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## Variability in PolyIC induced immune response: Implications for preclinical maternal immune activation models

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### Abstract

Maternal infection during pregnancy may increase the risk of offspring neurodevelopmental disorders. The preclinical Polyinosinic-polycytidylic acid (PolyIC) model has become one of the most widely used approaches in maternal immune activation (MIA) research. However, variability in molecular weight may impact the immune activating potential of PolyIC. Nulliparous rats injected with high molecular weight PolyIC exhibit pronounced cytokine response and sickness behavior that was not observed in rats injected low molecular weight PolyIC. Although an essential next step is to extend these studies to pregnant animals, the preliminary results suggest that PolyIC molecular weight is an important experimental design consideration.

### Keywords

Maternal immune activation; Polyinosinic-polycytidylic acid; PolyIC; Cytokine; Sickness behavior; Immune

## 1. Introduction

Exposure to a variety of viral and bacterial pathogens during pregnancy has been associated with an increased risk of neurodevelopmental or neuropsychiatric disorders in offspring, including schizophrenia (SZ) and autism spectrum disorder (ASD) (Patterson, 2009; Knuesel et al., 2014; Estes and McAllister, 2016). Maternal immune activation (MIA)

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Competing interests

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

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studies in pregnant animals indicate that the maternal cytokine response plays a critical role in the subsequent changes in brain and behavioral development of offspring (Meyer, 2014; Boksa, 2010; Meyer et al., 2009; Piontkewitz et al., 2012). Although early MIA models used live viral or bacterial pathogens to stimulate the maternal immune system (Shi et al., 2003; Fatemi et al., 1998), more recent efforts have utilized specific immune-activating agents to induce maternal cytokine responses (Smith et al., 2007; Meyer et al., 2005). One of the most widely used agents in MIA model research is Polyinosinic-polycytidylic acid (PolyIC) (Meyer and Feldon, 2012; Reisinger et al., 2015). PolyIC is a synthetic analog of double stranded RNA, consisting of a chain of double stranded inosine (I) and cytidine (C), which is detected by toll-like receptor 3 (TLR-3) (Medzhitov, 2001). Stimulation of these receptors leads to a potent immune response characterized by the production of inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF $\alpha$  as well as interferons (Alexopoulou et al., 2001; Takeuchi and Akira, 2009). During pregnancy, many of these cytokines may cross the placenta and potentially alter neurodevelopment by acting directly on the developing fetus or by indirectly altering placental endocrine or immune function (Deverman and Patterson, 2009; Hsiao and Patterson, 2011; Dahlgren et al., 2006; Zaretsky et al., 2004). The MIA model thus provides an experimental system to explore mechanistic links between changes in the maternal-fetal immune environment, altered fetal brain development and emergence of atypical offspring behavior (Carpentier et al., 2013; Lombardo et al., 2018; Arrode-Bruses and Bruses, 2012; O'Loughlin et al., 2017).

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The PolyIC MIA model is used extensively in both mice (Meyer et al., 2006a; Mandal et al., 2011; Hsiao et al., 2013; Malkova et al., 2012) and rats (Zhang et al., 2012; Dickerson et al., 2010; Zuckerman et al., 2003; Crum et al., 2017), and has recently been adapted for studies in nonhuman primates (Weir et al., 2015; Bauman et al., 2014; Machado et al., 2015; Rose et al., 2017). In spite of the strong interest in the PolyIC MIA model, methodological variability has yielded inconsistent results, making it increasingly challenging to replicate outcomes and translate results. Given that preclinical research is under increasing pressure to improve reproducibility (Collins and Tabak, 2014; Landis et al., 2012), the MIA model will undoubtedly benefit from a renewed interest in refining experimental design standards (Kilkenny et al., 2010). However, variability in PolyIC dose, route of administration, number of injections and gestational timing influences offspring outcomes and makes comparisons across studies challenging (Careaga et al., 2017). This, in turn, may have masked a major underlying issue with the MIA model. Specifically, despite the fact that PolyIC is a synthetic analog, variations in its manufacture can have significant effects on its ability to drive an immune response. These concerns stem from *in vitro* data demonstrating that PolyIC length varies among manufactures (Mian et al., 2013) and that this variability may affect the magnitude of immune responses (Zhou et al., 2013). This may explain the observation that three batches of PolyIC from the same vendor had significantly different abilities to induce an immune response (Harvey and Boksa, 2012). Collectively, these studies indicate that variability in PolyIC is emerging as a fundamental concern for the MIA model, which warrants further *in vivo* characterization.

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As an initial step in addressing PolyIC variability, we carried out a comparison of cytokine and sickness responses to two different molecular weights of PolyIC using the laboratory rat as an experimental system. Unlike the more varied approaches utilized in PolyIC-induced

mouse MIA models, the majority of rat MIA models have used a similar paradigm consisting of a 4 mg/kg single IV injection of PolyIC on gestational day (GD) 15 (Crum et al., 2017; Piontkewitz et al., 2011; Vernon et al., 2015; Wolff et al., 2011; Yee et al., 2012). Here we build upon previous rat PolyIC dosing studies (Fortier et al., 2007; Missault et al., 2014) and sickness behavior evaluations (Vorhees et al., 2012) by directly comparing 4 mg/kg IV injections of the high-molecular-weight (HMW) versus low-molecular-weight (LMW) PolyIC. Although previous rat MIA models have utilized these forms of PolyIC (Ballendine et al., 2015; Howland et al., 2012), we are unaware of any direct comparison between LMW-PolyIC and HMW-PolyIC. Thus, the goal of the present study was to evaluate the effects of different lengths of PolyIC on cytokine production and subsequent sickness behaviors and to determine if the results of previous in vitro cellular studies are reflected in vivo. Because these initial studies were carried out in nulliparous rats, an essential next step is to evaluate HMW- versus LMW-PolyIC in pregnant animals. While these experiments are underway, here we present the preliminary findings from nulliparous rats to highlight a methodological issue that has broad experimental design implications for the entire MIA model field.

## 2. Methods

All procedures were performed with approval by the University of California, Davis Institutional Animal Care and Use Committee and in accordance with the guidelines provided by the National Institutes of Health for the scientific treatment of animals. Data are available upon request.

### 2.1. Animals

Female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN), 8 weeks old, were maintained by the Teaching and Research Animal Care Services (TRACS), at University of California, Davis. All rats were housed in pairs in a temperature and humidity controlled vivarium and maintained at ambient room temperature of 22.9 °C and 53% humidity on a 12-h light/dark cycle (light on at 06:00). Standard home cage housing consisted of polypropylene Individually Ventilated Cage (IVC) housing systems from Lab Products measuring 30.5 cm × 35.6 cm × 20.3 cm with a high top wire lids, cob bedding, and nesting. Sentinel animals that are maximally exposed to all other animals in the vivarium are part of a comprehensive health-monitoring program employed to detect infections that may be present within the colony. Food and water were provided ad libitum throughout the study. Rats were allowed to habituate to the vivarium after arriving from Harlan laboratories for a minimum of two weeks before any procedures were performed.

### 2.2. CLAMS

CLAMS (Comprehensive Lab Animal Monitoring System) is a system of metabolic cages that allows for simultaneous measurement of numerous metabolic parameters including oxygen consumption, carbon dioxide production, food consumption, locomotor activity levels etc. The room housing the CLAMS was maintained at 24 °C and 55% humidity. The cages are enclosed in a light- and temperature-controlled environment, enabling the user to collect data at varied ambient temperatures. The environment in the CLAMS was maintained

at 24 °C on a 12-h light/dark cycle (light on at 06:00). Food and water were provided ad libitum. Water, food, O<sub>2</sub> consumption and CO<sub>2</sub> production and activity were monitored for 24 h before and for 24 h after Poly IC or vehicle injection. Data were recorded from the animals immediately after they were placed back into the CLAMS after injections.

### 2.3. Immune activation

Twenty-four hours before injections, rats were housed in the CLAMS chambers to allow them to habituate to the housing environment. After habituation, rats were removed one at a time from the CLAMS and randomized into three groups for tail vein injection (4 mg/kg, I.V.) with either low (0.2–1 kb) molecular weight Polyinosinic-polycytidylic acid (LMW-PolyIC), high (1.5–8 kb) molecular weight (HMW-PolyIC) Polyinosinic-polycytidylic acid (InvivoGen, San Diego, CA) or 0.9% saline vehicle (General Laboratory Products, Yorkville, IL). Although the majority of MIA models utilize PolyIC from Sigma, we selected InvivoGen which characterizes molecular weight and utilizes TLR2 and TLR4 cellular assays to confirm the absence of microbial contaminants (e.g. endotoxin or bacterial-membrane components). Injections for both HMW- and LMW-PolyIC were prepared using vials from the same respective lots following product sheet instructions: LMW-PolyIC was prepared by adding sterile endotoxin free 0.9% saline to the desired concentration and mixed gently for 10 min before being aliquoted and frozen at –80C. HMW-PolyIC was prepared by adding sterile endotoxin free 0.9% saline to the desired concentration and the heating the mixture for 10 min at 65–70 °C. The solution was then allowed to cool for 1 h at room temperature to ensure proper annealing before it was aliquoted and frozen at –80C until needed for injections. Rats were briefly restrained without anesthesia during the tail vein injections. Temperature at the time of injection was taken rectally and recorded. Immediately after injections, rats were returned to the CLAMS. The three treatment groups were: Saline (n = 12), PolyIC-LMW (n = 12), and PolyIC-HMW (n = 12). As described on the manufacturer's web site, “LMW-PolyIC comprises short strands of inosine poly(I) homopolymer annealed to strands of cytidine poly(C) homopolymer. The average size of LMW-PolyIC is from 0.2 kb to 1 kb. HMW-PolyIC comprises long strands of inosine poly(I) homopolymer annealed to strands of cytidine poly(C) homopolymer. The average size of HMW-PolyIC is from 1.5 kb to 8 kb.” Neither HMW- or LMW-PolyIC was further screened for endotoxins as both products are tested in TLR2 and TLR4 cellular assays to confirm the absence of microbial contaminants (e.g. endotoxin or bacterial-membrane components).

### 2.4. Blood collection

Blood was collected from rats at three time points; 3, 4.5, and 6 h post injection. Rats were individually removed from the CLAMS and restrained without anesthesia while 100 µL of blood was collected from the tail vein using a 26 g needle. Temperature was taken rectally and recorded immediately prior to blood collection. Blood collections were typically completed within 5 min. Blood was allowed to clot for at least one hour and then centrifuged at 1000g for 10 min. Serum was collected and stored at –80 °C until analyzed for cytokine levels. The 4.5 h time point samples were archived for future comparisons with samples collected from pregnant rats following PolyIC injections, thus only the 3 h and 6 h time points are included in the present study.

## 2.5. Cytokine analysis

The levels of serum cytokines IL-1 $\beta$ , IL-1 $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, IFN- $\gamma$ , and TNF $\alpha$  were determined using rat multiplexing bead immunoassays (Bio-Rad Laboratories, Hercules, CA). Samples were run in duplicate per manufacturer specifications. All samples were diluted 1:4 in sample diluent provided by the manufacturer. 50  $\mu$ L of diluted serum was incubated with antibody-coupled beads. After a series of washes, a biotinylated detection antibody was added to the beads, and the reaction mixture was detected by the addition of streptavidin-phycoerythrin. The bead sets were analyzed using a flow-based Luminex™ 100 suspension array system (Bio-Plex 200; Bio-Rad Laboratories). Unknown sample cytokine concentrations were calculated by Bio-Plex Manager software using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. A five-parameter model was used to calculate final concentrations. Concentrations obtained below the sensitivity limit of detection (LOD) were calculated as LOD/2 for statistical comparisons. Supernatant aliquots were free of any previous freeze/ thaw cycles. Only 2% of the samples exceeded the 15% CV variance allowance and were subsequently re-analyzed.

## 2.6. Statistical analysis

For each cytokine, a linear mixed effect model was used to evaluate the effect of treatment group, time and the interaction between these two factors. A random mouse effect was included to account for correlation of measurements between the two points. Models were fit using Proc Glimmix in SAS Software version 9.4. The interaction term was dropped if it was not significant, and a main effects only model used. For the main effects only models, if treatment group was a significant factor, post-hoc comparisons were conducted to determine if mean cytokine levels in the LMW and HMW groups differed significantly from the Saline group. If there was a significant interaction between treatment group and time, the following post-hoc comparisons were conducted: HMW vs. Saline at hour 3; HMW vs. Saline and hour 6; LMW vs. Saline at hour 3; LMW vs Saline at hour 6; Change in the difference between HMW and Saline from hour 3 to hour 6; Change in the difference between LMW and Saline from hour 3 to hour 6. The first four comparisons evaluate whether mean cytokine levels in each of the treatment groups differ from the Saline group at each time point. The last two comparisons evaluate whether there was a change in the differences between the each treatment group and the saline group over time. Adjusted p-values and confidence intervals for the post-hoc comparisons to control the type I error rate for the analysis of each cytokine at 0.05 were computed through simulations as provided for in Proc Glimmix. Five cytokines (IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$ ) were log transformed to meet model assumptions of normality and homogeneity of variances.

## 3. Results

### 3.1. Cytokine differences across groups

For all cytokines, mean levels differed significantly among treatment groups and between the two time points (Table 1). However, there was a significant interaction between treatment group and time for only four of the cytokines: IL-6 (Fig. 1), IFN- $\gamma$ , IL-1 $\beta$ , and TNF $\alpha$  (Fig. 2). For IL-6, post-hoc comparisons showed that mean levels in the HMW group were

significantly larger than the saline group at both hour 3 ( $t_{32} = 6.93$ , adj.  $p = 0.0001$ ) and hour 6 ( $t_{32} = 10.68$ , adj.  $p = 0.0001$ ), but were not statistically significantly different between the LMW and saline groups at either time point. Likewise for IFN- $\gamma$ , post-hoc comparisons showed that mean levels in the HMW group were significantly larger than the saline group at both hour 3 ( $t_{32} = 4.37$ , adj.  $p = 0.0006$ ) and hour 6 ( $t_{32} = 6.43$ , adj.  $p = 0.0001$ ), but were not statistically significantly different between the LMW and saline groups at either time point. For both cytokines, the difference between the HMW group and the saline group increased from hour 6 relative to hour 3 but this difference was only statistically significant for IL-6 ( $t_{32} = -4.57$ , adj.  $p = 0.003$ ). Based on the post-hoc comparisons, TNF $\alpha$  differed significantly between HMW and saline groups at 3 h ( $t_{32} = 11.93$ , adj.  $p = 0.0001$ ) and 6 h time points ( $t_{32} = 6.93$ , adj.  $p = 0.0001$ ), as did IL-1B for 3 h ( $t_{32} = 12.09$ , adj.  $p = 0.0001$ ) and 6 h ( $t_{32} = 7.80$ , adj.  $p = 0.0001$ ) time points. LMW had significantly different mean levels of TNF $\alpha$  at 3 h ( $t_{32} = 6.93$ , adj.  $p = 0.0001$ ) and IL-1B at hour 3 ( $t_{32} = 7.60$ , adj.  $p = 0.0001$ ) compared to saline controls. These differences between LMW and saline groups were no longer significant at the 6 h time point. Both HMW and LMW treatment groups showed a significant change in the difference in mean TNF $\alpha$  ( $t_{32} = 5.75$ , adj.  $p < 0.0001$ ,  $t_{32} = 7.37$ , adj.  $p < 0.0001$ , respectively) and IL-1B levels ( $t_{32} = 4.69$ , adj.  $p = 0.0005$ ,  $t_{32} = 4.39$ , adj.  $p = 0.0011$ , respectively) between the treatment and saline groups over time. In both treatment groups, the difference declined significantly from hour 3 to hour 6. For the remaining cytokines, with the exception of GM-CSF, mean levels differed significantly between HMW and saline but not between LMW and saline (Supplemental Tables). For GM-CSF neither HMW nor LMW differed from saline. Means of cytokines are summarized at 3 h (Table 2) and 6 h (Table 3) post injection and post hoc comparisons presented in Supplemental Materials.

### 3.2. Sickness behavior differences across groups

Sickness behavior is a coordinated set of adaptive behavioral changes that develop in individuals during the course of an infection (Dantzer, 2006). It is characterized by fever, lethargy, loss of appetite, as well as a myriad of other behavioral changes summarized in Table 4. Only animals that received HMW-PolyIC demonstrated weight loss, changes in  $O_2$  consumption/ $CO_2$  production and reduced feeding and drinking behaviors compared to saline injected animals (Fig. 3). Animals that received LMW-PolyIC did not differ from controls. Measures of core body temperature and activity did not differ among the three treatment groups.

## 4. Discussion

The maternal immune activation (MIA) model provides a powerful translational tool to explore the effects of prenatal immune challenge on the developing fetus. Mice, rat and nonhuman primate offspring born to MIA treated dams demonstrate significant alterations in brain and behavior development, which parallel features of human neurodevelopmental disorders (for reviews, (Patterson, 2009; Knuesel et al., 2014; Estes and McAllister, 2016; Meyer, 2014; Boksa, 2010; Meyer et al., 2009; Piontkewitz et al., 2012)). However, variability in the immune activating agents, such as PolyIC, commonly used to stimulate the maternal cytokine response, is emerging as a fundamental concern with the MIA model

(Kentner et al., n.d.). Here we report that PolyIC of varying molecular weights can induce significantly different cytokine responses and sickness behaviors in vivo. Although this initial step in addressing PolyIC variability was carried out in nulliparous animals, these novel findings have implications for the MIA model methodology and suggest an immediate need to: (i) report molecular weight of PolyIC in methods, (ii) validate immune activating agents prior to producing MIA-exposed offspring and (iii) report cytokine or sickness behavior data as an index of the maternal immune response.

The need for a more consistent MIA model is paramount, and in order to obtain such a model, certain limitations within the current paradigm need to be recognized and addressed. The two most commonly used immune activating agents, lipopolysaccharide (LPS) and PolyIC, present unique challenges. For example, the methods used for LPS extraction and purification make it inherently difficult to produce in a consistent or specific fashion (Ray et al., 1991). Many investigators instead choose to use the PolyIC model because it is synthetic in nature and therefore theoretically would avoid or minimize many of the shortcomings of LPS. However, despite its synthetic nature, PolyIC also varies substantially in its ability to stimulate an immune response depending on molecular length (Mian et al., 2013; Zhou et al., 2013). As most vendors do not regulate the molecular length, PolyIC can differ substantially from vendor to vendor, as well as between batches from the same vendors, with different lots having dramatically different immunological properties (Harvey and Boksa, 2012). In the present study, we demonstrated that PolyIC from the same supplier, and at the same dose, but of two different molecule weights lead to strikingly different immune responses and sickness behaviors in female rats.

MIA models have focused on changes in maternal IL-6 as a key cytokine associated with brain and behavioral impairments in offspring (Smith et al., 2007), though emerging evidence highlights a potential role for other cytokines, including IL-17 (Choi et al., 2016; Murray et al., 2015). We therefore utilized an expanded cytokine panel to evaluate serum cytokine levels of IL-1 $\beta$ , IL1- $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, IFN- $\gamma$ , and TNF $\alpha$  following injections of HMW- or LMW-PolyIC (Table 1). Significant interactions between treatment group and time were found for IL-6, TNF $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ . For both IL-6 (Fig. 1) and IFN- $\gamma$  (Fig. 2), HMW-PolyIC injected animals demonstrated significantly higher levels compared to saline controls at both 3 h and 6 h post injection. Although the IL-6 and IFN- $\gamma$  levels for the LMW-PolyIC injected animals were also higher than controls, these differences were not significant at either time point. TNF $\alpha$  and IL-1 $\beta$  (Fig. 2) differed significantly between HMW-PolyIC injected animals and controls at both time points, though LMW-PolyIC injected animals only differed from controls at 3 h, but not 6 h post injection. For the remaining cytokines that did not demonstrate a significant interaction between treatment group and time, mean levels differed significantly between HMW-PolyIC injected animals and controls but not between LMW-PolyIC injected animals and controls for all cytokines except GM-CSF (Tables 2,3; Supplemental Material).

Here we focus specifically on the molecular weight of PolyIC as one factor contributing to variability in the MIA model, but readily acknowledge that there are many other factors to take into consideration. Although exposure to HMW- versus LMW-PolyIC resulted in dramatically different immune responses and subsequent sickness behaviors, at this point we



do not know what impact this may have on the MIA model. Indeed, numerous MIA models that have used uncharacterized or LMW-PolyIC have yielded offspring with changes in brain and behavioral development (for reviews, (Meyer, 2014; Boksa, 2010; Meyer et al., 2009; Piontkewitz et al., 2012)). At present, it is not clear which maternal cytokines or what magnitude of cytokine change is causally associated with the cascade of altered brain and behavioral development described in previous MIA models (Konefal and Stellwagen, 2017). For example, a recent study found that vitamin D treatment during pregnancy eliminates behavior impairments in MIA-exposed mouse offspring, but surprisingly does not alter pro-inflammatory cytokine levels in dams or in fetal brains (Vuillermot et al., 2017). To further complicate comparisons across studies, doses of PolyIC in mouse MIA models typically range from 1 mg/kg to 20 mg/kg, which can result in a range of maternal immune response properties and subsequent brain and behavioral outcomes in the offspring. Although higher doses of PolyIC are associated with more pronounced behavioral deficits (as well as litter loss) (Shi et al., 2003; Meyer et al., 2005; 2006b), recent evidence indicates that even low doses of PolyIC can induce long-lasting changes in brain development (Goeden et al., 2016). Reinstating the use of dose response trials implemented by early PolyIC based MIA models (Shi et al., 2003; Meyer et al., 2005; Missault et al., 2014), reporting litter size/loss, and carrying out comprehensive evaluations of the maternal immune response will improve reproducibility efforts and provide insight into the mechanism by which prenatal immune challenge impacts fetal development.

Results from the present study support the need for greater standardization of MIA models and, in particular, the need to report maternal immunological measures in order to interpret offspring outcome measures. While many MIA model studies have reported intriguing alterations in offspring brain and behavioral development, the lack of maternal cytokine data hinders progress in understanding mechanistic links between specific cytokines profiles and patterns of aberrant behavior. Moreover, studies reporting negative offspring findings without maternal cytokine data are perhaps even more problematic because an insufficient or absent immunological response may be the reason for the negative effects in such studies. Although reporting cytokine data in future MIA models would help to address these issues, we recognize that repeated blood collection is not always compatible with experimental design. Quantification of sickness behavior may serve as an alternative read out of immune challenge (Dantzer, 2009) that can be used to improve the MIA model. Here we demonstrate that indices of sickness, such as weight loss, O<sub>2</sub>usage/CO<sub>2</sub> production, food/water consumption were present in the animals receiving HMW-PolyIC. Although fever response to inflammatory cytokines is well characterized, we found it to be an inconsistent measure in our current study, perhaps due to methodological limitations associated with our approach. This could relate to a lack of sensitivity in our thermometer because measured changes in core temperature were relatively small. Change in weight, previously highlighted as a key measure of sickness response in the MIA model (Missault et al., 2014; Vorhees et al., 2012; Bronson et al., 2011), was found to be as dependable a measure as any we collected using a sophisticated metabolic apparatus. Although the LMW-injected rats did not demonstrate robust sickness behaviors, this is consistent with previous rat MIA models utilizing uncharacterized PolyIC from Sigma-Aldrich that reported increase serum IL-6 two hours post-injection, but no subsequent changes in weight (Meehan et al., 2017). As previously

demonstrated (Fortier et al., 2004), food and water intake may also provide a useful index of sickness behavior and underlying cytokine response.

There are several limitations in this current study. First and foremost, the experiments were carried out in nulliparous animals. Although HMW-PolyIC was associated with a more pronounced immune response, it is plausible that the levels of cytokine changes in pregnant animals this could lead to litter loss. At present, we are unable to make recommendations regarding the use of HMW- versus LMW-PolyIC in pregnant animals, but would suggest that molecular weight should be factored into experimental design considerations that are essential to improving rigor and reproducibility in the MIA model. Moreover, we elected to utilize Sprague Dawley rats to initiate the MIA model as they exhibit robust social behavior and are amenable to genetic manipulations (Berg et al., 2018; Ku et al., 2016). It is, however, plausible that different strains of rats may respond differently to immune challenge (Schwartz et al., 2013). Although the focus of our study was a comparison between HMW- and LMW-PolyIC responses, it is also important to consider the potential impact of stress associated with blood sample collection and handling. As noted in Tables 2 and 3, the majority of cytokine levels increased between the 3 h and 6 h time points, even for the saline treated controls. Animals in the present study received multiple bouts of restraint and blood sample collection within 6 h, potentially inducing stress that may exacerbate cytokine response (Johnson et al., 2002; Cavigelli et al., 2018). Moreover, metabolic chambers, such as the CLAMS, have been associated with stress responses in rodents and may have contributed to the relatively high baseline cytokine levels in the saline group (Kalliokoski et al., 2013). While this does not alter the interpretation of the HMW- versus LMW-PolyIC comparison, it does raise issues regarding the use of metabolic chambers to evaluate sickness behaviors in the MIA model. Less invasive approaches (i.e, weight monitoring at the time of post-injection blood collection and 24 h later) may be preferable, especially for future studies with pregnant dams.

Our results indicate that PolyIC of varying molecular weights can induce significantly different cytokine responses and sickness behaviors *in vivo*, which may contribute to ongoing reproducibility issues of the MIA model (Begley and Ioannidis, 2015). These results are consistent with previous cell stimulation studies (Mian et al., 2013; Zhou et al., 2013), and provide insight into possible mechanisms underlying reports of variability in PolyIC immune activating properties (Harvey and Boksa, 2012). Given that pregnancy alters maternal immune responses, an essential next step is to characterize HMW-PolyIC and LMW-PolyIC cytokine responses and sickness behaviors in pregnant animals. While experiments in gravid animals are underway, here we present our preliminary results that have implications for the growing field of MIA model research, and may provide methodological insight needed to improve rigor and reproducibility of this valuable translational model.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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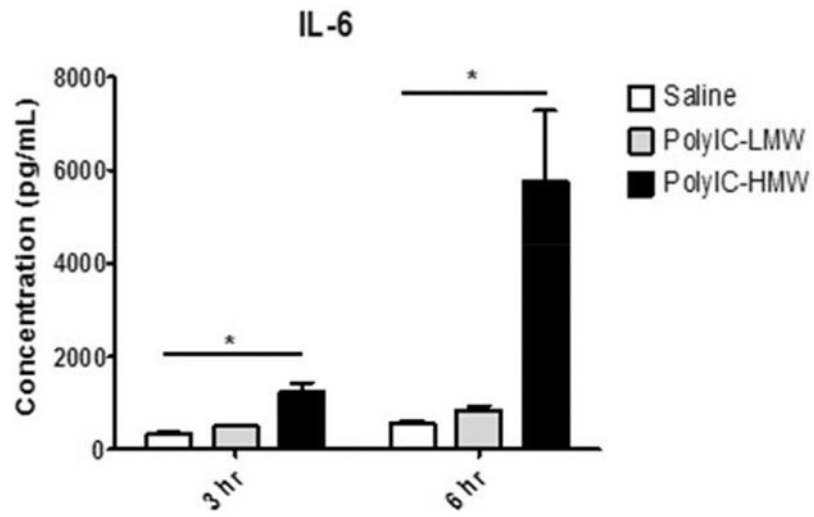
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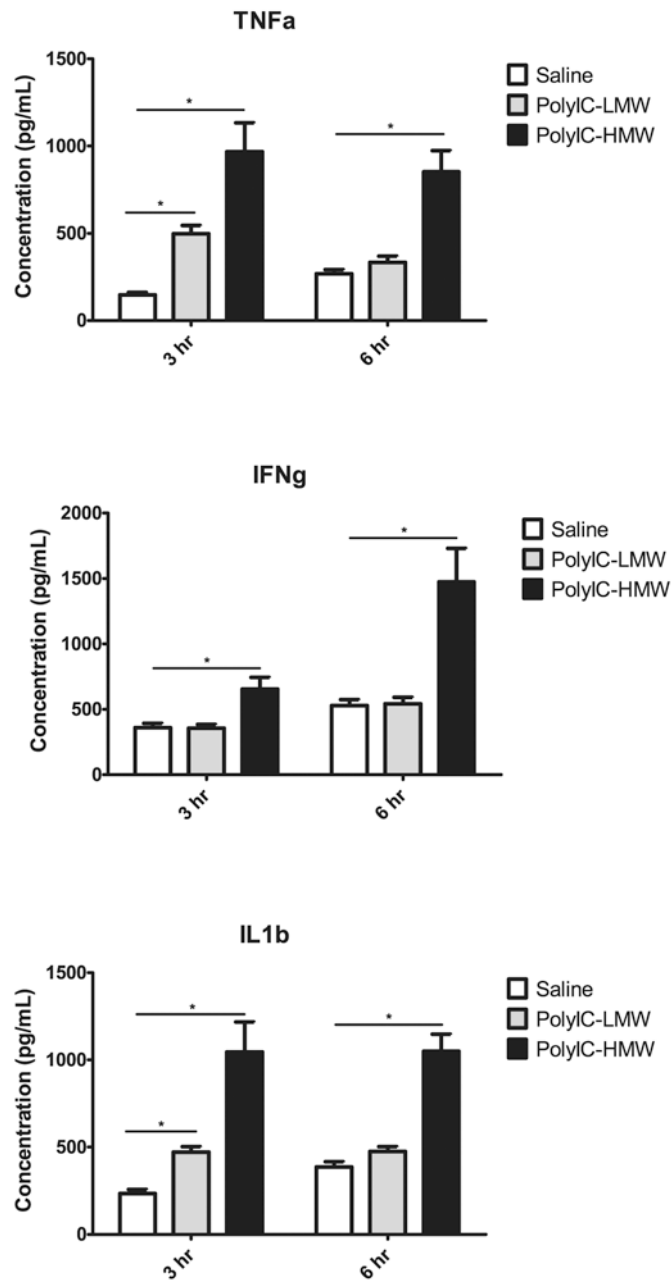
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**Fig. 1.** IL-6 Response following PolyIC Injection. Only animals that received HMW-PolyIC demonstrate elevated IL-6 levels compared to saline controls 3 h and 6 h after the injection. \* P < 0.05.

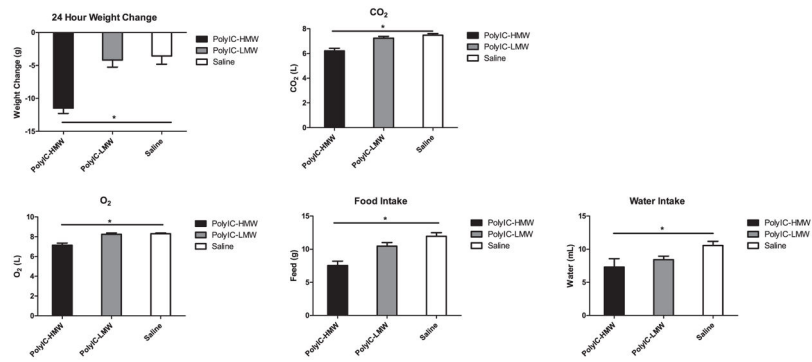


**Fig. 2.**

Expanded cytokine profiles of TNF $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , at 3 h and 6 h post PolyIC injection.

(A) At 3 h post injection animals that received either LMW- or HMW-PolyIC demonstrated elevated TNF $\alpha$  levels compared to saline controls. The HMW-PolyIC injected animals continue to demonstrate higher TNF $\alpha$  levels than controls 6 h post injection. (B) For IFN- $\gamma$ , only the HMW-injected animals differed from controls at both 3 h and 6 h post injection. (C) At 3 h and 6 h post injection animals that received HMW-PolyIC demonstrated elevated IL-1 $\beta$  levels compared to saline controls. LMW-PolyIC injected animals differed from controls at 3 h, but not 6 h post injection.





**Fig. 3.**

Rat receiving LMW-PolyIC showed no significant differences compared with rats receiving saline alone. However, rats receiving HMW-PolyIC showed significant differences in (A) weight loss (B) CO<sub>2</sub> production, (C) O<sub>2</sub> consumption, (D) Food intake and (D) Water intake compared with rats receiving saline. \* P < 0.05.

**Table 1**

Results of evaluation of effect of treatment group, time and the interaction between group and time on cytokine levels.

Cytokine	Group	Time	Interaction
GM-CSF	$F_{2,33} = 3.75$	$F_{1,34} = 17.75$	$F_{2,32} = 1.82$
	$p = 0.034$	$p = 0.0002$	$p = 0.178$
IFN- $\gamma$	$F_{2,33} = 22.76$	$F_{1,32} = 97.24$	$F_{2,32} = 3.65$
	$p < 0.0001$	$p < 0.0001$	$p = 0.0374$
IL-1 $\alpha$	$F_{2,33} = 11.84$	$F_{1,32} = 9.89$	$F_{2,33} = 1.28$
	$p = 0.0001$	$p = 0.0034$	$p = 0.291$
IL-1 $\beta$	$F_{2,33} = 65.13$	$F_{1,32} = 11.77$	$F_{2,33} = 13.86$
	$p < 0.0001$	$p = 0.0017$	$p < 0.0001$
IL-4	$F_{2,33} = 6.61$	$F_{1,32} = 34.86$	$F_{2,33} = 0.75$
	$p = 0.0039$	$p < 0.0001$	$p = 0.48$
IL-6	$F_{2,33} = 49.04$	$F_{1,32} = 146.21$	$F_{2,33} = 15.05$
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
IL-10	$F_{2,33} = 9.99$	$F_{1,32} = 59.14$	$F_{2,33} = 0.06$
	$p = 0.0004$	$p < 0.0001$	$p = 0.941$
IL-17	$F_{2,33} = 8.62$	$F_{1,32} = 13.75$	$F_{2,33} = 1.62$
	$p = 0.001$	$p = 0.0007$	$p = 0.214$
TNF $\alpha$	$F_{2,33} = 55.84$	$F_{1,32} = 0.08$	$F_{2,33} = 30.14$
	$p < 0.0001$	$p = 0.781$	$p < 0.0001$
IL-2	$F_{2,33} = 8.84$	$F_{1,32} = 28.16$	$F_{2,33} = 2.50$
	$p = 0.001$	$p < 0.0001$	$p = 0.098$
IL-5	$F_{2,33} = 4.34$	$F_{1,32} = 30.87$	$F_{2,33} = 1.22$
	$p = 0.021$	$p < 0.0001$	$p = 0.308$
IL-12	$F_{2,33} = 4.94$	$F_{1,32} = 13.95$	$F_{2,33} = 1.08$
	$p = 0.0133$	$p = 0.0007$	$p = 0.352$
IL-13	$F_{2,33} = 9.44$	$F_{1,32} = 28.41$	$F_{2,33} = 0.11$
	$p = 0.0006$	$p < 0.0001$	$p = 0.893$

**Table 2**Means  $\pm$  SD of cytokines 3 h post injection by treatment group.

Cytokine	Saline (pg/ml)	HMW (pg/ml)	LMW (pg/ml)
GM-CSF	178.74 $\pm$ 59	260.49 $\pm$ 59	162.63 $\pm$ 59
IFN- $\gamma$	359.52 $\pm$ 115	714.58 $\pm$ 115	356.54 $\pm$ 115
IL-1 $\alpha$	230.63 $\pm$ 128	378.56 $\pm$ 128	201.25 $\pm$ 128
IL-1 $\beta$	235.01 $\pm$ 82	1141.78 $\pm$ 82	471.95 $\pm$ 82
IL-4	40.34 $\pm$ 13	62.84 $\pm$ 13	39.86 $\pm$ 13
IL-6	331.35 $\pm$ 142	1348.14 $\pm$ 142	503.34 $\pm$ 142
IL-10	1235.56 $\pm$ 279	1842.25 $\pm$ 279	1308.96 $\pm$ 279
IL-17	56.21 $\pm$ 16	85.25 $\pm$ 16	53.95 $\pm$ 16
TNF $\alpha$	146.8 $\pm$ 48	1055.95 $\pm$ 48	497.31 $\pm$ 48
IL-2	271.56 $\pm$ 62	420.81 $\pm$ 62	259.93 $\pm$ 62
IL-5	473.55 $\pm$ 67	567.7 $\pm$ 67	461.55 $\pm$ 67
IL-12	127.75 $\pm$ 34	193.67 $\pm$ 34	126.9 $\pm$ 34
IL-13	71.91 $\pm$ 27	119.92 $\pm$ 27	71.64 $\pm$ 27

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**Table 3**Means  $\pm$  SD of cytokines 6 h post injection by treatment group.

Cytokine	Saline (pg/ml)	HMW (pg/ml)	LMW (pg/ml)
GM-CSF	286.46 $\pm$ 78	290.99 $\pm$ 78	249.25 $\pm$ 78
IFN- $\gamma$	529.54 $\pm$ 155	1473.26 $\pm$ 155	543.26 $\pm$ 155
IL-1 $\alpha$	291.21 $\pm$ 51	388.99 $\pm$ 51	292.22 $\pm$ 51
IL-1 $\beta$	387.09 $\pm$ 101	1049.5 $\pm$ 101	475.59 $\pm$ 101
IL-4	63.99 $\pm$ 18	78.08 $\pm$ 18	64.59 $\pm$ 18
IL-6	569.59 $\pm$ 212	5758.68 $\pm$ 212	857.12 $\pm$ 212
IL-10	1988.2 $\pm$ 467	2635.18 $\pm$ 467	2007.58 $\pm$ 467
IL-17	75.7 $\pm$ 17	89.96 $\pm$ 17	72.72 $\pm$ 17
TNF $\alpha$	267.81 $\pm$ 84	837.56 $\pm$ 84	333.26 $\pm$ 84
IL-2	402.11 $\pm$ 74	464.27 $\pm$ 74	383.96 $\pm$ 74
IL-5	597.741 $\pm$ 60	629.38 $\pm$ 60	577.33 $\pm$ 60
IL-12	187.41 $\pm$ 51	215.11 $\pm$ 51	186.5 $\pm$ 51
IL-13	118.95 $\pm$ 36	158.25 $\pm$ 36	116.35 $\pm$ 36

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**Table 4**

Sickness behavior following HMW-Poly versus LMW-PolyIC.

Sickness measure	Treatment effect	Post Hoc
Weight change	F <sub>(2,32)</sub> = 15.86; p < 0.001	HMW > saline (p < 0.001) LMW = saline (p = 0.898)
Temp (3h)	F <sub>(2,32)</sub> = 2.60; p = 0.09	-
Temp (4.5 h)	F <sub>(2,32)</sub> = 0.72; p = 0.50	-
Temp (6h)	F <sub>(2,32)</sub> = 2.32; p = 0.13	-
co <sub>2</sub>	F <sub>(2,32)</sub> = 17.82; p < 0.001	HMW < saline (p < 0.001) LMW = saline (p = 0.446)
O <sub>2</sub>	F <sub>(2,32)</sub> = 18.37; p < 0.001	HMW < saline (p < 0.001) LMW = saline (p = 0.945)
Feed	F <sub>(2,32)</sub> = 15.05; p < 0.001	HMW < saline (p < 0.001) LMW = saline (p = 0.130)
Water	F <sub>(2,32)</sub> = 3.89; p = 0.03	HMW < saline (p = 0.019) LMW = saline (p = 0.135)
Ambulation	F <sub>(2,32)</sub> = 1.47; p = 0.24	-

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