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TITLE:

A Murine Model of Fetal Exposure to Maternal Inflammation to Study the Effects of Acute Chorioamnionitis on Newborn Intestinal Development

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KEYWORDS:

Lipopolysaccharide (LPS), Fetal exposure to maternal inflammation (FEMI), Chorioamnionitis, Intestinal development, Paneth cell, Goblet cell

SUMMARY:

We developed a model of chorioamnionitis to simulate fetal exposure to maternal inflammation (FEMI) without complications of live organisms to examine the effects of FEMI on development of the offspring's intestinal tract. This allows for study of mechanistic causes for development of intestinal injury following chorioamnionitis.

ABSTRACT:

Chorioamnionitis is a common precipitant of preterm birth and is associated with many of the morbidities of prematurity, including necrotizing enterocolitis (NEC). However, a mechanistic link between these two conditions remains yet to be discovered. We have adopted a murine model of chorioamnionitis involving lipopolysaccharide (LPS)-induced fetal exposure to maternal inflammation (FEMI). This model of FEMI induces a sterile maternal, placental, and fetal inflammatory cascade, which is also present in many cases of clinical chorioamnionitis. Although models exist that utilize live bacteria and more accurately mimic the pathophysiology of an ascending infection resulting in chorioamnionitis, these methods may cause indirect effects on

development of the immature intestinal tract and the associated developing microbiome. Using this protocol, we have demonstrated that LPS-induced FEMI results in a dose-dependent increase in pregnancy loss and preterm birth, as well as disruption of normal intestinal development in offspring. Further, we have demonstrated that FEMI significantly increases intestinal injury and serum cytokines in offspring, while simultaneously decreasing goblet and Paneth cells, both of which provide a first line of innate immunity against intestinal inflammation. Although a similar model of LPS-induced FEMI has been used to model the association between chorioamnionitis and subsequent abnormalities of the central nervous system, to our knowledge, this protocol is the first to attempt to elucidate a mechanistic link between chorioamnionitis and later perturbations in intestinal development as a potential link between chorioamnionitis and NEC.

INTRODUCTION:

The chorionic membranes play an integral role in mammalian pregnancy. They include the chorion and amnion, which serve multiple functions. They surround and protect the fetus, facilitate paracrine signaling between the maternal and fetal compartments[1], and create local feedback loops within the chorionic membranes, which may be involved in initiating parturition[1]. Current understanding of the membranes indicates that the amnion provides structural barrier function, and the chorion provides an immunological buffer primarily to protect the developing fetus from the maternal immune system[2]. Inflammation of these membranes is known as chorioamnionitis. Historically, the diagnosis of clinical chorioamnionitis was made following the presence of maternal fever plus one or more fetal or maternal clinical findings[3,4]. However, while this definition is clinically useful, its lack of precision has made chorioamnionitis research challenging. In 2015, in an attempt to clarify the diagnosis, an expert panel workshop by the Eunice Kennedy Shriver National Institute for Child Health and Human Development defined chorioamnionitis as intrauterine inflammation, or infection, or both (triple I)[3]. This clarification is important because while microbial induced infection is an important cause of uterine/amniotic inflammation, it occurs less commonly than sterile uterine/amniotic inflammation[5-7]. Overall, chorioamnionitis remains a significant public health problem, as it is seen in 2–4% of term deliveries and 25–30% of preterm deliveries[8,9].

Chorioamnionitis can have significant effects on the fetus and neonate. It has been well documented in the literature that chorioamnionitis is associated with increased risk of many of the morbidities of prematurity, including bronchopulmonary dysplasia[10], cerebral white matter injury[11], intraventricular hemorrhage[12], retinopathy of prematurity[13], and both suspected and confirmed early onset neonatal sepsis[14,15]. As we are interested in injury and repair mechanisms of the immature intestinal tract, it is important to note that chorioamnionitis is also associated with later development of necrotizing enterocolitis (NEC)[15,16]. NEC is a devastating gastrointestinal disease of preterm infants that results in a dysregulated host response to inflammation and subsequent intestinal necrosis[17]. Each year, NEC affects over 4000 infants in the United States, and up to one third of these infants die from the disease[18]. The pathogenesis of NEC likely involves a combination of intestinal immaturity, dysregulation of the immature immune system, intestinal inflammation, and bacterial translocation[19], culminating in a final common pathway of intestinal necrosis. Importantly, the onset of NEC often occurs weeks after birth and potential exposure to chorioamnionitis, making the mechanistic link between

chorioamnionitis and subsequent development of NEC unclear[20]. One potential mechanism by which chorioamnionitis may contribute to the pathophysiology of NEC is through upregulation of the maternal immune system, subsequently producing a strong fetal inflammatory response which may disrupt normal fetal developmental patterns[21-23].

Multiple mammalian models of chorioamnionitis exist in rodents and sheep[24-32]. However, few data exist concerning the development of the intestinal tract beyond the initial newborn period following chorioamnionitis-induced fetal exposure to maternal inflammation (FEMI). In order to explore the relationship between FEMI and subsequent development of injury of the immature intestinal tract, we have adapted the lipopolysaccharide (LPS)-induced FEMI model. Lipopolysaccharides are a major component of the extracellular surface on gram negative bacteria and are a potent stimulant of the innate immune system of multiple eukaryotic species, including humans[33]. Maternal LPS injection results in a sterile inflammatory cascade without the confounding effects of live bacteria, and it is a well-established model for induction of preterm birth[34], as well as a model of acute chorioamnionitis and the fetal inflammatory response syndrome (FIRS), which is the most severe form of chorioamnionitis[24,35]. It has also been shown to induce both cerebral white and gray matter injury in a sheep model[36] and a murine model[37-40]. However, to our knowledge, we are the first to use this model of chorioamnionitis and FEMI to investigate its effects on the development of the gastrointestinal tract past birth, as well as to investigate a possible mechanistic link between chorioamnionitis and later development of NEC[41,42].

PROTOCOL:

All animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee (Protocol #8041401). All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved vivarium at the University of Iowa. All mice were wild type strain C57Bl/6J.

1. Establishment of FEMI in pregnant mice

1.1. LPS preparation

1.1.1. Use LPS derived from *Escherichia coli* O55:B5 (stock concentration 2 mg/mL).

1.1.2. Dilute LPS stock concentration 1:100 with sterile saline for a working concentration of 20 µg/mL.

1.2. Maternal LPS injection

1.2.1. Inject pregnant dams at gestation day e15. This timepoint is approximately 75% through the murine pregnancy, making this model developmentally similar to the early third trimester of human pregnancies, which is when the majority of preterm births due to chorioamnionitis occur.

1.2.2. Weigh pregnant mice immediately prior to injection to determine appropriate LPS dosing.

1.2.3. Calculate the dose of working concentration by using the following formula: 5 µL x gram body weight (gbw), for a total dose of LPS of 100 µg/kg. For control animals, use an equivalent volume of normal saline for injection.

1.2.4. Vortex LPS solution three times for 15 seconds on high prior to each injection.

1.2.5. Draw up LPS volume into a 1 mL syringe.

1.2.6. Restrain pregnant mouse with scruffing technique. Hold in dorsal recumbency position and perform the injection.

1.2.6.1. Insert a 30 gauge 8 mm needle bevel up overlying the right lower quadrant of the abdomen (to avoid bladder and abdominal vessels) at a 30–40° angle. Insert about ¼ to ½ the length of the needle.

1.2.6.2. Pull back on the syringe plunger to ensure negative pressure prior to injecting. Proceed with injection if negative pressure is present.

1.2.6.3. Following injection, monitor mice for approximately 30 min and then return to cages for the remainder of the pregnancy.

2. Delivery and care of offspring, and intestinal harvesting

2.1. Deliver pups normally via vaginal delivery at e20.

NOTE: This model does have an expected dose-dependent fetal loss rate that can be seen in **Figure 1** and is discussed in the results below.

2.2. Allow pups to remain with mothers and receive ad libitum feeds.

2.3. On the day of harvest, typically postnatal day 14 (P14), euthanize pups via cervical dislocation in compliance with Institutional Animal Care and Use Committee protocols.

2.4. Using scissors and forceps, make a vertical incision down the midline of the abdomen, through the skin and peritoneum, for the entire length of the abdomen. Excise the small intestine from the stomach to the cecum with scissors and remove the mesentery with forceps.

2.5. Isolate and keep the distal 1/3 of the small intestine (section representative of human ileum), discarding the proximal small intestine, the cecum, and the colon.

2.6. Divide the ileum portion in half using scissors.

2.7. Place the proximal half in an RNA stabilization solution for later RNA quantification.

2.8. Place the distal half in 10% neutral buffered formalin for slide preparation.

3. Intestinal injury scoring

3.1. Section paraffin embedded tissue into 5 µm thick slices and mount on glass slides.

NOTE: We send the specimens to a histology core for paraffin embedding, sectioning, and mounting onto slides.

3.2. Deparaffinize slides according to standard procedures.

3.3. Stain sections with hematoxylin and eosin according to standard procedures.

3.4. Score sections on a 3-point scale for intestinal injury as previously described[42,43].

3.4.1. Using light microscopy, assess generalized intestinal injury by two separate blinded investigators on a 3-point scale evaluating villus integrity and separation from the basement membrane[43] (**Supplementary Figure 1**). Intestinal injury is best assessed at 20x magnification and numerical aperture 0.50.

3.4.2. Assign a score of 0 to describe normal mucosa.

3.4.3. Assign a score of 1 describe mild injury which encompasses the development of subepithelial Gruenhagen's space, vacuolization or subepithelial lifting limited to the lamina propria or tips of villi.

3.4.4. Assign a score of 2 to describe severe injury, indicated by epithelial lifting and vacuolization greater than half of the villi, villi distortion, or mucosal ulceration and disintegration of the lamina propria.

4. Quantification of Paneth and goblet cells

4.1. Following deparaffinization, stain slides of tissue sections from Step 2.8 with Alcian Blue/Periodic Acid Schiff stain to denote both goblet and Paneth cells as previously described[44,45] according to the following steps.

NOTE: While Alcian Blue/Periodic Acid Schiff stain is not specific to either Paneth or goblet cells, in our experience, blinded experienced investigators have equivalent cellular quantification using this stain compared to cellular targeted antibodies, with significantly less background staining[46].

4.2. Deparaffinize, stain, and dehydrate slides as follows.

4.2.1. Submerge slides in xylene for 10 min twice.

CAUTION: Xylene should be used in a fume hood.

4.2.2. Rinse with 100% EtOH.

4.2.3. Submerge slides in 100 % EtOH for 3 min, then in 90% EtOH for 3 min, followed by 70% EtOH for 3 min, and lastly submerge slides in 50% EtOH for 3 min.

4.2.4. Wash under running tap water for 5 min.

CAUTION: Point the section away from the running water to prevent loss of tissue sample.

4.2.5. Filter Alcian blue stain solution with a standard coffee filter.

4.2.6. Stain slides in Alcian blue stain for 15 min and then wash under running tap water for 2 min.

4.2.7. Dilute 1 mg of periodic acid in 200 mL of double distilled water. Submerge slides in this solution for 5 min. Then wash under running tap water for 1 min.

4.2.8. Stain with Schiff's reagent for 10 min. Wash under running tap water for 5 min.

4.2.9. Stain the slides with hematoxylin for 1 min and then wash under running tap water for 2 min.

4.2.10. Submerge them in acid alcohol (1 mL of hydrochloric acid mixed in 99 mL of 70% EtOH) for 1 min.

4.2.11. Submerge in Scott's tap water (0.1% concentration of $NaHCO_3$ in tap water) for 1 min and then wash under running tap water for 1 min.

4.2.12. Dehydrate the slides.

4.2.12.1. Dip each slide 10 times in 70% EtOH, then dip 10 times in 90% EtOH, and 10 times in 100% EtOH.

4.2.12.2. Submerge slides in 100% EtOH for 10 min, followed by submerging twice in fresh xylene for 3 min each.

4.2.13. Place a drop of mounting media on the specimen and place a coverslip over it.

4.3. Goblet cell counting

4.3.1. Using light microscopy, count goblet cells (**Supplementary Figure 2**). For each piece of intestinal tissue, count the number of goblet cells and 500 epithelial cells and express goblet cell ratio as a ratio per 100 epithelial cells. Goblet cells are best counted at 20x magnification and numerical aperture 0.5.

4.4. Paneth cell counting

4.4.1. Using light microscopy, count Paneth cells (**Supplementary Figure 2**). For each piece of intestinal tissue, express as a ratio of Paneth cells per intestinal crypt. Count 100 intestinal crypts per each piece of intestinal tissue. Paneth cells are best counted at 20x-60x magnification and numerical aperture 0.50-1.30.

REPRESENTATIVE RESULTS:

Exposure to FEMI on embryonic day 15 leads to a dose-dependent loss of pregnancy and a dose dependent rate of preterm labor (**Figure 1**)[42]. For the experiments, we chose to use an LPS dose of 100 μg/kg to minimize pregnancy loss and prematurity (50% loss between both prematurity and intrauterine fetal demise) while exposing the fetuses to a significant inflammatory insult.

Using this approach, we next examined the effects of FEMI on subsequent injury of the offspring. Using a 3-point histologic scale to measure generalized intestinal injury, we found significant injury at birth (P0) and at adulthood (P56 or 8 weeks of life) (**Figure 2**). It is important to note that this injury occurred in the absence of any additional stimuli to the animals other than FEMI, suggesting that FEMI alone disrupts the normal homeostasis of the newborn murine intestinal tract. As the mouse is born with a relatively immature intestine that continues to develop during the first 4 weeks of life[47,48], this is relevant to preterm infants who also have immature intestinal tracts.

To further understand the effect of FEMI on both the normal development of the intestinal epithelium and on the defense mechanisms of the immature intestinal tract, we quantified the number of mucin-producing goblet cells and antimicrobial peptide-producing Paneth cells in the distal third of the small intestinal tract which is similar to the human ileum. We found that FEMI disrupted the normal composition of the intestinal epithelium by inducing loss of both goblet cells and Paneth cells compared to animals without FEMI (**Figure 3**).

To investigate the effects of FEMI on the neonatal inflammatory response, using ELISA with electrochemiluminescence, we quantified a variety of serum inflammatory markers, which included IL-1β, IL-10, KC-GRO (the murine equivalent of IL-8), and IL-6, from serum of pups with and without FEMI (**Figure 4**). We found that FEMI significantly increased the inflammatory cascade for all cytokines at P0. The inflammatory cascade at later ages (P7–P56) differed based on timepoint and cytokine. Most interestingly, for IL-6, there were similar levels at P7–P28 in the FEMI and sham groups, but despite no secondary intervention, there were significantly higher levels in the FEMI group at P56. This is especially important as we have demonstrated that IL-6 is a critical cytokine for the development of postnatal intestinal injury in the FEMI model.

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of FEMI dose on pregnancy outcomes. Survival of pregnancy litters is dose dependent with higher doses causing higher rates of pregnancy loss (**A**) and higher rates of premature birth (**B**). Figure is adapted with permission from Fricke et al[42]. FEMI using an LPS dose of 100 μg/kg creates a 50% survival for pups by one week of life. Each data point is representative of an n > 8 pregnancies and at least three individual experiments.

Figure 2: Effect of FEMI on distal small intestinal injury patterns over time. Intestinal samples were harvested at birth, 1 week of life, 2 weeks of life, and 8 weeks of life from mice exposed to FEMI (100 μg/kg of LPS) or sham control. Samples were scored using a 3-point injury scale by blinded investigators[42,43]. FEMI alone with no further insult induced significant amount of injury at birth, 1 week of life, and at 8 weeks of life. Figure has been adapted with permission from Fricke et al.[42]. Each data point is representative of an n > 10 pups and at least three individual experiments from at least 3 pregnant dams. Mann-Whitney non-parametric T-testing was used to compare intestinal injury scores at each timepoint. The asterisk indicates $p < 0.05$.

Figure 3: FEMI induces alterations of normal goblet and Paneth cell quantities in the small intestine during development. Intestinal samples were harvested at birth, 1, 2, 4, and 8 weeks of life from mice exposed to FEMI (100 μg/kg LPS) or sham control. Samples were stained with Alcian blue/Periodic Acid Schiff stain to detect both goblet and Paneth cells and these were quantified by a blinded investigator. Both goblet cells and Paneth cells from animals with FEMI showed either a trend or a significant decrease compared to sham controls at all ages. Figure adapted with permission from Elgin et al.[41]. Each data point is representative of an n > 10 pups and at least three individual experiments. Error bars represent standard error of the mean. Student T-testing was used to compare quantities of goblet and Paneth cells at each time point. The asterisk indicates $p < 0.05$.

Figure 4: FEMI induces a global neonatal inflammatory surge for all cytokines immediately after birth at P0, with a late surge of IL-6 at P56. Serum cytokines were quantified at P0, P7, P14, and P28 using ELISA with electrochemiluminescence according to the manufacturer's instructions, and plates were read at 620 nm. Cytokine values are represented here in a radar plot, and all cytokines are plotted as percent of maximal value. There were significant increases in all cytokines (IL-1β, IL-10, KC-GRO and IL-6) at P0 in the FEMI group compared to the control group (all $p < 0.05$). There was also a resurgent increase in IL-6 levels at P56 in the pups with FEMI compared to no FEMI ($p < 0.05$ by non-parametric Kruskal-Wallis testing), which was the only cytokine that was significantly elevated in the FEMI group compared to control at this late timepoint. Figure adapted with permission from Elgin et al.[41].

Supplementary Figure 1: Intestinal injury scoring of H&E stained ileal tissue. Injury scores are determined by a three-point intestinal injury scoring scale (0=normal, 1=mild injury, 2=severe injury) based on degree of villi vacuolization, mucosal ulceration, lamina propria damage, and presence of hemorrhage within villi as previously described[43]. Figure adapted with permission from Elgin et al.[41].

Supplementary Figure 2: Representative appearance of Alcian Blue/PAS staining of goblet and Paneth cells. Alcian Blue/PAS staining of intestinal tissues allows clear visualization of goblet cells, present in intestinal villi (top panel marked with white arrows, image taken at 20x magnification), and Paneth cells, present in crypts of Lieberkuhn, located below the intestinal villi in the lamina propria (bottom panel marked with yellow arrows, image taken at 60x magnification).

DISCUSSION:

Chorioamnionitis impacts 2–4% of term and 25–30% of preterm deliveries[8,9]. However, the impact of chorioamnionitis can extend long past birth as it has been shown to have significant effects on the fetus and neonate[10-16]. Importantly, chorioamnionitis has been shown to be associated with subsequent development of NEC[15,16]. While it is still incompletely understood, the pathogenesis of NEC likely involves a combination of intestinal immaturity, dysregulation of the immature immune system, intestinal inflammation, and bacterial translocation, culminating in a final common pathway of intestinal necrosis[19]. However, a mechanistic link between chorioamnionitis and subsequent development of NEC remains unclear[20], and previous animal models of chorioamnionitis have been insufficient to examine this relationship. To address this gap in knowledge, we modified the commonly used LPS-induced murine model of chorioamnionitis and preterm birth[34,37-40] to allow for neonatal birth and survival. In doing so, we have created a model that approximates the inflammatory condition seen in chorioamnionitis[42] to study the impact of fetal exposure to maternal inflammation (FEMI) on subsequent intestinal development.

With this protocol, we have demonstrated that this murine model of chorioamnionitis, using LPS-induced FEMI, results in both short and long-term intestinal injury as well as interruption of normal intestinal development, most notably downregulation of both goblet and Paneth cells, both of which provide a first line of innate immunity against intestinal inflammation. The intestinal injury and histologic cellular changes that are seen with this model indicates that it is an effective model to mimic the injury seen in NEC. This is primarily because the downregulation of Paneth cells and goblet cells both have been implicated in the pathogenesis of NEC, and the patterns of histologic injury are similar to what are seen in human cases of NEC[41,42,49]. Therefore, this LPS-induced FEMI model of chorioamnionitis is an ideal model for investigating the mechanistic link between chorioamnionitis and later intestinal injury, specifically the development of NEC, as well as potential effects of chorioamnionitis on the developing microbiome, which would not be possible with existing models that utilize live bacteria.

In vivo, chorioamnionitis often involves an ascending bacterial infection that often results in preterm rupture of membranes and the clinical phenotype of chorioamnionitis, and animal models of chorioamnionitis exist which more accurately reflect this pathophysiology[25,28,30,32]. However, because our laboratory studies intestinal development, including the developing microbiome, the presence of live bacteria in a model of chorioamnionitis would confound the microbiome analysis. Therefore, existing models of chorioamnionitis utilizing live bacteria are impractical for investigating the mechanistic link between chorioamnionitis and later

development of NEC. In addition, LPS-induced models of chorioamnionitis have already been effective in modeling postnatal white and gray matter brain injury[36], demonstrating that this method is an effective way to model morbidities of prematurity that are associated with exposure to chorioamnionitis at the time of birth.

It is also important to note that this model is highly dependent on the dose of LPS used to induce FEMI. The primary critical step in this protocol is induction of FEMI in the pregnant dams with intraperitoneal injection of LPS; therefore, not surprisingly, we have found that the dose of LPS used to induce FEMI is extremely important in the outcomes of these experiments. With initial experiments, a dose of 100 μg/kg of LPS was used, as this LPS dose was not associated with maternal mortality and resulted in approximately 50% neonatal survival as well as significant intestinal injury in offspring[41,42].

LPS binds to the Toll-like receptor 4 (TLR4) complex, resulting in aggregation of intracellular signaling proteins, cytokine production, and the initiation of pro-inflammatory signaling[50]. Toll-like receptors (TLRs) including TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 are important in the induction of the inflammatory response that is seen with a variety of pathogens and microorganisms such as viruses, bacteria, and fungi[51]. Interestingly, upregulation of TLR3 has also been shown to correlate with increased histologic intestinal damage and viral shedding in a neonatal murine rotavirus model; furthermore, knockout of TLR3 ameliorated these effects[52]. Thus, while the model makes use of TLR4 pathways, it is reasonable to assume that stimulation of other TLRs may confer similar findings.

Using this methodology, we have been able to show that LPS injection in pregnant dams causes increases in maternal, placental, and fetal inflammatory markers while sparing the amniotic fluid, as well as inducing direct damage to the placenta[42]. Interestingly, this methodology showed no alteration of resistance in the uterine arteries. The FEMI model also has a significant impact on the offspring as we have shown that exposed pups have significant intestinal injury[42] through an IL-6 dependent pathway[41] and can impact important defense mechanisms of the intestine such as goblet cells and Paneth cells[41]. This injury makes the neonatal offspring increasingly susceptible to subsequent LPS-induced intestinal injury and inflammation[41] which may explain why infants exposed to chorioamnionitis have an increased susceptibility to develop NEC.

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DISCLOSURES:

The authors have nothing to disclose.

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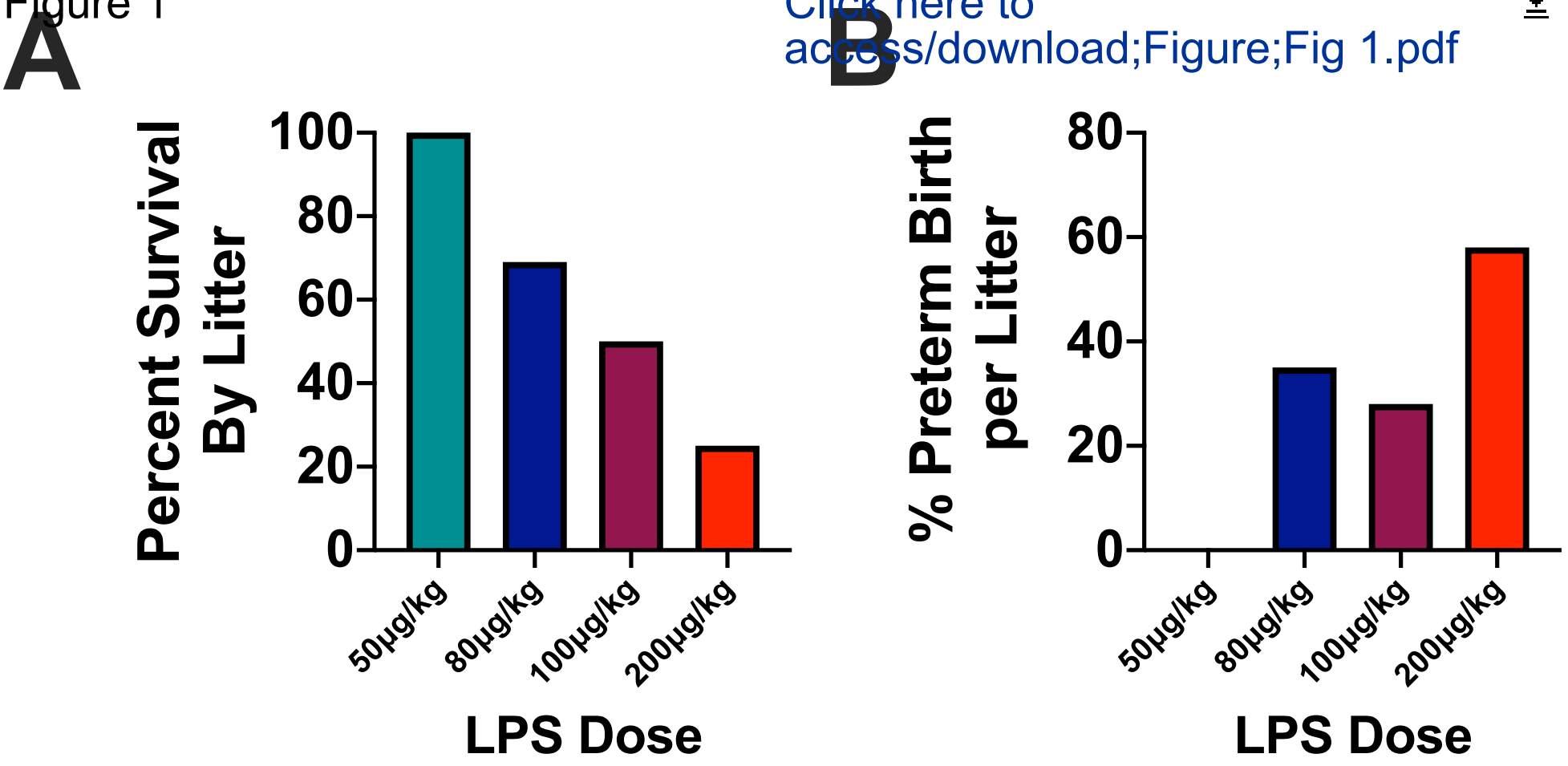

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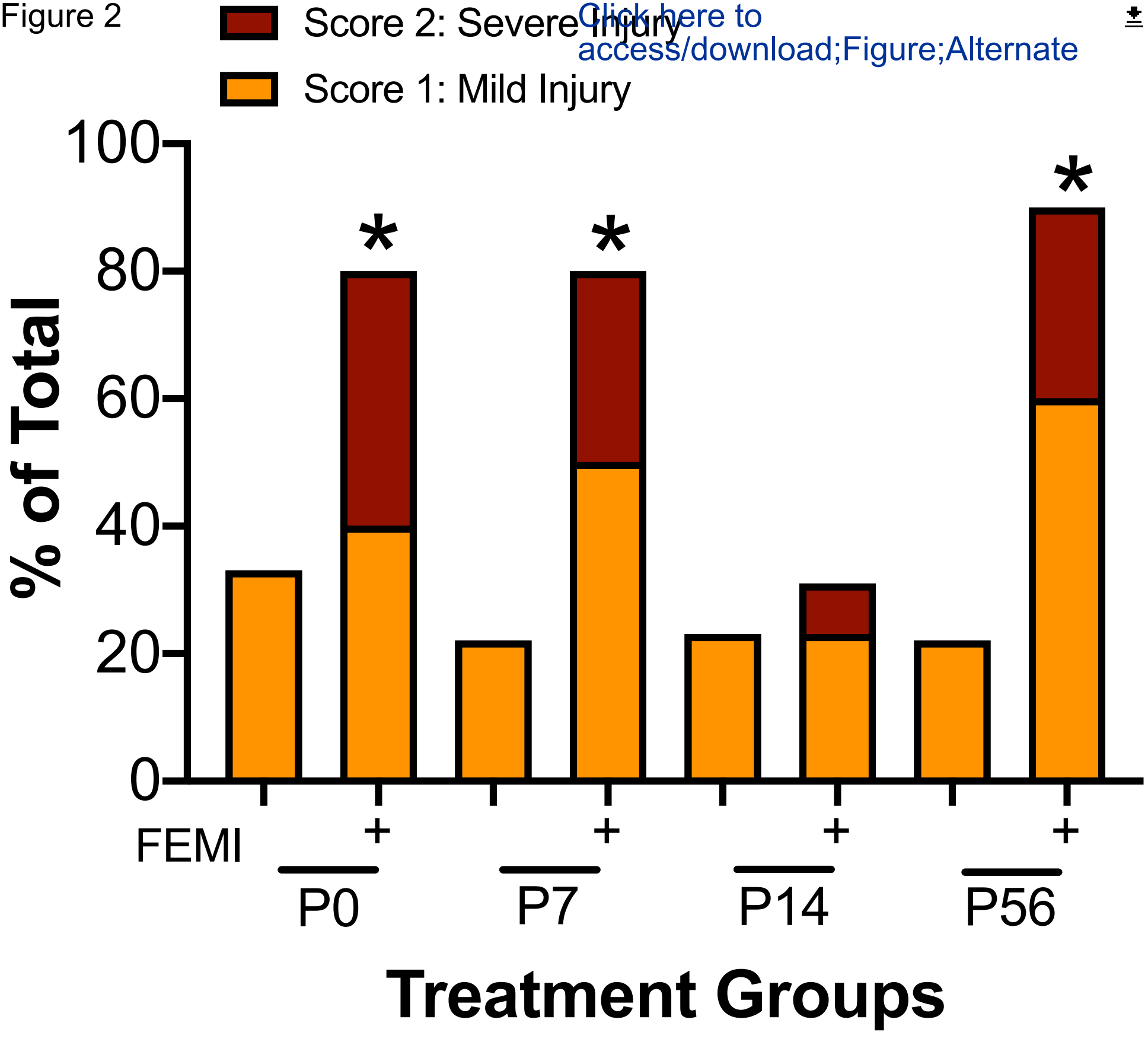

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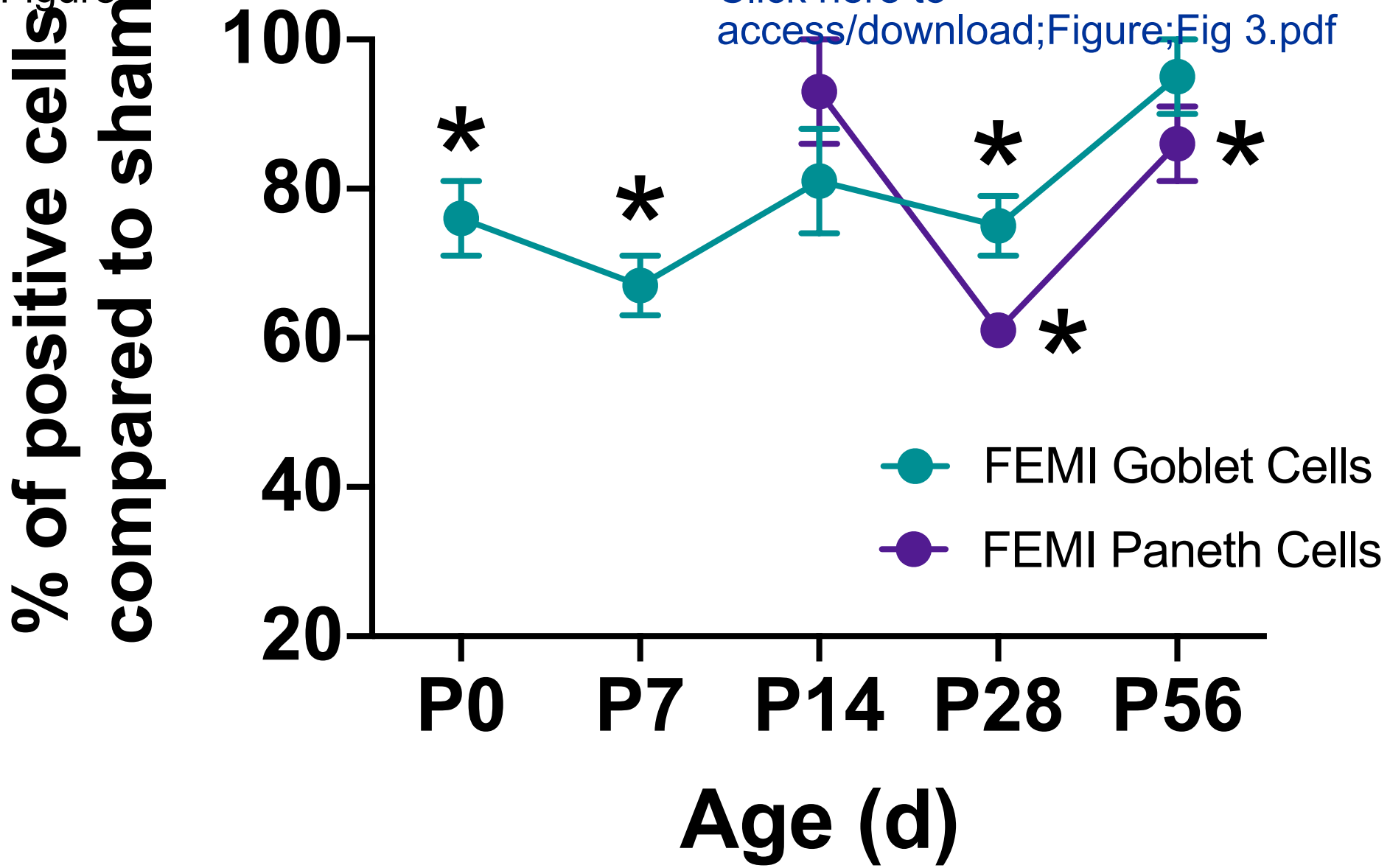

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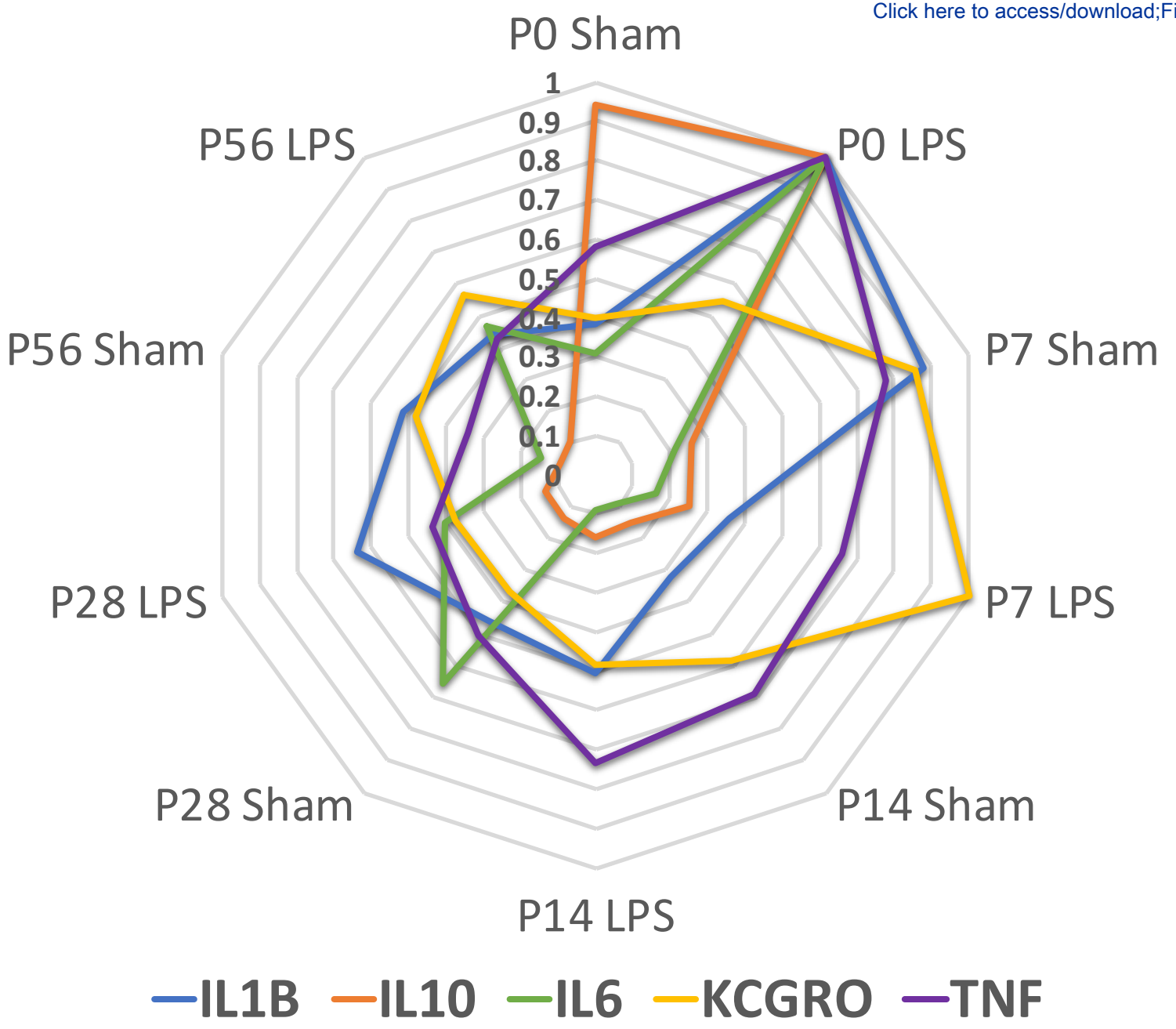

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% neutral buffered formalin	Sigma	HT501128	
Alcian blue stain	Newcomer supply	1003A	
C57Bl6/J mice	Jackson Laboratories	664	
Ethanol	Decon labs	2701	
HCl	Sigma	H1758	
Hematoxylin stain	Leica	381562	
LPS	Sigma	L2880	
$NaHCO_3$	Sigma	S6014	
Nikon Eclipse Ni-U Microscope	Nikon	2CE-MQVJ-1	
Periodic Acid	ACROS	H5106	CAS# 10450-59-9
RNAlater	Thermofisher	Am7021	
Schiff's reagent	Sigma	S5133	
Secor Imager 2400	Meso Scale Discovery (MSD)		
V-Plex Assay	Meso Scale Discovery (MSD)		
Xylene	Sigma	534056	

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May 6, 2020,

Dear Editorial staff of *Jove*,

Thank you for your reviews of our research manuscript entitled "*A murine model of fetal exposure to maternal inflammation to study the effects of acute chorioamnionitis on newborn intestinal development*" for consideration for publication. Below is a list of the issues raised and how we have addressed them in our revised manuscript. We appreciate the comments by both the editorial staff and the reviewers and believe that their input has made our manuscript much stronger. Please let us know if there are any other issues that you would like us to address.

Sincerely,

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Editorial Comments:

Thank you for your formatting comments and suggested edits. We have adjusted our text as requested. We have also updated our Table of Materials to include all relevant materials used in our experiments.

Reviewer Comments:

Reviewer #1: The unique aspect of the protocol rests on the assessment of intestinal injury. The authors could do a better job of outlining what the expected abnormalities are so that the reader can judge if their findings truely mirror the disease of interest. This was not clear in the intro or discussion as the description of NEC was somewhat vague re "perturbations" without specifying histiologic or cell specific expected findings. Further I would have expected some assessment of immune competence and am not certain if cell counts are a sensitive/specific finding.

Thanks for your comments. We have altered the text to describe NEC better and point out why our data matches what is seen in the disease process. We have also added additional data regarding serum markers of inflammation in a new Figure 4.

Reviewer #2:
Thanks for your kind comments and for your suggestions. Our response to your three suggestions are below.

1. Myeloid cell invasion of intestinal wall as a function of time after LPS ip injection to dams, starting (if possible) in the fetus.
This is a great comment and one which we are currently investigating but this data is outside the scope of the JOVE format.
2. Perhaps also add cytokine profile of intestinal wall as a function of time after LPS ip injection to dams, starting (if possible) in the fetus.
Thanks for this comment. We have added serum cytokine data to address this question. Unfortunately we do not have intestinal wall data at this time and are unable to generate this new data due to current COVID issues that have closed the lab.
3. In addition to TLR4 activation, discuss if stimulation of viral-activated TLRs, such as TLR3 (dsRNA), 7 (ssRNA), 9 (DNA) may be inducers of antenatal intestinal damage; this is particularly relevant as we face increasingly unknown viral pathogens.
Thanks for this suggestion. We have added text regarding this in the discussion.

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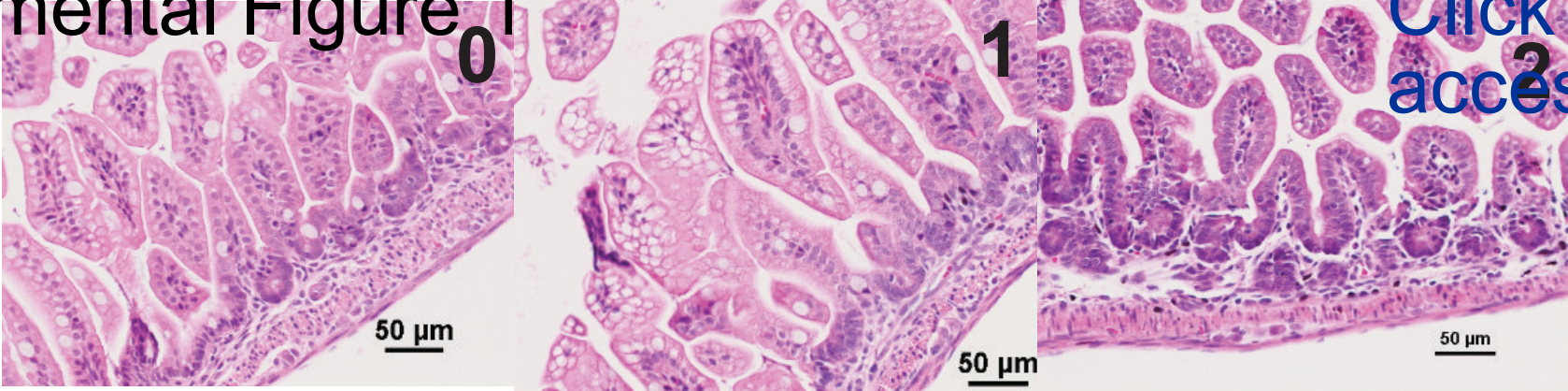
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