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Air pollution from traffic during pregnancy impairs newborn's cord blood immune cells: The NELA cohort

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ABSTRACT

Background: Hazards of traffic-related air pollution (TRAP) on the developing immune system are poorly understood. We sought to investigate the effects of prenatal exposure to TRAP on cord blood immune cell distributions; and to identify gestational windows of susceptibility.

Methods: In-depth immunophenotyping of cord blood leukocyte and lymphocyte subsets was performed by flow cytometry in 190 newborns embedded in the Nutrition in Early Life and Asthma (NELA) birth cohort (2015–2018). Long-term (whole pregnancy and trimesters) and short-term (15-days before delivery) residential exposures to traffic-related nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$ and PM_{10}), and ozone (O_3) were estimated using dispersion/chemical transport modelling. Associations between TRAP concentrations and cord blood immune cell counts were assessed using multivariate Poisson regression models.

Results: Mean number of natural killer (NK) cells decreased 15% in relation to higher NO_2 concentrations (≥36.4 μg/m3) during whole pregnancy (incidence relative risk (IRR), 0.85; 95% CI, 0.72, 0.99), with stronger associations in the first trimester. Higher $PM_{2.5}$ concentrations (≥13.3 $\mu g/m^3$) during whole pregnancy associated with a reduced mean number of cytotoxic T cells (IRR, 0.88; 95% CI, 0.78, 0.99). Newborns exposed to higher PM_{10} (≥23.6 $\mu g/m^3$) and $PM_{2.5}$ concentrations during the first and third trimester showed greater mean number of helper T type 1 (Th1) cells ($P < 0.05$). Decreased number of regulatory T (Treg) cells was associated with greater short-term NO_2 (IRR, 0.90; 95% CI, 0.80, 1.01) and PM_{10} (IRR, 0.88; 95% CI, 0.77, 0.99) concentrations.

Conclusions: Prenatal exposure to TRAP, particularly in early and late gestation, impairs fetal immune system development through disturbances in cord blood leukocyte and lymphocyte distributions.

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1. Introduction

In 2016 outdoor air pollution caused 4.2 million deaths worldwide (WHO, 2018). In many areas, traffic has become the principal source of most harmful air pollutants (Belis et al., 2013; HEI, 2010). The prenatal period represents a critical exposure window for air pollution effects on

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Abbreviations:

APC	Allophycocyanin
CCR4	C–C chemokine receptor type 4
CCR6	C–C chemokine receptor type 6
CD	Cluster of differentiation
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CXCR3	C–X–C motif chemokine receptor 3
FITC	fluorescein isothiocyanate
IL	Interleukin
NK	natural killer cells
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein complex
Tc	cytotoxic T cells
Th	helper T cells
Th1	helper T type 1 cells
Th17	helper T type 17 cells
Th2	helper T type 2 cells
Tim-3	T-cell immunoglobulin (Ig) and mucin domain-containing molecule-3
Treg	regulatory T cells
WRF-Chem	Weather Research and Forecasting model coupled with Chemistry

humans due to the plasticity and susceptibility of respiratory (Pinkerton and Joad, 2006), immune (Kuper et al., 2016; Palmer, 2011), and detoxification (McElroy et al., 1992) systems. Epidemiologic studies have shown prenatal exposure to outdoor air pollution to be associated with adverse respiratory health outcomes in childhood (Kim et al., 2018), including respiratory tract infections (Aguilera et al., 2013; Jedrychowski et al., 2013; Rice et al., 2015; Soh et al., 2018), impaired lung function performance (Bose et al., 2018; Bougas et al., 2018; Jedrychowski et al., 2010; Korten et al., 2017; Latzin et al., 2009; Morales et al., 2015), asthma (Jung et al., 2019; Subramanian and Khatri, 2019), and related allergic manifestations (Burbank et al., 2017; Cecchi et al., 2018; Deng et al., 2016). The biological mechanisms responsible for these long-lasting effects remain unclear, but disturbances in the developing immune system might play a key role (Ashley-Martin et al., 2016; Sun et al., 2020).

So far, results from limited studies on prenatal exposure to outdoor air pollution and immune cell subsets in cord blood are inconsistent. A first cross-sectional study showed that living in a highly polluted urban area (i.e. high particulate matter levels) was associated to an increased percentage of natural killer (NK) cells and a decreased percentage of T cells in cord blood of neonates from the Czech Republic (Hertz-Picciotto et al., 2002); however, no differences were found for either B cells or T cells, including helper T (Th) and cytotoxic T (Tc) subsets or their ratio (Hertz-Picciotto et al., 2002). In another study from the same group, short-term exposure to higher levels of air pollutants (i.e. mean particulate matter PM_{10} concentrations 30-days before delivery, and mean polycyclic aromatic hydrocarbons (PAHs) and $PM_{2.5}$ concentrations 14-days before delivery) were associated with a decrease in the percentages of T cells, including Th and Tc subsets, and an increase in the percentage of B cells in cord blood of neonates (Hertz-Picciotto et al., 2005); however, no associations were found for other immune cell subsets. A subsequent study conducted in the same population of Czech Republic focused on timing of exposure found higher ambient concentrations of PAH and $PM_{2.5}$ during early gestation (first three months) to be associated with increases in the percentages of T and Th cells and decreases in the percentages of B and NK cells (Herr et al., 2010). In contrast, exposures during late gestation (months seven to eight) were associated with decreases in T cells and Th cells percentages but

Table 1
Comparison of the distribution of main characteristics between included and excluded participants. The NELA study (2015–2018).

	Included (n = 190)	Excluded (n = 548)	p value*
Maternal age, years, mean (sd)	33.1 (4.2)	32.4 (4.8)	0.07
Parity, nulliparous, n (%)	99 (52.1)	275 (50.2)	0.65
Maternal education level, n (%)			
Incomplete secondary or less	32 (16.8)	114 (20.8)	0.46
Complete secondary	49 (25.8)	142 (25.9)	
University	109 (57.4)	292 (53.3)	
Maternal pre-pregnancy BMI (kg/m^2), mean (sd)	24.5 (4.8)	23.7 (4.3)	0.08
Normal, n (%)	124 (65.2)	389 (71.0)	0.28
Overweight, n (%)	44 (23.2)	112 (20.4)	
Obesity, n (%)	22 (11.6)	47 (8.6)	
Maternal asthma, yes, n (%)	26 (13.7)	55 (10.0)	0.17
Maternal history of atopy, yes, n (%)	88 (46.3)	222 (40.5)	0.162
Maternal smoking during pregnancy, yes, n (%)	31 (16.3)	97 (17.7)	0.66
Father age, years, mean (sd)	35.6 (5.2)	34.6 (5.4)	0.06
Father education level, n (%)			
Incomplete secondary or less	48 (25.3)	170 (31.1)	0.01
Complete secondary	75 (39.5)	154 (28.2)	
University	67 (35.3)	222 (40.7)	
Father asthma, yes, n (%)	17 (9.0)	49 (9.0)	0.99
Father history of atopy, yes, n (%)	70 (37.0)	193 (35.3)	0.66
Father smoking, yes, n (%)	71 (37.4)	187 (34.3)	0.44
Area of study, n (%)			
Urban	147 (77.4)	387 (70.6)	0.20
Residential	23 (12.1)	84 (15.3)	
Rural	20 (10.5)	77 (14.1)	
Metropolitan area, n (%)			
No	32 (16.8)	79 (14.4)	0.42
Yes	158 (83.2)	469 (85.6)	
Parental social class, n (%)			
I-II	96 (50.5)	271 (49.5)	0.55
III	47 (24.7)	115 (21.0)	
IV-V	43 (22.7)	148 (27.0)	
Unemployed	4 (2.1)	14 (2.5)	
Pets at home, yes, n (%)	93 (49.0)	248 (45.3)	0.38
Maternal contact with farming animals, yes, n (%)	38 (20.0)	94 (17.2)	0.38
Newborn's sex, female, n (%)	89 (46.8)	274 (51.7)	0.25
Gestational age, weeks, mean (sd)	39.7 (1.2)	39.6 (1.6)	0.32
Full-term (≥37 wks), n (%)	187 (98.4)	497 (93.8)	0.01
Preterm (<37 wks), n (%)	3 (1.6)	33 (6.2)	
Birth weight, g, mean (sd)	3261 (434.4)	3235 (489.0)	0.63
≥2500 g, n (%)	181 (95.3)	489 (93.7)	0.43
<2500 g, n (%)	9 (4.7)	33 (6.3)	
Season at birth, n (%)			
Autumn	48 (25.3)	154 (29.1)	<0.001
Spring	57 (30.0)	129 (24.3)	
Summer	36 (19.0)	172 (32.5)	
Winter	49 (25.8)	75 (14.2)	
Mode of delivery, n (%)			
Vaginal non-instrumental	110 (57.9)	299 (57.3)	0.98
Vaginal Instrumental	38 (20.0)	108 (20.7)	
Cesarean section	42 (22.1)	115 (22.0)	
Fever during labor, yes, n (%)	9 (4.7)	25 (5.0)	0.91
Use of antibiotics during labor, yes, n (%)	52 (27.8)	126 (28.1)	0.93
Residential air pollution (μg/m^3) during whole pregnancy			
All sources NO_2, median (iqr)	44.1 (38.4–47.1)	43.6 (38.2–47.4)	0.54
Traffic-related NO_2, median (iqr)	36.4 (23.1–40.0)	35.6 (25.2–39.6)	0.61
All sources $PM_{2.5}$, median (iqr)	17.4 (16.7–17.9)	17.5 (16.6–18.3)	0.06
Traffic-related $PM_{2.5}$, median (iqr)	13.2 (11.1–14.3)	13.2 (11.4–14.4)	0.35
All sources PM_{10}, median (iqr)	31.2 (29.4–33.0)	31.3 (29.0–33.1)	0.71
Traffic-related PM_{10}, median (iqr)	23.6 (18.9–26.0)	23.5 (19.8–26.0)	0.67
All sources O_3, median (iqr)			0.45

(continued on next page)

Table 1 (*continued*)

	Included (n = 190)	Excluded (n = 548)	p value*
	76.3 (69.3–88.5)	80.5 (68.5–88.6)	
Traffic-related O_3, median (iqr)	20.3 (14.5–27.1)	19.6 (14.3–27.7)	0.95

*p value derived from Kruskal-Wallis test for continuous variables and from Chi^2 test for categorical variables. Iqr: interquantile range.

increases in B cells and NK cells fractions (Herr et al., 2010). No effects were observed during the middle of gestation (months three to seven) (Herr et al., 2010). A French prospective birth cohort study found increased percentage of NK and Tc cells and decreased percentages of T cells, $CD4^+CD25^+$, and CD4+/CD8+ ratio in cord blood in association with higher maternal exposure to PM_{10} and NO_2 during the three-months period before and during pregnancy (Baïz et al., 2011). Finally, short-term residential exposure during late pregnancy (14-days before delivery) to higher NO_2 concentrations, but no PM_{10}, was associated with decreased leukocyte, neutrophil and monocyte counts in cord blood of neonates from Switzerland (Lurà et al., 2018).

Available studies have examined main lymphocyte subsets, overlooking potential developing effects of air pollutants on pivotal immune helper T (Th) subsets such as Th type 1 (Th1), Th type 2 (Th2), Th type 17 (Th17), and regulatory T (Treg) cells, which have distinct functional characteristics and are involved in clinical features and progression of conditions such as allergy and asthma (Abdel-Gadir et al., 2018; Lloyd and Hawrylowicz, 2009). Furthermore, potential critical windows of susceptibility during prenatal development remain largely unidentified.

Hence, we aimed to investigate the effects of exposure to residential traffic-related air pollutants during pregnancy on leukocyte and lymphocyte distributions in cord blood of neonates after performing a thorough immunophenotyping; and to identify gestational windows of susceptibility by examining the effects of exposure during different trimesters of pregnancy, as well as, the short- and long-term effects.

2. Material and methods

2.1. Study participants

Data come from participants embedded in the Nutrition in Early Life and Asthma (NELA) study (www.nela.imib.es), a prospective population-based birth cohort set up in Murcia, a south-eastern Mediterranean region of Spain. The main objective of NELA is to unravel the developmental origins and mechanisms of asthma and allergy.

Pregnant women who fulfill the inclusion criteria were invited to participate in the study at the time of the control ultrasound at 20 weeks of gestation at the Maternal-Fetal Unit of the Virgen de la Arrixaca University Hospital over a 36-month period from March 2015 to April 2018. The inclusion criteria were: women from Health Area I and certain districts of Health Areas VI and VII of the Region of Murcia; planning to live in the area of study during at least 2 years; intention to give birth at the reference hospital; Spanish Caucasian origin; 18–45 years of age; singleton pregnancy; non-assisted conception; and normal echography at 20 weeks of gestation (no major malformations). The exclusion criteria included: an existing chronic disease; pregnancy complications (except gestational diabetes and hypertensive disorders); and not intending to deliver in the reference hospital. Among the 1350 women invited to participate, 738 (54%) were finally enrolled in the study and 720 (97%) had data on exposure to air pollution during pregnancy (Supplementary Figure S1). Among these, umbilical cord blood samples were collected in 390 (53%) newborns. White Blood Cell (WBC) counts were determined in 257 (35%) newborns and immunophenotyping was conducted in 190 of them (26%). Compared with excluded participants, parents of those who were included in the present analysis tended to be older, fathers had low educational level, and neonates were born less frequently during the summer season; but did not differ in other main baseline characteristics (Table 1).

The study protocol was reviewed and approved by the Ethics Committee of the Virgen de la Arrixaca University Clinical Hospital in accordance with the guidelines of The Declaration of Helsinki. Written informed consents were obtained from parents at recruitment.

2.2. Exposure assessment to residential outdoor air pollutants

Geo-codification of residential address during pregnancy was performed using Geographic Information System (GIS). We estimated whole pregnancy, trimester-specific, and short-term exposure to mean concentrations of air pollutants (i.e. NO_2, $PM_{2.5}$, PM_{10} and O_3) for each participant using dispersion/chemical transport modelling. We defined whole pregnancy as from date of conception to birth; first trimester of pregnancy as weeks 1–13; second trimester as weeks 14–26; third trimester as the period from week 27 until birth; and short-term as 15-days before delivery. Date of gestation was calculated from the date of the last menstrual period reported at recruitment and confirmed using estimates based on ultrasound examination at 12 weeks of gestation.

The Weather Research and Forecasting (WRF)+CHIMERE modelling system was developed for generating the outdoor concentrations for air pollutants. The regional meteorological model Advanced Research Weather Research and Forecasting (WRF-ARW) Model v3.6.1 was used to provide the meteorology to the chemistry transport model (Skamarock et al., 2005). WRF has been coupled off-line on an hourly basis to CHIMERE chemistry transport model (Menut et al., 2013). WRF fields are interpolated to CHIMERE working grids. The chemistry transport model includes gas-phase chemistry and also aerosols and heterogeneous chemistry. The aerosol module was adapted to chemical species of health interest, distinguishing among different chemical aerosol components, namely nitrate, sulfate, ammonium, elemental and organic carbon with three subcomponents (primary, secondary anthropogenic and secondary biogenic), dust and marine aerosols.

Existing regional anthropogenic emission inventories were updated, improved and adapted to species of interest. Regional modelling (ranging from 9 km resolution in the Iberian Peninsula to 0.5 km for the NELA geographical area) was performed to analyze the spatial gradient in air pollution 3D maps at the urban scale. Several twin sensitivity simulations were conducted with the aforementioned modelling chain to measure how pollutant concentrations at receptors respond to perturbations at sources. The most straightforward sensitivity method (Brute Force Method, BFM) is to run a model simulation, repeat it with perturbed emissions, and compare the two simulation results. The zero-out method can be considered as an extreme case of variation of the BFM, and differs from the latter in that a specific emissions input is set to zero and the change in output measured (Koo et al., 2009). Therefore, we conducted simulations by using regional emission inventories, independently, for total emissions and isolating (setting to zero) the traffic emission. Hence, the difference between base-case simulations including total emissions in WRF + CHIMERE and simulations zeroing-out traffic emissions provided the levels of air pollution caused by traffic.

2.3. Immunophenotyping of cord blood immune cells

Blood samples were collected from the umbilical cord vein, transferred into sterile tubes with ethylenediamine tetraacetic acid (EDTA). WBCs were assayed immediately after delivery. After that and within 48 h, 50 μL of whole cord blood were incubated with the appropriate combination of fluorochrome-conjugated antibodies (Supplementary Table S1) for 15 min at room temperature in dark conditions. Then, red blood cells were lysed using Fixing/Lising solution (Becton Dickinson) for 5 min. Immune cells were analyzed using a BD FACSCanto™ flow cytometry system and immunophenotypes were defined based on previous studies

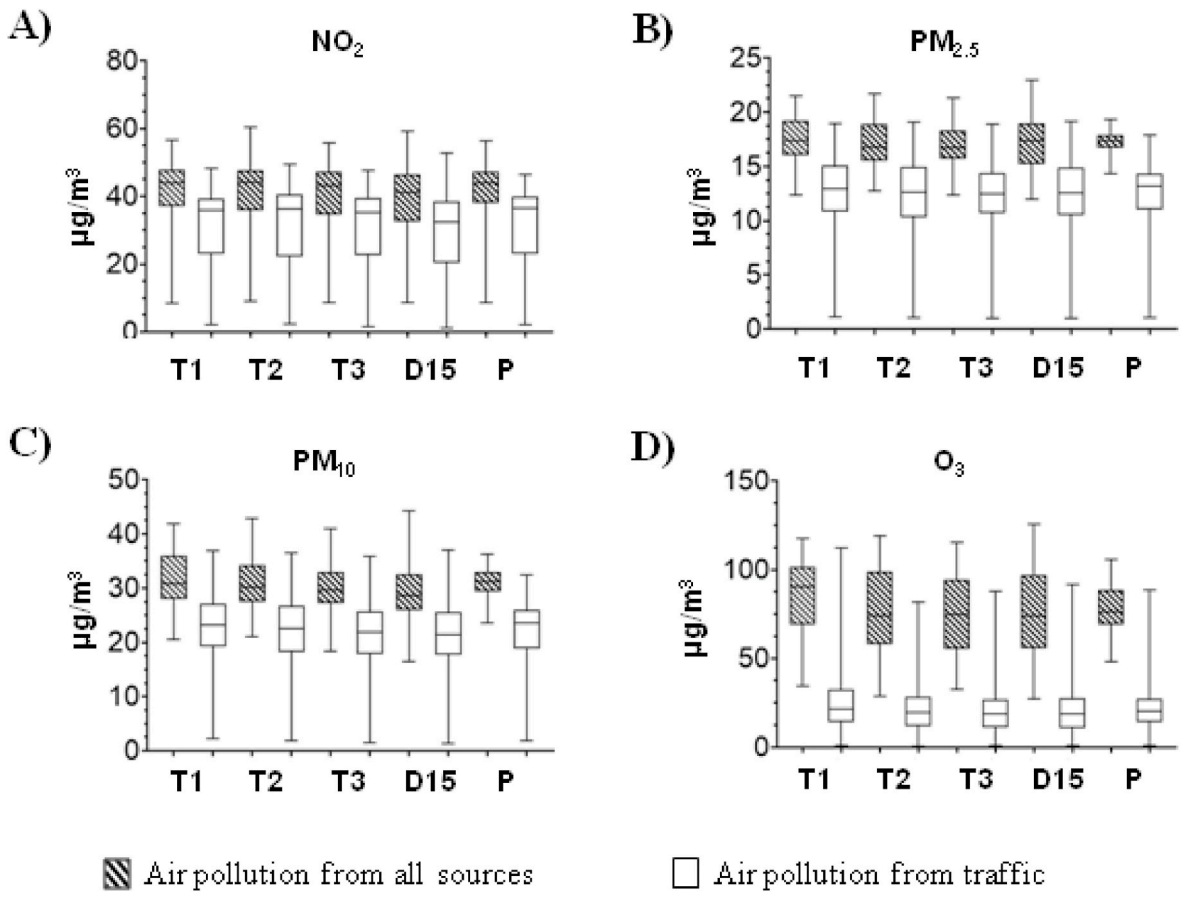

Fig. 1. Estimated residential exposure to outdoor air pollution during pregnancy in the NELA study (2015-2018).Box-plot of exposure concentrations to all sources and traffic-related air pollutants during each trimester of pregnancy (T1, T2, T3), whole pregnancy (P), and 15-days before delivery (D15) in the total sample of 190 mothers. Within the boxes, the median concentration is displayed as a horizontal line.

(Acosta-Rodriguez et al., 2007; Rivino et al., 2004; Singh et al., 2015), including lymphoid B ($CD19^+$), T ($CD3^+$), NK cells ($CD3^-CD16^+$), and main T cell subpopulations, including: Th cells ($CD3^+CD4^+$), Tc cells ($CD3^+CD8^+$), Th1-related cells ($CD3^+CD4+CXCR3+$), Th2-related cells ($CD3^+CD4+CCR4+$), Th17-related cells ($CD3^+CD4+CCR6+$ and $CD3^+CD4+CCR6+CD161+$), Th1Th2-related cells ($CD3^+CD4+CXCR3+CCR4+$) and Treg cells ($CD3^+CD4^+CD24^+CD127-$). Immune cells were gated according to size (Forward Scatter, FSC) and granularity (Side Scatter, SCC) parameters. Cell percentages were obtained according to the phenotype of different subpopulations and absolute cell numbers were calculated using total automatized blood cell counts (WBC/*ul*).

2.4. Other variables

Based on previous knowledge, the following variables were considered as potential confounder factors because of their possible associations with levels of outdoor air pollutants and assessed outcomes. We obtained information through questionnaires administered in person during pregnancy about area of residency (urban, residential, and rural); maternal and paternal age; parental social class (defined as maternal or paternal occupation during pregnancy based on the highest social class by using a widely used Spanish adaptation of the international ISCO88 coding system: I–II, managers/technicians; III, skilled; IV–V, semi-skilled/unskilled; and unemployed) (Domingo-Salvany et al., 2000); maternal and paternal educational level (incomplete secondary or less, complete secondary, and university); maternal and paternal reported history of asthma (yes/no) and atopy (yes/no); maternal smoking during pregnancy and paternal smoking (yes/no); parity (0, nulliparous; *vs*. 1 or more, no nulliparous); maternal pre-pregnancy body mass index (BMI) based on height and pre-pregnancy self-reported weight (kg/m^2) (categorized as normal BMI<25, overweight 25 < BMI<30, and obesity BMI≥30); pets at home (yes/no); and maternal contact with farming animals during pregnancy (yes/no). Information related to child's sex, birthweight (g), gestational age (weeks), mode of delivery (vaginal non-instrumental, vaginal instrumental, and cesarean section), fever (yes/no), and use of antibiotics during labor (yes/no) was obtained from clinical records. Season of birth (spring, March–May; summer, June–August; fall, September–November; and winter, December–February) was also considered.

2.5. Statistical analysis

Differences of main characteristics between included and excluded participants were assessed by the Chi-square test for categorical variables and the Kruskal-Wallis test for continuous variables. Spearman's rank correlation coefficient (ρ) was computed to evaluate correlations between air pollutants and between cord blood immune cells. Associations between exposure to traffic-related air pollutant concentrations and cord blood immune cells counts were examined using multivariate Poisson regression models since the data fit a Poisson distribution. Concentrations of air pollutants were dichotomized ("high" *vs*. "low") based on the median value of each pollutant to balance the number of subjects between the groups for comparison, and the low category (below the median value) was used as the reference. Coefficients of associations are presented as incidence relative risks (IRR) and their corresponding 95% confidence intervals (CI) and can be interpreted as the percentage change in the mean of cell counts. Variables were retained in the multivariate models only if they showed at least marginally

Table 2
Distribution of immune cell subsets in newborn's cord blood. The NELA study (2015–2018).

Immune cell subset	Surface markers	Frequency (%)	Absolute counts (cel/μl)
		Median (iqr)	Median (iqr)
Leukocytes		–	15500 (13090–188200)
Neutrophils		53.5 (48.6–58.8)	8230 (6530–10460)
Lymphocytes		33.4 (28.3–38.1)	5120 (4120–6320)
Monocytes		9.3 (8.0–10.9)	1440 (1120–1830)
Eosinophils		2.6 (1.8–3.9)	400 (290–620)
Basophils		0.6 (0.5–0.9)	100 (70–150)
B cells	$CD19^+$	12.6 (9.5–15.3)	645 (466–842)
NK cells	$CD16^+CD3^-$	18.9 (13.1–24.8)	993 (564–1527)
T cells	$CD3^+$	65.4 (56.6–73.3)	3270 (2669–4071)
Tc cells	$CD3^+CD8^+$	17.3 (14.4–20.5)	892 (688–1179)
Th cells	$CD3^+CD4^+$	46.1 (38.5–52.2)	2228 (1902–2835)
Th1-related cells[a]	$CD3^+$CD4+CXCR3+	2.7 (2.0–3.4)	58 (46–79)
	$CD3^+$CD4+CXCR3+CCR6-	1.9 (1.4–2.6)	41 (32–60)
Th2-related cells[a]	$CD3^+$CD4+CCR4+	6.0 (4.41–8.11)	130 (103–175)
Regulatory T cells[a]	$CD3^+CD4^+CD25^+CD127^-$	6.0 (4.8–6.9)	135 (105–167)
Th17-related cells[a]	$CD3^+$CD4+CCR6+CXCR3-	1.2 (0.9–1.7)	27 (19–39)
	$CD3^+$CD4+CCR6+CD161+	0.4 (0.3–0.6)	10 (7–15)
Th1Th2-related cells[a]	$CD3^+$CD4+CCR4+CXCR3+	1.4 (0.9–1.9)	29 (21–42)

[a] Frequency related to Th cells. Iqr: interqualtile range.

significant association ($p < 0.1$) or modified the coefficient of air pollutants by at least 5%. Final models were adjusted for parity; maternal pre-pregnancy BMI; parental social class; season of birth; mode of delivery; newborn's sex; gestational age; and birthweight. Furthermore, estimates for each trimester were mutually adjusted. Inclusion of other variables did not change the estimates. Linear dose–response relation between continuous air pollutant concentrations and cell counts was assessed using adjusted generalized additive models by graphical examination and likelihood ratio test (Hastie and Tibshirani, 1990). Data were analyzed in Stata Software (version 15.1, StataCorp, College Station, Texas, USA), RStudio (version 1.1.463, RStudio, Boston, Mass) and GraphPad Prism Software (version 8.0.2, GraphPad Software Inc., USA).

3. Results

The study population characteristics are presented in Table 1. The mean age of included pregnant women was 33.1 (±4.2) years (mean (±sd)); 52% were primiparous; 57% had a high educational level; and 16% reported to be active smokers during pregnancy. Overall, 23% of women were overweight and 11% obese before pregnancy; 14% reported to be asthmatic and 46% to have a history of atopy. The mean age of fathers was 35.6 (±5.2) years, 35% of them had a high educational level, and 37% reported to be active smokers. 9% of fathers reported to suffer from asthma and 37% from atopy. Most of the families resided in an urban area (77%), were from high social class (50%), and had pets at home (49%). The study population included 53% male newborns. Mean gestational age was 39.7 (±1.2) weeks and 4.7% of neonates had low birthweight. The rate of cesarean delivery was 22%.

Fig. 1 shows the estimated concentrations of air pollutants by exposure periods. Median levels during whole pregnancy of traffic-related NO_2 ranged between 35.3 and 36.4 $\mu g/m^3$; between 18.7 and 21.6 $\mu g/m^3$ for O_3; between 22.0 and 23.6 $\mu g/m^3$ for PM_{10}; and between 12.5 and 13.2 µg/m3 for $PM_{2.5}$. The medians did not change considerably across exposure periods. Positive strong correlations (Spearman's $\rho > 0.7$) were detected between traffic-related NO_2, PM_{10} and $PM_{2.5}$ (Supplementary Figure 2) with each other. Traffic-related O_3 showed moderate correlation with PM_{10} and $PM_{2.5}$ ($0.45 < \rho < 0.55$). Mothers with high education level and low pre-pregnancy BMI tended to live in areas with high traffic-related $PM_{2.5}$, PM_{10} and NO_2 concentrations (Supplementary Tables S2-S5). Births in autumn were more common in areas with high traffic-related $PM_{2.5}$, PM_{10} and O_3 levels.

Distributions of cord blood immune cells are shown in Table 2. We found a Th1/Th2 ratio that favored Th2 cells (2.7% of Th1-related cells *vs.* 6% of Th2-related cells). Leukocytes expressing Tim-3, IL23 and CD294 receptors were not detected (data not shown). As expected, very strong correlations were found between leukocytes and neutrophils ($\rho = 0.90$); and between T and Th cells ($\rho = 0.95$). Furthermore, strong correlations were found between lymphocytes and T cells ($\rho = 0.85$) and between T cells and Tc cells ($\rho = 0.83$) (Supplementary Table S6). Th1Th2-related cells correlated moderately with Th1 ($\rho = 0.70$) and Th2 cells ($\rho = 0.62$).

Adjusted Poisson regression models showed that newborns exposed to higher traffic-related NO_2 concentrations (≥36.4 µg/m3) in early pregnancy (first trimester) had a decreased mean number of leukocytes (12%), lymphocytes (14%), monocytes (15%) and basophils (25%) compared to those exposed to lower concentrations (Supplementary Table S7). The mean number of NK cells decreased 15% in relation to higher NO_2 concentrations during pregnancy (Table 3), showing a linear inverse relationship (Fig. 2A) and stronger effects for exposures in the first trimester and 15-days before delivery (Table 3). Conversely, the exposure to higher O_3 concentrations 15-days before pregnancy was associated with an increased mean number (25%) of NK cells. The mean number of Tc cells was 12% lower in newborns exposed to higher $PM_{2.5}$ concentrations (≥13.3 $\mu g/m^3$) during whole pregnancy as compared to those exposed to lower levels (Table 3), showing a linear inverse relationship (Fig. 2B). Counts of total T, Th and Tc cells decreased in newborns exposed to high NO_2 levels during first trimester but tended to increase in those exposed to high NO_2 levels during second trimester (Table 3). The mean number of total Th cells increased in relation to higher PM_{10} and $PM_{2.5}$ concentrations in early gestation (first trimester) (Table 3).

Newborns exposed to higher PM_{10} and $PM_{2.5}$ concentrations during first and third trimesters of gestation showed greater mean number of Th1-related cells (Table 4). Exposure to higher PM_{10} (Fig. 2C) and $PM_{2.5}$ concentrations during whole pregnancy were associated with a decreased mean number of Th1Th2-related cells, with stronger associations in the second trimester. Short-term exposure to higher NO_2 and PM_{10} concentrations was associated with a decrease in the mean number of Treg cells (Table 4). The mean number of Th17 (CD161+) cells tended to decrease in relation to greater O_3 concentrations in whole pregnancy (Table 4), showing a linear inverse relationship (Fig. 2D).

4. Discussion

This study shows that residential exposure to higher concentrations of traffic-related air pollutants during pregnancy alters the distribution of cord blood immune cells in neonates and identifies early and late gestation as windows of higher susceptibility. Greater NO_2 concentration in early gestation (first trimester) was associated with decreased mean number of leukocytes. The mean number of NK cells decreased in relation to higher levels of NO_2 during gestation, with stronger associations for exposures in the first trimester and 15-days before delivery.

Table 3
Association between residential traffic-related air pollution and lymphocyte subsets in newborn's cord blood. The NELA study (2015–2018).

Exposure	B cells		NK cells		T cells		Helper T cells		Cytotoxic T cells		CD4/CD8	
	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)
NO2												
T1	0.76	(0.57–1.00)*	0.77	(0.59–1.00)*	0.86	(0.76–0.98)**	0.88	(0.77–1.00)*	0.85	(0.72–0.99)**	1.01	(0.88–1.15)
T2	1.11	(0.87–1.41)	1.18	(0.93–1.51)	1.21	(1.05–1.40)**	1.25	(1.08–1.45)**	1.12	(0.95–1.33)	1.16	(1.01–1.34)**
T3	1.20	(0.87–1.65)	0.92	(0.74–1.15)	0.97	(0.86–1.09)	0.93	(0.82–1.06)	1.05	(0.91–1.22)	0.87	(0.75, 0.99)**
15-D	0.97	(0.83–1.14)	0.84	(0.71–0.99)**	0.99	(0.91–1.09)	0.99	(0.90–1.10)	0.99	(0.88–1.11)	1.01	(0.91–1.13)
P	1.09	(0.92–1.28)	0.85	(0.72–0.99)**	1.03	(0.93–1.13)	1.03	(0.94–1.14)	1.02	(0.90–1.14)	1.01	(0.91–1.12)
PM2.5												
T1	1.04	(0.84–1.28)	0.94	(0.74–1.19)	1.10	(0.96–1.26)	1.14	(0.99–1.32)*	1.02	(0.88–1.19)	1.06	(0.94–1.19)
T2	1.16	(0.94–1.42)	1.11	(0.90–1.38)	0.97	(0.87–1.07)	0.96	(0.86–1.07)	0.97	(0.85–1.12)	0.97	(0.85–1.12)
T3	0.96	(0.78–1.17)	0.82	(0.68–1.00)*	0.98	(0.89–1.09)	0.99	(0.89–1.10)	0.97	(0.84–1.12)	1.05	(0.92–1.20)
15-D	1.06	(0.90–1.25)	0.93	(0.76–1.13)	1.02	(0.93–1.13)	1.05	(0.95–1.16)	0.95	(0.85–1.08)	1.10	(0.99–1.22)*
P	1.07	(0.91–1.26)	0.87	(0.74–1.03)	1.00	(0.91–1.10)	1.06	(0.96–1.16)	0.88	(0.79–0.99)**	1.16	(1.05–1.28)**
PM10												
T1	1.07	(0.86–1.34)	1.02	(0.81–1.29)	1.14	(1.01–1.30)**	1.15	(1.01–1.31)**	1.13	(0.97–1.32)	0.97	(0.86–1.09)
T2	1.09	(0.85–1.39)	0.96	(0.78–1.19)	0.99	(0.87–1.14)	1.04	(0.90–1.20)	0.90	(0.77–1.05)	1.12	(0.99–1.28)*
T3	0.97	(0.79–1.18)	0.95	(0.79–1.14)	0.97	(0.87–1.08)	0.96	(0.86–1.07)	0.99	(0.85–1.15)	0.98	(0.87–1.11)
15-D	0.96	(0.82–1.12)	0.89	(0.75–1.05)	0.96	(0.87–1.05)	0.94	(0.86–1.04)	0.99	(0.87–1.11)	0.94	(0.85–1.05)
P	1.06	(0.89–1.26)	0.91	(0.76–1.08)	1.02	(0.92–1.12)	1.05	(0.95–1.17)	0.94	(0.83–1.06)	1.10	(0.99–1.22)*
O3												
T1	1.07	(0.88–1.30)	0.96	(0.79–1.16)	1.05	(0.93–1.18)	1.08	(0.95–1.22)	0.99	(0.85–1.14)	1.11	(0.98–1.24)*
T2	1.01	(0.82–1.23)	1.15	(0.92–1.45)	1.00	(0.88–1.13)	0.99	(0.87–1.12)	1.02	(0.87–1.21)	0.84	(0.73–0.98)**
T3	1.03	(0.82–1.29)	1.04	(0.83–1.29)	0.99	(0.87–1.12)	0.99	(0.87–1.12)	1.00	(0.84–1.19)	1.05	(0.92–1.21)
15-D	1.08	(0.91–1.28)	1.25	(1.05–1.48)**	1.01	(0.91–1.12)	1.00	(0.90–1.12)	1.02	(0.89–1.16)	0.96	(0.87–1.07)
P	1.04	(0.88–1.23)	1.04	(0.88–1.24)	1.00	(0.92–1.10)	1.01	(0.92–1.10)	0.99	(0.88–1.12)	0.96	(0.86–1.06)

Coefficients represent percentage change in mean cell counts comparing high (≥the median value) *vs.* low (<the median value) concentrations of each air pollutant. All model adjusted for parity, maternal pre-pregnancy BMI, parental social class, season of birth, mode of delivery, newborn's sex, gestational age and birthweight. Estimates for each trimester were mutually adjusted. IRR: incidence relative risk. CI: confidence interval. T1: first trimester. T2: second trimester. T3: third trimester. 15-D: 15-days before delivery. P: whole pregnancy.*p-value<0.1; **p-value<0.05.

Higher $PM_{2.5}$ concentration during pregnancy was associated with decreased mean number of Tc cells. Exposure to greater PM_{10} and $PM_{2.5}$ concentrations during specific trimesters of gestation was associated with increased mean number of total Th and Th1-related cells. Higher O_3 concentration during pregnancy was associated with a decreased mean number of Th17 cells. Finally, greater short-term (15-days before delivery) NO_2 and PM_{10} concentrations were associated with a reduced mean number of Treg cells.

We found decreased mean number of leukocytes, lymphocytes, monocytes and basophils in cord blood of newborns exposed to higher NO_2 concentrations in early pregnancy (first trimester). No previous studies have examined leukocyte and lymphocyte distributions in newborn's cord blood in relation to air pollution exposure through gestation, except for a previous study that reported decreased leukocytes, neutrophils and monocytes counts in newborns exposed to higher NO_2 concentrations 14-days before delivery (Lurà et al., 2018). To this regard, our findings suggest that early (first trimester) and late (15-days before delivery) gestation may represent developmental windows of higher susceptibility of the immune system to potential harmful effects of *in utero* exposure to TRAP. Air pollution toxicity could interfere immune cell development by affecting initiation of hematopoiesis, migration of stem cells, and expansion of progenitor cells that occur during early gestation (gestational weeks 4–16) (Kuper et al., 2016). Leukocyte development starts at gestational week 4 and neutrophils and monocytes are the first innate cells to appear at weeks 4–8 of gestation (Kuper et al., 2016).

We found NK cell counts decreased in newborns exposed to higher NO_2 concentration *in utero*, showing strong effects in early and late gestation. Associations between prenatal exposure to air pollutants and cord blood NK cells are inconsistent. Some studies found increased percentage of NK cells in cord blood associated with living in areas with higher PM levels during pregnancy (Hertz-Picciotto et al., 2002), exposure to PM_{10} during the first trimester of pregnancy (Baïz et al., 2011), and exposure in late pregnancy to PAH, $PM_{2.5}$, and NO_2 (Baïz et al., 2011; Herr et al., 2010). Other findings have showed reduced percentage of NK in cord blood associated with exposure to PAH and $PM_{2.5}$ during early gestation (first two months) (Herr et al., 2010). In addition, a decreased number of NK cells was found in children exposed to elevated air pollution in Mexico City (Calderón-Garcidueñas et al., 2009). In contrast to previous studies that used both CD56 and CD16 surface markers to characterize cord blood NK cells (Baïz et al., 2011; Herr et al., 2010; Hertz-Picciotto et al., 2002), we used only CD16 marker. This fact could explain some discrepancies. Nevertheless, NK cells play an important role in the early response to viral infections (Abel et al., 2018) and can also inhibit allergic eosinophilic airway inflammation (Ferrini et al., 2017; Simons et al., 2017). Thus, it is plausible that impairment of NK cell fetal development could mediate associations between prenatal exposure to air pollution and subsequent higher risk of respiratory infections (Rice et al., 2015; Soh et al., 2018) and asthma (Jung et al., 2019) in childhood.

Furthermore, we found that mean number of Tc cells decreased in newborns exposed to higher $PM_{2.5}$ concentration during whole pregnancy, although no specific windows of higher susceptibility were identified. Effects of air pollution on cord blood Tc cells are unclear. Two previous studies have reported a decreased percentage of Tc cells in cord blood of neonates associated with exposure to high levels of outdoor air pollution in late pregnancy, including exposure to PAHs during 14-days before birth (Hertz-Picciotto et al., 2002) and to PAH and $PM_{2.5}$ in month 9 of gestation (Herr et al., 2010). However, another study found increased percentage of Tc cells in newborns in relation to high NO_2 concentration during the first and second trimester of gestation but not in relation to PM_{10} (Baïz et al., 2011). In contrast to our study, previous ones have examined percentages of immune cells and findings should be interpreted with caution because changes in frequencies could be the result of an immune compensation but not a real change in the absolute cell number (Rivino et al., 2004). Nevertheless, we hypothesize that a reduction in Tc cells, which are involved in the response to virus infections (Schmidt and Varga, 2018), may play a role in predisposing to higher risk of respiratory viral infection in infants associated with air pollution exposure as previously reported (Aguilera et al., 2013; Jedrychowski et al., 2013; Rice et al., 2015; Soh et al., 2018).

Th-related cells could play an important role in the development of

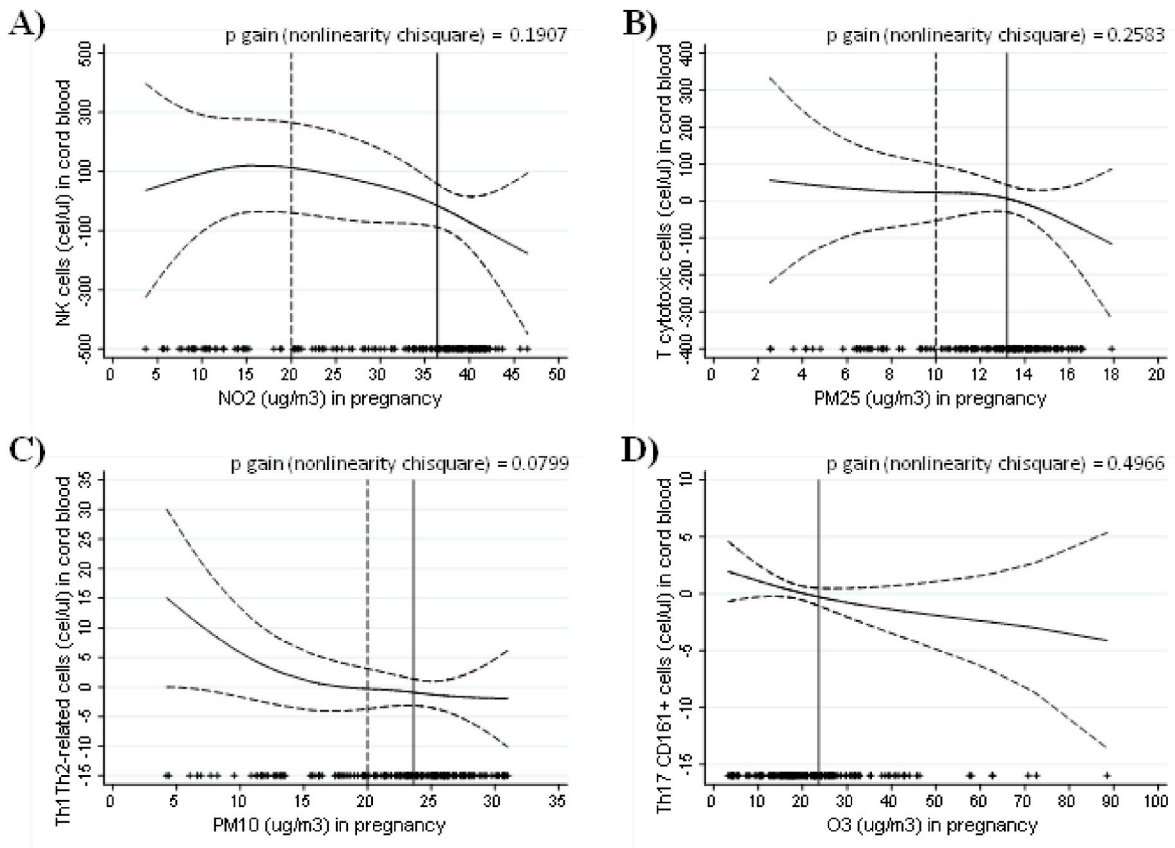

Fig. 2. The relation (and 95% confidence levels) of traffic-related air pollutant concentrations during pregnancy with selected immune cell counts in cord blood of neonates. General additive models adjusted for parity, maternal pre-BMI, parental social class, season of birth, mode of delivery, newborn sex, gestational age and birthweight for A) NO_2 and NK cell count; B) $PM_{2.5}$ and Tc cell count; C) PM_{10} and Th1Th2-related cell count; and D) O_3 and Th17 cell count. The symbols (+) on the X-axis indicate air pollutant observations. Vertical solid line indicates median concentration value in our study, and vertical dash line indicates WHO recommended air quality standards.

respiratory diseases, asthma and allergic manifestations (Fedele et al., 2018; Jung et al., 2019). Our results revealed an increased mean number of Th cells in newborns exposed to high PM concentration during early pregnancy and an increased mean number of Th1-related cells in newborns exposed to high PM concentration during early and late gestation. The influence of prenatal exposure to air pollutants on Th1- and Th2-related cells distribution in cord blood of neonates has not been investigated. Th1 cells generate important proinflammatory cytokines whose changes in serum may be indicative of increased systemic inflammation (Zhou et al., 2009). Our results were consistent with those of Latzin et al. who found that levels of IL-1β (a pro-inflammatory cytokine) increased in cord blood serum in association with PM_{10} exposure in late gestation (Latzin et al., 2011). Similarly, animal studies evidenced that exposure to PM_{10} could induce inflammatory and allergic immune responses by the upregulated release of Th1-related cytokines (TNF-α and IFN-γ) (Huang et al., 2017a,b). Thus, increased Th1-related cells in cord blood could reflect an inflammation stage in newborns prenatally exposed to higher PM concentration (Kuo et al., 2018).

Short-term exposure to higher NO_2 and PM_{10} concentrations were associated with decreased number of Treg cells in cord blood. These results are consistent with those previously found in newborns and children (Abdel-Gardir et al., 2018; Baïz et al., 2011). Interestingly, tolerance to allergens is critically dependent on the generation of allergen-specific Treg cells, which mediate a state of sustained non-responsiveness to allergens and suppress inflammatory Th2 responses that are responsible for allergic symptoms (Abdel-Gadir et al., 2018). Moreover, defective Treg cells are found in cord blood of neonates at hereditary risk of allergy (Haddeland et al., 2005; Schaub et al., 2008) and in allergic subjects including asthmatics (Huang et al., 2017a, b; Lloyd and Hawrylowicz, 2009).

Our study has some limitations. Firstly, the study was conducted in a subsample of the full cohort. Although the distributions of several variables differed in the study sample compared with the full cohort, these differences are not likely to have affected either the internal or external validity of the results, because the variables were controlled in the analysis by multivariate adjustment. Secondly, the somewhat small sample size could reduce the study power, affect the precision of the estimates, and limit our ability to evaluate potential effect modifiers and to apply multi-pollutant models. Thirdly, estimated individual exposure to TRAP was based on WRF-Chem models applied to geocoded home address under the assumption that pregnant women spend a larger fraction of time in their residence. We did not consider time-activity patterns as potential factors affecting exposure misclassification. Fourthly, WRF-Chem does not include unknown emission sources. Finally, implementation of statistical methods for allowing count outcome data is warranted to disentangle health effects of high correlated concurrent environmental exposures during different time windows (Bobb et al., 2018).

Among the strengths of this study are its population-based, prospective design, and the availability of extensive information on potential confounders. Our study distinguishes itself from previous studies due to in-depth immunophenotyping of cord blood lymphocyte subsets. Moreover, WRF-Chem models provide a high spatial and temporal resolution assessing pollutants not routinely monitored and modelling short- and long-term exposure periods (Hoek, 2017). Finally, models were evaluated drawing GAM representations, proving linear relation between exposures and outcomes.

Table 4
Association between residential traffic-related air pollution exposure and Th subsets in newborn's cord blood. The NELA study (2015–2018).

Exposure	Th1		Th1 CCR6-		Th2		Treg		Th17		Th17 CD161+		Th1Th2-related	
	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)	IRR	(95% CI)
NO2														
T1	0.88	(0.68–1.13)	0.94	(0.75–1.18)	0.80	(0.64–1.01)*	0.93	(0.77–1.13)	0.76	(0.51–1.14)	0.73	(0.54–0.98)**	0.96	(0.70–1.31)
T2	1.08	(0.84–1.39)	1.27	(0.97–1.68)	1.14	(0.91–1.41)	1.16	(0.96–1.40)	1.32	(0.76–2.31)	1.15	(0.84–1.56)	1.36	(0.93–1.99)
T3	1.11	(0.86–1.42)	0.84	(0.65–1.08)	1.03	(0.83–1.27)	0.86	(0.70–1.04)	0.87	(0.59–1.29)	1.11	(0.84–1.45)	0.73	(0.51–1.04)*
15-D	0.98	(0.87–1.10)	0.90	(0.79–1.03)	0.95	(0.83–1.09)	0.90	(0.80–1.01)*	0.82	(0.68–1.01)*	0.97	(0.80–1.18)	0.86	(0.74–1.01)*
P	1.06	(0.93–1.21)	0.99	(0.85–1.15)	0.99	(0.86–1.12)	0.98	(0.87–1.10)	0.90	(0.71–1.15)	0.99	(0.81–1.20)	0.90	(0.77–1.06)
PM2.5														
T1	1.20	(1.01–1.43)**	1.11	(0.90–1.37)	1.02	(0.86–1.21)	1.15	(0.99–1.34)*	0.94	(0.70–1.25)	0.92	(0.71–1.18)	0.98	(0.78–1.22)
T2	0.86	(0.75–0.99)**	0.89	(0.76–1.04)	0.88	(0.74–1.04)	0.94	(0.81–1.08)	0.87	(0.58–1.30)	0.96	(0.75–1.23)	0.79	(0.66–0.95)**
T3	1.15	(0.97–1.38)	1.06	(0.88–1.28)	1.03	(0.89–1.19)	0.99	(0.87–1.14)	1.36	(0.95–1.95)*	1.09	(0.89–1.33)	1.10	(0.90–1.35)
15-D	1.19	(1.03–1.37)**	1.01	(0.85–1.20)	1.00	(0.87–1.14)	1.02	(0.90–1.16)	1.01	(0.77–1.33)	0.98	(0.79–1.23)	0.94	(0.77–1.14)
P	1.08	(0.96–1.22)	0.95	(0.83–1.10)	0.95	(0.84–1.08)	1.02	(0.91–1.15)	1.06	(0.85–1.33)	0.93	(0.76–1.13)	0.87	(0.74–1.02)*
PM10														
T1	1.12	(0.93,1.35)	1.31	(1.04–1.66)**	1.09	(0.92–1.28)	1.13	(0.96–1.33)	0.99	(0.75–1.30)	1.00	(0.76–1.31)	1.32	(0.97–1.80)*
T2	0.91	(0.77,1.09)	0.77	(0.63–0.93)**	0.84	(0.71–0.99)**	1.01	(0.85–1.21)	0.92	(0.70–1.20)	0.80	(0.63–1.00)*	0.67	(0.53–0.85)**
T3	1.17	(1.01,1.36)**	1.07	(0.90–1.28)	1.03	(0.88–1.20)	0.95	(0.82–1.10)	1.23	(0.89–1.68)	1.10	(0.89–1.35)	1.04	(0.86–1.26)
15-D	1.11	(0.96–1.28)	1.05	(0.90–1.24)	1.00	(0.87–1.15)	0.88	(0.77–0.99)**	0.87	(0.66–1.14)	0.92	(0.75–1.12)	0.94	(0.78–1.14)
P	1.04	(0.91–1.18)	0.94	(0.81–1.10)	0.94	(0.83–1.07)	1.01	(0.90–1.14)	0.95	(0.76–1.19)	0.87	(0.71–1.07)	0.82	(0.69–0.98)**
O3														
T1	1.20	(1.02,1.40)**	1.25	(1.01–1.55)**	1.11	(0.96–1.30)	1.07	(0.92–1.25)	1.11	(0.76–1.64)	0.86	(0.69–1.06)	1.26	(0.94–1.70)
T2	1.00	(0.85–1.18)	0.88	(0.73–1.07)	0.98	(0.85–1.13)	0.99	(0.87–1.14)	0.79	(0.51–1.23)	0.84	(0.66–1.07)	0.82	(0.66–1.03)*
T3	0.94	(0.79–1.11)	0.90	(0.75–1.09)	0.91	(0.78–1.06)	0.98	(0.83–1.15)	1.05	(0.81–1.36)	1.16	(0.93–1.45)	0.87	(0.69–1.10)
15-D	1.09	(0.94–1.27)	1.13	(0.95–1.35)	1.01	(0.88–1.16)	1.08	(0.94–1.24)	0.91	(0.71–1.15)	0.92	(0.74–1.14)	1.04	(0.86–1.26)
P	1.04	(0.92–1.17)	0.95	(0.82–1.11)	0.99	(0.87–1.12)	0.98	(0.87–1.10)	0.92	(0.73–1.15)	0.83	(0.68–1.01)*	0.86	(0.71–1.04)

Coefficients represent percentage change in mean cell counts comparing high (≥the median value) *vs.* low (<the median value) concentrations of each air pollutant. All model adjusted for parity, maternal pre-pregnancy BMI, parental social class, season of birth, mode of delivery, newborn's sex, gestational age and birthweight. Estimates for each trimester were mutually adjusted. IRR: incidence relative risk. CI: confidence interval. T1: first trimester. T2: second trimester. T3: third trimester. 15-D: 15-days before delivery. P: whole pregnancy. *p-value<0.1; **p-value<0.05.

5. Conclusions

In conclusion, long- and short-term exposure to traffic-related air pollutants through fetal development impairs leukocyte and lymphocyte distributions in cord blood of neonates. Early and late gestation may represent windows of higher susceptibility of fetal immune system to air pollutants. Further research is warranted to unravel whether the potential fetal immunotoxicity of traffic-related air pollutants persists beyond birth and/or leads to adverse immune mediated diseases, including respiratory infections, asthma and other allergic manifestations.

Credit author statement

Eva Morales and Luis García-Marcos conceived and supervised the project and recruited the participants. Pedro Jiménez-Guerrero and María Muñoz-García provided air pollution data. Carmen Ballesteros-Meseguer and Irene Pérez de los Cobos collected the biological samples. Azahara M. García-Serna, Trinidad Hernández-Caselles, Esther Cantero-Cano, and Elena Martín-Orozco performed the experiments. Azahara M. García-Serna and Trinidad Hernández-Caselles analyzed flow cytometry data. Azahara M. García-Serna and Virginia Pérez-Fernández conducted the statistical analyses. Azahara M. García-Serna and Eva Morales conceived the study and wrote the paper. All authors have reviewed the results and contributed to the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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NELA study group

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