026	0.745503
1422707_at	BB205102	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	Pik3cg		0.0077	0.684983
1425871_a_at	AB007986	Similar to immunoglobulin light chain variable region	LOC384413		0.0094	0.634865
1424931_s_at	M94350	Immunoglobulin lambda chain, variable 1	Igl-V1		0.0074	0.574379
1452292_at	AV271093	Adaptor-related protein complex 2, beta 1 subunit	Ap2b1	Inracellular transport and cell metabolism	0.0028	1.487326
1450283_at	NM_007511	ATPase, Cu++ transporting, beta polypeptide	Atp7b		0.0064	1.471984
1434140_at	AV293368	mcf.2 transforming sequence-like	Mcf2l		0.0067	1.450361
1455285_at	BB771765	Solute carrier family 31, member 1	Slc31a1		0.0080	1.393235
1420959_at	NM_023066	Aspartate-beta-hydroxylase	Asph		0.0099	1.393208
1449943_at	NM_008494	Lunatic fringe gene homolog (Drosophila)	Lfng		0.0082	1.381953
1451424_at	BC027245	Gamma-aminobutyric acid (GABA-A) receptor, pi	Gabrp		0.0026	1.375588
1426343_at	AK018758	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	Stt3b		0.0075	1.347831
1434939_at	BB437522	Forkhead box F1a	Foxf1a		0.0056	1.3441
1444279_at	BB531571	HECT, UBA and WWE domain containing 1	Huwe1		0.0051	1.33872
1423368_at	BI695636	Lysosomal-associated protein transmembrane 4A	Laptm4a		0.0099	1.253873
1435432_at	BE688580	Centaurin, gamma 2	Centg2		0.0094	1.238072
1429325_at	BB667633	WD repeat domain 51B	Wdr51b		0.0093	1.230761
1423981_x_at	BC006711	Solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter), member 29	Slc25a29		0.0007	1.220603
1455711_at	AW122183	Deltex 4 homolog (Drosophila)	Dtx4		0.0088	1.204474
1427035_at	BB399837	Solute carrier family 39 (zinc transporter), member 14	Slc39a14		0.0018	1.184887
1435553_at	AV376136	PDZ domain containing 2	Pdzd2		0.0094	1.172223
1450395_at	NM_011396	Solute carrier family 22 (organic cation transporter), member 5	Slc22a5		0.0058	1.165831

(*Continued*)

TABLE 2. (CONTINUED)

Probe Set ID	Representative Public ID	Gene Title	Gene Symbol	Process	Adjusted *P* Value	Fold Ti > PBS
1436103_at	AV235634	RAB3A interacting protein	Rab3ip		0.0049	1.162922
1443830_x_at	AV337847	Ring finger protein 103	Rnf103		0.0052	1.156454
1443332_at	BB157520	Solute carrier family 12, member 2	Slc12a2		0.0040	0.877155
1439367_x_at	AV148210	ADP-ribosylation factor 4	Arf4		0.0049	0.819864
1448687_at	NM_026125	C1q domain containing 2	C1qdc2		0.0095	0.813187
1456071_a_at	AV155488	Cytochrome c, somatic /// similar to Cytochrome c, somatic /// similar to Cytochrome c, somatic	Cycs /// LOC670717 ///		0.0098	0.777892
1431705_a_at	AK014467	Mucolipin 2	Mcoln2		0.0055	0.749568
1424967_x_at	L47552	Troponin T2, cardiac	Tnnt2		0.0053	0.720553
1434342_at	BB316114	S100 protein, beta polypeptide, neural	S100b		0.0060	0.702169
1419063_at	NM_011674	UDP galactosyltransferase 8A	Ugt8a		0.0038	0.696748
1426225_at	U63146	Retinol binding protein 4, plasma	Rbp4		0.0084	0.648824
1451054_at	BE628912	Orosomucoid 1	Orm1		0.0098	0.25717
1426342_at	AK018758	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	Stt3b	Metabolism	0.0065	1.155948
1449417_at	NM_009664	Ameloblastin	Ambn		0.0069	0.864413
1430889_a_at	AK002335	Thiopurine methyltransferase	Tpmt		0.0054	0.848695
1422033_a_at	NM_053007	Ciliary neurotrophic factor /// zinc finger protein 91	Cntf /// Zfp91		0.0034	0.800306
1417741_at	NM_133198	Liver glycogen phosphorylase	Pygl		0.0075	0.78752
1460316_at	BI413218	Acyl-CoA synthetase long-chain family member 1	Acsl1		0.0048	0.767281
1430584_s_at	BB213876	Carbonic anhydrase 3	Car3		0.0089	0.662411
1415964_at	NM_009127	Stearoyl-Coenzyme A desaturase 1	Scd1		0.0049	0.595407
1460256_at	NM_007606	Carbonic anhydrase 3	Car3		0.0048	0.580866
1416487_a_at	NM_009534	Yes-associated protein 1	Yap1	Transcription,	0.0067	1.379506
1421604_a_at	NM_008453	Kruppel-like factor 3 (basic)	Klf3	DNA replication,	0.0070	1.376898
1418366_at	BC010564	Histone 2, H3c1, H2aa2, etc	Hist2h3c1 ///2	metabolism and repair	0.0024	1.337869
1428354_at	BM206907	Forkhead box K2	Foxk2		0.0068	1.328746
1450333_a_at	NM_008090	GATA binding protein 2	Gata2		0.0008	1.316352
1425988_a_at	AF071071	Homeodomain interacting protein kinase 1 /// similar to homeodomain-interacting protein kinase 1	Hipk1 /// LOC634033		0.0069	1.27636
1426358_at	BB272466	TAO kinase 1	Taok1		0.0054	1.266734
1415834_at	NM_026268	Dual specificity phosphatase 6	Dusp6		0.0031	1.224709
1454785_at	BE951717	Dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	Dusp11		0.0075	1.183246
1420628_at	NM_008989	Purine rich element binding protein A	Pura		0.0092	1.117373
1420811_a_at	NM_007614	Catenin (cadherin associated protein), beta 1	Ctnnb1		0.0005	1.114722
1435251_at	AV377013	Sorting nexin 13	Snx13		0.0080	1.114005
1452460_at	BF134412	Ankyrin repeat domain 26 /// similar to ankyrin repeat domain 26	Ankrd26 /// LOC669838		0.0002	0.918573
1450576_a_at	NM_013651	Splicing factor 3a, subunit 2	Sf3a2		0.0086	0.842407
1448986_x_at	NM_010062	Deoxyribonuclease II alpha	Dnase2a		0.0029	0.789776
1432646_a_at	BE859789	Hypothetical LOC640370 ///	LOC640370 ///	Other	0.0007	1.324163
1458358_at	BB402666	Pantothenate kinase 2 (Hallervorden-Spatz syndrome)	Pank2		0.0099	1.30126
1422609_at	BE648432	cAMP-regulated phosphoprotein 19	Arpp19		0.0060	1.267996
1437856_at	BM225636	Inositol polyphosphate multikinase	Ipmk		0.0078	1.224059
1451584_at	AF450241	Hepatitis A virus cellular receptor 2	Havcr2		0.0075	1.220239
1460580_at	BB772192	Pecanex homolog (Drosophila)	Pcnx		0.0079	1.219831
1429044_at	AK005444	Calmodulin regulated spectrin-associated protein 1-like 1	Camsap1l1		0.0019	1.194988
1424280_at	BC018329	Motile sperm domain containing 1	Mospd1		0.0050	1.189681
1447624_s_at	BB174262	Storkhead box 2	Stox2		0.0059	1.170559
1417965_at	NM_133942	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	Plekha1		0.0085	1.155582
1435096_at	BB667093	Resistance to inhibitors of cholinesterase 8 homolog B (C. elegans)	Ric8b		0.0027	1.155356
1436215_at	BB081797	Inositol polyphosphate multikinase	Ipmk		0.0091	1.133207
1428197_at	AK020159	Tetraspanin 9	Tspan9		0.0084	1.107396
1427773_a_at	L40934	Rab acceptor 1 (prenylated)	Rabac1		0.0035	1.079644
1458308_at	BF148215	Strawberry notch homolog (Drosophila)	Stno		0.0076	0.882994
1442786_s_at	BB461022	RUN and FYVE domain containing 3	Rufy3		0.0021	0.850871
1455927_x_at	AV216677	Similar to non-SMC element 1 homolog /// similar to non-SMC element 1 homolog	LOC623809 /// LOC677159		0.0047	0.81128
1417668_at	NM_130892	Reticulon 4 interacting protein 1	Rtn4ip1		0.0045	0.803591

Genes significantly different in the group Norm TiO2 versus Norm PBS with exclusion of those genes significantly different in Preg TiO2 versus Preg PBS as well as of genes significantly changed by pregnancy. See heatmap in the online supplement, *right*.

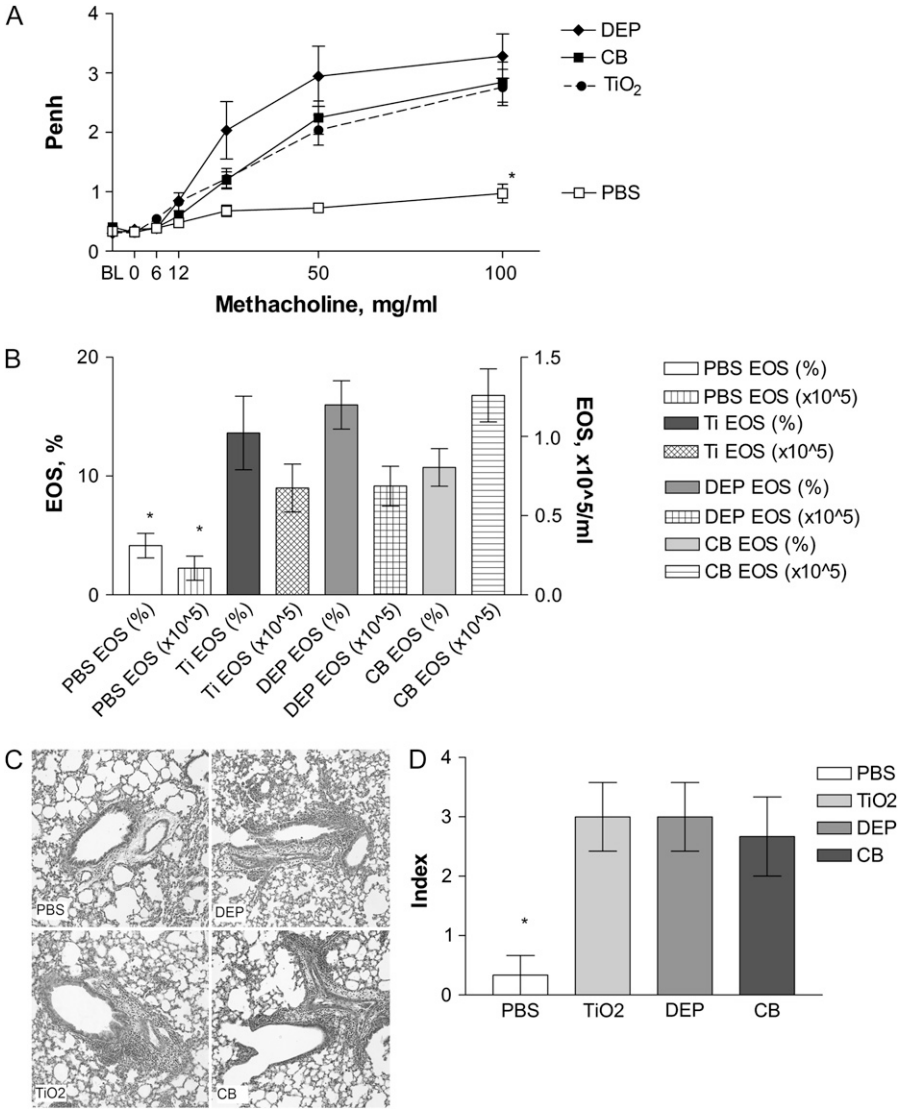

Figure 5. Neonatal susceptibility in OVA protocol. Mother mice were exposed during pregnancy to 50 μg/mouse of either DEP or TiO_2, or 250 μg/mouse of CB particle suspension or PBS (Protocol 1B, Figure 1). Newborns were injected once with 0.1 ml of 50 μg/ml OVA plus alum and challenged three times with 3% OVA aerosol. Offspring of mice exposed during pregnancy to DEP showed increased airway hyperresponsiveness (AHR) seen as response to methacholine via whole-body plethysmography (Penh at 100 mg/ml Mch of 3.3±0.4) (*A*) and increased eosinophilic AI in BAL (*B*) as well as increased pulmonary infiltration (*C*, *D*), indicating that allergic susceptibility was induced. Surprisingly, neonates from mice exposed to "inert" TiO_2 and CB also showed similarly increased AHR (Penh at 100 mg/ml Mch of 2.8 ± 0.3 and 2.8 ± 0.3, respectively, versus 1.0 ± 0.2 in PBS controls, $P < 0.05$) (*A*). BAL eosinophilia was also increased (TiO_2 13.6 ± 3.1% and CB 10.7 ± 1.2% versus 4.1 ± 1.0% in PBS controls) (*B*), as well as pulmonary inflammation (*C*, *D*). Mean ± SEM (n = 17–21 each group). *$P < 0.05$.

(*see* online supplement). We then applied a more selective approach using less stringent ANOVA-based analysis to identify genes that are only changed in pregnant mice upon exposure to TiO_2, as well as those that are only changed in normal mice upon TiO_2 exposure. While these gene lists somewhat overlapped, after mutual subtraction we identified two separate gene sets (*see* online supplement), which indicates that possibly different genes are responsible for lung TiO_2 response in pregnant and in normal mice. Further investigation including PCR validation and mechanistic studies is underway.

We found that newborns from DEP-exposed mothers had significantly higher AHR and AI (Figure 2) than PBS controls. Moreover, the offspring of TiO_2- and CB particle–exposed mothers (Figure 2) also showed increased susceptibility, an unexpected finding that was replicated in four separate experiments. It has been concurrently shown using the same model that maternal exposure to residual oil fly ash (ROFA) increases offspring susceptibility (22). Here, we demonstrate that maternal exposure to particles considered immunologically innocuous, TiO_2 and CB, can also cause increased allergic susceptibility in offspring. This finding identifies a functionally important consequence of the differential response to particles in pregnancy, and this may ultimately help identify mechanisms of the phenomenon. The data suggest that exposures of nonallergic pregnant females to environmental air particles under some conditions may cause increased allergic susceptibility in offspring.

The mechanisms by which pregnancy exposure caused increased susceptibility to allergy in offspring remain unknown. One possibility is suggested by previous findings that components of DEP can mediate pro-allergic effects. The organic components, especially polycyclic aromatic hydrocarbons (PAH), cause increased production of Th2 cytokines (e.g., IL-4), known to be important mediators of allergy and asthma (14–16). Studies found that pyrene, an abundant component in DEP, has caused specific and robust induction of IL-4 gene expression by T cells, but only as a co-factor in the presence of antigen (30). However, we sought but did not find evidence of Th2 cytokines in the lavage fluids and serum samples from DEP-treated pregnant mice (no detectable IL-4, -5, or -13; data not shown). Rather, multiplexed cytokine analysis of serum show that pregnant mice exposed to either DEP or TiO_2 had elevated levels of IL-1β, TNF-α, IL-6, and KC levels 48 hours after exposure, as opposed to nonpregnant controls (Figure 4). These findings are consistent with the greater acute cellular inflammation observed in BAL samples, including after treatment with the "inert" particle TiO_2. The discordance between similar levels of PMNs in the normal DEP versus pregnant DEP control groups and different levels of cytokines in these groups

may be caused by "saturation" of bronchoalveolar inflammation locally but not systemically. Further studies are necessary to address this issue in more detail.

Some limitations of our study merit discussion. First, we used a single bolus dose of particles via intranasal insufflation of pregnant mice. While this strategy provides proof-of-principle, additional studies using aerosol exposures and dose–response analysis would allow more realistic comparison to actual human exposures. Second, the study uses one strain of mice (BALB/c). Additional studies are needed to determine if similar findings occur in other mouse strains. Finally, in our mouse model, we use noninvasive plethysmography to evaluate pulmonary function in very young mice (15 days old). We are aware of the ongoing discussion in the literature about whether Penh measurement via whole-body plethysmography truly represents AHR and whether it is a valid technique for different strains of laboratory animals (23). However, it is worth noting that analysis of responses to aerosolized OVA in sensitized, BALB/c strain mice (i.e., as in our model) is the experimental setting in which Penh values correlate best and to an (arguably) acceptable degree with more invasive measures (24–29). We also point out that the more invasive testing is technically impractical, given the small size of young mice in our model. Finally, in an earlier study we were able to find similar trends in Penh and basal pulmonary function tests using the invasive Flexivent approach in older, larger mice studied in a similar protocol (8).

In conclusion, we have developed a mouse model for analysis of environmental exposures during pregnancy and their effect on susceptibility of offspring to allergy. We showed using this model that maternal exposure to TiO_2 and CB particles, previously considered immunologically "inert," causes enhanced immune response in pregnancy and, similarly to DEP exposure results in increased allergic susceptibility in offspring. This model may be useful for toxicology screening and for further mechanistic analysis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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