Chapter 7

Pro-Inflammatory Phenotype Induced by Maternal Immune Stimulation During Pregnancy

Nicholas M. Ponzio, Mili Mandal, Stella Elkabes, Pan Zhang, Junichi Sadoshima, Sayantani Basak, Peiyong Zhai and Robert Donnelly

Additional information is available at the end of the chapter

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1. Introduction

There is consensus among investigators studying Autism Spectrum Disorders (ASD) that the etiological basis involves environmental factors acting on the genetic susceptibility of the individual [1-5]. Over 100 candidate genes that may contribute to ASD susceptibility have been identified, and numerous environmental "triggers" have been suggested. Yet, the cause of ASD eludes clear definition and most likely is, as in most diseases, multi-factorial. However, several common immunological themes emerge from clinical and experimental studies of ASD, including persistent neuroinflammation, immune dysregulation, or autoimmune manifestations in many autistic children. Thus, in addition to genetic and environmental factors, there is compelling evidence that immune factors also play a role in ASD. Abnormalities consistent with immune dysregulation, including abnormal or skewed T helper (Th) cell subsets and cytokine profiles, decreased lymphocyte numbers, decreased T cell mitogen responses, and an imbalance of serum immunoglobulin levels have been reported in children with ASD [6-11].

Recent results of transcriptomic analysis of autistic brains [5] provides strong evidence supporting a gene-environment etiology for ASD. These authors demonstrated consistent differences in transcriptome organization in the cerebral cortex of autistic and normal brains, and identified two discrete modules of co-expressed genes associated with autism. The first, a neuronal module of 209 genes, was enriched for known autism susceptibility genes, and the second module of 235 genes was enriched for immune genes and glial markers. Gene enrichment analysis showed that genes in the neuronal module were downregulated and enriched

for gene ontology categories related to synaptic function, whereas the genes in the immune-glial module were upregulated, and showed enrichment for gene ontology categories implicated in immune and inflammatory responses. The finding of a genetic association for the neuronal module genes, but a non-genetic association for the immune-glial module genes strengthens the gene-environment etiology for ASD.

Compelling clinical data demonstrate that children of mothers exposed to certain infectious organisms during pregnancy have significantly higher frequencies of neurological disorders [12-21], including schizophrenia and ASD, the etiology of which have been linked to activation of the maternal inflammatory/immune responses (reviewed in [9, 22]). Rodent studies in which the maternal immune system is activated during pregnancy replicate these clinical findings, and provide validated mouse models of ASD [14, 15, 19, 23-33]. We have used a well-characterized prenatal mouse model to investigate questions related to the influence of maternal immune stimulation during pregnancy as an environmental risk factor that affects development of the brain and immune system in the offspring.

Injection of pregnant dams with polyclonal immune stimuli, [e.g., polyinosinic-polycytidylic acid (poly(I:C), lipopolysaccharide (LPS)] or direct injection of the pro-inflammatory cytokines these polyclonal stimuli induce (e.g., IL-1, IL-2, IL-6) cause immune dysregulation and behavioral abnormalities in their offspring in comparison to the offspring of pregnant dams given a control [i.e., Phosphate Buffered Saline (PBS)] injection [30, 34-39]. The underlying mechanisms that mediate these abnormalities have not been clearly defined, and are the focus of ongoing studies by us and others. A unique and powerful advantage of this model is the ability to examine subjects for the initiation and persistence of effects and mechanisms over a continuum of time and development from the earliest embryonic stages through the neonatal period and into adulthood.

While it is impossible for any animal model to completely replicate a human condition as complex as ASD, the mouse model of maternal immune stimulation with poly(I:C) has been recognized as an excellent prenatal model for numerous reasons presented in recent reviews by Meyer and Feldon [40] and Patterson [41]. These include (i) **face validity** (resemblance to the human symptoms) (ii) **construct validity** (similarity to the underlying causes of the disease) and (iii) **predictive validity** (expected responses to treatments that are effective in the human disease) [42]. Thus, offspring from poly(I:C)-injected dams exhibit behavioral anomalies reminiscent of those seen in autistic and schizophrenic individuals. In addition to their behavioral abnormalities, our studies show that as a result of in utero exposure to products of maternal immune stimulation these offspring also exhibit a "pro-inflammatory" phenotype that confers a vulnerability to develop immune-mediated pathology after birth and into adulthood [43-45].

In this regard, the results obtained from our investigation of the poly(I:C) mouse model have provided the scientific rationale for an ongoing translational research project to determine if similar molecular pathogenic mechanisms are involved in a cohort of ASD children who also exhibit diagnostic evidence of immune dysregulation [46]. Using DNA obtained from the Autism Genetic Resource Exchange (AGRE) database, we initiated a parallel study to determine if there were polymorphisms in selected maternal cytokine genes that occurred

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more frequently in mothers of these autistic children. Our results show that mothers of autistic children in this cohort have significant increases in pro-inflammatory cytokine gene polymorphisms, thereby conferring the genetic capability to respond more vigorously to immune stimulation by producing the types and amounts of cytokines that promote inflammatory reactions. Thus, results obtained from our investigation of the experimental prenatal mouse model of maternal immune stimulation during pregnancy have already shown biological relevance in humans.

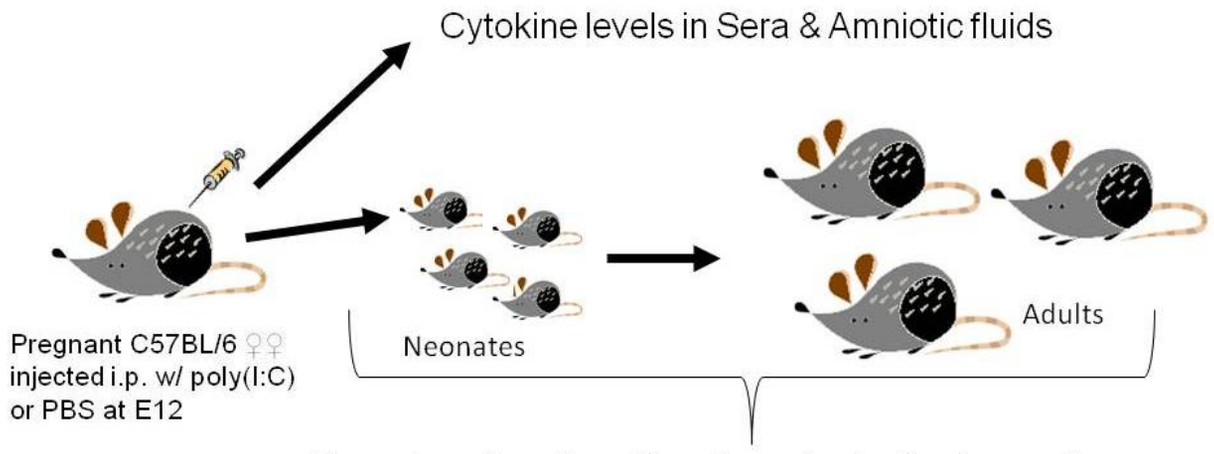
Th cell Type	Surface Markers	Signal Pathways	Transcription Factor	Inducing Cytokines	Cytokines produced
Th1	CD4; Tim-3	STAT1	T-bet	IL-12	IFN-γ, IL-2
Th2	CD4; T1/ST2	STAT3	GATA3	IL-4	IL-4,5,10,13
Th17	Not yet defined	STAT3	RORγt	IL-1, IL-6	TNF-α
				TGF-β	IL-1,6,17,21,22
T_{reg}	CD4; $CD25^{hi}$	FoxP3	FoxP3	TGF-β	IL-10, TGF-β

Table 1. Properties of T helper (Th) cell subsets

The hypothesis we are investigating in the prenatal mouse model is that maternal immune stimulation during pregnancy acts as a "first hit" that alters the developing immune system in ways that result in more robust pro-inflammatory immune responses by offspring upon subsequent (i.e. second hit) postnatal immune stimulation. Moreover, such fetal programming occurs in elements of both the innate and adaptive immune systems. Therefore, our experiments investigate how maternal immune stimulation during pregnancy influences the development and function of myeloid and lymphoid compartments of the immune system beginning at the level of the progenitor cells, and progressing to functional outcomes in neonates and adult offspring. In the myeloid compartment, we are focusing on the functions of Antigen Presenting Cells (APC), and on those innate immune elements that mediate acute inflammatory responses. With respect to the adaptive immune system, we are focusing on pro-inflammatory T helper (Th) cell subsets (Th1 and Th17) and anti-inflammatory Th cell subsets [T regulatory (T_{reg}) cells and Th2 cells]. To do this, we are using several well-characterized model systems to document the pro-inflammatory nature of the offspring of poly(I:C)-injected vs. PBS-injected pregnant dams. The results of these studies are forming a solid foundation to investigate how the pro-inflammatory phenotype exhibited by these offspring also contributes to the etiology and neuroinflammation associated with ASD.

Th cell subsets are induced by different cytokines, use different cell signaling pathways, and produce unique cytokine profiles mediated by cytokine-specific transcription factors (Table 1). Th17 and T_{reg} cells are dependent on cytokines for their development, maintenance, and function, and have been implicated in modulating the incidence and/or progression of various inflammatory and autoimmune phenomena, including rheumatoid arthritis (RA) [47], Experimental Autoimmune Encephalomyelitis (EAE) [48-50], Inflammatory Bowel Disease

(IBD) [51-53], diabetes [54, 55], and atherosclerosis [56, 57]. Thus far, however, little is known about the involvement of proinflammatory Th1 and Th17 cells in autism, and how Th cell subsets interact with microglial APC in the brain [58].



Pregnant Dams		Phenotype of Offspring
Immune Status	Injected with	
Non-immune	PBS	Non-immune Control
	poly(I:C)	Non-immune Pro-inflammatory
Immune	PBS	Immune Control
	poly(I:C)	Immune Pro-inflammatory

Figure 1. Prenatal models of maternal immune stimulation during pregnancy

2. Prenatal models of immune stimulation using poly(I:C)

In the prenatal model of maternal immune stimulation with poly(I:C) (Figure 1), C57BL/6 (B6) females and males are mated, and appearance of a vaginal plug is considered day zero of gestation (E0). At E12, pregnant females are given a single i.p. injection of poly(I:C) or PBS alone as a control. Sera and amniotic fluids are harvested, and stored at -80°C prior to measurement of cytokine levels by Luminex® bead-based multiplex assay [59] that measures up to 32 individual cytokines. In vitro and in vivo analyses are also performed on the neonatal and adult offspring of these poly(I:C)-injected and PBS-injected pregnant dams to assess the phenotype and function of their innate and adaptive immune system components. As also shown in Figure 1, we mate females that are immunologically naïve (i.e., non-immune), as well as females that possess immunological memory (i.e., immune) with immunologically naïve males. The phenotype of the offspring from these mating schemes reflects the immune status of the of the pregnant dams, and the nature of the prenatal stimulus. Our results demonstrate that offspring of both non-immune and immune poly(I:C)-in-

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jected dams exhibit a pro-inflammatory phenotype in comparison to offspring of PBS-injected dams. In addition, however, T helper (Th) cells from offspring of immune poly(I:C)-injected dams show a unique ability to preferentially differentiate to become pro-inflammatory Th17 cells.

Figures 2A and 2B show the significant increases in IL-6 at 2hr and 16hr after poly(I:C) injection, and similar differences were also seen in levels of IL-1β, IL-12, TNF-α, and GM-CSF in these samples [45, 60].

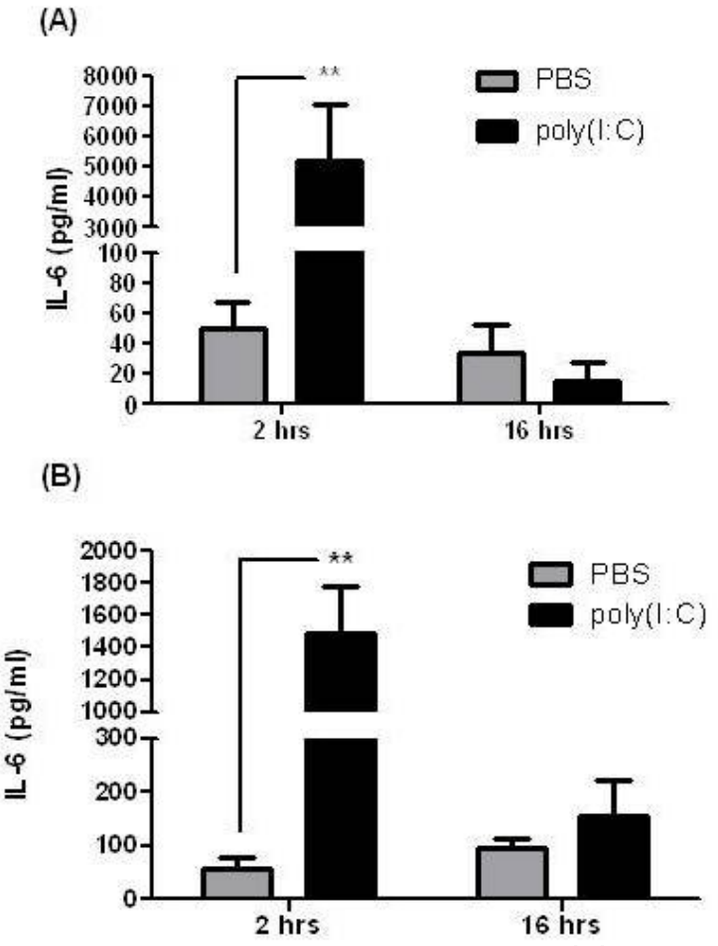

Figure 2. IL-6 levels in sera and amniotic fluids from B6 pregnant dams. Samples collected 2hrs after injection of poly(I:C) or PBS were tested for IL-6 in sera (A) and amniotic fluids (B) using Luminex bead-based multiplex assay. Data show mean ± SEM. (N=3-8; **p<0.01 using Tukey's HSD test)

In addition to testing sera and amniotic fluids from pregnant dams, we also analyze phenotypic and functional characteristics of lymphoid cells from offspring. To avoid bias due to "litter effects" [61], the number of subjects examined in our experiments not only reflects offspring within a litter, but also offspring from multiple dams, so that the "N" in our studies considers both the number of dams, as well as the number of offspring.

Use of immunologically naïve pregnant dams: Immunologically naïve mice are used by most investigators in the standard prenatal model of immune stimulation during pregnancy. These pregnant dams are injected with poly(I:C) on embryonic day 12 (E12), and control pregnant dams are injected with PBS. The embryos and offspring from these dams are then used experimentally to determine the influence of maternal immune stimulation on prenatal development and postnatal function. Since this model was originally developed to investigate neurodevelopmental disorders, such as schizophrenia and autism, a majority of the studies focus on the CNS and behavioral outcomes of offspring. These investigations have shown that maternal immune stimulation during pregnancy with polyclonal stimuli [e.g., poly(I:C) or LPS], infectious pathogens, or specific cytokines (e.g., IL-2 or IL-6) results in expression of ASD-like behavioral manifestations, as well as structural or functional changes in cells in the brain of the offspring [39-41, 61, 62].

However, in the prenatal models that use poly(I:C) as the immune stimulus, the type of poly(I:C) (i.e., sodium or potassium salt), dose of poly(I:C) (2-20 mg/Kg), and time of injection during pregnancy (E9 through E18) can influence some of the parameters that have been examined in these offspring, including open field exploration, sensorimotor gating (e.g., prepulse inhibition of the startle response), and repetitive/perserverative behavior ([63, 64]. It is thought that poly(I:C)-induced maternal cytokines are primarily responsible for the abnormalities seen in offspring. However, downstream effects induced by these maternal cytokines or trans-placental stimulation of fetal tissues by poly(I:C) itself have not been completely ruled out.

Use of pregnant dams with immunological memory: In addition to the existing model using immunologically naïve dams, we also modified this mouse model of neurodevelopmental disorders by using dams that possess immunological memory prior to mating [43, 44]. This experimental design more closely resembles the human scenario, where women possess immunological memory resulting from immunizations and natural exposure to environmental antigens prior to pregnancy. Using dams with immunological memory yields a more robust mouse prenatal model, which revealed outcomes in offspring that may be significant not only in the etiology and/or pathogenesis of schizophrenia and autism, but also in other disorders that are currently not being considered by use of these prenatal mouse models.

In both of these models, we and others have previously shown that following injection of poly(I:C), pregnant dams produce significantly higher levels of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-12, TNF-α, and GM-CSF) than PBS-injected dams in sera as well as amniotic fluids. Most of the studies involving structural/chemical changes and behavioral abnormalities that are observed after injection of poly(I:C) to pregnant dams have been performed on adult offspring from immunologically naïve pregnant dams. Recently, Hsaio, et al. [65] observed alterations in the peripheral immune system of these offspring. Our results indicate that the adult offspring of immunologically naïve poly(I:C)-injected pregnant dams also exhibit a more robust acute inflammatory response after injection of the TLR2 ligand, zymosan [45, 60].

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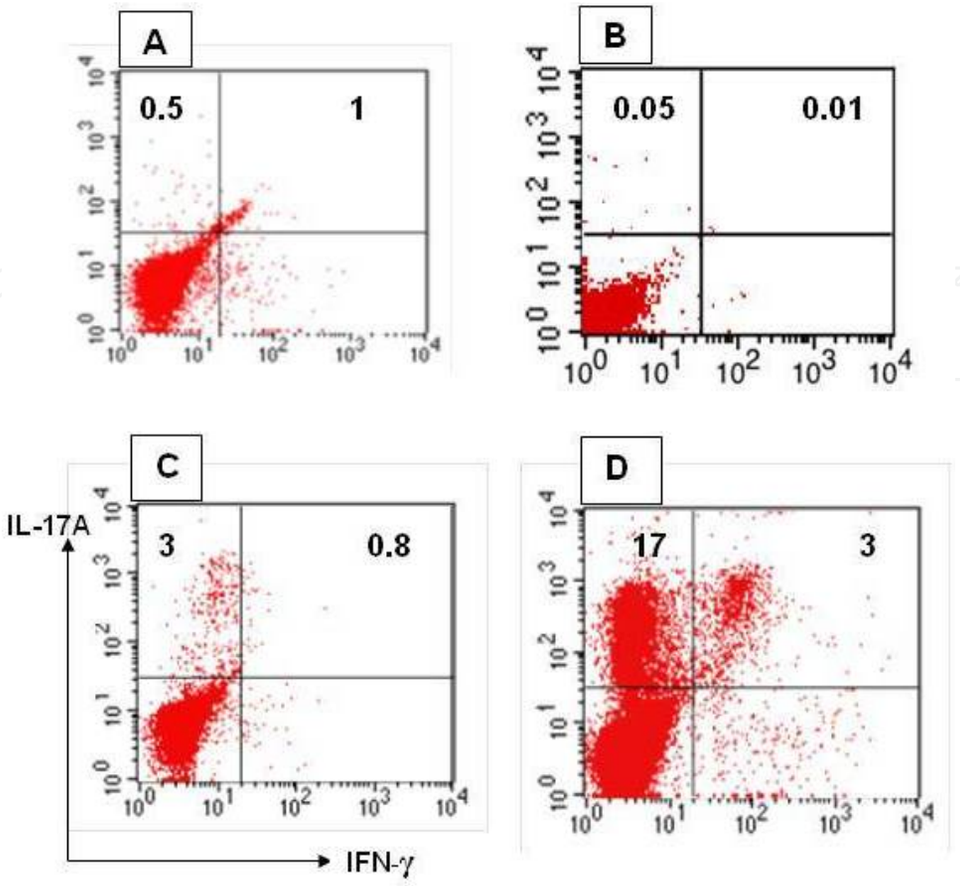

Figure 3. Enhanced production of Th17 cells in offspring poly(I:C)-injected (20mg/Kg) immune dams. Spleen cells from 3wk old offspring of poly(I:C)- and PBS-injected dams were stimulated with 3ng/ml PMA and 100ng/ml ionomycin for 16hr, the last 4hr of which were in the presence of 10ug/ml Brefeldin A to block cytokine secretion. Cells were harvested, and stained with fluorochrome-conjugated mAbs to detect cell surface molecules and intracellular cytokines by FACS analysis. The spleen cells analyzed in each of the panels were from offspring of PBS-injected immunologically naïve dams (A); offspring of poly(I:C)-injected immunologically naïve dams (B); offspring of PBS-injected immune dams (C), and offspring of poly(I:C)-injected immune dams (D). Numbers in upper left quadrants are percentages of IL-17A$^+$ (Th17) cells after gating on CD4+ cells. Results shown are representative of seven experiments comparing 18 offspring from 12 different dams. Overall results of percentages of Th17 cells were: 15.1±7.8 in offspring from immune poly(I:C)-injected dams vs. 0.8±0.5 in offspring from immune PBS-injected dams (p=0.05 using Tukey's HSD test)

The offspring of poly(I:C)-injected (vs. PBS-injected) pregnant dams who possess immunological memory prior to pregnancy exhibit a unique pro-inflammatory phenotype in which there is preferential development of Th17 lymphocytes after T cell activation (Figure 3) [43, 44]. This preferential Th17 cell development is not seen at all in offspring of immunologically naïve poly(I:C)-injected or PBS-injected pregnant dams. Given their role in immune-mediated disorders, it is likely that the potential to produce Th17 cells that we have discovered in offspring of poly(I:C)-injected pregnant dams with immunological memory may also be an important component in the neuroinflammatory pathogenesis of ASD-like changes that have been observed in this prenatal mouse model. Thus, one hypothesis we have tested is that the pro-inflammatory phenotype of offspring induced as a result of embryonic development in a pro-inflammatory cytokine environment in utero make them more susceptible

(i.e., vulnerable) to develop immune-mediated pathology. Indeed, we have obtained compelling results from in vivo experiments in adult offspring that strongly support this possibility. Using a model of EAE, in which mice are injected with an encephalogenic-peptide, Myelin Oligodendrocyte Glycoprotein peptide (MOG_{35-55}), we found that adult offspring of poly(I:C)-injected pregnant dams exhibited a significantly higher frequency and earlier onset of clinical symptoms of EAE compared to offspring of PBS-injected pregnant dams [45, 60]. Our zymosan induced results and the EAE experiments are described in subsequent sections of this chapter.

Maternal vs. fetal sources of cytokines: In this prenatal model, a single i.p. injection of poly(I:C) (or control PBS) is given on gestational day 12 (E12). Convincing evidence from this model by us and others [30, 43, 44, 61, 66-68] has shown that pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNF-α, GM-CSF) produced as a result of maternal immune stimulation during pregnancy induce changes in the development of the immune system and the brain of offspring that result in immunological and behavioral manifestations similar to those seen in individuals with ASD. To what degree these changes are induced by cytokines produced by the mother or fetus has not been fully defined. However, our results using IL-6 knock-out (KO) dams mated with wild type males [44], suggests that there is a fetal source for at least some of the cytokines detected in the amniotic fluid of poly(I:C)-injected pregnant dams.

In these experiments, our results from mating IL-6 knock-out (KO) B6 females ($IL\text{-}6^{-/-}$) and wild-type (WT) B6 males ($IL\text{-}6^{+/+}$) show that despite a maternal genetic deficiency for IL-6 production, fetal components of the heterozygous $IL\text{-}6^{+/-}$ placenta are a source of this cytokine (Figures 4 and 5), and heterozygous neonates can also produce IL-6 [44]. Using similar mating schemes in this prenatal model, however, Hsiao, et al. [66] did not find IL-6 in amniotic fluid of poly(I:C)-injected pregnant IL-6 KO B6 dams. Our results suggest that poly(I:C) (a TLR3 agonist) stimulates fetal placental tissues directly, and contributes to the levels of IL-6 found in amniotic fluid. This is relevant to the interpretation of data about the source of IL-6 (as well as other cytokines) found in the amniotic fluids and fetal tissues, such as the brain [9, 63, 69], and also because TLR3 is expressed in the brain during fetal development [70].

Pregnant dams injected with	Sickness behavior ratio	N
PBS	1.00 ± 0.10	9
Poly(I:C)	0.40± 0.02*	21

Pregnant dams were analyzed for sickness behavior before and 2 and 24 hrs after poly(I:C) injection. All mice in a group were analyzed by calculating a ratio, where each post injection sickness behavior score was divided by its pre-injection score. The individual ratios were then used to calculate the means, standard errors, and significance values. *$p < 0.0001$ (Tukey's HSD test).

Table 2. Sickness behavior scores of immune poly(I:C)- and PBS- injected pregnant dams

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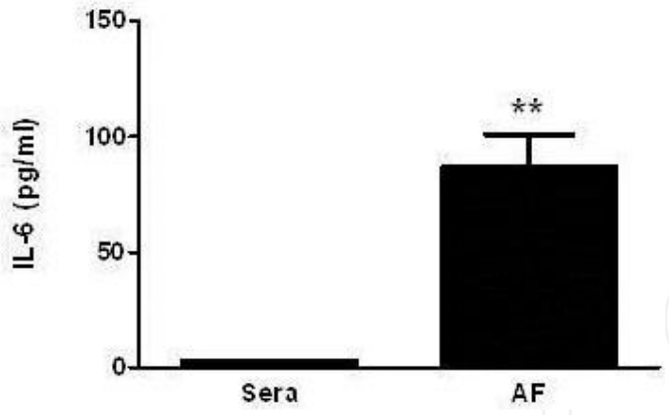

Figure 4. Source of cytokines in pregnant dams. Sera (N=6) and amniotic fluids (N=17) from IL-6 KO pregnant dams were collected 24 hrs after injection of poly(I:C), and IL-6 levels determined by Luminex assay. (*p<0.0001 Tukey's HSD test).

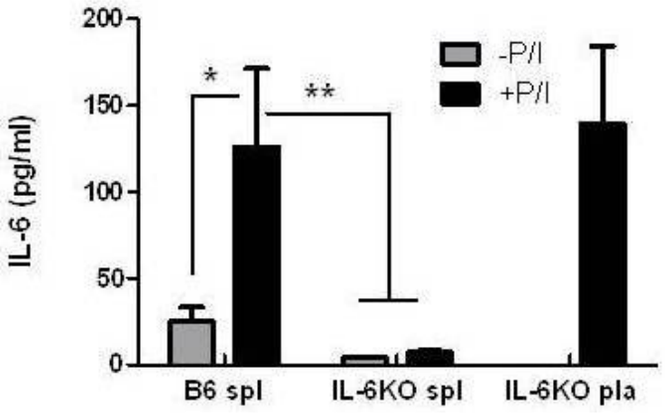

Figure 5. Source of cytokines in offspring. Spleen cells from WT B6 (N=5) and spleen and placental cells from poly(I:C)-injected IL-6 KO females (N=5) were cultured with/without PMA and Ionomycin (P/I). Supernatants were collected 24hrs later, and tested for the presence of IL-6 by Luminex assay. (*p<0.02; **p<0.01; Tukey's HSD test)

Regardless of source, however, since in utero exposure to the products of maternal immune stimulation during pregnancy appear to be part of the underlying mechanisms responsible for the changes observed in offspring, it is important to be sure that the pregnant dam responds to the immune stimulus if their offspring are used for experiments. We have addressed this issue by monitoring locomotor activity in a novel environment in every pregnant dam before, and at 2hrs and 16hrs after injection as a reliable, non-invasive measure of response to poly(I:C). We opted to use this method in lieu of more invasive procedures that would jeopardize pregnancy in these dams and/or add a level of stress that could influence the cytokine levels and/or fetal development. As shown in Figure 6 and Table 2, there is a consistent and dramatic decrease in activity (indicative of "sickness behavior – [71-74]) in poly(I:C)-injected pregnant dams at 2hrs post injection that is not seen in PBS-injected dams. Moreover, sickness behavior at 2hrs post poly(I:C) injection correlates very nicely with the increased levels of pro-inflammatory cytokines seen at 2hrs in the sera and amniotic fluids of pregnant dams (Figure 2). Activity scores

are measured in every pregnant dam, including those that are brought to term and give birth. In this way, we are confident that the offspring used for subsequent in vivo and in vitro experiments to characterize phenotypic and functional immunological parameters were exposed in utero to a pro-inflammatory cytokine milieu.

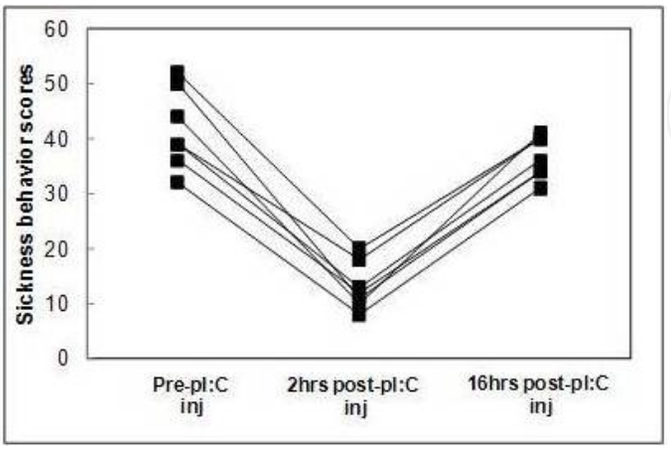

Figure 6. Sickness behavior scores in poly(I:C)-injected pregnant dams. Dams were tested before, and at 2hrs & 16hrs after poly(I:C) or PBS injection. Activity was monitored in a novel environment, and mice were given positive scores for locomotion, rearing, grooming, sniffing, and negative scores for periods of inactivity. The data in this figure show scores for 6 individual poly(I:C)-injected dams at the 3 time periods.

Effects of maternal immune stimulation on Toll-Like Receptors (TLR): We are also examining the effects of poly(I:C) exposure during pregnancy on the expression and function of TLRs during fetal development and in neonates and adult offspring. TLRs are part of a larger family of membrane bound cell surface and intracellular Pattern Recognition Receptors (PRR). Eleven TLRs have been discovered in humans (TLRs 1-11), and 12 TLRs have been found in mice (TLRs 1-12). First discovered in Drosophila [75], this family of molecules is very heterogenous, complex, and highly conserved among species. Individual TLRs bind to particular microbial products, such as LPS, peptidoglycan, lipoproteins, and flagellin on bacteria, as well as viral fusion protein, unmethylated CpG motifs, double- and single-stranded RNAs [76, 77]. TLR expression by cells of the innate and adaptive immune systems allows these cells to recognize and respond to extracellular and intracellular microbial pathogens. Downstream cell signaling pathways are initiated when ligands bind to TLRs, leading to activation of different transcription factors (e.g., NF-κB and others), which stimulate expression of pro-inflammatory cytokine genes (e.g., IL-1, IL-6, TNF, interferons).

Thus, TLRs play an early and important role at the interface between the environment and host tissues by initiating immune responses against pathogens. In addition to expression on cells of the innate and adaptive immune systems, TLRs are also expressed in/on many cell types in various other tissues of the body, including the placenta, embryonic brain, and hematopoietic progenitor cells. In the context of our experimental model of maternal immune stimulation during pregnancy, how the maternal response to poly(I:C) (a TLR3 agonist) during pregnancy affects the normal expression and function of TLR3, as well as other

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TLRs in the developing embryos and offspring is an important question that allows the design of experiments that address underlying mechanisms.

Modulation and desensitization of TLR expression, as well as cross-talk among TLRs has been shown in cells from humans and rodents. There is increasing evidence of TLR expression during embryonic development in the placenta, fetal brain and hematopoietic stem cells [78-81]. In this regard, we have obtained results indicating modulation of TLR expression in 4wk old neonates from poly(I:C)-injected dams (Figure 7). Using pathway-focused gene expression profiling qRT-PCR arrays, spleen cells from offspring of pregnant dams injected with the TLR3 agonist, poly(I:C), showed a 3.3 to 4.7 -fold increase in constitutive expression levels of TLR2, 4 and 7 over those seen in age-matched control B6 offspring. In contrast, expression levels of TLR3 and 9 were <2-fold greater than controls. These results indicate that exposure to poly(I:C) (or poly(I:C)-induced cytokines) during fetal development results in altered TLR expression that persists after birth, consequences of which may relate to differential immune responses to micro-organisms and auto-antigens. These data also suggest that TLR modulation, desensitization, and cross-talk also occur when fetal tissues are exposed to TLR agonists in utero.

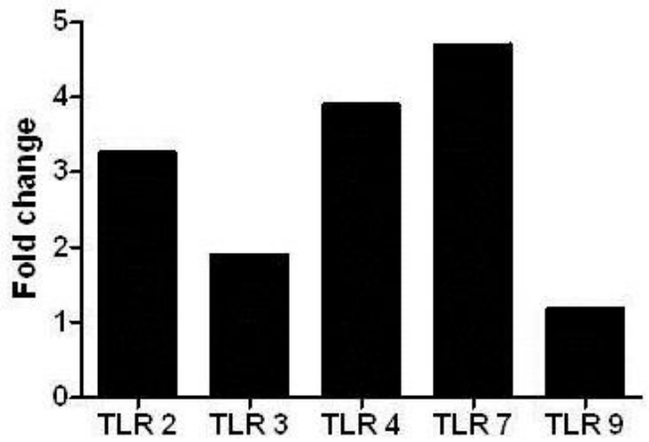

Figure 7. Altered constitutive expression of TLRs in offspring of poly(I:C)-injected dams. RNA was extracted directly from unstimulated spleen cells of 4wk old offspring of poly(I:C)- and PBS-injected dams, and tested for expression of TLRs using the RT2 Profiler PCR Array system. Data are expressed as fold-changes in gene expression for TLRs in offspring of poly(I:C)-injected dams over PBS-injected dams. The results show >3-fold upregulation for TLR2, 4, and 7 and <2-fold increase for TLR3 and 9 in offspring of poly(I:C)- compared to PBS-injected dams.

Recent studies have shown expression of TLRs by neural progenitor cells (NPC), neurons and glial cells in the adult brain, which may be important in the responses of these cells to injury or infection [76, 82-88]. Studies of TLR expression in the developing rodent brain have revealed that TLR3 appears as early as embryonic day 12.5 (E12.5) in mouse cortices, but declines over time. By contrast, TLR2 expression appears around E15.5 and increases with time [70, 88]. Moreover, in standard in vitro neurosphere assays (used to assess developmental potential of NPCs), both TLR2 and TLR3 activation appear to regulate NPC proliferation. These studies raise questions regarding the expression of other TLRs during brain develop-

ment, and how that expression pattern is altered when dams are exposed to TLR agonists, such as poly(I:C), during pregnancy. The structural and/or functional abnormalities seen in the brains of offspring from poly(I:C)-injected pregnant dams may correlate with alterations of the normal patterns of TLR expression in the developing brain. Therefore, disruption of the normal TLR expression pattern might be involved in the observed structural and/or functional changes in the brain of individuals with neurodevelopmental disorders, such as schizophrenia and autism.

Cell Marker	Percent Positive Cells in Fetuses from			
	PBS-injected dams		poly(I:C)-injected dams	
	#1	#2	#1	#2
Sca-1$^+$, c-kit$^+$ (HSC)	0.3	0.3	1.5	1.3
Sca-1$^-$, c-kit$^+$ (CMP)	24.0	25.9	21.6	24.5
Sca-1$^+$, c-kit$^-$ (CLP)	9.6	8.0	23.1	23.1

Pregnant dams were injected at E12 and fetuses were obtained 24 hrs later. Fetal liver cells from individual fetuses were analyzed by FACS for expression of markers that define HSCs and early common progenitor cells.

Table 3. Hematopoietic Stem Cells in Fetal Liver

TLR expression on hematopoietic stem cells (HSCs): As previously mentioned, maternal exposure to poly(I:C) during pregnancy induces production of pro-inflammatory cytokines, including significant increases in IL-6 in maternal circulation, amniotic fluid, placenta, and fetal brain [51, 89-93]. Direct injection of IL-6 to pregnant dams also results in consequences for the offspring, including structural abnormalities in the brain, as well as behavioral and cognitive abnormalities [30, 34-36, 38]. However, IL-6 also affects the immune system; it is an autocrine growth factor for thymic epithelial cells [94], stimulates fetal hematopoiesis [95], and can alter the balance of T_{regs} and Th17 cells towards the pro-inflammatory Th17 phenotype [96-101]. Thus, IL-6 is a key player in the differentiation of cells in the immune system, and may play a role in the immune dysregulation seen in ASD.

Recent studies have also revealed that HSCs not only respond to cytokine signaling to initiate myelopoiesis and lymphopoiesis, but also can sense microbial pathogens directly via TLR signaling [78]. Administration of nanomolar concentrations of the TLR4 agonist, LPS, triggers emigration of monocytes from the BM into the bloodstream, indicating that circulating levels of TLR ligands can also stimulate HSCs within hematopoietic tissues [102]. Additionally, treatment of mice with TLR3 agonist poly(I:C) activates HSCs to proliferate [103]. Therefore, it is likely that in the prenatal model we are studying, HSCs are influenced not only by the poly(I:C) induced cytokines elicited during pregnancy, but also by this TLR3 agonist as well. Therefore, we have examined placentas, fetal livers, and neonatal bone marrow from poly(I:C)-injected (vs.PBS-injected) pregnant dams and offspring to characterize the changes in HSCs, as well as lineage-specific progenitor cells. We examined cells from

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these tissues for surface markers (Sca-1 and c-kit) that define HSCs, and the lineage-specific progenitors for T cells (TCR, CD3), B cells (sIg, CD19), and myeloid cells (CD11b, CD11c).

An example of our results for HSCs in fetal liver is presented in **Table 3**. Pregnant dams were injected at E12 with either PBS or poly(I:C), and fetuses were examined 24 hrs later. The data show that in comparison to fetuses from PBS-injected dams, fetal livers from poly(I:C)-injected dams had a 4- to 5-fold increase in the percentage of HSCs that were double-positive for Sca-1 and c-kit, and almost a 3-fold increase in the percentage of HSCs that expressed only Sca-1, which are early **C**ommon **L**ymphoid **P**rogenitors (CLP). By contrast, the percent of HSCs that expressed only c-kit, which are early **C**ommon **M**yeloid **P**rogenitors (CMP), was similar in all fetal livers. These results are intriguing because they indicate hyper-proliferation of HSCs and early CLP, which may forecast the preferential changes we have observed in mature T lymphocytes in the adult offspring of poly(I:C)-injected dams [43-45, 60].

3. *In vivo* proof-of-concept experiments

In addition to our investigation of the consequences of maternal immune stimulation to pregnant dams, embryonic tissues, and 2-4 wk old neonates, we have also extended our studies to adult offspring of poly(I:C)-injected (vs. PBS-injected). Our guiding hypothesis is that as a result of in utero exposure of the fetus to cytokines elicited by maternal immune stimulation (acting as a "first hit"), developmental programming of the immune system occurs in offspring, which persists postnatally and into adulthood. In the case of this prenatal model, such fetal programming results in development of a "pro-inflammatory" phenotype, such that upon subsequent postnatal exposure to an immune stimulus (i.e., second hit) the offspring of poly(I:C)-injected pregnant dams exhibit exacerbated responses in comparison to offspring of PBS-injected dams. Such a scenario is also consistent with the "multiple hit" concept of mental disorders [104, 105]. In the context of ASD, this would mean that abnormalities of behavior and immune dysregulation in some children with ASD could reflect such developmental programming during embryonic development that is manifested postnatally upon encounter with a second hit to their immune system. We tested this hypothesis by using adult offspring of poly(I:C)-injected (vs. PBS-injected) pregnant dams in selected in vivo experimental models that involve activation of their innate and/or adaptive immune systems.

Inflammatory response to TLR2 agonist, zymosan: We induced an antigen non-specific acute inflammatory response in the peritoneal cavity with zymosan (TLR-2 agonist), and assessed the qualitative and quantitative nature of the inflammatory response 4 hrs later [106].

Adult offspring from immunologically naïve poly(I:C)-injected dams were injected i.p. with PBS (control) or zymosan. Adult offspring from immunologically naïve PBS-injected dams were also injected with PBS or zymosan for comparison. Mice were euthanized at 4 hrs, and 2ml of cold PBS was used to flush their peritoneal contents. The number and type of peritoneal exudate cells were determined by manual counting and FACS analysis, and the peritoneal fluid was analyzed for the presence of cytokines.

As shown in Table 4, the >2 fold increase in total Peritoneal Exudate Cell (PEC) count in the zymosan-injected poly(I:C) offspring was significantly higher than the count recovered from zymosan-injected PBS offspring. In contrast, there were no significant differences in absolute PEC numbers in control PBS-injected adult poly(I:C) or PBS offspring. The peritoneal cellular infiltrate in offspring injected with PBS was primarily mononuclear cells (monocytes and lymphocytes) (Figure 8A). In contrast, the acute cellular inflammatory response in the peritoneal cavity of zymosan-injected offspring was mostly neutrophils (Figure 8B).

Offspring		Total PEC ($x10^6$)	Neutrophils		IL-6 (pg/ml)	
From	Injected with		Percent	Absolute number ($x10^6$)	Sera	Peritoneal Fluid
PBS-injected dams	PBS	0.8 ± 0.3	<5.0	<0.004	4 ± 0.6	15 ± 7.9
	Zymosan	6.3 ± 1.8	70.0 ± 10	4.40 ± 1.9	420 ± 200	1176 ± 586
Poly(I:C)-injected dams	PBS	1.0 ± 0.4	<5.0	<0.005	8 ± 2.7	6 ± 2.5
	Zymosan	13.3 ± 2.1**	81.0 ± 6	10.8 ± 2.0**	2692 ± 514*	7808 ± 1306*

Adult offspring from immunologically naïve poly(I:C)-injected dams were injected i.p. with PBS (control) or zymosan. Adult offspring from immunologically naïve PBS-injected dams were also injected with PBS or zymosan for comparison. Mice were euthanized at 4 hrs, and 2ml of cold PBS was used to flush their peritoneal contents. The number and type of peritoneal exudate cells (PEC) were determined by manual counting and FACS analysis, and sera and peritoneal fluids were analyzed for the presence of cytokines. N = 5-8, ** P=0.016 (student's t-test). *P< 0.05 (student's t-test)

Table 4. Zymosan-induced acute inflammatory responses in offspring

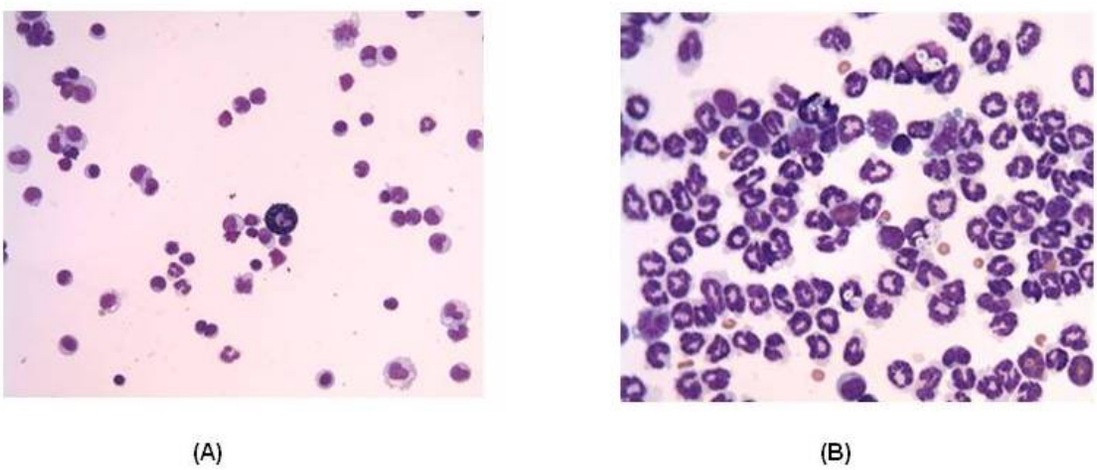

Figure 8. Acute inflammatory response in zymosan-injected adult offspring. Adult offspring from poly(I:C)-injected non-immune dams were injected i.p. with 1 ml of zymosan suspension or PBS (control). Four hours after injection, peritoneal cavities were flushed with ice cold PBS. Cytospin slides were made from peritoneal exudate cells, and stained with Wright's/Giemsa stain. (A) PBS-injected offspring from poly(I:C)-injected non-immune dams at 630X. (B) zymosan-injected offspring from poly(I:C)-injected non-immune dams at 630X.

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The percentage of neutrophils in zymosan-injected offspring from PBS-injected and poly(I:C)-injected offspring (Table 4) were similarly high (i.e., 70% and 81%, respectively). However, because the total number of PEC recovered from zymosan-injected offspring from poly(I:C)-injected (vs. PBS-injected) dams was significantly higher, the absolute number of neutrophils from zymosan-injected offspring was also significantly greater in offspring from poly(I:C)-injected dams. Given the huge infiltration of neutrophils into the peritoneal cavity in zymosan-injected offspring, we also examined the bone marrow for evidence of increased myeloid activity, and found evidence of increased myeloid activity in mice showing PEC counts in excess of 10×10^6 cells. As also shown in Table 4, significantly higher levels of IL-6 were observed in fluid obtained from the peritoneal cavity of zymosan-injected poly(I:C) offspring vs. PBS offspring at 4 hrs after zymosan injection. Although not shown in the table, levels of TNF-α and IL-10 were also significantly higher in these zymosan-injected poly(I:C) offspring.

Results from the myocardial Ischemia/reperfusion model: Based on the results we obtained using injection of zymosan to mimic the acute inflammatory response induced by an infectious organism, we wished to determine if the offspring of immunologically naïve poly(I:C)-injected dams would also mount a more robust inflammatory responses to endogenous molecules created by non-infectious tissue injury. The persistent neuroinflammation observed in brains of individuals with autism and in rodents from experimental models of neurodevelopmental disorders may be triggered by such endogenous stimuli. For these experiments, we selected a well-characterized cardiac model in which ischemia/reperfusion causes a "sterile" inflammatory response. After an acute myocardial infarction, reperfusion (by thrombolytic therapy or primary percutaneous intervention) is currently the most effective strategy to minimize myocardial damage and improve clinical outcome [107]. Paradoxically, restoring blood flow to the ischemic heart tissue can also induce injury – a phenomenon called myocardial reperfusion injury (R/I). The modes of myocardial cell injury and death following myocardial R/I are apoptosis, autophagy, and necrosis, and several underlying mechanisms have been identified or proposed [108-112]. However, one well-studied cause of myocardial R/I is the host inflammatory response that occurs during reperfusion. Despite the fact that ischemia and reperfusion takes place in a sterile environment, activation of innate and adaptive immune responses occurs and contributes to injury (reviewed in [112]). Contributing factors of reperfusion-induced inflammation include activation of Toll-like Receptors (TLRs), complement activation, free radical generation, cytokine cascade initiated by release of pro-inflammatory cytokines, and chemokine upregulation [113-116]. The presence of these immune mediators leads to recruitment of neutrophils to the ischemic myocardium, which exert cytotoxic effects themselves by release of proteolytic enzymes. Another paradox of myocardial reperfusion is that it may also significantly enhance a healing process. Studies have shown that Monocyte Chemoattractant Protein-1 (MCP-1) is also induced in the infarcted area, which may regulate myeloid cell recruitment, leading to accumulation of macrophages and mast cells that secrete angiogenesis-stimulating factors, which facilitate myocardial repair [117, 118].

For these experiments, adult offspring of immunologically naïve poly(I:C)-injected and PBS-injected dams were anesthetized, intubated and ventilated; the heart was exposed by a thor-

acotomy through the 4th and 5th ribs, and a suture was passed under the left coronary artery. The left coronary artery was occluded for a period of 20 min, and reperfusion applied for 24 hrs. Reperfusion was achieved by removal of the occlusion, the thoracotomy incision was closed, and mice were allowed to recover under monitoring in an incubator. After 24 hr of reperfusion, mice were assessed for cardiac injury as previously described [119, 120].

As shown in Figure 9, significantly greater cardiac damage was observed in offspring from immunologically naïve poly(I:C)-injected dams than in offspring from control PBS-injected dams. We are currently assessing the underlying mechanisms responsible for the difference in levels of damage in experimental and control offspring. Myocardial I/R induces infiltration of inflammatory cells, such as neutrophils that secrete cytokines/chemokines, including IL-6 and TNFα, which in turn contribute to cell death, fibrosis and reduced myocardial contractility [121]. Therefore, it is likely that similar underlying inflammatory mechanisms also occur in the myocardial I/R model as those described above for the acute inflammation induced by zymosan. In the zymosan and myocardial I/R models, the response is measured within hours of the immune stimulus. This indicates that elements of the innate immune system are the primary mediators of the pathology, and suggest that modification of these components has occurred as a result of maternal immune stimulation during pregnancy.

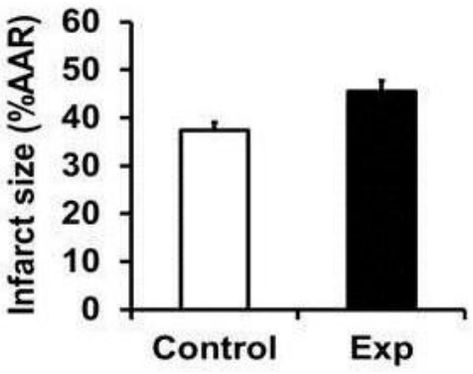

Figure 9. Cardiac damage in offspring of poly(I:C)-injected vs. PBS-injected immunologically naïve pregnant dams. Adult offspring from poly(I:C)-injected (Exp) and PBS-injected (Control) dams were subjected to cardiac ischemia (20 min) and reperfusion (24 hrs), and assessed for cardiac damage (indicated by infarct size). Offspring from poly(I:C)-injected dams exhibited significantly greater cardiac damage (p=0.011; student's t-test) than control offspring. (N = 8 mice/group; 8 weeks of age)

Response to auto-antigens: In order determine if components of the adaptive immune system also mount more robust responses following immune stimulation we chose a well-characterized EAE model of an immune-mediated disease, and used adult offspring of pregnant dams with immunological memory. We have previously shown that T cells from offspring of poly(I:C)-injected pregnant dams with immunological memory (i.e., immune) preferentially differentiate to become Th17 cells after in vitro activation [43-45, 60]. In contrast, T cells from offspring of poly(I:C)-injected immunologically naïve pregnant dams (i.e., non-immune) do not show such Th17 cell preferential differentiation. Th17 cells have been shown to be involved in the neuropathology responsible for the clinical symptoms that develop in Experimental Autoimmune

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Encephalomyelitis (EAE), a mouse model of multiple sclerosis [122-128]. Female offspring of poly(I:C) (vs. PBS) -injected pregnant dams were injected s.c. in each hind flank with an encephalogenic-peptide (MOG_{35-55}) in Complete Freund 's adjuvant (CFA). I.p. injections of pertussis toxin were given after MOG immunization to enhance the immune response and promote T cell migration into the brain [129]. Typically, 10 – 12 days after injection of MOG and pertussis, 90% of B6 mice develop progressively: weakness and paralysis in their tail, hindlimb paresis and finally hindlimb paralysis. Controls that receive CFA and pertussis toxin, but no MOG, do not develop clinical signs of EAE.

However, offspring of poly(I:C)-injected immune pregnant dams exhibited clinical signs of EAE significantly earlier and with higher frequency than offspring of poly(I:C)-injected non-immune dams (Figure 10). More than 70% of poly(I:C) immune offspring began to show clinical signs of EAE by day 4 after MOG_{35-55} immunization compared to none seen in poly(I:C) non-immune offspring. On day 7 after immunization, 25% of poly(I:C) non-immune offspring began to show symptoms, but this was still significantly lower than the >70% seen in poly(I:C) immune offspring. By day 9, >60% of poly(I:C) non-immune offspring showed clinical signs of EAE. In addition to the higher frequency of clinical signs of EAE, poly(I:C) immune offspring also had significantly higher disease severity from days 4-7 post MOG_{35-55} immunization compared to poly(I:C) non-immune offspring [45, 60]. From days 9-20, the development of EAE among offspring was very similar in both groups. However, the earlier appearance of clinical symptoms affords a window of opportunity to investigate underlying mechanisms in the EAE model that can be applied in future studies of mechanisms of neuroinflammation and pathogenesis in experimental models of autism.

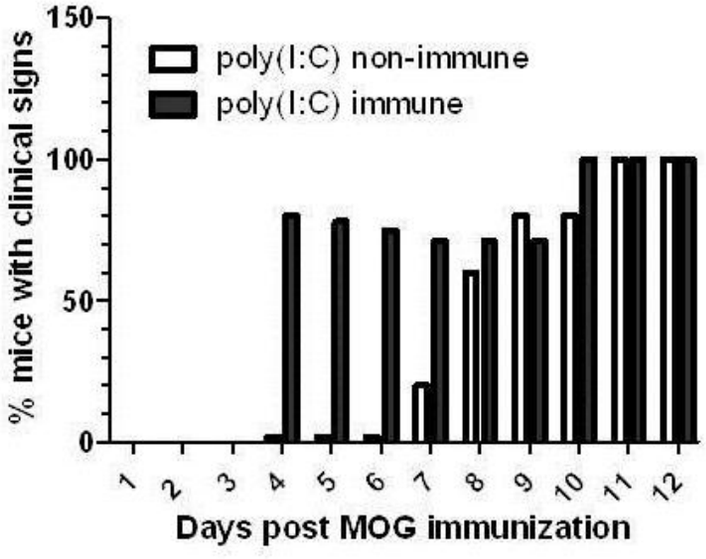

Figure 10. Frequency of mice showing clinical signs of EAE. Adult offspring of of poly(I:C)-injected dams with immunological memory [vs. immunologically naïve poly(I:C)-injected dams] were injected s.c. with MOG in CFA and i.p. with pertussis toxin. Mice were scored for clinical signs of neurological impairment, and the percent of mice showing clinical signs at the indicated times after immunization is shown in this figure.

These results are consistent with our hypothesis of fetal programming due to effects of maternal immune stimulation during pregnancy, leading to increased susceptibility of offspring to a "second hit" postnatal stimulus. This is likely due to an overall heightened immune responsiveness to develop EAE by MOG-specific Th cells that preferentially differentiate to become Th17 cells in these pro-inflammatory mice and/or a lower antigen threshold for initiation of an immune response. Another contributing factor could be differential responses of these pro-inflammatory offspring to TLR agonists on the mycobacteria in CFA (TLR2) and pertussis toxin (TLR4) used as part of the MOG immunization protocol [130, 131]. Support for this possibility is shown in Figure 7, where offspring of poly(I:C)-injected dams who possess immunological memory showed >3-fold higher expression of TLR2 and TLR4 compared to controls.

Overall, our results are consistent with the concept of developmental programming of the immune system [71, 132-136]. These changes persist into adulthood, and increase the vulnerability of offspring from poly(I:C)-injected dams to develop immune-mediated diseases when exposed to subsequent antigen specific, as well as antigen non-specific immune challenges. There is considerable plasticity of the developing immune system, and maternal stressors, such as immune stimulation during pregnancy, can modulate normal development [137]. Immune stimuli during the perinatal period of life can also act as a vulnerability factor for later-life alterations of immune responsiveness [132]. Such fetal programming has been described in relation to abnormalities of metabolism, growth, and behavior in offspring [138-140], as well as in relation to allergic and autoimmune disorders [133-135, 141-143]. The fetal programming of the developing immune system in this prenatal mouse model described herein is most likely mediated by cytokines and/or other inflammatory mediators produced by immune stimulation in response to poly(I:C) given to the pregnant dam. However, as we have previously shown, the sources of these products of immune stimulation are of both maternal and fetal origin [44].

4. Summary and conclusions

The results from our investigation of the poly(I:C)-induced prenatal model of neurodevelopmental disorders further identifies and characterizes gene-environment interactions (i.e., maternal immune response genes vs. environmental antigens) that influence fetal development in ways that have consequences for health and disease of offspring. Further characterization of this model presents excellent opportunities to define the underlying mechanisms responsible for the alterations that occur during embryological development, which persist and are manifested in adult offspring. We are using this model to examine the peripheral immune system of offspring to identify mechanisms that explain the immune dysregulation that is characteristic in a significant cohort of children with Autism Spectrum Disorders (ASD). The immunological changes we find in offspring of dams that receive immune stimulation during pregnancy involve significant differences in cyto-

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kines and T helper (Th) lymphocyte subsets. Our investigation of this mouse model has also provided a scientific basis for an ongoing translational research project to determine if similar molecular pathogenic mechanisms are involved in the cohort of ASD children who also exhibit evidence of immune dysregulation. Thus, mothers of autistic children in this cohort have polymorphisms in the same cytokine genes that promote inflammatory reactions in our mouse model, and their children with autism and immune dysregulation inherit the maternal pro-inflammatory phenotype.

Convincing evidence from this model has shown that pro-inflammatory cytokines produced by maternal immune stimulation during pregnancy induce changes in the development of the immune system and brain of offspring that result in similar immunological and behavioral manifestations as those seen in individuals with ASD. Therefore, our results are relevant to the concept of developmental programming of the immune system. In utero exposure to these cytokines produces offspring that exhibit a pro-inflammatory phenotype, which persists throughout the neonatal period and into adulthood. Subsequently, upon postnatal exposure to agents that stimulate the immune system, offspring that exhibit this phenotype mount a more robust immune response in which pro-inflammatory immune elements (i.e., Th17 cells and cytokines) predominate. Th17 cells have been shown to mediate immunopathology in numerous disorders that model human diseases, such as multiple sclerosis, arthritis, inflammatory bowel disease, atherosclerosis, and diabetes. Our use of offspring that have Th cells with the potential to preferentially differentiate into Th17 cells will also determine the contribution of Th17 cells to the etiology and pathogenesis of ASD.

The nature and timing of this second hit to the immune system may also be a critical determining factor in the manifestation of immune outcomes. Thus, if immune stimulation occurs very early in life when organ systems, such as the brain, are still developing, it may lead to neurodevelopmental disorders like ASD. Contrastingly, if the second hit occurs later in life, the outcome may be manifested as an autoimmune disorder. However, possession of a pro-inflammatory phenotype as described in our model is not necessarily a disadvantage. In certain clinical scenarios, such as malignancy or infection with pathogenic micro-organisms, a more robust immune response may provide survival advantage. Indeed, our preliminary results in an infection model indicate that the offspring of poly(I:C)-injected pregnant dams that exhibit a pro-inflammatory phenotype show increased survival time and lower pathogen burden than control offspring from PBS-injected pregnant dams.

As with many other components of the immune system, the effector functions resulting from developmental programming induced by maternal immune stimulation during pregnancy have the potential to be a double-edged sword with outcomes that can be either detrimental or beneficial. The future challenge in studying this prenatal model system will be to sufficiently understand the underlying cellular and molecular mechanisms to enable the design of effective therapeutic interventions to inhibit outcomes that are harmful, and enhance those that are beneficial.

Author details

Nicholas M. Ponzio[1], Mili Mandal[2], Stella Elkabes[3], Pan Zhang[4], Junichi Sadoshima[5], Sayantani Basak[6], Peiyong Zhai[7] and Robert Donnelly[8]

*Address all correspondence to: ponzio@umdnj.edu

1 Department of Pathology & Laboratory Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School and the Graduate School of Biomedical Sciences, Newark, USA

2 Department of Pathology & Laboratory Medicine, University of Medicine and Dentistry of New Jersey – Graduate School of Biomedical Sciences, Newark, USA

3 Department of Neurological Surgery, University of Medicine and Dentistry of New Jersey – New Jersey Medical School and the Graduate School of Biomedical Sciences, Newark, USA

4 Department of Pathology & Laboratory Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School, Newark, USA

5 Department of Cell Biology & Molecular Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School and the Graduate School of Biomedical Sciences, Newark, USA

6 Department of Pathology & Laboratory Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School, Newark, USA

7 Department of Cell Biology & Molecular Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School, Newark, USA

8 Department of Pathology & Laboratory Medicine, University of Medicine and Dentistry of New Jersey – New Jersey Medical School and the Graduate School of Biomedical Sciences, Newark, USA

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