Maternal obesity alters uterine NK cell activity through a functional KIR2DL1/S1 imbalance

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Running Title: Maternal obesity drives uNK KIR-related imbalances

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3 **CONFLICT OF INTEREST**

4 The authors declare no conflict of interest.

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1 **ABSTRACT**

2 In pregnancy, uterine natural killer cells (uNK) play essential roles in coordinating uterine
3 angiogenesis, blood vessel remodeling, and promoting maternal tolerance to fetal tissue. Deviances
4 from a normal uterine microenvironment are thought to modify uNK function(s) by limiting their
5 ability to establish a healthy pregnancy. While maternal obesity has become a major health concern due
6 to associations with adverse effects on fetal and maternal health, our understanding into how obesity
7 contributes to poor pregnancy disorders is essentially unknown. Given the importance of uNK in
8 pregnancy, this study sets out to examine if obesity affects uNK function. Using a cohort of pregnant
9 women, we show that baseline activity of uNK from obese women is elevated, but that enhanced
10 activity does not equate to increased killing potential. Instead, obesity associates with altered uNK
11 production of angiogenic VEGF-A and PlGF. These changes coincide with alterations in $NKp46^+$ and
12 $NKG2A^+$ uNK subsets and elevated expression of KIR2D(L1/S1/S3/S5) receptors. Detailed
13 examination revealed that obesity leads to imbalances in KIR2DL1/S1 expression that together instruct
14 altered responses to HLA-C2 antigen, including increased production of TNFα. Together, these
15 findings suggest that maternal obesity modulates uNK function by altering angiokine/cytokine
16 production and the response to HLA-C2 antigen.

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INTRODUCTION

Establishment of the fetal-maternal interface in early pregnancy requires coordinated interactions between fetal and maternal cells that help direct placentation and ensure an adequate blood supply to the developing fetus. Early stages of uterine angiogenesis, arterial remodeling, trophoblast invasion & survival, as well as maternal tolerance toward the fetal semi-allograft are controlled in part by maternally-derived uterine immune cells[1,2]. In particular, uterine natural killer cells (uNK), identifiable as $CD56^{bright}/CD16^{-}$ cells in humans, constitute the most abundant leukocyte population within the uterus[2,3]. Unlike peripheral blood NK cells (pbNK), uNK do not normally mount cytotoxic responses, but instead help coordinate placental development by controlling uterine neo-angiogenesis and spiral artery remodeling, as well as limiting the immune response against fetal antigen[4].

NK activity (i.e. cytotoxicity, cytokine & angiokine production) is controlled in part by activating and inhibiting natural killer receptors (NKRs). NKRs bind epitopes expressed on major histocompatibility (MHC) class I and class I-like molecules, stress-related molecules and cytokines[5–7]. Generally, signaling via activating receptors stimulate cytolytic programs whereas signaling via inhibitory receptors drives immuno-tolerance through mechanisms that dampen activating NKR signals[8]. uNK are phenotypically distinct from pbNK ($CD56^{dim}/CD16^{+}$) in that they express a distinct repertoire of NKRs that facilitate interaction with trophoblast-derived MHC class I molecules (HLA-C, HLA-E, and HLA-G) but not HLA-A or HLA-B[9]. Depending on the activating/inhibitory NKR balance, interaction of uNK with MHC ligand can promote or restrain uNK activity. Inadequate (not enough) or inappropriate (too much) uNK activation may contribute to preterm birth, recurrent miscarriage, and preeclampsia by limiting angiogenesis/artery remodeling or over-sensitizing uNK to being cytotoxic[10–13].

1 Obesity, with a prevalence between 13-18% in women of reproductive age[14], correlates with
2 higher incidences of poor pregnancy outcomes[15]. For example, pre-existing obesity associates with
3 recurrent miscarriage[16], gestational diabetes[17], preterm birth[18], and preeclampsia[19]. Studies designed to
4 generate insight into how maternal obesity contributes to pregnancy disorders have shown in rodents
5 that high-fat diet exposure prior to and during pregnancy results in impaired uterine artery
6 remodeling[20,21]. Notably, vascular defects of this type are also observed in rats and mice deficient in
7 uNK[22]. Previous work from our group shows that maternal obesity in women associates with impaired
8 uterine artery remodeling[23]. Importantly, we show that vascular changes coincide with fewer uNK and
9 altered global uNK gene expression[23]. Altogether, these studies suggest that obesity may modify pro-
10 angiogenic and vascular-remodeling programs controlled by uNK within the maternal-fetal interface.

11 In this study we examine if maternal obesity alters uNK activity and function. We show that
12 uNK from obese women are more active and differentially secrete angiogenic factors PlGF and VEGF-
13 A. Phenotypic analyses show altered expression of NKRs in obese women compared to lean women,
14 where differences in activating (NKp46, KIR2DS1) and inhibitory (NKG2A, KIR2DL1) NKR cell
15 populations are observed. Importantly, we provide evidence that the increase in obesity-linked uNK
16 activity is in part due to changes in expression of the NKRs KIR2DL1 and KIR2DS1. We show that
17 upon HLA-C2 stimulation, KIR2DS1-expressing uNK from lean and obese women differentially
18 regulate TNFα production. Together, this work provides insight into how maternal obesity affects uNK
19 function. Further, this work identifies uNK-directed processes modified in obesity that may contribute
20 to impaired vascular remodeling, placental function and pregnancy outcome.
21

1 **RESULTS**

2 **Maternal obesity promotes uNK activation**

3 As a first step to examine if maternal obesity affects uNK function, we compared
4 degranulation/activation responses in uNK isolated from lean (BMI 20-24.9 kg m^{-2}) and obese (BMI
5 ≥30 kg m^{-2}) women in their first trimester of pregnancy (Table 1). Following culture in the presence or
6 absence of phorbol 12-myristate 13-acetate/ionomycin (PMA), uNK activity was measured via surface
7 expression of the degranulation marker CD107a[24]. For these studies, uNK were defined as
8 $CD56^{bright}/CD9^{+}/CD16^{-}/CD3^{-}$ $CD45^{+}$ cells (Figure 1a). As expected, PMA stimulation resulted in
9 potent uNK activation in both BMI groups (Figure 1b,c). However, proportions of $CD107a^{+}$ uNK from
10 obese women were more abundant in both unstimulated (median = 5.4% *vs* 2.6%) and stimulated
11 (median = 11.8% *vs* 5.9%) conditions (Figure 1c). These initial findings indicate that maternal obesity
12 associates with a heightened state of uNK activation.

13

14 **Obesity associates with altered uNK angiokine production**

15 To gain insight into how obesity-linked differences in uNK activity translate into uNK
16 functional differences, we measured by flow cytometry the intracellular production of tumor necrosis
17 factor α (TNFα) and interferon gamma (IFNγ), two cytokines expressed by uNK known to play central
18 roles in uterine blood vessel remodeling[25]. In line with higher $CD107a^{+}$ uNK proportions, a non-
19 significant but slight increase in baseline TNFα production was observed in uNK from obese women (P
20 = 0.05); baseline IFNγ levels between BMI groups were similar (Figure 2a-c). Following PMA
21 stimulation, uNK from both groups markedly enhanced production of TNFα and IFNγ (Figure 2c).
22 Although we did not detect a significant difference in cytokine expression between BMI groups, uNK

from obese women again showed a slight tendency to produce more TNFα (median = 19.4% *vs* 9.9%, P = 0.05) (Figure 2c).

To complement the above intracellular cytokine measurements, we also measured the secretion of 8 factors from conditioned media (CM) generated by uNK-enriched *ex vivo* cultures (> 60% uNK) from lean and obese women. These factors included: granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 10 (IL10), interferon-γ inducible protein 10 (IP10), macrophage inflammatory protein 1β (MIP1β), placental growth factor (PlGF), TNFα, and vascular endothelial growth factor A and C (VEGF-A, VEGF-C). These cytokines were chosen based on prior studies showing their production by uNK and their importance in controlling pregnancy-related processes[26,27]. uNK CM from obese women secreted lower amounts of VEGF-A (median = 6.8 pg/ml *vs* 18.4 pg/ml) and higher amounts PlGF (median = 1.0 pg/ml *vs* 0.3 pg/ml) (Figure 2d). However, secretion of TNFα, VEGF-C, GM-CSF, IL10, IP10 and MIP1β were comparable between uNK CM from either BMI group (Figure 2d-f). Our results suggest that obesity-linked changes in uNK activity translate into differences in angiokine secretion, reflecting possible modifications to angiogenic processes.

Maternal obesity associates with phenotypical differences in NKR expression

Our finding that maternal obesity associates with heightened uNK activity and altered angiokine production indicates that differences in NKR expression/abundance may exist. To examine this, we compared the expression of 8 NKRs in uNKs from lean and obese women using a flow cytometry approach outlined in Supplementary Figure 1a. Specifically, we measured activating (NKG2D) and inhibitory (NKG2A) CD94/NKG2 family members, natural cytotoxicity receptors NKp30, NKp44 and NKp46, inhibitory leukocyte immunoglobulin-like receptor (LILRB) 1, and members of the killer-cell

immunoglobulin-like receptor (KIR)2D subfamily [using antibodies targeting KIR2DL4 and KIR2D(L1/S1/S3/S5)]. These NKRs were selected in part due to their importance in controlling NK cell activation[5,6,8], their prior description in uNK[26,28], or their association with pregnancy-related outcome[13,29,30]. uNK from lean women ubiquitously expressed inhibitory NKG2A and activating NKp30 and NKp46, while varied proportions of NKG2D$^+$ (median = 58.9%), LILRB1$^+$ (median = 30.1%), and KIR2DL4$^+$ (median = 62.0%) uNK were observed (Figure 3a-d, and S1b-e). Between BMI groups, proportions of NKG2D$^+$, NKp30$^+$, NKp44$^+$, LILRB1$^+$, and KIR2DL4$^+$ uNK were comparable, however, maternal obesity associated with a decrease in NKG2A$^+$ (median = 93.3% *vs* 96.6%) and NKp46$^+$ (median = 68.3% *vs* 96.0%) cell proportions (Figure 3a,b). Interestingly, the decrease in NKp46$^+$ cell proportions in obese women was characterized by a distinct bimodal distribution (Figure 3b). Additionally, uNK from obese women had elevated proportions of KIR2D$^+$ [L1/S1/S3/S5; herein referred to as KIR$^+$] uNK (median = 46.0% *vs* 35.6%) (Figure 3c). These differences in NKR proportions in obese women also associated with decreases in cell surface levels (median fluorescence intensity; MFI) of NKp46 and NKG2A (NKp46: 1529 arbitrary units (AU) *vs* 2567 AU; NKG2A: 1349 AU *vs* 2051 AU) (Figure 3a,b). These results suggest that uNK from obese women show a distinct NKR phenotype defined by changes in both activating and inhibitory NKRs.

Maternal obesity drives KIR2DL1/S1 NKR imbalances

KIRs are involved in NK cell education, promotion of fetal tolerance and contribution to successful placentation[30,31]. Because previous studies have highlighted strong associations with pregnancy outcome and KIR2DL1/S1 expression[30], and because we showed that maternal obesity associates with increased proportions of KIR2D$^+$ uNK, we next set out to examine if maternal obesity leads to imbalances in inhibitory KIR2DL1 (2DL1) or activating KIR2DS1 (2DS1). Gene analysis

1 using a qPCR strategy revealed that maternal obesity correlates with higher mRNA levels of *KIR2DS1*
2 in uNK (purified by negative selection), while levels of inhibitory *KIR2DL1* and activating
3 *KIR2DS3/S5* did not differ between BMI groups (Supplementary Figure S2). Using a flow cytometry
4 approach that identifies distinct 2DL1 and 2DS1 single-positive (sp) and mixed double-positive (dp)
5 uNK populations (Figure 4a), we measured proportions and surface expression levels of these KIRs in
6 lean and obese women. As the presence or absence of 2DS1 depends on inherent maternal and paternal
7 haplotype combinations [two main haplotypes exists: haplotype A (HA), primarily comprised of
8 inhibitory receptors (i.e. lacking 2DS1), and haplotype B (HB), containing both activating and
9 inhibitory receptors, including 2DS1[32]], we sub-stratified lean and obese cohorts into KIR HB^-
10 ($2DL1^+/S1^-$) and HB^+ ($2DL1^+/S1^+$) by PCR genotyping (Supplemental Table 1). Flow cytometry
11 analysis revealed that HB^- uNK are negative for 2DS1, whereas mixed $2DL1^+/2DS1^+$ populations are
12 seen within HB^+ cells in both lean and obese women (Figure 4a,b). Among HB^- individuals (> 95% are
13 allotypically 2DL1[33]), total proportions of $2DL1^+$ uNK were similar between lean and obese women
14 (Figure 4c). Interestingly, uNK from obese HB^+ women showed reduced proportions of $2DL1^+$ cells
15 (gated on total $CD56^{bright}$ uNK; median = 23.8% *vs* 34.5%). In either haplotype, 2DL1 MFI did not
16 differ between lean and obese groups (Figure 4d).

17 Focusing on only HB^+ (containing *2DS1* gene) uNK, we again examined 2DL1/S1 subsets
18 within KIR^+ cells in both BMI groups. Proportions of 2DS1sp and dp populations did not differ
19 between lean and obese groups (Figure 4e). However, 2DS1 surface expression (MFI) within the
20 2DS1sp population in obese women was approximately 2-fold higher; levels of 2DL1 within
21 2DL1/S1dp and 2DL1sp populations did not differ (Figure 4e). Measuring 2DS1 MFI on all KIR^+ uNK
22 (combined 2DS1sp and 2DL1/S1dp populations) showed that, similar to expression within the 2DS1sp
23 population, 2DS1 levels are elevated in uNK from obese women (Figure 4f). Together our results

1 indicate that maternal obesity instructs changes in KIR2D expression to preferentially favour 2DS1,
2 potentially conferring functional changes towards increased cell activation.

3

4 **KIR2DS1$^+$ uNK from obese women show enhanced activity**

5 Because KIR expression is influenced by maternal KIR haplotype, we next revisited our
6 findings (Figure 1) that examined if differences in uNK activity exist between lean and obese women
7 by stratifying women into HB$^-$ and HB$^+$ haplotypes. In non-stimulated conditions, the proportion of
8 CD107a$^+$ HB$^-$ uNK was not different between BMI groups [1.8% (obese) *vs* 2.1% (lean)]. However,
9 within HB$^+$ uNK, maternal obesity associated with higher proportions of CD107a$^+$ cells (6.7% *vs* 2.7%;
10 Figure 5a). Following PMA stimulation, uNK from obese women of either haplotype degranulated at
11 higher frequencies (HB$^-$: 8.2% *vs* 5.2%; HB$^+$: 17.1% *vs* 8.2%), where HB$^+$ uNK from obese women
12 showed modestly higher degranulation rates than HB$^-$ cells from obese women (Figure 5b).
13 Surprisingly, proportions of TNFα-expressing uNK were higher in PMA-primed HB$^-$ cells from obese
14 women, where BMI-associated differences were not seen in HB$^+$ cells (Figure 5c). Consistent with our
15 previous results, maternal obesity did not affect proportions of IFNγ expressing uNK, regardless of
16 KIR haplotype (Figure 5c).

17

18 **Obesity, combined with maternal HLA-C2 allotype, potentiates uNK activity**

19 KIR expression can be affected by gestational age[34] and maternal HLA-C[35]. To determine if
20 KIR changes may relate to these BMI-independent variables, we next examined if gestational age and
21 maternal HLA-C affects 2DL1/S1 uNK proportions or expression levels. uNK 2DL1/S1 frequency and
22 expression level was comparable amongst different gestational ages (Supplementary Figure S3).

1 Homozygous C1/C1 subjects showed an inverse correlation in 2DS1 and 2DL1 frequency depending
2 on BMI. Specifically, uNK from obese C1/C1 women exhibited higher proportions of 2DS1$^+$ cells and
3 lower proportions of 2DL1$^+$ cells (Supplementary Figure 4b,c). Contrary to these KIR imbalances, we
4 also observed that the presence of maternal HLA-C2 associates with an increase in degranulation in
5 non-stimulated uNK from obese women (compared to C1/C1); this association was not maintained
6 following PMA stimulation (Figure 6a,b). The impact of C2 allele on HB$^+$ cells was clear after PMA-
7 priming (Figure 6c). However, HLA-C2-associated heightened activity did not translate into an
8 increase in PMA-stimulated cytokine production, although a modest trend was observed (Figure 6d).
9 These results demonstrate that in the context of obesity, maternal HLA-C2 allele equates to heightened
10 activity in HB$^+$ uNK.

11

12 **uNK from obese women show enhanced HLA-C2-induced activity**

13 To examine if KIR2DL1/S1 alterations in obese/HB$^+$ uNK translate into functional changes, we
14 measured uNK responsiveness towards ectopically expressed HLA-C2 (Supplementary Figure S5a). To
15 this end, HLA class I-deficient K562 cells stably expressing the HLA-Cw*0602 allele were subjected
16 to uNK activity/killing assays using KIR-genotyped uNK from lean and obese groups. HLA-Cw*0602
17 is the fourth most frequent HLA-C2 allele[36] and its specificity for 2DL1/S1 allowed us to minimize
18 confounding effects elicited by other HLA-C2-receptors (i.e. LILRB1, KIR2DL2/L3, and KIR2DS4)[37].
19 To control for KIR-HLA-C2 interaction and to enable differentiation between 2DS1/2DL1-directed
20 responses against HLA-C2, CD107a surface mobilization in CD56^{bright}/KIR$^-$ cells and HB$^-$ uNK were
21 compared to KIR$^+$/HB$^+$ cells (Figure 7a and S5b). As expected, CD107a mobilization within
22 CD56^{bright}/KIR$^-$ uNK (HB$^-$ and HB$^+$) in response to HLA-C2-expressing K562 cells was minimal
23 (Supplementary Figure S5b). Similarly, HB$^-$ KIR$^+$ uNK (lacking 2DS1) from both BMI groups were

1 also minimally responsive towards HLA-C2 (Figure 7b). Importantly, $2DS1^+$ cells from HB^+ obese
2 women showed significantly higher CD107a mobilization than $2DS1^+$ cells from lean women in
3 response to high levels of HLA-C2 expression (4.2% *vs* 0.3%). While low-level HLA-C2 expression in
4 target cells showed a trend for increased degranulation in obese 2DS1-expressing uNK, this was not
5 significant. Together, these findings suggest that obesity alters the 2DL1/S1 balance in favour of
6 instructing an activating signal (Figure 7b). Interestingly, HB^+ uNK from obese C1/C1 women, which
7 have lower levels of 2DL1 and increased proportions of 2DS1 (Supplementary Figure S4b,c), had
8 higher degranulation rates compared to HB^+ uNK from lean C1/C1 women after HLA-C2 stimulation
9 (Supplementary Figure 5c). This suggests that the absence of a maternal HLA-C2 allele may affect
10 KIR2DS1/L1 responsiveness (education/licensing[38]) to its cognate ligand.

11 Activation of KIR2DS1 strongly triggers cytolysis and cytokine production in pbNK, and this
12 engagement in cytotoxicity is more striking in the absence of KIR2DL1[39]. As our above finding shows
13 that HLA-C2 engagement enhances degranulation in HB^+ uNK from obese women, we next examined
14 whether HLA-C2 interactions also induce cytotoxicity. As expected, HLA-C2 interaction with HB^+
15 uNK resulted in increased early apoptosis (measured by annexin V) of K562 target cells compared to
16 culture with HB^- uNK (K562: 14.9% *vs* 9.8%; K-C2 Lo: 26.5% *vs* 14.2%; K-C2 Hi: 28.9% *vs* 16.0%);
17 however we did not observe differences in late apoptosis based on KIR haplotype (Figure 7c,d).
18 Moreover, within HB^+ uNK, maternal obesity seemed to associate with impaired HLA-C2 instructed
19 K562 killing, although low sample size/power prevented conclusive interpretation (Figure 7c,d). To
20 complement annexin V killing assays, we also analyzed whether secreted perforin levels were different
21 among lean and obese HB^+ subjects. In agreement with the annexin V results, the levels of secreted
22 perforin were comparable among BMI groups (Figure 7e). However, obesity appeared to dampen
23 perforin secretion, suggesting that maternal obesity results in impaired uNK cytotoxicity. To exclude

1 the contribution of other KIR-expressing cells in HLA-C2 directed cytolysis, we also analyzed
2 proportions of T ($CD3^+$) and NKT ($CD56^+/CD3^+$) cells and their degranulation upon HLA-C2
3 stimulation. We found the contribution of these cells on cytotoxicity was negligible (Supplementary
4 Figure S6). These findings suggest that although HB^+ uNK from obese women show enhanced HLA-
5 C2 instructed activity (CD107a mobilization), this does not translate into enhanced cytotoxicity.
6

7 **Skewing of 2DS1 in lean and obese women results in dichotomous TNFα production**

8 To investigate if the obesity-linked KIR2D-associated increase in uNK activity may also
9 translate into altered production of cytokines/angiokines important in pregnancy, we measured factors
10 from our cytokine 8-plex panel in conditioned media of HB^+ and HB^- uNK from lean and obese women
11 stimulated with a KIR2DL1/S1 crosslinking/activating antibody (clone 11BP6). Changes in cytokine
12 secretion in uNK activated with 11BP6 were compared to responses from isotype-matched IgG1
13 antibody treatment within paired uNK cultures. As expected, 11BP6 treatment in HB^+ uNK led to
14 CD107a surface induction, whereas crosslinking did not lead to degranulation responses in HB^- cells;
15 these findings indicate that 11BP6 is only capable of inducing an activating response in uNK
16 expressing 2DS1 (Figure 8a). Within lean HB^+ uNK, 11BP6 crosslinking resulted in an overall
17 decrease in TNFα; one 2DS1sp-containing uNK preparation responded to HLA-C2 by promoting
18 TNFα secretion (Figure 8b). As expected, secretion of TNFα in HB^- uNK from either lean or obese
19 groups was not affected (Supplementary Figure 7a). Additionally, we did not observe consistent
20 changes in PlGF, VEGF-A, VEGF-C, GM-CSF, IL10, IP10 or MIP1β following 2DL1/2DS1
21 crosslinking in uNK from lean $HB^{+/-}$ women (Figure 8b-d and S7). While a trending increase in GM-
22 CSF production was observed in HB^+ uNK from obese women following crosslinking, penetrant and
23 consistent increases in TNFα production were observed (Figure 8b,d). Notably, a significant interaction

1 between KIR2DL1/S1 crosslinking and BMI was identified, where 11BP6 had opposing effects on
2 TNFα production in uNK from lean and obese women (Figure 8b). Taken together, KIR2DL1/S1
3 crosslinking in uNK cultures from obese/HB^+ women promoted TNFα secretion and also appeared to
4 dampen the inhibitory effect of cytokine secretion (i.e. PlGF and TNFα) that was observed in lean HB^+
5 cells. These data suggest that the uNK KIR2DL1/S1 imbalance in obese women, potentially favouring
6 2DS1 activity, differentially regulates HLA-C2-induced secretion of key factors important in vascular
7 remodeling.
8
9

1 **DISCUSSION**

2 In the present study we examined the effects of maternal obesity on uNK activity and function. 3 We show that maternal obesity links with heightened uNK activation/degranulation, and that this 4 change associates with differences in multiple NKRs known to play roles in uNK biology. Specifically, 5 we found that obesity correlates with reductions in NKp46 and NKG2A cell frequencies, while in HB^+ 6 obese women, exacerbated uNK activity is driven in part by an imbalance in activating KIR2DS1 and 7 inhibitory KIR2DL1 receptor expression. This altered obesity-related KIR2D phenotype, when 8 challenged with ectopic HLA-C2, led to a selective increase in TNFα production and the blunting in 9 inhibition of other key soluble factors. These findings highlight how the condition of obesity is able to 10 instruct functional changes in uNK and establishes insight into how uNK respond and/or adapt to an 11 obesogenic environment.

12 Our finding that maternal obesity associates with increased uNK activity is both novel and 13 complex. While uNK have a complete arsenal of lytic granules (perforin, granzyme A/B, granulysin), 14 their ability to mount cytotoxic responses against target cells is largely impaired compared to their 15 peripheral NK counterparts[28]. This difference is most likely due to the robust expression of inhibitory 16 NKRs (KIR2DL1, KIR2DL4, NKG2A, LILRB1) in uNK and a hampered ability for uNK to form 17 effective effector-target cell synapses[28]. Inhibitory NKRs facilitate uNK interaction with trophoblast 18 MHC-I antigen (i.e. HLA-C, HLA-E and HLA-G) to generate tolerogenic signals towards the fetus[40]. 19 Even though our work shows that obesity associates with enhanced uNK activity/degranulation and 20 links with altered NKR expression that might seemingly equate to increased cytotoxicity (i.e. decreases 21 in NKG2A & KIR2DL1; increase in KIR2DS1), these changes did not lead to enhanced K562 target 22 cell killing. Instead, heightened uNK activity correlated with altered angiokine (increased PlGF, 23 decreased VEGF-A) and KIR2DS1/L1-instructed TNFα production. These findings indicate that

1 although obesity correlates with a shift towards activating NKRs, inhibitory mechanisms preserving
2 immuno-tolerance/impaired cytotoxicity are largely maintained. Therefore the outcome of increased
3 uNK activity resulting from obesity most likely affects uterine vascular remodeling and angiogenesis.
4 Although our study does not examine this, prior studies showing impaired blood vessel remodeling in
5 rodents subjected to high-fat diets[20,21] and in obese pregnant women[19,23] indicate that these vascular
6 defects may be due to obesity-directed changes in uNK function. Further work is required to decipher
7 these interactions.

8 Our focus on KIR2D(L1/S1) stems from prior research showing the importance of these two
9 receptors in controlling aspects of placental development and in their associations with poor pregnancy
10 outcomes[11,30,41]. Notably, HLA-C2 directed activation of KIR2DS1[42] and the resulting expression of
11 GM-CSF in 2DS1sp uNK was shown to promote trophoblast invasion[30]. Moreover, a KIR2DS1$^+$
12 containing genotype (i.e. *KIR B* haplotype), in association with fetal HLA-C2 allotype, provides
13 protection from pregnancy disorders like recurrent miscarriage, preeclampsia, and intrauterine growth
14 restriction[31]. In contrast to this, a maternal *KIR AA* genotype (containing 2DL1, but not 2DS1)
15 combined with fetal HLA-C2 associates with increased risk[31,41]. Our finding that maternal obesity
16 correlates with decreased proportions of 2DL1$^+$ uNK and increased expression of 2DS1 in only *KIR B*
17 haplotype (HB$^+$) women was somewhat perplexing due to obesity's predisposition towards having a
18 poor pregnancy outcome[19,43]. Our results indicate that, with respect to KIR2DL1/S1 composition, HB$^+$
19 uNK from obese women may be more capable of promoting healthy placentation and may be
20 protective against aberrant pregnancies. It may therefore be that HB$^+$ uNK establish an active
21 phenotype as a compensatory mechanism to facilitate successful placentation in obesity. In contrast,
22 HB$^-$ uNK (containing 2DL1, but not 2DS1), in the context of obesity, may show aberrant/insufficient
23 activation leading to compromised vascular remodeling and/or inadequate placentation, and thus

1 contribute to or potentiate poor outcomes in these women. Indeed, HB^- uNK from obese women were
2 more efficient producers of TNFα following stimulation suggesting these cells may have enhanced
3 cytotoxic potential and/or an impaired ability to promote trophoblast invasion.

4 Comparison of cytokine/angiokine secretion profiles following KIR2DS1/L1 activation in HB^+
5 uNK ($2DL1^+$/$2DS1^+$) identified TNFα as a cytokine that is differentially regulated with respect to
6 KIR2DL1/S1 balance. Within the context of HB^+ lean women where $2DL1^+$ uNK proportions are
7 approximately 1.5-fold higher compared to HB^+ obese women, 2DL1/S1 activation results in decreased
8 TNFα production. In contrast, 2DL1/S1 activation with uNK from obese women who have fewer
9 $2DL1^+$ cells and also express higher levels of surface 2DS1 led to an enhancement in, or at the very
10 least, the maintenance of TNFα production. These findings indicate that 2DL1 and 2DS1 play opposing
11 roles in controlling TNFα secretion, a finding that is consistent with previous work showing that 2DS1
12 engagement with HLA-C2 in $CD8^+$ γδT cells induces TNFα[44]. Surprisingly, HLA-C2 interaction did
13 not potentiate an increase in CD107a expression over baseline levels observed in obese uNK exposed
14 HLA-C2-deficient target cells. This inconsistency could be explained by the presence of other
15 activating NKRs responding to target cell exposure (independent of HLA-C2). Moreover, our findings
16 also illustrate how environmental factors, such as obesity, can differentially modulate KIR2D receptor
17 activity following HLA-C interaction. Since factors that regulate KIR2DL1/S1 expression include prior
18 exposure to HLA-C and DNA methylation, it would be important to examine if obesity regulates KIR
19 expression via epigenetic mechanisms.

20 In addition to KIR2DL1/S1 alterations, our findings also indicate that maternal obesity
21 associates with changes in other non-KIR NKRs that may play roles in modifying uNK function.
22 Specifically, we show that both inhibitory NKG2A and activating NKp46 expression levels and uNK
23 proportions are decreased in obesity. Within the uterine environment, NKG2A's primary ligand is

1 fetal/maternal derived HLA-E, and this interaction is thought to promote strong tolerogenic/inactivating
2 signals. Thus a loss or reduction in NKG2A signal may be a contributing factor in increased uNK
3 activity in obesity. Our observation of a bimodal distribution of NKp46^{+} uNK in obese women was
4 interesting, and was in stark contrast to the near ubiquitous nature of NKp46 in uNK from lean women.
5 Given that NKp46 is a member of the natural cytotoxicity receptor family and is directly involved in
6 target cell recognition and cytolysis[45], our finding that NKp46 proportions and expression levels
7 decrease in obesity is contradictory to our finding that obesity also correlates with enhanced surface
8 CD107a/degranulation. However, in pbNK, lower surface levels of NKp46 associate with enhanced
9 activity and exposure to hCMV infection[46,47]. Other studies have shown that increases in NKp46^{+} NK
10 proportions align with enhanced cytotoxicity[48]. How NKp46 modifications in obese uNK contribute to
11 overall activity and function at this point is unclear. It is tempting to speculate that the combined
12 decrease in NKG2A in uNK may translate to enhanced NKp46 function, a relationship that has been
13 previously identified[48]. Alternatively, loss of the mouse NKp46 orthologue (NCR1) results in penetrant
14 uterine vascular defects defined by impaired angiogenesis, delayed conceptus growth and increased
15 resorption frequencies[49], findings that are consistent with rodent and human studies showing obesity-
16 related uterine blood vessel defects[23,20,21]. Additionally, NKp46 antibody crosslinking in human uNK
17 induces production of angiogenic VEGF-A and PlGF[26,50], a finding somewhat consistent with our
18 observation that uNK from obese women secrete less VEGF-A, albeit higher amounts of PlGF.

19 uNK are specialized cells that are thought to play essential roles in coordinating and controlling
20 critical processes in pregnancy. While uNK bear some similarities to cytotoxic pbNK counterparts (i.e.
21 contain cytolytic granules), their primary role does not appear to involve the induction of cell-mediated
22 killing. While our study provides evidence that changes within the maternal environment resulting from
23 obesity lead to profound phenotypic and functional differences in uNK, these changes appear to impact

1 predominately angiogenic pathways and do not instruct heightened killing activity. Moving forward, it
2 will be important to examine how the condition of obesity instructs altered uNK function. Identification
3 of obesity-enriched or depleted factors within the maternal-fetal interface that contribute to uNK
4 dysregulation will be important in generating an understanding into the etiology of obesity-related
5 pregnancy disorders.
6
7

1 **METHODS**

2 Additional methods are provided in Supplementary Materials.

3 **Patient recruitment and tissue collection**

4 Informed written consent was obtained from women (19 to 35 years of age) that were 5 undergoing elective pregnancy termination at British Columbia's Women's Hospital, Vancouver, 6 Canada. This study was conducted with approval from the Research Ethics Board on the use of human 7 subjects, University of British Columbia (H13-00640). Fresh first trimester decidual tissues (5 to 12 8 weeks of gestation) and whole blood (N = 184) were collected from participating women having 9 confirmed viable pregnancies indicated by ultrasound-measured fetal heartbeat. Decidual tissue 10 samples were selected based on the presence of a smooth uterine epithelial layer and a course/textured 11 thick spongy underlayer. Patient clinical characteristics i.e. height and weight were additionally 12 obtained to calculate body mass index (BMI: kg/m^2). Samples were classified as lean (BMI 20-24.9 13 kg/m^2; n = 104) or obese (BMI ≥30 kg/m^2; n = 80). Further information regarding the studied cohorts is 14 depicted in Table 1.

15 Decidual tissues collected after elective termination of pregnancy where washed extensively in 16 ice-cold phosphate buffered saline (PBS; pH 7.4) after which tissue was finely minced using sterile 17 razor blades and subjected to enzymatic digestion. Decidual leukocyte-enrichment was performed 18 using methods previously described in Perdu *et al*[27].

19

20 **Viral infection of K562 cells with HLA-Cw*0602**

21 Lentiviral vector pHRSIN-HLA-Cw*0602 (kindly donated by Professor John Trowsdale from 22 University of Cambridge, UK) was transiently co-transfected into the HEK293T packaging cell line 23 (kindly provided by Dr. John Priatel, BC Children's Hospital Research Institute, Canada) using

Lipofectamine® 2000 (Thermo Fisher Scientific), together with psPAX2 and pMD2.G vectors (gifted by Dr. Christopher Maxwell, BC Children's Hospital Research Institute, Canada). Supernatants were harvested 48 hours post-transfection and used to infect K562 cells (provided by Dr. John Priatel, BC Children's Hospital Research Institute, Canada). Positive clones were sorted based on the expression level of an Emerald green fluorescent protein (eGFP) reporter gene using a BD FACSAria™ IIu cell sorter (BD Biosciences). Cells were sorted directly into growth media.

Functional assays

An enriched decidual uNK fraction was used for all functional assays (> 60% uNK).

Degranulation/activity assays were assessed by flow cytometry as described previously[23]. *Apoptosis Annexin V/FVD780* assay was performed using Annexin V-PeCy7 antibody (Thermo Fisher Scientific). Culture conditions were the same as described for the K562 target cell degranulation assay (Supplementary Methods), without the addition of brefeldin A or monensin. After 4 hours of co-culture, decidual leukocytes and K562 target cells stained for FVD780 and CD45-ef450 for 30 minutes at 4°C. Following washes with Binding Buffer (Thermo Fisher Scientific), cells were stained for Annexin V for 10 minutes at RT, washed in in 200 µl of Binding Buffer 1X, and data was acquired on an LSRII FACS (BD Biosciences); at least 20,000 events were collected.

Perforin ELISA. Cell culture supernatants were harvested after 4 hours of decidual leukocyte co-culture with K562 target cells, centrifuged, and snap frozen in liquid nitrogen. Secreted perforin measurements were conducted using Human Perforin ELISA kit (Origene, Rockville, MD, USA) following manufacturer's instructions. ELISA plates were read using a FLUOstar Optima plate reader (BMG LabTech).

1 **Multiplex cytokine/angiokine/chemokine array**

2 Decidual leukocytes (DL; > 60% uNK) were stimulated by KIR2DL1/S1 receptor ligation using
3 antibody-coated V-bottom 96-well plates (Corning, NY, USA). KIR2DL1/S1 antibody (clone 11PB6;
4 Miltenyi Biotec, Bergisch Gladbach, Germany) or IgG1 (Thermo Fisher) were coated at a
5 concentration of 2.5 μg/ml in 50 mM HEPES buffer at 4°C overnight. Following antibody coating,
6 wells were washed three-times with PBS and $5x10^5$ DL were seeded in RPMI1640 medium containing
7 10% FBS, 1 mM sodium pyruvate (Life Technologies), 55 nM βME (Sigma), 1%
8 penicillin/streptomycin (Life Technologies), and 1% anti antimycotic (Life Technologies) at 37°C 5%
9 CO_2. After 4 hours, cell supernatants were collected and analyzed for secreted factors using a custom
10 V-PLEX human biomarkers multiplex assay (PlGF, VEGF-A, VEGF-C, TNFα, GM-CSF, IL10, IP10,
11 and MIP1β) according to manufacturer's procedures (Meso Scale Discovery, Rockville, MD, USA).

12

13 **Statistical analysis**

14 Quantitative PCR (qPCR) gene expression data are presented as mean values ± standard
15 deviation (SD). Flow cytometry and cytokine array data are presented as median values and inter-
16 quartile ranges (IQRs). Differences in continuous variables between two groups were analyzed for
17 statistical significance by non-parametric two-tailed Mann-Whitney U test. Statistical comparisons of
18 uNK frequencies and MFIs among (BMI, HLA-C, HB) groups were analyzed using a non-parametric
19 Kruskal-Wallis test; multiple comparisons were controlled for using Dunn's post test. For identification
20 of BMI-related interactions with KIR2DL1/S1 crosslinking cytokine production, a two-way repeated
21 measures ANOVA was performed. Statistical analyses were performed with GraphPad Prism software
22 (La Jolla, CA, USA).

23

AUTHOR CONTRIBUTIONS

AGB designed the research. BC, SP, YM, KC, MM, JA, and AGB, performed experiments and analysed data. BC and AGB wrote the paper. All authors read and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

1 **REFERENCES**
2

3 1. Lee, J. Y., Lee, M. & Lee, S. K. Role of endometrial immune cells in implantation. *Clin. Exp.*
4 *Reprod. Med.* (2011).doi:10.5653/cerm.2011.38.3.119
5 2. Zhang, J., Dunk, C., Croy, A. B. & Lye, S. J. To serve and to protect: the role of decidual innate
6 immune cells on human pregnancy. *Cell Tissue Res.* (2016).doi:10.1007/s00441-015-2315-4
7 3. Moffett-King, A. Natural killer cells and pregnancy. *Nat. Rev. Immunol.* **2**, 656–663 (2002).
8 4. Manaster, I. & Mandelboim, O. The Unique Properties of Human NK Cells in the Uterine
9 Mucosa. *Placenta* (2008).doi:10.1016/j.placenta.2007.10.006
10 5. Long, E. O., Kim, H. S., Liu, D., Peterson, M. E. & Rajagopalan, S. Controlling natural killer
11 cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* **31**, 227–
12 58 (2013).
13 6. Vacca, P. *et al.* Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the
14 interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of
15 decidual versus peripheral NK cells. *Int. Immunol.* **20**, 1395–1405 (2008).
16 7. Vacca, P., Mingari, M. C. & Moretta, L. Natural killer cells in human pregnancy. *J. Reprod.*
17 *Immunol.* (2013).doi:10.1016/j.jri.2012.10.008
18 8. Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K. & Kershaw, M. H. Activating and
19 inhibitory receptors of natural killer cells. *Immunol. Cell Biol.* **89**, 216–224 (2010).
20 9. Moffett, A. & Colucci, F. Uterine NK cells: Active regulators at the maternal-fetal interface. *J.*
21 *Clin. Invest.* **124**, (2014).
22 10. Hiby, S. E. *et al.* Combinations of maternal KIR and fetal HLA-C genes influence the risk of
23 preeclampsia and reproductive success. *J. Exp. Med.* **200**, 957–65 (2004).
24 11. Dambaeva, S. V. *et al.* Recurrent Pregnancy Loss in Women with Killer Cell Immunoglobulin-
25 Like Receptor KIR2DS1 is Associated with an Increased HLA-C2 Allelic Frequency. *Am. J.*
26 *Reprod. Immunol.* **75**, 94–103 (2016).
27 12. Murphy, S. P. *et al.* Evidence for participation of uterine natural killer cells in the mechanisms
28 responsible for spontaneous preterm labor and delivery. *Am. J. Obstet. Gynecol.* **200**, 308.e1-9
29 (2009).
30 13. Wallace, A. E., Whitley, G. S., Thilaganathan, B. & Cartwright, J. E. Decidual natural killer cell
31 receptor expression is altered in pregnancies with impaired vascular remodeling and a higher

1 risk of pre-eclampsia. *J. Leukoc. Biol* **18**, 79–86 (2014).
2 14. Ogden, C. L., Carroll, M. D., Kit, B. K. & Flegal, M. Prevalence of Childhood and Adult
3 Obesity in the United States, 2011–2012. *Jama* **311**, 806–814 (2016).
4 15. Leddy, M. A., Power, M. L. & Schulkin, J. The Impact of Maternal Obesity on Maternal and
5 Fetal Health. *Rev. Obstet. Gynecol. [Rev Obs. Gynecol* **1701**, 170–178 (2008).
6 16. Lashen, H., Fear, K. & Sturdee, D. W. Obesity is associated with increased risk of first trimester
7 and recurrent miscarriage: Matched case-control study. *Hum. Reprod.* **19**, 1644–1646 (2004).
8 17. Chu, S. *et al.* Maternal Obesity and Risk of Gestational DIabetes Mellitus. *Diabetes Care* **30**,
9 2070–2076 (2007).
10 18. McDonald, S. D., Han, Z., Mulla, S. & Beyene, J. Overweight and obesity in mothers and risk of
11 preterm birth and low birth weight infants: systematic review and meta-analyses. *Bmj* **341**,
12 c3428–c3428 (2010).
13 19. Spradley, F. T., Palei, A. C. & Granger, J. P. Immune Mechanisms Linking Obesity and
14 Preeclampsia. *Biomolecules* **5**, 3142–3176 (2015).
15 20. Parker, V. J., Solano, M. E., Arck, P. C. & Douglas, A. J. Diet-induced obesity may affect the
16 uterine immune environment in early–mid pregnancy, reducing NK-cell activity and potentially
17 compromising uterine vascularization. *Int. J. Obes.* **38**, 766–774 (2014).
18 21. Hayes, E. K. *et al.* Trophoblast invasion and blood vessel remodeling are altered in a rat model
19 of lifelong maternal obesity. *Reprod. Sci.* **21**, 648–57 (2014).
20 22. Ratsep, M. T. *et al.* Uterine natural killer cells: supervisors of vasculature construction in early
21 decidua basalis. *Reproduction* **149**, R91–R102 (2014).
22 23. Perdu, S. *et al.* Maternal obesity drives functional alterations in uterine NK cells. *JCI Insight* **1**,
23 (2016).
24 24. Alter, G., Malenfant, J. M. & Altfeld, M. CD107a as a functional marker for the identification of
25 natural killer cell activity. *J. Immunol. Methods* (2004).doi:10.1016/j.jim.2004.08.008
26 25. Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z. & Sun, P. D. Natural killer cell-produced IFN-
27 and TNF- induce target cell cytolysis through up-regulation of ICAM-1. *J. Leukoc. Biol.* **91**,
28 299–309 (2012).
29 26. Hanna, J. *et al.* Decidual NK cells regulate key developmental processes at the human fetal-
30 maternal interface. doi:10.1038/nm1452
31 27. Lash, G. E. *et al.* Expression of angiogenic growth factors by uterine natural killer cells during

1 early pregnancy. *J. Leukoc. Biol.* **80**, 572–80 (2006).
2 28. Kopcow, H. D. *et al.* Human decidual NK cells form immature activating synapses and are not
3 cytotoxic. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15563–15568 (2005).
4 29. Fukui, A. *et al.* Correlation between natural cytotoxicity receptors and intracellular cytokine
5 expression of peripheral blood NK cells in women with recurrent pregnancy losses and
6 implantation failures. *Am. J. Reprod. Immunol.* **62**, 371–380 (2009).
7 30. Xiong, S. *et al.* Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *J.*
8 *Clin. Invest.* **123**, 4264–4272 (2013).
9 31. Hiby, S. E. *et al.* Maternal activating KIRs protect against human reproductive failure mediated
10 by fetal HLA-C2. *J. Clin. Invest.* (2010).doi:10.1172/JCI43998
11 32. Middleton, D. & Gonzelez, F. The extensive polymorphism of KIR genes. *Immunology* **129**, 8–
12 19 (2010).
13 33. Single, R. M. *et al.* Global diversity and evidence for coevolution of KIR and HLA. *Nat. Genet.*
14 **39**, 1114–9 (2007).
15 34. Sharkey, A. M. *et al.* Killer Ig-like receptor expression in uterine NK cells is biased toward
16 recognition of HLA-C and alters with gestational age. *J. Immunol.* **181**, 39–46 (2008).
17 35. Yawata, M. *et al.* Roles for HLA and KIR polymorphisms in natural killer cell repertoire
18 selection and modulation of effector function. *J. Exp. Med.* **203**, 633–45 (2006).
19 36. Martin Maiers, Loren Gragert, W. K. High-resolution HLA alleles and haplotypes in the United
20 States population. *Hum. Immunol.* **68**, 779–788 (2007).
21 37. Chazara, O., Xiong, S. & Moffett, A. Maternal KIR and fetal HLA-C: a fine balance. *J. Leukoc.*
22 *Biol.* **90**, 703–16 (2011).
23 38. Pittari, G. *et al.* NK Cell Tolerance of Self-Specific Activating Receptor KIR2DS1 in
24 Individuals with Cognate HLA-C2 Ligand. *J. Immunol.* **190**, 4650–4660 (2013).
25 39. Sivori, S. *et al.* Natural killer cells expressing the KIR2DS1-activating receptor efficiently kill T-
26 cell blasts and dendritic cells: implications in haploidentical HSCT. *Blood* **117**, 4284–92 (2011).
27 40. Apps, R. *et al.* Human leucocyte antigen (HLA) expression of primary trophoblast cells and
28 placental cell lines, determined using single antigen beads to characterize allotype specificities
29 of anti-HLA antibodies. *Immunology* **127**, 26–39 (2009).
30 41. Hiby, S. E. *et al.* Combinations of Maternal KIR and Fetal HLA-C Genes Influence the Risk of
31 Preeclampsia and Reproductive Success. *J. Exp. Med.* **200**, 957–965 (2004).

1 42. Chapel, A. *et al.* Peptide-specific engagement of the activating NK cell receptor KIR2DS1. *Sci.*
2 *Rep.* **7**, 2414 (2017).
3 43. Gaillard, R. *et al.* Risk factors and outcomes of maternal obesity and excessive weight gain
4 during pregnancy. *Obesity* **21**, 1046–1055 (2013).
5 44. Poggi, A. *et al.* Immunomodulation Regulation of cd T cell survival by soluble HLA-I :
6 Involvement of CD8 and activating killer Ig-like receptors. 2670–2678
7 (2005).doi:10.1002/eji.200526177
8 45. Schilham, M. W., Egeler, R. M. & Tol, M. J. D. Van Role of NKG2D , DNAM - 1 and natural
9 cytotoxicity receptors in cytotoxicity toward rhabdomyosarcoma cell lines mediated by resting
10 and IL - 15 - activated human natural killer cells. 573–583 (2015).doi:10.1007/s00262-015-
11 1657-9
12 46. Viel, S. *et al.* Alteration of Natural Killer cell phenotype and function in obese individuals. *Clin.*
13 *Immunol.* 5–10 (2016).doi:10.1016/j.clim.2016.01.007
14 47. Siewiera, J. *et al.* Human Cytomegalovirus Infection Elicits New Decidual Natural Killer Cell
15 Effector Functions. *PLoS Pathog.* **9**, (2013).
16 48. Costa, H. El *et al.* Critical and differential roles of NKp46- and NKp30-activating receptors
17 expressed by uterine NK cells in early pregnancy. *J. Immunol.* **181**, 3009–3017 (2008).
18 49. Felker AM, C. B. Natural cytotoxicity receptor 1 in mouse uNK cell maturation and function.
19 *Mucosal Immunol.* (2017).doi:10.1038/mi.2016.126
20 50. Siewiera, J. *et al.* Natural cytotoxicity receptor splice variants orchestrate the distinct functions
21 of human natural killer cell subtypes. *Nat. Commun.* **6**, 10183 (2015).
22
23

1 **FIGURE LEGENDS**

2 **Figure 1.** Maternal obesity associates with heightened uNK activity. **(a)** Flow cytometry gating
3 strategy used to analyze degranulation in uNK defined as $CD56^{bright}/CD9^{+}$ cells. **(b)** Representative
4 flow cytometry plots of CD107a in uNK at baseline (non-cultured), or cultured in the absence (non-
5 stimulated) or presence (stimulated) of PMA/ionomycin for 4 hours. Baseline measurements were
6 determined from *ex vivo* non-cultured uNK. Percentages of $CD56^{bright}/CD9^{+}$ cells positive for CD107a
7 are indicated within the plots. **(c)** Scatter plots depicting percentages of $CD56^{bright}/CD9^{+}$ uNK from lean
8 (black circles; n = 30) and obese (green squares; n = 26) expressing CD107a following
9 PMA/ionomycin treatment. *P* values (nonparametric two-tailed Mann-Whitney *t* test) are shown.

10

Figure 2. Maternal obesity leads to alterations in uNK cytokine production. Representative flow cytometry plots of **(a)** TNFα and **(b)** IFNγ expression in uNK from lean (n = 30) and obese (n = 26) women at baseline (non-cultured) or cultured in the absence (non-stimulated) or presence (stimulated) of PMA/ionomycin for 4 hours. Percentages of $CD56^{bright}$ cells positive for TNFα and IFNγ are indicated within plots. **(c)** Scatter plots showing median and IQR values of proportions of $CD56^{bright}$ uNK expressing TNFα and IFNγ from lean (black circles) and obese (green squares) women. Scatter plots depicting levels of secreted **(d)** angiogenic and remodeling [PlGF, VEGF-A, VEGF-C, TNFα], **(e)** placental development [GM-CSF], and **(f)** immunomodulation [IL10, IP10, MIP1β] factors from decidual leukocytes (>60% uNK) derived from lean (black; n = 15) and obese (green; n = 13) women. *P* values (nonparametric two-tailed Mann-Whitney *t* test) are shown.

1 **Figure 3.** Maternal obesity correlates with changes in activating and inhibitory Natural Killer
2 Receptors (NKRs). Scatter plots show flow cytometry-derived proportions of uNK (gated on $CD56^{bright}$
3 cells) expressing **(a)** inhibitory NKG2A (n = 9 lean *vs* 10 obese) and activating NKG2D (n =14 lean *vs*
4 18 obese) CD94/NKG2 family receptors, **(b)** activating natural cytotoxicity receptors NKp30 (n = 14
5 lean *vs* 18 obese), NKp44 (n = 14 lean *vs* 18 obese), and NKp46 (n = 11 lean *vs* 12 obese), **(c)**
6 inhibitory KIR2DL4 (n = 9 lean *vs* 10 obese) and KIR2DS1/S3/S5 (n = 28 lean *vs* 34 obese) killer-cell
7 immunoglobulin-like receptor (KIR)2D subfamily receptors, and **(d)** the leukocyte immunoglobulin-
8 like receptor LILRB1 (n = 9 lean *vs* 10 obese), as well as their surface expression levels (MFIs). On the
9 right of each scatter plot, representative histograms display uNK surface levels of individual NKRs.
10 Grey area indicates unstained control, where black and green histograms indicate representative lean
11 and obese subject expression levels. MFIs and standard deviation (SD) of lean (L) and obese (O)
12 samples are shown above the histogram. Activating (black) and inhibitory (red) receptors are color-
13 coded. *P* values (nonparametric two-tailed Mann-Whitney *t* test) are shown.
14

1 **Figure 4.** Obesity links with imbalances in uNK KIR2DS1/L1 expression. **(a)** Shown is the flow
2 cytometry gating strategy for KIR2DL1/S1 analysis. CD3/FVD780 exclusion identifies live $CD3^-$ cells
3 that are further selected on CD45, CD56, and KIR2D(L1/S1/S3/S5) positivity. Within the KIR^+
4 population, uNK are analyzed for KIR2DS1 and KIR2DL1 expression using antibodies directed against
5 KIR2DL1 and KIR2DS1/L1 where three subsets (KIR2DS1sp, KIR2DL1/S1dp, KIR2DL1sp; sp,
6 single-positive; dp, double-positive) are identified. **(b)** Representative flow cytometry plots of
7 KIR2DS1 and KIR2DL1 uNK populations from a $KIR2DS1^-$ haplotype B negative subject (HB^-; left)
8 and $KIR2DS1^+$ haplotype B positive (HB^+) subjects from lean (n = 9) and obese (n = 15) women. The
9 percentage of cells is shown in each gated area. **(c-d)** Scatter plots show total KIR2DL1 proportions of
10 uNK (gated on $CD56^{bright}$ cells) and expression levels (MFI) from HB^- and HB^+ lean and obese women.
11 **(e)** Scatter plots show KIR2DS1 proportions (2DS1sp, 2DL1/S1dp, and 2DL1sp) and surface levels
12 (MFI) in uNK (gated on $CD56^{bright}/KIR^+$) in HB^+ lean and obese women. Representative flow
13 cytometry histogram (right) shows KIR2DS1 levels (MFI) in KIR2DS1sp uNK. **(f)** Percentage and
14 expression of total KIR2DS1 (combined KIR2DS1sp and KIR2DL1/S1dp populations) in HB^+ lean and
15 obese subjects. MFI and standard deviation (SD) of KIR2DS1 is shown: solid grey area indicates the
16 fluorescence minus one (FMO) baseline signal; black and green histograms indicate 2DS1 MFI in lean
17 and green subjects. *P* values (nonparametric two-tailed Mann-Whitney *t* test) are shown.
18

1 **Figure 5.** Maternal obesity and KIR haplotype interact to potentiate uNK activity. **(a)** Scatter plots
2 show flow cytometry-derived proportions of CD107a cells in **(a)** non-stimulated and **(b)** PMA-
3 stimulated uNK cultures from $HB^{-/+}$ lean (black: HB^- n = 22; HB^+ n = 7) and obese (green: HB^- n = 15;
4 HB^+ n = 9) subjects. **(c)** Scatter plots show proportions of uNK from $HB^{-/+}$ lean and obese subjects
5 producing TNFα and IFNγ following 4 hours of PMA stimulation. *P* values (nonparametric two-tailed
6 Mann-Whitney *t* test) are shown.
7

1 **Figure 6.** Maternal HLA-C2 and KIR2DS1 correlate with an increase in uNK activity. Scatter plots
2 show proportions of uNK from $HB^{-/+}$ and HLA-C1/C2 genotyped lean (HB^{-} n = 6; HB^{+} n = 8) and
3 obese (HB^{-} n = 8; HB^{+} n = 6) women expressing CD107a following **(a)** no treatment (non-stimulated)
4 and **(b)** PMA/ionomycin (PMA) stimulation. Cx indicates that the individual is either HLA-C2
5 homozygous or heterozygous. uNK from lean or obese subjects are indicated as black circles or green
6 squares, while HB haplotype are indicated as red outline (HB^{-}) or grey outline (HB^{+}). **(c)** As above,
7 except scatter plots show comparisons of $CD107a^{+}$ proportions of uNK from only HB^{-} or HB^{+} obese
8 subjects. **(d)** Proportions of $TNF\alpha^{+}$ and $IFN\gamma^{+}$ uNK from HLA-C1/C2 and $HB^{-/+}$ genotyped lean and
9 obese women after PMA treatment. NS = non-stimulated. *P* values (One-way ANOVA-Dunn's
10 multiple comparisons- [panels **a,b**] test and nonparametric two-tailed Mann-Whitney *t* test [panels **c,d**])
11 are shown.
12

Figure 7. HLA-C2 potentiates heightened activity in KIR2DS1$^+$ uNK from obese women. **(a)** Representative flow cytometry plots showing proportions of uNK from lean (HB$^-$ n = 13; HB$^+$ n= 12) and obese (HB$^-$ n= 5; HB$^+$ n= 10) women expressing CD107a following 4 hours of co-culture with K562 target cells (5:1 E/T) ectopically expressing HLA-C2-eGFP (HLA-Cw*0602). K562 clones expressing ectopic HLA-C2 at low (K-C2 Lo) or high (K-C2 Hi) levels were established; K562 cells transduced with an empty vector (K562) served as a control. **(b)** Scatter plots show proportions of CD56^{bright}/KIR$^+$ uNK from HB$^-$ (left) and HB$^+$ (right) lean and obese women expressing CD107a. **(c)** Shown is the gating strategy for analyzing early and late apoptosis in K562 cells co-cultured with decidual leukocytes (> 60% uNK) for 4 hours. Early and late apoptosis in K562 cells is measured by annexin V and fixable viability dye (FVD) single or dual positivity. **(d)** Scatter plots depict the proportion of early (left) and late (right) apoptotic K562 target cells following co-culture with HB$^-$ (red outline; n = 13) or HB$^+$ (grey outline; n = 13) uNK from lean (black filled) or obese (green filled) women. **(e)** Scatter plot show levels of secreted perforin from decidual leukocytes from lean and obese women in response to co-culture with HLA-C2-expressing K562 target cells. One-way ANOVA (Dunn's multiple comparisons test) were performed for panels **(b-d)**. *P* values (nonparametric two-tailed Mann-Whitney *t* test) are shown for panel **(e)**.

1 **Figure 8.** Targeted KIR2DL1/S1 activation differentially controls uNK cytokine production in lean and
2 obese women. **(a)** Representative histograms show CD107a expression in HB^- and HB^+ uNK following
3 4 hours of KIR2DL1/S1 antibody cross-linking (via clone 11PB6); IgG1 was used a control KIR^+ cells
4 by flow cytometry. **(b-d)** Scatter plots showing quantification of secreted factors within conditioned
5 media generated from HB^+ uNK from lean (n = 8) and obese (n = 8) women following KIR2DL1/S1
6 antibody cross-linking. Secreted factors include **(b)** PlGF, VEGF-A, VEGF-C, TNFα, **(c)** GM-CSF,
7 and **(d)** IL10, IP10, and MIP1β. Significant interactions following crosslinking between uNK from lean
8 and obese subjects were determined via paired repeated measures statistics (two-way ANOVA). Int =
9 Interaction; Ab = 11BP6 Antibody.
10
11

1 **SUPPLEMENTAL FIGURE LEGENDS**

2 **Supplementary Figure S1**. Multicolor flow cytometry analysis of uNK NKRs in lean and obese
3 subjects. **(a)** Flow cytometry gating strategy for NKR analysis. Decidual leukocytes were gated on live
4 (FVD780$^-$)/CD3$^-$ cells, then gated on their positivity for CD45 and further CD56^{bright}/CD16$^-$ to examine
5 different NKR composition. LILRB1-expressing CD56^{bright}/CD16$^-$ is shown as an example.
6 Representative flow cytometry plots of uNK proportions of **(b)** NKG2 family receptors (NKG2A,
7 NKG2D), **(c)** natural cytotoxicity receptors (NKp30, NKp44, NKp46), **(d)** killer immunoglobulin-like
8 receptors (KIR2DL4, KIR2D(L1/S1/S3/S5), and **(e)** the leukocyte immunoglobulin-like receptor
9 LILRB1. Activating (black) and inhibitory (red) receptors are denoted by color. Median values of
10 CD56^{bright} cells are shown within plots.

11

12 **Supplementary Figure S2.** Maternal obesity associates with increased expression of KIR2DS1 on
13 uNK. qPCR analysis of *KIR2DL1*/*S1* (left) and KIR2DS3/S5 (right) mRNA levels in uNK from lean (n
14 = 14) and obese (n = 14) women. uNK from lean (n = 26) and obese women (n = 21) were
15 KIR2DL1/S1 phenotyped without considering their KIR haplotype status

16

17 **Supplementary Figure S3.** Expression levels of KIR2DL1/S1 in uNK remain comparable during first
18 trimester of pregnancy. Linear regression between **(a)** KIR2DS1sp, **(b)** KIR2DL1sp, and **(d)** total
19 KIR2DS1 CD56^{bright}/KIR$^+$ uNK (left) or MFI (right) and gestational age (5 to 12 weeks). Correlation
20 between percentage of CD56^{bright} uNK (left) or MFI (right) during first trimester of pregnancy for **(c)**
21 total KIR2DL. Lean (black) and obese (green) subjects were color code. Shown are the coefficient of
22 determination (*r*2) and the *P* value for each plot.

1

2 **Supplementary Figure 4.** Maternal HLA-C does not influence the overall frequency or expression of
3 KIR2DL1/S1 in uNK. Scatter plots depict frequencies (left) and expression levels by MFI measurement
4 (right) for **(a)** KIR2DS1sp, **(b)** total KIR2DS1, **(c)** KIR2DL1sp, and **(d)** total KIR2DL1 in uNK in lean
5 and obese subjects stratified according to the maternal HLA-C type [homozygous C1/C1 (lean n = 10;
6 obese n = 8) and C2/Cx (lean n = 12; obese n = 9)]. One-way ANOVA (Dunn's multiple comparisons
7 test) were performed. When significant, *P* values are shown.

8

9 **Supplementary Figure 5.** Low degranulation in KIR^- uNK in response to HLA-C2-expressing K562
10 cells. K562 cells infected with HLA- Cw*0602 were selected to express low and high levels of surface
11 HLA-C2. **(a)** Histogram plot shown the different levels of expression of HLA-C2 on K562 cells. Grey
12 shadow line represents empty vector infected. HLA-C2 low expression on K562 (green line) and high
13 expression (red line) are shown. **(b)** Scatter plots illustrate degranulation response of $CD56^{bright}/KIR^-$
14 cells in lean (HB^- n = 12; HB^+ n = 7) and obese (HB^- n = 5; HB^+ n = 10) subjects. **(c)** HB^+ subjects were
15 stratified according to their maternal HLA-C [C1/C1 (lean n = 6, obese n = 7) and C2/Cx (lean n = 5,
16 obese n = 7). Scatter plots show CD107a levels of KIR^+ subset of uNK from HB^+ subjects. *P* values
17 One-way ANOVA (Dunn's multiple comparisons test) were performed. When significant, *P* values are
18 shown.

19

20 **Supplementary Figure 6.** NKT cells have a negligible effect on overall degranulation rates in HLA-
21 C2 activation assays. **(a)** Scatter plots show proportions of T ($CD3^+$) and natural killer T (NKT;
22 $CD3^+/CD56^+$) cells within decidual leukocyte isolates from HB^- (n = 22) and HB^+ (n = 26) women. **(b)**

1 Proportions of T and NKT cells in lean (n = 26) and obese (n = 22) women. Scatter plots showing
2 proportions of CD107a NKT in **(c)** HB^- [left, (lean n = 8, obese n = 3)] and **(d)** HB^+ [right, (lean n = 11,
3 obese n = 12)] women. *P* values (nonparametric Mann-Whitney *t* test [panels a,b] are shown. One-way
4 ANOVA was performed on panels **c,d.**

5

6 **Supplementary Figure 7.** Scatter plots showing levels of secreted factors by HB^- uNK after the
7 crosslinking assay with KIR2DL1/S1 mAb (11BP6) or isotype control IgG1 antibody. **(a)** Angiogenic
8 and remodeling [PlGF, VEGF-A, VEGF-C, TNFα], **(b)** placental development [GM-CSF], and **(c)**
9 immunomodulating [IL10, IP10, MIP1β] factors from decidual leukocytes (>60% uNK) derived from
10 lean (n = 6) and obese (n = 5) women. Significant interactions following crosslinking between uNK
11 from lean and obese subjects were determined via paired repeated measures statistics (two-way
12 ANOVA). Int = Interaction; Ab = 11BP6 Antibody.

13

14 **Supplementary Figure 8.** Flow cytometry gating strategy to analyze KIR2DL1/S1 subsets in uNK. **(a)**
15 Comparison of our gating strategy (right) and the previously published method (left), where total uNK
16 are gated against KIR2DL1 and KIR2DL1/S1. **(b)** Flow cytometry analysis of KIR2DL1/S1 subsets in
17 $CD56^{bright}$ cells from lean (n = 26) and obese (n = 21) women. *P* values (nonparametric two-tailed
18 Mann-Whitney *t* test) are shown.