

BASIC SCIENCE: OBSTETRICS

Smoking during pregnancy influences the maternal immune response in mice and humans

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OBJECTIVE: During pregnancy the maternal immune system has to adapt its response to accommodate the fetus. The objective of this study was to analyze the effects of smoking on the maternal immune system.

STUDY DESIGN: First-trimester decidua and peripheral blood of smoking and nonsmoking women were analyzed by real time reverse transcription–polymerase chain reaction (RT-PCR) and flow cytometry. A mouse model was used to further analyze the effects of smoking. Murine tissue was analyzed by flow cytometry, real-time RT-PCR, and immunohistochemistry.

RESULTS: Smoking caused lower percentages of viable pups in mice and lower birthweights in humans. Smoking mothers, both mice and

human, had more natural killer cells and inflammatory macrophages locally, whereas systemically they had lower percentages of regulatory T cells than nonsmoking controls.

CONCLUSION: Maternal smoke exposure during pregnancy influences local and systemic immune responses in both women and mice. Such changes may be involved in adverse pregnancy outcomes in smoking individuals.

Key words: macrophages, maternal immune system, natural killer cells, smoking

Cite this article as: Prins JR, Hylkema MN, Erwich JJHM, et al. Smoking during pregnancy influences the maternal immune response in mice and humans. *Am J Obstet Gynecol* 2012;207:76.e1-14.

Despite the well-known fact that smoking adversely affects pregnancy outcome,¹ about 30% of pregnant women still smoke in The Netherlands.^{2,3} Fetal reactions to cigarette smoke were already described in 1935.⁴ After a mother started smoking a cigarette, an increase in fetal heart rate was found, and it was concluded that a toxic agent, probably nicotine, crossed the placenta to the fetus.⁴ Currently, it is clear that smoking during pregnancy causes lower birth weights, an increased incidence of spontaneous abortions and preterm birth, increased

placental pathology, and increased stillbirth rates.⁵⁻⁹ Interestingly, it has also been shown that smoking women have a lower incidence of pregnancies complicated by preeclampsia.¹⁰ The mechanisms responsible for the toxic and protective effects of smoking on pregnancy and preeclampsia are not yet understood.

Tobacco smoke contains many substances and may affect the fetus directly, but it may also affect placental development and decidualization.^{1,11,12} Alternatively, smoking may achieve its effects by modulation of maternal immunological and endocrine param-

eters. During pregnancy the maternal immune system plays a key role in the success of pregnancy by adapting to accommodate the semiallogeneic fetus.¹³ Adaptations of the maternal immune system take place locally at the implantation site as well as in the peripheral circulation. Disturbances in this adaptation toward maternal tolerance have been associated with adverse pregnancy outcomes and other reproductive pathologies.^{13,14} Indeed, in nonpregnant women and animals, smoking alters immune responses.¹⁵⁻¹⁹ Therefore, adverse pregnancy outcomes in smoking women could be the result of immunological changes caused by smoking.

The exact influence of smoking on the maternal immune system during pregnancy is not known. Only 1 study investigated the effects of smoking on circulating maternal leukocytes during pregnancy, showing that smoking pregnant women had higher percentages of CD3+ cells, lower percentages of CD56+ cells, and a lower expression of CD54 (activation marker) on monocytes in peripheral blood in early pregnancy compared with nonsmoking pregnant women.²⁰ It was concluded that smoking acts both as an inflammatory and antiinflammatory stimulus on the maternal immune system during pregnancy.²⁰

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Received Feb. 2, 2012; revised April 3, 2012; accepted April 9, 2012.

This study was supported by the Dutch Kidney Foundation (grant 2266, to B.N.M.), Jan Kornelis de Cock Foundation, and Guide Graduate School.

The authors report no conflict of interest.

Presented, in part, at the 57th Annual Scientific Meeting of the Society for Gynecologic Investigation, March 24-27, 2010, Orlando, FL, and at the 11th International Congress of Reproductive Immunology, August 15-19, 2010, Cairns, Queensland, Australia.

Reprints not available from the authors.

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Important players in the adaptation of the maternal immune response are T cells (especially regulatory T cells), natural killer (NK) cells, and monocytes and macrophages.^{13,21-24} Regulatory T (Treg) cells are a subpopulation of T cells that regulate immune responses. They are important in the process of tolerance to self and foreign antigens, and they therefore play a role in autoimmune diseases, inflammatory diseases, transplantation, and pregnancy.^{13,25,26} NK cells are key players in the regulation of remodeling of spiral arteries and also have a role in regulation of invasion of trophoblasts.²⁷ Functional and numerical changes of NK and Treg cells are both associated with adverse pregnancy outcomes.^{13,23,28}

Monocytes and macrophages are also involved in remodeling of spiral arteries and placental development.^{29,30} It has become clear that there are specific subsets of monocytes and macrophages, which are thought to have specific roles in inflammation, tissue remodeling, and vascularization, respectively, M1 (inflammatory) and M2 (remodeling/regulatory) macrophages.^{24,31,32} Macrophages in the placenta during healthy pregnancy appear to have a remodeling/regulatory role and are thus mainly of the M2 subtype.²⁴

The aim of this study was to analyze the effects of smoking on the maternal immune system during pregnancy locally in the uterus and systemically. In humans, smoking effects on the local and systemic maternal immune system were investigated in first-trimester decidual tissue and peripheral blood of smoking women. In this study, we used unique decidual material, collected during routine chorionic villous sampling between 10 and 12 weeks of pregnancy, which allowed us to study smoking effects in deciduas of ongoing pregnancies with known pregnancy outcomes. We furthermore used a mouse model to compare the results with human pregnancy and more specifically study changes in peripheral and local immune responses.

MATERIALS AND METHODS

Study design

First-trimester decidual tissue with a known pregnancy outcome of 42 women, 21 smoking women, and 21 control women

was selected from a tissue collection and analyzed by real-time reverse transcription–polymerase chain reaction (RT-PCR). In addition, data on peripheral blood T cell subsets of 6 smoking and 12 nonsmoking pregnant women were selected from a control cohort of a previously published study.³³ To more specifically study changes in peripheral blood and local immune responses, we used a mouse model. Therefore, 6 pregnant mice were exposed to smoke and 6 control pregnant mice were exposed to fresh air.

To analyze the effects of smoking on the local maternal immune system, decidual tissue was analyzed by immunohistochemistry and real-time RT-PCR, and uterus-draining lymph nodes were analyzed by flow cytometry. For the analysis of the systemic immune response, systemic lymph nodes and spleens were analyzed by flow cytometry.

Human study

Collection of human decidual tissue. During routine chorionic villus sampling, performed vaginally between 10 and 12 weeks of gestation for maternal age, a previously chromosomal abnormal child, or serum screening-related risk for Down syndrome, surplus material, which was not needed for karyotyping, was obtained. Immediately after sampling, decidual tissue was mechanically separated from villi under a microscope by a qualified, experienced laboratory technician to minimize trophoblast contamination. The chorionic villi appeared as free-floating white structures with fluffy, filiform branches, whereas decidual tissue had a more amorphous appearance and lacked distinct branches. Earlier studies showed that trophoblast cells were seldom found in decidual samples.^{34,35} Tissue was stored immediately at -20°C until further processing as described before.³⁴

Patients were informed that surplus material could be used for research. Follow-up of these pregnancies was available by a questionnaire returned by the patient postpartum. From this material, we selected decidual tissue from smoking women, and for each case we used a nonsmoking control woman matched for maternal age, parity, and gestational age at time of sampling. Information about smoking was obtained by medical history

taking. For cases and controls, only pregnancies with a known pregnancy outcome longer than 30 weeks' gestation were selected. In total, decidual tissue of 21 smoking and 21 nonsmoking women was selected for analysis (Table 1).

To determine whether smoking had a dose-dependent effect, we divided the total smoking group into a heavy (≥ 10 cigarettes per day) and moderate smoking group (< 10 cigarettes per day). The low percentage of male offspring is within normal variation and may have been influenced by the relatively high maternal age because there is evidence that a higher maternal age is associated with a shift from a male to a female majority in newborns.^{36,37}

PCR on human decidual tissue. Total ribonucleic acid (RNA) was isolated from decidual tissue using a RNA isolation Trizol kit (Invitrogen, Carlsbad, CA). To further purify RNA, a Turbo DNA free kit and column chromatography were used according to the manufacturer's instructions (respectively, Applied Biosystems, Foster City, CA, and Bio-Rad Laboratories, Hercules, CA). Complementary deoxyribonucleic acid (cDNA) was reverse transcribed using a Superscript-II reverse transcriptase kit (Invitrogen).

As a housekeeping gene (HPRT), we used the forward primer 5'-GGCAGTA-TAATCCAAAGATGGTCAA-3'-GTCTGGCTTATATCCAACACTTCGT-3' (Invitrogen), and probe: 6-FAM 5'-CAAGCTTGCTGGTGAAAAGGACCCC-3' TAMRA (Eurogentec, Herstal, Belgium). For the other genes, interleukin (IL) 6, IL10, Tbet21 (Th1 response), Gata 3 (Th2 response), Ror γ t (Th17 response), Foxp3 (Treg cell), CD56 (NK cell), CD68 (pan macrophage marker), NOS2 (iNOS, M1 macrophage), and CD206 (MRC-1, M2 macrophage), On-Demand gene expression assays were used (Applied Biosystems). PCR reactions were performed in triplicate in a volume of 10 μL consisting of 0.1 μL of MilliQ water, 5 μL PCR mix (Eurogentec), 1 μL of reverse and forward primer each, 0.4 μL probe, and 2.5 μL cDNA for the housekeeping gene.

For the other genes, the total volume of 10 μL contained 2 μL of MilliQ water, 5 μL PCR mix (Eurogentec), 0.5 μL assay

TABLE 1
Characteristics of pregnant women for the decidual mRNA expression data

Characteristic	Nonsmoking (n = 18)	Smoking (n = 20)	<10 cigarettes per day (n = 11)	≥10 cigarettes per day (n = 9)
Age, y	37.6 ± 2.1	36.8 ± 4.3	37.5 ± 2.8	35.8 ± 5.7
Primiparity, %	20.0	25.0	27.3	22.2
Birthweight, g	3388.6 ± 591.6	3339.4 ± 600.2	3612.0 ± 574.8	3165.0 ± 548.5 ^a
Sex of child (% male)	28.6	35.0	36.4	33.3
Duration of pregnancy, d	273.1 ± 18.1	270.9 ± 31.1	268.6 ± 41.8	273.6 ± 14.3
Smoking average, (cigarettes per day)	0	9.2 ± 6.3 ^b	4.8 ± 2.6 ^b	15.0 ± 4.3 ^b

Data are mean ± SD.

^a $P < .001$ compared with controls when analyzing birthweight percentiles for gestational age; ^b $P < .001$ compared with controls.

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mix, and 2.5 μ L cDNA. Runs were performed by a 7900HT Fast real-time PCR system (Applied Biosystems), and RNA data were normalized to HPRT messenger RNA (mRNA) expression using $2^{-\Delta Ct}$. After the run, it appeared that HPRT mRNA expression of 4 samples (3 controls, 1 case) was undetectable; these cases were excluded from this study. Undetectable cycle threshold (Ct) values of the gene of interest (more than 40) were interpreted as the maximum Ct value (40).

Human flow cytometry. To analyze the influence of smoking on the systemic maternal immune system in humans, data on T-cell subsets were selected from of a previously published control pregnant cohort and reanalyzed for effects of smoking.³³ In short, peripheral blood samples were obtained from pregnant women well before the onset of labor, and T-cell subsets were analyzed using fluorescently labeled antibodies against CD4, CD25, and Foxp3 as described before.³³ In total, 6 smoking pregnant women were selected and matched by gestational age, parity, and maternal age to 2 nonsmoking control pregnant women per case, leading to a control group of 12. Results obtained by flow cytometry were reanalyzed using Winlist software and cell subsets were calculated. Lymphocytes were selected based on forward and side scatter plots. Additional gates were used based on fluorescence emission wavelengths of the antibodies, and negative isotype controls were used to set the gates (Supplemental Figure 1).

Animal study

Animals. Female and male C57Bl6 mice, aged 8-10 weeks, were obtained from Harlan (Horst, The Netherlands). All mice were held under specific pathogen-free conditions on a 12:12 light-dark cycle and were administered food and water ad libitum. All animal protocols were approved by the local committee on animal experimentation (University of Groningen, Groningen, The Netherlands) and were performed under strict governmental and international guidelines on animal experimentation.

For experimental purposes, female mice were treated with 1.5 IU pregnant mare's serum gonadotropin and 1.25 IU human chorionic gonadotrophin to induce simultaneous cycling. To induce pregnancy, 2 females were housed with 1 male. Mating was confirmed by vaginal plug detection. All female mice had been mated, 4 of the 6 smoking females (pregnancy rate 67%), and 5 of the 6 nonsmoking females (preg-

nancy rate 83%) ended up being pregnant (Table 2). The morning after mating was called day 0.5 of gestation. Mated females were separated from males and were killed at day 11.5 of gestation. At day 11.5 paraaortic lymph nodes (uterus-draining lymph nodes), inguinal (systemic) lymph nodes, and spleens were removed. In addition, implantation sites were removed and fixed in 4% paraformaldehyde, zinc fixative³⁸ or immediately snap frozen at -80°C for immunohistochemical and real-time RT-PCR analysis.

Cigarette smoke exposure. Cigarette smoke was generated using a TE-10 smoke exposure system of Teague Enterprises Smoke Exposure System (Woodland, CA). Female mice were exposed to fresh air or smoke in sessions of 7 hours continuously per day, from 7 days before mating until the day the animals were killed. Mice were exposed to 5 cigarettes the first day, 10 cigarettes the second day, 25

TABLE 2
Pregnancy outcome in smoke-exposed and control mice

Variable	Nonsmoking (n = 6)	Smoking (n = 6)
Pregnancy rate, %	83	67
Number of implantations	14.4 ± 2.1	14.0 ± 3.4
Number of resorptions	0.60 ± 0.6	3.4 ± 1.5 ^a
Viable pups, %	96	74 ^b
Maternal weight, g	21.5 ± 1.4	19.6 ± 0.8

Data are mean ± SD.

^a $P < .01$ in a Student *t* test; ^b $P < .05$.

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cigarettes the third day, 50 cigarettes the fourth, and 60 cigarettes on the fifth day and thereafter. Total particulate matter counts were at least 100. Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) were used.

Mouse decidual real-time RT-PCR. Frozen implantation sites were sectioned transversely at 4 μm and stained with hematoxylin. Frozen tissue of 1 control mouse was not available. Decidual and placental tissue was identified, selected, and cut from the slides using laser dissection microscopy (Leica, Rijswijk, The Netherlands). Decidual and placental tissue was collected separately. Total RNA was isolated from decidual and placental tissue using an mRNA easy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. cDNA was reverse transcribed using a Superscript-II reverse transcriptase kit (Invitrogen).

For the housekeeping gene (B2M) and for the other genes (IL6, IL10, Tbet21 [Th1 response], Gata 3 [Th2 response], Ror γ t [Th17 response], Foxp3 [Treg cells], Nk1.1 [NK cells], CD68 [pan macrophage marker], NOS2 [M1- macrophages; iNOS], and CD206 [M2-macrophages; MRC-1]), On-Demand gene expression assays were used (Applied Biosystems). PCRs were performed in triplicate in a volume of 10 μL consisting of 2 μL of MilliQ water, 5 μL PCR mix (Eurogentec), 0.5 μL assay mix, and 2.5 μL cDNA. Runs were performed by a 7900HT Fast real-time PCR system (Applied Biosystems), and mRNA data were normalized to B2M mRNA expression using $2^{-\Delta\text{Ct}}$. Undetectable Ct values (greater than 40) were interpreted as the maximum Ct value (40).

Mouse flow cytometry. Immediately after the animals were killed, paraaortic lymph

nodes (uterus-draining lymph nodes), inguinal (systemic) lymph nodes, and spleens were removed and thoroughly dispersed into single cell suspensions in fluorescence-activated cell sorting (FACS) buffer (consisting of PBS with 2% fetal calf serum). Red blood cells in cell suspensions were lysed using NH_4Cl solution.

For flow cytometric analysis, 1 million cells were stained according to standard methods. In short, cells were incubated for 5 minutes with 10% normal mouse serum to block a-specific binding. Cells from the lymph nodes were then incubated with a cocktail of fluorescently labeled antibodies for 30 minutes, including the following: Pacific Blue-labeled anti-CD3 (17A2; BioLegend, San Diego, CA), PerCP-labeled anti-CD4 (RM4-5; BD Pharmingen, San Diego, CA), Alexa 700-labeled anti-CD8 (53-6.7, Biolegend), allophycocyanin (APC)-labeled anti-CD25 (3C7; BD Pharmingen), and PE-Cy7-labeled anti-NK1.1 (PK 136; BioLegend). After surface staining, intracellular staining was performed according to the Foxp3-staining kit instructions (eBioscience, San Diego, CA) using fluorescein isothiocyanate (FITC)-labeled anti-Foxp3 (FJK-16s; eBioscience).

Cells from the spleen were not only incubated with the T-cell antibody cocktail described in the previous text but were also incubated with a monocyte antibody cocktail: PerCP-labeled anti-CD4 (RM4-5; BD Pharmingen), APC-labeled anti-CD11b (M1/70; BD Pharmingen), phycoerythrin (PE)-labeled anti-CD43 (1B11; Biolegend), PE-Cy7-labeled anti-Gr-1 (RB6-8C5; Biolegend), Pacific Blue-labeled anti-F4/80 (BM8; Biolegend), and FITC-labeled anti-MHC-II (2G9; BD Pharmingen).

Cells were analyzed on a LSRII (Beckton Dickinson, Lincoln Park, NJ) flow cytometer. Results were analyzed with Facs Diva software (Beckton Dickinson).

Lymphocytes were selected based on forward and side scatter plots and subsequently staining for CD3, CD4, CD25, and Foxp3. To identify cells positive for the labeled antibodies, additional gates were set based on fluorescence emission wavelengths of the used antibodies and negative controls (Supplemental Figure 1). For monocytes all viable cells were gated for CD11b and F4/80. CD11b-hi and F4/80-intermediate cells were considered monocytes, and these were gated for CD43 and GR1 to distinguish the GR1-hi and GR1-lo subset (Supplemental Figure 2).

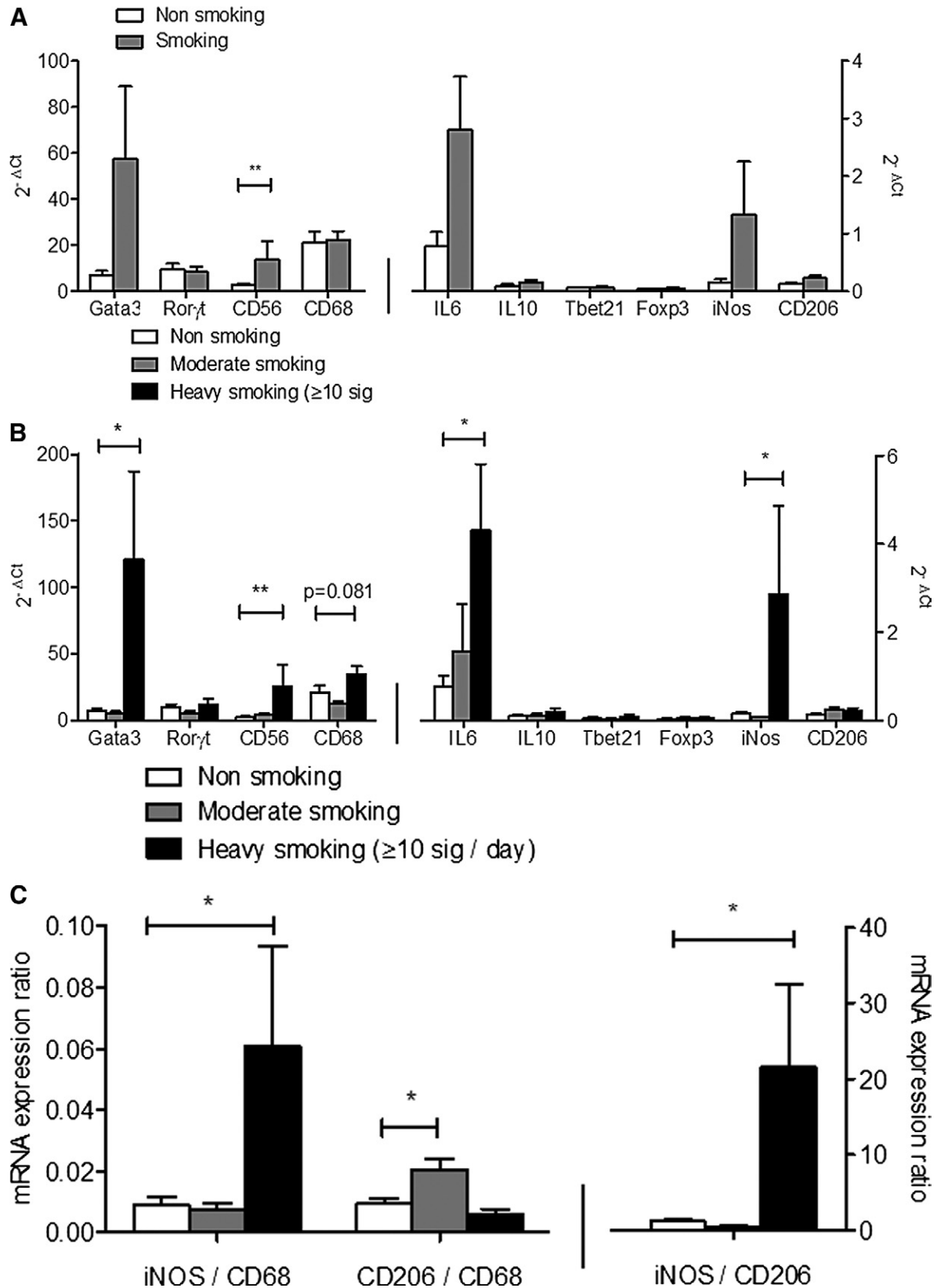
(Immuno)histochemical analysis mice uterine tissue. Implantation sites were fixed in paraformaldehyde and zinc fixative³⁸ for 24 hours, transferred into 70% ethanol, and embedded in paraffin according to standard methods and sectioned transversely at 7 μm . Slides were dewaxed in xylene and rehydrated. For general histological examination, sections were stained with hematoxylin-eosin. First, myometrial, decidual, and placental sizes, and ratios of decidual spiral artery vessel to lumen area were measured.³⁹ T cells and Foxp3+ cells were stained using primary antibodies against CD3 (ab16669; Abcam, Cambridge, MA), and Foxp3 (FJK16s; eBioscience). Antigen retrieval was performed by incubating sections (15 minutes at 400 W) in citrate buffer.

Sections were incubated with primary antibodies diluted 1:400 (CD3) or 1:50 (Foxp3) overnight at 4°C. Thereafter sections were incubated for 30 minutes with the appropriate secondary and tertiary peroxidated antibodies (CD3), or slides were incubated with a biotinylated secondary antibody and were incubated thereafter with a streptavidin-peroxidase complex (Foxp3). Positive staining was localized using diaminobenzidine tetra-

A, Higher CD56 mRNA expression in smoking pregnant women (*gray bars*, $n = 20$) compared with nonsmoking pregnant women (*white bars*, $n = 18$). **B**, When further analyzing dose effects of smoking on mRNA expressions in nonsmokers (*white bars*, $n = 18$), moderate smokers (<10 cigarettes per day, $n = 11$, *gray bars*), and heavy smokers (≥ 10 cigarettes per day, $n = 9$, *black bars*), a higher expression of Gata3, CD56, CD68, IL6, and iNOS was found in heavy smoking pregnant women compared with nonsmoking pregnant women. **C**, There was a dose effect of smoking on mRNA expressions of macrophage subset markers, with higher ratios of M1 macrophage markers in heavy smokers. Data are expressed as mean \pm SEM, statistical analysis was performed using 1-way analysis of variance and Dunnet post hoc testing on log transformed data. Asterisk indicates $P < .05$. Double asterisks indicate $P < .01$.

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FIGURE 1
mRNA expression in first-trimester decidual tissue



chloride (DAB). NK cells were stained using biotinylated lectin for dolichos biflorus (Sigma, St. Louis, MO) diluted 1:800 and were incubated overnight at 4°C. Sections were then incubated with a streptavidin-peroxidase complex for 30 minutes at room temperature, and positive staining was localized using 3-amino-9-ethyl-carbazole (AEC).

Subsets of macrophages were stained using primary antibodies against F4/80 (pan macrophage marker) (MCA497; Serotec, Raleigh, NC), IRF5 (M1 macrophage lineage marker) (10547-1-AP; Proteintech, Manchester, UK), and YM-1 (M2 macrophage lineage marker) (Mouse Chitinase 3-like; R&D Systems Europe Ltd, Abingdon, UK). Antigen retrieval was performed by incubating sections (15 minutes at 400W) in citrate buffer. Sections were incubated with diluted antibody solution, 1:50 (F4/80), 1:100 (IRF5), or 1:400 (YM-1) for 1 hour at room temperature (IRF5, YM-1) or were incubated overnight at 4°C (F4/80). Thereafter sections were incubated for 30 minutes with the appropriate secondary and tertiary peroxidated antibodies (IRF5, YM-1), or sections were incubated with a biotinylated secondary antibody and were incubated with ABC elite kit (Vector Laboratories, Burlingame, CA) (F4/80). Positive staining was localized using DAB (IRF5) or AEC (F4/80, YM-1). All sections were counterstained using hematoxylin and all incubation steps were followed by 3 washes in PBS for 5 minutes.

Except for the Foxp3 staining, which was performed in triplicate, every staining was performed in duplicate with at least 7 sections in between to avoid duplicate counting of cells. Sections were scanned using a NanoZoomer (Hamamatsu, Shizuoka, Japan). Morphometric analysis was performed by setting a staining threshold manually, based on negative control sections, and measuring the total stained area using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical analysis

For statistical analysis, SPSS (SPSS, Inc., Chicago, IL) was used. All real-time RT-

TABLE 3

Characteristics of pregnant women for the peripheral blood data

Characteristic	Nonsmoking (n = 12)	Smoking (n = 6)
Age, y	32.8 ± 4.6	30.0 ± 4.2
Primigravidity, %	25.0	50.0
Birthweight, g	3798.6 ± 465.5	3273.0 ± 637.6
Sex of child (% male)	53.0	60.0
Duration of pregnancy, d	261.7 ± 22.5	265.3 ± 23.3

Data are mean ± SD.

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PCR data were log transformed before statistical analysis. Statistical comparisons between human smoking and nonsmoking groups were performed using 1-way analysis of variance and Dunnett post hoc testing. Statistical comparisons between smoke-exposed and control mice groups were performed using Student *t* tests. Pearson correlation coefficients were calculated to analyze the correlation between number of cigarettes and mRNA expression of several genes. *P* < .05 was considered to be significant, and *P* < .10 was considered a statistical trend.

RESULTS

Smoking influences the human maternal immune system locally and systemically

Smoking affects immune parameters in human decidua. To investigate the effect of human maternal smoking on local immune parameters during pregnancy, first-trimester decidua tissue was analyzed for mRNA expression of different immune markers. When comparing the total smoking group with the control group, a significantly higher CD56 mRNA expression in

smoking women was found as compared with nonsmoking women. No significant differences in expression of the other genes were found (Figure 1, A). However, when further dividing the smoking women into a moderate (<10 cigarettes per day) and a heavy smoking (≥10 cigarettes per day) subgroup, a significant increase was seen in IL6, Gata 3 (Th2 response), CD56 (NK cells), and iNOS (M1 macrophages) and a trend toward higher CD68 (pan macrophages) mRNA expression in heavy smoking women compared with nonsmoking women (Figure 1, B). Because iNOS (M1 macrophages) was increased in smoking women, we analyzed iNOS/CD68 and CD206/CD68 ratios to identify M1 and M2 macrophages as well as the iNOS/CD206 ratio as a measure for the ratio of M1/M2 macrophages (Figure 1, C). In moderate smokers a significant higher CD206/CD68 ratio was found, whereas in heavy smokers significantly increased iNOS/CD206 and iNOS/CD68 macrophage ratios were found (Figure 1, C). Interestingly, when calculating correlation coefficients (CC), significant positive correlations be-

TABLE 4

Percentages of T-cell subsets in human peripheral blood

Variable	Nonsmoking (n = 12)	Smoking (n = 6)
CD4+CD25+	36.1 ± 9.5	40.7 ± 9.9
CD4+CD25+Foxp3- (Teff cells)	38.4 ± 9.8	30.8 ± 9.4
CD4+Foxp3+ (Treg cells)	11.9 ± 5.6	6.1 ± 4.7 ^a

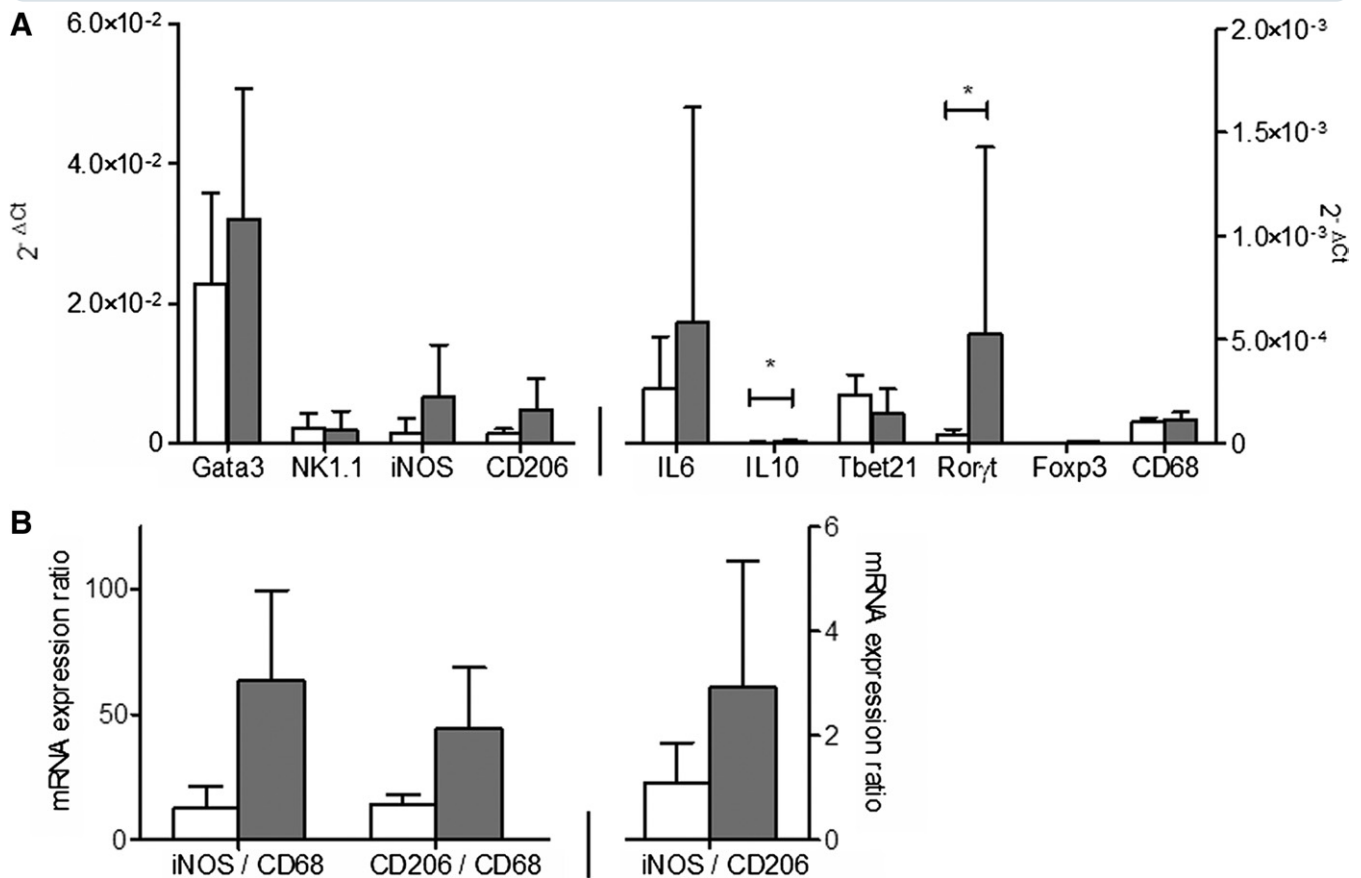
Data are mean ± SD. Subsets are expressed as a percentage of CD4+ cells.

^a *P* < .05.

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FIGURE 2

Expression of mRNA in decidual tissue of smoke-exposed and control pregnant mice



A, Higher expressions of IL10 and Ror γ t were observed in smoke-exposed mice (gray bars) compared with control mice (white bars). **B**, No statistical differences were found in macrophage subset ratios. Data are expressed as mean \pm SEM, and statistical analysis was performed using Student *t* test on log-transformed data. Asterisk indicates $P < .05$.

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tween the number of cigarettes and mRNA expressions were found for IL6 (CC 0.64 $P < .001$), CD56 (CC 0.58, $P < .001$), and CD68 (CC 0.55, $P < .001$) (data not shown).

Lower percentages of Treg cells in peripheral blood of smoking pregnant women. To analyze the influence of smoking on the systemic human maternal immune response, data of a previously published study were reanalyzed (Table 3).³³ Smoking pregnant women had significantly lower percentages of Treg cells (CD4+Foxp3+) than nonsmoking pregnant women ($P < .05$) (Table 4). No differences in percentages of effector T cells (Teff) (CD4+CD25+Foxp3-) cells were found between smoking and nonsmoking pregnant women (Table 4).

Smoking adversely affects pregnancy in mice and humans

Effects of smoking on pregnancy outcome in mice and humans. It is well established that smoking during pregnancy adversely affects human pregnancy outcome.^{1,7} In this study, no differences in birthweights were found between the total human smoking group and control group (Table 1). However, percentile birthweights in the heavy smoking group were significantly ($P < .001$) lower than birthweights in the control group (Table 1, not all data shown). Our results show that smoke exposure to pregnant mice induced a significantly higher number of resorptions (Table 2) and significantly lower percentages of viable pups. Numbers of implantations were similar in

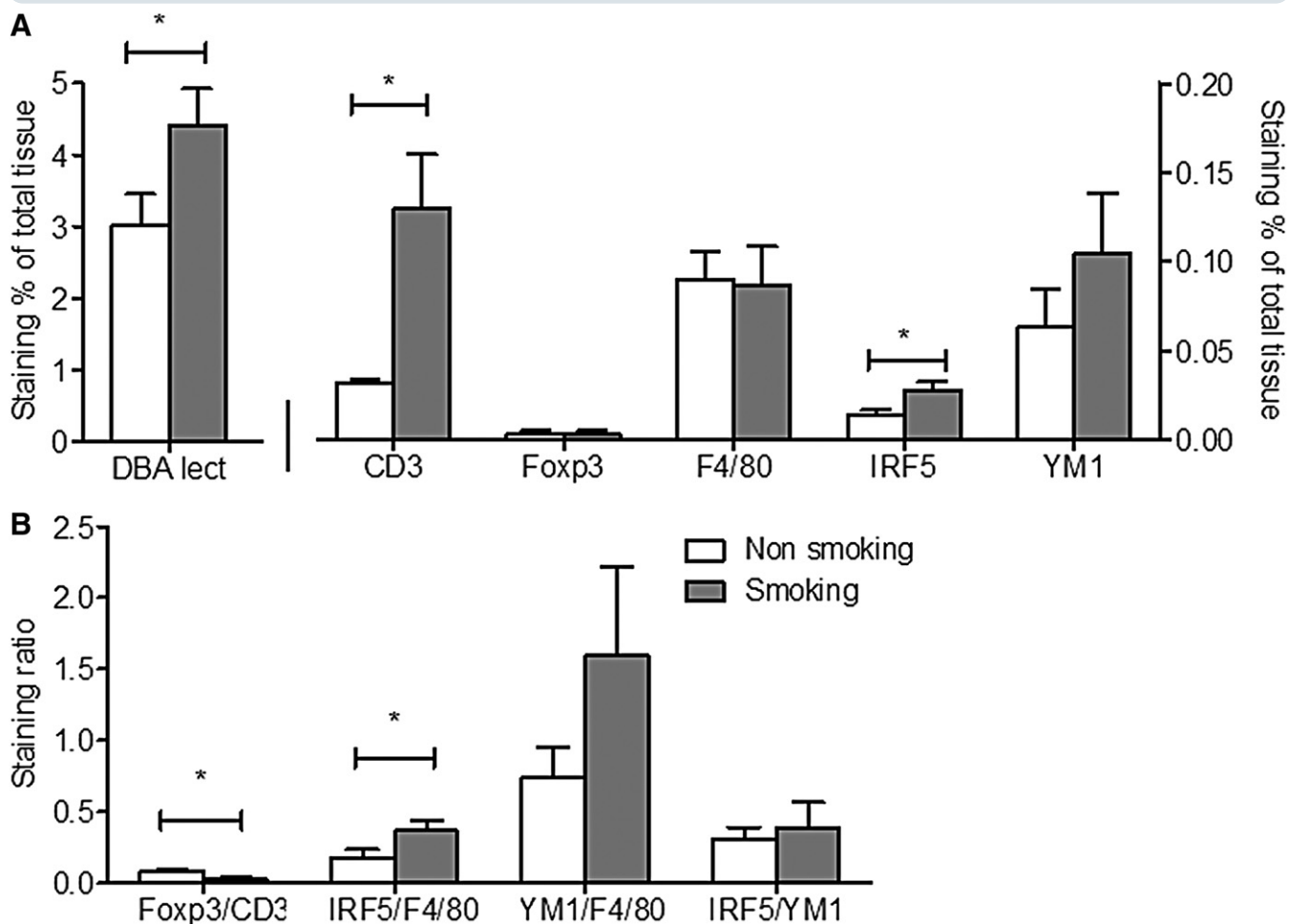
smoke- and air-exposed mice, suggesting that smoking does not adversely affect implantation.

Smoking influences the maternal immune system locally and systemically in mice

Smoking affects immune parameters in murine decidual tissue. In contrast to the human decidual PCR data, mRNA expressions of IL10 and Ror γ t (Th17 response) were significantly increased in smoke-exposed mice (Figure 2, A). There were no significant differences in macrophage subset ratios between smoke-exposed and control mice (Figure 2, B). The mRNA expressions of all tested genes in placental tissue were similar in smoke-exposed and control mice (results not shown). We further analyzed T-cell sub-

FIGURE 3

Quantification of immune parameters after immunohistochemical staining of decidual tissue of smoke-exposed and control mice



A, Higher numbers of CD3+ cells, natural killer cells (DBA lectin), and M1 macrophages (IRF5) in smoke-exposed mice (gray bars) as compared with control mice. **B**, Lower Foxp3/CD3 and higher IRF5/F4/80 ratios in decidual tissue of smoke-exposed mice ($n = 4$) compared with control mice. Data are expressed as mean \pm SEM, and statistical analysis was performed using Student *t* test on log-transformed data. Asterisk indicates $P < .05$.

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sets locally in uterus-draining lymph nodes using flow cytometry. Our data show that locally, in the uterine draining lymph nodes, smoking had no significant effect on percentages of any T-cell subsets in mice (results not shown).

To further analyze whether smoking caused local changes in placentas and deciduas of mice, we histologically and immunohistochemically analyzed murine uterine tissue. Histological analysis showed comparable myometrial, decidual, and placental area sizes and similar ratios of decidual spiral artery vessel to lumen area in smoke-exposed and control mice (results not shown). Immunohistochemi-

cal staining of deciduas showed presence of T cells (CD3), Treg cells (Foxp3), NK cells (DBA lectin), macrophages (F4/80), and macrophage subsets (IRF5 [M1] and YM-1 [M2]). All negative controls did not show staining. Decidual tissue of smoke-exposed mice showed significantly more cells positive for CD3 (T cells), DBA (NK cells), and IRF5 (M1 macrophages) and as a result of that a higher IRF5/F4/80 ratio (Figure 3).

Smoking influenced the systemic percentages of different T cell subsets in pregnant mice. To analyze the systemic effects of smoking on the immune response of preg-

nant mice, we analyzed changes in T-cell subsets in inguinal lymph nodes and spleens of pregnant mice. Percentages of Teff (CD3+CD4+CD25+Foxp3-) were significantly lower in systemic lymph nodes of smoke exposed mice as compared with control mice. Furthermore, these Teff cells showed a trend to be lower in the spleens of smoke-exposed mice ($P = .05$) (Table 5). Our data also showed that smoke-exposed mice show a trend toward lower percentages of Treg cells in the spleens ($P = .06$) (Table 5). Percentages of CD8+ cells showed a trend toward higher percentages in smoke-exposed mice in both inguinal lymph nodes and spleen (Table 5).

Smoking only influences numbers of Gr1-lo monocytes in pregnant mice. Our data show that smoke-exposed mice had a trend toward lower numbers of monocytes (Figure 4, A), mainly caused by lower numbers of the GR1-lo monocyte subset, the nonclassical monocyte subset (Figure 4, B). There was no difference in numbers of GR1-hi monocytes, the classical monocyte subset (Figure 4, C). There were no other differences in expression of activation markers on the monocyte subsets (data not shown).

COMMENT

It has been known for a long time that smoking during pregnancy is associated with adverse pregnancy outcomes.^{1,9} In this study, we analyzed the effects of smoking on the local and systemic human maternal immune system during human pregnancy. To further analyze the effects of smoking on the maternal immune system, we used a mouse smoking model. We indeed found that smoke exposure during pregnancy adversely affects pregnancy outcome in human and mice as shown by higher numbers of resorptions in pregnant mice and lower birthweights in heavy smoking women.

In this study, we further showed that smoking affects the maternal immune system locally and systemically in the same way in pregnant humans and mice. In both humans and mice, decidual inflammatory macrophages (M1) and NK cells were higher in smokers as compared with nonsmoking controls. Systemically, percentages of Treg cells were lower in smoking mice and women than in controls.

In this study, we found higher numbers of DBA lectin-positive NK cells in decidual tissue of smoke-exposed mice and higher mRNA expression of NK cell marker CD56 in decidual tissue of smoking women. The higher numbers and higher mRNA expression of NK cell markers might be involved in the adverse pregnancy outcomes in smoking mice and women. NK cells are 1 of the key immune players during pregnancy and are regarded as especially important in supporting vascular remodeling and decidualization.^{23,40}

TABLE 5

Percentages of peripheral T-cell subsets in mice

Variable	Nonsmoking (n = 5)		Smoking (n = 4)	
	Inguinal	Spleen	Inguinal	Spleen
CD4+	51.8 ± 1.3	57.1 ± 2.6	49.2 ± 2.4 ^a	54.0 ± 3.2 ^a
CD4+CD25+	6.0 ± 1.2	7.4 ± 1.2	5.0 ± 1.5	6.2 ± 0.9
CD4+CD25+Foxp3- (Teff cells)	1.3 ± 0.2	1.5 ± 0.5	0.93 ± 0.09 ^b	0.92 ± 0.2 ^a
CD4+Foxp3+ (Treg cells)	5.2 ± 1.2	7.5 ± 0.5	4.4 ± 1.6	6.7 ± 0.6 ^a
CD8+	47.4 ± 1.5	35.8 ± 3.2	50.6 ± 1.9 ^a	40.2 ± 2.1 ^b

Data are mean ± SD. All T-cell subsets are CD3+ and expressed as a percentage of CD3+ cells.

^a $P < .1$; ^b $P < .05$.

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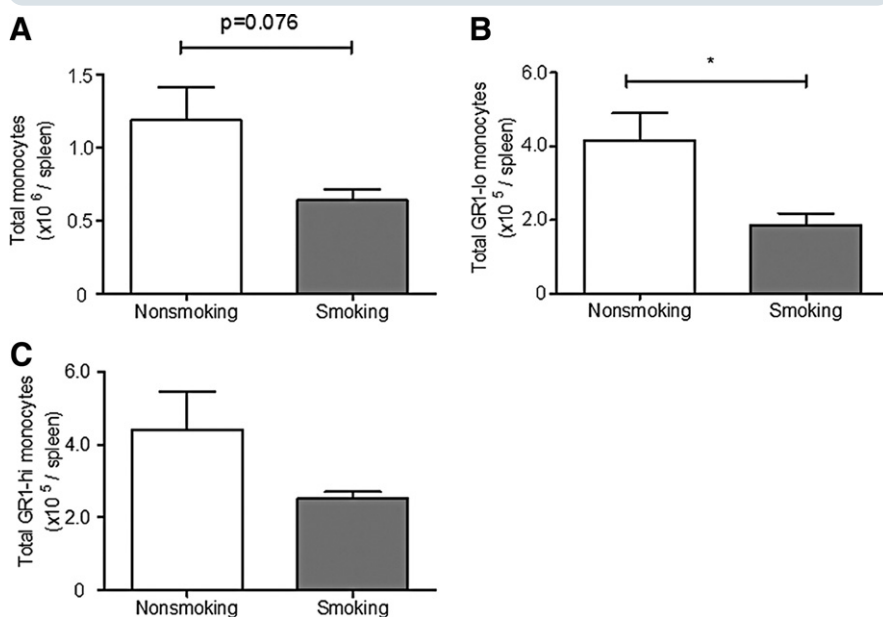
Higher numbers of NK cells are associated with adverse pregnancy outcomes.^{28,41} The mechanism by which smoking affects uterine NK (uNK) cell numbers remains unknown from this study. Theoretically, numbers of NK cells could increase in the deciduas of smoking women and smoke-exposed mice as a response to oxidative stress,

caused by impaired vascularization, which is caused by smoking.⁴²

The higher numbers of NK cells in decidual tissue of smoking mice and women could also be a compensatory response for impairment of these NK cells by smoking as in a nonpregnant population, it has been reported that smoking impairs NK cell cytotoxic activity.⁴³ Al-

FIGURE 4

Absolute numbers of monocyte subsets in spleens of smoke-exposed and control mice



A, A trend ($P = .076$) toward lower absolute numbers of monocytes in spleens of smoke-exposed mice compared with control mice. **B**, Significantly lower numbers of GR1-lo monocytes and **C**, no difference in numbers of GR1-hi monocytes in spleens of smoke-exposed mice compared with control mice. Data are expressed as mean ± SEM, and statistical analysis was performed using Student *t* test on log-transformed data. Asterisk indicates $P < .05$.

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though this compensatory increase may be necessary to stimulate vascularization of the decidua and placenta, it might have adverse effects on inflammatory parameters and thereby cause adverse pregnancy outcome. Whether smoking indeed affects uNK cell function is unknown. Effects of smoking on uNK cells may be different from effects of smoking on NK cells in nonpregnant women because uNK cells produce cytokines and regulate their actions differently from systemic NK cells.⁴³

Interestingly, contrary to the higher local numbers of NK cells, systemically we found lower percentages of NK cells in spleens of smoke-exposed mice. These systemically lower percentages of NK cells in smoke-exposed mice are in line with previous human research, showing lower percentages of CD56+ NK cells in peripheral blood of smoking pregnant women.²⁰ However, as explained above, systemic and uterine NK cells have different functions and capacities. Future research focusing on the influence of smoking on the function of decidual and systemic NK cells during is needed to elucidate these intriguing findings.

Other findings that might be evidence for an altered decidual and placental vascularization in smoke-exposed mice and smoking women would be the changed ratios of inflammatory (M1)/regulatory (M2) macrophage subsets in the decidua of smoke-exposed mice and smoking women. It has been shown that in decidual tissue there are 2 different subsets of macrophages.^{24,31,44} These subsets are activated in different ways and have different functions.⁴⁴ Higher expression levels for M2 macrophage markers have been found in first-trimester decidua.⁴⁵ This regulatory, tissue-repairing macrophage subset may be higher during pregnancy to promote maternal tolerance and coregulate vascular remodeling. The greater presence of M1 subset markers in the decidua of smoke-exposed mice and smoking women indicates a skewing towards an inflammatory environment in the decidua, and furthermore, it might indicate impaired vascularization caused by smoke exposure.

Interestingly, systemically, we found lower numbers and percentages of

GR1-lo monocytes in smoke-exposed mice. A previous study showed angiogenic properties of these GR1-lo monocytes and also showed that GR1-lo monocytes can transform into (M2) macrophages regulating tissue remodeling and angiogenesis.⁴⁶ The lower numbers of the M2 precursor monocytes and higher mRNA expression of M1 markers might both reflect the same change from a regulatory remodeling macrophage environment toward an inflammatory macrophage environment. This could lead to altered decidual remodeling and vascular changes leading to adverse pregnancy outcome.

We found significantly lower percentages of Treg cells in peripheral blood of smoking women. In line with this lower percentage in human peripheral blood, smoke exposure in mice did result in a statistical trend ($P = .085$) toward lower percentages of Treg cells in spleens of smoke-exposed mice. Furthermore, locally, we found a lower Foxp3+/CD3+ cell ratio in deciduas of smoke-exposed mice. Because it is known that Treg cells are essential for pregnancy success,¹³ the lower systemic percentages of Treg cells and lower Foxp3+Treg/CD3+ T-cell ratios in smoking pregnant women and in smoke-exposed mice are possibly involved in the increase in adverse pregnancy outcomes in smoking women and mice. The higher Ror γ t expression, suggesting increased Th17 responses, in the decidua of smoke-exposed mice is in line with previous research showing induction of a Th17-like inflammatory environment in smoke-exposed nonpregnant mice.¹⁹ It is known that increases in the Th17 cell subset and imbalances between the different T-cell subsets can lead to adverse pregnancy outcomes.⁴⁷ The higher Ror γ t mRNA expression in mice could therefore also reflect a mechanism responsible for the adversely affected pregnancy outcomes in smoke-exposed mice.

The lower numbers of Treg cells and higher expression of Ror γ t could be explained by the higher IL6 mRNA expression because it is known that IL6 has an important role in the differentiation of naïve T cells into Treg or Th17 cells.^{48,49} Our data of increased IL6 in the human

decidua is in line with the previously described effect of smoking on IL6 levels in nonpregnant mice.¹⁹ During pregnancy, IL6 is produced systemically and also locally by uterine, decidual cells and macrophages, and it has been shown that IL6 has constant levels during pregnancy with a slight increase at the end of pregnancy.⁵⁰⁻⁵²

Interestingly, it is further shown that levels of IL6 are higher in placental and decidual tissue from abortion-prone CBA/J \times DBA/2J mice compared with normal pregnant mice.⁵¹ In human pregnancy, higher amniotic levels of IL6 are associated with preterm birth.⁵³ Therefore, the higher mRNA expression of IL6 we found could be responsible for adverse pregnancy outcomes. This could be the result of higher numbers of M1 (inflammatory) macrophages because M1 macrophages are known to produce IL6. However, whether higher expression of IL6 is a cause or consequence of the more inflammatory macrophages remains to be answered.

The gestational time points used in our experiments were different for mice and humans. In humans, we analyzed first-trimester decidual tissue, and peripheral blood was taken from third-trimester pregnant women, whereas murine decidual tissue, lymph nodes, and spleens were obtained midpregnancy. Although, we did find similar changes in numbers of NK cells, macrophage subsets, and Treg cells, we could not confirm the higher mRNA expression levels of IL10 and Ror γ t that were found in smoke exposed mice. Furthermore, the lower percentages of Treg cells in smoke-exposed mice and higher expression of IL6 mRNA in smoking women did not reach significance in the comparable human/mouse group.

The different gestational time points might be responsible for differences we found in the effects of smoking between mice and human because it is known that immunological parameters vary during pregnancy.¹³ In addition, the small number of mice used in this experiment (6 per group) could explain some of the differences. Furthermore, the fact that humans are continuously challenged by antigens, contrary to the mice used in

our experiments, and the fact that we used syngeneic mating couples in our mice experiments, which is contrary to the human situation, might also be responsible for differences in immunological findings. Our mice and human data, however, clearly show the adverse effects of smoking.

In summary, smoke exposure during pregnancy influences the local and systemic maternal immune response in both human and mice and does this in a dose-dependent manner. The higher numbers and/or expression of IL6, decidual NK cells, M1 macrophages, and lower Treg/CD3 cell ratio in addition to the systemically lower percentages of regulatory T cells in smoke-exposed mice and smoking women may be responsible for the increase in adverse pregnancy outcomes. If these differences in peripheral and local immune responses after smoking could also explain the lower incidence of preeclampsia in smoking women remains an open question. However, the immunological changes seen in smoke-exposed pregnant women and mice will help us define the mechanisms causing complicated pregnancies and ultimately finding novel therapies. ■

ACKNOWLEDGMENTS

We would like to acknowledge A. Lech (PhD student) and M. Geerlings (technician) for helping with the animal experiments and J. Seinen and J.D. Heeringa (biology students) for their help with the PCR experiments. The acknowledged persons did not receive any compensation.

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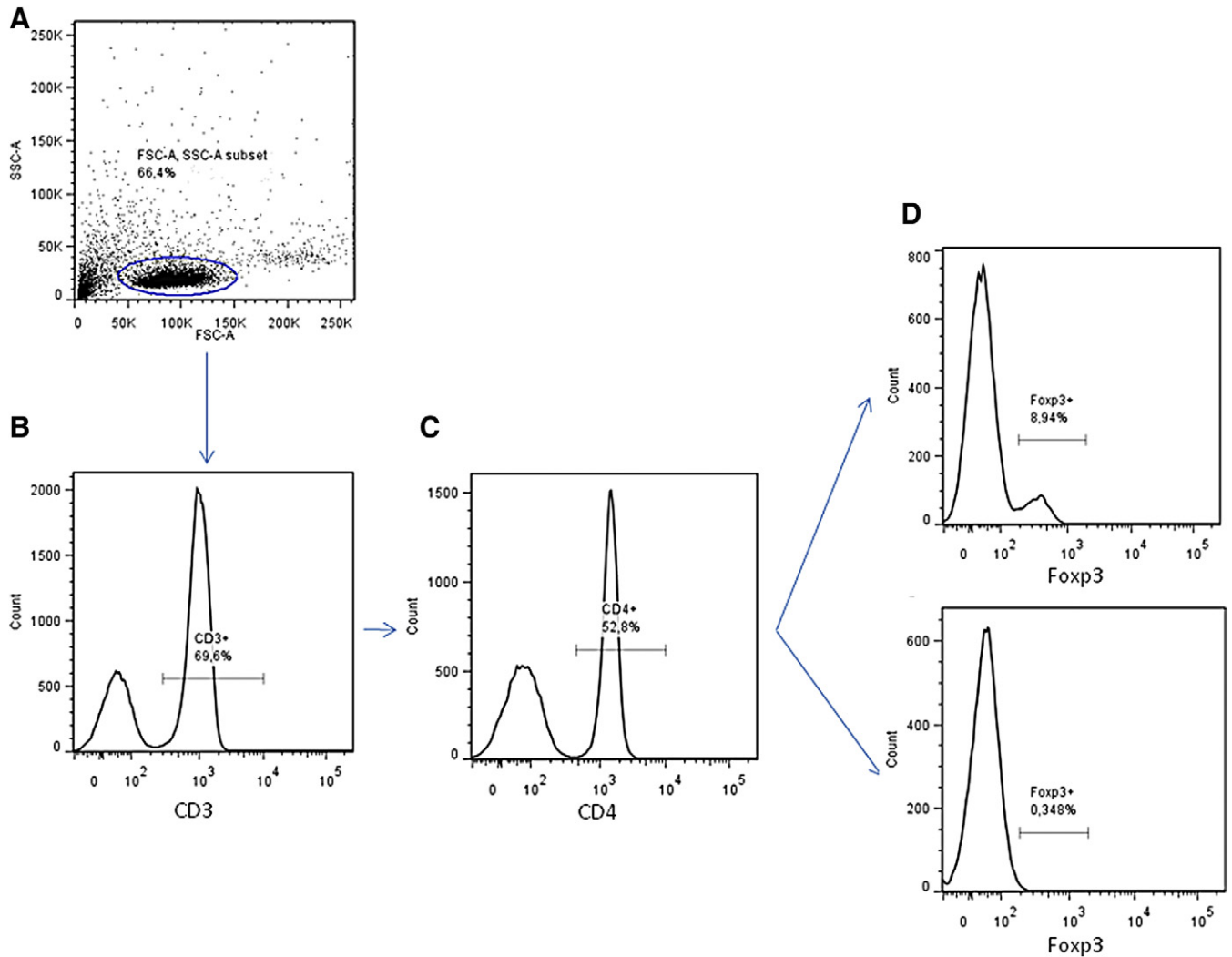
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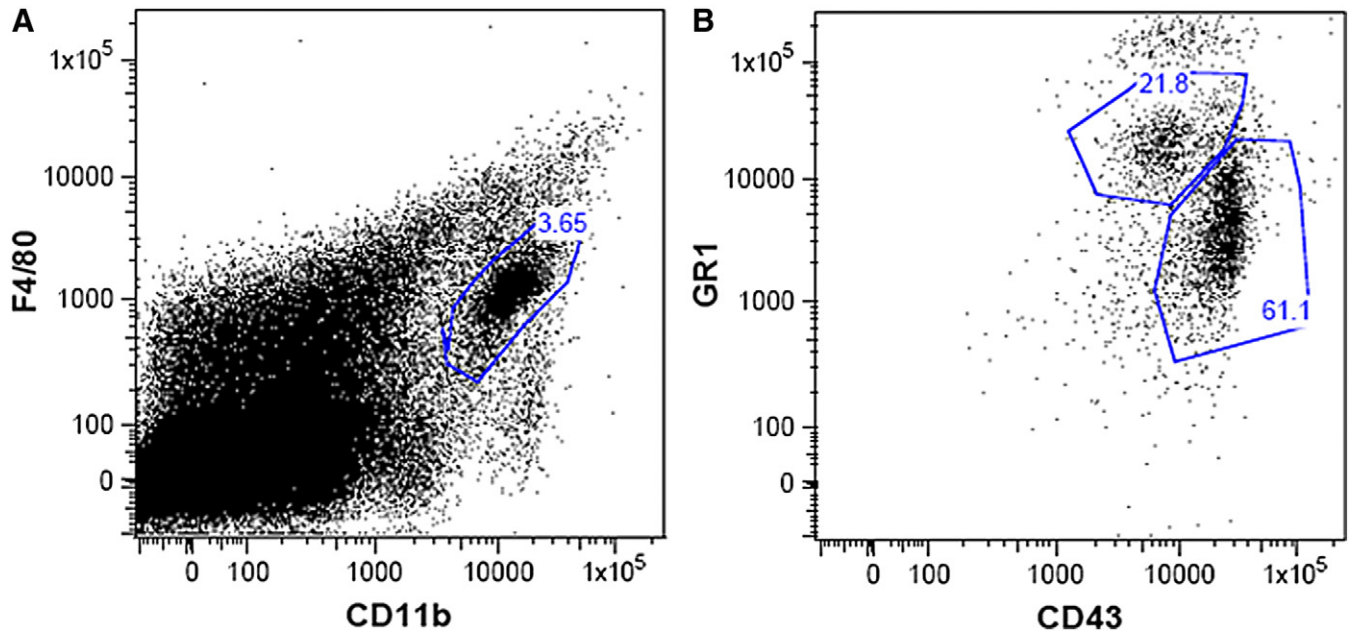
APPENDIX

SUPPLEMENTAL FIGURE 1
Gating strategy lymphocytes



A, Lymphocytes were selected based on forward and side scatter plots. Further T-cell subsets were defined using additional gates. For example, CD3+CD4+Foxp3+ Treg cells were gated based on positive staining for antibodies against **B**, CD3, **C**, CD4, and **D**, Foxp3. **E**, Isotype controls were used to more precisely define positions of gates.

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SUPPLEMENTAL FIGURE 2
Gating strategy monocytes

A, Monocytes were selected based on CD11b and F4/80. CD11b-hi and F4/80-intermediate cells were considered monocytes. **B**, The CD11b-hi and F4/80-intermediate monocytes were gated for CD43 and GR1 to distinguish the GR1-hi and GR1-lo subset.

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