Neuroprotection in preterm birth by modification of astrocyte polarization

SIGNIFICANCE:
Between 28 and 34 weeks gestation, the major axon tracts of the developing human brain are exquisitely vulnerable to injury. For reasons that are incompletely understood, repeated inflammatory and ischemic insults during this period irreversibly damage the myelinating cells of these axon tracts, leading to defects in subcortical myelination (white matter injury, WMI) and to a consequent constellation of long-term neurological disability known clinically as Cerebral Palsy (1-3). Most cases of WMI occur in infants born preterm as a result prenatal, intrapartum, and/or postnatal insults during this window of white matter vulnerability (3,4). Treatments to rescue or inhibit this brain damage are extremely limited, due to poor understanding of underlying disease pathophysiology. For treatments that do exist, the mechanisms of action remain unclear, making optimization of therapy exceedingly difficult (4,5). Given the rising rate of preterm birth worldwide, more effective neuroprotective therapies are desperately needed (6).

While reactive astrocytes have long been recognized as hallmarks of preterm WMI, what role they play remains unclear. Evidence highlights different reactive astrocyte profiles that are dependent on the mode of initial insult, and these reactive astrocytes can be beneficial/neuroprotective (termed ‘A2 reactive astrocytes’) or detrimental/neurodegenerative (‘A1 reactive astrocytes’) (7,8). I have recently generated preliminary data showing the formation of A1 astrocytes in a rodent model of WMI. This proposal focuses on neurodegenerative (A1) reactive astrocytes as putative key early mediators of WMI and a novel therapeutic target in infants born preterm. In the experiments described, we set out to define the precise timing of A1 formation relative to brain insults in WMI, to test the ability of in utero versus postnatal blockade of A1 astrocyte formation to improve disease outcomes, and to design a therapeutic approach to WMI targeting these cells in the preterm fetus.

Figure 1: Features and Multiple-hit Etiology of Preterm White Matter Injury
A. Injury occurs in the white matter adjacent to the lateral ventricles and is characterized acutely by diffuse gliosis and in extreme cases, areas of focal cystic necrosis.
B. Multiple hits of inflammatory and ischemic nature during the peak period of pre-oligodendrocyte vulnerability 24-32w gestation underlie cases of preterm WMI. In this figure, an example of a series of inflammatory and ischemic events leading to perinatal WMI. Modified from (23).
**BACKGROUND:**

**WMI results from multiple inflammatory / hypoxic / ischemic insults to the developing brain.**

White matter injury is a major complication in infants born prematurely. In terms of neuropathology, the disease is most often characterized by injury, death, and failed maturation of myelinating cells, diffuse white matter gliosis involving activated microglia and reactive astrocytes, and in some cases, small regions of focal necrosis (Figure 1). Both inflammatory and hypoxic/ischemic insults are recognized to play an important role in the development of these features (1). In addition to postnatal and intrapartum inflammation/hypoxia/ischemia, the contribution of intrauterine events such as maternal infection and intrauterine growth restriction to the pathophysiology of WMI is increasingly recognized (1-3, 9).

Along these lines, exposure to *multiple* perinatal inflammatory and/or hypoxic/ischemic “hits” is central to the development of WMI, with a first insult sensitizing the developing preterm brain to subsequent insults that worsen injury (1,10). This concept is supported by data from clinical studies showing that exposure to multiple insults greatly increases the risk of white matter abnormalities (10). The mechanisms linking repeated inflammation/hypoxia/ischemia to myelination failure are incompletely understood, but candidate mechanisms include inflammatory cytokine production, free radical generation, and glutamate excitotoxicity (1-3).

**Effective neuroprotection for infants born preterm is lacking.**

At present, magnesium sulfate (MgSO₄) infusion before birth is the only available therapy to combat WMI in infants born preterm. Meta-analysis of multiple randomized controlled trials has demonstrated a neuroprotective effect of antenatal MgSO₄ infusion on Cerebral Palsy at two
years of age. As a result, antenatal MgSO4 infusion is currently recommended in women ≤ 32 weeks gestation at risk for preterm. However, the efficacy of this therapy is limited and impact on neurological performance (motor, cognitive, and behavioral) has not been demonstrated into later childhood. The mechanism through which MgSO4 works also remains unclear, making further optimization of this therapy difficult (11,12). Neonatal cooling, although an option for term infants, is not currently approved for preterm infants (4).

**A1 reactive astrocytes are a novel target for neuroprotection in preterm WMI.**
Whether reactive astrocytes play a beneficial or detrimental role after brain injury is a long-standing controversy. One reason for this controversy is likely heterogeneity of the astrocyte response to injury (8,13). Indeed, recent work demonstrates that distinct sub-types of reactive astrocytes form in response to different types of injury (8). So far, two different types of reactive astrocytes are recognized in Central Nervous System injury. A1 astrocytes are induced by inflammation and form in response to specific factors released by reactive microglia, namely complement protein C1q and cytokines IL-1α and TNF (8,13). A1s kill neurons, delay the maturation of myelinating cells, and are increasingly recognized to play a role in neurodegenerative disease (8,15). In contrast, A2 astrocytes form predominately in response to ischemia and appear to be supportive of neuronal survival and tissue repair (8). The specific nature of astrocyte reactivity after WMI (A1s, A2s, or other) is unclear (11, Figure 2).

Given that A1 reactive astrocytes inhibit the maturation of myelinating cells and lead to neurodegeneration, outcomes characteristic of preterm WMI, we hypothesized that A1s form in preterm WMI and drive WMI outcomes. In support of our hypothesis, I have recently generated preliminary data in a combined inflammation/ hypoxia/ ischemia rat model of preterm WMI indicating the formation of A1 astrocytes within 24 hours of injury (Figure 3). This finding opens the door to a series of experiments aimed to develop a new neuroprotective therapy targeting the formation of these cells.

According to my preliminary experiments, A1 astrocytes form within 24 hours of initial inflammatory injury at postnatal day 2. This timecourse suggests that in the many cases of
preterm WMI in which an initial “hit” to the developing brain, such as maternal infection, occurs prenatally, A1 formation occurs before birth. The question then arises whether disease outcomes can be improved by preventing formation of A1 astrocytes in the fetal brain before preterm delivery. In Specific Aims 1 and 2, our goal is to precisely define the timing of A1 formation in a rodent model of preterm WMI and to understand when during the disease process (prenatally or postnatally) A1 formation must be inhibited in order to provide optimal neuroprotection.

Exosomes derived from hWJ-MSCs are a promising candidate for inhibiting A1 astrocyte formation

In animal studies of preterm WMI, delivery of human Wharton’s Jelly derived Mesenchymal Stem Cells (hWJ-MSCs) has been shown to mitigate myelination defects, to decrease microglia and astrocyte reactivity, to induce neuroregeneration, and to ameliorate behavioral function (16,17). Our lab has been a key player in advancing understanding of the mechanisms through which these cells exert their effects and in innovating ways to deliver these cells therapeutically (13,16,17,19,20). Interestingly, the major therapeutic effect of these cells appears to act in a paracrine fashion, mediated at least in part through the release of extracellular vesicles called exosomes (18,19). In terms of clinical application, these exosomes hold great therapeutic promise because they can achieve the same regenerative effects as their cells of origin, without the safety concerns of cell-therapies (20).

The optimization of exosomes as a therapeutic tool in WMI has been limited thus far by incomplete understanding of their mechanism of action. Prenatal delivery of exosomes in WMI has not yet been investigated. Of particular relevance to the mission of inhibiting A1 astrocyte formation, our lab has just published data demonstrating that hWJ-MSC-derived exosomes delivered intranasally in a rodent model of WMI significantly reduce microglia-mediated inflammation (20). Given that A1 astrocytes form in response to inflammatory cytokines secreted by activated microglia, this finding suggests that inhibition of A1 astrocytes may be a crucial step underlying the known therapeutic effects of hWJ-MSCs and their derived exosomes in preterm WMI. (20, 8). In Specific Aim 3, we test the hypothesis that hWJ-MSC-derived exosomes block A1 astrocyte reactivity in vitro and in vivo. Finally, using the window for optimal therapeutic efficacy determined in Specific Aim 2, we test the ability of these vesicles to improve outcomes in preterm WMI. Throughout the proposed experiments we use a fetal inflammation plus postnatal hypoxia rat model of preterm WMI in which we can detect the timing of formation of A1 astrocytes relative to multiple insults, study the efficacy of prenatal versus postnatal inhibition of A1 formation, and test a prenatal delivery strategy for hWJ-MSC-derived exosomes (10).

SPECIFIC AIMS

Objective: Define a clinically-feasible and mechanistically-informed therapeutic strategy for fetal neuroprotection in cases of preterm birth that targets the formation of A1 reactive astrocytes (A1s). Treatment options for WMI are currently extremely limited, due in large part to incomplete understanding of disease pathophysiology. For treatments that do exist, the mechanisms of action remain unclear, making optimization of therapy exceedingly difficult. This proposal focuses on A1 reactive astrocytes as putative key early mediators of disease in WMI. In Specific Aims 1 and 2, we elucidate critical parameters of A1 formation and link to disease outcomes. In Specific Aim 3, we test whether exosomes derived from Wharton’s Jelly Mesenchymal Stem Cells (WJ-MSCs) inhibit A1 formation, and, informed by the mechanistic insight gained in Specific Aims 1 and 2, test the potential of these vesicles to mitigate WMI outcomes.
**Hypothesis 1 (Aim 1):** We will use gene expression analysis methods to test the hypothesis that \( \text{A1 reactive astrocytes form within 24 hours of an inflammatory insult.} \)

**Hypothesis 2 (Aim 2):** Using transgenic mice and neutralizing antibodies to inhibit the formation of A1s, we will test the prediction that prenatal inhibition of A1 astrocyte formation is more neuroprotective than postnatal inhibition.

**Hypothesis 3 (Aim 3):** Through a combination of in vitro and in vivo experiments, we will determine if hWJ-MSC-derived exosomes inhibit A1 astrocyte formation and if these secreted vesicles can be delivered prenatally to ameliorate disease outcomes in preterm WMI.

**SPECIFIC AIM 1:** Identify the timing of A1 astrocyte formation in preterm WMI initiated by in utero inflammation.

When, where, and for how long do A1 astrocytes form after prenatal inflammation
- In a combined fetal inflammation plus postnatal hypoxia rat model of preterm WMI?
- In postmortem human neonatal brains following preterm delivery with confirmed intra-uterine infection or confirmed white matter injury?

**SPECIFIC AIM 2:** Determine the optimal window for therapeutic modulation of A1 reactivity with respect to preterm WMI disease outcome.

How are WMI disease outcomes affected when A1 astrocyte formation is blocked
- Prior to inflammatory insult?
- Prenatally, at various timepoints before and after A1 formation?
- Postnatally?

**SPECIFIC AIM 3:** Test the hypothesis that WJ-MSC-derived exosomes can inhibit A1 astrocyte formation and provide neuroprotection to infants born preterm.

- How do hWJ-MSC-derived exosomes modulate astrocyte reactivity in vitro?
- How do hWJ-MSC-derived exosomes modulate astrocyte reactivity and WMI disease outcomes in vivo?

**SPECIFIC AIM 1:** Identify the timing of A1 astrocyte formation in a combined fetal inflammation + postnatal hypoxia model of preterm birth with WMI

**Figure 4:** Combined fetal inflammation/postnatal hypoxia rat model of preterm WMI.
A1 formation in this model will be evaluated at multiple pre- and postnatal timepoints after intraperitoneal maternal LPS injection. Disease outcome will be evaluated using MBP immunofluorescence. Modified from (10).
Knowledge of the precise timing of A1 formation is critical to developing therapies aimed to inhibit or modulate A1 astrocyte reactivity. In a fetal inflammation + postnatal hypoxia model of preterm WMI (Figure 4, (15)), we test the hypothesis that A1 astrocytes form within 24 hours of inflammatory stimulus. We will 1) use A1-specific in situ hybridization (ISH) probes to establish the timecourse and distribution of A1 astrocyte formation after inflammatory stimulus, 2) use qRT-PCR of astocytes acutely purified from injured brain tissue to establish the state of astrocyte polarization (A1s/A2s) over the same timecourse and 3) correlate our rodent timecourse with (ISH) studies of A1 formation in post-mortem human fetal brains.

The rat model of preterm WMI chosen for this study involves repeated maternal intraperitoneal LPS injection at embryonic day E18 and E19 followed by exposure to 8% O2 for 140 minutes on postnatal day 4 (P4). This model of WMI results in sustained myelination defects. The model is relevant to human preterm WMI because between E18 and P 4, the rodent brain is comparable developmentally to the human brain at 24-32 weeks gestation (10). In our lab, we have many years of experience using LPS and hypoxia to induce preterm WMI in rodents. We have all of the equipment and expertise necessary to use this rodent model for the proposed experiments.

For in situ hybridization experiments, co-localization of Slc1a3 (astrocyte marker) and C3 (A1 marker) probe signal as well as exclusion of Aif1 signal (microglia marker) will be used to identify A1 astocytes (Figure 2). A1 astrocyte formation will be quantified in the corpus callosum, internal capsule, and external capsule regions (Figure 2). Animals will be sacrificed for brain tissue collection at E20, E22/P0, P5, P8, and P12 (Figure 4).

To examine A1 astrocyte gene expression in WMI more deeply and to gain a picture of astrocyte polarization (A1 versus A2) at the same timepoints, astrocytes will be purified from naive and injured rat cortex and subcortical white matter using immunopanning. Purified RNA from these cells will be analyzed using qRT-PCR. The protocol for acute purification of astrocytes from the rat brain using immunopanning has been used successfully in our hands (Figure 5). We are currently optimizing the purity of our immunopanned astrocytes. For qRT-PCR analysis, we have established a collaboration with Shane Liddelow, Assistant Professor at New York University, who has extensive experience performing microfluidics gene expression analysis of reactive astrocytes (see letter of support).
In order to confirm the relevance of our findings in this Aim to human disease, we propose to examine A1 astrocyte formation in post-mortem human fetal brain tissue. The tissue for these studies will be obtained through collaboration with the Institute for Neuropathology at the University of Zurich (Professor Adriano Aguzzi). Ethics approval is pending.

**SPECIFIC AIM 2: Determine the optimal window for therapeutic modulation of A1 reactivity with respect to neuroprotection in cases of preterm birth.** Studies of WMI indicate that the occurrence of multiple perinatal hits plays a crucial role in disease pathophysiology, with a first insult sensitizing the developing brain to subsequent insults (10). In our rodent model of preterm WMI (fetal inflammation plus postnatal hypoxia), we test the hypothesis that inhibiting A1 astrocyte formation prenatally, prior to subsequent intrapartum or neonatal insults, can protect the brain from sequelae of these later hits and is more protective than postnatal inhibition of A1 formation. We will assess myelination outcomes in our preterm WMI model 1) in triple knockout mice (Il1a-/ Tnf-/C1q-) in which A1 astrocytes are not generated, and 2) following intraventricular injection of neutralizing antibodies (anti-C1q, anti-TNF, and anti-Il-1α) to inhibit the formation of A1s at multiple prenatal and postnatal timepoints. (Figure 6)

The efficacy of neutralizing antibodies (anti-C1q, anti-TNF, and anti-Il-1α) as inhibitors of A1 formation has been demonstrated in the rodent retina, where intra-vitreal injection of these antibodies blocked A1 formation at the optic nerve head (8). For in utero intraventricular injections, we will use light-guided antibody delivery into the rat fetal lateral ventricle (just adjacent to the targeted white matter tracts) after surgical exposure of the uterus. For intraventricular antibody delivery to postnatal pups, we will use stereotaxic methods.

Triple knockout mice (Il1a-/ Tnf-/C1q-) lacking the ability to form A1 astrocytes will be obtained and bred in-house at the University of Bern in collaboration with Shane Liddelow at New York University (see letter of support). These mice mature and reproduce normally.

Depletion of A1 astrocytes using the methods just discussed will be assessed using in situ hybridization. MBP immunofluorescence in subcortical white matter tracts will be used to assess an effect on WMI disease outcome.
SPECIFIC AIM 3: Test the hypothesis that WJ-MSC-derived exosomes can inhibit A1 astrocyte formation and that in utero delivery of these vesicles mitigates disease outcomes in preterm WMI.

MSCs harvested from the Wharton’s Jelly of the human umbilical cord (hWJ-MSCs) have well-established therapeutic properties in perinatal brain injury (16,17,19). While their mechanism of action is incompletely understood, the therapeutic effect of these cells is mediated at least in part through the release of extracellular vesicles called exosomes (20). Very recent work from our lab indicates that when delivered to the neonatal brain, the purified exosomes released from WJ-MSCs reduce microglia-mediated inflammation, targeting the same pathways that are known to be upstream of A1 astrocyte formation (Figure 7 A, B) (8,20). In order to assess hWJ-MSC-derived exosomes as a potential neuroprotective therapy for preterm WMI, we will test whether these exosomes can prevent or revert the generation of A1s 1) in vitro using primary rodent astrocytes and an established in vitro model for A1 astrocyte formation and 2) in vivo following intra-amniotic delivery of exosomes to the fetal rodent brain.

Our lab has extensive experience isolating hWJ-MSCs from human umbilical cords and purifying the exosomes produced by these cells (20). Ethics approval has been obtained for this procedure.

For in vitro studies, resting astrocytes will be purified through immunopanning (Figure 5) and maintained in tissue culture. Microglia-derived factors C1q, TNF, and Il-1α will be added to the astrocytes in culture in order to induce A1 astrocyte formation. The formation of A1 astrocytes in vitro through these means has been established (8). To test whether hWJ-MSC-derived exosomes can prevent the formation of A1 astrocytes, purified exosomes will be added to the culture media before the addition of the activated microglia-derived factors. To test whether hWJ-MSC-derived exosomes can revert A1 formation, addition of purified exosomes will be carried out after exposure of the
astrocytes to the microglia-derived factors. Astrocyte polarization (expression of A1/A2 genes) will be assessed using qRT-PCR of purified astrocyte RNA.

For in vivo studies, we will test the ability of hWJ-MSC-derived exosomes to ameliorate WMI disease outcomes when delivered during the therapeutic window defined in Specific Aim 2. Our lab has already established that exosomes delivered intranasally at postnatal day 2 reach the brain and exert anti-inflammatory effects on microglia in white matter tracts (20,22). For prenatal delivery, we will probe our ability to accomplish intranasal delivery of exosomes to the fetal brain through injection of hWJ-MSC-derived vesicles into the amniotic fluid (Figure 7C). Delivery of exosomes to the fetal brain through physiologic fetal inhalation of amniotic fluid will be assessed using infrared dye-labeling of the exosomes and subsequent infrared scanning of the brain. Effect on A1 astrocyte formation will be determined through in situ hybridization. As in Specific Aim 2, neuroprotective impact will be evaluated through MBP immunohistochemistry at postnatal day 18.

**TIMELINE:**
**Year 1:** Experiments described in Specific Aim 1. Acquisition of human fetal tissue. Acquisition of reagents and transgenic mice needed for Specific Aim 2. Begin in vitro component of Specific Aim 3.
**Year 2:** Experiments described in Specific Aim 2. Complete in vitro component of Specific Aim 3.
**Year 3:** In vivo component of Specific Aim 3.

**REFERENCES**

11. Fetal Neuroprotection by Magnesium Sulfate: From Translational Research to Clinical Application. Front Neurol. 2018 16(9):247


