**α-actin Down Regulation and Perforin Loss in Uterine Natural Killer Cells From LPS-Treated Pregnant Mice**

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**Summary**

One of the most abundant immunologic cell types in early decidua is the uterine natural killer (UNK) cell that despite the presence of cytoplasmic granules rich in perforin and granzymes does not degranulate in normal pregnancy. UNK cells are important producers of angiogenic factors that permit normal dilation of uterine arteries to provide increased blood flow for the growing fetoplacental unit. Gram-negative bacteria lipopolysaccharide (LPS) administration can trigger an imbalance of pro-inflammatory and anti-inflammatory cytokines impairing the normal immune cells activity as well as uterine homeostasis. The present study aimed to evaluate by immunohistochemistry the reactivity of perforin and α-actin on UNK cell from LPS-treated pregnant mice. For the first time, we demonstrate that LPS injection in pregnant mice causes α-actin down regulation, concomitantly with perforin loss in UNK cells. This suggests that LPS alters UNK cell migration and activates cytotoxic granule release.

**Key words**

α-actin • Perforin • UNK • Lipopolysaccharide • Pregnancy • Mouse

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UNK cells are present in the uterus during pregnancy in animals with hemochorial placentation, such as humans and rodents (Tayade et al. 2007). In mice, the precursor of these cells migrates from the bone marrow (Peel et al. 1983, Lysiak and Lala 1992) and from secondary lymphoid organs such as the spleen (Moore et al. 1996, Chantakru et al. 2002, Zavan et al. 2012) arriving in the uterus during early pregnancy (Kruse et al. 2002) displaying an immature cell morphology with few cytoplasmic granules (Paffaro et al. 2003). These cells develop and proliferate in this environment (Stewart and Peel 1981), reaching the maximum number of cells around gestational day (gd) 10. According to Paffaro et al. (2003) during this period of pregnancy most of these cells show mature cell morphology, filled with cytoplasmic granules that could be identified by periodic-acid Schiff (PAS) histochemistry due to the amylase resistant glycoprotein content in their granules (Peel 1989, Paffaro et al. 2003, Zhang et al. 2009). Some of these PAS⁺ UNK cells are negative to Dolichos biflorus agglutinin lectin (DBA⁻) (Zhang et al. 2009). However, DBA lectin can selectively label (Paffaro et al. 2003) the angiogenic subtype of these cells in the pregnant mouse uterus (Chen et al. 2012). DBA⁺ UNK cells become the dominant subset in mid-pregnancy (Chen et al. 2012) reaching their peak in number (Ashkar and Croy 1999) in a gestational period where the opening of utero-placental circulation occurs (Aasa et al. 2013). After this period, UNK cells begin a senescent process and decrease in quantity until the end of pregnancy (Delgado et al. 1996, Rajagopalan and Long 2012).

Inflammation can be induced in experimental animals by exposure to gram-negative bacteria cell walls, specifically lipopolysaccharide (LPS), mimicking the acute phase of the inflammatory response, without...
causing infection in the host (Burrell 1994). Sepsis during pregnancy is recognized as the primary cause of preterm birth (Goldenberg et al. 2000) and the major cause of human neonatal morbidity and mortality (Slatterly et al. 2002). There is considerable interest in the development of novel therapeutic strategies that could contribute to a reduction of preterm birth (Pizarro and Troster 2007).

In normal pregnancy, despite the presence of cytoplasmic granules rich in perforin and granzymes (Kopcow et al. 2005, Smyth 2005), UNK cells do not degranulate, having as their main function the production of vasoactive agents such as interferon gamma (IFN-γ), vascular endothelial growth factor (VEGF) and nitric oxide (NO) that promote vascular remodeling, an essential process to ensure healthy pregnancy development (Ashkar and Croy 1999, Hanna et al. 2006). There are no evidences in literature on LPS changing UNK migration or causing lytic granule release.

In many secretory cell types, the fusion of lytic granules with the plasma membrane requires the disassembly of cortical actin, since this structure can function as a barrier to exocytosis (Trifaro et al. 1992, Sugawara et al. 1993, Roth and Burgoyne 1995, Chowdhury et al. 2000, Gil et al. 2000, Yoneda et al. 2000, Lyubenchenko et al. 2003). Moreover, in circulating NK cells actin polymerization for filamentous actin (F-actin) formation (Carpen et al. 1983) is required for cytotoxic function (Katz et al. 1982).

Actin cytoskeletal rearrangements probably precede perforin mobilization (Orange et al. 2002); however, little is known about contractile actin expression (α-actin) and cell migration during the cytotoxic activation process in UNK cells during pregnancy.

For evaluation of the LPS effects during mouse pregnancy with focus on the UNK, forty virgin female Swiss Webster mice (8-12 weeks age) were mated with congenic males and the morning of vaginal plug detection was called gd1. Mice were maintained in the Central Animal Facility of the Federal University of Alfenas (Unifal-MG, Brazil) and housed under controlled light and temperature conditions with access to food and water ad libitum as approved by the Ethics Committee on Animal Experimentation of UNIFAL-MG.

Mice at mid pregnancy (gd10) received an intraperitoneal (ip) injection of LPS serotype 026:B6 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterile isotonic saline (100 µg/kg). Implantation sites were collected 30 min, 1 h and 2 h after treatment (n=10 each group). The control group (n=10) received only saline (0.9 % NaCl).

Mice were anesthetized with Ketamine hydrochloride solution and Xylazine hydrochloride (#K-002 and #X1251, Sigma-Aldrich) before perfusion fixation with 4 % paraformaldehyde (#P614, Sigma-Aldrich) in PBS (50 mM). The sites of developing embryos were dissected from the uterus and processed for paraffin embedding. Tissue was cut at 7 µm thickness, mounted on poly-L-lysine coated slides for Dolichos biflorus agglutinin (DBA) lectin histochemistry and immunohistochemistry for α-actin and perforin.

Histological sections were deparaffinized, hydrated and treated with 1 % hydrogen peroxide (#216763, Sigma-Aldrich) for 30 min. After washing with 50 mM PBS, sections were incubated with 1 % bovine serum albumin (BSA) (#05470, Sigma-Aldrich) in PBS for 30 min, and 3 µg/ml biotinylated DBA lectin (#I9142, Sigma-Aldrich) or 5 µg/ml polyclonal rabbit anti-mouse α-actin (#A2103, Sigma-Aldrich) in 1 % PBS/BSA overnight at 4 °C. When using the α-actin antibody the sections were incubated with 2 µg/ml goat biotinylated anti-rabbit (#B8895, Sigma-Aldrich). After washing with PBS, sections were incubated with streptavidin-peroxidase (Chemicon International, Temecula, CA, USA); 1 h, room temperature and visualized with 3,3-diaminobenzidine (#D12384, Sigma-Aldrich) in 50 mM TBS containing 0.1 % hydrogen peroxide. Sections were counterstained with Harris’s hematoxylin, mounted with Entellan (Merck kGaA 64271 #HX378130 Darmstadt, Germany) and observed by light microscopy (Nikon Eclipse 80i, Tokyo, Japan).

The double staining for DBA lectin and anti-perforin was developed in deparaffinized sections, and after washing with PBS, incubated with 1 % PBS/BSA for 30 min and 1 h incubation with FITC conjugated DBA lectin 18 µg/ml (#L9142, Sigma-Aldrich). After washing with PBS, the sections were incubated overnight at 4 °C with 5 µg/ml polyclonal rabbit anti-mouse perforin (#PA1-22489, Thermo Fisher Scientific Inc, Waltham, MA, USA). Sections were incubated with 2 µg/ml sheep anti-rabbit conjugated with Cy3 (#C2306, Sigma-Aldrich) for 1 h. All negative controls were developed by omitting the DBA lectin and/or the primary antibody during the procedure. Sections were mounted with anti-fading medium (#H-1000 Vectashield, Vector Co. Burlingame, CA, USA) and observed with fluorescence (λ=494 and λ=488 for FITC/ λ=520 and λ=560 for Cy3) microscopy (Nikon Eclipse 80i Japan).
Fig. 1. Photomicrographs of implantation sites histological sections from normal pregnant mice at gd10 showing UNK cells (A). Note the DBA lectin reactivity (brown color) on the membrane surface and granules of these cells. DBA lectin/peroxidase-DAB/Harris hematoxylin. Negative control for both α-actin and DBA lectin cytochemistry (B). α-actin positive UNK cells can be observed in mice treated with saline (C). After 30 min (D), 1 h (E) and 2 h (F) of the LPS injection, a reduction in α-actin expression in UNK cells can be observed. α-actin/peroxidase-DAB and Harris hematoxylin. Double fluorescent staining of DBA lectin and perforin can be observed (G-L). Note the negative control for DBA lectin (G), the DBA lectin positive reaction at UNK cell granules (H), and UNK DBA staining loss (I) after LPS. Note the negative control for perforin (J), the positive reaction (perforin) to the granule content (K) and the non-reactivity for perforin (L) in UNK cells from 1 h post-LPS. UNK cells (arrows). Red blood cells (arrowheads). DBA FITC-lectin (green). Perforin-Cy3 (red).
At the time of sample collection there was no macroscopic evidence of conceptus death at 30 min, 1 h and 2 h after LPS. However, the results described below refer to all implantation sites analyzed by histochemical and immunohistochemical technique. The DBA lectin reactivity on the membrane surface and granules, widely described in the literature, allowed us to identify the uterine natural killer cells (Fig. 1A) and no positive reaction was found in the developed negative control (Fig. 1B).

Felker and colleagues (2013) have recently described for the first time that DBA- UNK cells in normal mice are smaller throughout pregnancy than DBA+ cells. In addition, Zhang and colleagues (2009) showed that the DBA- UNK cells account for just 10 % of total UNK cells in mid pregnant mouse uterus. In this context, even without UNK cells double staining for α-actin and DBA lectin in this study, the large size, incidence, morphology and location of the α-actin positive cells observed in control mice strongly suggests that these cells are DBA- UNK cells (Fig. 1C). Thirty minutes after LPS injection, these cells showed a decrease of α-actin labeling (Fig. 1D), followed by the total loss of α-actin reactivity after 1 h (Fig. 1E) and 2 h (Fig. 1F) treatment.

It was possible to observe strong DBA lectin (Fig. 1H) and perforin (Fig. 1K) staining on the granules of the same UNK cells from control mice, while 1 h after LPS injection there was only weak DBA lectin staining of these granules (Fig. 1I) that was concomitant with the loss of perforin labeling (Fig. 1L) in the same cell. No reaction was observed in either negative controls, DBA lectin (Fig. 1G) or perforin staining (Fig. 1J).

Interestingly the positive reaction for α-actin that was found in UNK cells was polarized toward the direction of the implanted embryo. These results suggest the α-actin polarization on UNK cells may be to assemble the focal contacts likely to migrate toward the region near the implanted embryo. This corroborates studies that suggested mice UNK cells migrate from the myometrium to areas close to the embryo during mouse pregnancy on a fibronectin rich substrate (Paffaro et al. 2003, Zavan et al. 2010), where they became differentiated and play important roles in vessel dilation and embryo nutrition (Rätsep et al. 2015) by preparing for the opening of mouse utero-placental circulation by gd10 (Aasa et al. 2013). In addition, the decrease of α-actin labeling at 1 h post-LPS and total loss of α-actin labeling 2 h after treatment occurred over the same period that perforin expression became undetectable.

Therefore, we hypothesize that LPS ip injection has indirectly activated UNK cells, supported by the literature which shows that many immune cell types recognize LPS through toll-like receptor 4 (TLR4) assisted by LPS binding protein (LBP) (Gerondakis et al. 2001, Fernández-Pérez et al. 2005, Sabroe et al. 2005, Guinn et al. 2007) causing a intracellular signaling cascade resulting in NF-kB activation (Hirsch and Wang 2005), which leads to transcription of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 and interleukin-6 (Buhimschi et al. 2003, Schmitz et al. 2007). The increase in pro-inflammatory cytokines at the uterus, especially TNF-α after 2 h of LPS administration (Faas et al. 2004, Cotechini et al. 2014), can be harmful for the pregnancy by activating UNK cells (Murphy et al. 2005, 2009, Erlebacher et al. 2013) to release their granules while having their migration impaired.

Therefore, this short and prospective study suggests that LPS ip injection during mid-gestation in mice may indirectly and very early promote disorganization of α-actin filaments and disassembly of focal contacts of UNK cells impairing their migration while activating their cytotoxicity.

Conflict of Interest
There is no conflict of interest.

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References


