Hematopoietic Stem Cells are Resistant to Transient Alterations Induced by Maternal Immune Activation

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Maternal immune activation (MIA) increases the risk for schizophrenia and symptoms of autism spectrum disorders (ASD) in humans and mice. In pregnant mice, MIA can be induced by respiratory infection or by the administration of poly(I:C), a synthetic, double-stranded RNA that triggers an anti-viral inflammatory response (Shi et al., 2003). The offspring of mothers injected with poly(I:C) at E12.5 exhibit peripheral immune dysregulation and behavioral deficits associated with schizophrenia and autism. In addition, Smith et al. revealed that the pro-inflammatory cytokine IL-6 is both necessary and sufficient to induce behavioral and transcriptional changes in the offspring (Smith et al, 2007). As IL-6 can cross the placenta and as E12.5 marks the onset of placental hematopoietic stem cell (HSC) expansion (Mikkola et al.,2006), we sought to determine whether the placenta could be a site of early peripheral immune dysregulation in an MIA mouse model. Our studies show that MIA upregulates IL-6 in the E13.5 placenta and that HSCs in the placenta at this time express TLR3. Thus, HSCs could respond to MIA induced IL-6 through cytokine receptors and/or to poly(I:C) directly through the TLR3 receptor. Real-Time PCR on CD34 dim c-Kit high HSCs isolated from E13.5 and E15.5 embryos of pregnant mothers injected with poly(I:C) reveals MIA induced trends in gene expression among saline versus poly(I:C) groups. This finding was supported by methylcellulose cultures in which the introduction of poly(I:C) to primary cells led to increased numbers of myeloid colonies. This data would suggest that poly(I:C) induced MIA may alter the fate of HSCs and progenitors in vivo. However, the observed changes in these populations are temporary; examination of overall cell populations in the spleens of adult mice whose pregnant mothers were injected with poly(I:C) or saline revealed no differences in abundance of general cell types between poly(I:C) and saline groups. Thus, it appears that HSCs and progenitors are resistant to transiently induced changes in gene expression.

Introduction

Schizophrenia and Autism Spectrum Disorders (ASD) include severe behavioral and developmental deficits (DSMIV TR). Schizophrenia affects roughly 1.0% of the population over the age of eighteen while the diagnoses of ASD cases are rising at an alarming rate. Also of concern are the high annual costs of these disorders. There is therefore a great need to further our understanding of the development of schizophrenia and ASD in order to provide better treatment and/or prevention of these disorders.

While there are a number of factors that have been shown to contribute to the pathology of ASD and schizophrenia, a significant environmental influence is the infection of mothers during pregnancy (Togichi et al., 2004). In humans, infection during the second trimester increases the risk for schizophrenia and symptoms of ASD in the offspring. Epidemiological studies show that there is increased incidence of schizophrenia following outbreaks of influenza with the second trimester being especially significant (Brown and Susser, 2002; Brown et. al, 2004; Brown, 2006,). In mice, poly(I:C) induced MIA is used as

a model of influenza infection (Smith et al, 2006; Patterson, 2009; Urs et al, 2008, Shi et al, 2003). A single intraperitoneal injection of poly(I:C) on embryonic day E12.5 induces behavioral deficits in the offspring that are associated with schizophrenia and ASD. Shortly after MIA, cytokine levels in the placenta, maternal serum, amniotic fluid and fetal brain are altered. The cytokine IL-6 is induced in maternal serum by MIA and is critical for mediating the behavioral and transcriptional changes observed in these offspring (Smith et al., 2007).

In addition to behavioral deficits, the adult offspring display dysregulated peripheral immunity. Elevated levels of IL-17 and IL-6 are produced by CD4+ T cells from lymph nodes and spleens of adult MIA offspring (Patterson, unpublished). Similar abnormalities are observed in CD4+ T cells isolated from spleens of young and newborn MIA offspring. Likewise, T cell dysregulation has been reported in humans with autism. Mostafa et al. showed that children with autism have lower levels of CD4+CD25+ T cells with a positive correlation between the severity of symptoms and the deficiency of this cell population (Mostafa et al., 2010). In addition, it has been shown that children with ASD have altered levels of naïve CD8+ cells (CCR7+ CD45Ra+), CD8+ effector memory (EM) cells (CCR7-/CD45RA-), and CD4+ terminally differentiated (TD) cells (CCR7-/CD45RA+) (Sarsella et al., 2009).

While the above studies shed further light on the complexities of ASD and schizophrenia, there is much information that remains to be discovered about the etiology of these disorders. We have investigated the effects of MIA on the development of HSCs in the placenta and fetal liver as well as possible subsequent alterations in myeloid and lymphoid populations. The development of HSCs is of particular interest as several reports demonstrate that the time of poly(I:C) injection, E12.5, marks the onset of placental hematopoietic stem cell (HSC) expansion (Mikkola et al., 2005) as well as HSC seeding of the fetal liver (Mikkola et al., 2006). In addition, embryonic HSCs express cytokine and toll-like receptors and may therefore respond to poly(I:C) directly or to poly(I:C)-induced proinflammatory cytokines (Dorner et al., 2009; McKinstry et al., 1997; Nagai et al., 2006). Thus, MIA may initiate the dysregulation of peripheral immunity in the offspring by affecting the development of the immune system at key sites during embryogenesis.

Pregnant mice were injected with poly(I:C) at E12.5, and IL-6 levels were evaluated at 24 and 72 hours post-injection in the placentas and fetal livers. HSCs were then isolated from placentas and fetal livers of E13.5 and E15.5 embryos. Real-Time PCR of these cells revealed that HSCs express TLR3 and could therefore be responding directly to poly(I:C). To examine aberrations in lineage potential, expression of transcription factors for myeloid and lymphoid progenitors were evaluated in the isolated HSCs. This data shows trending differences in gene expression of GATA-1 and IL-7Rα placental and fetal liver HSCs from poly(:C) versus saline injected mothers. Methylcellulose assays were also used to examine effects of poly(I:C) in vitro on lineage potential of fetal liver cells. To further evaluate alterations of lineage potential in vivo, abundance of adult MIA offspring spleen cell populations was evaluated for differences in saline and poly(I:C) groups.

Materials and Methods

Mice

Female C57BL/6 mice were maintained at the in-house breeding facility at Caltech University. All mice were weighed and were randomly assigned to either poly(I:C) or control groups. At E12.5, mice were injected i.p. with 20mg/kg poly(I:C) (potassium salt; Sigma, St. Louis, MO) dissolved in saline or with saline alone. Mice were sacrificed by i.p. injection with 200uL of sodium pentobarbital (Nembutal).

ELISAs for IL-6 and IL-17

ELISAs were performed on fetal liver and placenta tissues and serum from E13.5 and E15.5 embryos whose mothers were injected with either saline or poly(I:C) at E12.5. Serum refers to supernatant collected during the HSC isolation described below. Cytokine levels of serum samples were normalized to cell count of the suspension. Tissues were frozen in liquid nitrogen, and cytokine levels were normalized to total protein of the tissues with BCA assay.

Immunohistochemistry

Whole Placentas from E13.5 embryos were fixed in 4.0% PFA for 2 hours at 4°C. Tissues were then washed with PBS and left overnight at 4°C covered in 30.0% sucrose. Sucrose was removed and OCT added, and tissues were left for 1 hour at 4°C to equilibrate. Tissues were removed and placed into plastic molds, covered with OCT, frozen in liquid nitrogen, and stored at -80°C until sectioning.

For TLR3 staining, tissues were cut into 20um sections. Antigen retrieval and endogenous peroxidase blocking was performed prior to blocking in normal goat serum. Primary antibody was then added at a final concentration of 1ug/mL diluted in blocking buffer, and tissues were left overnight at 4°C in a humidified chamber. The following day the slides were washed, and a 1/400 dilution of the secondary antibody goat anti-rabbit IgG was added. Slides were incubated with the primary antibody for 1 hour at room temperature. The slides were washed and the substrate was added (ABC solution, CAT) and left to develop for 30 minutes at room temperature.

For HSC staining, we followed the procedure described in *Current Protocols* (Mikkola et al., 2008).

Serum Collection and Isolation of Fetal Liver HSCs

Mice were injected with either poly(I:C) or saline on E12.5 as previously described. At E13.5 and E15.5 mice were sacrificed with 200uL Nembutal. Conceptuses were removed and washed in HBSS with Calcium and Magnesium and 5%FBS and DNase (Sigma 9003-98-9). Fetal livers were then removed from the embryos and placed in falcons containing the above media. For the E13.5 time point, 6 saline livers from one mouse were placed into a tube with 6 livers from another mouse for a total of 12 tissues per tube. For the

E15.5 time point, 5 saline livers from one mouse were placed into a tube with 5 livers from another mouse for a total of 10 tissues per tube. Only 10 tissues total were used per E15.5 group because of the expected higher HSC yields at this time (Mikkola et al., 2005). Single cell suspensions were prepared by filtering the tissues through sterile mesh filters into new falcons. All falcons were centrifuged 10'at 1200rpm at 4°C. Supernatant was removed and stored at -80°C for ELISAs. RBC lysis was then performed on the cells, and the remaining cells were washed and filtered. Cell counts were done using 0.25% trypan blue. Cells were then incubated in FC Block and dead and lineage positive cells were depleted using Miltenyi Biotec cell columns and reagents. The remaining lineage negative cells were again incubated with FC block and stained with various antibodies diluted in PBS with Magnesium and Calcium at 50uL final volume. Cells were filtered immediately before sorting, and 20uL 7ADD was added to the cell solution. Cells were gated into 4 groups: $CD34^{high}$, $Ckit^{high}$, $CD34^{dim}$ Ckit ^{high}, and $CD34^{high}$ $Ckit^{high}$. Sorted cells were stored in RNase/DNase free eppendorfs with 0.5mL RNA Cell Protect at -80°C.

Serum Collection and Isolation Placental HSCs

Mice were injected with either poly(I:C) or saline on E12.5 as previously described. At E13.5 or E15.5 mice were sacrificed with 200uL Nembutal. Conceptuses were removed and washed in HBSS with Calcium and Magnesium and 5%FBS and DNase (Sigma 9003-98-9). Placentas were separated and the deciduas and spongiotrophoblasts were removed. Placentas were then placed in falcons containing the above media: 6 saline/poly(I:C) placentas from one mouse were placed into a tube with 6 saline/poly(I:C) placentas from another mouse for a total of 12 tissues per tube. All falcons were centrifuged 10'at 300xg at 4C, and the supernatant removed. Cells were then resuspended in 5.0mL of a 0.1%(w/v) collagenase solution diluted in PBS with 10%FBS and 1%P/S. Mechanical dissociations were performed on the cells using a 16G needle followed by an 18G needle. The cell suspensions were then incubated 30' at 37°C. Following the incubation, the cells were centrifuged 10'at 1200rpm and resuspended in 2.0mL HBSS. The cells were then mechanically dissociated using a 20G needle, 2.0mL 0.25% trypsin was added, and the cells were incubated 10' at 37°C. HBSS with 5%FBS was added to the cell solutions to deactivate the trypsin, and cells were centrifuged 10'at 1200rpm and resuspended in HBSS with 5% FBS. The cells were then dissociated further with 22G and 25G needles, centrifuged, and the supernatant was stored at -80°C for ELISAs. RBC lysis, cell counts, and dead cell removal was then performed as described above. The lineage depletion was also done as previously described with the exception of an additional incubation with a cytokeratin Ab to deplete trophoblasts. Cell staining and sorting was done as described above.

Antibodies and Reagents for FACS

CD34/Alexa Fluor 647 1/75 dilution (eBioscience #51-0341), Terr119/PerCPCy5.5 1/250 (Biolegend #116227), SA/PerCPCy5.5 1/100 (Biolegend #116227), CkitACK45/PE 1/75 for livers (BD Pharmingen #553869 clone ACK45,), Ckit2B8/PE 1/50 for placentas (BD Biosciences clone 2B8 #553355, BD Biosciences), CD41/FITC 1/150 (CAT), 7AAD (BD Pharmingen #559925), Cytokeratin 18/Biotin (abcam #ab27553), CD16/32 (eBioscience #14-0161), Lineage Cell Depletion Kit (Miltenyi Biotec #130-090-858), Dead Cell Removal Kit (Miltenyi Biotec 130-090-101)

RTPCR

Total RNA was isolated with the RNeasy QIAGEN kit, and cDNA was synthesized with the iscript cDNA synthesis kit (Biorad). cDNA was purified with PCR purification kit (Qiagen). Real-Time PCR was done using SYBR green with ROX (Roche) on the ABI Prism 4300.

Primer Sequences:
GATA-1 sense, TCCTCTGCATCAACAAGCCCA
GATA-1 anti-sense GTTGAGCAGTGGATACACCTG
β-actin sense AGAGGGAAATCGTGCGTGAC
β-actin anti-sense CAATAGTGATGACCTGGCCGT
IL-7Rα sense GGATGGAGACCTAGAAGATG
Il-7Rα anti-sense GAGTTAGGCATTTCACTCGT
TLR3 sense ACCTTTGTCTTCTGCACGAACCT
TLR3 anti-sense AGTTCTTCACTTCGCAACGCA

GEMM Methylcellulose Assays

GEMM methylcellulose assays refer to lineage potential assays for colony forming units of granulocytes, erythrocytes, monocytes, and macrophages. Two types of methylcellulose assays were performed. The first assay will be referred to as the in vitro assay. In this assay, fetal liver tissues were isolated from E12.5 C57BL/6 mice and roughly minced. Equal numbers of tissues from 2 mice were combined on a 6cm petri and left overnight at 37°C with IMDM supplemented with 2%FBS and 1%P/S. Half of the dishes also had 25ug/mL poly(I:C) added to the media while other dishes half of the dishes had extra media. After 24 hours, tissues were mechanically dissociated to make single cell suspensions. Cells were plated on 35mm petris (Stem Cell Technologies #27110) with methylcellulose (Methocult 3434, Stem Cell Technologies) supplemented with 5ng/mL TPO (recombinant mouse TPO #488-TO, R&D Systems) at the density of 2x10^4 cells per plate. Colonies were scored at days 5 and 12.

The second type of assay will be referred to as the in vivo assay. In this assay, pregnant C57BL/6 mice were injected at E12.5 with 20mg/kg saline or poly(I:C). Mice were sacrificed at E13.5, and single cell suspensions were made from fetal livers. Cells were then plated as described above and scored at days 5 and 12.

Flow Cytometry

Flow Cytometry was performed on cells isolated from spleens of C57BL/6 adult mice whose pregnant mothers were injected at E12.5 with saline or with poly(I:C). Spleens were removed, and single cell suspensions prepared by mashing the spleens through 0.2um cell filters and washing with CLB buffer. RBC lysis was performed, and cells were fixed and stained at a final count of 2x10^6 cells per trial.

Antibodies for Flow Cytometry

IL-17A/APC (eBioscience #17-7177-81), CD62L/APC (Biolegend # 104411), CD44/PE (Biolegend #103007), CD4/FITC (Biolegend # 100405), CD4/PE (Biolegend # 100407), CD8/FITC (Biolegend # 100705), Gr-1/APC (Biolegend # 108411), CD11b/APC (Biolegend # 101211), B220/FITC (Biolegend # 103205), NK1.1/PE (Biolegend # 108707)

Results

MIA elevates IL-6 levels in the Placenta and Fetal Liver

As IL-6 has was previously shown to be crucial in mediating the effects of MIA, we examined overall levels of IL-6 in the tissues and supernatant of E13.5 and E15.5 fetal livers and placentas of poly(I:C) or saline injected pregnant mothers. IL-6 is significantly upregulated in the placentas of poly(I:C) embryos at E13.5. The fetal liver shows a similar trend, but the results are not significant. By E15.5 these changes are no longer apparent in either the placenta or the fetal liver (figure 1).

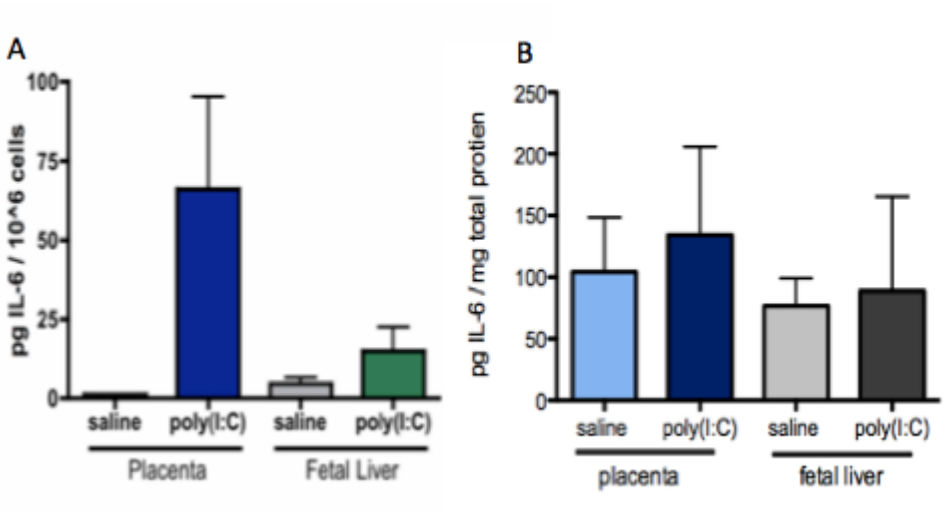

Figure 1. MIA Induced IL-6 in the Placenta and Fetal Liver: (A) IL-6 levels in the E13.5 placental and fetal liver supernatants collected from HSC isolation preparations. IL-6 is significantly upregulated in the placenta, but the upregulation in the fetal liver is not significant. (B) E15.5 IL-6 levels in placenta and fetal liver tissues. There are no longer any differences in IL-6 levels between saline and poly(I:C) groups.

MIA has no Effect on IL-17 levels in the E13.5 and E15.5 Placenta or Fetal Liver

We were also interested in examining IL-17 levels in the placenta and the fetal liver as IL-17 has been implicated in the dysregulated peripheral immunity of MIA in mice. ELISA was used to assess IL-17 levels in whole placenta and fetal liver tissues, and expression was normalized to total protein of the tissues. No significant differences were observed in IL-17 levels at E13.5 or E15.5 in either the placenta or the fetal liver.

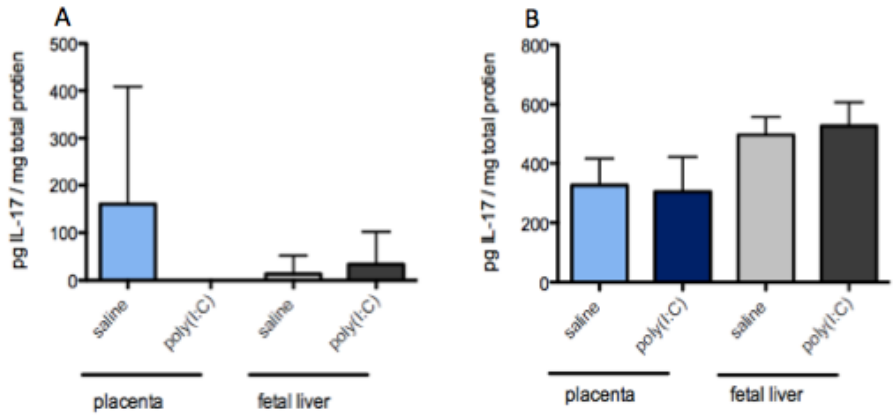

Figure 2. MIA has no effect on Il-17 levels in the placenta or fetal liver
(A) IL-17 levels in the placenta and fetal liver at E13.5 (B) IL-17 levels in the placenta and fetal liver at E15.5

HSCs express toll-like receptor 3

HSCs have various cytokine receptors, including the receptor for IL-6 (McKinstry et al, 1997). Thus, HSCs can respond directly to IL-6 through the IL-6 receptor. In addition, HSCs have various toll-like receptors (Nagai et al., 2006). As poly(I:C) signals through toll-like receptor 3, we were interested in whether or not placental CD34 dim c-Kit high HSCs have toll-like receptor 3. We used the markers CD34 and c-Kit as opposed to SLAM family markers, because Daley et al showed that while the placenta contains a large number of HSCs at E12.5, these cells most likely lack the SLAM family markers CD48 and CD150 as evidenced by repopulation assays (Daley et al., 2009). After cell sorting Real-Time PCR was used to evaluate TLR3 expression. TLR3 expression was observed in both fetal liver and placental HSCs. Although not significant, the TLR3 expression is lower in HSCs embryos from poly(I:C) versus saline injected mothers. This difference suggests that poly(I:C) may be inducing a downregulation of TLR3 in HSCs, perhaps through a negative feedback mechanism (figure 3). In order to visualize TLR3 in the placenta, we performed IHC on E13.5 saline tissues (figure 4).

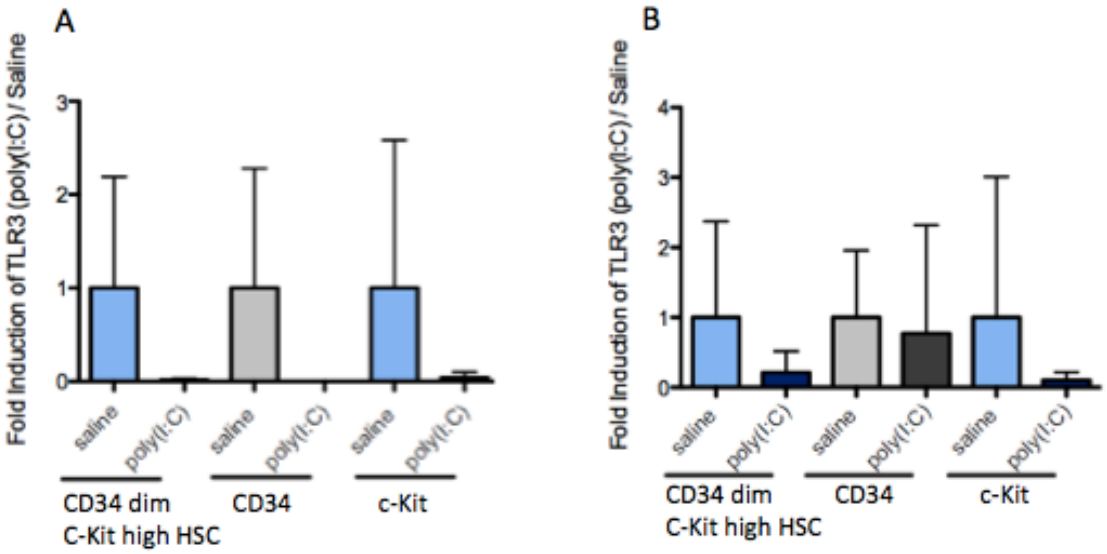

Figure 3. TLR3 expression in E13.5 Placenta and Fetal Liver HSCs (A). Fold induction of TLR (poly(I:C)/saline) in E13.5 placental HSCs (B) Fold induction of TLR (poly(I:C)/saline) in E13.5 fetal liver HSCs

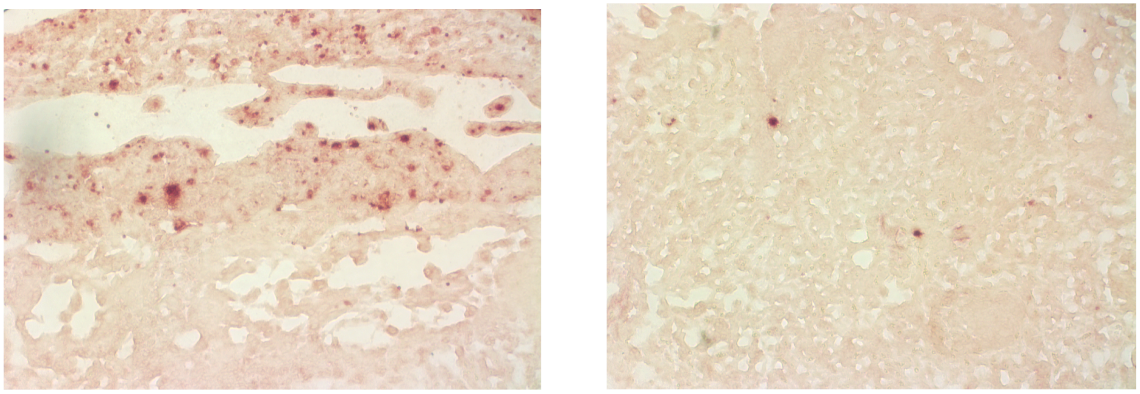

Figure 4. TLR3 staining in E13.5 Placenta, 10X objective (A). TLR3 in the decidua (B) TLR3 in the labyrinth

MIA has no Effect on the Abundance of HSCs in the Placenta or Fetal Liver

As IL-6 is upregulated at E13.5 and as HSCs express TLR3, we examined potential effects of MIA on abundance of HSCs in the placenta and fetal liver 24 hours post-poly(I:C) injection. Previous studies have shown that IL-6 and poly(I:C) alters the differentiation and/or proliferative capacities of HSCs (Essers et al., 2009; Suzuki et al., 1989). HSCs were isolated from the placenta and fetal liver using fluorescence activated cell sorting (figure 5). HSCs were CD34 dim c-Kit high and progenitors were single positive for either CD34 or c-Kit. Percent totals of cell populations reveals no differences in saline versus

poly(I:C) samples (figure 5). IHC for the nascent HSC and progenitor marker CD41 also reveals no differences in saline versus poly(I:C) tissues (figure 6).

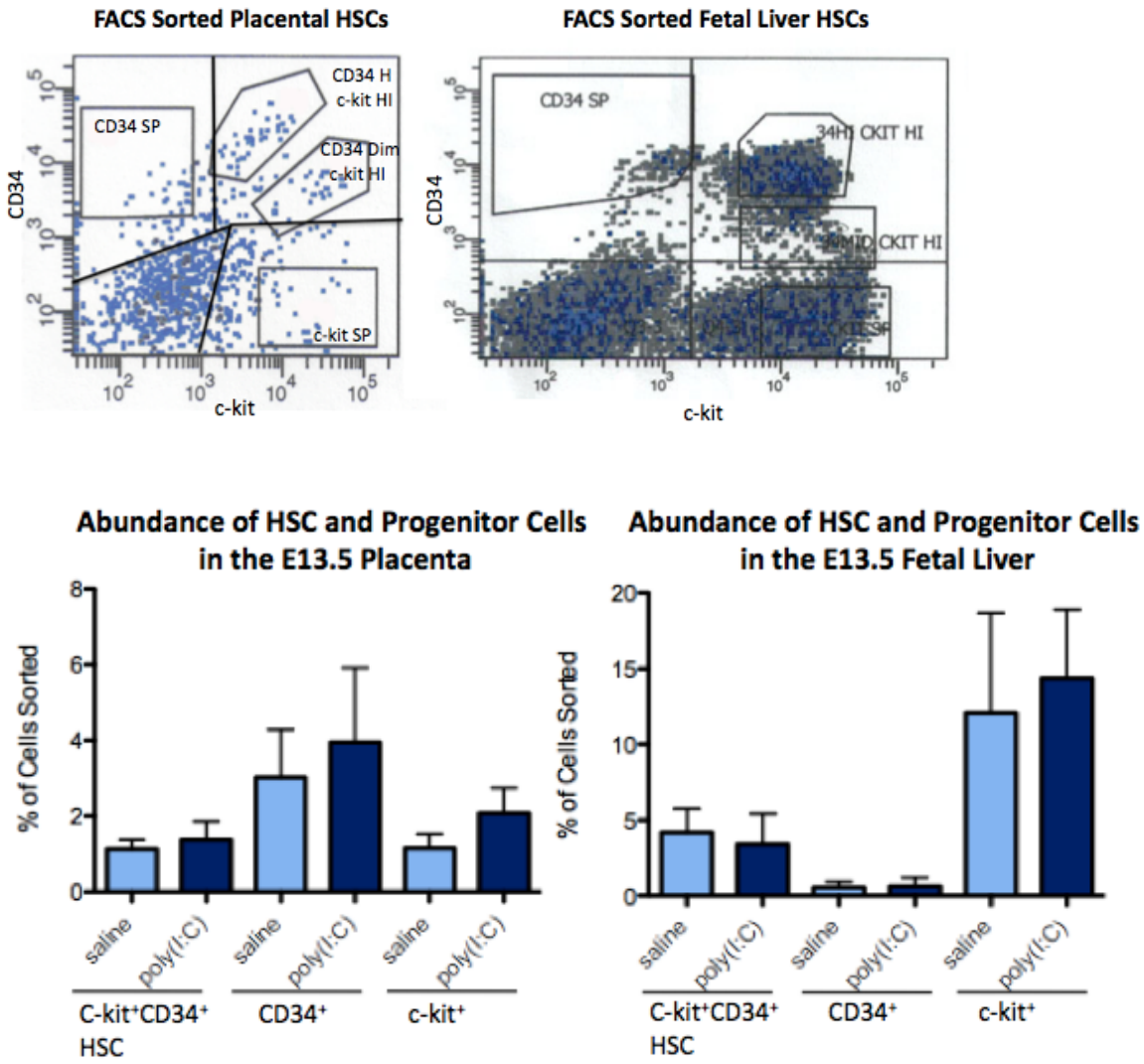

Figure 5. (Top) Representative FACS plots of HSCs (CD34 dim c-Kit High) and progenitors (CD34+ or c-Kit+) in the placenta and fetal liver
(Bottom) Percent total HSC and progenitor placenta and fetal liver cells sorted at E13.5. Percent of cells sorted refers to the percent of a specific cell type within the parent population.

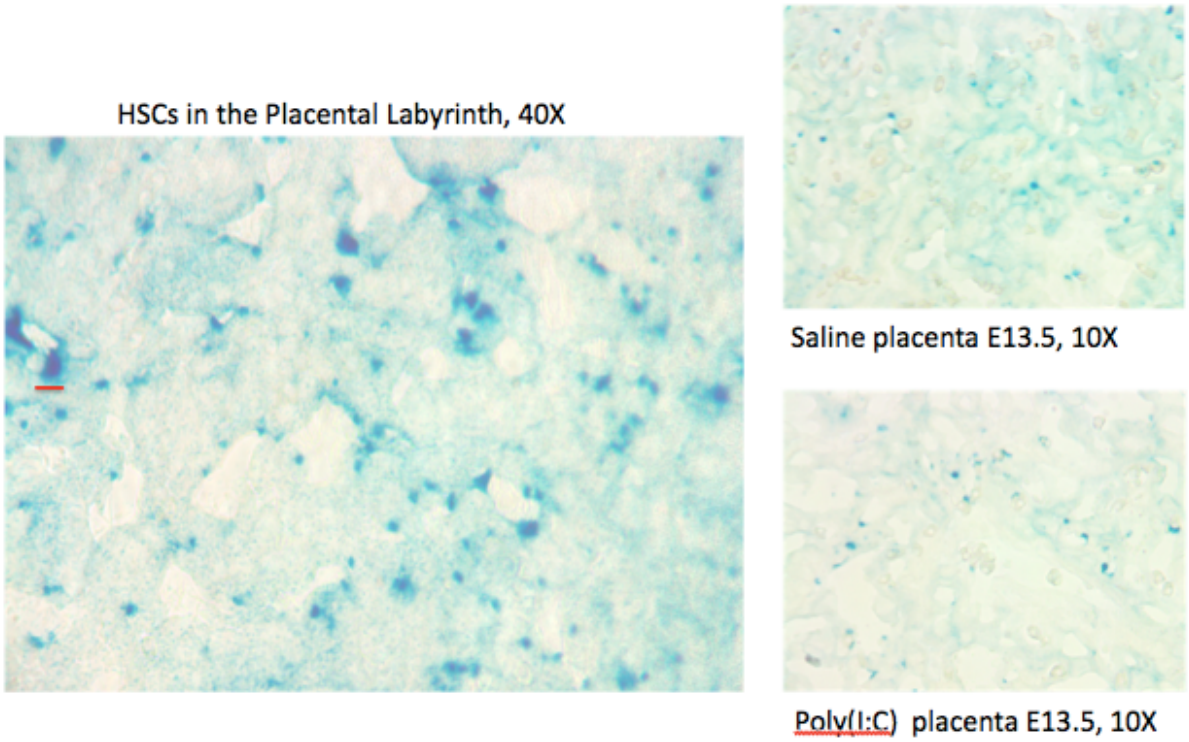

Figure 6. (Left) Red dash indicates a CD41+ HSC in the placental labyrinth at E13.5. HSCs range in size from about 8-12um, and the dash represents 10um. CD41 also stains platelets, but these are relatively smaller in size than HSCs. (Right) E13.5 CD41+ HSCs in the placental labyrinth of saline and poly(I:C) tissues. There is no difference in the abundance of HSCs in these tissues as indicated by IHC.

Poly(I:C) Induces Myeloid Lineage Potential In Vitro but not In Vivo

To evaluate potential effects of poly(I:C) on differentiation potential, colony forming methylcellulose assays for myeloid potential were done in the presence or absence of increasing concentrations of poly(I:C). E12.5 fetal liver were roughly minced and left overnight at 37°C with the indicated concentrations of poly(I:C) or vehicle. Single cell suspensions were prepared on E13.5, and cells were plated on methylcellulose (Stem Cell Technologies) supplemented TPO (5ng/mL). Myeloid colonies were scored on day 5 according to morphological differences. The results indicate that in vitro, poly(I:C) enhances the lineage potential of fetal liver cells (figure 7A). These results then led us to investigate the effects of poly(I:C) in vitro over a longer time. For this experiment we used the same procedure, but poly(I:C) was only added at the concentration of 25ug/mL, and colonies were scored at day 12. The effect of poly(I:C) on total colony count was no longer apparent by day 12 (figure 7B). We then repeated a similar experiment, but poly(I:C) was administered in vivo by i.p. injection of E12.5 pregnant mothers rather than being added to the culture medium. On E13.5, fetal liver cells were made into single cell suspension and plated on myeloid promoting methylcellulose. However, no differences in total colony counts were observed at days 5 or 12 (figure 8).

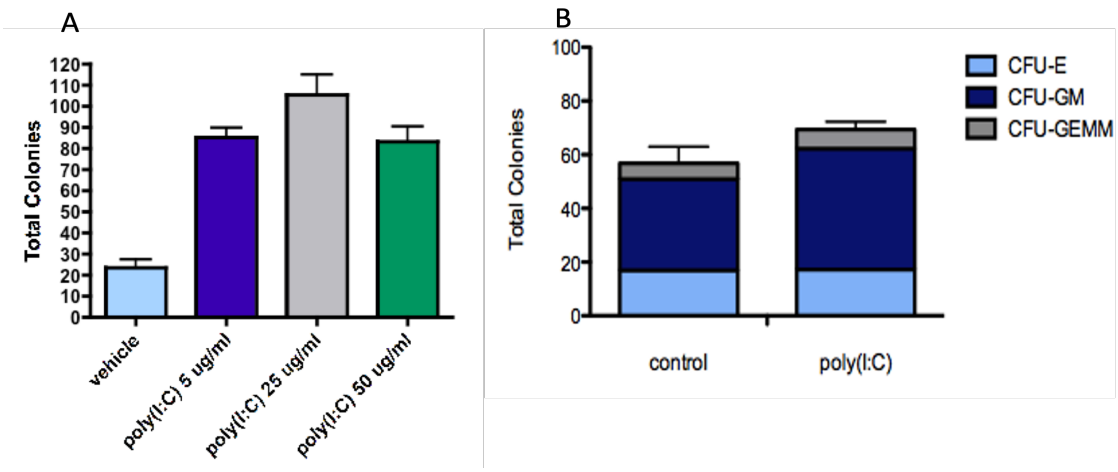

Figure 7. (A) Total Myeloid Colonies of Fetal Liver Primary Cells In Vitro Day 5. (B) Total Myeloid Colonies of Fetal Liver Primary Cells In Vitro Day 12
CFU-E (Erythroid), CFU-GM (granulocyte, macrophage), CFU-GEMM (granulocyte, erythroid, monocyte, macrophage)

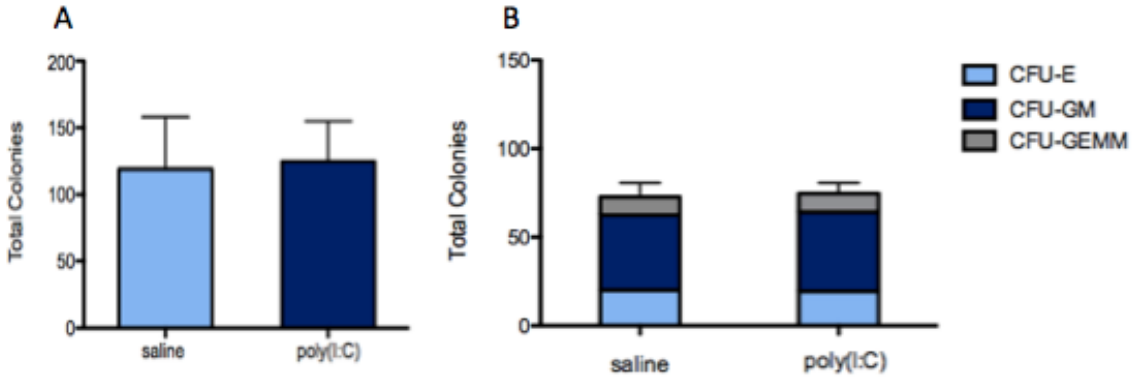

Figure 8. (A) Total Myeloid Colonies of Fetal Liver Primary Cells In Vivo Day 5. (B) Total Myeloid Colonies of Fetal Liver Primary Cells In Vivo Day 12

Alterations in Gene Expression of MIA HSCs and Progenitors

The in vitro assays with poly(I:C), the upregulation of IL-6, and the alterations in TLR3 expression led us to further investigate if MIA causes changes in HSC lineage potential in vivo. Pregnant mice were injected with 20mg/kg poly(I:C) or vehicle at E12.5. On E13.5 placental and fetal liver HSCs and progenitors were isolated by FACS as previously described. Real-Time PCR was used to evaluate gene expression levels of the common myeloid marker GATA-1 and the common lymphoid marker IL-7Rα. HSCs are CD34 dim c-Kit high and progenitors are either CD34+ or c-Kit+. We also sorted the CD34 high c-Kit high population. There are some trends that indicate MIA induced alterations in gene expression. However, these changes are not significant. The figures below show if expression of GATA or IL-7Rα is higher in the saline or poly(I:C) groups. N/A indicates

that no expression level was detected. Consistencies among time points are highlighted in red.

GATA Fetal Liver	E13.5	E15.5
HSC	same	Same
CD34	Same	Poly(I:C) up
c-Kit	Same	Same
CD34 high c-Kit high	Same	Same
GATA Placenta		
HSC	Poly(I:C) up	Same
CD34	Saline up	Saline up
c-Kit	Poly(I:C) up	Saline up
CD34 high c-Kit high	Saline up	Saline up

IL-7Rα Fetal Liver	E13.5	E15.5
HSC	same	same
CD34	Poly(I:C) up	Saline up
c-Kit	Poly(I:C) up	Saline up
CD34 high c-Kit high	same	same
IL-7Rα Placenta		
HSC	Saline up	Poly(I:C) up
CD34	N/A	N/A
c-Kit	N/A	Poly(I:C) up
CD34 high c-Kit high	N/A	Saline up

Spleen Cell Populations in Adult MIA Offspring

The observed changes in GATA-1 and IL-7Rα is not significant as there is high variation among the samples. In some cases, the fold induction of the relative expression of poly(I:C) over saline for a sample is 20 fold and in other instances there is no difference. Thus, in order to determine if these gene expression changes were leading to long-term alterations in cell populations, we evaluated overall cell populations in the adult offspring of mothers that were injected with either saline or poly(I:C) at E12.5. Again, these results are not significant.

Discussion

Poly(I:C) induced MIA is a useful model for studying symptoms of ASD and schizophrenia in mice. However, the mechanisms by which these disorders develop remain unclear. Previous studies have linked poly(I:C) induced MIA to behavioral deficits associated with ASD and schizophrenia and to dysregulated peripheral immunity. As the cytokine IL-6 is crucial to poly(I:C) induced MIA and as IL-6 can cross the placenta, we hypothesized that MIA could aberrantly affect the development of HSCs during embryogenesis. ELISAs confirmed the transient upregulation of IL-6 following MIA in the placenta at E13.5. In

addition, we were able to observe that TLR3 is expressed in placenta tissue, and more specifically in HSCs. Thus, it is possible that placental HSCs can respond not only to the effects of MIA induced IL-6 but also to the introduction of poly(I:C) via TLR3. Furthermore, methylcellulose assays in which poly(I:C) was introduced into the media for a 24 hour period prior to plating revealed that poly(I:C) can influence the lineage potential of HSCs for up to 5 days in vitro. We did not observe similar results in the "in vivo" methylcellulose experiments. However, as there are many inconsistencies between cell culture experiments and in vivo work, we decided to investigate potential differences in lineage potential of HSCs and progenitors isolated from E13.5 and E15.5 placentas and fetal livers. While alterations in gene expression for the myeloid marker GATA-1 and the lymphoid marker IL-7Rα were revealed, these changes were transient and insignificant. Likewise, no differences in spleen cell populations of adult MIA offspring were observed. Thus, it appears that while MIA is capable of inducing short-term changes in HSCs and progenitors, the significance of these changes is highly variable among samples. It may also be that HSCs harbor resistance to transient changes as they migrate between niches, as suggested by Kiel et al., 2005. Their study revealed that unlike stem cells of the nervous system, HSCs do not retain phenotypic, functional, or gene expression differences as they migrate between various niches (Kiel et al, 2005). Mikkola et al. showed that while the placenta supports HSCs, it does not promote their differentiation. However, the fetal liver does promote the differentiation of HSCs. Thus, a more significant injection time might be at E15.5, when the fetal liver HSC population reaches its peak (Mikkola et al., 2005). Another possibility is that while overall cell type is not affected, cell functioning may remain altered. Such deficiencies may not become apparent without additional insult, and further investigation would be needed to fully explore these possibilities.

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