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Both maternal and placental toll-like receptor activation are necessary for the full development of proteinuric hypertension in mice

Piyali Chatterjee[a], Valorie L. Chiasson[a], Shelley E. Kopriva[a], Kelsey R. Bounds[a], M. Karen Newell-Rogers[b], Brett M. Mitchell[a,b,*]

[a] Department of Internal Medicine, Texas A&M Health Science Center/Baylor Scott and White Health, Temple, TX, United States
[b] Department of Medical Physiology, Texas A&M Health Science Center/Baylor Scott and White Health, Temple, TX, United States

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ABSTRACT

Objective: Innate immune system activation and excessive inflammation contributes to hypertension during pregnancy (HTN-preg). Activation of Toll-like receptors (TLRs), the primary innate immune system sensor, is evident in women with HTN-preg and is sufficient to induce pregnancy-dependent, proteinuric hypertension in animals. However, whether HTN-preg is a maternal disease, a placental disease, or both is unclear. We hypothesized that activation of TLR3, the double-stranded RNA sensor, in both maternal systemic and placental cells would be necessary for the full development of HTN-preg in mice.

Study design: Various mating schemes generated pregnant mice that lacked TLR3 in maternal cells, paternally-derived placental cells, and both. Mice were then injected with a TLR3 agonist on days 13, 15, and 17 of pregnancy.

Main outcome measures: Blood pressure, urinary protein excretion, fetal development, maternal vascular endothelial function, and immune system activation were all assessed and compared between groups.

Results: Pregnant mice lacking TLR3 in maternal cells as well as pregnant mice lacking TLR3 in placental cells had significantly attenuated increases in systolic blood pressure, urinary protein excretion, fetal demise, and endothelial dysfunction compared to wild-type pregnant mice following TLR3 activation. Pregnant mice lacking TLR3 in both maternal systemic and placental cells were completely resistant to the hypertension, proteinuria, fetal demise, endothelial dysfunction, splenomegaly, and increases in pro-inflammatory immune cells induced by TLR3 activation.

Conclusions: These data suggest that both maternal and placental TLR3 activation are crucial for the full development of HTN-preg and that TLR3 antagonists may be beneficial in some women with HTN-preg.

1. Introduction

Hypertension during pregnancy (HTN-preg) is a multisystem disorder that complicates ~15% of all pregnancies, and the incidence has increased over the past decade. Several mechanisms suggested to play a role in the pathogenesis of HTN-preg include an abnormal immune response, pathogens, defective placentation, placental ischemia, and oxidative stress [1]. The development of HTN-preg involves a shift from protecting the mother and fetus as immune privileged sites to a destructive response that includes excessive inflammation and generalized endothelial cell activation [2–4]. Regardless of the initiating insult, the maternal innate immune system plays a central role in HTN-preg [5,6]. Toll-like receptors (TLRs) are innate immune system receptors expressed on or in most immune cells that recognize characteristics of extracellular endogenous ligands or pathogens, and their activation leads to a pro-inflammatory immune response. We and others have reported that excessive activation of TLRs causes pregnancy-dependent HTN in animals and is associated with HTN-preg in women [7–16]. However, whether TLR activation of cells is important on the maternal side, the placental side, or both is unclear.

Activation of TLR3 by polyinosinic:polycytidylic acid (poly I:C) mimics the innate immune system activation by the endogenous danger signal double-stranded RNA (dsRNA) and dsRNA viruses that women who develop HTN-preg encounter during pregnancy. While a TLR3-induced anti-viral response is important for fighting infections, prolonged and aberrant immunity results in excessive inflammation that

Abbreviations: dsRNA, double-stranded RNA; HTN-preg, hypertension during pregnancy; KO, knockout; Poly I:C, polyinosinic:polycytidylic acid; TLR, toll-like receptor; WT, wild-type

* Corresponding author at: Texas A&M University Health Science Center, Department of Medical Physiology, 361A Reynolds Medical Building, College Station, TX 77843-1114, United States.
E-mail address: bmitchell@tamhsc.edu (B.M. Mitchell).

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damages organs leading to dysfunction and HTN-preg. This "immunologic" etiology represents 1 of 3 distinct subclasses of human HTN-preg as determined by recent gene expression profiling [17]. We have demonstrated previously that immunosuppression reduces HTN-preg in animals, however the "blunt instrument" of immunosuppression is not feasible in pregnant women [18]. Understanding the source of TLR3 activation is important in an effort to design safe and effective therapeutics for HTN-preg [10,19]. Additionally, understanding whether HTN-preg is a maternal disease, a placental disease, or both may aid therapeutic targeting.

Given that many placental cells are of paternal origin, we used various mating schemes to develop pregnant mice that were deficient of TLR3 in either maternal cells, placental cells, or both to determine where TLR3 activation "needs" to occur for the development of HTN-preg in mice. We hypothesized that both maternal and placental TLR3 activation are necessary, and that HTN-preg mice lacking TLR3 on the maternal side or the placental side would have an attenuated phenotype.

2. Methods

2.1. Animals and treatments

Male C57BL/6J mice and TLR3 KO mice on a C57BL/6J background (crossed > 10 generations internally), used for mating purposes only, and female C57BL/6J and TLR3 KO mice (crossed > 10 generations internally) were purchased from Jackson Laboratories (Bar Harbor, ME). Female mice 10–12 weeks of age were assigned randomly to 1 of 5 groups with 6 females in each group: (1) pregnant (P) = WT male X WT female + saline; (2) pregnant + poly I:C (PPIC) = WT male X WT female + injections of the TLR3 agonist poly I:C; (3) PPIC Ma T3KO = WT male X TLR3 KO female + injections of poly I:C; (4) PPIC Pa T3KO = TLR3 KO male X WT female + injections of poly I:C; (5) PPIC MaPa T3KO = TLR3 KO male X TLR3 KO female + injections of poly I:C. Poly I:C (20 mg/kg/day) or saline vehicle were injected ip on gestational days 13, 15, 17 as described previously [9,10,13,19]. Although various parenteral routes of administration can be chosen for mice, we chose i.p. injection of substances into the peritoneal cavity, a common technique in laboratory rodents. This type of delivery is advantageous over i.v. which is more challenging in C57Bl/6J mice and it can be used to administer large volumes of fluid safely. In addition, absorption of material delivered i.p. is typically much slower than for i.v. injection before reaching the systemic circulation. Our study also needed repeated injections of poly I:C and i.p. is more suitable for pregnant female mice. We chose the dose at 20 mg/kg as i.p. doses are typically higher than other routes to be effective. This dose was also based on prior optimization experiments performed in pregnant mice in our lab [9,13]. All procedures performed in mice were approved by the Texas A&M Health Science Center/Baylor Scott and White Health IACUC in accordance with the *NIH Guide for the Care and Use and Care of Laboratory Animals*.

2.2. Tissue collection

After weighing the mice on day 18 of pregnancy, they were euthanized by prolonged anesthesia and blood, urine, and tissues were obtained. Spleens were removed and weighed and the spleen weight/body weight ratio (mg/g) was determined. The intact uterine horn containing pups, placentas, and amniotic fluid was removed and pups and placentas were then removed and counted, and the number of pups experiencing fetal demise due to resorption or malformations was also counted. Fetal demise was assessed by an obvious lack of development and viability due to resorption, which is detected by a nodule in the line of pups in the uterine horn, or malformation, markedly smaller in size compared to other pups in the litter. Pups were then euthanized by decapitation with sharp scissors. The number of mice in each group was 6.

2.3. Blood pressure measures

Tail-cuff systolic blood pressures (IITC, Inc.) were assessed at baseline and on day 17, prior to injections, as described previously [9,13]. Mice were trained to enter restrainers and undergo the procedure for 3 days prior to measures. They were warmed to 32 °C and 10 readings were recorded and averaged for each mouse.

2.4. Urinary protein excretion

Urine obtained on gestational day 18 by squeezing the bladder under anesthesia was used to measure urinary protein and creatinine using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method, Sigma) according to the manufacturer's instructions [9,13]. Creatinine was using by commercially available kit (R&D Systems) according to the manufacturer's directions. Total protein was normalized to total creatinine and the ratios were averaged for each group.

2.5. Vascular reactivity

Relaxation responses were assessed by wire myography of isolated endothelium-intact aortic rings (2 mm in length) obtained from mice on day 18 as described previously [9,10,13,19]. Briefly, aortas were mounted on stainless steel pins in a myograph (Danish Myo Technology) and were warmed to 37 °C, oxygenated with 95% O_2/5% CO_2, and set at a passive tension of 0.75 g of force. Following the determination that the vessel was viable, vessels were contracted with an EC_{70} concentration of phenylephrine (1 μM) followed by cumulative concentrations of the endothelium-dependent dilator acetylcholine. Vessels were then washed until passive tension returned to baseline followed by phenylephrine-induced contraction and the addition of cumulative concentrations of the endothelium-independent dilator sodium nitroprusside. Concentrations-response curves were created based on the percent relaxation from the phenylephrine-induced contraction.

2.6. Flow cytometry

Flow cytometry was performed as we have reported previously [9–13,20]. Briefly, spleens were collected from all groups of mice and homogenized by passing through a 70 μM cell strainer to obtain single cell suspensions. BD Pharm Lyse Buffer was used to lyse red blood cells and Fc receptors were blocked using Fc Block (BD Bioscience) on ice for 15 min prior to staining with cell surface markers following the manufacturer's instructions. All antibodies were purchased from BD Bioscience/eBiosciences. The following cell types were gated based on isotype controls and identified using these cell surface markers: CD11c + /B220 + dendritic cells, CD11b + /CD14 + monocytes, CD19 + /CD23 + B cells, CD3 + /gamma-delta T cell receptor + T cells, and CD4 + /CD25 + T regulatory cells. Flow cytometry was performed on a BD FACS Canto II and analyses were performed using Flow Jo (Treestar; Ashland, OR). Compensation and quadrants were set based on the isotype control data and/or negative staining controls and lymphocyte gating in the forward x side scatter plot as before [10,13,20]. Lymphocytes were quantified and averaged. Data are expressed as percent of total splenocytes.

2.7. Statistics

Results are presented as mean + or ± standard error of the mean. Normality was tested prior to the one-way analysis of variance followed by the Student's-Newman-Keuls *post hoc* test when necessary. This was performed for all measures including the vascular dose-response curves. SigmaStat software was used and the significance level was 0.05.