Jan 23 2018

Re: Preeclampsia Foundation Canada Vision Grant - Final Progress Report

Dear Violet and Members of the Preeclampsia Foundation Canada Advisory Board,

Thank you once again for supporting our project entitled "*Natural Killer Cells and Preeclampsia".* Our goal was to decipher the contribution of natural killer (NK) cells to inflammation-induced placental maldevelopment and fetal distress. ***Our hypothesis was that inflammation disrupts the normal behaviour of NK cells, which are the main immune cells in the uterus, and leads to abnormal development of the placenta via enhanced production of inflammatory mediators such as interferon-gamma (IFNG)****.* Since placental maldevelopment during early pregnancy is the key pathological lesion in preeclampsia and fetal growth restriction, we believe that this research is a stepping stone that will ultimately help us devise strategies targeting NK cells that will improve both maternal and fetal health in pregnancies at high-risk of developing preeclampsia.

Funding from the Preeclampsia Foundation Canada was instrumental in getting these novel ideas and experiments "off-the-ground", and has led to some surprising discoveries about the role of NK cells in the regulation of placental development and fetal health. There were two Aims in this project. **Aim 1** sought to determine the effect of abnormal uterine NK cell activation on placental and fetal development in rats. **Aim 2** was to determine the role of IFNG signaling via activation of the transcription factor *Signal Transducer and Activator of Transcription 1* (STAT1) in the regulation of human trophoblast invasion. Progress made on each Aim during the grant period is described below:

Aim 1: To determine the effect of abnormal uterine NK cell activation on placental and fetal development in rats.

The goal of this Aim was to determine the effect of the inflammatory stimulus and potent NK cell activator polyinosinic:polycytidilic acid (polyI:C) on placental and fetal development in rats. Rats were used for these experiments because they exhibit many similarities with humans in terms of placental development, including abundant uterine NK cells during early pregnancy, as well as extensive trophoblast-directed uterine vascular remodelling.

***Results*:**

Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), we determined that systemic exposure of pregnant rats to polyI:C on embryonic day (E)8.5 caused robust production of transcripts encoding a variety of inflammatory cytokines and chemokines within the uterus, including interferon gamma (IFNG), tumour necrosis factor alpha (TNF), interleukin-6 (IL6), CCL2, CXCL10, and perforin (**Figure 1A**). Thus, systemic administration of an inflammatory stimulus caused inflammation within the uterus adjacent to where the placenta develops. Using immunohistochemistry for perforin – a protein most prominently expressed by NK cells – we determined that polyI:C caused a robust increase in perforin expression within the uterus (**Figure 1B**). This finding was confirmed using qRT-PCR and western blotting (**Figure 1A, C**). Perforin-positive cells were located in closer proximity to the developing placenta following polyI:C exposure (**Figure 1B**). PolyI:C also caused a robust decrease in fetal growth, including a 22% overall decrease in fetal weight (73% of pups below the tenth

percentile) and a 9% decrease in fetal weight on E18.5 (43% below the tenth percentile). Comparable decreases in fetal brain weight and liver weight were observed, indicating that polyI:C-exposed fetuses exhibited symmetrical growth restriction.

Next, we compared placental morphology on E13.5 following maternal exposure to saline or polyI:C on E8.5. The extent of trophoblast invasion in both saline and polyI:C-exposed placentation sites was variable, with some placentation sites showing extensive invasion and some exhibiting limited invasion. However, using vimentin immunostaining to demarcate the different zones of the placenta, we observed significant differences in placental zonal morphology, including a ~25% increase in the size of the junctional zone (which harbours the invasive trophoblast lineage), and a ~25% decrease in the size of the labyrinth zone (site of maternal-fetal nutrient exchange; **Figure 2**). These discoveries indicate that polyI:C, or polyI:C-induced cytokines, disrupt placental development possibly by interfering with trophoblast lineage allocation. The effect of polyI:C-induced cytokines on trophoblast development is the subject of future investigations.

Our next goal was to determine the role of NK cells on polyI:C-induced deficiencies in fetal growth and viability. Initially an immunodepletion approach was used to transiently deplete NK cells. Immunodepletion was achieved by injecting pregnant rats with anti-asialo GM1 antibodies four days prior to polyI:C administration (**Figure 3**). We determined that expression of some (perforin, IFNG), but not all, of these cytokines is abrogated in uteri lacking NK cells. This data suggests that NK cells respond to polyI:C, but they are not the only cells in the uterus that respond to polyI:C. Interestingly, depletion of NK cells *exacerbated* fetal growth restriction following polyI:C (**Figure 4**). Likewise, in a different strain of rats in which NK cells are absent due to a genetic deficiency of interleukin-15 (a cytokine vital for NK cell development), the absence of NK cells was associated with an 80% loss of viability after polyI:C exposure, in comparison to only a moderate (20%) loss of viability in wild-type rats (**Figure 5**). Thus, NK cells may have a protective role in pregnancy, which is consistent with previously published genetic associations showing that women harbouring NK cell activation have a decreased risk of preeclampsia. We are currently determining the effect of NK cell deficiency on placental structure after polyI:C exposure, and exploring mechanisms by which NK cells have a protective effect on inflammation-induced pregnancy complications.

Significance:

There are several exciting new discoveries from these experiments. First, polyI:C is capable of eliciting a localized immune response in the uterus during early pregnancy. Second, polyI:C-induced uterine inflammation is associated with major changes in placental structure. Third, NK cells may have a protective role in inflammation-induced pregnancy complications and may thus be therapeutic targets to enhance placental development in pregnancies at-risk of preeclampsia. These findings are the subject of follow-up investigations.

Aim 2: To determine the role of IFNG signaling on human trophoblast invasion.

IFNG is a major cytokine produced by uterine NK cells, and functions by stimulating phosphorylation and activation of the transcription factor *Signal Transducer and Activator of Transcription-1* (STAT1). The goal of this Aim was to delineate the role of STAT1 activation in human trophoblast cell motility/invasion.

Results:

Using a scratch-wound assay to assess migratory potential, and Matrigel-based invasion assays to analyze cellular invasiveness, we noted a substantial reduction in HTR8 trophoblast

migration and invasion following exposure to IFNG (**Figure 6**). Additionally, we used another trophoblast cell line, JEG3, and noted a similar reduction in invasiveness after IFNG (**Figure 6**). We also determined that IFNG induces STAT1 phosphorylation and nuclear translocation in human trophoblast cells in a time- and dose-dependent fashion (**Figure 7**). We did not observe changes in proliferation or viability in trophoblasts following IFNG exposure. We then used a lentiviral approach to introduce *STAT1*-targeted short hairpin RNAs into trophoblast cells. Using this strategy, we successfully knocked-down *STAT1* expression in human trophoblasts using two different shRNAs (**Figure 8**). Interestingly, we found that STAT1-deficient trophoblasts were completely protected from the anti-migratory effects of IFNG (**Figure 8**). We are in the process of determining whether STAT1 deficiency prevents the inhibitory effect of IFNG on trophoblast invasiveness, and also which genes are directly affected by IFNG-STAT1 signaling.

Finally, we assessed STAT1 expression in placental tissue collected from preeclampsia with intra-uterine growth restriction (n=6, mean gestational age 31.9 weeks), preeclampsia with HELLP syndrome (n=4, mean gestational age 34.15 weeks), term control placenta (n=3, mean gestational age 39.6 weeks), or preterm normotensive controls (n=3, mean gestational age 34.7 weeks). Tissues were obtained from the Research Center for Women's and Infant's Health Biobank at Lunenfeld-Tanenbaum Research Institute. However, we did not observe a change in STAT1 expression either by immunohistochemistry or by western blotting. These results may indicate that alterations in STAT1 expression are not involved in the pathogenesis of preeclampsia, although we cannot exclude the possibility that STAT1 activity (phosphorylation and/or nuclear translocation) may be perturbed in preeclampsia. Additionally, it is possible that STAT1 expression and/or activity are dysregulated during early gestation in pregnancies predisposed to develop preeclampsia, and thus STAT1 may be involved in the ontogeny of preeclampsia rather than disease progression.

Significance:

Discoveries in this Aim have revealed a novel role of IFNG-STAT1 signaling in the regulation of trophoblast migration and invasion, and have identified STAT1, or genes regulated by STAT1, as an intriguing target to enhance trophoblast invasion in high-risk pregnancies. However, changes in placental STAT1 expression do not appear to be involved in the late stage pathogenesis of preeclampsia.

Training of high-quality personnel:

Funds from the Preeclampsia Foundation Canada have been used to support experiments undertaken by a PhD candidate in the lab, Kelly Baines. Additionally, these funds were used to support the initial training of a MSc candidate, Amanda Rampersaud, and a summer and Honours research project student, Grace Grafham.

Knowledge dissemination:

The Preeclampsia Foundation Canada has been acknowledged for supporting experiments in the following abstracts and presentations (reverse chronological order). The Preeclampsia Foundation Canada will continue to be acknowledged in additional knowledge dissemination activities (abstract, publication) that arise as a result of funded experiments.

- Baines KJ and Renaud SJ. *The Role of Uterine Natural Killer Cells in Immune-Mediated Intrauterine Growth Restriction in Rats.* To be presented as an oral presentation at the Canadian National Perinatal Research Meeting, Feb 15 2018, Banff, AB. *Oral presentation.*

- Baines KJ and Renaud SJ. *Investigating the role of uterine natural killer cells in immune-mediated fetal growth restriction in rats.* Anatomy and Cell Biology Research Day, London, ON, Canada. October 29 2017. *Poster presentation.*

- Renaud SJ. *Uterine Natural Killer cells: Friends or Foes for the Fetus.* Platform presentation at Developmental Biology Research Day, London, ON, Canada. June 1, 2017. *Oral presentation.*

- Renaud SJ. *Building a Placenta (Lab)*. Children's Health Research Institute – Division of Genetics and Development. London, ON, Canada. May 20, 2017. *Oral presentation.*

- Baines KJ and Renaud SJ. *Investigating the role of uterine natural killer cells in immune-mediated fetal growth restriction in rats.* Southern Ontario Reproductive Biology, London, Canada, May 12 2017. *Oral presentation.*

- Baines KJ and Renaud SJ. *The role of aberrant natural killer cell activation on placental development in rats.* Experimental Biology, Chicago, IL, USA, Apr 22-26 2017. *Poster presentation.*

- Baines KJ and Renaud SJ. *The role of aberrant natural killer cell activation on placental development in rats.* London Health Research Day, London, Canada. March 31, 2017 *Poster presentation.*

- Baines KJ and Renaud SJ. *The role of natural killer cells in immune-mediated intra-uterine growth restriction in rats.* Canadian National Perinatal Research Meeting, Montibello, QC, Canada. Feb 18-22 2017. *Oral presentation.*

PolyI:C induces the production of various cytokines, chemokines, and perforin in the pregnant uterus

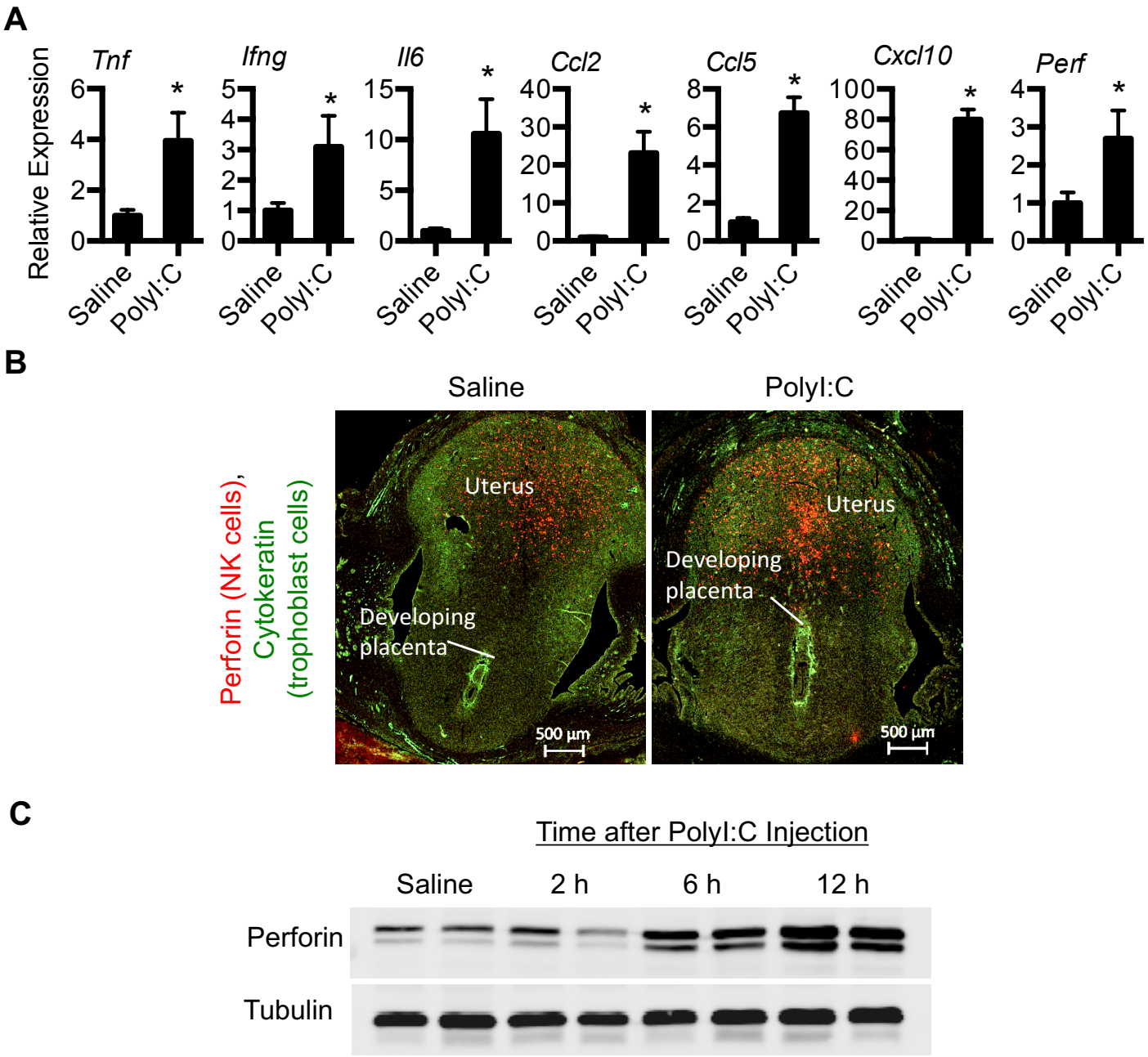

Figure 1. A) Transcript expression of various cytokines, chemokines, and perforin in decidua 6-h after injection of 10 mg/kg polyI:C in pregnant rats. Perforin expression in uterus was assessed by immunofluorescence (**B**) and western blotting (**C**) after saline or 10 mg/kg polyI:C injection. Please note: perforin identifies both NK cells and T cells, but the uterus contains relatively few T cells compared to NK cells, so increased perforin expression after polyI:C is interpreted as enhanced NK cell activation. (*, P<0.05)

PolyI:C disrupts placental development

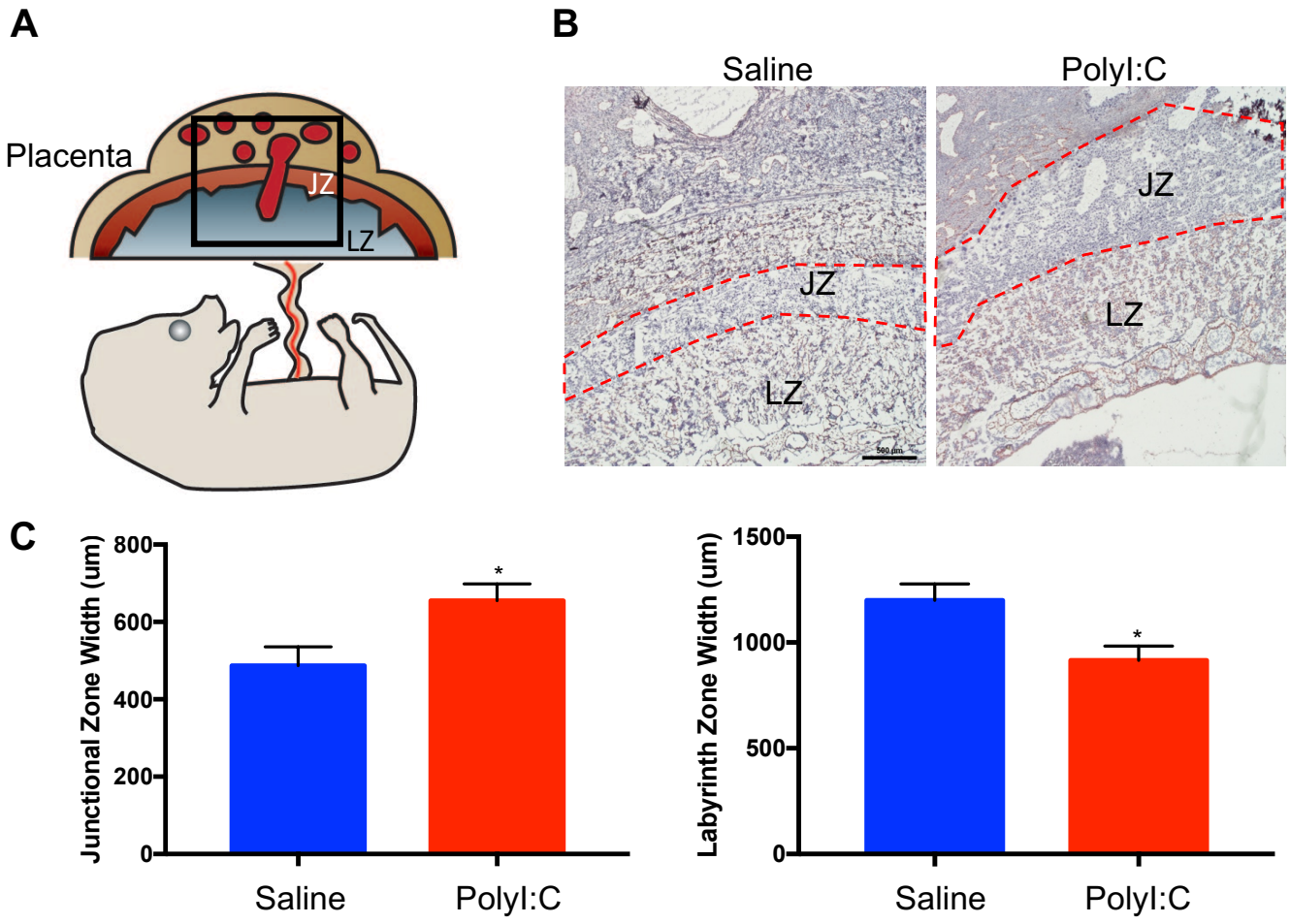

Figure 2. (A) Schematic depiction of the structure of the rodent placenta. The junctional zone (JZ) is coloured maroon; whereas the labyrinth zone (LZ) is coloured teal. Immunohistochemistry for vimentin in rat placenta on embryonic day (E)13.5, five days after saline or 10 mg/kg polyI:C administration. The red dashed line demarcates the location of the JZ (vimentin-negative). (**C**) Quantification of the thickness of the JZ and LZ on E13.5, five days after saline or 10 mg/kg polyI:C administration. (*, P<0.05)

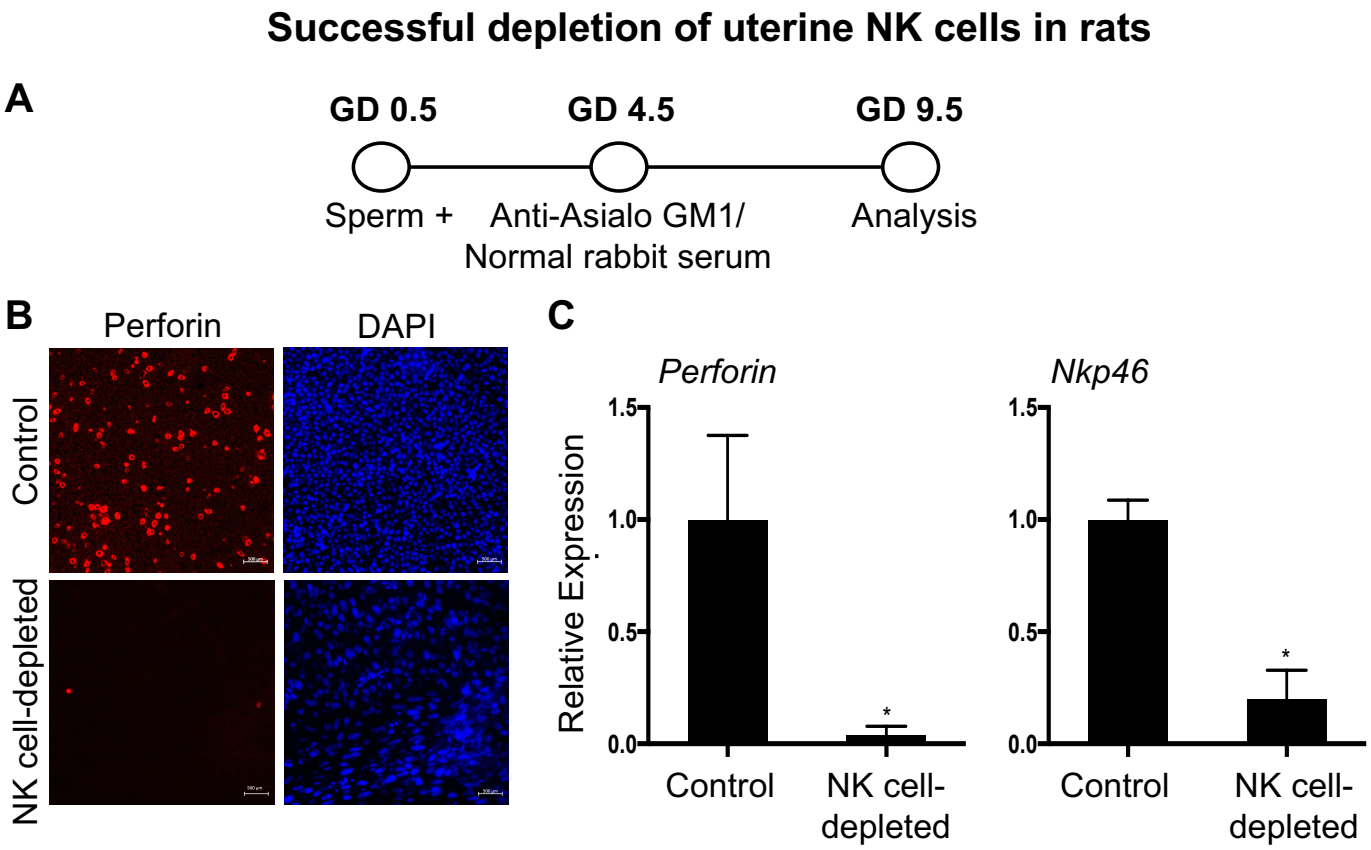

Figure 3. A) Schematic representation of timeline. **B**) Immunohistochemistry for perforin in uterine tissue of control (normal rabbit serum-injected) and NK cell-depleted (anti-asialo GM1-injected) rats on gestational day (GD) 9.5. DAPI was used to identify nuclei. **C**) Quantitative RT-PCR showing relative expression of perforin and the NK cell-specific receptor, NKp46, in uterine tissue of control and NK cell-depleted rats. (*, P<0.05)

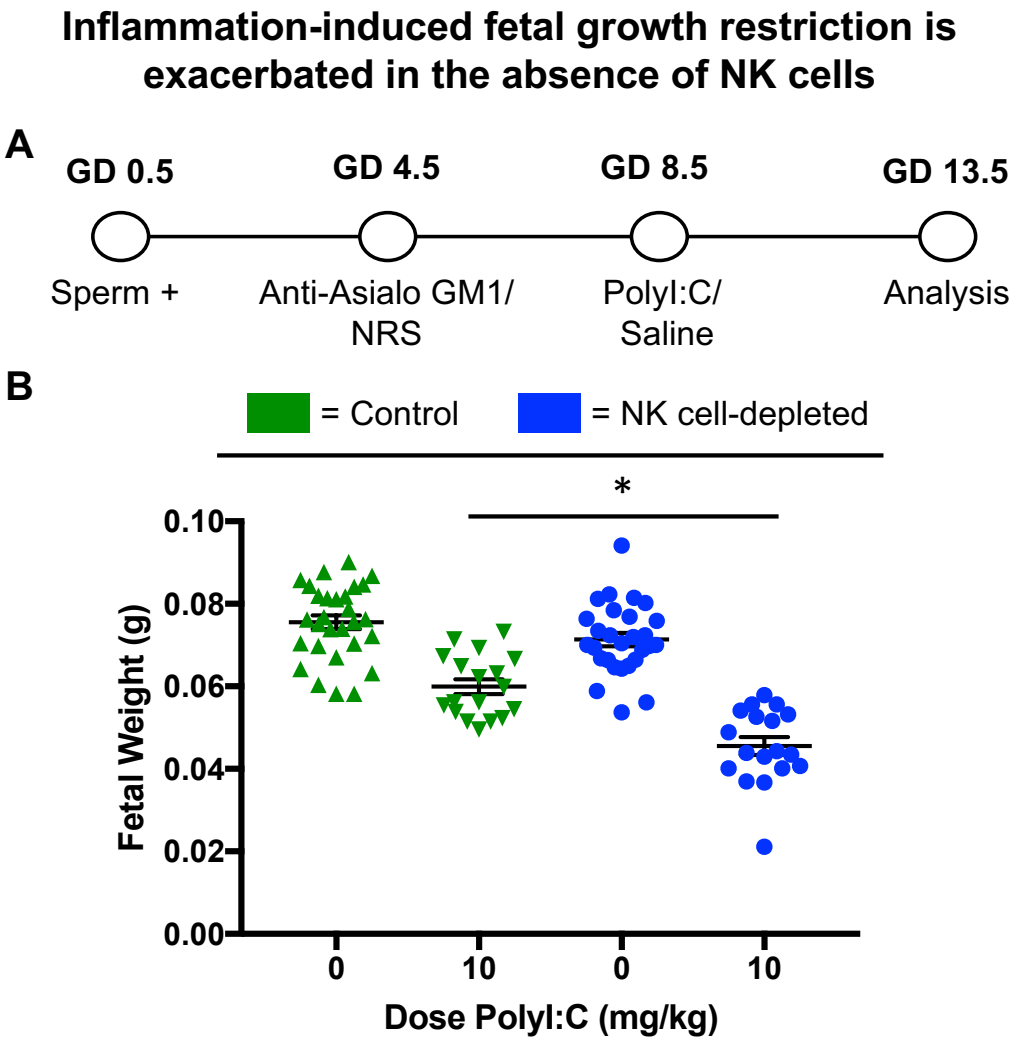

Figure 4. A) Schematic representation of timeline. **B**) Fetal weight on gestational day (GD) 13.5 in control (normal rabbit serum-injected) and NK cell-depleted (anti-asialo GM1-injected) rats following exposure to saline or the inflammatory stimulus polyI:C. Note that polyI:C administration reduces fetal weight, and this effect is exacerbated in rats lacking NK cells. (*, $P<0.05$)

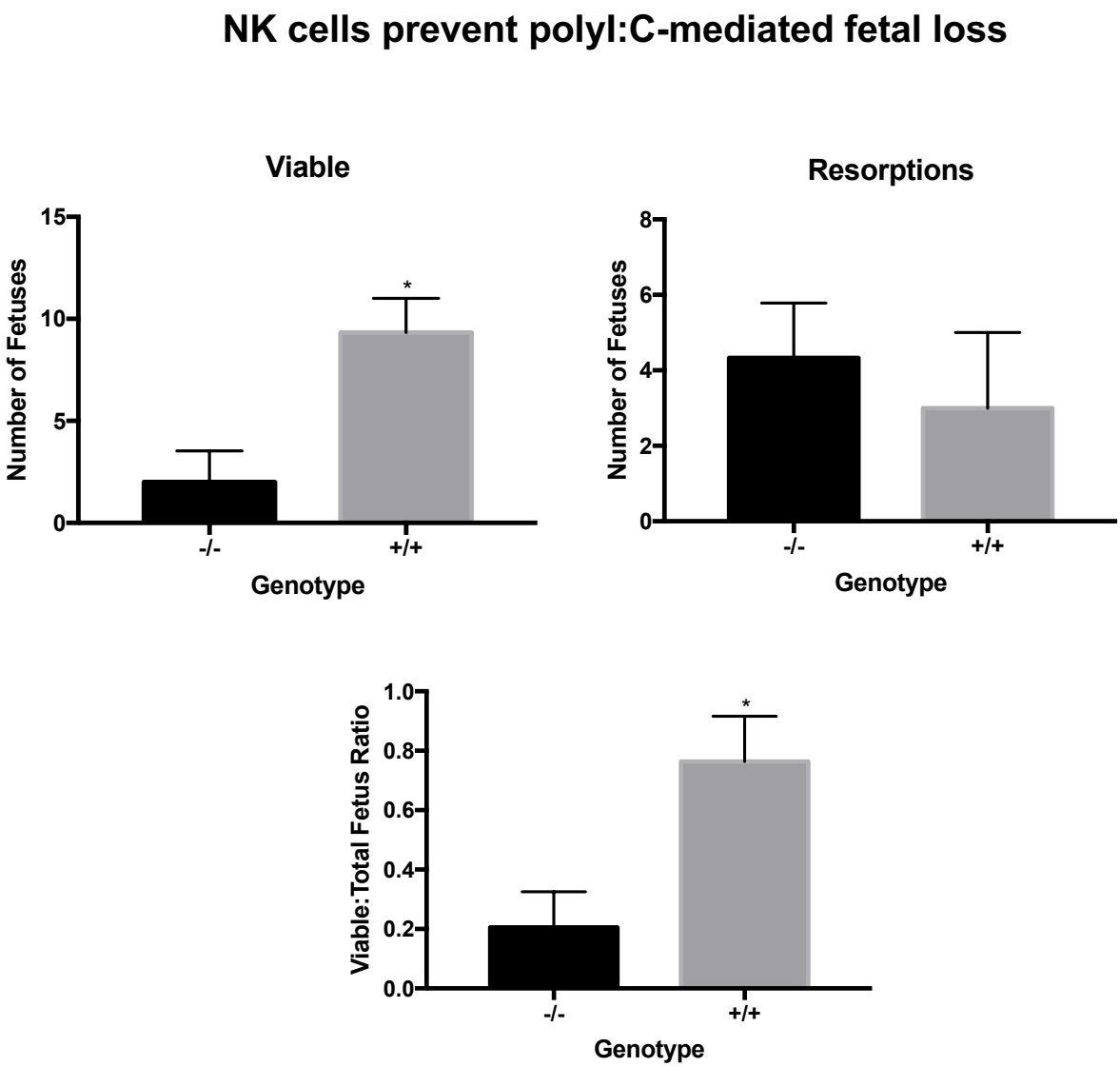

Figure 5. Wild-type (+/+) or NK cell-deficient (-/-) rats were administered 10 mg/kg polyI:C on embryonic day (E)8.5, and fetal viability was assessed five days later. (*, $P<0.05$)

IFNG reduces trophoblast migration and invasion in human trophoblast cells

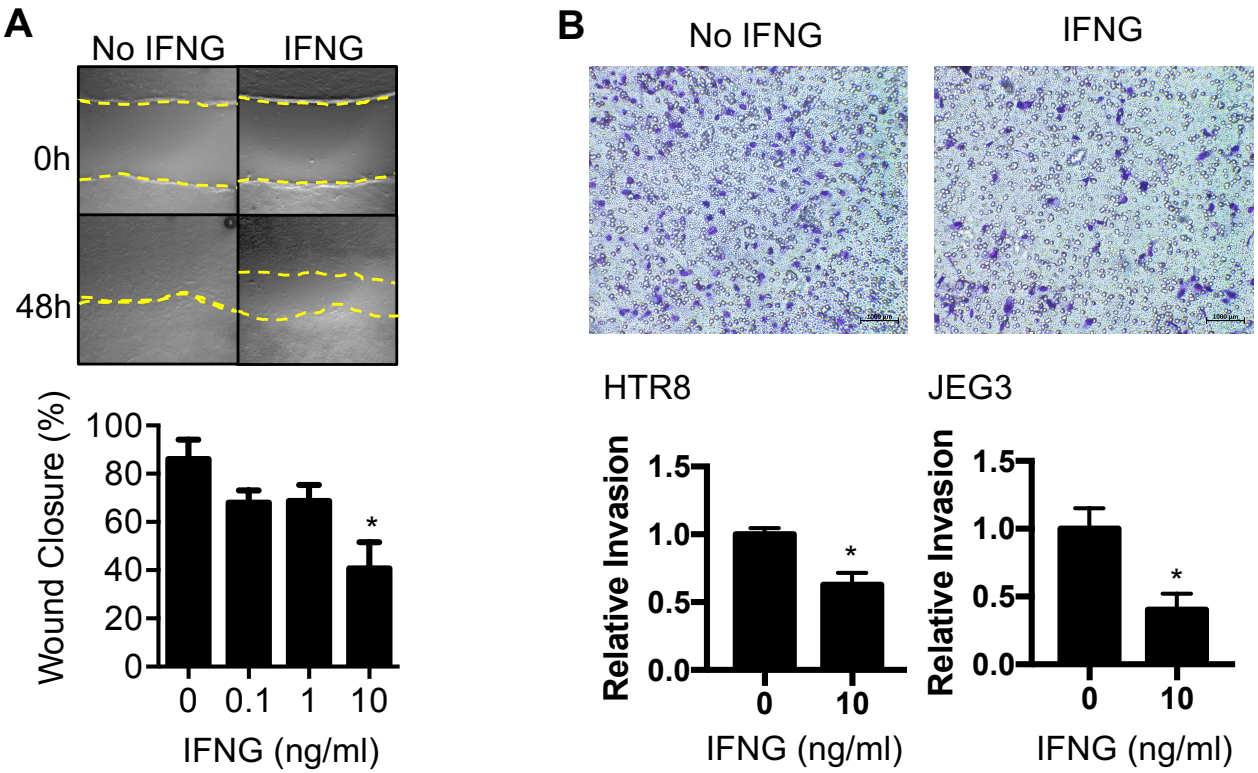

Figure 6. A) Scratch-wound assays comparing the effect of multiple doses of IFNG on migration of human HTR8 invasive trophoblasts *in vitro*. **B**) Matrigel-based cell invasion assays comparing the effects of 0 and 10 ng/ml IFNG on HTR8 trophoblast invasiveness. Representative images of HTR8 cells (purple) exposed to 0 and 10 ng/ml IFNG are shown in the images above the histograms (*, $P<0.05$).

IFNG stimulates STAT1 phosphorylation and nuclear accumulation in trophoblasts

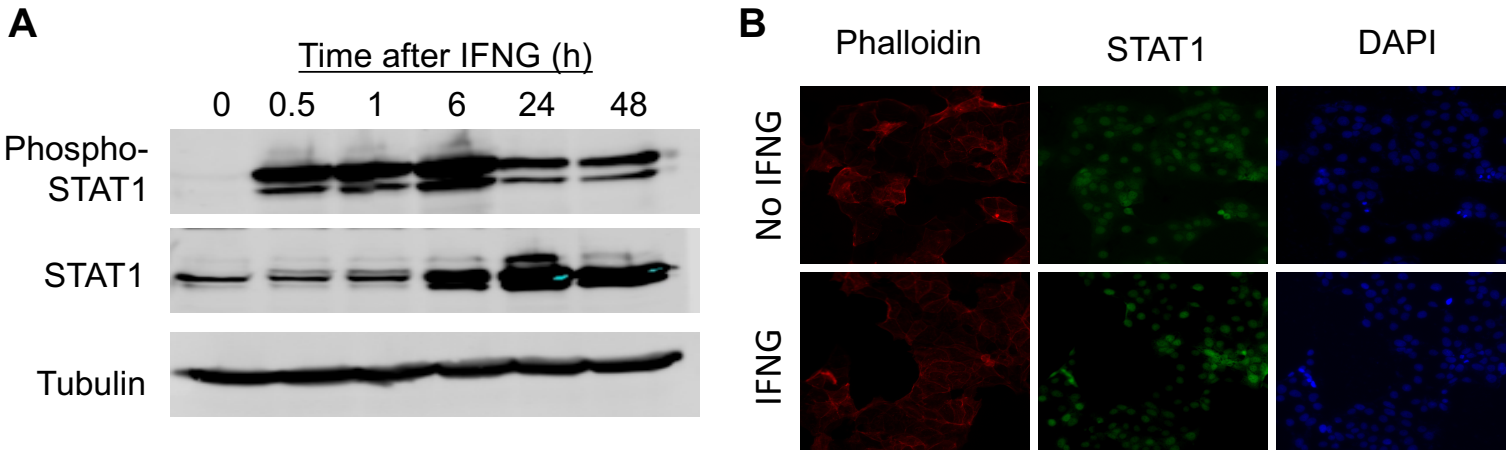

Figure 7. (**A**) Western blot using lysates of HTR8 trophoblasts exposed to 10 ng/ml IFNG for various times. Note that IFNG stimulates STAT1 phosphorylation and accumulation in HTR8 trophoblasts. Tubulin was used as a loading control. **B**) Immunofluorescence detecting STAT1 in HTR8 trophoblasts under control conditions or after exposure to 10 ng/ml IFNG for 6 h. Phalloidin (red) was used to identify the cytoskeleton; DAPI was used to denote nuclei. Please note that IFNG exposure stimulated STAT1 translocation to the nucleus.

Inhibiting STAT1 using lenti-shRNA restores trophoblast motility after IFNG exposure

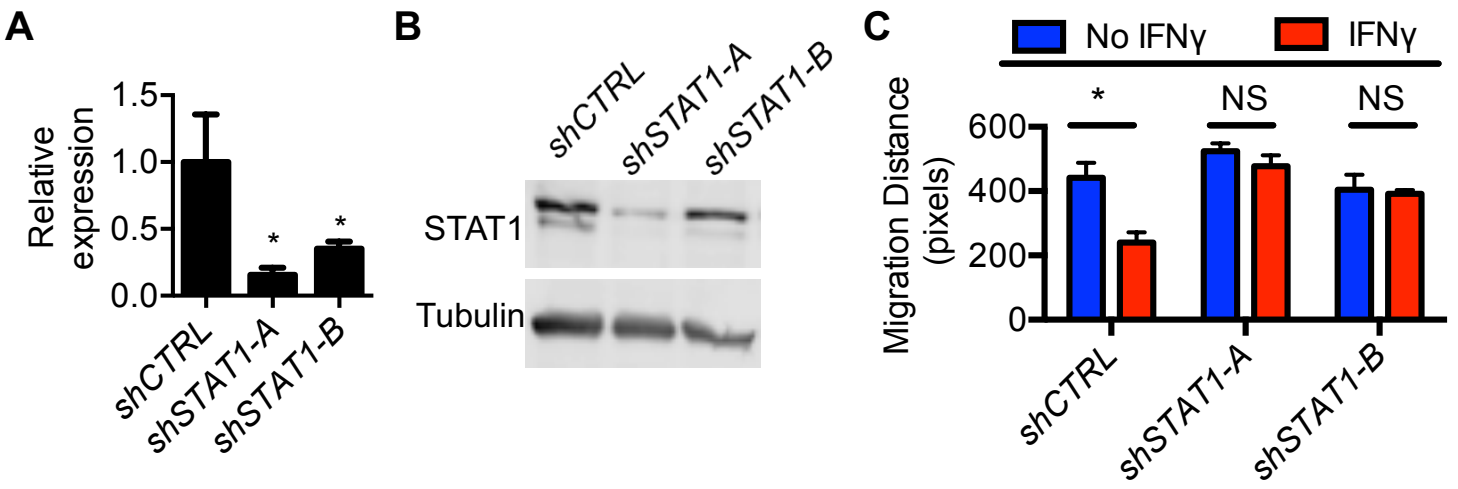

Figure 8. Knockdown of STAT1 measured by qRT-PCR (**A**) and western blotting (**B**) in human trophoblasts using a lentivirus-shRNA delivery approach. Two different shRNAs were used: *shSTAT1-A* and *shSTAT1-B.* **C**) Scratch-wound assay showing that STAT1-deficient human trophoblasts are protected from the anti-migratory effects of IFNG. (*, P<0.05).