Learning from experience: cellular and molecular bases for improved outcome in subsequent pregnancies

Debra Goldman-Wohl, PhD; Moriya Gamliel, PhD; Ofer Mandelboim, PhD; Simcha Yagel, MD

The frequencies of preeclampsia, fetal growth restriction, fetal demise, and low birthweight are lower in subsequent pregnancies. Enhanced maternal cardiovascular adaptation, shorter first and second stages of labor, and more robust lactation also have been observed in subsequent as compared with first pregnancies. We sought to investigate the cellular and molecular bases for better outcomes in subsequent pregnancies. Based on the knowledge that specialized immune cells at the maternal—fetal interface, decidual natural killer cells, promote development of the placental bed and conversion of the spiral arteries by secreting a myriad of angiogenic and growth factors, we asked whether decidual natural killer cells differ in subsequent as compared with first pregnancies. This idea stemmed from recent studies suggesting that natural killer cells, although part of the innate immune system, possess some features of adaptive immunity, including a certain type of immune cell memory, termed trained immunity. We found that decidual natural killer cells from parous women "remember pregnancy" and differ from decidual natural killer cells of primigravidae. Compared with the decidual natural killer cells of first pregnancy, these cells, that we termed pregnancy-trained decidual natural killer cells, express greater levels of the natural killer receptors NKG2C and leukocyte immunoglobulin-like receptor B1, which interact with ligands expressed on invasive trophoblasts. Furthermore, they secrete greater levels of several growth factors, including vascular endothelial growth factor α as well as interferon-γ, augmenting remodeling of the placental bed. We propose that this pregnancy-trained memory dwells in the epigenome, where memory of stimuli is known to persist even when the stimulus is no longer present. This epigenetic memory apparently resides in endometrial natural killer cells between pregnancies. We suggest that this trained memory, which we coined pregnancy-trained decidual natural killer cells, may be the missing link in the immune basis for enhanced subsequent pregnancy. Epigenetic memory (chromatin modification) also may afford a global explanation for additional findings of enhanced maternal cardiovascular adaptation, shorter first and second stages of labor, and more robust lactation. Understanding the molecular and cellular bases of improved outcomes of subsequent pregnancy may lead to the development of treatment modalities designed for women at high risk for pregnancy disorders originating at the maternal—fetal interface.

Key words: decidual NK cells, DNA methylation, epigenetics, fetal growth restriction, FGR, IFN-γ, multigravida, NK cells, preeclampsia, primigravida, trained memory, VEGF

Consider the following scenario: gravida 2 para 1 vs gravida 1 para 0: Two patients of similar age and sociodemographic status visit a prenatal clinic. Both are apparently healthy nonsmokers who do not report any significant history. Patients are 6 weeks by dates with confirmatory ultrasound demonstrating fetal viability. The first patient is gravida 2 para 1, following an uncomplicated first pregnancy that resulted in the normal vaginal delivery of a healthy term infant, now 2.5 years old. The next patient is gravida 1 para 0. As these 2 patients carry different risks for pregnancy complications, consider the different degrees of caution and circumspection that will come into play as you follow these 2 gravidae through the coming months.

There is general agreement that pregnancy outcomes are more favorable for parous as compared with nulliparous women.[1] Differences between first vs subsequent pregnancies have been widely investigated through multiple approaches: from population-based epidemiologic studies to histologic analysis of the maternal—fetal interface, to molecular biology—based examination of epigenetic memory. First pregnancies are associated with a 2.5-fold greater stillbirth risk,[2,3] and placental and newborn weights are greater in parous as compared with primiparous women.[4,5–7] Indeed, first deliveries are associated with lower birthweight and greater rates of small for gestational age.[8–10] Further examples of these observations would be the reduction in preeclampsia risk in subsequent pregnancies as a result of upgraded implantation/placentation;[11] enhanced maternal cardiovascular adaptation[12–14] observed in increased end-diastolic volume, stroke volume, and cardiac output, and decreased vascular resistance;[13] decreased mean arterial pressure and

From the Magda and Richard Hoffman Center for Human Placenta Research, Department of Obstetrics and Gynecology (Drs Goldman-Wohl and Yagel), and The Concern Foundation Laboratories at the Lautenberg Centre for Immunology and Cancer Research, IMRIC, Faculty of Medicine (Drs Gamliel and Mandelboim), Hebrew University Hadassah Medical Center, Jerusalem, Israel.

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Corresponding author: Simcha Yagel, MD. simcha.yagel@gmail.com

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reduced arterial stiffness;[14] as well as better outcomes of first and second stages of labor and delivery;[15] and more successful lactation, characterized by an improved nursing experience and increased milk supply[16] in repeat pregnancy.[17] Here we review the evidence of better outcomes of subsequent pregnancies by showing that the natural killer (NK) cells of the decidua remember pregnancy, enhancing development of the placental bed with epigenetic memory residing in the NK cells of the endometrium, between pregnancies.[18]

Many of the "great obstetrical syndromes" have their biological basis in defects of deep placentation and development of the placental bed,[11] and some are more prevalent in nulliparous women. There is evidence for augmented deep placentation in subsequent pregnancies. It has been shown that the proportion of decidual vessels undergoing trophoblast invasion is greater in first-trimester decidua isolated from parous as compared with nulliparous women.[19] Khong et al showed that an "experienced" parous uterus differs from the nulliparous, lending histologic support to the notion that the uterus does not return to its nulliparous state following delivery,[20] while suggesting that the uteri of parous women are "primed" for future pregnancies. They demonstrated that the elastic tissue and musculature surrounding the uterine arteries in the nulliparous uterus can be distinguished from that of the altered uterine architecture found in the parous uterus[20] and that the parous uterus does not revert to the nulliparous tissue architecture. Enhanced placentation is further exhibited by differing levels of circulating angiogenic factors (greater angiopoietin-2 at 8 weeks) and more rapid placental development, as measured by ultrasound scan, in the early first trimester of parous as compared with nulliparous women.[4]

An unexpected finding, that parity negatively affects the success rate of first-trimester medical abortion, is described in several studies.[21–24] Investigators examined different end points to determine successful medical abortion, such as β-human chorionic gonadotropin levels, ultrasound-based imaging, and the need for surgical intervention, and no specific mechanism has been offered to elucidate this observation. However, Bartley et al comment that this finding is suggestive of a difference in the placental bed in parous vs nulliparous women, ie, the placental bed of parous women may be sounder early in pregnancy, resulting in less efficacious medical abortion.[21]

One of the most striking examples of contrasting risks in first and subsequent pregnancies is the increased risk for the development of preeclampsia.[25] Primiparity differs from the myriad identified risk factors for preeclampsia, which implicate fetal, maternal[26] and environmental factors[27] in the development of the syndrome. The rate of preeclampsia in primiparae is more than double that observed in parous women.[28] Yet while some of these risk factors seem to have obvious connections with the disorder (such as metabolic syndrome, for example) first pregnancy seems to defy most logical explanations for its association with preeclampsia, unless we look for some underlying immune component. First pregnancy as a risk factor for preeclampsia was recognized as early as the 17th century by the Frenchman Francois Mauriceau, who was the first to systematically describe eclampsia and to note that primigravidae were at greater risk for convulsions as compared with multigravidae[29,30] (reported by Mcmillen 2003).[31] Preeclampsia is thought to be initiated early in pregnancy with ineffective spiral artery conversion mediated by shallow trophoblast invasion, leading to inefficient utero placental blood flow.[32–34]

To further categorize preeclampsia, attempts have been made, with some success, to connect specific histopathology of the placenta and gene expression profiles.[35] Burton et al have extensively studied and modeled blood flow to the intervillous space, including when the spiral arteries are narrow and have not undergone appropriate conversion. They reason that oxygenation and reoxygenation malperfusion resulting in oxidative stress and damage to syncytiotrophoblast would be a leading factor in the development of preeclampsia.[36–38] This defective placentation, coupled with an imbalance between antiangiogenic and angiogenic factors expression[39–41] and a maternal inflammatory stress response,[42,43] subsequently lead to endothelial cell dysfunction[44,45] and the maternal cascade of the syndrome.[46]

These observations of remembered development of the fetal–maternal interface in first pregnancy, persevering to enhance subsequent pregnancies, led us to seek a mechanism on a molecular and cellular level retaining the memory of pregnancy, thereby leading to more efficient or more rapid development of the placental bed and hence better pregnancy outcomes in parous women. We predict that this example of enhanced subsequent pregnancy, for which we demonstrate a molecular epigenetic model of NK cells at the maternal–fetal interface, will provide the basis for investigation into other, similar mechanisms leading to improved pregnancy, labor, and delivery outcomes and more robust lactation, in other cell types and tissues, as this field develops.

In this review, we will focus on the possible cellular and molecular bases for sounder subsequent pregnancies as compared with first pregnancies. We will discuss the evidence for enhanced adaptation to pregnancy through cellular epigenetic memory in decidual natural cell (dNK)-trained memory.[18]

Epigenetic Memory

When we discuss memory and the experience of pregnancy and childbirth, we can paraphrase the Japanese writer Haruki Murakami, who said that no matter how much suffering you went through, you never want to let go of those memories.

Epigenetics is defined as modification of chromatin but not the DNA code itself (reviewed in Dor et al[47]). This epigenetic modification is in part facilitated through methylation of cytosine nucleotides usually found within a framework of CpG (cytosine phosphate guanine) in the chromosomal DNA. The process is controlled in part by enzymes, DNA methyltransferases, responsible for

adding a methyl group to cytosine residues in cytosine/guanine-rich regions of DNA (called "CpG islands"). The methylated DNA is generally more tightly wrapped around nucleosomes that pack the DNA with histone proteins and restrict accessibility of the transcriptional machinery inhibiting gene expression, whereas unmethylated DNA is more accessible to the cellular proteins responsible for activation of gene expression,[47] commonly referred to as "open" DNA.

Epigenetic modification of chromosomal DNA affords an explanation of how organs develop and maintain their identity, albeit with the same DNA sequence in each cell of the body.[48] Based on the understanding that a specific pattern of methyl groups added to chromosomal DNA may affect gene activity, hypomethylated regions of chromosomal DNA are thought to be more accessible to transcriptional machinery. This stable, mitotically inheritable pattern of "open" and "closed" DNA able to activate or silence transcription, has implications to cancer biology, developmental biology, and aging and could explain how an experienced cell lineage may be more readily activated when encountering a repeat of previous stimuli. This system affords a molecular mechanism for long-term memory of transcriptional events when the stimulus is no longer present.[47] We will discuss how this epigenetic mechanism functions as memory of previous pregnancy at the maternal–fetal interface.

dNK Cells Benefit the Establishment of a Healthy Placental Bed

dNK cells are in direct contact with trophoblast cells that have anchored the placenta and invaded the uterus and spiral arteries. This is a unique challenge to the maternal immune system: although it is essential to protect the fetus from infectious agents, there must be a mechanism that allows the semiallogenic trophoblast cells to invade the maternal tissues without being eliminated by the maternal immune cells. NK cells are part of the innate immune system, accounting for 5%–15% of total lymphocytes in the peripheral blood (pbNK). Since their first characterization more than 30 years ago, these innate immune lymphocytes have been found to serve as a first line of defense against a variety of infections.[49] The NK cells that are recruited to or proliferate in the decidua, the dNK, function not in the typical NK cytotoxic fashion but rather secrete factors that facilitate the remodeling of the placental bed.[50] This field of investigation will likely rapidly advance with the application of the technology of single-cell RNA sequencing to analyze RNA expression at the maternal–fetal interface of individual cell types.[51–53]

One of the possible differences between the parous and nulliparous uterine environments could rest in the dNK cells found in the uterus during pregnancy. These cells enrich the maternal–fetal environmental interface as they produce chemokines, growth factors and angiogenic factors, including isoforms of vascular endothelial growth factor (VEGF), placental growth factor, and NKG5, an alternative splicing product of the granulysin gene that stimulates mitogenicity of endothelial cells that promote spiral artery remodeling and development of the placental bed.[50] Furthermore, specific chemokines produced by dNKs, notably interleukin-8 and interferon-inducible protein-10, can attract trophoblasts expressing the appropriate chemokine receptors to the maternal–fetal interface.[50,54] Concomitantly, extravillous trophoblasts expressing the chemokine CXCL12 can attract dNKs expressing the appropriate chemokine receptor, CXCR4, to the maternal fetal interface.[55] Phenotypically, dNK cells express a different repertoire of cell-surface receptors from the vast majority of NK cells in the peripheral blood (ie, pbNK).[56]

The pioneering work of Croy and colleagues, employing alymphoid mice, revealed a role for uterine NK cells in remodeling the uterine vascular environment (Ashkar et al,[57] reviewed by Ratsep et al[58]), with interferon-γ (IFN-γ) enlarging the diameter of the lumen of the uterine arteries.

Highlighting the importance of NK cells at the fetal–maternal interface, recent investigations using rat models of NK cell deficiency and of hypertension of pregnancy address a dual role for NK cells in both promoting and inhibiting excessive trophoblast invasion, and in the development of vasculopathy.[59,60]

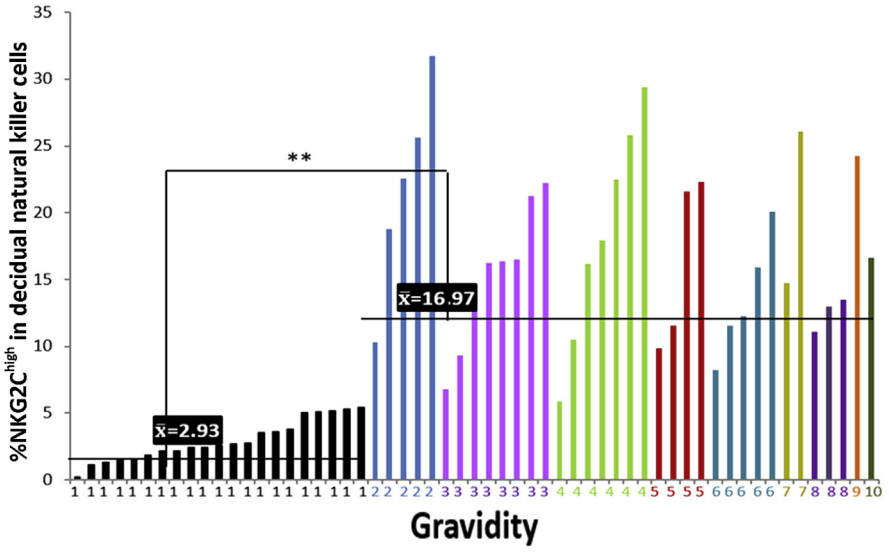
In summary, dNK cells are major players in placental bed remodeling, through secretion of growth factors, angiogenic factors, and chemokines and cytokines. Furthermore, dNKs are in active cross talk with trophoblasts migrating into the decidua and spiral arteries[55] but do not kill these invaders.[50,61] Rather, they attract the extravillous trophoblasts essential for remodeling and conversion of the spiral arteries.[55] For these reasons, we propose that dNK cells may act as a reservoir of memory of pregnancy.

Memory-Like Properties of NK Cells

NK cells in mice and humans may possess memory-like properties. As NK cells are part of the innate rather than the adaptive immune system, finding NK cell memory was in some ways revolutionary. Memory in the immune system is composed of several steps: antigen specificity, clonal expansion, generation of long-lived memory cells, and the mounting of recall responses.[62,63] In mice, these stages of memory have been demonstrated in peripheral blood NK cells in several experimental systems, including exposure to viral infections.[62,64] Antigen-specific memory of NK cells to murine cytomegalovirus infection and expansion of these NK cells in subsequent murine cytomegalovirus exposure, along with mounting more efficient secondary and tertiary recall responses, have been demonstrated.[65] Thus, the criteria for immune memory have been met in the circulating pbNK cells in the murine system.

In humans, the evidence of memory in NK cells is derived mainly from the finding that a subset of human NK cells expressing the CD94/NKG2C receptor expands in response to human cytomegalovirus (CMV) antigens[66,67] (reviewed in Rolle et al[62]). In these studies of pbNK cells, expansion of the CD94/NKG2C NK cells receptor population may indicate a first step in the generation of NK cell memory. Evidence

FIGURE 1
dNK from multigravid women have a distinct population



Summary of the percentages of dNK cells with high NKG2C expression that discriminates between dNK derived from primigravid (gravidity 1, *black bars*) and multigravid (gravidity 2—10, *colored bars*) women, independent of the order of gravidity. Modified from Gamliel et al.[18]

dNK, decidual natural killer.

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of this was found in recipients of hematopoietic cell transplantation, where expansion of NK cells with the receptor NKG2C was observed when the donors were CMV positive.[68] Recently, a mechanism has been elucidated demonstrating that a viral peptide (UL40) found on HCMV infected cells is recognized by the NK receptor NKG2C. This interaction confers NK cell expansion, an altered receptor expression profile, and a modified global epigenetic signature.[69] Evidence continues to accumulate, suggesting that pbNK cells acquire a form of trained memory. These findings are of clinical relevance, as maternal human CMV infection during pregnancy is a major cause for concern. However, whether dNK cells also possess trained memory remains to be investigated.

Memory of Pregnancy Resides with NK Cells of the Decidua

We have discussed first pregnancy as an outlier risk factor for the development of preeclampsia, the basis of epigenetic memory, the beneficial properties of dNKs at the fetal—maternal interface, and the recent observations that NK cells, although part of the innate immune system, possess memory-like properties. We hypothesized that dNK cells may possess memory of past pregnancies, lending cellular and molecular support to the observations that subsequent pregnancies generally have better outcomes.

To ascertain this, we isolated dNK cells from the deciduae of primigravid and parous women, obtained from dilation and curettage procedures of elective terminations of pregnancy.[18] By comparing the expression of 10 candidate NK cell receptors on the dNKs of primigravid vs parous women, we revealed that the activating receptor NKG2C was significantly upregulated in the dNKs of parous women (Figure 1). This expansion was independent of the order of gravidity and maternal age. Notably, in women who had conceived but whose pregnancies did not reach term (parity 0), the expansion of NKG2C was also detected.[18] This result suggests that pregnancy itself, not necessarily childbirth, is sufficient to induce memory in the dNK cells.

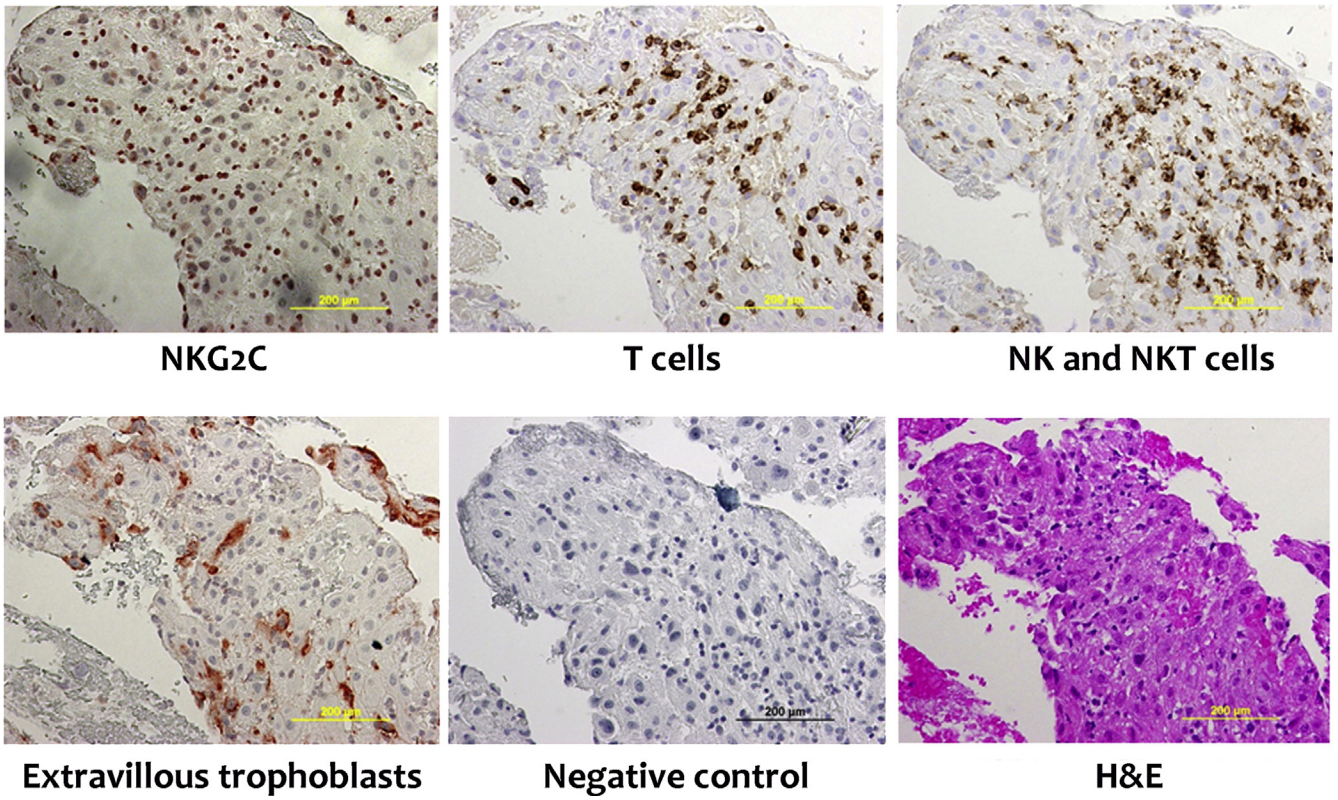
NKG2C expression is not limited to dNK cells. As shown in Figure 2, immunohistochemistry of first trimester decidua indicates expression of NKG2C in T cells, NK, and natural killer T cells (which share properties of NK and T cells). However, only in dNKs was NKG2C elevated when comparing parous with nulliparous women. This differential expression was not found in other decidual lymphocytes.[18] Our observation that the dNK cells of first as compared with subsequent pregnancies are distinguishable through their expression of a particular receptor, NKG2C, provided the first evidence of cellular and molecular difference in dNKs between these 2 groups.

Notably, NKG2C is a marker for NK cells that "remember" CMV infection (see Memory-Like Properties of NK Cells).[62,66—68,70] We therefore determined the women's CMV antibody status and found that the difference found in NKG2C receptor expression in primigravid compared with parous women was independent of CMV exposure,[18] as all women included in the study were positive for previous CMV exposure.

We then asked whether the dNK cells that had high NKG2C expression (as found in parous women) as compared with low NKG2C expression (as found in primigravidae) had different cellular properties. By analyzing global RNA expression with RNA seq technology, we found differences in RNA of the high NKG2C as compared with the low NKG2C expression. These differences included genes with functions related to NK-cell activation (NKG2C), chemokine ligands (ie, CXCL5, CXCL12, CXCL11, CX3CL1) and chemokine receptors (ie, CXCR2, CCR10), interleukins (IL-3, IL-5), hormone regulation (ie, ESRRG, PAQR5), and growth factors and immune modulation (ie, LILRB1, CSF1, EFEMP1, CSH1).

In addition to the upregulation of the NK cell receptor NKG2C, initially found in parous dNK samples, an additional receptor, LILRB1, which most notably interacts with the extravillous trophoblast non-classical HLA class I molecule

FIGURE 2
Decidual lymphocyte immunohistochemistry profile



Immunohistochemistry of human decidual formalin-fixed, paraffin-embedded serial sections. Individual panels show immunohistochemistry for NKG2C expression, and identification of T, NK, NKT cells, and trophoblasts. A negative (no primary antibody) control and H&E staining are included. Immunohistochemistry was performed as routinely performed in our laboratory.[50] Antibodies used were antiCD56 and anti-CD3 (Zymed, South San Francisco, CA; Ventana automated strainer performed by our Pathology Department, Hadassah University Medical Center, Ein Kerem). Antibodies for trophoblast detection: HLA-G (a kind gift from Professor Mike McMaster, UCSF, clone 4H84), and for NKG2C bs 2416R (Bioss Antibodies, Woburn, MA). Immunohistochemistry detection was either with AEC (reddish-colored stain) or DAB substrate (brownish-colored stain) (Zytomed Systems, Berlin, Germany).

AEC, 3-amino-9-ethylcarbazole; *DAB*, 3,3′ diaminobenzidine; *H&E*, hematoxylin and eosin; *NK*, natural killer; *NKT*, natural killer T.

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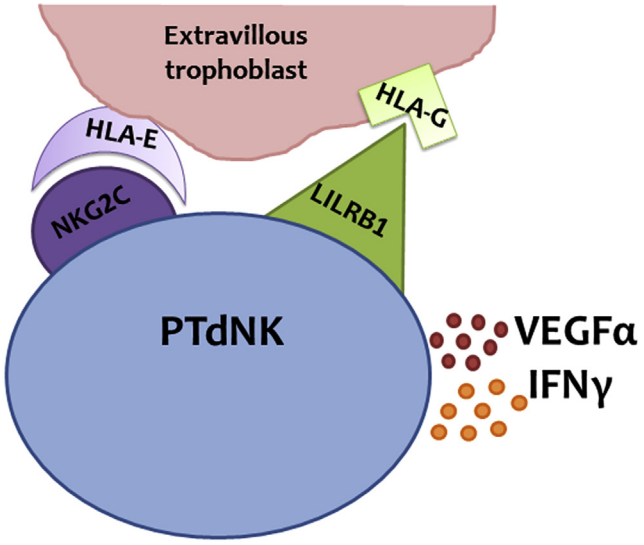
HLA-G,[71–74] also was found to be upregulated in parous samples.

Because dNKs produce abundant chemokines, cytokines, and growth factors,[50] we explored whether growth factors were differentially secreted in dNKs of parous as compared with nulliparous women. As trophoblasts express the ligand HLA-E that engages with the dNK receptor, NKG2C, as well as the ligand HLA-G that engages with the dNK receptor, LILRB1, we asked whether the dNK with upregulated receptor expression (as in parous women) also secreted more growth factors when incubated with cells expressing their ligands (NKG2C/HLA-E engagement and/or LILRB1/HLA-G engagement) (Figure 3). We found that secretion of VEGF and IFN-γ is induced on interaction of the extravillous trophoblast expressing ligands, HLA-E and HLA-G, with NKG2C and LILRB1 receptors of pregnancy-trained decidual natural killer (PTdNK), respectively. Indeed, there was significantly more secretion of the angiogenic factor VEGFα as well as IFN-γ in the cell population with the upregulated dNK receptors (as in parous women) (Figure 4[18]). These factors have been shown to be essential for angiogenesis and to possess properties necessary for appropriate placental bed formation.[75] On finding that the dNK of parous women, as compared with primigravidae, express greater levels of NKG2C and LILRB1, possess a unique transcriptome and produce and secrete increased VEGFα and IFN-γ, we termed these "pregnancy-trained decidual natural killer" (ie, PTdNK) cells. Functionally, the supernatants derived from dNK cells of parous (with high levels of VEGFα) as compared with primiparous women promoted angiogenesis in both aortic ring outgrowth and tumor development assays.[18] These experiments demonstrate,

FIGURE 3
EVT interacting with PTdNK induces secretion of growth factors



When EVTs expressing the ligands HLA-E and HLA-G interact with the receptors NKG2C and LILRB1, respectively, on PTdNK, both IFN-γ and VEGFα are secreted.

EVT, extravillous trophoblast; *IFN-γ*, interferon-gamma; *PTdNK*, pregnancy-trained decidual natural killer; *VEGFα*, vascular endothelial growth factor α.

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for the first time, enhanced function in normal physiological memory of dNK cells in humans.

Epigenetic Memory of PTdNK Cells

To determine how this pregnancy-trained memory could be maintained, we proceeded to investigate the epigenome of PTdNKs as compared with primigravid dNKs, using the ATAC-seq (Assay for Transposase-Accessible Chromatin sequencing) technique, which allows one to identify regions of chromatin accessibility. In this assay, sequencing of DNA is performed on areas of chromatin that are accessible and exposed to enzymatic cleavage because the region is nucleosome free. These sequenced areas are thought to be available for active RNA transcription. The number of times a specific area of chromatin is sequenced is an indicator of its accessibility to proteins activating transcription, thus reflecting potential gene expression. The assay employs a mutated hyperactive transposase enzyme that cuts DNA at transcriptionally active areas equivalent to DNAse 1 hypersensitive sites. We used this technology on DNA isolated from the 2 classes of dNKs: those with high levels of NKG2C vs those negative for NKG2C expression. Overall, the PTdNKs were found to have a unique epigenetic profile. More specifically, focusing on IFNG and VEGFα, the NKG2C^{high} cells were shown to have a more "open" chromatin configuration,[18] allowing for active transcription. These data corroborate the uniqueness of the PTdNK cell population and the observation of increased cytokine secretion from these cells as compared to primigravid dNKs (Figure 5).

PTdNK Cells Appear to Reside in the Endometrium Between Pregnancies

In light of these findings, we explored where PTdNKs might reside between pregnancies. Initially we considered whether the reservoir of PTdNKs was to be found in the pbNK cells of women who had been pregnant as compared with pbNKs of nulligravid women, but this was not observed to be significant. Knowing that NK cells reside in the endometrium (eNK),[76] we investigated whether the PTdNKs were present in the uterus between pregnancies. Menstrual blood was collected and the eNK cells from women who had been pregnant in the past and from nulligravidae were compared. Both NKG2C and LILRB1, receptors that were elevated in the decidua of parous women, were significantly increased in the eNKs isolated from the menstrual blood of parous as compared with nulligravid women. Thus, the 2 receptors whose expansion we found in parous women also were found elevated in the eNK of women between pregnancies, as compared with low levels in the nulliparous women. These results suggest that the memory of PTdNK cells likely resides in eNK cells.

To characterize these cells on the epigenetic level and determine chromatin areas "open" for transcription, we performed ATACseq on the NKG2C^{high} cells isolated from (menstrual) eNK cells (nulligravidae vs parous) and dNK cells (primigravidae vs parous). We were taken aback when we noted that the eNK epigenetic profile of parous women was strikingly similar to dNKs and quite dissimilar to eNK of nulligravidae. Thus, the PTdNK memory is found in the epigenome of NK cells of the endometrium, as depicted in Figure 5.

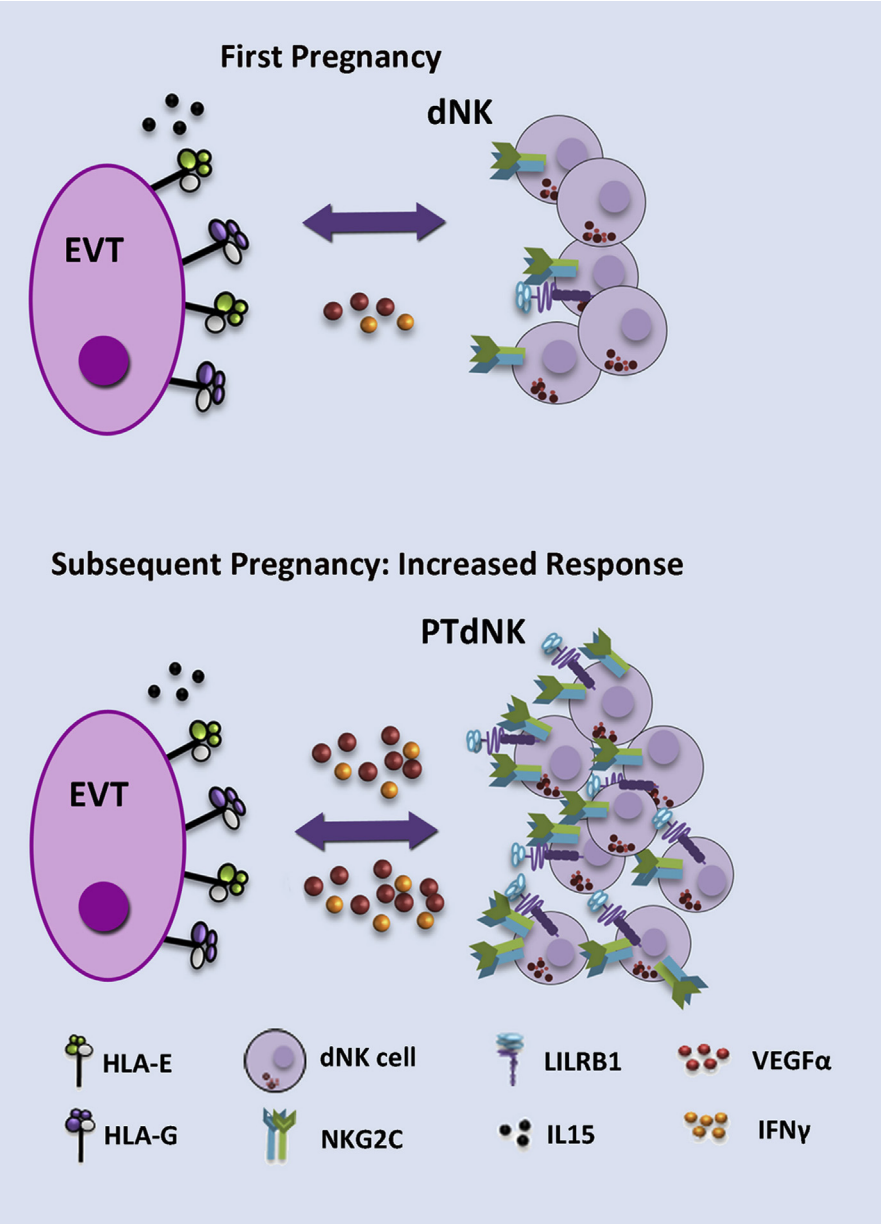
Linking Memory of dNK Cells with Reduced Preeclampsia Risk

Our findings indicate that dNK cells are primed and established in the first trimester of first pregnancy and subsequently possess properties of trained memory, hence PTdNK cells. These dNK cells provide beneficial factors necessary for the development of the placental bed and the maintenance of pregnancy.[50,57,77] These PTdNKs, residing in the endometrium between pregnancies, have memory that allows them to expand more easily or earlier

than in first pregnancy, thus enhancing their functions (Figures 4 and 5). Placentation would be augmented and spiral artery conversion would be more easily achieved in parous women; consequently, pregnancy disorders with an origin in the placental bed would be less likely in these women. We propose that memory of pregnancy generated by dNK cells may be the missing link joining the immunological underpinning of preeclampsia with the additional risk of preeclampsia in first pregnancy.[78–80] We suggest that on interaction with the decidual environment, including but not limited to trophoblasts, the PTdNKs, by producing greater levels of factors known to be essential for successful remodeling of the maternal–fetal interface (chemokines, cytokines, growth and angiogenic factors), enhance placentation/implantation and thus reduce the risk of preeclampsia.

A caveat to these findings is the lack of data concerning paternity in subsequent pregnancies. Given that there is a primipaternity model, as compared with primigravidity as the risk factor for the preeclampsia, it would be of interest to investigate PTdNK from pregnancies with a change in paternity or with a long interval between pregnancies.[81–83] Perhaps the extravillous trophoblast interaction with PTdNK, which results in enhanced expression of proteins related to the placental bed, may be influenced by a change in paternity. An additional avenue of future investigation may be to characterize the eNK in menstrual blood in women suffering from recurrent spontaneous abortion. It is possible that their eNK "do not remember" pregnancy and hence their subsequent pregnancies are not at an advantage for placentation and success. Translational research may be along the lines of finding a way to prime the eNK of these women toward eNK of the type observed between pregnancies. Furthermore, given that particular combinations of maternal KIR receptors on dNK and fetal expression of HLA-C influence the risk for preeclampsia,[84] it will be interesting to continue this investigation, as a subset of PTdNK expresses KIR2DL2-3.[18]

FIGURE 4
Proposed model



HLA-E and HLA-G are expressed on the EVT of the placenta. During first pregnancy, we assume that a priming process is initiated. NK cells of first pregnancies express low levels of NKG2C and LILRB1. In subsequent pregnancies, the decidual cells are already primed, thus rapidly expand expression of NK receptors NKG2C and LILRB1 and secrete greater amounts of VEGFα and IFN-γ, which enhance vascularization and proliferation. Modified from Gamliel et al.[18]

EVT, extravillous trophoblast; *IFN-γ*, interferon-gamma; *NK*, natural killer; *VEGFα*, vascular endothelial growth factor.

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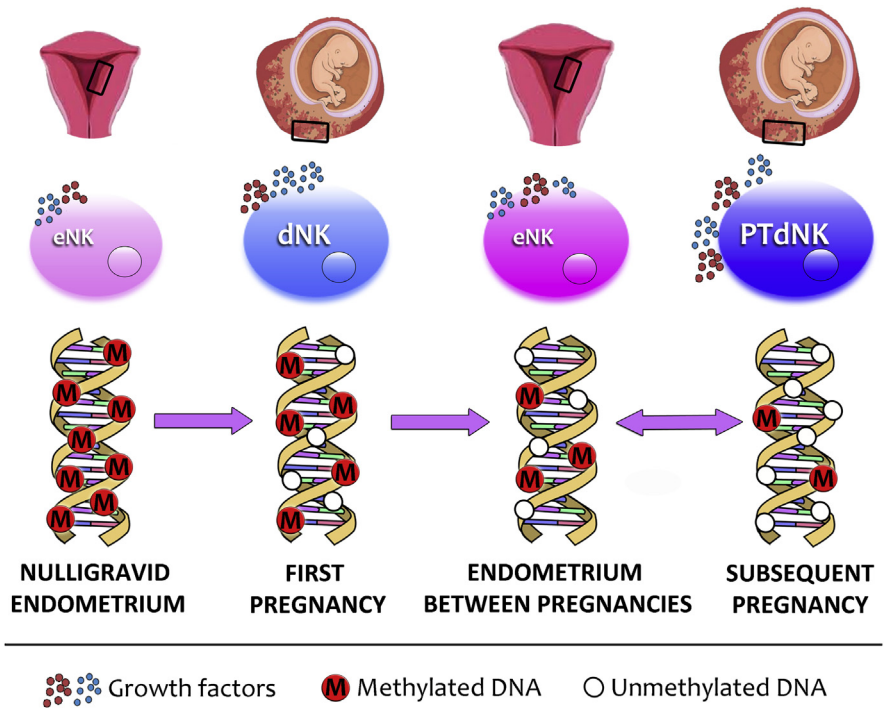
We can also speculate that as abnormal invasive placentation is associated with pregnancies following uterine surgery and damaged or deficient decidua, lack of sufficient PTdNK interaction with trophoblasts may be part of the puzzle of abnormal invasive trophoblast.

FIGURE 5
Summary model



Nulligravid women have a relatively "closed" epigenetic profile of specific genes in their eNK cells (M in *red circle*, methylation). In first pregnancy, there is enhanced expression of dNK cell receptors and growth factors (*colored red* and *blue dots*) as well as a more "open" (*white circles* on chromatin) epigenetic configuration. Some of this epigenetic memory of hypomethylation remains in eNK between pregnancies. In subsequent pregnancies, the educated PTdNK produce significantly greater amounts of growth factors, thereby enhancing development of the placental bed with the epigenetic signature found in a more "open" configuration.

dNK, decidual natural killer; *eNK*, endometrial natural killer; *PTdNK*, pregnancy-trained decidual natural killer.

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Epigenetic Memory May Explain a Wide Range of Observed Improved Outcomes in Subsequent Pregnancy and Lactation

We have provided cellular and molecular epigenetic evidence supporting the notion that the immune cells of the fetal–maternal interface recall passed pregnancies and maintain characteristics that enhance future pregnancies. This may have bearing on other, often observed instances of better outcomes in subsequent pregnancies.

Maternal cardiovascular system adaptations to pregnancy also have been investigated. Clapp and Capeless showed that measurable changes to cardiac functional parameters: increased end-diastolic volume, stroke volume, and cardiac output and decreased vascular resistance are observed in first pregnancies, persist post-partum, and are enhanced in subsequent pregnancies.[13] More recently, Morris et al showed similar persistent changes to vascular compliance: decreased mean arterial pressure and reduced arterial stiffness during and following first pregnancy.[14] Cardiac adaptation to subsequent pregnancy was compared in women who were preeclamptic in their first pregnancy.[12] The investigators found that women in whom preeclampsia recurred had lower left ventricular mass and stroke volume than those in whom preeclampsia did not recur, during the interpregnancy interval; however, adaptation to pregnancy was similar in the 2 groups.[12] Although these studies focused on physiologic response to pregnancy without corroboration from molecular epigenetic investigation, it is tempting to speculate whether the memory of pregnancy is recalled by myocardial or vascular endothelial cells or others.

Another example would be the well-known differences between the length of the first and second stages of labor in first vs subsequent deliveries. Evidence for epigenetic memory in the uterus or cervix that could provide a molecular basis for the observed greater efficiency of labor and delivery remains to be described. However, Seaborne et al observed muscle memory of exercise on both the epigenetic and consequently the transcriptional level in humans. They showed that induced muscle hypertrophy invoked a "signature" of epigenetic memory by hypomethylation of specific genes in human skeletal muscle.[85] Although the study by Seaborne et al refers to skeletal muscle and not uterine muscle, we may conjecture that shorter first and second stages of labor and better outcome in subsequent deliveries may be related to epigenetic memory in uterine and cervical muscle.

Lactation is yet another aspect of reproduction in which subsequent pregnancies have an advantage over first. Significantly more milk is produced in second as compared with first pregnancies.[16] Although in humans epigenetic memory for more robust lactation in subsequent pregnancies has not been found, such epigenetic memory was found in the mouse mammary gland.[17] In the parous mouse, a more-robust response of the mammary glands to subsequent pregnancy has been shown.[17] The physiological response involves both the expansion of ductal structures and synthesis of milk proteins earlier in pregnancy, in parous as compared with primiparous mice. The rapid response of gene expression is conferred though epigenetic memory of pregnancy by master regulators of mammary gland transcription. Hypomethylated regions of transcriptional regulation persist as stable changes. They are first induced in the mammary epithelium by pregnancy primed genes

and produce greater responses to hormone exposure. This may in turn result in a mammary gland that functions more effectively during subsequent pregnancies. These targeted experiments demonstrated that genes primed by parity-associated epigenomic changes are poised for more rapid reactivation in a subsequent pregnancy, supporting the existence of an epigenetic memory of past pregnancies.[17]

In conclusion, we have delineated some of the many aspects of pregnancy, childbirth, and lactation wherein subsequent pregnancies have been shown to be more robust than first. Our investigations have shown that NK cells at the maternal−fetal interface act as builders of the maternal−fetal interface, promoting angiogenesis, attracting trophoblasts and producing chemokines, cytokines, and growth factors essential for conversion of the spiral arteries. Our most recent work has further shown that "primed" PTdNK cells recall pregnancy on an epigenetic level, which may contribute to their more rapidly and successfully remodeling the placental bed. This offers an explanation as to why first pregnancy, where dNK cells are possibly not yet primed in their functions, is a risk factor for the development of preeclampsia, and others of the "great obstetrical syndromes." Molecular epigenetic evidence in humans for other examples of enhanced pregnancy and lactation outcomes remain to be investigated. For now, we speculate that investigating the activation of dNK may yield treatment modalities for women at risk for disorders of primigravidity originating at the placental bed. ■

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