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Maternal immune stimulation during pregnancy shapes the immunological phenotype of offspring

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ABSTRACT

Epidemiological studies have associated infection during pregnancy with increased risk of neurodevelopmental disorders in children, which is modeled in rodents by stimulating the immune system of pregnant dams with microorganisms or their mimics, such as poly(I:C) or LPS. In two prenatal mouse models, we show that in utero exposure of the fetus to cytokines/inflammatory mediators elicited by maternal immune stimulation with poly(I:C) yields offspring that exhibit a proinflammatory phenotype due to alterations in developmental programming of their immune system. Changes in the innate and adaptive immune elements of these pro-inflammatory offspring result in more robust responses following exposure to immune stimuli than those observed in control offspring from PBS-injected pregnant dams. In the first model, offspring from poly(I:C)-injected immunologically naïve dams showed heightened cellular and cytokine responses 4 h after injection of zymosan, a TLR2 agonist. In the second model, using dams with immunological memory, poly(I:C) injection during pregnancy produced offspring that showed preferential differentiation toward Th17 cell development, earlier onset of clinical symptoms of EAE, and more severe neurological deficits following immunization with MOG_{35-55}. Such "fetal programming" in offspring from poly(I:C)-injected dams not only persists into neonatal and adult life, but also can have profound consequences on health and disease.

1. Introduction

The epidemiological and clinical evidence for a causal relationship between bacterial or viral infection of women during pregnancy and increased risk of neurodevelopmental disorders [such as Autism Spectrum Disorders (ASD) and schizophrenia] in their children is compelling (Brown, 2006; Brown and Derkits, 2010; Hagberg and Mallard, 2005; Pardo and Eberhart, 2007; Sorensen et al., 2009). Moreover, the etiology of these disorders appears to involve, in part, pro-inflammatory mediators produced as a result of maternal infection (Jonakait, 2007; Patterson, 2011). The mechanisms that underlie these clinical observations have been studied in prenatal rodent models, in which pregnant dams are injected with either infectious pathogens or synthetic agents that mimic viral or bacterial infections [namely, lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly(I:C)], or cytokines they induce (e.g., IL-1, IL-2, and IL-6) (Gilmore et al., 2005; Meyer and Feldon, 2012; Nawa and Takei, 2006; Ponzio et al., 2007; Smith et al., 2007). Offspring of these immunostimulated dams exhibit behavioral abnormalities, as well as, chemical, and structural anomalies of the brain, which are similar to those seen in individuals with autism and schizophrenia (Fatemi et al., 2002; Makinodan et al., 2008; Meyer et al., 2008; Shi et al., 2009).

While much has been published on immunological parameters exhibited by children diagnosed with ASD (Arrode-Bruses and Bruses, 2012; Ashwood et al., 2011, 2006; Ashwood and Van de Water, 2004; Brown and Mehl-Madrona, 2011; Jyonouchi et al., 2011, 2012; Michel et al., 2012), much less has been published about how maternal immune stimulation of pregnant rodent dams affects the development and function of the peripheral immune system in their offspring. We have previously shown, however, that offspring of immunostimulated pregnant dams exhibit accelerated development and heightened responsiveness of Th1, Th17, and cytotoxic effector T cell subsets, indicating a pro-inflammatory phenotype (Mandal et al., 2010, 2011; Ponzio et al., 2007). Others have now also shown effects on T cells in offspring of pregnant dams that receive immune stimulation (Hsiao et al., 2012).

Two of our previously published observations provided a rationale for the experimental design described in this study. These are

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the influence of maternal immune stimulation during pregnancy on (a) T cell differentiation following in vitro activation, and (b) upregulated expression of TLR in offspring. Spleen cells from offspring of immunized, poly(I:C)-injected pregnant dams preferentially differentiate to become Th17 cells following in vitro activation. Such preferential differentiation is not seen in offspring of poly(I:C)-injected immunologically naïve pregnant dams or in offspring of PBS-injected dams regardless of their immune status (Mandal et al., 2010, 2011). In addition, the constitutive expression of TLRs (including TLR2, 4, and 7) are upregulated in offspring of poly(I:C)-injected pregnant dams in comparison to offspring of PBS-injected pregnant dams (Ponzio et al., 2013).

Thus, in order to document that these offspring possess and exhibit a proinflammatory phenotype, we chose two in vivo experimental models for further examination. The first was an acute inflammatory response based on stimulation of the innate immune elements with TLR2 agonist, zymosan. The second was the well-characterized EAE model, which includes inoculation with an emulsion containing the encephalitogenic peptide MOG_{33-55} that stimulates antigen-specific T cells (including Th17 cells), as well as bacterial components that act as TLR agonists.

In addition to using the existing model with immunologically naïve dams, we also modified this mouse model of neurodevelopmental disorders to more closely resemble the human scenario, where women possess immunological memory resulting from immunizations and natural exposure to antigens prior to pregnancy. To replicate the human condition, we immunized B6 females to induce immunological memory before they were mated. By doing this, we developed a more robust mouse prenatal model, which revealed factors that may be significant not only in the etiology and/or pathogenesis of autism, but also in other disorders that are currently not being considered by investigators using animal models.

We present evidence herein that offspring from poly(I:C)-injected dams exhibit a pro-inflammatory phenotype that is not seen in offspring from PBS-injected dams. This phenotype, which is already present in neonates and persists into adulthood, is a result of fetal programming of the developing immune system caused by products of the maternal and/or fetal responses induced by poly(I:C). As a result of their pro-inflammatory phenotype, these adult offspring possess the potential to mount a more robust response when faced with stimuli to cells of both the innate and adaptive immune systems. The manifestations of heightened immune responsiveness exhibited in these offspring demonstrate the significant and persistent influence that fetal programming can have on health and disease in adult life.

2. Materials and methods

2.1. Mice and breeding protocol

Wild type (WT) C57BL/6 (B6) and Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed and bred in a specific pathogen-free animal facility at University of Medicine and Dentistry of New Jersey (UMDNJ, NJMS, Newark, NJ). Food and water was provided *ad libitum*. Experimental procedures using mice were approved by the Institutional Animal Care and Use Committee of UMDNJ, NJMS, Newark.

Immunologically naïve B6 females (hereafter referred to as "naïve") were bred with WT B6 males and presence of vaginal plug was considered embryonic day 0 (E0). Naïve pregnant B6 females received a single intra-peritoneal (i.p.) injection of poly(I:C) (10 mg/kg; Sigma; St. Louis, MO) or vehicle control injection of PBS on embryonic day 12 (E12).

To induce immunological memory, B6 females received an i.p. injection with 2×10^7 γ-irradiated (3000R) allogeneic, RBC depleted Balb/c spleen cells from female donors. One month later, these "immunized" B6 females were mated with naïve WT B6 males. As previously shown (Mandal et al., 2011), immunized dams exhibited allospecific humoral and cell-mediated memory. Except for their immunological memory phenotype, these immunized dams were bred and injected with poly(I:C) or PBS exactly as described for the naïve dams.

This created four experimental groups for comparison, based on immunological phenotype (immunized or naïve) and type of injection given during pregnancy [poly(I:C) or PBS]. There were no differences in the ages of naïve and immunized dams used for breeding, which were all between 3 and 6 months old. All pregnant dams were housed individually post poly(I:C) or PBS injection, and whole-body weights of pregnant females were recorded before and 24 h after injections given on E12. Some pregnant dams were bled from the retro-orbital plexus under anesthesia for collection of sera, and then immediately sacrificed to obtain amniotic fluids. Other pregnant dams were brought to full-term and allowed to give birth. Their offspring were weaned at 4 weeks, and housed until 8–10 weeks of age for further experimental use.

Except for EAE experiments [in which we used only female mice by design in conformity with methodology used previously in the laboratory of co-author Elkabes (Nicot et al., 2005)], we used male and female offspring for all other experiments. In these experiments, we did not find a difference between results obtained from males vs. females. For some experiments (e.g., zymosan-induced acute inflammation), blood was obtained from the retro-orbital plexus and collected in tubes with anticoagulant in order to prepare blood smears for differential counts. In these experiments, plasma was examined instead of sera for the presence of cytokines.

2.2. Sickness behavior

Following poly(I:C) or PBS injection, mice were analyzed for "sickness behavior" (Dantzer, 2004; Dantzer et al., 2008; Fortier et al., 2004b; Gandhi et al., 2007) before, and at 2 and 24 h post injection. Mice were placed in a new cage (i.e., novel environment), and scored for several activities, including: exploration, rearing, grooming, sniffing, digging, and periods of inactivity. Mice received positive scoring for all activities except for periods of inactivity, where negative scoring was done. Each mouse was analyzed for one 3 min. interval, following which, scores were totaled to get an overall sickness behavior score. Low scores demonstrated the presence of sickness behavior, whereas high scores indicated its absence. Since poly(I:C)-induced sickness behavior in rodents has been shown to be cytokine-mediated, we used sickness behavior as a surrogate marker for maternal response to poly(I:C), and to ensure that neonatal and adult offspring used for experiments had received fetal exposure to products of maternal immune stimulation.

2.3. Flow cytometry

Spleen cells from adult (8–10 weeks old) offspring of poly(I:C)-injected and PBS-injected immunized and naïve dams were stimulated in vitro with anti-CD3 (1.25 μg/ml) and anti-CD28 (2 μg/ml) alone or in combination with TLR agonists, R837 (100 μg/ml; Sigma) and poly(I:C) (100 μg/ml). Cells were cultured for 3 days, after which they were re-stimulated with PMA (3 ng/ml) and ionomycin (100 ng/ml) for 16 h. Such stimulation has been shown to facilitate the ability of T-cell receptor-activated Th cells to fully express their cytokine-producing capabilities (Richter et al., 1999; Sester et al., 2000). Brefeldin A (10 μg/ml, Invitrogen; Carlsbad, CA) was added to cultures during the last 4 h to block cytokine secretion.

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