REVIEW ARTICLE

Prenatal maternal stress exposure and immune function in the offspring

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Abstract

The intra-uterine environment provides the first regulatory connection for the developing fetus and shapes its physiological responses in preparation for postnatal life. Psychological stress acts as a programming determinant by setting functional parameters to abnormal levels, thus inducing postnatal maladaptation. The effects of prenatal maternal stress (PNMS) on the developing immune system have been documented mostly through animal studies, but inconsistent results and methodological differences have hampered the complete understanding of these findings. As the immune system follows a similar ontogenic pattern in all mammals, a translational framework based on the developmental windows of vulnerability proposed by immunotoxicology studies was created to integrate these findings. The objective of this review is to examine the available literature on PNMS and immune function in the offspring through the above framework and gain a better understanding of these results by elucidating the moderating influence of the stressor type, timing and duration, and the offspring species, sex and age at assessment. The evaluation of the literature through this framework showed that the effects of PNMS are parameter specific: the moderating effects of timing in gestation were relevant for lymphocyte population numbers, Natural Killer cell function and mitogen-induced proliferation. The presence of an important and directional sexual dimorphism was evident and the influence of the type or duration of PNMS paralleled that of stress in non-pregnant animals. In conclusion, PNMS is a relevant factor in the programming of immune function. Its consequences may be related to disorders with an important immune component such as allergies.

Introduction

The important influence that psychological stress exerts on human health (Cohen et al., 2007) is significantly related to its close interaction with inflammation and immunity (Padgett & Glaser, 2003). However, this relationship is modulated in the maternal-fetal ensemble by several factors unique to this context. During pregnancy, important changes are needed to allow the support and immune tolerance of the fetus. These adaptations, largely mediated by the placenta, include modifications in the neuroendocrine response to stress and in immune function. Of particular relevance are the hypothalamic–pituitary–adrenal axis (HPA) hyporesponsiveness to stressors in late pregnancy (Brunton et al., 2008), and complex immunomodulatory changes necessary to avoid fetal rejection by the maternal immune system (Munoz-Suano et al., 2011). In addition, there is an important body of evidence indicating that the prenatal environment shapes developmental outcomes, programming postnatal function to be adapted to adverse conditions and that the appearance of maladaptive

physiological responses might occur when these conditions differ between pre- and postnatal life (Kuzawa, 2004).

Currently, the consequences of prenatal maternal stress (PNMS) exposure on the developing fetal immune system are not completely understood. Nevertheless, multiple lines of evidence are shedding some light on this issue. Human epidemiological research has demonstrated a link between PNMS and immune-mediated disorders such as asthma (Fang et al., 2011), and animal studies show that programming and long-lasting structural effects arise in individuals exposed in utero (Charil et al., 2010). However, animal studies looking at immune function after PNMS have been conducted in a variety of different species using diverse stressors and measuring heterogeneous parameters, complicating the translation of these findings to humans. Yet, the immune system of mammals across species has a similar organization, cell composition and developmental plan that allows for the creation of comparative models. The objectives of this review are: (1) to create a framework to compare the effects of PNMS on the offspring's immune system across species based on the chronology of developmental milestones; (2) to qualitatively analyze the available studies through this framework to elucidate the influences of the factors that modulate the effects of PNMS on offspring immunity: timing, type and duration of the prenatal stressor and the age, sex and species

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of the offspring and (3) use these results as a guideline for future research, and set the basis for a translational approach that advances the understanding of the effects of prenatal stress on human immunity.

Background

Windows of vulnerability (WOV) in the development of the immune system

The ontogeny of the immune system consists of a series of coordinated and consecutive processes of progressive complexity. During development, gene expression is continually changing, modifying the profile of receptor molecules in all cells and hence their responsiveness to environmental influences such as hormones, cytokines, toxicants and other signaling molecules (Munitic et al., 2004). This results in specific time frames in which some cellular elements become more sensitive than others. The recognition of these windows of vulnerability (WOV) is fundamental, not only for immunotoxicology and prenatal stress research, but also for the determination of the underlying molecular processes. In all mammals, the development of the immune system

follows a similar pattern of orderly successive events and reaches a mature state that is comparable in terms of organs, cells, receptors, cytokines, intracellular messengers and transcription factors among species, making extrapolation models possible. To analyze the effects of timing, we devised a timetable comparing the ontogeny of the immune system among the species used for PNMS studies and humans (Figure 1 and Supplementary Table 1). Due to the similarities in the duration of pregnancy and in developmental milestones between mice and rats, these two species were considered together (murine). Similarly, the duration of pregnancy is similar between macaques and squirrel monkeys, thus, these species were also analyzed together (non-human primates). However, there is little information on the developmental milestones of the immune system in the squirrel monkey, and the observations made using this species are approximate.

The timetable is divided into the five WOV proposed by immunotoxicological studies (Dietert et al., 2000; Holsapple et al., 2003). These windows represent the main organizational states through which the immune system goes from the appearance of the first hematopoietic stem cells (HSC) to the

Timing	WOV	Stem Cell: HSC generation	Hepatic: Liver hematopoiesis	Myeloid: Transition to bone marrow hematopoiesis	Immunocompetence: Functional maturation	Memory: Complete maturation
Species comparison	Human	First trimester		Second trimester	Third trimester	Birth to ~5 years
	Non-human primate	1st third		2nd third	3rd third	Postnatal to ~4 years
	Swine	1st third		2nd third	3rd third	Postnatal to ~7 months
	Murine	21 day gestational period		Early suckling (PND ~10)	Late suckling to weaning (21 PND)	Weaning to 8 weeks
WOV description	Milestones delimiting initiation of the WOV	Begins with the formation of angio-hematopoietic stem cells in mesoderm and yolk sac (all studied species).	Established by the migration of pluri-potent HSC to the liver, thus initiating liver hematopoiesis (all studied species).	Begins with the transition to, and establishment of the bone marrow as the primary and definitive lymphopoietic site (humans and murines). Beginning coincides with the appearance of proliferative capacities (response to mitogens) in T cells (swine) and the detection of B cells expressing sIgM (macaques).	Begins after the establishment of the first lymphocytic pool (end of lymphocyte burst: humans, murines and swine). Beginning coincides with demarcation of splenic architecture (macaques).	Characterized by the progression from a naïve immune response to mature levels of immunocompetence including immune memory (all studied species). The production of adult levels of IgG marks the maturity limit and the end of this WOV (all studied species).
	Key ontogenic events	Formation of lymphocyte (T and B) and granulo-macrophage cell progenitors. Organogenesis begins, e.g. formation of thymic rudiment and hepatic plate.	Definition of medulla and cortex in thymus. First lymphocyte gene rearrangements. M chain from IgM appears in cytoplasm of B cell precursors.	Gradual decline in hepatic hematopoietic function. Rapid initial expansion of T-cell populations (lymphocyte burst). Positive and negative selection in the thymus begins: first T-cell pool selection.	Acquisition of immunocompetence begins. NK cells become functional. Expansion of cellularity in the thymus. Th2 shift in humans, detected at birth.	Gradual acquisition of immune memory. Th1/Th2 cytokine homeostasis.

Figure 1. Timetable comparing the ontogeny of the immune system in humans and other species reviewed (non-human primates, swine and murines [mouse, rat]) with developmental windows of vulnerability (WOVs) as common reference points. Occurrence of WOVs are shown relative to gestation length for each species. The five progressive WOVs are indicated in the first row as column headings. The next four rows display gestational timing in each species for comparison. Gestation was divided into trimesters in humans, into thirds in non-human primates and swine, and into gestational and postnatal periods in murines. The sixth row lists the ontogenic milestones that delimit the WOVs. These milestones were used to match and extrapolate the gestational or postnatal ages at which these events occur in all the included species. The bottom row includes key ontogenic immune events occurring within each WOV that also guided the extrapolation of gestational ages, and can be used to understand some of the conclusions stemming from this review. Please note that this list is not comprehensive. The lengths of the column headings for the first four WOVs correspond to their relative lengths in human gestation; the proportional length of the Memory WOV is compressed in the illustration. Because ontogenic events in the immune systems of animal species take place at different times and at different rates compared to humans, gestation divisions in animals appear asymmetrical, despite representing equivalent proportions of gestation (i.e. thirds in non-human primates and swine). For example, the transition between the Hepatic and the Myeloid WOV occurs at the beginning of the second trimester in humans, but later in the second third of the non-human primate gestation, indicating that human immune ontogeny is comparatively faster at this point (in proportion to the complete length of gestation) despite the fact that the pregnancies of macaques and squirrel monkeys are shorter than human pregnancies. Note that the Myeloid and Immunocompetence WOVs take place postnatally in murines, in contrast to the other species. Timetables illustrating each study by species can be found in the Supplementary Material. This figure was inspired by figures found in Dietert et al. (2000), Buse (2005), Burns-Naas et al. (2008) and Šinkora & Butler (2009). Key references regarding the milestones and key ontogenic events: Bona (2005), Burns-Naas et al. (2008), Buse (2005), Holsapple et al. (2003), Šinkora & Butler (2009), West (2002). Abbreviations: CD3: Cluster of Differentiation 3; HSC: Hematopoietic Stem Cells; IgM and sIgM: Immunoglobulin M and Soluble Immunoglobulin M; PND: Postnatal day; Th1: T helper 1; Th2: T helper 2; WOV: Window of Vulnerability; in last column, rows 2,3,4, and in Myeloid column, Murine row~ indicates approximate age.

achievement of a complete and mature organizational ensemble. The boundaries of each window were assigned based on the available information, and using as many common reference points (milestones) as possible. The development of the immune system during fetal life has been reviewed by various authors and is beyond the scope of the present article. We refer readers to the works from which we have extracted this information (Bona, 2005; Burns-Naas et al., 2008; Buse, 2005; Holsapple et al., 2003; Šinkora & Butler, 2009; Tavian & Péault, 2005; West, 2002). In the present review, we will briefly describe main ontogenic events, especially those important to define these vulnerability windows. In chronologic order, the windows are: (1) Stem cell, (2) Hepatic, (3) Myeloid, (4) Immunocompetence and (5) Memory (Figure 1). It is important to note that although the events occurring during each WOV are similar, the degree of immune development at birth is different across species. For reference, the lengths of pregnancy are: 280 days (40 weeks) in humans, 168 days in macaques, 155 days in the squirrel monkey, 114 days in swine and 21 days in murines.

Stem cell WOV

The Stem cell WOV starts with conception and comprises the period of embryogenesis when totipotent stem cells are formed. During this period the first cell, the zygote, starts dividing and differentiating, going through a series of phases including the formation of the germ layers that give rise to the embryonic tissues, leading to organogenesis. The primordial liver appears during the 5th week in humans and 10th gestational day in mice (Houssaint, 1980), and the thymic rudiment is first observed at 6 weeks in humans, 10 days in murines, 38 days in swine and 35 days in non-human primates. All immune cells originate from HSC derived from the yolk sac and the embryo proper. HSC in the yolk sac are limited to production of myeloid precursors while in the embryo these cells also have lymphoid potential (Bona, 2005). This WOV ends with the migration of HSC to the liver, its colonization and establishment as a hematopoietic organ.

Hepatic WOV

The Hepatic WOV is marked by the beginning of hepatic hematopoiesis; the timing of this event has been determined in all four species, occurring from 7 weeks of pregnancy in humans (Holsapple et al., 2003), 10 days in murines (Cumano & Godin, 2007; Holladay & Smialowicz, 2000), 20 days in swine (Šinkora & Butler, 2009) and 35–40 days in macaques (Buse, 2005). This is a crucial period during which the first pools of T and B lymphocytes start to rearrange their genes, acquiring unique antigen specificity. Thus, this period marks the beginning of the formation of the repertoire of adaptive immune cells, documented by the expression of the specific antigen receptors in the surface of T (TCR) and B (sIgM) lymphocytes (~10–12 weeks in humans). In addition, this period is characterized by the appearance of secondary lymphoid organs such as the spleen, lymph nodes, tonsils, mucosa-associated lymphoid tissue (MALT) and Peyer's patches.

This period ends with the transition to bone marrow hematopoiesis which begins between 13 and 14 weeks in humans (Holsapple et al., 2003) and around birth in murines (West, 2002). There are no definite data on the initiation of this latter process in swine or non-human primates. To guide the selection of this cut-off point in swine, the capacity of T cells to proliferate after mitogen stimulation was chosen here because it occurs approximately at the same time as the beginning of bone marrow hematopoiesis in both humans and murines. This capacity appears in swine ~50 days (Trebichavský et al., 1996). Correspondingly, the assignment of this period in non-human primates was estimated from other concomitant developmental milestones. In macaques, immature B cells, expressing surface IgM, appear at 65 days of gestation (Bona, 2005), an event that occurs at 12 weeks in humans (Holt & Jones, 2000), 17 days in murine species (Holladay & Smialowicz, 2000; Holsapple et al., 2003) and 40 days in swine (Šinkora & Butler, 2009), just preceding bone marrow hematopoiesis. In addition, T cells express CD3 (common T lymphocyte marker, indicating differentiation) at 60 days (Buse, 2005) in macaques and at 10 weeks in humans (Holt & Jones, 2000). Given this information, the cut-off point for this WOV was arbitrarily established at 10–11 weeks (70–77 days) for macaques (and by extension, for squirrel monkeys). During this WOV, a defined cortex and medulla can be observed in the thymus (~12 weeks in humans and 65 days in non-human primates).

Myeloid WOV

The Myeloid WOV corresponds to the transition from hepatic to bone marrow hematopoiesis and its establishment as the definitive hematopoietic locus. Despite the fact that the presence of HSC in specialized mesodermal structures in the primitive bone marrow can be observed during the Hepatic WOV (~11 weeks in humans), it is only during the Myeloid WOV when cells with genuine hematopoietic potential start providing the fetus with immune cells (Tavian & Péault, 2005). As such, it is only during this phase that the bone marrow progressively assumes control of hematopoiesis. In contrast, thymic maturity is attained early in this WOV through the complete formation of its medulla (15 weeks in humans). This event concurs with the identification of clonal deletion of auto-reactive lymphocytes, and the detection of CD4+ and CD8+ cell exportation. By the end of this stage the first lymphocytic pool is formed. During this period, a rapid expansion of lymphocytic populations occurs. This event takes place in humans between 14 and 26 weeks of gestation, and provides a time reference for the delimitation of this WOV. Similar patterns have been described in mice, with this rapid population growth peaking at postnatal day 10 (Fagoaga et al., 2000), and in swine occurring between 60 and 80 gestational days (Šinkora & Butler, 2009). The end of this burst in T-cell numbers marks the end of the Myeloid WOV. This event has not been studied in non-human primates. For this reason, the demarcation of splenic architecture taking place at 26 weeks in humans (around the same time-point as the end of the described lymphocytic expansion) was used to delimit the end of this period in macaques, which takes place ~17 weeks of gestation (Buse, 2005). It is important to note that this and subsequent windows of vulnerability take place postnatally in murines.

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Immunocompetence WOV

At the beginning of the Immunocompetence WOV, the immune system is almost completely constituted at the anatomical and cellular levels. However, functional development is still taking place. The innate immune system shows functional capabilities before the adaptive immune system. For example NK cells at the beginning of this stage display some degree of cytolytic capacity against target cells (28 gestation weeks in humans and 3 postnatal weeks in mice; Holladay & Smialowicz, 2000). In contrast, the adaptive immune system is just starting to gain complex functions, and only later in this WOV (towards the end of the third trimester in humans), T lymphocytes acquire the capacity to produce some cytokines. In a human study comparing cytokine production in cord blood mononuclear cells (CBMC) induced by phytohemagglutinin (PHA, a T-cell mitogen) between fetuses and neonates, CBMC from fetuses were unable to produce interleukin-2 or -4 (IL-2, IL-4) or interferon gamma (IFN-γ), whereas CBMC from neonates were able (Zhao et al., 2002). These results indicate that some functional properties only appear close to birth in humans. Another factor that might contribute to the progression of maturity is the gradual expression of MHC class II molecules in the surface of monocytes in the same period of time (Jones et al., 2002). The capacity of the immune system to elicit an immune response, present at birth in humans, was used to determine the extent of this window in the other three species. This ability appears in the murine immune system around weaning (postnatal day 21, approximately) (Holsapple et al., 2003) and is assumed to be present soon after birth in macaques (Bona, 2005) and between birth and weaning in swine (Butler et al., 2009).

Memory WOV

The onset of the Memory WOV is characterized by a functional but naïve immune system. It is during this period when immunologic memory, the capacity to induce a faster and stronger response against a microorganism after a previous encounter, is progressively acquired. This WOV begins with birth in humans, after a short and variable postnatal period in non-human primates and swine, and after weaning in murines. The state of immunity at the onset of this period is better characterized by studies in human neonates. At this point there is a qualitative deficiency in immunocompetence as a result of the immaturity of some innate and adaptive processes, and a lack of adaptive memory cells. Mononuclear cells have reduced phagocytic capacities and cytokine production; there are fewer NK cells, which are hyporesponsive and show attenuated cytotoxicity compared to adult cells. T cells are functional and capable of responding to antigen stimulation, but with a reduced ability to proliferate and produce cytokines. CD8+ cells can have adult levels of cytotoxicity and other effector functions, and CD4+ cells exhibit a bias towards the generation of Th2 responses (Adkins et al., 2004). Humoral responses are characterized by a low production of IgG and IgA antibodies, and by a deficit in the response to antigens that does not depend on the interaction between T and B cells (''T-independent''), such as capsular polysaccharide antigens. This deficit explains the increased susceptibility of newborns and infants to some

bacterial infections, for example to group B streptococci. These deficiencies are gradually corrected with age.

The limits of this WOV are harder to define due to intraspecies fluctuation. The acquisition of adult levels of IgG was used as a proxy to set the end cut-off point. This occurs in humans ∼5 years of age (Millet et al., 1999), 45–56 days in mice (Silva-Lima et al., 2010), ∼7 months in swine (Klobasa et al., 1985), 4 years in macaques and after 2 years in the squirrel monkey (Coe et al., 1988)[1].

Considerations of the factors modulating prenatal maternal stress (PNMS)

The term ''stressor'' encompasses a broad range of potentially noxious stimuli that engage the central nervous system (CNS) in an integrated response directed to preserve or restore homeostasis. These stimuli may be of endogenous origin and produced by physiological processes (e.g. hypoglycemia), or exogenous and transmitted through processing in the CNS (e.g. maternal psychosocial stress). Although the neural circuits that process these signals overlap, the former primarily engage brainstem structures while the latter recruit limbic structures. Stress processing from these sources will ultimately be integrated in the hypothalamus, especially in the paraventricular nucleus, but the neuroendocrine response to stress would depend on the nature of the stressor (Ulrich-Lai & Herman, 2009). Exogenous/processive stressors may be further differentiated as neurogenic, i.e. those originating through physical stimuli (e.g. footshocks) or psychogenic, i.e. those elicited via psychosocial changes (e.g. housing with an unfamiliar conspecific) (Anisman & Matheson, 2005).

Neurogenic and psychogenic stressors not only have distinctive neuroendocrine responses, but also induce differential neuroimmune changes. For example, exposure to psychogenic stress increased the inflammatory response and mortality after lipopolysaccharide (LPS) injection in mice, whereas neurogenic stress did not (Quan et al., 2001).

Apart from the influence of the type of PNMS, the duration of the stress response has differential effects on the immune system. In general, acute stressors enhance innate immunity (Galon et al., 2002) and drive cytokine secretion towards an anti-inflammatory Th2 shift (Segerstrom & Miller, 2004), whereas chronic stress favors inflammation (Hänsel et al., 2010) and suppresses both Th1 and Th2 responses (Segerstrom & Miller, 2004). Given these differences occurring in non-pregnant animals, we sought to analyze the influence of the factors that modulate processive stress responses during pregnancy on offspring immunity.

Immunological consequences of PNMS exposure: animal studies

Description of the studies

Selection criteria

The present review focuses on the developmental immune outcomes in offspring caused by exposure to exogenous/

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[1]The memory WOV, taking place postnatally, cannot be considered here for the analysis of prenatal stress effects. This WOV was included in the framework to keep consistency with the definitions proposed by immunotoxicologists.

processive stressors during pregnancy, since these stressors are surrogates for psychosocial stress in humans. As such, endogenous/systemic stressors (e.g. hypoxia) were not considered. Similarly, pharmacological interventions (e.g. glucocorticoid injection) were also excluded because these models exclusively activate one of the several pathways recruited during the stress response and do not reflect the complete array of neuroimmune changes resulting from exogenous stressors. Moreover, as some authors have pointed out, the correlation between maternal glucocorticoids and stress in pregnancy is not strong (O'Donnell et al., 2009). In addition, stressors that are likely to activate additional homeostatic systems (different from the ones classically associated with psychological stress), such as the thermoregulatory response, were also excluded. For example, cold stress not only stimulates the HPA axis and sympatho-adreno-medullary axes, but also triggers the hypothalamic–pituitary–thyroid axis (Fiedler et al., 2006), which in turn induces metabolic changes specific to this response. Two human studies have assessed offspring immunity after PNMS exposure, which showed the induction of cytokine changes compatible with a Th2 shift in adult women (Entringer et al., 2008) and in neonatal cord blood (Wright et al., 2010). However, these important studies were not included in the analysis because they lack information about the timing or frequency of PNMS exposure (WOV) and include multiple types of PNMS in the same category.

Methods and analyzed factors

A literature search was conducted using four databases (MEDLINE, EMBASE, PsycINFO and AGRICOLA) to identify relevant publications dating between January 1990 and January 2012. Thirty-two animal studies that directly addressed the question of whether exogenous/processive PNMS (neurogenic or psychogenic) is capable of programming immune function in offspring were selected. The effects of PNMS on offspring immunity were qualitatively analyzed using factors from the prenatal stressor and the subject (animal), and immune outcomes from the offspring (not the mother). Thus, we assessed the influence of the type, timing (WOV) and duration/frequency from the prenatal stressor, and the species, sex and age at assessment for the subject (animal) on different offspring immune outcomes. Offspring immune outcomes were analyzed individually and clustered into eight categories to look for general patterns: cytokines (pro-inflammatory and immunoregulatory), cell populations, mitogen-induced proliferation, humoral immunity, cellular immunity, innate immunity and other findings (Supplementary Table 1). This categorization is based on function; however some categories are complementary (e.g. pro-inflammatory cytokines enhance cellular immunity).

The effects of PNMS on different immune outcomes in offspring

All studies exploring the same immune outcome in offspring were analyzed together by comparing the influence of the different PNMS factors searching for common patterns (Figure 2). For example, for a given outcome, changes might only be observed in offspring exposed to neurogenic

PNMS. Within those affected, the response could follow a sexually dimorphic pattern.

Pro-inflammatory cytokines

TNF-α was the most frequently studied cytokine; only the characteristics of the stressor appeared as relevant moderators of the effects of PNMS. Three studies in swine using psychogenic stress paradigms (crowded housing or with unfamiliar conspecifics) measured TNF-α levels; only one found reduced levels in PNMS-exposed offspring after blood culture with LPS (Couret et al., 2009a), while the other two studies did not find any changes *in vivo*, either basally or after LPS injection (Couret et al., 2009b; Sorrells et al., 2006). Three additional studies using neurogenic stressors (daily restraint, noise or both) showed a reduction in TNF-α production that was independent of WOV, stressor duration/frequency, species or offspring age and of whether TNF-α was measured *in vivo* (basal or after LPS injection) or *in vitro* (cultures with LPS, dendritic cells or saline-control) (Coe et al., 2002; Collier et al., 2011; Pincus-Knackstedt et al., 2006). When sex differences were investigated, TNF-α levels (basal or post-LPS injection) in males appeared more sensitive than females to PNMS (Collier et al., 2011). Overall, neurogenic PNMS had a more consistent dampening effect on TNF-α production than psychogenic stressors across multiple measuring conditions, with males being more sensitive.

Studies measuring IL-6 produced mixed results with an undefined response pattern. Effects were found after PNMS was induced during the Hepatic, Myeloid and Immunocompetence WOVs in rats, swine and macaques. Changes in IL-6 levels were found in most of the experiments, which mostly used neurogenic stressors. However, IL-6 levels were found to be either increased (Collier et al., 2011; Vanbesien-Mailliot et al., 2007), decreased (Coe et al., 2002) or unchanged (Couret et al., 2009b; Reyes & Coe, 1997). Methodological parameters could have accounted for these differences. For example, in one report, in which IL-6 production was induced with an intravascular infusion of LPS in PNMS-exposed swine during the Immunocompetence WOV, the response was enhanced by PNMS with a greater effect in females (Collier et al., 2011), while in macaques exposed in the same WOV, whole blood cultures stimulated with LPS yielded a decreased production of IL-6 (Coe et al., 2002).

Two murine studies that administered intense PNMS during the Hepatic WOV found opposite IL-2 responses: short-term continual PNMS (24 h constant noise for two non-consecutive days) lowered the IL-2 response in culture with dendritic cells (Pincus-Knackstedt et al., 2006), while long-term daily PNMS (3 times/day, 45 min, 12 consecutive days) elevated the basal mRNA levels for this cytokine (Vanbesien-Mailliot et al., 2007). Administering PNMS during the Myeloid and Immunocompetence WOVs did not produce any IL-2 differences basally, after LPS injection or in culture with lymphocytes (Coe et al., 1999; Collier et al., 2011), suggesting an early sensitivity period for the establishment of the mechanisms underlying the production of this cytokine.

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The same murine studies that explored IL-2 also measured IFN-γ levels. The levels of IFN-γ (in culture with dendritic cells) were not affected in the study that implemented a short-term, continual PNMS protocol (Pincus-Knackstedt et al., 2006). Nonetheless, the long-term daily PNMS exposure (Vanbesien-Mailliot et al., 2007) induced increased secretion, but only in adults after PHA stimulation, and not after LPS or when measuring IFN-γ mRNA. This change was not present in pre-pubertal animals when measured either basally or after PHA stimulation, suggesting an age-dependent effect.

IL-1β production seems to be refractory to PNMS. Two studies explored its production after an LPS challenge *in vivo* (basal or after LPS injection). IL-1β levels remained unchanged despite the fact that both experiments used restraint as a short or long-term stressor in different WOVs (Collier et al., 2011; Kohman et al., 2008).

Immunoregulatory cytokines

Immunoregulatory cytokines were also studied in the two murine models that applied PNMS in the Hepatic WOV. Short-term continual PNMS enhanced IL-4 production after stimulation (in culture with ionomycin or dendritic cells), although not basally (Pincus-Knackstedt et al., 2006). IL-10 was assessed by only one study, which concluded that it is not affected by long-term, daily PNMS (Vanbesien-Mailliot et al., 2007). Remarkably, both short- and long-term PNMS procedures increased IL-5 production (except in culture with PHA). Finally, in one study a pooled ratio of Th1 over Th2 responses (measured as TNF-α + IFN-γ/IL-4 + IL-5) showed that the short-term semi-constant PNMS protocol produced a lower Th1/Th2 index when these cytokines were measured after culture with no effect on baseline levels, thus indicating an overall imbalance towards Th2 responses (Pincus-Knackstedt et al., 2006).

In summary, the effects of PNMS on offspring cytokine production are evidently specific for each mediator, with some responses being more sensitive or refractory, and others being dose-dependent. Although more studies are needed, the available data suggest that PNMS may dampen Th1-related, and/or enhance Th2-related, responses. As both cytokine types are antagonistic and in equilibrium, this leads to a global imbalance in cytokine production in favor of Th2 cytokines: a Th2 shift. IL-4 and IFN-γ are two of the main inducers of CD4+ lymphocyte differentiation towards Th2 or Th1 patterns, respectively. Production of the two cytokines was enhanced by PNMS, but since IL-4 seemed to be more sensitive than IFN-γ, the influence of IL-4 might prevail over that of IFN-γ, thus driving differentiation of more T cells to a Th2 phenotype. However, with results from only two studies it is too soon to draw strong conclusions about such an effect. In addition, cytokines more directly involved with effector functions also followed this pattern: production of TNF-α was decreased while IL-5 was increased after PNMS exposure, making it more difficult to discern in which component of the immune response a Th2 shift would be generated. In the case of IL-6, the finding of contradictory results is not surprising due to its pleiotropic and cell-specific effects. It is still unknown in which layer of the immune response, innate or adaptive, this phenomenon is generated.

Cell populations

The effects of PNMS on cell populations varied depending upon the type of cells studied, the WOV, and the type and frequency of the stressor. Five studies implementing PNMS from the second half of the Hepatic WOV or through the Myeloid WOV failed to show changes in any of the cell populations measured regardless of the species, type and duration of the stressor or the assessment age of the offspring (Coe et al., 2002, 2007; Kay et al., 1998; Lay et al., 2008, 2011), suggesting a stress-refractory timeframe during and after mid-ontogeny of the immune system.

Nevertheless, PNMS induced changes in cell populations when implemented in the Stem cell and Hepatic WOVs. These effects might depend on an interaction of the timing and the frequency of the stressor since all four studies that induced PNMS on a daily basis (psychogenic or neurogenic) in this time-frame found differences (Götz & Stefanski, 2007; Götz et al., 2007; Llorente et al., 2002; Vanbesien-Mailliot et al., 2007), while the other two studies that implemented PNMS 2 or 3 times a week did not (Couret et al., 2009b; Mayer et al., 2011). It is important to mention that one of these studies found differences, but only after induction of an acute postnatal stressor (Mayer et al., 2011). Two studies by the same authors using the same psychogenic stressor protocol (resident-intruder confrontation) found a decrease in CD4+ lymphocytes, and no changes in B, NK, monocyte or neutrophil populations (Götz & Stefanski, 2007; Götz et al., 2007). Numbers of CD8+ lymphocytes remained unchanged but in one of these reports a decreasing trend was observed ($p = 0.06$) as well as a reduction in total lymphocyte and leukocyte counts (Götz & Stefanski, 2007). In the studies using neurogenic stressors, results appear to be contradictory: while CD4+ lymphocytes were not affected, CD8+ lymphocytes decreased (Llorente et al., 2002) or increased (Vanbesien-Mailliot et al., 2007). Apart from the type of stressor, the main differences between these studies were the frequency and length of the stress protocol: once a day for 8 days (Llorente et al., 2002) versus 3 times a day for 12 days (Vanbesien-Mailliot et al., 2007). Other cell populations were not included in either study and cannot be compared, but increases in NK cells (Vanbesien-Mailliot et al., 2007) and neutrophils (Llorente et al., 2002) have been reported.

A different picture emerges from the studies that induced PNMS in the Immunocompetence WOV. In one study on swine, total leukocyte, lymphocyte and granulocyte counts were decreased by PNMS (Couret et al., 2009a), but the same authors were unable to replicate these findings using identical protocols (Couret et al., 2009b). Two other studies in non-human primates were also unable to detect such differences (Coe et al., 2002, 2007). Finally, sex differences were not found in any of the aforementioned studies.

In summary, the most sensitive period in which white blood cell populations are affected by PNMS corresponds to the Stem cell and the early Hepatic WOVs. In contrast, in mid-ontogeny these blood cells are probably refractory. These changes seem to depend on the frequency of the stressor, occurring only if the intervention was applied on a daily basis. The most sensitive cells appear to be T lymphocytes. Interestingly, each subpopulation appeared to have a selective

sensitivity to the type of stressor; while CD4+ lymphocytes are more susceptible to psychogenic PNMS, CD8+ lymphocytes are susceptible to neurogenic PNMS.

Mitogen-induced proliferation

Mitogens activate specific lymphocyte populations independently of their antigen specificity. Hence, mitogens are commonly used to mimic an immune challenge and to assess how all the lymphocytes (T or B) in the target population respond to it.

Lymphocytic proliferation was explored in the reviewed studies using five different mitogens. To analyze the effects of PNMS on mitogen-induced proliferation in offspring, mitogen responses were clustered in two groups: T-cell predominant mitogens including concanavalin A (ConA), PHA and the Mixed Lymphocyte Reaction (MLR); and B-cell predominant mitogens such as pokeweed mitogen (PWM) and LPS (Andersson et al., 1977; Wimer & Mann, 2002). At the innate level, LPS is a major activator of monocytes/macrophages.

T-cell mitogens. The proliferative response of T-cells to ConA is generally not affected by PNMS exposure in the Hepatic WOV. In adult rats (Götz & Stefanski, 2007; Götz et al., 2007; Klein & Rager, 1995) or pre- or post-weaned swine (Couret et al., 2009b), proliferation levels were comparable to those of controls. In one study with rats between birth and weaning, this response was reduced but only at one assessment age, 14 postnatal days, while at 1, 7 or 21 postnatal days the effect was not observed (Sobrian et al., 1997). In addition, PNMS during the Myeloid WOV also had no effect (Couret et al., 2009b).

In contrast, when pregnant swine were exposed during the Immunocompetence WOV, the effects of PNMS on ConA-induced proliferation depended on its type, and the age of the offspring at the time of testing. Restraint, a neurogenic stressor, depressed proliferation only during the first week of life and not after three or five postnatal weeks (Otten et al., 2000; Tuchscherer et al., 2002). Conversely, psychogenic PNMS (conflict) enhanced this response consistently throughout the first four postnatal weeks (Couret et al., 2009a,b). This effect was sex specific, with females being more sensitive. These observations clearly suggest that the sensitivity period of ConA-induced proliferation in T-cells is limited to the Immunocompetence WOV.

All three studies that explored proliferation with PHA were performed in murines exposed to PNMS in the Hepatic WOV. One study, inducing PNMS with noise and light on three intermittent days, found a non-significant dampening trend (Kay et al., 1998). When chronic (7 or 12 days) daily neurogenic PNMS was imposed, an increased proliferative T-cell response was observed in adult (Vanbesien-Maillot et al., 2007) and weaning rats (Sobrian et al., 1997) but not in neonates (Sobrian et al., 1997).

The MLR was used as another measure of T-cell proliferation in one study with macaques. Earlier PNMS (Hepatic and Myeloid WOVs) amplified, while later PNMS (Immunocompetence WOV) minimized, this response (Coe et al., 1999).

In summary, changes in ConA-induced proliferation depended on the WOV, the type of stressor and the sex and age of the offspring, suggesting that while psychogenic prenatal stressors might enhance proliferation, neurogenic stressors produce an opposite effect and that a specific sensitivity period exists during the Immunocompetence WOV. PHA-induced T cell proliferation changes were influenced by the duration and/or frequency of PNMS and the age of the offspring. A threshold might exist, indicated by the finding that only prolonged exposures were able to elicit a significant effect. In addition, results using the MLR showed that opposite effects could arise from PNMS in different WOVs. When sex differences were explored, females appeared to be more susceptible to PNMS than males regarding lymphocyte proliferation.

B-cell mitogens. The proliferative response to PWM after PNMS exposure during the Stem-cell and Hepatic WOVs has been studied only in rats. The duration or type of PNMS had no influence on the proliferative response to PWM in the offspring. For example, a long-term psychogenic (resident-intruder confrontation, daily throughout pregnancy) (Götz & Stefanski, 2007; Götz et al., 2007) and a short-term neurogenic (noise and light on three separate days) (Kay et al., 1998) PNMS exposure dampened this response, while a long-term neurogenic protocol (noise, daily for 1 week) enhanced the response in the offspring (Sobrian et al., 1997). The only salient factor that could account for these differences is the age of the offspring at assessment: when rats were of an age corresponding to the human premature and neonatal periods, lymphocyte proliferation after PWM was increased (Sobrian et al., 1997), whereas in adolescent or adult rats this response was decreased (Götz & Stefanski, 2007; Götz et al., 2007; Kay et al., 1998).

In contrast, PWM proliferation after PNMS exposure during the Immunocompetence WOV has been assessed only in neonatal and suckling swine. In this case, the most important factor was the type of PNMS. In two studies inducing psychogenic PNMS the response was enhanced (Couret et al., 2009a,b), whereas in two other studies implementing neurogenic PNMS a reduction was documented (Otten et al., 2000; Tuchscherer et al., 2002). Sex differences were detected in the Hepatic and Immunocompetence WOVs, with females being more sensitive than males to the effects of PNMS regardless of the direction of the effect (enhancing or dampening). These findings indicate that PWM-induced proliferation is affected by PNMS through a complex interaction among the sex of the offspring, the type of stressor and its timing. In addition, the age of the offspring might have an influence as well, as the effect of PNMS on PWM-induced proliferation seemed to disappear with increasing age in some models (Otten et al., 2000; Tuchscherer et al., 2002).

Lipopolysaccharide-induced proliferation was measured in two studies. In the first, pregnant rats were restrained and exposed to light 3 times a day (45 min) from the end of the Stem cell WOV and throughout the Hepatic WOV (Vanbesien-Maillot et al., 2007). This intense procedure was unable to produce any proliferative changes in the offspring. In the second study, swine were restrained daily

(5 min) for the majority of the Immunocompetence WOV resulting in a decrease of this response (Tuchscherer et al., 2002), strongly suggesting that this WOV is indeed a vulnerability period for those processes related to LPS signaling, such as Toll-like receptor function.

In summary, proliferation stimulated by both B cell mitogens was influenced by the WOV, and in the case of PWM, by the type of stress and the age of the offspring at the time of assessment.

In conclusion, lymphocyte proliferation in offspring is affected by PNMS and its effects are importantly moderated by the timing and type of stressor, and the sex and age of the offspring: females appeared to be more vulnerable, the Immunocompetence WOV seemed to be the most sensitive period, the type of stressor defined the direction of the response and the age at assessment indicated that some responses were transient while others lasted until adulthood. No patterns arose when considering T or B lymphocyte mitogen responses as a whole; this might be a reflection of the different pathways activated by each mitogen.

Humoral immunity

Humoral immunity refers to the arm of the immune system whose effector elements are immunoglobulins (Ig), acting as antibodies. Five different types can be distinguished, and measured by immunoassays: IgG, IgM, IgA, IgE and IgD.

Passive immunity

Passive immunity, the passage of antibodies from the mother to the fetus through the placenta or to the infant via breastfeeding, substantially varies among species. First, differences exist in placental functioning and antibody permeability between species (Pentšuk & van der Laan, 2009). Second, murines are born in a much more immature stage than swine and non-human primates. These factors have to be considered for the analysis of the effects of PNMS on this parameter. In addition, since passive immunity depends on the maternal (not fetal) production of antibodies, here the concept of WOV is replaced by gestational timing, which influences placental permeability and maternal immune function across pregnancy. The most important factor that influences passive immunity is the type of stressor: studies implementing prenatal neurogenic stressors were more effective in decreasing offspring antibody levels than psychogenic stressors. Also, the effects on the offspring of psychogenic stressors last longer than neurogenic stressors in both rats and swine despite the fact that rats and swine have different types of placentation. One study in rats that implemented PNMS in the third week of pregnancy (fetal Hepatic WOV) used both types of PNMS: neurogenic (foot shock) and psychogenic (bystander stress: witnessing foot-shocks applied to another animal without receiving them itself). IgG levels in offspring of footshock-stressed dams were decreased in neonates, 7-day sucklings and at weaning (28 days) but not in 14- or 21-day sucklings. In contrast, levels of IgG in offspring of dams that witnessed the foot-shocked rats, without experiencing the footshocks directly, were decreased only in neonates, but no differences were found at other assessment ages (Sobrian et al., 1992). Interestingly, the same authors were unable to

replicate this pattern when using noise as a prenatal stressor (Sobrian et al., 1997). In swine stressed during the last third of pregnancy (fetal Immunocompetence WOV), a similar pattern emerged. Two studies using a neurogenic stressor (restraint) showed an antibody level decrease in neonates and sucklings in the first week, but not in the second or third weeks of life (Otten et al., 2000; Tuchscherer et al., 2002). A third study using a psychogenic stressor (housing with an unfamiliar conspecific) also in the last third of pregnancy (fetal Immunocompetence WOV) was unable to find these differences in the first week of life or after weaning (Couret et al., 2009a).

Two different studies implemented psychogenic PNMS protocols throughout pregnancy. Swine exposed to PNMS by living in crowded housing conditions had no antibody changes from birth to weaning (Sorrells et al., 2006), whereas squirrel monkeys prenatally stressed by being housed with unfamiliar conspecifics had higher IgG levels, but only in females (Coe & Crispen, 2000). The latter effect was not observed when these non-human primates were stressed only during mid-gestation (fetal Myeloid WOV). It is unknown how much of this difference depended on inter-species factors; however, since neurogenic stressors altered passive immunity independently from the species, it could also be possible that the contrast between these two studies reflects, at least in part, a difference within psychogenic PNMS protocols. The lack of effect when PNMS was implemented during mid-pregnancy in the squirrel monkey is congruent with the progression of antibody transfer to the fetus, which increases considerably ∼26 weeks of pregnancy in humans (Chucri et al., 2010).

In conclusion, passive immunity is altered by PNMS, and its effects depend mostly on the type of stressor but also on the duration of the stressor and the sex of the offspring. Neurogenic protocols were noxious, indicated by the longer duration of these changes. However, a prolonged exposure to psychogenic stress induced an antibody level increase only in female offspring, indicating a complex interaction between the type and duration of the stressor, and the sex of the offspring. The moderating influence of sex is in line with other findings indicating a sexual dimorphism in placental immunity (Clifton, 2010). The timing in pregnancy (not the WOV) also influenced the effect of PNMS on passive immunity. The temporal aspect of these changes may be related to a transient immunosuppression of the mother's antibody production in the later period of pregnancy. The long-term consequences of this temporary deficiency remain unknown.

Mature production of antibodies.   Fewer studies have explored antibody production beyond weaning. The study that compared both types of PNMS during the Hepatic WOV (foot shock and bystander stress) found differences in humoral function. When offspring from these pregnancies reached 5 weeks of age (34 days), males born from footshock-stressed dams, and females from dams that were stressed as bystanders had significantly lower total basal IgG levels after postnatal stress than their same-sex non-stressed counterparts (Sobrian et al., 1992). However, it is still possible that these differences depend, at least partially, on the remaining levels of maternal

antibody, since weaning occurred at 28 days. The half-life of rat polyclonal IgG is 63 h (Peppard & Orlans, 1980); this means that after 6 days ~20% of the initial maternal level of antibody was still circulating. In both rats and swine exposed to PNMS and assessed as juveniles or adults, basal levels of total IgG (Couret et al., 2009a; Lay et al., 2011) and IgG1, IgG2, IgM or IgA (Vanbesien-Mailliot et al., 2007) remained unchanged. Specific antibody responses after immune challenge were also studied. Some anti-immunogen titers were enhanced by PNMS in females (intraperitoneal Type-14 pneumococcal polysaccharide) (Bakker et al., 1998) or in both sexes (subcutaneous keyhole limpet hemocyanin) (Klein & Rager, 1995) whereas others were decreased (intraperitoneal herpes simplex virus-1) (Sobrian et al., 1997). Finally, in one study, specific anti-immunogen IgE titers remained unchanged while total IgE increased (intraperitoneal ovalbumin) (Pincus-Knackstedt et al., 2006).

In summary, the effects of PNMS on mature offspring humoral immunity are complex and depend on the type of stressor, the sex of the offspring and the immunological context in which antibodies were measured. The current number of studies is insufficient to draw definitive conclusions; however, some parameters were enhanced and in one case females seemed more responsive.

Cellular immunity

Cellular immunity is tested through the induction of immune reactions known to involve CD4-Th1 and CD8 cell activation. For example, in the delayed-type hypersensitivity reaction (DHR), an immunogenic adjuvant (e.g. tuberculin) is injected into the skin. After 48–72 h, T cells that recognize the immunogen infiltrate the injection site producing inflammation, which can be measured (e.g. degree of erythema). Despite the advent of more detailed ways of measuring cellular immune responses, data using these assays provide valuable information on the state of this arm of immunity.

Three similar studies with murines that implemented PNMS in the Hepatic WOV explored cellular immunity. The most critical factor appears to be the duration of the stressor. One study using acute neurogenic PNMS (saline injection, a procedure lasting a few minutes) on two non-consecutive days induced an increase in the DHR (Type IV) (Bakker et al., 1998). The other two studies using lengthier stress protocols found dampening responses (Gorczynski, 1992; Sobrian et al., 1997). Conditioned stress (transfer to a cage with cues associated with rotational stress) during three non-consecutive days of pregnancy suppressed skin allograft rejection in offspring, which substantially depended on Th1 cytokine secretion (Gorczynski, 1992). Noise stress for seven consecutive days decreased the DHR (Type IV) and suppressed a model of adjuvant induced arthritis, again showing that Th1/cellular responses were weakened (Sobrian et al., 1997). These results could mean that shorter exposures to PNMS enhance, while longer exposures depress, T-cell dependent reactions in offspring. In addition, the enhanced reaction that was associated with the use of a saline injection as a stressor in pregnant dams was observed only in their male offspring, indicating that a sex-specific sensitivity also exists for the cellular components of immunity. Since all three

protocols were implemented in the Hepatic WOV, it is still unknown if other WOVs are also sensitive.

Innate immunity

Innate immunity has been explored by measuring the functional activity of two of its main cell components: NK cytotoxicity and macrophage activity. NK cell cytotoxicity is assessed by measuring isotope or enzyme release from labeled tumor target cells in contact with NK cells. The influence of PNMS on NK cells is dependent on the timing of the stressor and the sex and age of the offspring. Two studies with rats using different PNMS protocols during the Hepatic WOV showed a reduction in NK cytotoxicity in the offspring, and in both studies males were more sensitive (Kay et al., 1998; Klein & Rager, 1995). In addition, this effect was observed in infancy and puberty, but not in adulthood (Klein & Rager, 1995). In macaques, the same effect on NK cells was observed when PNMS was induced during the Myeloid, but not the Immunocompetence, WOV (Coe et al., 2007). This latter observation was replicated in swine, which had normal NK cytotoxicity levels after PNMS during the Immunocompetence WOV, delimiting the sensitivity period for NK cells to earlier stages (Hepatic-Myeloid) (Tuchscherer et al., 2002).

Four aspects of macrophage activity have been explored: spreading and phagocytosis with microscopy; and nitric oxide (NO) and H_2O_2 liberation through spectrophotometry. Three different studies from the same laboratory assessed macrophage function, and all applied similar neurogenic PNMS protocols in mice during the Hepatic WOV. The first study found that PNMS depressed spreading and phagocytosis in 60-day-old offspring (Palermo Neto et al., 2001). The second study confirmed the first finding but showed that these alterations were not present at an earlier assessment in 37-day-old mice (Fonseca et al., 2002). In a third paper using more sensitive techniques, the authors were again able to demonstrate alterations in phagocytosis in both sexes and in oxidative burst in females, when measured in 30-day-old offspring (Fonseca et al., 2005).

In summary, NK cell and macrophage functions are sensitive to the effects of PNMS. Evidence from rats, swine and macaques indicate that NK cells are affected by earlier PNMS exposure. For both types of innate cells an interaction of PNMS with sex was evident: NK cells in males, and macrophages in females, were most sensitive to the effects of PNMS.

Other findings

The intestinal microbiota, which has a symbiotic relation with gut-associated immune cells, is also affected by PNMS. Bacterial counts were found to be decreased following PNMS exposure during the Myeloid and Immunocompetence WOVs, showing that the effects of PNMS might also alter mucosal immunity dynamics (Bailey et al., 2004). PNMS exposure during the Hepatic WOV reduced the severity of the Arthus reaction (Sobrian et al., 1997), a hypersensitivity response involving the formation and deposition of immune complexes with the participation of multiple inflammatory mediators, such as the C5a complement protein, chemokines (IL-8;

monocyte chemoattractant protein-1, MCP-1) and Th1 cytokines (IL-1, TNF-α). Animal models of immunity in asthma have demonstrated that exposure to early PNMS increased airway hyper-responsiveness (Pincus-Knackstedt et al., 2006) and leukocyte infiltration (Nogueira et al., 1999) (Hepatic or both Stem cell and Hepatic WOVs, respectively) after allergen sensitization, a finding compatible with human epidemiological data on this disease. A tumor model in mice evidenced increased neoplastic growth after exposure to PNMS in the Hepatic WOV (Palermo Neto et al., 2001). An additional study with rats demonstrated a dampening effect on stress-induced hyperthermia when PNMS was induced in the Hepatic WOV, indicating an alteration of basic neuroimmune responses (Hashimoto et al., 2001).

In summary, taking all these findings together, it is clear that the sum of immunological changes produced by PNMS can induce alterations in complex immune responses, consisting of either the restriction of normal responses or the promotion of abnormal ones. In some cases, for example when testing a model of an immune-related disorder, specifically asthma, these findings reinforce the notion that PNMS enhances Th2 immunity. In other cases, a dampening of Th1 responses was observed.

Analysis of the factors associated with PNMS

The analysis of the influence of the different PNMS factors was performed by integrating these from the initial analysis, controlling for other factors when possible, and searching for interactions among them (Table 1). This is illustrated in Figure 2, which shows how factors that depend on the prenatal stressor or the offspring might interact to program a specific immune outcome after PNMS.

Type of stressor

As discussed above, the two types of exogenous/processive stressors, neurogenic and psychogenic, induce distinct types of neuroendocrine responses (Figure 2, Maternal compartment). As such, divergent neuroimmune programming patterns after PNMS exposure by type were expected. Both types of PNMS are capable of influencing offspring immune function, but in general, neurogenic PNMS seemed consistently more effective in inducing an effect (e.g. in TNF-α production) than a psychogenic stressor. However, the picture is far more complex and the effects of the type of PNMS depended on interactions with other factors, in particular sex and the WOV. More often, the effects of neurogenic PNMS are detrimental while psychogenic PNMS has either stimulating or dampening effects. These differences indicate that the nature of the response produced by each type of prenatal stressor has a specific influence on ontogenic processes in the immune system, thus inducing distinct developmental changes.

Timing of stressor: WOVs

The use of WOVs was supported by the results of our review of the literature, which in some cases clearly showed well-defined thresholds for the emergence (or not) of a PNMS effect. The influence of the timing of PNMS exposure was

Table 1. Analyzed prenatal maternal stress (PNMS), factors and properties.

Factors	Properties	Examples
Stressor		
Type	Neurogenic	Light
		Restraint
		Electric shocks
		Saline injection
		Rotation
		Tail-suspending
		Chronic mild unpredictable
	Psychogenic	Housing unfamiliar conspecific
		Crowded housing
		Resident-intruder confrontation
		Witness of stress induction (bystander)
		Conditioned stress
Timing	Stem cell	
(immune system	Hepatic	
development	Myeloid	
window of	Immunocompetence	
vulnerability,	Memory*	
WOV)		
Duration/frequency	Daily	
	Intermittent	
	Constant	
Animal		
Species	Non-human primate	*Macaca mulatta*
		Saimiri boliviensis
	Swine	*Sus scrofa*
	Murines	*Rattus norvegicus*
		Mus musculus
Sex	Male	
	Female	
Age at assessment	Neonate	
	Infant	
	Puberty	
	Adult	

*The Memory WOV is exclusively postnatal, thus it was not included in the analysis.

relevant for several immunological outcomes, indicating that PNMS is able to program immune function depending on its timing (Figure 2, Embryo-fetal compartment). CD4+ populations (and perhaps other lymphocytic populations) appeared to be sensitive only during the Stem Cell WOV. Our analysis corroborates findings from other authors indicating that cell numbers are established very early in ontogeny, and that PNMS is able to alter this neuroimmune set-point (Coe & Lubach, 2000), implying that PNMS can affect developmental processes as early as the stem cell WOV. Hence, if PNMS exposure is able to create an imprint on multi-potent stem cells, this homeostatic change could be transmitted to their respective daughter cells, which would carry this alteration along the whole lifespan. Other outcomes with a well-defined WOV were IL-2 production and NK cytotoxicity. The latter is not surprising given that NK cell progenitors in mice are first recognized in the liver during postnatal day 13, corresponding to the Hepatic WOV (Bona, 2005). In addition, the first functional NK elements in humans can be demonstrated at the beginning of the Immunocompetence WOV (Uksila et al., 1983).

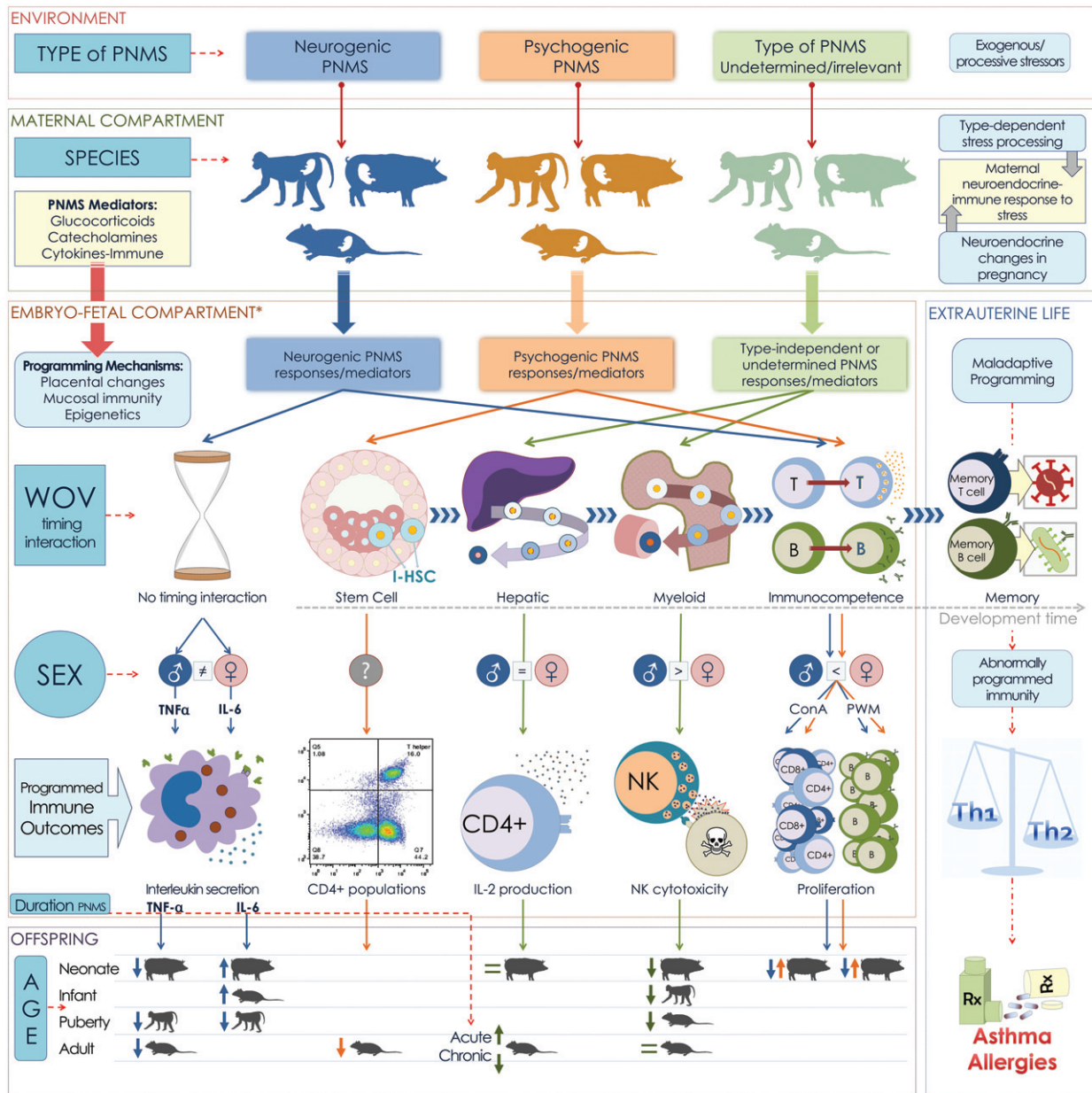Finally, various proliferative responses are modulated in important ways during the Immunocompetence WOV,

Figure 2. Summary of the results of this analytical review, showing the influence of the different prenatal maternal stress (PNMS) factors (type, window of vulnerability [WOV], and duration of the prenatal stressor; and the species, sex and age at assessment of the offspring), their interactions and the resulting programming changes occurring in different immune parameters in the offspring. The figure is divided into five different compartments to organize the sequence and localization of these events: Environment, Maternal compartment, Embryo-fetal compartment, Offspring and Extrauterine life. Box A, Environment: Different types of stressors arising from the environment affect pregnant animals. Box B, Maternal compartment: Depending on the type of stressor, the pregnant animal's nervous system differentially processes these noxious stimuli and induces a distinctive neuroendocrine response. This response is modulated by the changes induced by pregnancy on the neuroendocrine-immune response mechanisms. Box C, Embryo-fetal compartment: These PNMS type-specific mediators then directly or indirectly (e.g. through placental changes) reach the developing immune system. Influences arising from these types of PNMS and their interactions with other factors can be tracked by following the corresponding color-coded arrows: Blue: neurogenic, Orange: psychogenic, Green: type of stress not determined, or irrelevant. Influence of the different types of PNMS (through its mediators) depends on: Window of vulnerability (WOV; the developmental stage) at which the exposure occurs, or not: No timing interaction; on any sex-specific susceptibility of the developing offspring and on the extent (Duration PNMS) of the PNMS exposure. Analysis of resulting interaction of these factors showed some specific combinations can program a particular immune outcome in the offspring. Box D, Offspring compartment: During postnatal life, age of offspring when assessed influenced expression of some immune outcomes; these ages (neonate, infant, puberty and adult) are displayed by species. Most effects were detrimental, but depending on the offspring's age or the PNMS duration (Acute or Chronic) some immune parameters were enhanced, as indicated by symbols left of the animal icon (small arrows: ↑, increase, ↓, decrease; =: no effect). Box E, Extrauterine life. Overall PNMS could result in maladaptive programming of immune function, and disease, as PNMS increases production of Th2 relative to Th1 cytokines, dampening cellular immunity. Asthma and other allergies can result. Box C shows seven immune outcomes: changes in TNFα, IL-6 and IL-2 production, CD4+ populations, NK cytotoxicity and mitogen-induced proliferation, e.g. in Box A psychogenic PNMS induces specific PNMS mediators (thick orange arrow Box B to Box C); psychogenic PNMS during the Stem cell WOV programs (thin orange arrow) CD4 (T-helper) lymphocyte populations (see flow cytometry plot[#]) regardless of the sex of the developing offspring; the effect was documented in adult offspring (Box D) and was detrimental (small orange arrow) (Götz & Stefanski, 2007; Götz et al., 2007). More details are in in Supplementary Table 1. #CD3-CD4 flow cytometry plot for the detection of CD4+ T cells; these cells have high levels CD3 and CD4 surface markers (*x* and *y* axis, respectively), and CD4+ T cells are in the top right quadrant (positive for CD3 and CD4). Abbreviations: CD: Cluster of differentiation; CD4+: CD4 positive cell (T helper lymphocyte); ConA: Concanavalin A; I-HSC: immune hematopoietic stem cells; IL-2: interleukin 2; IL-6: interleukin 6; NK: natural killer cell; PNMS: prenatal maternal stress; PWM: pokeweed mitogen; Rx: prescription medicine; Th1: T helper 1; Th2: T helper 2; TNF-α: Tumor necrosis factor-alpha; WOV: Window of vulnerability. NOTE: *The Myeloid and Immunocompetence WOVs occur during extrauterine life.

suggesting a common mechanism sensitive to the effects of PNMS during this period.

Duration/frequency of stressor

Differences between the effects of acute and chronic stress on immunity have been widely documented despite a lack of consensus on how to (rigorously) define this distinction (Kusnecov & Rossi-George, 2002). Most of the studies included in this review employed chronic PNMS protocols, which facilitated our analysis of other factors, but made it more difficult to analyze the influence of the duration and frequency of PNMS. Despite the paucity of relevant studies, the influence of the duration of PNMS was evident, suggesting that acute (or at least brief) PNMS has enhancing effects while chronic stress is detrimental in most, but not all, cases (Figure 2, Offspring compartment). In addition, our review suggests that higher frequency PNMS exposures might be more effective in inducing an effect than less frequent exposures, regardless of the type of stress.

In summary, the frequency and duration of a PNMS protocol influence its capacity to program offspring immunity and the direction of its effects.

Species

By identifying the analogous milestones that define the boundaries of the same WOVs across different species, we expected to highlight any patterns in the influence of PNMS on offspring immunity that are dependent upon the timing of the stressor during specific stages of immune development. (Figure 2, Maternal compartment). In those cases when we were able to make a comparison controlling for other factors, the species did not affect the immune outcomes studied. These observations indicate that despite important disparities in placentation, pregnancy duration and fecundity, the mechanisms involved in the neuroimmune response to stress during pregnancy are preserved across the species studied. As such, animal models of PNMS make valid approximations to neuroimmune interactions occurring in humans.

Sex of offspring

The sex of the offspring is an important determinant of the effects of PNMS on the majority of outcomes except for total immune cell counts (Figure 2, Embryo-fetal compartment). In some cases, the effects of PNMS were observed only in males or only in females, while in other cases sex modulated the intensity or the direction of the response. Female offspring appear to be more susceptible to the effects of PNMS on lymphocyte proliferation, macrophage activity, passive immunity and specific humoral responses; some of these responses are enhanced. In contrast, males appear to be more sensitive to the effects of PNMS regarding TNF-α production, NK cytotoxicity and cell-dependent hypersensitivity reactions; these responses are more consistently suppressed. This pattern might be explained at the intracellular level by the direct interaction of sex hormones with immune-inflammatory pathways (Biswas et al., 2005) or by hormone-independent intrinsic sex-dependent differences (Khan et al., 2010).

Age of offspring at assessment

The effects of PNMS on some measures were detected only in younger offspring (Figure 2, Offspring compartment). This phenomenon might be partly explained by the constant renewal of cells occurring in the immune system and the phenotypic differences between fetal and adult-derived immune cells (Montecino-Rodriguez & Dorshkind, 2012). This finding suggests that the constant cell production process would eventually eliminate cells programmed by PNMS, replacing them with new, non-exposed leukocytes except in the case when this programming is induced early in pluripotent stem cells. A similar pattern can be observed in human diseases, where some immune-related disorders, such as rheumatic fever and asthma, are more prevalent during childhood (Carapetis et al., 2005; Myers & Tomasio, 2011). This argues for the presence of a developmental component in the etiology of some immune-related disorders. However, other changes persisted until adulthood, indicating a profound and long-lasting programming mechanism. The nature of such alterations might be explained by epigenetic modifications (O'Sullivan et al., 2012).

Discussion

Use of WOVs to analyze the effects of PNMS

Matching the gestational timing of exposure to noxious agents such as stress during pregnancy with underlying ontogenic events is fundamental to understanding which cellular and molecular processes are susceptible to the effects of PNMS on immune system ontogeny (Figure 1). Previous work has arbitrarily divided pregnancy into ''trimesters'' in swine or non-human primates, or matched human weeks with days in murines (2:1, respectively). Two observations illustrate that this approach has serious shortcomings. First, the speed of development varies widely across species, making the direct comparison of such periods among species impractical (Figure 1). Second, and most importantly, as immune ontogeny stretches well into postnatal life, the physiological impact of the events that occur during and after birth on immune development vary across species, especially for murines.

Overall conclusions

Analysis of the literature, using the WOV framework, produced three main conclusions. First, the available research supports the existence of a PNMS-sensitive homeostatic set-point during embryonic life (Stem cell WOV) that determines postnatal lymphocyte numbers, as previously proposed by other authors (Coe & Lubach, 2000). This suggests that PNMS has the potential to inhibit stem cell proliferation, a phenomenon already documented after stress exposure in postnatal life (Schraml et al., 2009).

Second, the presence of specific vulnerability periods in immune ontogeny for various processes was supported. These observations could provide clues about the underlying mechanisms responsible for PNMS-induced changes. For example, during the Hepatic WOV, thymic cortico-medullary specialization arises (Millet et al., 1999) and by the beginning of the Myeloid WOV the medulla is fully formed and

thymocytes respond to mitogens (West, 2002). This implies that the effects of PNMS on lymphocyte proliferation, which were more consistent in the Immunocompetence WOV, would not depend on an alteration of the organization of thymic architecture but on processes related to thymocyte maturation, during or after positive and negative selection

Finally, multiple findings in the literature suggest that PNMS is able to program an immune imbalance, skewing the system towards the production of Th2 responses. The most consistent finding, the dampening of TNF-α production observed despite the use of different measuring conditions/techniques, attests to this hypothesis. Concurrently, the Th2 cytokines IL-4 and IL-5 were increased in PNMS-exposed animals. The attenuation of Th1-dependent responses (e.g. DHR, Arthus reaction and adjuvant induced arthritis), and the enhancement of Th2 immune processes (e.g. increased IgE levels associated with intensified airway reactivity), further reinforces this notion. These findings are in line with the results from the two human studies that are available to date exploring the association between PNMS and postnatal cytokine production. Both found an enhancement of Th2 cytokine production after PNMS exposure (Entringer et al., 2008; Wright et al., 2010).

Underlying mechanisms potentially mediating the effects of PNMS

The pathways through which PNMS induces developmental changes in the immune system are not completely understood. Nevertheless, multiple mediators have the potential to convey this influence through various mechanisms (Figure 2, maternal compartment).

Mediators

Although the stress response is comprised of a broad array of mediators, glucocorticoids play a predominant role. Multiple lines of evidence exploring the programming role of stress in adult disease point towards critical aspects in the biology of glucocorticoids in pregnancy, suggesting that these hormones act as the principal pathophysiological mediators in this relationship. Such aspects include alterations of the placental 11β-hydroxysteroid dehydrogenase Type-2 enzyme, changes in the fetal HPA axis activity, and imbalances between the glucocorticoid and mineralocorticoid receptors (Harris & Seckl, 2011). Moreover, exposure in pregnancy to excess levels of glucocorticoids through injection or induced stress has already proven to program blood pressure levels, glucose homeostasis, muscle and fat metabolism, and behavior (Drake et al., 2007), indicating that a wide variety of cells can be programmed during development. Additionally, sexual dimorphism is one of the observed patterns in our analysis that might be explained by glucocorticoid biology. Glucocorticoid signaling in adulthood has sexually dimorphic effects on the expression of inflammatory genes, having a more extensive influence in males (Duma et al., 2010). Thus, given the close relation between these hormones and the immune system, and the documented effects of glucocorticoids on the development of other tissues, the programming of glucocorticoid-related factors by PNMS might explain in part why the sex differences in prevalence rates are more

pronounced in conditions for which the immune system plays a central role, such as allergies (Kurukulaaratchy et al., 2011) and autoimmune disorders (Beeson, 1994).

Catecholamines mediate the sympatho-adrenal response to stress (Ulrich-Lai & Herman, 2009) and directly regulate multiple aspects of immunity. In particular, these mediators are strong promoters of Th2 immunity (Elenkov, 2007), a phenomenon which we propose is induced by PNMS. Whether and how catecholamines, as part of the stress response in pregnancy, could program immune ontogeny in the fetus, is still an unanswered question. Nevertheless, despite a lack of innervation of the placenta, noradrenaline is capable of crossing the placental barrier (Morgan et al., 1972).

An alternate pathway through which PNMS might affect fetal development is via the maternal immune system. One compelling example is IL-6. This versatile cytokine, which is induced by stress (Steptoe et al., 2001), has inflammatory, anti-inflammatory (Scheller et al., 2011), neurotrophic (Spooren et al., 2011) and lymphocyte differentiation effects (Diehl & Rincón, 2002). It has been demonstrated that IL-6 is capable of crossing the placenta although, interestingly, only during mid-gestation (Dahlgren et al., 2006). Additionally, developmental changes in the brain have been proven to occur after prenatal induction of IL-6 and to be prevented by blocking this mediator (Aguilar-Valles et al., 2012). Taken together, these data show that IL-6 has the potential to act as a PNMS-associated agent in the programming of immune ontogeny.

Mechanisms

Another important question is how PNMS mediators influence immune development. The extent to which these mechanisms are adaptational or pathological is still the focus of active investigation and at the core of discussion of the developmental origins of health and disease (McEwen & Wingfield, 2010).

The mediators through which PNMS reaches to the developing immune system require altering and/or overriding the placental mechanisms shielding the fetus from external influences (Figure 2, embryo-fetal compartment). As an example, PNMS downregulates the placental expression of the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which shelters the fetus from excessive glucocorticoid exposure (O'Donnell et al., 2012).

In addition, there is a close relationship between commensal bacteria and cells of the innate immune system, where signals from the former regulate the latter. Reductions in gut microbiota resulted in increased numbers of basophils having an abnormal Th2 activation state leading to allergic inflammation (Hill et al., 2012). Such reductions in microbiota were observed in PNMS exposed monkeys, measured as late as 24 weeks of age (Bailey et al., 2004). These discoveries combine to suggest a new potential pathway linking PNMS with Th2 responses and allergy.

Finally, epigenetic changes provide an important molecular substrate that explains prenatal programming of disease in adult offspring. In rats, induction of neurogenic PNMS resulted in CpG site-specific methylation changes in the promoter of the 11β-HSD2 gene in the placenta and fetal

hypothalamus (Peña et al., 2012), demonstrating the capacity of PNMS to induce epigenetic alterations within and beyond the placenta. In humans, severe stress during childhood (e.g. parental loss) was associated with increased methylation in the promoter of the glucocorticoid receptor gene (Tyrka et al., 2012), linking early-life stress with long-lasting epigenetic changes in leukocytes. In addition, atopic dermatitis patients show, relative to control subjects, significantly decreased methylation in the promoter of the high-affinity immuno-globulin-E receptor in monocytes. Moreover, an inverse correlation exists between methylation levels and receptor protein levels (Liang et al., 2012) illustrating an epigenetic-dependent immune alteration related to an allergic disorder. The above examples strongly suggest that PNMS may act via epigenetic modulation to disrupt immune homeostasis.

Implications and future perspectives

A comprehensive elucidation of the effects of PNMS on immunity would have indirect and direct influences on various fields, potentially improving human health. Indirectly, reducing stress in pregnant farm animals will make their offspring less susceptible to subsequent disease in postnatal life. This would subsequently decrease the need for pharmaceutical treatments in these animals, in turn decreasing the levels of such compounds in consumed animal products. Directly, if current evidence indicating that allergies and other immune-mediated disorders are enhanced in PNMS exposed individuals is fully confirmed, a new level of insight into the molecular and pathophysiological aspects of these conditions could be attained. Such an advance would be reflected in better preventive strategies, improved personalized healthcare and possibly in the form of new treatments. In addition, the promotion of a healthy stress-free environment in pregnancy could reduce the personal, social and economic burden of allergic disorders.

Forthcoming studies on the effects of PNMS on immunity could benefit by taking into account the influence of timing and other potential moderating factors. The identification of vulnerability periods is central to understanding the environmental-genetic dynamics that impact intrauterine development. In addition, the marked sexual dimorphism observed in some outcomes should encourage researchers to design studies capable of testing sex differences. The use of such methods would facilitate directing newer efforts to the unmasking of the mechanisms responsible for the programming of immunity during development. In particular, epigenetic changes provide a molecular foundation capable of explaining current psychoimmunological models.

Concluding remarks

Prenatal maternal stress has proven to be an important programming factor for many aspects of postnatal immunity in the offspring of all the species in which this relationship has been studied. Multiple characteristics of the stressor and the stressed offspring intervene in the modulation of the effects of PNMS on the developing immune system, in some cases completely determining the programming (or not) of an outcome. In particular, the timing of the stressor, expressed in relation to WOVs, delimits the capacity of PNMS to have an

influence on most outcomes of the offspring's immunity, and in some cases modifies the quality of the response. This factor was the backbone of our analysis. Through the use of a framework that corrects for the differences created by different maturational timing in the studied species using WOVs, it was possible to evaluate the extent of the influence of other factors. The sex of the offspring and the type of the stressor also proved to be relevant. In contrast, the species of the animal had little influence, implying that animal models of PNMS exposure and postnatal immunity in offspring might be partially applicable to humans. Finally PNMS, by virtue of its specific effects on postnatal immunity, in particular through its promotion of Th2 responses, is a candidate factor in the prenatal programming of allergic disease during postnatal life (Figure 2, Extrauterine life).

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