Supplemental Materials

Asthma Susceptibility through Prenatal Exposure to Diesel Exhaust

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Supplemental Methods

NK cell depletion in vivo

The experiment involved OVA-immunized offspring of three DEP-exposed mice. Each litter was divided into two groups. Pups from the first group were intraperitoneally injected with 200 μg of the anti-NK1.1 antibody (clone PK136; BioLegend, San Diego, CA)[E1-E3] . Pups from the second group received 200 μg of the mouse IgG2a isotype control antibody (BioLegend). The injections were performed on PND 20, 21 and 22. All pups were intranasally challenged with OVA on PND 23, 24 and 25 as described in the parent protocol.

Measurement of airway hyperresponsiveness

Lung resistance was measured using the FlexiVent apparatus (Scireq, Montreal, CA) as described[E4-E7]. Resistance values for each methacholine dose were expressed as a percent of baseline (saline inhalation) resistance.

Histology and morphometric analysis

Paraffin-embedded lungs were sectioned and stained with hematoxylin and eosin[E4-E7]. The MetaMorph software was used to measure areas of the peribronchial cellular infiltrate and a perimeter of the bronchial epithelial basement membrane (proxy for airway perimeter)[E4-E8]. Results are expressed as an inflammation area per 1 μm of airway perimeter.

OVA-specific IgE ELISA

OVA-specific IgE was measured in serum as described[E4, E9]. Purified OVA-specific mouse IgE (AbD Serotec, Raleigh, NC) was used for standard curve.

Cytokine ELISA

Cytokine concentration was measured in cell-free supernatants of BALF[E5]. For IFNγ, IL-4, IL-5, IL-6 and TNFα multiplex ELISA was used (Quansys Biosciences, Logan, UT). Concentrations

of IL-13 and IL-17 were determined using traditional ELISA (eBioscience, San Diego, CA and R&D Systems, Minneapolis, MN, respectively).

Real time PCR

RNA was extracted from Trizol lysates using PureLink RNA Mini Kit (Life Technologies, Grand Island, NY). cDNA was synthesized using ImProm-II reverse transcriptase and random primers (all from Promega, Madison, WI). cDNA was amplified and quantified using transcript-specific primers (Table EI in the Online Repository), the Absolute qPCR SYBR Green mix (Thermo Scientific) and the ABI Prism 7000 Sequence Detection System (Life Technologies)[E4].

Flow cytometry

Splenocytes were treated with OVA at 100 μg/ml for 30h (and monensin at 2 μM for final 6h), stained with fluorescently-labeled antibodies against CD3, CD4, NK1.1, NKp46 and a cytokine (IL-5, IL-13 or IL-17). Separate splenocyte samples were treated with OVA without monensin and immunostained for CD3, NK1.1, NKp46 and CD69. Cells were analyzed using CyAn ADP cytometer (Beckman-Coulter, Fullerton, CA).

Statistical analyses

Statistical analyses were done using the GraphPad Prism 6 software. Following tests were performed: one-way ANOVA, two-way repeated measures ANOVA and the Holm-Sidak's multiple comparison test. Results are shown as mean ± standard error of the mean. $P<0.05$ was considered statistically significant.

Supplemental References

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Table EI: Sequences of transcript-specific primers

Transcript Name	Forward Primer	Reverse Primer
AhRR	GTGTACATACGCCGGTAGGA	GGTGCCGTTTGGAAGGATTT
Cyp1a1	GACCTTCCGGCATTCATCCT	GCCATTCAGACTTGTATCTCTTGTG
Cyp1b1	AGGATGTGCCTGCCACTATT	CACAACCTGGTCCAACTCAG
Hmox1	GAGCAGAACCAGCCTGAACT	TCAAGGCCTCAGACAAATCCT
IFNγ	GCGTCATTGAATCACACCTGA	CTGGACCTGTGGGTTGTTGA
IL-4	CCATATCCACGGATGCGACA	GACGAGCTCACTCTCTGTGG
IL-5	GAGGCTTCCTGTCCCTACTC	CCCACGGACAGTTTGATTCTT
IL-6	TGTTCTCTGGGAAATCGTGGA	TTCTGCAAGTGCATCATCGT
IL-13	GAGCAACATCACACAAGACCA	GATGTTGGTCAGGGAATCCAG
IL-17	AGACTACCTCAACCGTTCCAC	CACTGAGCTTCCCAGATCACA
Nrf2	CTCAGCATGATGGACTTGGAG	GCCTCCAAAGGATGTCAATCAA
TNFα	CTCTTCTCATTCCTGCTTGTGG	GATGATCTGAGTGTGAGGGTCT
18S rRNA	CGGCGACGACCCATTCGAAC	GAATCGAACCCTGATTCCCCGT

Table EII: Previous prenatal DEP/DE exposure models

		Model by Fedulov et al.[E10]	Model by Auten at al.[E11]	Model by Reiprich et al.[E12]	Model by Sharkhuu at al.[E13]	Model by Corson et al.[E14]
Mouse strain		BALB/c	C57BL/6	BALB/c	BALB/c	BALB/c
Maternal exposure	**DEP/DE**	*Agent:* DEP *Dose:* 50 µg *Route:* i.n. *Frequency:* once (GD 14)	1) *Agent:* DEP *Dose:* 50 µg *Route:* o.p. *Frequency:* twice weekly for 3 weeks of pregnancy 2) *Agent:* DE *Dose:* 0.5-2 mg/m^3 4 hours/day *Route:* inhalation *Frequency:* daily (from GD 9 to GD 17)	*Agent:* DEP *Dose:* 20 µg (30 min before LPS exposure) *Route:* i.n. *Frequency:* 3 times per week from GD 7 until delivery	*Agent:* DE *Dose:* 0.8 or 3.1 mg/m^3, 4 hors/day *Route:* inhalation *Frequency:* daily (from GD 9 to GD 18)	*Agent:* DE *Dose:* 1.09 mg/m^3, 5 hours/day *Route:* inhalation *Frequency:* daily, Monday to Friday during second and third week of pregnancy
	Other treatments			*Agent:* LPS *Dose:* aerosolized solution at 1 µg/10 ml, 10 min/day (30 min after DEP exposure) *Route:* inhalation *Frequency:* 3 times per week from GD 7 until delivery		*Agent:* A.fumigatus extract *Dose:* 62.5 µg *Route:* i.n. *Frequency:* seven times (days -20, -16, -12, -8 and -4 before mating, GD 7, GD 14)
Offspring exposure	**Immunization with allergen**	*Agent:* OVA and alum *Dose:* OVA: 5 µg; alum: 1 mg *Route:* i.p. *Frequency:* once (PND 4)		*Agent:* OVA and alum *Dose:* OVA: 20 µg; alum: 2 mg *Route:* i.p. *Frequency:* twice (postnatal week 6 and 8)	*Agent:* OVA *Dose:* 50 µg *Route:* i.n. *Frequency:* twice (PND 42 and 43)	*Agent:* A. fumigatus extract *Dose:* 62.5 µg *Route:* i.n. *Frequency:* 5-6 times (4 days apart, beginning at week 9-10 of age)
	Airway challenge with allergen	*Agent:* OVA *Dose:* aerosolized 3% solution *Route:* inhalation *Frequency:* Daily for 3 days (PND 13, 14, 15)		*Agent:* OVA *Dose:* 20 µg *Route:* i.n. *Frequency:* 6 times (days 14-16 and 21-23 after initial immunization)	*Agent:* OVA *Dose:* 5 µg *Route:* i.n. *Frequency:* three times (PND 54, 55, 56)	
	Other treatments		*Agent:* ozone *Dose:* 1 ppm, 3 hours/day *Route:* inhalation *Frequency:* 3 days per week for 4 weeks	*Agent:* LPS *Dose:* aerosolized solution at 1 µg/10 ml, 10 min/day *Route:* inhalation *Frequency:* 3 times per week for first 4 weeks of life		
Effect of maternal DEP/DE exposures		Eosinophilic inflammation of airways, airway hyperreactivity	Augmentation of ozone-induced airway hyperreactivity	Inhibition of LPS-mediated protection against asthma	No effect	Protection from airway eosinophilia

Abbreviations used in the Table EII:
DE, diesel exhaust
DEP, diesel exhaust particles
GD, gestational day
i.n., intranasal
i.p., intraperitoneal
LPS, lipopolysaccharide
o.p., oropharyngeal
OVA, ovalbumin
PND, postnatal day

Supplemental Figure Legends

Figure E1: Expression of cytokine mRNA in the lung. Three offspring groups (PBS-PBS, PBS-OVA and DEP-OVA) were studied. Values for each cytokine transcript were normalized to housekeeping 18S rRNA and expressed as a fraction of this rRNA. Mean values (± SEM) shown from 6-13 mice/group; *, P<0.05; **, P<0.01; ***, P<0.001

Figure E2: CD69 expression on NK cells. OVA-stimulated and immunostained (CD3, NK1.1, NKp46 and CD69) splenocytes were analyzed by flow cytometry. CD69+ NK cells (CD3-NK1.1+NKp46+) are expressed as a percentage of all NK cells. Mean values (± SEM) shown from 6 mice/group; *, P<0.05

Figure E3: Expression of AhR-regulated transcripts. Lungs (A-C) and livers (D-F) from four offspring groups (as in Fig 2) were analyzed for relative expression of AhRR, Cyp1a1 and Cyp1b1 mRNA. Values for each AhR-regulated transcript were normalized and expressed as in Fig E1. Mean values (± SEM) shown from 6-13 mice/group; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001

Figure E4: Expression of oxidative stress-regulated transcripts. Lungs (A-B) and livers (C-D) from three offspring groups (as in Fig 3 and E1) were analyzed for relative expression of Hmox1 and Nrf2 mRNA. Values for each oxidative stress-regulated transcript were normalized and expressed as in Fig E1. Mean values (± SEM) shown from 6-13 mice/group; *, P<0.05; **, P<0.01