Cellular senescence induced by CD158d reprograms natural killer cells to promote vascular remodeling

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Natural killer (NK) cells, which have an essential role in immune defense, also contribute to reproductive success. NK cells are abundant at the maternal–fetal interface, where soluble HLA-G is produced by fetal trophoblast cells during early pregnancy. Soluble HLA-G induces a proinflammatory response in primary, resting NK cells on endocytosis into early endosomes where its receptor, CD158d, resides. CD158d initiates signaling through DNA-PKcs, Akt, and NF-κB for a proinflammatory and proangiogenic response. The physiological relevance of this endosomal signaling pathway, and how activation of CD158d through soluble ligands regulates NK cell fate and function is unknown. We show here that CD158d agonists trigger a DNA damage response signaling pathway involving cyclin-dependent kinase inhibitor p21 expression and heterochromatin protein HP1-γ phosphorylation. Sustained activation through CD158d induced morphological changes in NK cell shape and size, and survival in the absence of cell-cycle entry, all hallmarks of senescence, and a transcriptional signature of a senescence-associated secretory phenotype (SASP). SASP is a program that can be induced by oncogenes or DNA damage, and promotes growth arrest and tissue repair. The secretome of CD158d-stimulated senescent NK cells promoted vascular remodeling and angiogenesis as assessed by functional readouts of vascular permeability and endothelial cell tube formation. Retrospective analysis of the decidual NK cell transcriptome revealed a strong senescence signature. We propose that a positive function of senescence in healthy tissue is to favor reproduction through the sustained activation of NK cells to remodel maternal vasculature in early pregnancy.

KIR2DL4 | microarray

Natural killer (NK) cells are lymphocytes that participate in immune responses through their cytotoxic activity and secretion of cytokines. NK cells have also a nonimmunological function during early pregnancy (1–3). The presence of maternal genes encoding activating receptors of the killer cell Ig-like receptor (KIR) family in combination with HLA-C alleles of the fetus correlates with successful pregnancies and reduced risk of preeclampsia and recurrent miscarriage (4, 5). Conversely, a particular combination of fetal HLA-C and maternal inhibitory NK cell receptor is associated with predisposition for preeclampsia (6). Human NK cells are abundant in uterus, where they remain after embryo implantation and through the first trimester of pregnancy (1). However, the role of NK cells in reproduction and their regulation in the decidua basalis of pregnant uterus is not well understood.

Typically, NK cells are activated by interaction with ligands on target cells or by soluble mediators such as cytokines and chemokines. In addition, soluble HLA-G, a nonclassical major histocompatibility complex molecule secreted by fetal trophoblast cells during early pregnancy (1), stimulates resting NK cells to secrete proinflammatory and proangiogenic factors (7). Its receptor, CD158d (KIR2DL4) is an unusual member of the KIR family (8). Other members of the KIR family are expressed at the NK cell surface, bind to classical HLA class I molecules, and regulate NK cell cytotoxicity and cytokine secretion through tyrosine kinases and phosphatases (9, 10). In contrast, CD158d resides in early endosomes. On endocytosis of either soluble HLA-G or agonist antibody, CD158d initiates a signaling pathway through the serine/threonine kinases DNA-PKcs and Akt, and NF-κB (7, 11). We set out to study why endosomal signaling by CD158d uses a kinase involved in DNA repair to produce a proinflammatory and proangiogenic response. We have identified the induction of cellular senescence in NK cells by DNA damage response (DDR) signaling as a molecular switch by which CD158d generates the sustained secretion of factors that can promote vascular remodeling.

Results and Discussion

DDR Signaling Results in $p21^{Cip1/Waf1}$ Induction and Phosphorylation of HP1-γ. We investigated why an NK cell receptor would co-opt DNA-PKcs, a kinase essential for DNA repair (12), to deliver its signal. DDR signaling is mediated primarily by proteins of the phosphoinositide 3-kinase-like kinase family, including ATM, ATR, and DNA-PK, to coordinate cellular responses such as survival, apoptosis, or senescence of damaged cells (12). Phosphorylation of Akt at Ser-473 by DNA-PKcs was implicated in signaling by CD158d in NK cells (11). DNA-PKcs also activates Akt during ionizing radiation-induced DDR, leading to the up-regulation of the cyclin-dependent kinase inhibitor p21 (also known as Cip1 or Waf1) (13). To test if p21 is up-regulated on CD158d engagement, primary human NK cells were stimulated with agonist antibody to CD158d or with soluble HLA-G (sHLA-G). Up-regulation of p21 with agonist antibody or sHLA-G was seen after 16 h (Fig. 1*A*). Stimulation by sHLA-G was blocked in the presence of the HLA-G antibody G233. To test if p21 up-regulation was DNA-PKcs dependent, NK cells were pretreated with NU7026, a DNA-PKcs inhibitor, before stimulation via CD158d (Fig. 1*B*). Inhibition of DNA-PKcs blocked p21 induction, suggesting that DDR signaling was required to induce p21, a key negative regulator of cell-cycle progression after DNA damage (14).

We also tested for phosphorylation of heterochromatin protein 1-γ (HP1-γ), a nonhistone protein involved in gene silencing and chromatin organization that is phosphorylated in response to DNA damage to initiate the DDR (15). In cells entering senescence, phosphorylation of HP1-γ is thought to be required for its efficient incorporation into specialized domains of facultative heterochromatin called senescence associated heterochromatin foci (16). We show HP1-γ phosphorylation at Ser-83 in response to CD158d engagement by agonist antibody and by sHLA-G (Fig. 1*C*). Thus, in the absence of DNA damage, activation of NK cells by CD158d results in DDR signaling to induce p21 expression and the phosphorylation of HP1-γ.

NK Cells Acquire Features of Senescence Upon CD158d-Mediated Activation. Because p21 up-regulation and HP1-γ phosphorylation are DDR signaling events implicated in the induction of senescence,

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo/ (accession no. GSE35330).

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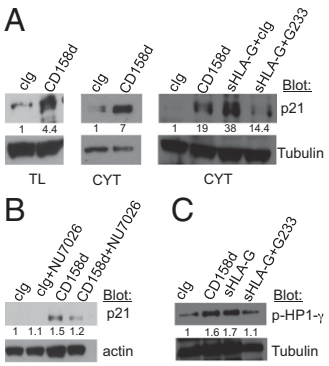


Fig. 1. DDR signaling results in p21 induction and phosphorylation of HP1-γ. (*A*) Western blot analysis of total lysate (TL) or cytoplasmic (CYT) proteins from resting NK cells stimulated with control Ab (clg), a mAb to CD158d or sHLA-G in the presence of clg or anti-HLA-G (G233). Blots were probed with antibodies to p21 or tubulin (loading control). (*B*) Western blot analysis of p21 expression in resting NK cells stimulated with CD158d mAb in the presence or absence of DNA-PK inhibitor, NU7026. (*C*) Western blot analysis of phosphorylated HP1-γ in resting NK cells stimulated with CD158d mAb or sHLA-G in the presence of clg or anti-HLA-G (G233). Normalized intensities of p21 and phosphorylated HP1-γ relative to the tubulin or actin loading controls are listed. Each experiment was performed using NK cells from at least three independent donors.

we tested if NK cells activated by CD158d showed any of the morphological features of senescent cells. Primary NK cells had a characteristic flattened and enlarged morphology after activation through CD158d (Fig. 2*A*). The average 2D cell area of NK cells activated by CD158d was $19.5 \pm 11\ \mu m^2$ ($n = 23$), up from $6.6 \pm 2.4\ \mu m^2$ ($n = 22$) for cells stimulated with control Ab. There was also an increase in nucleus size (Fig. 2*B*) from $2.9 \pm 0.3\ \mu m^2$ ($n = 29$) with control Ab to $4.95 \pm 1.2\ \mu m^2$ ($n = 15$) in cells activated by CD158d. There was an increase in senescence-associated β-galactosidase (SA-β-gal) activity, a widely used senescence biomarker (Fig. 2*C*), indicative of increased lysosomal biogenesis in senescent cells (17). Taken together, we show that NK cell activation by CD158d induces morphological changes and SA-β-gal expression in line with the requirement for p21 induction for the morphological changes associated with senescence (18).

NK cells activated by CD158d were then tested for survival, proliferative capacity, and sensitivity to apoptosis. CD158d-stimulated cells survived better than unstimulated cells or cells stimulated through other NK activation receptors such as CD16 and 2B4 (Fig. 2*D*). To test if this CD158d-induced survival involved cell proliferation, freshly isolated human NK cells were stimulated with soluble antibodies to CD158d and to CD16 or with IL-2, a cytokine that stimulates NK cell proliferation. Lack of proliferation on day 2 and day 4 poststimulation was evident from the very low BrdU incorporation by NK cells stimulated via CD158d (Fig. 2*E*). In contrast, IL-2 induced robust NK cell proliferation by day 4. Furthermore, NK cells activated by CD158d were arrested at the G0/G1 stage of the cell cycle (Fig. 2*F*), whereas IL-2–activated NK cells, which proliferate actively and progress through the cell cycle, showed a decrease in the percent of cells in G0/G1 over time. Thus, the cell-cycle arrest and the lack of proliferation are consistent with features of senescent cells (19, 20). In addition to cell-cycle arrest, senescent cells are resistant to apoptosis (19). NK cells were tested for their ability to undergo apoptosis in response to activation through CD158d (Fig. 2*G*). Although the majority of NK cells showed surface expression of Annexin V in response to CD16 over a 72-h period, only a few NK cells stimulated by CD158d did, similar to unstimulated cells or cells stimulated with IL-2. Thus, activation of NK cells by CD158d does not promote cell-cycle progression and does not induce apoptosis, consistent with induction of senescence in these cells.

Transcriptional Response to Activation by CD158d Reveals a Senescence Signature. We set out to obtain a comprehensive view of the transcriptional response to CD158d-mediated signals. Transcriptional profiling was performed on primary quiescent NK cells that were stimulated with an agonist mAb to CD158d for 4 h, 16 h, and 64 h. Changes in gene expression that were >2.5-fold for each time point are shown in Tables S1, S2, and S3. The peak response was seen at 16 h where we identified 1,186 genes (selected for $P < 0.05$) that were up-regulated in cells stimulated with a CD158d mAb. Because there is no gene ontogeny term for senescence, the gene

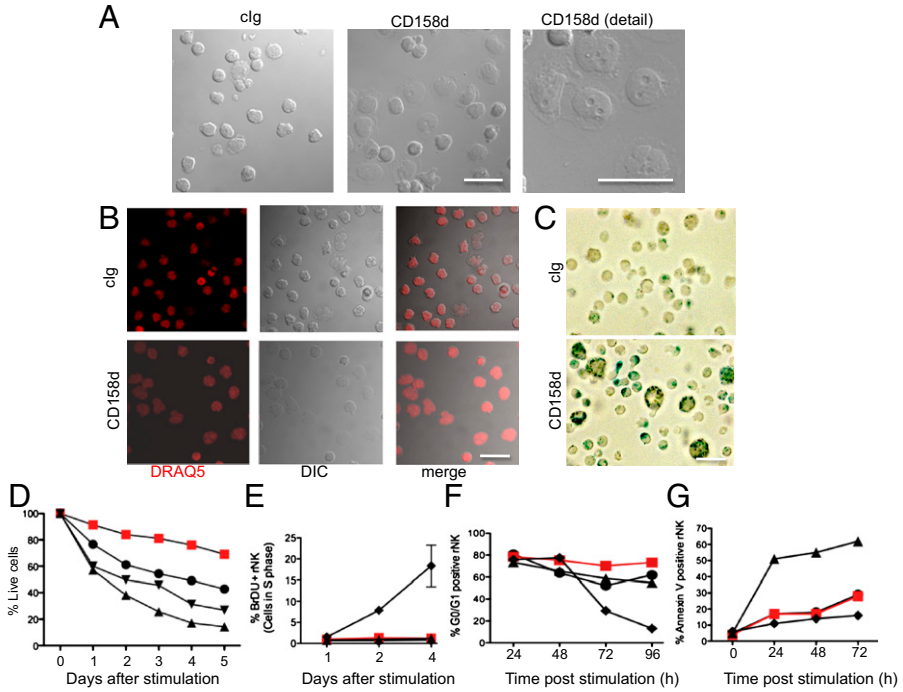


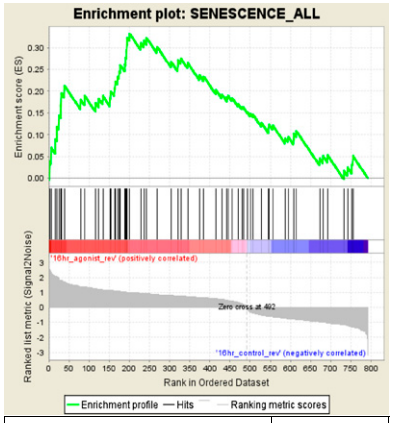
Fig. 2. NK cells acquire features of senescence on CD158d-mediated activation. (*A*) Differential interference contrast images of resting NK cells stimulated with clg or CD158d mAb for 16 h. The DIC image on the right (Magnification 2×). (*B*) NK cells treated as in (*A*) were stained with DRAQ5 (nuclei in red). (*C*) NK cells treated as in (*A*) and stained for SA-β–gal at 48 h. (*D*) Resting NK cells stimulated with clg (circle), CD158d (red square), 2B4 (inverted triangle), and CD16 (triangle) were counted for live cells on a hemacytometer using Trypan Blue exclusion of dead cells (Scale bar, 10 μm). (*E, F,* and *G*) Resting NK cells stimulated with clg (circle), CD158d mAb (red square), CD16 mAb (triangle), and IL-2 at 100 U/mL (diamond) were tested at the indicated time points for BrDU incorporation (*E*), G0/G1-positive NK cells (*F*), and Annexin V–positive NK cells (*G*). All data are representative of experiments using NK cells from at least three normal donors.

expression profile induced by CD158d at 16 h was compared with the molecular signature of oncogene-induced senescence (21). Gene set enrichment analysis (GSEA) revealed a significant (P = 0.004) enrichment of up-regulated senescence genes (Fig. 3*A*) with a positive enrichment score of 0.33. The top 115 up-regulated genes at 16 h (>2.7 fold induction) were analyzed for their status at 4 h and 64 h, as depicted in a heat map (Fig. 3*B*). Among the top 30 most up-regulated genes, 13 have a documented role in either the induction or maintenance of senescence, as identified in large-scale screens for genes involved in senescence or by antibody arrays (Fig. 3*C*) (22–24).

We compiled a set of genes from our microarray that had been shown previously to be up-regulated during the induction of senescence (Table S4). As shown in Fig. 3*D*, within this set of 40 genes, there is some overlap with genes involved in proinflammatory responses and wound healing that is consistent with a role for senescence in tissue repair (25, 26). Recent studies have shown that expression of a subset of proinflammatory cytokines is essential to trigger and sustain the senescent phenotype (20, 27). These include IL-6, IL-8, IL-1α, and IL-1β, for which the gene up-regulation and protein secretion was detected on CD158d engagement in peripheral blood and decidual NK cells (7, 28). Collectively, the secretion of a variety of immune modulators and inflammatory cytokines by senescent cells is referred to as a senescence-associated secretory phenotype (SASP) (19). We validated a panel of 23 genes that constitute a senescent signature of primary NK cells by RT–quantitative PCR (Fig. 3*E*). Furthermore, in addition to stimulating NK cells with agonist antibody to CD158d, we also stimulated cells with sHLA-G. The IL6, IL8, and IL1B genes were strongly up-regulated (Fig. 3*F*) and this up-regulation was blocked in the presence of mAb to HLA-G. Thus, the transcriptional profile induced on activation of NK cells by CD158d is consistent with the induction and maintenance of senescence in these cells.

The SASP has profound effects on the surrounding tissue microenvironment, which can be either detrimental [e.g., tumor

A



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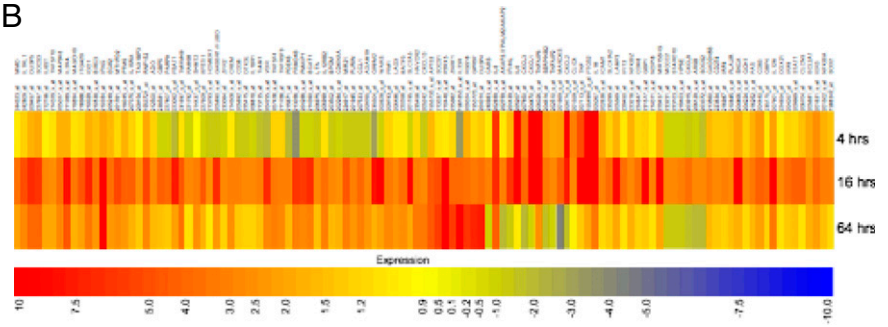
FWER p-Value	0.417

B



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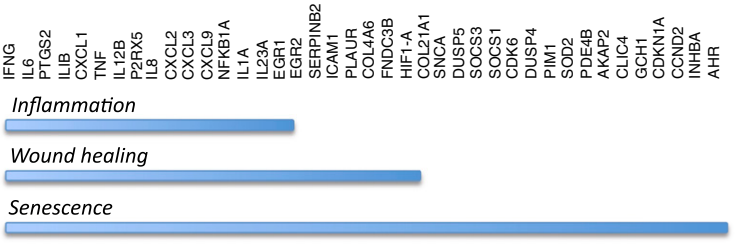
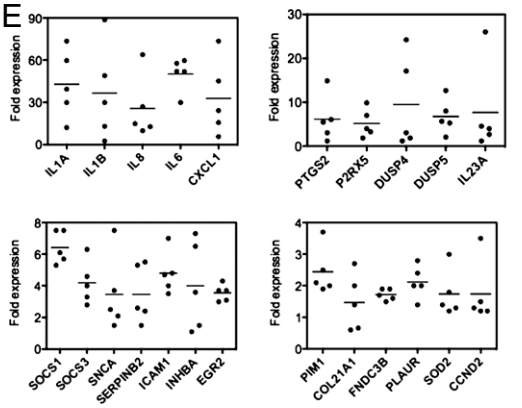


C

#	Gene Symbol	Gene Title	Fold Increase	Function
1	IL12B	IL12p40	70.09	S/I/WH
2	IFNG	IFN-gamma	66.55	S/I/WH
3	IL6	Interleukin 6	51.83	S/I/WH
4	PTGS2	COX-2	39.15	S/I/WH
5	IL1B	interleukin 1 beta	26.66	S/I/WH
6	SNCA	synuclein alpha	20.4	S
7	CXCL1	CXC ligand 1	15.45	S/I/WH
8	TNF	TNF-alpha	12.36	S/I/WH
9	P2RX5	P2X purinergic receptor 5	11.46	RS/I/WH
10	SLC7A5	solute carrier family 7 member 5	11.42	
11	MIR155HG	BIC transcript	11.27	
12	TNFAIP6	TNF-a induced protein 6	10.55	I
13	WARS	tryptophanyl tRNA synthetase	9.91	
14	GBP1	guanylate binding protein 1	9.59	I
15	LAMP3	CD208	8.66	
16	IL2RA	IL2 receptor alpha	8.31	I
17	GREM2	gremlin 2	8.25	A
18	PSAT1	phosphoserine aminotransferase1	7.93	
19	CXCL2	CXC ligand 2	7.68	S/I
20	IDO-1	indoleamine-pyrole 2,3 dioxygenase	7.56	
21	VDR	Vitamin D receptor	7.12	
22	UBD	ubiquitin D	7.1	
23	IL8	interleukin 8	6.82	S/I/WH
24	BCAT1	branched chain aminotransferase 1	6.81	
25	DUSP5	dual specificity phosphatase 5	6.78	S
26	FRMD4B	FERM domain containing 4B	6.45	
27	IFI44L	IFN-induced protein 44-like	6.42	I
28	SOCS3	Suppressor of cytokine signaling 3	6.32	S
29	PMAIP1	PMA-induced protein 1	6	
30	RNF152	ring finger protein 152	5.64	

E



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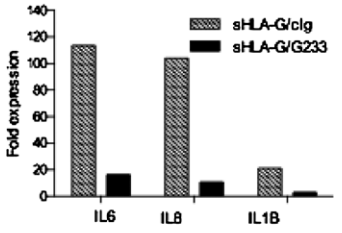


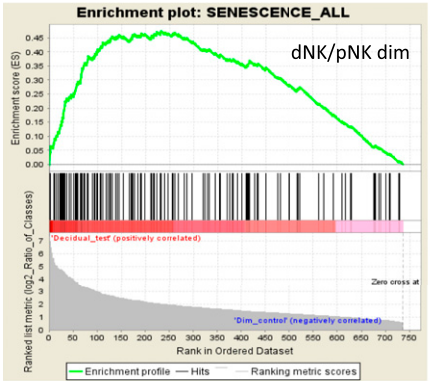
Fig. 3. The transcriptional response to activation by CD158d reveals a senescence signature of up-regulated genes. (*A*) GSEA of microarray data of CD158d-induced gene expression compared with the molecular signature of oncogene ras-induced senescence. (*B*) Heat map showing relative expression of the top 115 up-regulated genes at 16 h compared with their expression at 4 h and 64 h. A high resolution image is shown in Fig. S1. (*C*) List of top 30 up-regulated genes at 16 h. Genes known to be involved in senescence are highlighted in blue. Function: S, senescence; RS, replicative senescence; I, inflammation; WH, wound healing; A, gremlin 2 is unique to angiogenesis. (*D*) The senescence signature after CD158d stimulation includes 40 genes at 16 h (Table S4). The schematic shows the overlap of genes involved in inflammation, wounding healing, and senescence responses. (*E*) Fold increase in gene expression values (CD158d/clg stimulation) for the senescence signature at 16 h from resting NK cells of five different donors. Each panel has a different scale based on strength of gene expression. (*F*) Fold gene expression values for IL6, IL8, and IL1B from resting NK cells stimulated with sHLA-G in the presence of clg or anti-HLA-G (G233).

progression (20) and age-related tissue dysfunction (29)] or beneficial [e.g., tissue repair during wound healing (25, 26)]. What are the physiological implications of the SASP induced by a specific ligand in NK cells? Although NK cells constitute only 2–5% of peripheral blood lymphocytes, they are the most abundant lymphocyte subset (~70%) in the uterus during early pregnancy (1). Microarray profiling of decidual NK cells isolated between 6–12 wk of pregnancy had revealed >3,000 up-regulated genes ($P < 0.05$) compared with resting, peripheral blood NK cells (30). Another study of decidual NK cells suggested that they secrete factors that may influence vascular remodeling after stimulation in vitro (3). Although the ability of NK cells from early pregnancy to promote vascular remodeling has been documented, little is known about the regulation of this process.

Because we have shown here that a soluble agonist of CD158d alone triggers a major reprogramming of resting NK cells to a senescent state, we asked if decidual NK cells isolated from abortions (gestational age 6–12 wk) (30) displayed an up-regulation of genes involved in senescence and SASP compared with resting peripheral blood NK cell samples. Using GSEA, a statistical method to detect if a set of genes is enriched in an independent expression data set, to compare decidual NK cells with the molecular signature of oncogene-induced senescence (21), we found that the senescence signature was highly enriched in decidual NK cells compared with either CD56 bright or CD56 dim peripheral blood NK cells (Fig. 4*A* and *B*). Therefore, the increased expression of genes involved in senescence and SASP in NK cells stimulated by CD158d is also a feature of NK cells isolated directly from the decidua basalis of the pregnant uterus. That decidual NK cells have already been reprogrammed to a senescent state during the first trimester of pregnancy precludes ex vivo stimulations of these cells as an informative approach to understand their regulation. CD158d-stimulated cells and decidual NK cells share expression of a panel of genes implicated in senescence (Table S4), including genes known to be critical for inducing and reinforcing the senescent state, such as IL6, IL8, IL1B, and p21 (Fig. 4*C*). Notably, p21 (CDKN1A), a major determinant of senescence, was highly up-regulated ($P < 0.001$) throughout the gestational period studied (6–12 wk).

Senescence Secretome Induced by CD158d in NK Cells Increases Vascular Permeability and Promotes Angiogenesis. The gene for CD158d (KIR2DL4) is expressed in all human NK cells (8) and is up-regulated fivefold in decidual NK cells (30). Because CD158d

A



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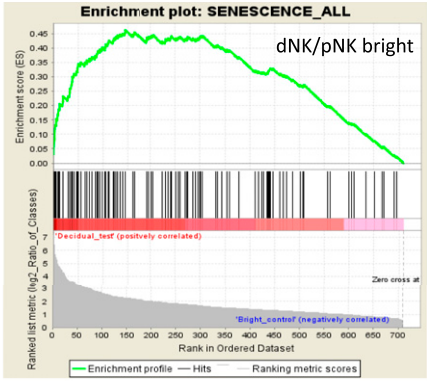
FWER p-Value	0.044

B



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FDR q-value	0.037544757
FWER p-Value	0.038

C

Gene	F.C. (dNK/pNK^{dim})	F.C. (dNK/pNK^{bright})
AKAP2	9.14	7.21
CCND2	3.5	3.16
CDK6	2.00	1.25
CDKN1A	9.83	8.3
CLIC4	3.02	4.57
COL4A6	2.15	1.59
CXCL2	9.78	10.41
DUSP4	4.35	ND
DUSP5	2.00	2.57
EGR1	2.54	2.04
EGR2	2.22	1.63
GCH1	1.58	1.55
ICAM1	1.91	ND
IL1B	5.78	7.6
IL6	10.86	6.75
IL8	34.4	13.32
IL23A	1.79	2.82
PTGS2	6.30	1.29
SERPINB2	13.72	15.81
SOD2	13.50	7.63
SOCS1	4.16	2.66
SOCS2	1.99	2.49

Fig. 4. Up-regulation of senescence-associated genes in decidual NK cells. (*A* and *B*) GSEA of microarray data of decidual NK cells (30) compared with the molecular signature of oncogene ras-induced senescence (21). The transcriptional up-regulation of decidual NK cells versus peripheral blood CD56^{dim} NK cells (*A*) and peripheral blood CD56^{bright} NK cells (*B*) were analyzed separately. (*C*) List of senescence-associated genes up-regulated in decidual NK cells and in CD158d-stimulated resting NK cells, presented as fold change (F.C.) of expression in decidual NK (dNK) compared with peripheral blood NK (pNK) belonging to CD56^{dim} or CD56^{bright} subsets. ND, not detected.

engagement induces an SASP, we reasoned that this secretory activity of NK cells could regulate their microenvironment at sites of HLA-G expression, such as in the first weeks of pregnancy. On implantation of the blastocyst in the uterine wall, HLA-G–expressing fetal trophoblast cells (derived from the external trophectoderm of the blastocyst) invade the maternal decidua and are intermingled with NK cells at this site (1). To assess if the CD158d-mediated SASP could contribute to remodeling by enhancing the vascular permeability of the endothelium, we tested its effect on a monolayer of human umbilical vein endothelial cells (HUVEC). Supernatants from primary NK cells stimulated by CD158d for 16 h increased the vascular permeability of the HUVEC monolayer, as determined by the ability of FITC-dextran to permeate through the monolayer after 8 h (Fig. 5*A*). The ability of the CD158d-mediated SASP to increase HUVEC vascular permeability was similar to that seen with two known inducers, the vasoactive cytokines IL-1β and TNF-α (Fig. 5*B*).

We also tested the ability of the SASP of NK cells activated by CD158d to induce angiogenesis, a highly ordered and regulated process. This was examined using the endothelial cell tube formation assay, which recapitulates many of the steps in angiogenesis, such as endothelial cell adhesion, migration, protease activity, alignment, and tubule formation. Supernatants from primary NK cells stimulated through CD158d were added to basement membrane extracts overlaid with HUVEC. Tube formation by HUVEC was observed at 3 h and 6 h after stimulation (Fig. 5 *C* and *D*). Taken together with the enhanced vascular permeability, a prerequisite for angiogenesis, these data show that the SASP induced by NK cells could play a favorable role in promoting neovascularization and tissue remodeling at sites of HLA-G expression.

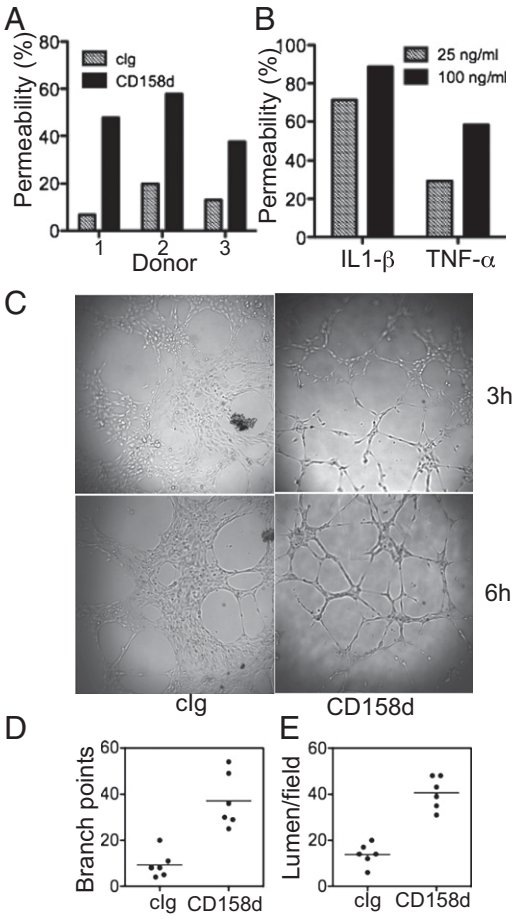

Fig. 5. The senescence secretome induced by CD158d in NK cells increases vascular permeability and promotes angiogenesis. (*A*) Effect of CD158d-induced senescence secretome on vascular permeability. HUVEC were incubated with supernatants from resting NK cells stimulated with control or CD158d mAbs and permeability of the HUVEC monolayer is shown. (*B*) Effect of recombinant IL-1β and TNF-α on the permeability of HUVEC monolayers. (*C*) Representative images from the tube formation assay to test the effect of the CD158d-induced secretome. HUVEC seeded on a gel matrix were evaluated for their ability to form tubes in the presence of supernatants of NK cells stimulated for 16 h with either clg or CD158d mAb. (*D*) Quantification of matrigel-tube formation assay in (*C*). The data are representative of experiments using NK cells from three independent donors.

Conclusions

This work has identified senescence as a mechanism leading to a regulated, specific, and sustained NK cell response after activation through CD158d. Sustained signals, a feature of endosomal signaling, occur when CD158d signals via a DNA-PKcs–Akt–NF-κB pathway. This senescence response is not initiated by one of its well-known inducers such as DNA damage or oncogenes, but by activation of an endosomal receptor with its soluble ligand. Nevertheless, this CD158d-induced pathway uses elements of the DDR signaling cascade to yield a senescent phenotype. We show that DDR signaling initiated by CD158d is a molecular switch for an SASP in NK cells, which is likely to promote vascular permeability and angiogenesis at sites of HLA-G expression. Furthermore, a retrospective analysis of the decidual NK cell transcriptome has revealed a strong senescence signature. It is rather remarkable that CD158d signaling alone is sufficient to trigger a DDR and to reprogram NK cells to a senescent state, similar to that of decidual NK cells.

The best validated physiological expression of sHLA-G is by fetal trophoblast cells, although de novo expression of sHLA-G has been detected in some cases during malignant transformation, transplantation, infections, and autoimmunity (31, 32). How CD158d activation through HLA-G influences NK cell functions on de novo HLA-G expression such as during transplantation or during infections remains to be understood. This study reveals a physiological setting where the SASP produced by CD158d–sHLA-G interactions could contribute to normal vascular adaptations that occur to support the development of the fetus during pregnancy. Here, senescence involves an active genetic reprogramming of NK cells in response to a fetal HLA-G signal. A role for SASP in the normal vascular adaptations and remodeling of healthy tissue during pregnancy validates the evolution of senescence as more than a tumor-suppressive mechanism and stands in contrast to the deleterious effects of the senescence secretome in settings such as age-related tissue dysfunction (29) and the development of cancer through malignant transformation (20).

Materials and Methods

Primary NK Cell Isolation and Stimulation. Human blood samples from anonymous healthy donors were drawn for research purposes at the National Institutes of Health (NIH) Department of Transfusion Medicine under an NIH Institutional Review Board–approved protocol with informed consent. Human polyclonal NK cells were isolated from peripheral blood lymphocytes using the Negative Selection Human NK Cell Enrichment Kit (Stem Cell Technologies). NK cells were greater than 97% $CD3^-$ and $CD56^+$. These freshly isolated NK cells ("resting NK") were cultured in Iscove's modified DMEM containing 10% (vol/vol) human serum without added IL-2 or feeders. Resting NK cells were incubated with the following mAbs: control IgG1 (MOPC21), agonist anti-CD158d (#33), anti-CD16 (3G8), and anti-2B4 (C1.7) at 10 μg/mL for 16 h unless indicated otherwise. For soluble HLA-G stimulation of resting NK cells, sHLA-G purified from 721.221 cells stably transfected with HLA-G5 (33) (Fig. S2) was used at 10 μg/mL together with either control IgG2a (MOPC173) or anti-HLA-G mAb G233 at 30 μg/mL.

Western Blotting. Total cell lysates or immunoprecipitated proteins from these lysates were subject to Western blotting according to standard methods. Blotting antibodies for phospho-HP1-γ (Cell Signaling), p21 (BD), actin (BD), and tubulin (BD) were used.

Flow Cytometry. G0/G1 cell-cycle analysis of NK cells was done using staining with propidium iodide for 40 min to mark DNA content. Annexin V (BD) staining was done to identify apoptotic cells by flow cytometry. After 1.5 h of labeling of NK cells with bromodeoxyuridine, staining for $BrdU^+$ NK cells was performed using a BrdU Flow Kit (BD). Samples were acquired on a FACSort (BD) and analyzed using CellQuest software.

Microarray Experiments and Analysis. Primary resting NK cells from four donors were stimulated with either cIg (MOPC21) or CD158d mAb (#33) for 4 h, 16 h, or 64 h. Total RNA was isolated with an RNeasy Mini Kit (QIAGEN). RNase free DNaseI (Amersham) was used to remove remaining DNA. cDNA was synthesized and labeled according to manufacturers' instructions for Affymetrix genome arrays (Affymetrix). Hybridization, fluidics, and scanning were performed according to standard Affymetrix protocols (www.affymetrix.com) at the National Institute of Allergy and Infectious Diseases (NIAID) microarray facility at Rocky Mountain Labs. GeneChip Operating Software (GCOS v1.4, www.Affymetrix.com) was used to convert the image files to cell intensity data (CEL files). All CEL files, representing individual samples, were normalized by using the scaling method within GCOS to a target of 1,250 to produce the analyzed CEL files (chp files) along with the report files. The pivot table was imported into GeneSpring GX 7.3 (www.chem.agilent.com), and used to create a hierarchical clustering (condition tree) using a Pearson correlation similarity measure with average linkage to produce the dendrogram indicating the clusters from the comparisons of interest. The CEL files were input into Partek Genomics Suite software (Partek, Inc.) and quantile normalized to produce the principal components analysis graph. A three-way ANOVA was performed within Partek to obtain multiple test corrected *P* values using the false discovery rate method (34) at the 0.05 significance level and was combined with fold change values, select quality measurements of signal, and call consistency as calculated using custom Excel (Microsoft Corp.) templates for each comparison of interest. GSEA (35, 36) was performed by appending the gene set database to include the oncogene-induced senescence set from Mason et al. (21). For the dataset of Koopman et al. (30), GSEA of decidual NK cells versus $CD56^{dim}$ peripheral blood NK cells and decidual NK cells versus $CD56^{bright}$ peripheral blood NK were performed against the oncogene-induced senescence set from Mason et al. (21).

Real-Time PCR. Total RNA from resting NK cells stimulated with mAbs or soluble ligand for 16 h was isolated using the RNeasy Mini Kit (Qiagen) and real-time PCR was performed using the iQ-SYBR Green SuperMix Kit (Bio-Rad) with the iCycler sequence detection system (Bio-Rad) using specific primers (IDT) (Table S5). Real-time PCR data were quantified using GAPDH as the internal control.

Vascular Permeability Assay. The In Vitro Vascular Permeability Kit (Trevigen) was used according to the manufacturer's instructions. Briefly, HUVEC were grown as a tight monolayer on collagen-coated membranes. Supernatants from NK cells stimulated with either cIg or CD158d mAb were added to the wells and after 8 h, the ability of FITC-dextran to diffuse through the HUVEC barrier was quantified to detect vascular permeability. Recombinant IL-1β and TNF-α were obtained from Millipore.

Tube Formation Assay. The ability of HUVEC (BD) to form tubes was evaluated using the BioCoat Angiogenesis System-Endothelial cell tube formation assay (BD). Briefly, HUVEC were overlaid on a matrigel matrix in the presence or absence of supernatants from NK cells stimulated with either cIg or CD158d mAb. Cells were assessed for tube formation at 3 h and 6 h after treatment. The number of branch points and lumen were calculated for each field.

Statistical Analysis. This was performed using GraphPad Prism 5.0.

SI Materials and Methods. Tables S1, S2, and S3 list the genes that were differentially regulated in the microarray analysis of the transcriptional response to activation via CD158d at 4 h, 16 h and 64 h. Table S4 contains references for the senescence list generated in Fig. 3*D*. Table S5 lists the primers used for real time PCR in Fig. 3. Fig. S1 is a high resolution image of Fig. 3*B*. Fig. S2 shows a Coomassie stain of soluble HLA-G (from 221-HLA-G5 cells) used to stimulate NK cells.

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