Angiogenic responses in the pregnant mouse uterus under uNK cell deletion and hypoxia

Eliana Mara Oliveira Lippe

Integrated Colleges of Bauru (FIB). Rua José Santiago, S/No. Vila Ipiranga. Bauru-SP, Brazil (CEP 17056-120). Email: elianalippe@yahoo.com.br.

Abstract. Inadequate uterine vascular remodeling is associated with pregnancy complication. Pregnancy specific uterine Natural Killer (uNK) cells produce angiogenic factors such as vascular endothelial growth factor (VEGF), nitric oxide (NO) and interferon-γ (IFNγ), but hypoxic effects of uNK cell functions are unknown. Here, we investigated the effects of hypoxia on murine uNK cell driven vascular remodeling and on pregnancy outcome. At gestational day (gd) 7.5, normal CD1 mice and genetically-modified $IL-15^{-/-}$ mice that lack uNK cells were exposed to 48 h of 420 Torr hypoxia. Pregnancies were then assessed at gestation day (gd) 9.5 for decidual gene and protein expression, histopathology or litter size at birth. Hypoxia delayed CD1 fetal development, but term deliveries occurred, with the exception in $IL-15^{-/-}$ uNK deficient mice. VEGF protein and gene expressions were down-regulated, but not abrogate in the uNK deficient mice, suggesting an alternative source of this angiogenic factor independent of uNK cell. High *eNOS/iNOS* expression seems to be uterine compensatory response in the uNK deficient mice and it is probably by increasing NO release to stimulate local angiogenesis. The reduced successful pregnancy outcome is not related to uNK cell cytotoxic activity. Otherwise, the high incidence of pregnancy failure of $IL-15^{-/-}$ animals confirmed the benefit of uNK cells in the homeostasis of pregnant uteri.

Keywords: Angiogenesis; Hypoxia; uNK cells; Uterine plasticity.

ORCID
0000-0003-4802-8830
Eliana Mara Oliveira Lippe

Introduction

Hormone and cytokine driven morphological and physiological changes occur in the uterus during interstitial embryo implantation and development of the hemochorial placenta. Stromal decidualization and vascular remodeling, including spiral arterial distention at implantation sites, play key roles supporting adequate trophoblast invasion and placental organogenesis (Burton et al., 2009; Liu et al., 2017). In humans, failure of spiral arterial adaptation is associated with complications that include recurrent miscarriages, intra-uterine growth restriction (IUGR) and preeclampsia (Burton et al., 2009; Kalkunte et al., 2010; Avagliano et al., 2011; Alecsandru and Garcia-Velasco, 2017; Franasiak and Scott, 2017). Spiral arterial modification is thought to be initiated in humans and mice by unique lymphocytes, the uterine Natural Killer (uNK) cells.

A shared feature of human and mouse pregnancy specific uNK cells is their ability to produce angiogenic and

vasoactive molecules, in particular, vascular endothelial growth factor-VEGF (Chen et al., 2012; Lima et al., 2012; Liu et al., 2017), angiotensin I/II (Hatta et al., 2011), nitric oxide-NO (Hunt et al., 1997) and interferon-γ-IFNγ (Ashkar et al., 2003), especially during the first half of pregnancy. Early implantation sites are physiologically hypoxic and, as in other hypoxic tissues, stimulate mediators of vascular remodeling (Zamudio et al., 2007; Leno-Durán et al., 2010; Possomato-Vieira and Khalil, 2016). However, the consequences of hypoxia on uNK cell activities have not been investigated experimentally. The other suggested major *in vivo* function of uNK cells is promotion of trophoblast invasion.

These studies underlie the current paradigm that suggests uNK cells have critical angiogenic and vascular remodeling functions that promote successful pregnancy. Importantly, pregnancy with litters of normal size progress to term in uNK cell deficient mice, a finding attributed to maternal cardiovascular adaptations.

The present work assessed effects of 48 h hypoxia during the post-implantation, pre-placental stages between gd 7.5 to 9.5 on angiogenic and vasoactive molecules on normal CD1 and NK cell deficient *IL15* mice pregnancy outcomes. 420 Torr hypoxia provoked developmental delay in conceptuses that was reversed and not fatal when the dams were returned to normoxia. However, in mice with uNK cell deficiency, recovery was not observed. The uNK deficient *IL-15-/-* mice down-regulated uterine VEGF expression and increased the *Nos2* and *Nos3* expression in comparison with CD1 mice. This aberrant local compensatory response may adequate local vascular remodeling to support at least 60% of successful pregnancies.

Materials and methods

Animals

Randombred +/+ (CD1) and *IL15-/-* mice on the CD1 background were purchased from Charles River (USA) and syngeneically mated. The morning of vaginal plug presentation was considered gestational day (gd) 0.5. All procedures for animal handling and experimental protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee (Protocol No. 2007-1647) and by Committee of Experimental Animal Care and Use, Institute of Biology, University of Campinas (Protocol No. 2111-2).

Normoxic and hypoxic conditions

The first study manipulated pregnant CD1 and *Il15-/-* mice (n = 10/genotype) on gd 7.5. Mated females pregnant were placed in hypobaric chambers (Biospherix Instruments, USA) and exposed to 420Torr (11% oxygen) for 48 h as reported by Ho-Chen et al. (2006). Pair-fed control gd 7.5 mice (at least five/group) were held in the same chamber at normoxia (760 Torr, 21% oxygen) for 48 h. These animals were immediately sacrificed by cervical dislocation for uterine tissue collection. The second group of pregnant CD1 and *IL-15-/-* mice received the same treatments but were allowed to give birth to quantify litter size and neonatal viability.

Tissue sampling

Implantation sites were enumerated and classified as normal (N), atrophic (Atr) or hyperemic (Hym). Atrophic sites were that 20%-40% smaller in size than normal embryo implantation sites and of normal or pale

color while hyperemic sites were dark red. Implantation sites with normal appearance were dissected to isolate fetal tissue-free mesometrial endometrium for RNA and protein assays or for freezing (-8 °C) in dry ice-cooled heptane (Fisher Scientifics, USA) for subsequent cryosectioning.

Cytochemistry and immunocytochemistry

Uterine cryosections (10 μm thick) collected on glass slides were fixed with cooled (-4 °C) 4% paraformaldehyde in 0.1 M PBS pH 7.4.

For uNK cell identification and quantification *Dolichos biflorus* (DBA) lectin cytochemistry (Paffaro Jr. et al., 2003) and perforin (PRF) immunocytochemical staining (rabbit polyclonal anti-*perforin*; Torrey Pines Biolabs, Houston, TX) (Ain et al., 2003) were used. Endoglin (EDG) (rat polyclonal anti-endoglin; Developmental Hybridoma, Iowa, USA) immunostaining was used to identify proliferating endothelial cells and TROMA-I immunostaining (mouse monoclonal anti-TROMA-I, Developmental Hybridoma, Iowa, USA) was used to identify trophoblast cells (Gomes et al., 2012). Analyses used a Leica MZFLIII stereomicroscope (Leica Mycrosystems GmbH, Welzlar, Germany) for bright field or Nikon Eclipse 55i microscope with CCD camera (Nikon Instruments Inc., Melville, NY) for fluorescence photomicroscopy.

Morphometry

DBA[+] or PRF^{+} cells in the mesometrial lymphoid aggregate of pregnancy (MLAp) and decidua basalis (DB) were quantified at 400x magnification. Cells were counted from 3 different sections from every animal in each group. The means and standard deviations of percentage of DBA[+] and PRF^{+} cells distributed in the MLAp and DB were obtained from animals of 3 different experiments.

In sections placed with the mesometrial side of the uterus to the top, the placental area delimited at the bottom by the amniotic cavity and at the top by TROMA-I[+] trophoblast giant cells facing the decidualized endometrium was measured using *Image J software* (NIH, USA). Taking CD1 placental areas as reference, ratios of *IL-15^{-/-}* placental areas were calculated and used as trophoblast invasive indices. The statistical analysis from 3 independent assays of each experimental group was determined by ANOVA.

Enzyme-Linked-Immuno Sorbent-Assay (ELISA)

VEGFA and IFNγ concentrations were measured in homogenates of fetal tissue-free mesometrial tissue using mouse VEGFA-ELISA KIT and IFNγ-ELISA KIT (cat. KMG0112 and KMC4021, Invitrogen, Carlsbad, CA, respectively), according to the manufacturer's instructions. The statistical significance of data obtained from 5 independent assays of each experimental group was determined by ANOVA.

Real time-PCR

RNA was extracted from mesometrial endometrium (decidua + MLAP) with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Random primers (Promega, Madison, WI, USA) were used for cDNA synthesis using the manufacturer's protocol.

The Real-Time PCR (ABI7500/ 7500 software) transcriptome amplifications were performed using SYBR Green PCR Kit (Qiagen, USA) and reactions according to manufacturer's instructions for *Nos2*, *Nos3*, *Tnfα*, *Tnfr1*, *Tnfr2*, *Vegfa*, *Vegfc* and *Perforin (Prf1)* genes using 18S RNA as the endogenous housekeeping gene. Sense and anti-sense primer sequences were designed using the Primer 3 software program (Table 1). Quantitative RT-PCR data from four independent experiments of each group were used to assess statistical analysis by ANOVA.

Table 1. Primers sequences us ed in real-time PCR and respective amplicon length.

Gene	Sense primer	Anti-sense primer	Amplicon (bp)
Vegf-a	CACGACAGAAGGAGAGCAGAA	CTATGTGCTGGCTTTGGTGA	265
Vegf-c	ACCTCCATGTGTGTCCGTCTAG	GCCTGACACTGTGGTAATGT	255
Nos2	AAAGGAGAACGTTGGATTTG	GTGGCCTTGTGGTGAAGAGT	339
Nos3	AGAACCTGTGGCTGTGGAAC	GTGCGTAGCAGTCGAAGGA	164
Perforin	ATTACGGTGATATTGATTACG	AATCGGTAGCGGTAAGTTAC	257
Tnf-α	CCGATGGGTTGTACCTTGTC	TGGCGTACAAGTGACGGTAC	232
Tnfr1	GGAGGACCGTACCCTGATCT	GTTCCTTTGGACTTGGTTAG	233
Tnfr2	GCCCACTTGTTAAGGCGCTG	CAGCCATTGTTCACATCCAC	241
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA	122

Source: Primer3Plus.

Results

Gross postmortem assessments of hypoxic gd 9.5 implantation sites does not coincide with litter sizes at birth in deficient in Il15 mice

The ratios between normal (N) and abnormal (either Atr or Hym) gd 9.5 implantation sites in CD1 and *Il15*$^{-/-}$ pregnancies are given in Table 2. Using CD1 in normoxia as reference, the ratio of Atr-sites doubled in CD1 by hypoxia.

No Hym-sites were present in CD1 suggesting onset of conceptus delay was recent. In contrast, no Atr sites but only Hym sites were present in *Il15*$^{-/-}$ mice. For uNK cell deficient strain, the proportion of failed sites was 5 to 6 fold greater than in CD1 under normoxia and hypoxia. These data suggest that conceptus delay or failure was more advanced when uNK cells were absent from decidua whether or not hypoxic insult was additionally present.

Table 2. Frequency rate of atrophic (Atr) and hyperemic (Hym) abnormal embryo developing site at gd 9.5 in the pregnant uterus of CD1and *IL15*$^{-/-}$ animals under normoxia (N) and hypoxia (H).

	CD1		*IL15*$^{-/-}$	
	Atr	**Hym**	**Atr**	**Hym**
N	1:30.5	0	0	1:5.0
H	1:16.1	0	0	1:4.6

The ratio represents the proportion of number of normal sites presenting 1 abnormal site accounted from n = 5 animals of each experimental group.

Pregnancy success evaluated as litter size at term compared to CD1 was reduced significantly under normoxic conditions in the gene-deleted strain (Table 3). Hypoxia did not alter CD1 litter size, but significantly reduced in the absence of uNK cells. The reduced number of pups in the *IL-15*$^{-/-}$ animals coincides with high proportion of Hym-sites found at gd 9.5. Similarly, the same litter size between normoxia and hypoxia conditioned CD1 animals suggest the embryo from Atr-site affected by 48 h hypoxia at gd 9.5, survive and growth to the term.

Table 3. Litter size of CD1 and *IL15$^{-/-}$* mice at term under normoxia (N) and hypoxia (H).

	CD1	***IL15$^{-/-}$***
N	13.94 ± 1.69 (n = 17)	9.12 ± 1.96* (n = 8)
H	14.0 ± 2.23 (n = 13)	7.75 ± 1.29# (n = 5)

Significant differences (p < 0.005) between mouse strains if compared with CD1 in normoxia (*) and hypoxia (#). n = number of animals.

Influence of hypoxia and IL-15 deficiency on implantation site histopathology

Both DBA lectin and perforin immunocytochemistry independently identified uNK cells distributed in the MLAP and DB of uterine embryo developing sites of CD1 mice. As expected, uNK cells were completely absent in the *IL-15$^{-/-}$* pregnant uterus at gd 9.5 (Figure 1).

Analyses of DBA$^+$ or PRF$^+$uNK cells (Table 4) revealed greater numbers of DBA$^+$ or PRF$^+$uNK cells in the DB under hypoxia than normoxia and lower numbers in the MLAP. This shift suggests recruitment of premature uNK cells into decidual regions to supplement the function of the decidual uNK cell subset.

Table 4. DBA positive- and perforin (PRF) positive-uNK cells percentage in the MLAP and DB of pregnant mice uterus at gd9,5 in normoxia (N) and hypoxia (H).

CD1 DBA$^+$uNK		**CD1** PRF$^+$uNK	
MLAP	**DB**	**MLAP**	**DB**
95.0 ± 5.0	67.5 ± 2.5	98.0 ± 2.0	73.7 ± 3.9
81.3 ± 9.7	77.4 ± 4.7	91.7 ± 1.0	89.4 ± 5.3

The percentage and standard deviation represents quantification from five independent experiments.

Based on endoglin+ endothelial cell distributions the endometrial vascular networks in CD1 mice did not show marked differences between normoxia and hypoxia. Endoglin-defined vascular networks were poorly remodeled in normoxic *IL-15$^{-/-}$* endometrium. The fetal blood vessels of labyrinthine placental layer were also endoglin-reactive.

Trophoblast giant cells (TGC) are the outermost layer TROMA-I positive cells of placenta in contact with decidua in all strains. The placental area of TROMA-I positive cells were measured and the trophoblast invasiveness index was established using CD1 in normoxia as a reference. All mutant placentas were significantly larger than CD1 placenta and those from IL-15 deficient mice being 4 fold larger than CD1 placenta under normoxic conditions. TROMA-I$^+$TGC intruded deeply into the mesometrial endometrium of *IL-15$^{-/-}$* animals both in normoxia and hypoxia, and corresponding trophoblast invasiveness index was 3 and 5 folds higher, respectively. Hypoxia did not statistically alter placental size within a strain. Taking into account the limited development of decidua and poor endoglin expression in the endometrium of uNK deficient *IL-15$^{-/-}$* mice, these abnormal conditions at the maternal-fetal interface seems to increase

trophoblast invasion and placenta hypertrophy.
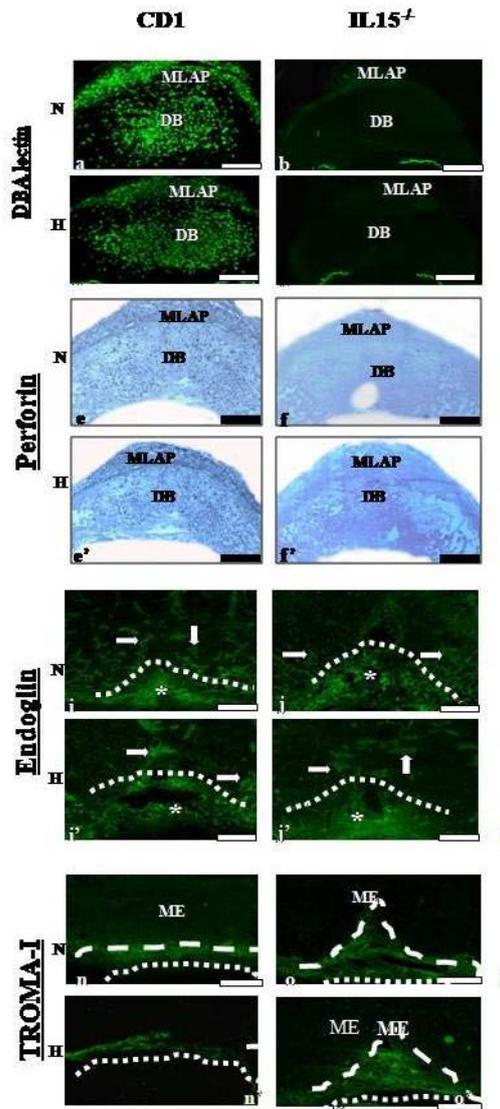
Figure 1. Influence of hypoxia and IL-15 deficiency on implantation site histopathology.

Influence of hypoxia and IL-15 deficiency on levels of IFNγ and VEGF in implantation sites

VEGF and IFNγ are molecules related to angiogenesis and produced by uNK cells in normal pregnancy (Ashkar et al., 2003; Lima et al., 2012). Protein quantification of VEGF showed the absence of uNK cells reduced the VEGF at the maternal-fetal interface to 23% if compared to CD1 mice in normoxia. The hypoxia did not change significantly the IFNγ concentration in the maternal-fetal interface of CD1 mice. Unexpectedly, the uNK cells deficiency increased significantly the amount of IFNγ in the uterus both in normoxia and hypoxia, suggesting the uNK cell is not the main source of this cytokine in the pregnant uterus at gd10th (Figure 2).
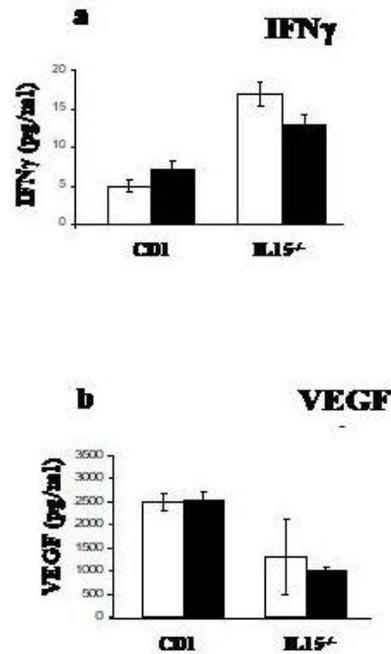
Figure 2. Influence of hypoxia and IL-15 deficiency on levels of IFNγ and VEGF in implantation sites.

Influence of hypoxia and IL-15 deficiency on gene expression in implantation sites

The effect of hypoxia on the expression of selected genes expressed by uNK cells (*Vegfa, Vegfc*, *Nos2* (iNOS), *Nos3* (eNOS), *Prf1* (perforin), *Tnfa*, *Tnfr1*, *Tnfr2* was evaluated in gd 9.5 decidual homogenates from normal mice and
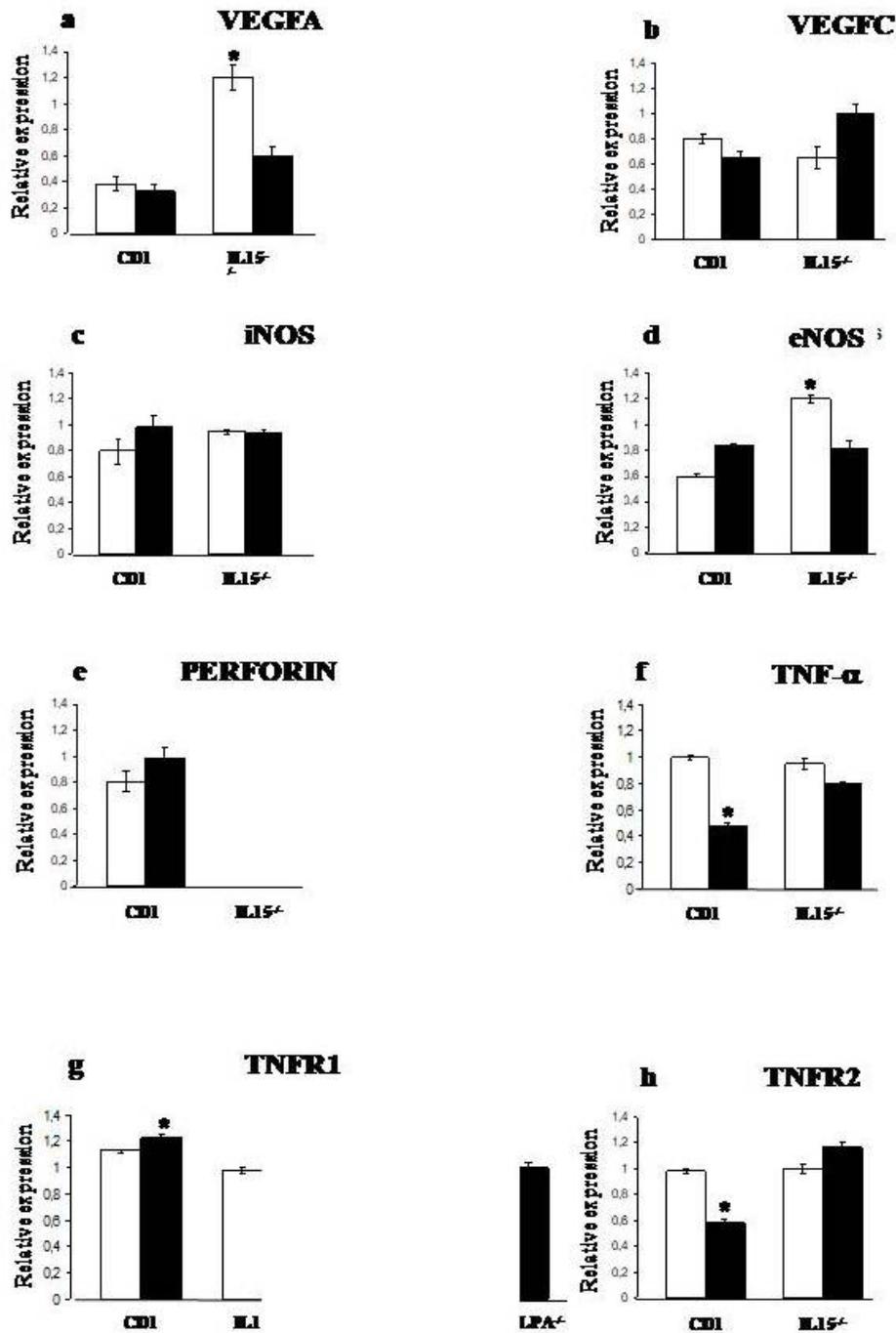
Figure 3. Influence of hypoxia and IL-15 deficiency on gene expression in implantation sites.

from mice lacking either uNK cells (*IL15⁻ᐟ⁻*). Deficiency of uNK cells gave relative elevation of VEGF in decidua. Hypoxia did not alter the *Vegfa* transcripts in control mice but affected differently in the mutant mice. Hypoxia reduced *Vegfa* expression in uNK cells depleted *IL15⁻ᐟ⁻* mice. Meanwhile, *Vegfc* expression was lower under hypoxic conditions in wild type mice and in the

uNK cell mice. It suggests different regulatory mechanism affecting the expressions of *Vegfa* and *Vegfc* isoforms in the uNK cell-independent alternative route of VEGF synthesis and releasing in the pregnant uterus.

The *Nos2* isoform gene expression showed down regulation under hypoxia in all groups. The expression of the *Nos3* isoform gene was higher in the absence of uNK cells while under hypoxic conditions it was down-regulated. These data clearly show the dual influence of oxygen tension and seems mobilize alternative cells.

The *Tnf-α* transcript expression of CD1 was similar to uNK cells deficient mice in normoxia. Under hypoxia the transcript level of this pro-inflammatory cytokine was down-regulated in the uNK cells deficient mice, suggesting no involvement in the increasing pregnancy loss of these experimental groups. However, the expression of the TNF-α receptor *Tnfr1* that induces apoptosis of target cell was similar to CD1 or was down-regulated in the uNK cells deficient uterus. The expression pattern of receptor *Tnfr2* looks like to seek undo the TNF-α and TNFR-1 activity in the pregnant uterus of uNK deficient and hypoxia experimental groups.

Discussion

The present work demonstrated experimentally the absence of uNK cell and 48h continuous threshold hypoxia during pre-placentation stage deeply affects vascular remodeling and increase trophoblast invasiveness at mid gestation, but compromise only partially the pregnancy outcome. The reduced deleterious effects of isolated or combined adverse conditions can be attributed to the finely orchestrated compensatory capability of the utero-placental tissues response to regulate angiogenic molecules at the maternal-fetal interface.

The 420Torr oxygen concentration corresponds to hypoxic conditions of altitude higher than 4500 meters (m) and doubled the incidence of abnormal hypotrophy of embryo implantation sites in the CD1 pregnant uterus. Hypoxia has been associated with preeclampsia in high altitude human pregnancy in which increases nitrative stress (Zamudio et al., 2007; Deng et al., 2018; Schoots et al., 2018) and increases uterine vascular eNOS and NO production in the ovines and ewes (Hempstock et al., 2003; Fujita et al., 2010). Uterine-NK cells, uterine endothelial cells, macrophages, stromal and epithelial cells and TGC among other cells, produce NO in the mouse pregnant uterus (Chen et al., 2017; Fortis et al., 2018). The up-regulation of *Nos2* (iNOS) and *Nos3* (eNOS) found in the CD1 endometrium under hypoxia could be either due to increasing numbers of DBA+/PRF+uNK cells in the DB and/or activation of nitrative stress pathway of uNK cells to increase NO concentration available in the uterus. On gd 9.5 the MLAP region usually contains small agranular DBA+uNK cells and the mature granulated forms dominates in the DB (Croy et al., 2010; Lima et al., 2012). Conversely, the expression of *Prf*, *Tnf-α* and *Ifn-γ* genes of molecules related to cytotoxicity of uNK cell (Croy et al., 2010; Chen et al., 2012; Lima et al., 2012), were not up-regulated under hypoxia in the CD1 mice. Therefore, these cytolytic and pro-inflammatory proteins are not the main cause of increasing of abnormal Atr-implantation sites. Intriguingly, despite the high incidence of Atr-implantation sites at gd 9.5 under hypoxia, this does not affect the litter sizes at term in CD1 animals and presumes the embryo in the Atr-sites at gd 9.5 by 48h hypoxia can be rescued to normalcy at term by restoring the normal oxygen supply.

Expectedly, IL15 gene deletion abrogate DBA+ and PRF+uNK cell incidence as has been reported by Ahskar et al (2003). The high proportion (5-6 folds) of abnormal Hym-embryo developing sites at gd 9.5 seems to be

irreversible, different from those Atr-sites of CD1, and results in reduced litter size of these animals at term independently of oxygen tension influence. These data suggest the impact of uNK cell deficiency deeply affecting normal utero-placental onset. In fact, those apparently normal *IL-15⁻/⁻* embryo developing sites showed poor endoglin positive endothelial cells distribution in the mesometrial endometrium and hypertrophic placenta at gd 9.5. Consistent to this, uNK cell deficiency including the *IL15⁻/⁻* mice encompass hypocellular, edematous and incomplete uterine spiral artery wall remodeling of mesometrial endometrium (Croy et al., 2003; Wu et al., 2006). Endometrial vascular remodeling is essential for normal pregnancy and dependent of angio-modulatory factors like NO, angiotensin-I and -II, VEGF and IFNγ produced by human and mouse uNK cells (Chen et al., 2012; Lima et al., 2012).

VEGF, IFNγ and, eNOS and iNOS to produce nitric oxide (NO) are all attributed as key molecule of uNK cells related angiogenesis in the pregnant uterus (Fortis et al., 2018) were not abrogated in the uNK cell deficient *IL15⁻/⁻* animals. Otherwise, low VEGF protein concentration in the *IL15⁻/⁻* pregnant uteri confirms the uNK cells as putative main source of this angiogenic factor, but not exclusive (Ain et al., 2003) is not the only mechanism regulating VEGF expression in the pregnant mouse uterus. This alternative uNK independent pathway ensures constitutive VEGF expression as compensatory mechanism driving the utero-placental vascular remodeling.

Endoglin is consistently present in the proliferating endothelial cell and it is stimulated by VEGF. The reduced endoglin positive endothelial cells in the *IL15⁻/⁻* mice endometrium matches to the low VEGF concentration. It means poor endometrial vascular remodeling and consequently low blood and oxygen supply to the placenta that stimulate

increasing of trophoblast invasiveness (Zhang et al., 2011), as were unveiled by deeply intruded TROMA-I positive TGC into the mesometrial endometrium and enlarged placenta in the *IL-15⁻/⁻* pregnant uteri. This hypertrophy of placenta could mechanistically ensure the necessary blood exchange area to the embryo in the poorly remodeled endometrial vascular bed. High incidence of abnormal Hym-embryo implantation detected at gd 9.5 of uNK deficient uteri looks like consequence of increased trophoblast invasiveness with disruption of utero-placental unit (data not shown) and impaired gestation.

Furthermore, more than 40% of the pregnancy failures in the IFNγ null mice reported by Ashkar et al (2003) demonstrated the importance of this cytokine in the pregnancy. One of the potential route of IFNγ mediated uNK cells gene expression regulation is the iNOS expression to produce NO (Frérat et al., 2009). Interestingly, the uterine eNOS expression is sensitive to hypoxia and is not dependent of uNK cells, while iNOS expression is only partially dependent of uNK cells affected by low oxygen tension.

The high concentration of IFNγ in the *IL-15⁻/⁻* mice is an unexpected, contradictory result to the established paradigm attributing the uNK cell as main source of this cytokine in the pregnant uterus (Ashkar et al., 2003). This discrepancy could be due to the high reproductive performance of CD1 mice strain used as background for *IL15⁻/⁻* mice instead of C57/Bl6. Previous reports using C57/Bl6 background *IL15⁻/⁻* mice conclude no difference of litter size if compared to B6 wild type (González et al., 2012) while our data detected Hym-uterine sites during mid-gestation and significant lowering of litter size in the CD1 background *IL15⁻/⁻* mice. Further investigations regarding influence of mouse strains in the reproductive performance should be carried out since most of the inbred strains have small litter sizes if compared to CD1 outbred

strains and probably influences uterine response capability to support pregnancy.

Conclusions

In conclusion, this study confirms the benefit of uNK cell present at the maternal-fetal interface as key modulatory cell working for adequate uterine tissue remodeling and supporting the successful pregnancy (Burkle et al., 2010; Lima et al., 2012). Secondly, despite the combined severe adverse conditions of uNK deletion and 48h hypoxia used in the present experiment to compromise angiogenesis and vascular remodeling in the pregnant uterus, at least 60% of implanted embryo were preserved or rescued to reach the successful gestation. This robust uterine support arise from the capability of uterine tissue compensatory response, namely the molecules essential for vascular remodeling to provide the blood supply for growing placenta. It also means the essential regulatory pathway necessary for successful pregnancy is not restricted or dependent of one single molecule, nor these are dependent of single sources in the pregnant uterus.

Acknowledgements

Conflicts of interest

The author declares that have no conflict of interests.

References

Ain, R.; Tash, J. S.; Soares, M. J. Prolactin-like protein-A is a functional modulator of natural killer cells at the maternal-fetal interface. **Molecular and Cellular Endocrinology**, v. 204, no. 1/2, p. 65-74, 2003. https://doi.org/10.1016/S0303-7207(03)00125-4

Alecsandru, D.; Garcia-Velasco, J. A. Why natural killer cells are not enough: a further understanding of killer immunoglobulin-like receptor and human leukocyte antigen. **Fertilily and Sterility**, v. 107, no. 6, p. 1273-1278, 2017. https://doi.org/10.1016/j.fertnstert.2017.04.018

Ashkar, A. A.; Black, G. P.; Wei, Q.; He, H.; Liang, L.; Head, J. R.; Croy, B. A. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. **The Journal of Immunology**, v. 171, no. 6, p. 2937-2944, 2003. https://doi.org/10.4049/jimmunol.171.6.2937

Avagliano, L.; Bulfamante, G. P.; Morabito, A.; Marconi, A. M. Abnormal spiral artery reodeling in the decidual segment during pregnancy: from histology to clinical correlation. **Journal of Clinical Pathology**, v. 64, no. 12, p. 1064-1068, 2011. https://doi.org/10.1136/jclinpath-2011-200092

Burton, G. J.; Charnock-Jones, D. S.; Jauniaux, E. Regulation of vascular growth and function in the human placenta. **Reproduction**, v. 138, no. 6, p. 895-902, 2009. https://doi.org/10.1530/REP-09-0092

Chen, Z.; Zhang, J.; Hatta, K.; Lima, P. D. A.; Yadi, H.; Colucci, F.; Yamada, A. T.; Croy, B. A. DBA-lectin reactivity defines mouse uterine natural killer cell subsets with biased gene expression. **Biology of Reproduction**, v. 87, no. 4, p. 1-9, 2012. https://doi.org/10.1095/biolreprod.112.102293

Chen, D. B.; Feng, L.; Hodges, J. K.; Lechuga, T. J.; Zhang, H. Human trophoblast-derived hydrogen sulfide stimulates placental artery

endothelial cell angiogenesis. **Biology of Reproduction**, v. 97, p. 478-489, 2017. https://doi.org/10.1093/biolre/iox105

Croy, B. A.; Esadeg, S.; Chantakru S.; Van den Heuvel, M.; Paffaro Jr., V. A.; He, H.; Black, G. P.; Ashkar, A. A.; Kiso, Y.; Zhang, J. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. **Journal of Reproductive Immunology**, v. 59, no. 2, p. 175-191, 2003. https://doi.org/10.1016/S0165-0378(03)00046-9

Croy, B. A.; Zhang, J.; Tayade, C.; Colucci, F.; Yadi, H.; Yamada, A. T. Analysis of uterine natural killer cells in mice. **Methods in Molecular Biology**, v. 612, p. 465-503, 2010. https://doi.org/10.1007/978-1-60761-362-6_31

Deng, Q.; Yin, N.; Chen, Y.; Shan, N.; Liu, X.; Qi, N. Downregulated N-acetylglucosaminyl-transferase III is involved in attenuating trophoblast migration and invasion under hypoxia-reoxygenation condition. **The Journal of Maternal-Fetal & Neonatal Medicine**, v. 21, p. 1-7, 2018. https://doi.org/10.1080/14767058.2018.1438392

Fortis, M. F.; Fraga, L. R.; Boquett, J. A.; Kowalski, T. W.; Dutra, C. G.; Gonçalves, R. O.; Vianna, F. S. L.; Schuler-Faccini, L.; Sanseverino, M. T. V. Angiogensis and oxidative stress-related gene variants in recurrent pregnancy loss. **Reproduction, Fertility and Development**, v. 30, no. 3, p. 498-506, 2018. https://doi.org/10.1071/RD17117

Franasiak, J. M.; Scott, R. T. Contribution of immunology to implantation failure of euploid embryos. **Fertilily and Sterility**, v. 107, no. 6, p. 1279-1283, 2017. https://doi.org/10.1016/j.fertnstert.2017.04.019

Frérat, F.; Lobysheva, I.; Gallez, B.; Dessy, C.; Feron O. Vascular caveolin deficiency supports the angiogenic effects of nitrite, a major end product of nitric oxide metabolism in tumors. **Molecular Cancer Research**, v. 7, no. 7, p. 1056-1063, 2009. https://doi.org/10.1158/1541-7786.MCR-08-0388

Fujita, D.; Tanabe, A.; Sekijima, T.; Soen, H.; Narahara, K.; Yamashita, Y.; Terai, Y.; Kamegai, H.; Ohmichi, M. Role of extracellular signal-regulated kinase and AKT cascades in

regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line. **Journal of Endocrinology**, v. 206, p. 131-140, 2010. https://doi.org/10.1677/JOE-10-0027

Gomes, S. Z.; Lorenzon, A. R.; Vieira, J. S.; Rocha, C. R. R.; Bandeira, C.; Hoshida, M.; Lopes, L. R.; Bevilacqua, E. Expression of NADPH oxidase by trophoblast cells: Potential implications for the postimplanting mouse embryo. **Biology of Reproduction**, v. 86, no. 2, p. 1-11, 2012. https://doi.org/10.1095/biolreprod.111.094748

González, I. T.; Barrientos, G.; Freitag, N.; Otto, T.; Thijssen, V. L.; Moschansky, P.; von Kwiatkowski, P.; Klapp, B. F.; Winterhager, E.; Bauersachs, S.; Blois, S. M. Uterine NK cells are critical in shaping DC immunogenic functions compatible with pregnancy progression. **PLoS One**. v. 7, no. 10, e46755, 2012. https://doi.org/10.1371/journal.pone.0046755

Hatta, K.; Carter, A. L.; Chen, Z.; Leno-Durán, E.; Ruiz-Ruiz, C.; Olivares, E. G.; Tse, M. Y.; Pang, S. C.; Croy, B. A. Expression of the vasoactive proteins AT-1, AT-2, and ANP by pregnancy-induced mouse uterine natural killer cells. **Reproductive Sciences**, v. 18, no. 4, p. 383-390, 2011. https://doi.org/10.1177/1933719110385136

Hempstock, J.; Jauniaux, E.; Greenwood, N.; Burton, G. J. The contribution of placental oxidative stress to early pregnancy failure. **Human Pathology**, v. 34, no. 12, p. 1265-1275, 2003. https://doi.org/10.1016/j.humpath.2003.08.006

Ho-Chen, J. K.; Ain, A. R.; Wood, J. G.; Gonzalez, N. C.; Soares, M. J. Hypobaric hypoxia as a tool to study pregnancy-dependent responses at the maternal-fetal interface. **Methods in Molecular Medicine**, v. 122, p. 427-434, 2006. https://doi.org/10.1385/1-59259-989-3:427

Hunt, J. S.; Miller, L.; Vassmer, D.; Croy, B. A. Expression of the inducible nitric oxide synthase gene in mouse uterine leukocytes and potential relationships with uterine function during pregnancy. **Biology of Reproduction**, v. 57, no. 4, p. 827-836, 1997. https://doi.org/10.1095/biolreprod57.4.827

Kalkunte, S.; Boji, R.; Norris, W.; Friedman, J.; Lai, Z.; Kurtis, J.; Lim, K. H.; Padbury, J. F.; Matthiesen, L.; Sharma, S. Sera from preeclampsia patients elicit symptoms of human disease in mice and provide a basis

for an in vitro predictive assay. **The American Journal of Pathology**, v. 177, no. 5, p. 2387-2398, 2010. https://doi.org/10.2353/ajpath.2010.100475

Leno-Durán, E.; Hatta, K.; Bianco, J.; Yamada, A. T.; Ruiz-Ruiz, C.; Olivares, E. G.; Croy, B. A. Fetal-placental hypoxia does not result from failure of spiral arterial modification in mice. **Placenta**, v. 31, no. 8, p. 731-737, 2010. https://doi.org/10.1016/j.placenta.2010.06.002

Lima, P. D. A.; Croy, B. A.; Degaki, K. Y.; Tayade, C.; Yamada, A. T. Heterogeneity in composition of mouse uterine natural killer cell granules. **Journal of Leukocyte Biology**, v. 92, no. 1, p. 195-204, 2012. https://doi.org/10.1189/jlb.0312136

Liu, S.; Diao, L.; Huang, C.; Li, Y.; Zeng, Y.; Kwak-Kim, J. Y. H. The role of decidual immune cells on human pregnancy. **Journal of Reproductive Immunology**, v. 124, p. 44-53, 2017. https://doi.org/10.1016/j.jri.2017.10.045

Paffaro Jr., V. A.; Bizinotto, M. C.; Joazeiro, P. P.; Yamada, A. T. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. **Placenta**, v. 24, no. 5, p. 479-488, 2003. https://doi.org/10.1053/plac.2002.0919

Possomato-Vieira, J. S.; Khalil, R. A. Mechanism of endothelial dysfunction in hypertensive pregnancy and preeclampsia. **Advances in Pharmacology**, v. 77, p. 361-431, 2016. https://doi.org/10.1016/bs.apha.2016.04.008

Primer3Plus. Available from: <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. Accessed on: Feb. 22, 2018.

Schoots, M. H.; Gordijn, S. J.; Scherjon, S. A.; van Goor, H.; Hillebrands, J. L. Oxidative stress in placental pathology. **Placenta**, in press, 2018. https://doi.org/10.1016/j.placenta.2018.03.003

Wu, X.; Wei, H.; Zhang, J.; Tian, Z. Increased uterine NK-derived IFN-gamma and TNF-alpha in C57BL/6J mice during early gestation. **Cellular & Molecular Immunology**, v. 3, no. 2, p. 131-137, 2006. Available from: <http://www.cmi.ustc.edu.cn/3/2/131.pdf>. Accessed on: Feb. 22, 2018.

Zhang, J.; Chen, Z.; Smith, G. N.; Croy, B. A. Natural killer cell-triggered vascular transformation: maternal care before birth? **Cell & Mollecular Immunology**, v. 8, p. 1-11, 2011. https://doi.org/10.1038/cmi.2010.38

Zamudio, S.; Kovalenko, O.; Vanderlelie, J.; Illsley, N. P.; Heller, D.; Belliappa, S.; Perkins, A. V. Chronic hypoxia in vivo reduces placental oxidative stress. **Placenta**, v. 28, no. 8/9, p. 846-53, 2007. https://doi.org/10.1016/j.placenta.2006.11.010