Prenatal maternal stress predicts reductions in CD4+ lymphocytes, increases in innate-derived cytokines, and a Th2 shift in adolescents: Project Ice Storm

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HIGHLIGHTS

- Prenatal maternal stress (PNMS) programs offspring immunity as far as adolescence.
- PNMS decreases the proportion of T-helper (CD4+) lymphocytes in offspring.
- PNMS increases the secretion of pro-inflammatory and Th2 cytokines in offspring.
- The observed immune changes could potentially link allergy susceptibility with PNMS.

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ABSTRACT

The relationship between psychological stress and immunity is well established, but it is not clear if prenatal maternal stress (PNMS) affects the development of the immune system in humans. Our objective was to determine the extent of this influence in a sample of teenagers whose mothers were pregnant during the 1998 Quebec ice storm. As part of a longitudinal study of PNMS, we measured the objective stress exposure and subjective distress of the women soon after the disaster. We obtained blood samples from 37 of their children when they were 13 years old to measure cell population percentages and mitogen-induced cytokine production. We found that the mothers' objective degree of PNMS exposure significantly predicted reductions in total and CD4+ lymphocyte proportions, increases in TNF-α, IL-1β, and IL-6 levels, and an enhancement of the Th2 cytokines IL-4 and IL-13. Sex and timing of PNMS exposure during gestation were also associated with some outcomes. These results show that PNMS is a programming factor that can produce long-lasting consequences on immunity, potentially explaining non-genetic variability in immune-related disorders. This information contributes to the understanding of the mechanisms underlying the influence of PNMS on immune-mediated disorders in humans.

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1. Introduction

There is increasing evidence documenting the pervasive influence of psychological stress on multiple levels of immune function [1]. However, evidence of effects of prenatal maternal stress (PNMS) exposure, that is, stress in the pregnant mother, on the developing immune system of the fetus and its long-term consequences is still scarce. PNMS is now recognized as an important programming factor or fine-tuning mechanism that adapts physiological development in preparation for postnatal life. The offspring's physiological responses are programmed during fetal life to perform under the environmental conditions experienced by his/her mother during pregnancy (e.g., high stress). However, if during postnatal life these conditions change (e.g., low stress), the programmed responses

of the individual's physiology will be maladaptive (e.g., altered stress reactivity) [2]. Such maladaptive changes would, in turn, exacerbate the risk of adverse health outcomes during childhood or adulthood.

Pregnancy modifies the maternal physiological response to stress, and thus the developing embryo–fetal immune system is protected in a number of ways from the influences of PNMS. First, stress responsiveness in the pregnant mother decreases with pregnancy progression [3]. Second, the placenta shields the fetus from glucocorticoids, the most well-characterized hormonal response to stress [4]. Finally, embryonic and fetal cells have different molecular receptor repertoires compared to adult cells [5], thus altering their susceptibility to these mediators.

Despite these protective mechanisms, PNMS has already proven to be a programming factor for other physiological parameters such as insulin secretion in humans [6], HPA axis reactivity [7] and brain development in animals [8], among others. In addition, recent epidemiological evidence strongly suggests a relationship between PNMS exposure and

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the development of immune-related disorders in postnatal life such as asthma [9].

If the changes in maternal physiology and the placenta only shield the fetus from the deleterious effects of glucocorticoids to a certain extent, alternative pathways might also play a role in the transmission of PNMS-programming signals. Mechanistically, the maternal stress response could reach the fetus through alternative pathways via non-classical mediators, for example via IL-6. This cytokine is induced by psychological stress [10], is able to cross the placental barrier during mid-gestation [11], and has hematopoietic and differentiation functions on multiple cell precursors [12].

To date, various animal experiments and two human studies have assessed the relationship between PNMS and postnatal immunity. We previously reviewed all available animal experiments (32 in total) [13], borrowing the concept of Windows of Vulnerability (WOV) from immunotoxicology [14,15] to analyze the effects of the timing of the stressor on immune outcomes. Likewise, we assessed the influence of the stressor's type and duration, the species, and the offspring's sex and age. The most replicated findings were a decrease in TNF-α, particularly in males, and sex-dependent changes in IL-6, both pro-inflammatory cytokines chiefly produced by immune cells from the innate arm of immunity. In the case of cytokines derived from cells of the adaptive arm, PNMS enhanced production of IL-4 and IL-5, which mobilize the immune system against extracellular parasites. In addition, cell population proportions – T cells in particular – were found to be susceptible only if PNMS occurred during the first WOV, which takes place in the early first trimester (i.e., "Stem Cell WOV"). When taken together, these results suggest that PNMS exposure induces a bias in the way T helper cells regulate their response following immune challenge, predisposing them to differentiate into type 2 cells (Th2). Th2 cells activate and regulate eosinophils, basophils, and certain subgroups of B cells, which are responsible for confronting extracellular parasites. However, abnormal Th2 activation has been associated with allergies, suggesting that this "Th2 shift" induced by PNMS could be a pathophysiological mechanism linking maternal distress and postnatal immune disease in offspring. This finding goes in line with the conclusions from both previous human studies [16,17]. Despite this body of evidence, there are still some gaps that need to be addressed. First, animal experiments require confirmation by human studies. Second, one of the aforementioned human studies collected PNMS information retrospectively (after at least 20 years), and only included women [16]. The other human study came from a disadvantaged urban population, in which the influence of asthma-linked exposures such as air pollutants, domestic allergens, and poor nutrition was not corrected for. Immune responses were measured in neonatal cord blood which is still under placental influence, and thus does not represent an independent picture of the state of the offspring's own immune system.

Our prospective longitudinal study, Project Ice Storm, is well-positioned to address some of these gaps in the knowledge on the role of PNMS on offspring immunity in humans. The objective of the present study was to analyze the long-term influences of PNMS exposure on immunity in adolescents. Two aspects of immune function were selected based on their importance and the weight of the experimental evidence found in our previous review of the animal literature. First, we sought to measure the proportions of different immune cells, focusing on lymphocyte populations, which would reflect a detrimental effect of PNMS on precursor immune cells. Second, we wanted to measure the capacity to produce cytokines, which reflects the ability of immune cells to communicate with each other. We tested cytokine responses after cell culture with two different mitogens: Lipopolysaccharide (LPS) and Phytohemagglutinin (PHA). LPS is derived from the cell wall of gram-negative bacteria, and mainly activates cells from the innate arm of the immune system (e.g., monocytes). PHA is a lectin extracted from the bean Phaseolus vulgaris, which activates T cells by agglutinating them with antigen-presenting cells. T cells are the leading cells in the adaptive arm of the immune system. Thus, the use of both

models of immune challenge allowed us to assess the state of intercellular communication in the two different layers of the immune system. In addition, we tested if the sex of the child or the timing at which the exposure occurred (WOV), contributed to explaining variance in these immune outcomes.

2. Materials and methods

2.1. Participants and recruitment

Project Ice Storm includes a cohort of women from Quebec, Canada who were pregnant during the 1998 ice storm crisis [18]. In January 1998, large parts of southeastern Canada were struck by a storm that brought heavy amounts of freezing rain. Massive layers of ice built up on the electrical transmission infrastructure, ultimately leading to its collapse. Depending on electricity for heating, many residents were displaced from their homes, and more than 27 people died from direct or indirect consequences of the storm, including hypothermia or CO_2 poisoning. Many others were injured, lost their jobs or their property. As such, these calamitous conditions generated different degrees of stress exposure in those affected. Women from the area that was most severely affected and who were pregnant during the storm (or became pregnant within 3 months after it) were recruited. This population is composed mostly of French-Canadian, upper-middle class families. The average age of the mothers at their child's birth was 30.2 (SD = 4.8 years). We invited all families who were still in the study (n = 89) to participate in this branch of the project. Of those, a subsample of 37 adolescents provided fasting blood samples: 16 females and 21 males (mean age 13.4 ± 0.1 years). The mothers of those who participated did not differ from those who did not, in any aspect of their ice storm experience (objective or subjective stress, days without power), nor in terms of socioeconomic status. On average, the families in the current project were without electricity for 16 days. Informed parental consent and child assent were obtained from all participants, and monetary compensation was offered. Blood draws took place in regional colleges by trained phlebotomists. The Research Ethics Board of the Douglas Mental Health University Institute approved all phases of the study.

2.2. PNMS study variables

Five months after the storm, on June 1, 1998, objective and subjective measures of the affected mothers' stress levels were determined from our initial postal questionnaire. The degree of objective exposure to the storm (the stressor) was assessed using a questionnaire that determined the degree of hardship in four categories: threat, loss, scope, and change [19]. The Scope category included items about the number of days the family went without electricity and telephone. Items regarding Loss asked about the amount of lost income, damage to the home, etc. Items in the Change category asked about the number of house moves the family made, the number of guests taken in, whether the family stayed together, etc. Threat items asked about the extent to which the woman and her family were in danger from a number of different hazards, if she or her loved ones had been injured, etc. Each of the four scales was designed to have a maximum value of 8 points, which are then summed to produce a total score with a maximum value of 32 points. A full presentation of the Storm32 items and scoring has been presented elsewhere [20]. We have shown that Storm32 objective hardship scores predict cognitive development [21], body mass index and obesity [22], and insulin secretion [6] in this cohort.

The subjective distress reaction to the storm was assessed using a French adaptation [23] of the Impact of Event Scale—Revised (IES-R) [24], a scale designed to reflect posttraumatic stress symptoms: intrusive thoughts or images, hyperarousal, and avoidance. The total score, being the sum of scores on all three scales, was used in all analyses. In

Project Ice Storm, maternal IES-R scores predict growth outcomes at birth [25], dermatoglyphic asymmetry [26], asthma risk in girls [20], and behavioral outcomes [27].

To determine the timing of exposure during pregnancy, we determined the embryonic days of each participant's exposure to the ice storm crisis, and extrapolated the concept and timing of WOV [15,28] from our previous analysis of the animal literature [13]. Briefly, the ontogeny of the immune system can be divided into five major WOVs corresponding to successive organizational/maturational stages: Stem Cell, Hepatic (hematopoiesis), Myeloid (hematopoiesis), Immunocompetence, and Memory. Because the review concluded that the effects of PNMS on cell populations were observed only during the Stem Cell WOV, a dichotomous variable (yes/no), "exposure during the Stem Cell WOV" (SC-WOV), was created to analyze the influence of timing on cell populations. Our initial questionnaire to the mothers after the ice storm included questions about the dates the power in their homes was lost and regained. Those participants whose mothers' first eight weeks of pregnancy (14 to 62 clinical days, or conception to 56 embryonic days, corresponding to the SC-WOV) overlapped with the days of the storm or the family's own period without electricity, were considered to be exposed regardless of whether the exposure started before conception or extended into the Hepatic (next) WOV. Twenty-four percent (nine participants) met these criteria (i.e., exposed during the SC-WOV). This was also carried out to enhance statistical power given the final number of participants.

2.3. Blood sampling and exclusion criteria

Th1 responses are enhanced in the summer [29] and T cell responsiveness is decreased during the winter [30]. In addition, cytokine production is sensitive to pollen production, which peaks in the spring and continues throughout the summer [31]. Blood samples for the current study were thus collected in October and November to avoid circannual influences on cytokine secretion. The sampling procedures took place between 7:00 and 9:00 am; all participants had been fasting for at least 8 h. Participants were queried on the day of the blood draw for the presence of exclusion criteria: active systemic inflammatory or obstructive respiratory symptoms (e.g., fever, malaise, sore throat) in the previous two weeks, and use of any cold medicine, analgesics/antipyretics, antibiotics, or inhaled or oral corticosteroids in the past two months. Ten mL of blood were collected by venipuncture in heparinized tubes and refrigerated at 4 °C until processing on the same day.

2.4. Flow cytometry immunophenotyping

An eight-color immunophenotyping panel was designed to assess major cell populations in circulating blood. The panel consisted of a combination of CD3/V500 (BD Biosciences), CD4/FITC, CD8/PerCP-Cy5.5, CD14/APC-eFluor780, CD16/PE-Cy7, CD19/PE, CD45/eFluor450, and CD56/APC (eBioscience). A direct staining procedure was performed based on the manufacturer's protocol. Briefly, 100 μL of whole blood were incubated with a mixture containing the complete panel of antibodies for 30 min in the dark at 4 °C. For compensation controls, extra samples were stained either with each single antibody or the respective eight Fluorescence-minus-one (FMO) combinations. Following this incubation, 1× red blood cell lysis buffer (eBioscience) was added for a reaction period of 15 min. After this procedure, samples were centrifuged at 350 g and 4 °C for 5.5 min. Supernatants were then extracted and cells were washed twice with 2 mL of FACS buffer, repeating the same centrifuge procedure. Samples were finally resuspended and fixed in FACS buffer with 1% Paraformaldehyde (PFA). Data were acquired from samples and compensation controls in a LSR-II cytometer (BD Biosciences™, San Diego, CA), using FACSdiva software (BD Biosciences™, San Diego, CA). The generated flow cytometry files were analyzed using FlowJo software (Tree Star, Ashland, OR).

The gating approach was performed using a combination of strategies and recommendations extracted from the literature [32]. An initial CD45/SSC plot was used to exclude remaining debris that was not discarded by hardware gating [33]. This was followed by the drawing of a lymphocyte-monocyte gate [34]. From this gate, monocytes were separated by CD14 levels, leaving an exclusive lymphocyte population. Next, B cells were separated using CD19 levels. The remaining types of cells were analyzed using CD3/CD4 and CD3/CD8 plots for T cells and a CD56/CD16 plot for NK-CD56dim and NK-CD56bright cells as described elsewhere [35].

2.5. Whole-blood cultures

To assess the functional state of cytokine function, whole-blood stimulated cultures were used to induce cytokine production; cytokines were then measured using multiplex bead-based immunoassays. Mitogen-stimulated whole-blood cultures are reliable and reproducible methods with multiple advantages [36]. The combination of whole-blood cultures and multiplex bead-based immunoassays has been previously endorsed [37]. Two different mitogens, LPS and PHA, were used to induce cytokine responses in whole-blood cultures, testing the two main arms of immunity. LPS is a constitutive macromolecule of the outer membrane of gram-negative bacteria. LPS signals via the Toll-like receptor 4, abundantly expressed in macrophages and polymorphonuclear cells, thus mainly activating innate cytokine responses [38]. PHA is a glycoprotein with strong mitogenic and agglutinating capacities which targets the adaptive immune system through its capacity to bind to the T cell receptor. PHA induces unspecific (antigen independent) T-cell activation, proliferation and cytokine secretion [39].

Succinctly, heparinized blood (0.5 mL) was diluted 1:5 with RPMI 1640 medium containing 2 μM L-glutamine (Sigma-Aldrich, Canada), 100 UI/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Canada), and LPS in 5 mL cell-culture tubes (BD Falcon). Samples from each participant were processed in three different culture tubes corresponding to the described experimental conditions: LPS (final concentration 10 ng/mL, extracted from E. coli, 42 K4120, serotype: 0111:B4 Sigma-Aldrich), PHA (final concentration 10 μg/mL, Calbiochem, San Diego, CA), and saline (controls). Tubes were incubated for 24 h under standard conditions (37 °C, 5% CO2, 95% humidity). After incubation, tubes were vortexed and centrifuged; supernatants were retrieved and stored at −80 °C until further analysis.

2.6. Cytokine bead arrays

Cytokine levels were measured using bead-based immunoassays (CBA: Cytometric Bead Array Flex-sets, BD Biosciences™, San Diego, CA) following the manufacturer's protocol. Two different cytokine panels were selected for each culture condition. The innate cytokine panel consisted of TNF-α, IL-1β, IL-6, IL-10, and IL-12, and was measured in the supernatants of LPS-stimulated cultures. The adaptive cytokine panel was comprised of IFN-γ, IL-4, IL-13, and IL-12, and was measured in the supernatants of PHA-stimulated cultures. Briefly, mixtures of standards, capture beads, and PE-detection antibodies were initially prepared. Assay tubes were then filled with 50 μL of sample (supernatants from whole-blood cultures) and standards. Fifty μL of mixed capture beads were added to both sample and standard tubes, and were incubated for one hour at room temperature. This was followed by the addition of 50 μL of the mixture of PE-detection antibodies for an incubation period of two hours at room temperature. After incubation, samples and standards were then rinsed in 1 mL of washing buffer and centrifuged at 200 g for 5 min. Next, samples and standards were fixed with 1% PFA followed by two washing steps with the same centrifuge parameters. Flow cytometric analysis was performed in a FACScalibur cytometer (BD FACStation™ v6.0x for Mac OS® X; BD Biosciences™, San Diego, CA) acquiring samples with the Cell Quest™

Pro software (BD Biosciences™, San Diego, CA). Beads corresponding to each cytokine cluster according to their fluorescence levels in the FL3–FL4 channels, and antigen-binding detection (corresponding to cytokine levels) is measured in the FL2 channel. For each sample (and standards), a maximum 300 events per cytokine cluster were acquired (1500 in total for 5 cytokines). Cytokine levels were then resolved by analyzing the generated flow cytometry files in FCAP array software (BD Biosciences™, San Diego, CA) matching mean fluorescence values against the respective standard curves.

2.7. Statistical analyses

We approached the data first by assessing the normality of the distributions of the variables using the Shapiro-Wilk test; variables that were not normally distributed were log-transformed, which yielded a better normal distribution fit. Next, we tested the zero-order correlations between the log-transformed main PNMS predictor variables (objective hardship and subjective distress scores) and the outcome variables (cell populations and cytokines). We then used multiple linear regression analyses to assess the influence of all predictor variables hypothesized to have an influence on the outcomes (Storm32, IES-R, sex, and exposure during the Stem Cell WOV). The limited number of participants restricted the inclusion of predictors to only three variables per model. Thus, to maintain statistical power, the interactions between independent variables were not included in the models. A priori significance levels were established at <0.05, although p-values of <0.10 were also noted. Data were analyzed using IBM SPSS Statistics 20 (2012, IBM Corp. Armonk, NY).

3. Results

We measured major lymphocytic populations and monocytes by using an eight-color flow cytometry panel for immunophenotyping that allowed us to acquire these values from the same blood sample. We also stimulated leukocytes in whole-blood culture to induce the production of innate-derived or adaptive-derived cytokines. We then analyzed both cell population and cytokine values using multiple linear regression models in which the levels of PNMS, being exposed during the Stem Cell WOV, and the sex of the participant were entered as predictors.

3.1. Descriptive statistics

The total number of participants was 37: 16 female and 21 male adolescents. From the total sample, 9 participants (2 female and 7 male) were exposed to the storm during the SC-WOV. The mean objective hardship (Storm32) score was 10.95 (SD = 4.19), and the mean subjective distress (IES-R) score was 9.58 (SD = 9.07). There were no significant differences in the mean objective hardship score between mothers of females (M = 10.69, SD = 4.62) and of males (M = 11.14, SD = 3.93), $t(35) = -0.32$, $p = 0.74$, or between SC-WOV exposure groups (exposed: M = 12.33, SD = 4.82, unexposed: M = 10.50, SD = 3.95, $t(35) = 1.15$, $p = 0.25$). Similarly, mean subjective distress scores had a comparable level between mothers of female (M = 8.55, SD = 8.07) and of male adolescents (M = 10.38, SD = 9.89), $t(35) = -0.60$, $p = 0.55$, as well as between SC-WOV exposure groups (exposed: M = 8.22, SD = 6.77, unexposed: M = 10.03, SD = 9.77, $t(35) = -0.51$, $p = 0.61$). The results from the Shapiro–Wilk test, assessing the normality in the distributions of the variables, indicated that the PNMS and cytokine variables were not normally distributed, as is often the case for immunological processes [40]. Thus, these variables were log-transformed. The improvement in the fit statistics observed in the models supported this measure. The same test indicated that the cell population variables were normally distributed.

3.2. Zero-order correlations between PNMS variables and outcome variables

The zero-order correlations between the predictor and outcome variables are shown in Table 1. Objective hardship was significantly negatively correlated with the percentages of total lymphocytes and CD4+ lymphocytes (Fig. 1), and there was a trend ($p < 0.10$) for a negative correlation between objective hardship and B cells. A marked and generalized tendency for a positive association between objective hardship and innate-derived cytokine levels was found, in which TNF-α (Fig. 2), IL-1β, and IL-6 were significantly positively correlated with logStorm32, and IL-10 reached borderline significance ($p < 0.10$). Levels of IL-12 were uncorrelated with objective hardship scores. In contrast, there was a differential association between objective stress and adaptive-derived cytokines depending on their type. Both Th2 cytokines, IL-4, and IL-13, had a strong positive correlation with objective hardship, while in the case of Th1 cytokines, the correlation was borderline significant for IL-2 and not significant for IFN-γ. None of the cell population or innate-derived cytokine variables were significantly correlated with IES-R subjective distress levels, which showed only a marginally significant correlation with logIFN-γ. In sum, maternal objective hardship, and not subjective distress in response to the storm, appears to be the active ingredient in the effect of PNMS on the development of immunity in the offspring, detectable in early adolescence.

3.3. Multiple linear regression models predicting outcomes using PNMS, exposure during the Stem Cell WOV and sex

Multiple regression analyses were conducted to predict cell population percentages and cytokine levels. As shown in Table 2, for cell populations, only the regression models of total and CD4+ lymphocytes (helper) were significant, explaining 22% and 21% of the variance, respectively. In both models, the level of objective hardship was a significant predictor, controlling for sex and Stem Cell WOV exposure, indicating a selective susceptibility of CD4+ cells during development to PNMS exposure. The effect of the exposure during the Stem Cell WOV was borderline significant only for total lymphocytes, while the sex of the participants had no influence in this sample. In contrast, CD8+ lymphocytes

Table 1
Correlations between predictor and outcome variables.

	Objective hardship	Subjective distress	N
Cell population percentages			
Total lymphocytes	−0.4268**	−0.2097	37
CD4+	−0.3821*	−0.1804	37
CD8+	−0.1863	0.0098	37
B cells	−0.3054†	0.0476	37
NK-dim cells	0.0413	0.0506	37
NK-bright cells	0.0691	−0.0823	36
Monocytes	0.0960	0.1167	37
Innate cytokine levels: pro-inflammatory			
logTNF-α	0.4567**	0.0776	36
logIL-1β	0.4171*	0.0073	37
logIL-6	0.4273**	0.0159	37
logIL-12	0.1394	0.0254	37
Innate cytokine levels: anti-inflammatory			
logIL-10	0.3065†	−0.0784	37
Adaptive cytokine levels: Th1			
logIFN-γ	0.2263	0.3165†	36
logIL-2	0.2957†	0.0081	37
Adaptive cytokine levels: Th2			
logIL-4	0.4370**	0.0507	36
logIL-13	0.4902**	−0.0255	35
Mean	10.95	9.58	
Standard deviation	4.19	9.07	

† $p < 0.10$.
* $p < 0.05$.
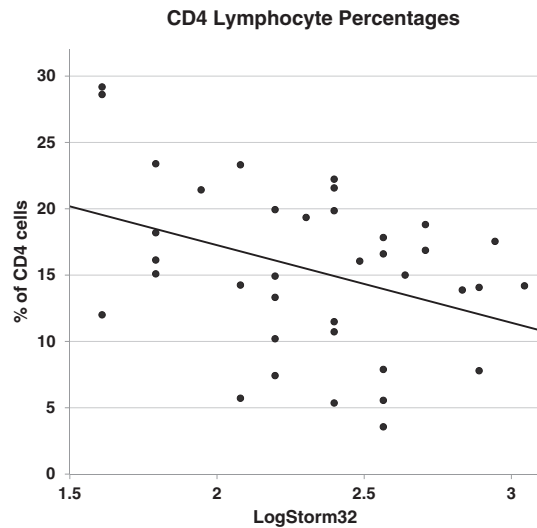** $p < 0.01$.

CD4 Lymphocyte Percentages

Fig. 1. Effect of exposure to objective hardship during prenatal life (LogStorm32) on the percentage of CD4 + cells at age 13½. Scatter plot showing the correlation between objective hardship (LogStorm32) and CD4 + cell percentages; r = −0.38, p < 0.05.

(cytotoxic), B lymphocytes, Natural Killer cells (dim and bright), and Monocytes appeared to be refractory to the effects of PNMS.

The regression models predicting the LPS-stimulated levels of innate-derived cytokines were significant for TNF-α, marginally significant for IL-1β and IL-6, and non-significant for IL-10 and IL-12 (Table 3). In the TNF-α model, the degree of objective hardship and the sex of the participant were significant predictors, and the model explained 27% of the variance. This result goes in line with prior animal and human studies, in which TNF-α secretion appears to be especially susceptible to the effects of PNMS.

The regression models predicting the PHA-induced production of adaptive-derived cytokines were significant for IFN-γ, IL-4, and IL-13, and marginally significant for IL-2 (Table 3). However, for IFN-γ, neither the level of objective hardship nor the exposure during the Stem Cell WOV were significant predictors. Thus, it was only the influence of the
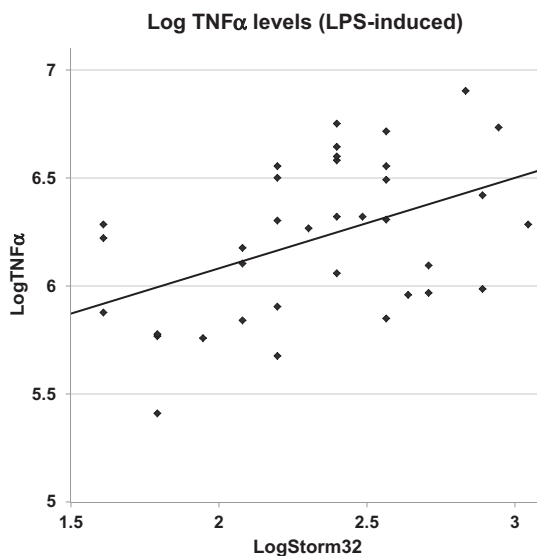
Log TNFα levels (LPS-induced)

Fig. 2. Effect of exposure to objective hardship during prenatal life (LogStorm32) on the production of TNF-α in culture with LPS at age 13½. Scatter plot showing the correlation between objective hardship (LogStorm32) and TNF-α levels (LogTNF-α); r = 0.46, p < 0.01.

sex of the participant which explained this association (higher levels in males). For IL-2, only the effect of the exposure during the Stem Cell WOV added to the marginal significance: levels were higher in exposed subjects. In contrast, the level of objective hardship was robustly significant for both IL-4 and IL-13, and the effect of the exposure during the Stem Cell WOV was borderline for the former and significant for the latter. The models as a whole explained 29% of the variance of IL-4 production and 51% of IL-13. These results strongly suggest that there is an enhanced susceptibility for the programming of Th2 cytokine responses by objective PNMS. Finally, the magnitude of subjective distress in response to the storm was not a significant predictor in any models (data not shown).

4. Discussion

PNMS is increasingly recognized as a programming factor for various physiological functions related to disease in postnatal life. The goal of the present study was to determine the long-term consequences of PNMS exposure on immune cell populations and stimulated cytokine production, which may shed some light on the mechanisms underlying the epidemiological association between PNMS and immune-mediated disorders, particularly asthma. The results presented here demonstrate that the degree of maternal exposure to objective hardship (but not maternal subjective distress) predicts the proportion of total and CD4+ lymphocytes, and cytokine production capacity at the innate and adaptive immunity levels, detected in early adolescence.

With respect to cell populations, PNMS significantly reduced the proportions of total and CD4 + lymphocytes, demonstrating this influence for the first time in humans. The effect of being exposed to PNMS during the Stem Cell WOV was marginally significant for the proportion of total lymphocytes (greater PNMS predicted fewer total lymphocytes), suggesting the existence of a period of enhanced susceptibility in the establishment of lymphocytic populations in humans, previously proposed by other authors [41], and expected from our review of the animal literature [13]. Removing sex from the regression model of CD4 + (thus enhancing power), increased the magnitude of the beta coefficient of SC-WOV from −0.226 to −0.309, and brought it close to significance (p = 0.063). This gives supporting evidence to indicate that CD4 + cell development in particular is sensitive to the effects of PNMS exposure during the SC-WOV. However, the limited number of participants might have hampered the clear-cut confirmation of this hypothesis; an effect of similar magnitude was found for NK bright cells.

We also identified a generalized enhancement of innate cytokine production in response to mitogens, which depended upon the magnitude of the exposure to objective PNMS, and was observed on both pro-inflammatory (TNF-α, and IL-1β) and anti-inflammatory (IL-10) cytokines. In the case of TNF-α, the model (including objective hardship, sex, and SC-WOV) explained 27% of the variance with a robust significance (p = 0.014), a result that goes in line with previous animal and human reports, confirming the particular susceptibility of this cytokine to the effects of PNMS. Regarding IL-1β and IL-10, the magnitudes of their objective hardship beta coefficients (0.306, 0.298) were comparable to the one observed in TNF-α (0.306), but having marginal significances (p = 0.066 and p = 0.074 respectively).

Finally, we showed that there was a significant positive association between objective PNMS and the production of the Th2 cytokines IL-4 and IL-13. In contrast, for the Th1 cytokines IFN-γ and IL-2, the effect was not significant. These four cytokines are the main determinants of the polarization of naïve CD4 + cells towards a Th1 or Th2 pattern, determining the type of adaptive immune response elicited after antigenic challenge. Therefore, our findings strongly suggest that PNMS induces a bias towards an increased Th2 responsiveness. Since abnormally active Th2 immunity is a central pathophysiological component of allergy [42], PNMS might enhance the risk for the development of allergic disorders during childhood and adolescence. The effect of the exposure during

Table 2
Multiple regression analyses (standardized coefficients): cell populations by sex, exposure during the Stem Cell WOV and objective PNMS.

	Total lymph.	CD4+	CD8+	B cells	NK-dim	NK-br	Mono
Predictors (beta):							
Sex (female)	−0.046	0.117	−0.046	−0.057	−0.238	−0.206	−0.260
SC-WOV	−0.276[†]	−0.226	−0.156	−0.016	0.112	−0.272	0.057
Objective hardship	−0.343[*]	−0.324[*]	−0.127	−0.268	−0.003	0.187	0.093
Model:							
N	37	37	37	37	37	36	37
R	0.467	0.461	0.212	0.272	0.286	0.339	0.303
R^2	0.218	0.212	0.045	0.074	0.082	0.115	0.092
F	3.071	2.967	0.518	0.876	0.979	1.388	1.112
p	0.041	0.046	0.672	0.463	0.415	0.264	0.358

[†] $p < 0.10$.
[*] $p < 0.05$.

the SC-WOV came close to significance for IL-2 and IL-4, and was strongly significant for IL-13, indicating a time-dependent programming effect on the cytokine production capacity of CD4+ cells.

The aforementioned results suggest that the development of the human immune system is susceptible to modification by PNMS. Moreover, such modifications may induce maladaptive programming of immune responses lasting into the second decade of life. This is the most profound implication, since the associations found in this study can establish a pathophysiological link between PNMS and postnatal deviations in immune function. Our results go in line with those from studies of early postnatal stress in laboratory animals showing a reduction in T lymphocyte populations: monkeys exposed in early life to prolonged stress (maternal separation for several months) had reduced levels of CD4+ and CD8+ cells [43]. This suggests that the impact of environmental stress on the immune system is amplified if the exposure occurs during early ontogenic periods.

The magnitude of the immune alterations documented in the present study might be regarded as limited since such changes would not result in immunodeficiency, and thus will not pose an immediate threat to the capacity of the immune system to withstand infections. However, the constant presence of a minor immune dysregulation might disrupt the individual's health in the long-term. In past decades, it has become evident that mild chronic inflammation is associated with a multitude of disorders such as cancer, obesity, diabetes, and heart disease, which are among the principal contributors of the mortality burden in western societies [44]. Exactly how the changes induced by PNMS contribute to the appearance of chronic disease is still unknown. However, some mechanisms can be hypothesized. In the case of the reduction in T lymphocyte populations, specific reductions in T cell subtypes have been associated with chronic disease. This is illustrated by the repercussions arising after the deterioration of the CD4+ cell pool occurring with HIV infection progression. The prolonged survival brought about by combined antiretroviral therapies has shown that there is an increased risk for the development of some infection-associated cancers not directly caused by HIV. This is the case of hepatocellular carcinoma caused by the Hepatitis B virus, which would be generally deterred by an intact immune system [45]. In addition, a recent epidemiological study has demonstrated a link between maternal bereavement and childhood cancer [46], providing additional evidence for the relationship between maternal stress and offspring immunity. Another mechanism that could link PNMS-induced reductions in T cells and chronic disease is inflammation. As mentioned above, chronic low-grade inflammation has been associated with metabolic disease. Reductions in specific types of T cell subpopulations might hamper the capacity of the immune system to control inflammation. In a recent study conducted in Denmark with subjects at risk of developing diabetes mellitus, a subpopulation of T cells bearing the Toll-like receptor 4 (TLR4+) was found to be (significantly) negatively correlated with fasting plasma glucose. Since TLR4+ T cells stimulate regulatory T cells, the authors propose that this change hampers immune counter-regulation and could be partially responsible for the increase in immune activation found in type 2 diabetes patients [47].

Regarding the exposure to PNMS during the Stem Cell WOV, the experience stemming from immunotoxicology studies [15,48] clearly indicates that the timing of exposure during development is a critical factor that determines the presence or the extent of some abnormal postnatal

Table 3
Multiple regression analyses (standardized coefficients): cytokines by sex, exposure during the Stem Cell WOV and objective PNMS.

	LPS-stimulated levels of innate-derived cytokines					PHA-induced adaptive-derived cytokines			
	Pro-inflammatory				Anti	Th1		Th2	
	logTNF-α	logIL-1B	logIL-6	logIL-12	logIL-10	logIFN-γ	logIL-2	logIL-4	logIL-13
Predictors (beta):									
Sex (female)	−0.421[**]	−0.180	−0.251	−0.231	0.038	−0.391[*]	−0.078	0.167[†]	0.122
SC-WOV	−0.100	0.121	0.135	−0.248	0.235	0.195	0.299[†]	0.272[†]	0.535[**]
Objective hardship	0.307[*]	0.306[†]	0.262	0.067	0.298[†]	0.176	0.209	0.417[**]	0.395[**]
Model:									
N	36	37	37	37	37	36	37	36	35
R	0.521	0.415	0.434	0.298	0.403	0.533	0.417	0.536	0.712
R^2	0.272	0.172	0.188	0.089	0.162	0.284	0.174	0.287	0.507
F	4.104	2.286	2.554	1.070	2.131	4.228	2.315	4.302	10.629
p	0.014	0.097	0.072	0.375	0.115	0.013	0.094	0.012	<0.001

[†] $p < 0.10$.
[*] $p < 0.05$.
[**] $p < 0.01$.

immune outcomes for specific environmental immunotoxicants. This aspect was evident in the present study, and is a step forward towards the documentation that specific periods of enhanced vulnerability to PNMS exposure exist in human immune ontogeny. This adds to the understanding of underlying pathophysiological mechanisms, and supports the use of WOVs in the design of future studies.

However, the most important implication is perhaps the documentation of the cytokine changes induced by PNMS: an enhancement in the production of TNF-α, and the induction of a Th2 shift. Both findings are of particular importance for asthma. As mentioned, one of the main pathophysiological components in allergic disorders is an immune Th2 imbalance. Second, TNF-α overactivity has been identified as an important component in a subgroup of asthmatic patients with severe refractory asthma [49]. Thus, this could be one of the potential missing pathophysiological links explaining the atypical characteristics observed in this subgroup of asthmatic patients, a condition proposed to be a distinct condition that forms part of the asthma syndrome [50].

There are some contrasting differences between our findings and those from the animal literature. Animal studies point to a reduction in CD4+ and increases or decreases in CD8+ cell percentages only when PNMS was induced during the SC-WOV. As indicated above, our results suggest that CD4+ cells are more sensitive during the SC-WOV, but we also observed this effect across gestation depending on the level of objective PNMS exposure. This disparity could arise from differences in the frequency or continuity of the stressor. While animal studies demonstrating this effect implemented a discrete, repetitive, and clustered psychosocial PNMS protocol (resident-intruder confrontation) [51,52], the stressor in our study (the ice storm) had a sudden onset, was intense, and in some cases the exposure extended for up to several weeks. Repetitive exposure to the same stressor (as was the case for the majority of animal studies used to determine a role for the SC-WOV) induces habituation [53], so its effects are likely to diminish over time. Thus, the important load of stress generated by the ice storm might have been of greater magnitude, and capable of overriding higher thresholds existing in the other WOVs.

The human study that tested the effects of PNMS on lymphocyte subpopulations in adult women [16] found no effects. This discrepancy might be explained either because they incorporated stressors with dissimilar magnitudes in the study group (e.g., death of partner, or being unmarried and the father not accepted by family), or by our use of a multiplex panel that allowed us to record all cell populations at the same time in the same sample.

Another puzzling contrast between our study and animal models of PNMS is the directional outcome observed in TNF-α production. A decrease in TNF-α levels after PNMS exposure has been consistently documented in macaques, swine, and mice regardless of the time of exposure and in different experimental conditions [54–57]. In contrast, we found that greater PNMS was associated with increases in TNF-α; this coincides with the results from the human study measuring cord blood cytokine responses which also detected increased levels associated with PNMS [17]. Given the similarities in the organization and development of the immune system between humans and other mammals, it is reasonable to believe that these disparities derive from differences in higher-order stress processing in the central nervous system. Such paradoxical results highlight the advantages and limitations of animal studies and underscore the need for human studies to ascertain the associations that exist between immunity and stress. The notion that the levels of stress produced by the ice storm are larger than those induced in animal studies is also supported by our cytokine results. We showed a significant correlation between PNMS and IL-1β, and a borderline significant increase in IL-10, which contrast with the negative associations found for these cytokines in animal studies of PNMS.

The small number of participants in our study was a limitation. Several correlations and regression coefficients with magnitudes as high as 0.30 would have been significant with a slightly larger sample. This is also illustrated by the magnitude of the beta coefficients of SC-WOV for CD4+ and NK-br cells, and of objective hardship for IL-1β and IL-10 in the whole models, which all had moderate effect sizes but were only borderline significant. Our study was also underpowered for testing interactions between predictors (e.g., sex or SC-WOV-by-PNMS). However, despite the modest number of participants, we had sufficient power to clearly demonstrate an important relationship between PNMS and the immune outcomes we studied. This indicates that the size of the effect of PNMS on the developing immune system is substantial. In addition, the general trend followed by innate and Th2 cytokine responses suggests that our findings were not spurious but the result of a pervasive influence of PNMS on common molecular mechanisms. Our results contribute to bringing our understanding of the implications of PNMS for immunity one step further by directly linking the levels of exposure to a specific event with two different immune parameters in postnatal life.

The conclusions we draw from our results are supported by the design of Project Ice Storm. First, a prospective determination of the influence of the two dimensions of PNMS – objective and subjective – was possible. Secondly, since exposures were randomly distributed across the affected population (correlations between Storm32 and socioeconomic status are < 0.20), associations between objective hardship and outcomes are unlikely to be confounded by any demographic or temperamental characteristics of the families; thus the ice storm acted as a "natural experiment". Unfortunately, the sample size limited our ability to control for any postnatal stressors statistically. In our analyses of the effects of PNMS on risk of asthma in this cohort, life events assessed at both 6 months and at 11 years were significant predictors; however, the inclusion of life events in the models did not reduce the effect size of PNMS, suggesting that prenatal stress and postnatal life events combine in an additive fashion to influence health outcomes. Furthermore, previous human studies were limited by the lack of information about the timing of exposure [16,17] or by the inclusion of only females [16], which are not limitations of the current study. The techniques we selected to assess immunity are simple yet robust, and have been previously validated. Whole blood cultures have the advantage of preserving the natural milieu in which immune cells exist, and better reflect the global individual state of immune responsiveness. The improvements in flow cytometry technology allowed for the measurement of multiple parameters in the same sample (leukocytes or cytokines) reducing added variability and increasing accuracy.

5. Conclusion

Exposure to PNMS affects the developing human immune system, precipitating long-lasting changes that can be observed in early adolescence. The present study documented a reduction in the proportion of the lymphocytic population, an enhancement of innate cytokine production, particularly TNF-α, and an imbalance in adaptive cytokine responses towards a Th2 pattern. As predicted by our analysis of the animal literature, the timing of exposure expressed as WOV contributed to explain individual variation in lymphocyte percentages. However, discrepancies with results from animal models regarding the direction of the results emphasize the need for additional human studies. The changes observed in the immune function of individuals exposed to PNMS could potentially exacerbate the postnatal development of immune-mediated disorders or diseases where immune impairments are central for their pathophysiology.

Abbreviations

APC	allophycocyanin
CD	cluster of differentiation
FITC	fluorescein isothiocyanate
IL	interleukin
LPS	lipopolysaccharide
mL	milliliter

NK-dim   natural killer cell CD56 dim
NK-br   natural killer cell CD56 bright
PE   phycoerythrin
PerCP   peridinin chlorophyll protein
PFA   paraformaldehyde
PNMS   prenatal maternal stress
SC-WOV   Stem Cell window of vulnerability
Th   T helper
TNF-α   tumor necrosis factor alpha
WOV   window of vulnerability

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