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Identification of a Novel Neutrophil Population: Proangiogenic Granulocytes in Second-Trimester Human Decidua

Hagai Amsalem,*‡ Melissa Kwan,*‡ Aleah Hazan,*‡ Jianhong Zhang,* Rebecca L. Jones,§ Wendy Whittle,¶ John C. P. Kingdom,¶ B. Anne Croy,‖ Stephen J. Lye,*‡,¶,§ and Caroline E. Dunk*‡

The maternal leukocytes of the first-trimester decidua play a fundamental role in implantation and early development of the fetus and placenta, yet little is known regarding the second-trimester decidual environment. Our multicolor flow cytometric analyses of human decidual leukocytes detected an elevation in tissue resident neutrophils in the second trimester. These cells in both human and murine samples were spatially restricted to decidua basalis. In comparison with peripheral blood neutrophils (PMNs), the decidual neutrophils expressed high levels of neutrophil activation markers and the angiogenesis-related proteins: vascular endothelial growth factor-A, Arginase-1, and CCL2, similarly shown in tumor-associated neutrophils. Functional in vitro assays showed that second-trimester human decidua conditioned medium stimulated transendothelial PMN invasion, upregulated VEGFA, ARG1, CCL2, and ICAM1 mRNA levels, and increased PMN-driven in vitro angiogenesis in a CXCL8-dependent manner. This study identified a novel neutrophil population with a physiological, angiogenic role in human decidua. The Journal of Immunology, 2014, 193: 000–000.

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Abbreviations used in this article: ARG1, Arginase-1; Ct, cycle threshold; DCM, decidual conditioned medium; dN, decidual neutrophil; dNK, decidual NK; EBM-2, endothelial basal medium; EVT, extravillous trophoblast; gd, gestational day; NE, neutrophil elastase; PMN, peripheral blood neutrophil; SFM, serum-free control RPMI medium; TAN, tumor-associated neutrophil, USMC, uterine microvascular endothelial cell; VEGF-A, vascular endothelial growth factor-A.

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dNK cell and macrophage dominance. In the second trimester, dominance is skewed toward CD4 Th cells, regulatory T cells, and a population of alternatively activated M2 macrophages (4, 18). In this study, we describe a novel tissue resident neutrophil population appearing in the second trimester.

Neutrophils are a major component of the innate immune system. They are short-lived cells equipped with numerous antibacterial effector mechanisms. Usually neutrophils do not reside in healthy peripheral tissues; rather, they are recruited from the circulation to sites of infection by tissue resident macrophages that have encountered a pathogen (19). Interestingly, inflammatory diseases such as chronic obstructive pulmonary disease are known to harbor organ-infiltrated neutrophils that express different chemokine receptors than peripheral blood neutrophils (PMNs) (20).

This has suggested a role for chemokines in mediating neutrophil recruitment and differentiation within inflamed tissue (20). Indeed, the chemokines CXCL8 (formerly known as IL-8) and CCL5 (formerly known as RANTES) are known to be potent activators of neutrophil recruitment (21, 22).

Little is known regarding the role of neutrophils in human reproduction. It is postulated that neutrophils, chemotrafficked from the circulation by endometrial secretion of CXCL8, function in the vascular breakdown leading to shedding of the endometrium during menstruation (23–25). Milne et al. (26) showed neutrophil infiltrate into human decidua after administration of a progesterone antagonist (RU-486) and concluded that neutrophil accumulation was responsible for the vascular breakdown and decidual shedding during pharmacological and, perhaps, natural miscarriage. In contrast, several authors have shown that, similar to the M2 tumor-associated macrophages (27), infiltrated neutrophils can have angiogenic properties and have a role in the late stages of tumor progression through enhancement of angiogenesis and vascular remodeling (28, 29). Recently, two tumor-associated neutrophil (TAN) populations were identified in a murine tumor model: N1 antitumorigenic neutrophils, with cytotoxic and immunostimulatory potential; and N2 protumorigenic neutrophils, with immunosuppressive and angiogenic properties, but lacking cytotoxic potential (30). Similarly, tissue resident neutrophils in nonpregnant human fallopian tube tissue are reported to be less cytotoxic than PMNs and have higher cytokine production, particularly of vascular endothelial growth factor (VEGF) (31).

We report the first description, to our knowledge, of a novel population of second-trimester decidual neutrophils (dNs). Under the influence of the decidual microenvironment, mainly decidual macrophages (27), infiltrated neutrophils can have angiogenic properties and have a role in the late stages of tumor progression through enhancement of angiogenesis and vascular remodeling (28, 29). Recently, two tumor-associated neutrophil (TAN) populations were identified in a murine tumor model: N1 antitumorigenic neutrophils, with cytotoxic and immunostimulatory potential; and N2 protumorigenic neutrophils, with immunosuppressive and angiogenic properties, but lacking cytotoxic potential (30). Similarly, tissue resident neutrophils in nonpregnant human fallopian tube tissue are reported to be less cytotoxic than PMNs and have higher cytokine production, particularly of vascular endothelial growth factor (VEGF) (31).

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Methods and Materials

Primary tissues

Decidual tissues were obtained following informed consent from healthy women undergoing elective termination of pregnancy between 6 and 20 wk of gestation. Peripheral blood from other healthy, ongoing second-trimester pregnant women (15–18 wk of gestation) was collected for some experiments. The study protocol was approved by the Morgantaler Clinic and the Research Ethics Board of Mount Sinai Hospital (Toronto, ON).

Isolation of primary decidual cells

Decidual tissue was collected after elective termination of pregnancy during the first (n = 10) and second trimesters (n = 14). In brief, the isolation protocol involved the tissue being washed extensively in HBSS with calcium and magnesium (HBSS+), and minced with sterile scalpels to pieces <1 mm3. Subsequently, the tissue was flushed repeatedly with HBSS+ using a wide-bore 25-ml pipette, to mechanically release cells that were collected, and then filtered through a 40-µm mesh. The cell suspension was incubated in erythrocyte lysis buffer (Qiagen) for 30 min at 4°C to decrease erythrocyte contamination, washed twice with HBSS+, and resuspended in serum-free RPMI 1640 (Invitrogen). Cells were cultured in 10 ml tissue culture plates in RPMI 1640 with 10% FBS for 30 min to remove decidual fibroblasts by plastic adherence. Nonadhering cells were then collected, washed by centrifugation (4°C, 400 × g), and resuspended in Dako protein free blocking solution (2 × 105 cells/sample; Dako) for flow cytometry.

Isolation of peripheral blood neutrophils

Blood samples from healthy second-trimester pregnant women were collected in Vacutainer sterile tubes containing K2 EDTA (7.2 mg/ml; Becton Dickinson) and were processed immediately. PMNs were isolated using a dual-density gradient separation kit (Histopaque 1119/1077; Sigma-Aldrich) according to the manufacturers’ protocol and centrifuged (30 min, 700 × g). Granulocytes were collected, resuspended, washed in HBSS+, and spun again (10 min, 700 × g). Purity of the PMN population was determined by flow cytometry. Samples having ≥95% purity were included in this study (n = 26 in total).

Endothelial cell culture

Normal human myometrial uterine microvascular endothelial cells (UtMECs; Clonetics, Lonza) were maintained in endothelial basal medium (EBM-2) with 5% FCS and endothelial growth medium supplement bullet kit (EGM-2) containing growth factors (hydrocortisone, human epidermal growth factor, VEGF, human fibroblast growth factor-B, R3-insulin growth factor-1, ascorbic acid, heparin, and gentamicin/amphotericin B; Lonza). Cells were grown to confluence in T25 flasks before passaging and were used for experiments between passages 4 and 9.

Decidual conditioned medium

Decidual conditioned media (DCM) were prepared as previously described (32). A constant second-trimester decidual tissue (15–18 wk of gestation)–to-medium ratio of 2.5 g/10 ml was used (n = 9). The decidual cells were plated in 10-cm2 tissue culture plates for 48 h at 8% O2/5% CO2 and 37°C. The cells were washed with warm HBSS+ before serum starvation for a further 48 h in serum-free RPMI medium containing 1/1000 Normocin (Invitrogen). 1 mM Hepes, 5 mM nonessential amino acids, and 5 mM sodium pyruvate (Wisent). After incubation, DCM were collected and spun at 4000 rpm for 10 min to remove cells, transferred to a new tube, and stored at −20°C for further use. Cell viability was routinely assessed at the end of the culture by trypan blue exclusion assay. No increase in number of dead cells was shown. To ensure standard conditions for all treatment groups per experiment, DCM from three samples were thawed on ice and pooled before use.

Flow cytometric analyses and gating strategy

PMNs and dNs were immunostained for flow cytometry according to the manufacturer’s instructions (BD Pharmingen). Preblocked cells were incubated for 45 min with an mAb mixture containing anti–CD145-allophycocyanin-Cy7, anti–CD15-allophycocyanin or anti–CD16-PF, anti–CD66b–FITC, and Abs to the chemokine receptors CD181-allophycocyanin, CD182-PE Cy5 or CD183-allophycocyanin, CD184-PE Cy5 or CD191-Alexa 647, and CD195-PE. All Abs were manufactured by Becton Dickinson and were titrated for optimal staining. After labeling, cells were washed and resuspended in a stabilizing fixative solution (Becton Dickinson), and stored at 4°C until analysis. Unstained cells were used to determine autofluorescence. Isotype control mixtures were established for each panel using isotype-matched, nonspecific Abs at the same concentration as the Ag-specific Abs. The PMN and dN populations were analyzed using either a FACSaria flow cytometer with FACS Diva software, version 6.1 (Becton Dickinson), or a Gallios flow cytometer with Kaluza software (Beckman Coulter). Flow cytometry data were collected as .FCS 3.0 data files and analyzed using both FlowJo (Tree Star) and Kaluza software (Beckman Coulter). Threshold gates were set on forward side scatter and CD45 fluorescence to exclude debris and non-leukocyte events. Positive leukocyte subpopulations were identified upon comparison of fully stained samples with Fluorescence Minus One and isotype controls.

Animal model

Uterine tissues from timed matings (gestational days [gd] 6, 8, 10 and 12, copulation plug counted as 0) BALB/cJc × BALB/cJ or Rag2−/− IL2rg−/− × Rag2−/− IL2rg−/− on the BALB/c background) were prepared at Queen’s University using perfusion fixation (4% paraformaldehyde in PBS) and...
standard paraffin embedding (33). Midline tissue sections of at least two implantation sites per mouse (n = 3 mice per day of gestation) were cut at 5 μM and placed on SuperFrost plus slides. The mice were housed under specific pathogen-free conditions and maintained on a 12/12-h light/dark cycle with food and water ad libitum. All mouse handling was in accordance with approved animal care protocols at Queen’s University.

Neutrophil transendothelial invasion assay

The ability of the decidua to stimulate PMN extravasation was tested using a transcellular endothelial invasion assay (n = 4). In brief, Transwell polycarbonate inserts with a 3-μm pore membrane (Becton Dickinson) were seeded with the UtMECs (1 × 10^5 cells/insert) and cultured (48 h, 37˚C, 5% CO2). The confluence of the endothelial cell monolayer was verified by testing their permeability to a trypan blue/BSA solution (3.6 mg: 80 mg; 10 ml HBSS) incubated at 37˚C for 10 min to yield a stable complex with maximum absorption at 590 nm. Confluent inserts were washed twice with warm medium and preincubated with the experimental conditions for 2 h. After isolation, PMNs were incubated (37˚C, 30 min, in the dark) in serum-free RPMI containing 2.5 μM calcein-AM (Biotium). The cells were twice washed with PBS and resuspended in serum-free RPMI. Prelabeled 4 × 10^5 neutrophils in serum-free RPMI were added to the endothelial cell–coated inserts and incubated under the same conditions for another hour. Migrated PMNs were then collected from the lower wells. The remaining monolayer cells were processed for the fluorescent intensity (FI) of the cells, as measured using an Infinite M200 microplate reader (TECAN US), excitation 488 nM, emission 525 nM. Results are presented as the percentage of cells migrating toward serum-free RPMI (baseline migration, arbitrary 100%).

Immunohistochemistry

First- and second-trimester human decidua tissues were fixed in 4% paraformaldehyde for 1 h and processed to paraffin blocks. For immunohistochemistry of human decidual tissue, 5-μm sections mounted on glass slides were deparaffinized in xylene and rehydrated through a gradient series of ethanol in PBS. Endogenous peroxidase activity was blocked (3% hydrogen peroxide in methanol, 40 min). Primary Abs (Table I) were diluted in PBS and incubated (overnight, 4˚C). Negative controls used nonimmune mouse IgG. Slides were washed in PBS and then incubated with either anti-mouse biotin Ab (1:300; Dako) or a combination of anti-mouse Alexa 488 Ab plus anti-rabbit biotin Ab (Dako) for 1 h. This was followed by further washes and 1-h incubation in streptavidin–HRP, or for dual immunofluorescence, streptavidin–Alexa 546 (Invi- trogen). Slides were washed in PBS and developed in 0.075% (v/v) 3,3-diaminobenzidine in water containing 0.002% (v/v) H2O2 (Vector Laboratories). After counterstaining with hematoxylin or nuclear staining with DAPI (Sigma), slides were dehydrated and covered. 3,3-Diaminobenzidine images were captured using an Olympus BX61 upright, motorized microscope with Olympus DP72 digital color camera operated with CellSens Standard proprietary acquisition software (Olympus Canada, Markham, ON). Dual-fluorescence images were captured using the Quorum Wave FX spinning disc confocal system comprising a Leica DMI 6000B microscope with a Yokogawa Spinning Head, Image EM Hame matsu EMCCD camera, and Velocity imaging software. To eliminate signal bleed through between the fluorophores used (Alexa-488 and Alexa-546), we captured images using a spectral separation protocol (Velocity).

Image analysis

Quantification of numbers of dNs in sections of second-trimester human decidua basalis (n = 12) and parietalis (n = 11) and murine uterine tissues (decidua and mesometrial triangle days 6–12, n = 6 per day of gestation) was performed using newCAST software (Visiopharm). Counts were performed using a standard protocol that assigned random counting frames covering 5% of the total mapped tissue area. A positively stained ratio was generated by dividing the numbers of positively stained cells by the total number of negative cells.

Reverse transcription real-time SYBR green PCR analysis

RNA was extracted using TRIzol LS (Invitrogen) from second-trimester PMNs cultured under experimental conditions for 5 h at 8% O2, 5% CO2, 37˚C (n = 4 in each group). Genomic DNA contamination was eliminated using DNase I and the RNA concentrated using an RNeasy Minelute kit (Qiagen). A total of 0.5 μg RNA was used to synthesize single-strand cDNAs in a total reaction volume of 20 μl using the I Script cDNA synthesis kit (Bio-Rad). Real-time SYBR green PCR was performed to detect the mRNA expression levels of ICAMI, VEGFA, CCL2, and ARG1. The thermal cycling parameters of the I Script reverse tran-
scription were primer incubation (25˚C, 10 min), reverse transcription (42˚C, 30 min), and reverse transcriptase inactivation (95˚C, 5 min). Ten nanograms of each cDNA was subjected to real-time PCR using specific sets of primers for the genes of interest and two housekeeping genes YWHAZ and SDHA (see Table II for primer sequences) in a 10-μl reaction volume containing 3 μM primers and SYBR green PCR mix (Sigma).

Real-time PCR was performed in a Bio-Rad CFX96 real-time PCR system using the run protocol: heat activation of Taq and denaturation (95˚C, 2 min) followed by 40 cycles of amplification (95˚C, 10 s and 60˚C, 30 s). After PCR, a dissociation curve was constructed by increasing temperature from 65˚C to 95˚C for detection of PCR product specificity. A cycle threshold (Ct) value was recorded for each sample. PCRs were conducted in triplicates and the mean of the three Cts was calculated. An arithmetic formula from the comparative Ct method was applied to the average raw Ct values to extract relative gene expression data in the treated samples as compared with the respective control samples. The mRNA level of the gene of interest from each sample was normalized to the geometric mean of the housekeeping genes mRNA levels. Validation experiments were performed to ensure the PCR efficiencies between all primer sets were approximately equal.

In vitro angiogenesis

Isolated PMNs were incubated under experimental conditions for 5 h. Neutrophils were then collected and centrifuged (700 × g for 5 min) and resuspended in EBM-2 5% FCS, and 50,000 cells/well were added to matrigel (Becton Dickinson)-coated wells containing 50,000 UtMECs (n = 4). After 16 h of incubation, photographs were captured using a Leica DMIL LED inverted microscope (Leica) with micropublisher camera 5.0 RTV (Q Imaging). Angiogenesis was assessed by measurement of tube lengths between branch points, and the number of branch points using Angiogenesis analyzer software from Image J (http://image.bio.methods.free.fr/ImageJ/Angiogenesis-Analyzer-for-ImageJ).

Statistical analysis

Statistically significant differences between dN and PMN groups were determined by independent unpaired t test. Differences within dN and PMN groups and in experimental treatments were assessed by one-way ANOVA followed by Bonferroni t test. All data were analyzed using Prism software. Data are presented as mean ± SD. A p value <0.05 was considered significant. All experiments were repeated at least three times and performed in duplicate.

Results

Neutrophils in second-trimester decidua

Leukocytes from decidual tissue (6–20 wk) and from peripheral blood (different women at 15–18 wk gestation) were identified using multicolor flow cytometry. Decidual and blood neutrophils (dNs and PMNs, respectively) were identified by side and forward scatter and the relative expression of the granulocyte markers CD15 and CD66b in the CD45+ leukocyte population (Fig. 1). A significantly increased proportion of CD45+CD15+ neutrophils was present in second- as compared with first-trimester decidua (Fig. 1a, 1b). Numbers of dN increased most rapidly over weeks 11–15 of pregnancy and reached a maximum at 16–20 wk (Fig. 1c). dNs also stained strongly for the mature infiltrated neutrophil marker CD66b (Fig. 1d) and showed a much higher mean fluorescent intensity than PMNs (Fig. 1e). To eliminate potential blood, and thus PMN contamination of second-trimester decidua samples, we also assessed the NK cell subpopulations in each sample. Only samples with a clear decidua dNK cell distribution (>95% CD56brightCD16low) were used for further study (Fig. 1f).

dNs are restricted to the midgestational decidua basalis

Immunostaining for the neutrophil markers neutrophil elastase (NE) and CD66b confirmed the presence of resident neutrophils within second-trimester decidua (n = 23 wk 12–19 specimens) (Abs used for immunohistochemistry are detailed in Table 1). Neutrophils were usually localized to discrete areas of decidua stroma (Fig. 2a), often close to a decidual spiral artery (Fig. 2b). Occasionally, clusters or aggregates of neutrophils were seen.
within the decidual stroma (Fig. 2b). In support of our hypothesis of PMN recruitment by the decidua, neutrophils were also observed adhered to venous endothelium and traversing the vascular wall (Fig. 2c, 2d). Of 23 decidual samples, only 14 were reactive for neutrophils. Using the presence or absence of EVT to separate decidua basalis from parietalis, neutrophils were present in 12 decidua basalis and 2 decidua parietalis samples. No neutrophils were present in the remaining nine parietalis samples. Dual-fluorescent immunohistochemistry colocalized NE in the dN (green) and CK-8 in EVT (red) in the decidua basalis samples (Fig. 2e). The dual reagent negative control showed no staining (Fig. 2f). Quantification was performed according to stereological principles to assess the number of positive NE-stained decidual cells. More dNs were present in decidua basalis as compared with parietalis (decidua basalis 9.90 ± 0.65% versus parietalis 1.01 ± 0.6%, p < 0.0001).

**FIGURE 1.** A mature dN population accumulates in the second-trimester decidua. Multicolor flow cytometry of decidual-derived leukocyte populations identified a CD45+ CD15+ neutrophil population that increased in the second trimester. Representative pseudo-dot plots of first- (7 wk, a) and second-trimester (19 wk, b) CD45+ CD15+ DN population. Numbers of dNs increased as gestation progressed from 6–10 to 11–15 and 16–20 wk (n = 10 in each group, c). (d) Representative pseudo-dot plot demonstrating high CD66b+-expressing dNs. (e) Comparison of mean fluorescence intensity (MFI) for CD66b between dN and pregnant PMN populations. (f) Only decidual samples that displayed a clear dNK distribution >95% CD56brightCD16low were used to exclude potential peripheral blood contamination. **p < 0.01, ***p < 0.0001.

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Chemokine receptor profiles and CXCL8 function in dNs and PMNs

To assess potential differences between second-trimester dNs and PMNs, we conducted flow-cytometric analyses of surface chemokine receptors. In comparison with second-trimester PMNs, second-trimester CD45+CD66b+ dNs downregulated surface expression of the CXCL8 receptors CD181 and CD182 (CXCR1 and CXCR2; 3-fold reduction, \( p < 0.0001 \)). Conversely, CD183 (CXCR3), CD184 (CXCR4), CD191 (CCR1), and CD195 (CCR5), which respectively bind CXCL10, CXCL12, CCL2-5 chemokines present in decidua, were expressed by more dNs (13.6–39.8% of the total CD66b population) than PMNs (3.8–8.5%, \( p < 0.012 \) in all cases, \( n = 3 \); Fig. 3a, 3b). Using an in vitro invasion assay, second-trimester DCM stimulated PMN invasion of a monolayer of UtMECs (\( p < 0.0001 \); Fig. 3c). DCM-stimulated invasion was 60% inhibited by anti-CXCL8 neutralizing Ab (\( p < 0.0001 \)). CXCL8 was used as a positive control and stimulated a similar extent of invasion, but its effect was completely neutralized by the anti-CXCL8 Ab. Thus, decidual CXCL8 could be a primary recruiting stimulus for PMN into the decidua.

Defective dN recruitment in Rag2\(^{-/-}\)IL2rg\(^{-/-}\) mice

To investigate the potential roles of other decidual immune cells in the recruitment and function of dNs, we compared implantation sites between barrier-raised normal and Rag2\(^{-/-}\)IL2rg\(^{-/-}\) mice that lack NK, T, and B cells with defective spiral arterial angiogenesis (34). We first investigated the distribution of neutrophils in gd6–12 BALB/c decidua and mesometrial triangle using immunodetection of LY6G (GR1)-positive cells. LY6G\(^{+}\) neutrophils were present in mouse decidua and reached peak levels during the

**FIGURE 2.** dNs infiltrate the decidua basalis. Representative photomicrographs of immunohistochemical staining of dN in the second-trimester decidua stained with NE (a, c, and d) or CD66b (b). dNs were observed distributed across the decidua (a) and also in clusters (b). DNs were often observed in association with decidual blood vessels, attached to endothelial cells and extravasating from the circulation into the decidua (c and d, arrows). (c, inset) Rabbit IgG negative control. (e) Dual-fluorescent immunohistochemistry showed that dNs stained with NE (green) localized to decidua basalis as shown by co-staining of EVT cells with cytokeratin 8 (CK8, red). (f) Negative control dual nonimmune guinea pig IgG and mouse IgG. Scale bars, 250 \( \mu m \) (a and c), 100 \( \mu m \) (b), 25 \( \mu m \) (d–f).

**FIGURE 3.** Differential chemokine receptor expression in dNs and PMNs. dNs were isolated from second-trimester decidual samples and PMNs from peripheral blood collected from pregnant women in the second trimester (weeks 15–18). Multicolor flow cytometry of the neutrophil populations was performed to assess cell-surface expression of the CXCL8 receptors CD181 and CD182 (CXCR1 and CXCR2; 3-fold reduction, \( p < 0.0001 \)). Conversely, CD183 (CXCR3), CD184 (CXCR4), CD191 (CCR1), and CD195 (CCR5), which respectively bind CXCL10, CXCL12, CCL2-5 chemokines present in decidua, were expressed by more dNs (13.6–39.8% of the total CD66b population) than PMNs (3.8–8.5%, \( p < 0.012 \) in all cases, \( n = 3 \); Fig. 3a, 3b). Using an in vitro invasion assay, second-trimester DCM stimulated PMN invasion of a monolayer of UtMECs (\( p < 0.0001 \); Fig. 3c). DCM-stimulated invasion was 60% inhibited by anti-CXCL8 neutralizing Ab (\( p < 0.0001 \)). CXCL8 was used as a positive control and stimulated a similar extent of invasion, but its effect was completely neutralized by the anti-CXCL8 Ab. Thus, decidual CXCL8 could be a primary recruiting stimulus for PMN into the decidua.

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time of spiral artery angiogenesis (gd8–10; p = 0.0001; Fig 4a). LY6G+ neutrophils were localized surrounding the developing uterine spiral arteries of the mesometrial triangle, suggesting an active role in angiogenesis (Fig. 4b, 4c, black arrows). The same stereological assessment of LY6G+ neutrophil numbers was then undertaken in BALB/c Rag2−/− IL2rg−/− mice. Very few neutrophils were seen in the mesometrial triangle on any day of pregnancy (Fig. 4a, 4e, 4f), although PMNs were observed in decidual blood vessels attached to the vascular endothelium (Fig. 4g, yellow arrow). The red lines demarcate the increase in vascular wall thickness of the spiral arteries in the Rag2−/− IL2rg−/− mice. Negative controls using a rat IgG showed no specific staining of neutrophils in serial sections of Balb/c day 10 mice (Fig. 4d).

CXCL8 drives the differentiation of an angiogenic dN population

Dual-fluorescent immunohistochemistry on human decidua basalis samples revealed that dNs (green) express the angiogenic factors VEGF-A (Fig. 5a), Arginase-1 (ARG1; Fig. 5b), and CCL-2 (Fig. 5c, red). These molecules are known to be increased in tumor-associated N2 cells in mice (30). Importantly, PMNs within the decidual blood vessels did not demonstrate angiogenic factor immunostaining in these same sections (Fig. 5d inset). Staining was absent from the dual negative controls (Fig. 5d).

We next assessed the potential peripheral blood origin of dNs and the role of CXCL8 in PMN differentiation. PMNs were isolated from blood collected from second-trimester women (weeks 15–18) and cultured with either serum-free control RPMI medium (SFM) or second-trimester DCMs ± the neutralizing anti-CXCL8 Ab for 5 h. RNA was isolated, and expression of the angiogenic growth factors listed earlier along with levels of ICAM1 were quantified by real-time PCR (primer sequences for genes of interest are listed in Table II). The PMNs cultured with DCM had a significant increase in the relative expression of VEGFA (127-fold), CCL2 (545-fold), ARG1 (5-fold), and ICAM1 (5-fold) mRNA levels as compared with control medium cultured PMNs (p < 0.0001). In all cases, addition of the anti-CXCL8 Ab significantly abrogated the DCM-mediated increase in gene expression (VEGFA 50.0 ± 8.0%; CCL2 41.1 ± 10.2%; ARG1 46.8 ± 6.9%; and ICAM1 79.0 ± 7.7%; p < 0.01). Lastly, to assess the angiogenic capability of DCM-stimulated PMNs, we performed in vitro angiogenesis assays using the UtMEC line. PMNs stimulated as described earlier were collected, suspended in EBM-2 5% FCS and added to UtMECs cocultured with PMNs preincubated in SFM displayed tube formation and early sprouting angiogenesis (Fig. 5e). However, in UtMECs cocultured with PMNs preincubated with DCM, extensive well-branched networks with a more mature vascular phenotype including lumen formation were seen (Fig. 5f). Tube length was significantly increased in comparison with SFM control (p < 0.0001; Fig. 5i). Addition of the neutralizing anti-CXCL8 Ab to the DCM treatment significantly reduced network formation and tube length as compared with DCMs alone (p < 0.0001), but had little effect on branch points (p < 0.05; Fig. 5g, 5i). When recombinant human CXCL8 (100 ng/ml) was added to the SFM control, PMNs were able to stimulate robust angiogenesis similar to that observed with DCM (Fig. 5h, 5i).

Discussion

Although numerous studies have demonstrated active roles for maternal decidual leukocytes in early placentation, this study shows for the first time, to our knowledge, that the second-trimester decidua harbors a novel population of angiogenic N2-like neutrophils. This observation suggests that dNs may have a physiological, rather than immunological, role in the later stages of uterine vascular remodeling. Supportive of this finding are the reports of the role of PMNs in wound healing and tumor growth and invasiveness that have been documented (35–37), in addition to their classical roles in pathogen defense. It is likely that the second-trimester dNs differentiate from PMNs recruited from the maternal circulation, as they increase in number during weeks 11–
15 of gestation. This is the same period when the intervillous space opens to the maternal uterine blood flow (38). This is further supported by our observation of adherent neutrophils extravasating from the maternal circulation into the decidual stroma. The preferential localization of the dNs at this time was within the decidua basalis (the site of placental implantation). The dNs also expressed much higher levels of the mature neutrophil marker CD66b than the PMNs, suggesting a more mature/differentiated phenotype. This is analogous to the maturation of the dNK cells, which show an increase in cell size and granularity in comparison with peripheral NK cells. The infiltration of dNs, in association with the decidual spiral arteries, was also seen in the mesometrial triangle of pregnant control BALB/c mice where an increase in dN number was observed at gd8–10. This time corresponds to the period of spiral arterial angiogenesis in the mouse, as well as the peak of dNK cell numbers (39, 40). This is of relevance as interactions between NK cells and neutrophils are known to significantly extend the neutrophil life span (41). Further evidence of the role of the dN population and interactions with dNK cells in spiral artery remodeling was provided by our immunohistochemical analysis of Rag2−/−IL2rg−/− mice. dNs were absent from the decidual tissue in these mice, although neutrophils were present in

**FIGURE 5.** dN- and DCM-treated PMNs are angiogenic. (a–d) Representative photomicrographs of dual fluorescent immunohistochemistry demonstrating colocalization of VEGF-A (a), ARG1 (b), and CCL2 (c) (red) with dN (green) in second-trimester decidua basalis sections (n = 5). (a–c, insets) Original magnification ×3 of the area marked by the dashed lines. (d) Dual negative control mouse and rabbit IgG. (d, insert) PMNs in blood vessels do not express ARG1. Scale bars, 25μm. DCM drives PMN-mediated in vitro angiogenesis. Control SFM-treated PMNs stimulate early sprouting angiogenesis of the UtMEC endothelial cell line (e). In contrast, DCM-stimulated PMNs stimulate the growth of an extensive mature vascular network (f), whereas addition of a neutralizing anti-CXCL8 Ab (0.5μg/ml) inhibits endothelial extension and results in an immature network (g). Recombinant human CXCL8 (100 ng/ml)-treated neutrophils also stimulate the formation of a well-branched vascular network (h). All images were captured at original magnification ×40. Quantification of branch points and tube length is shown in (i). n = 4, p < 0.0001 between a and b, p < 0.05 between b and c.
the lumen of the blood vessels. This defective dN recruitment may be associated with the absence of dNK cells, and thus the absence of recruiting stimuli for dNs. Alternatively, dNK cells may be required for differentiation of mature decidual stromal cells, which are required for the recruitment of PMNs to the decidua. The lack of dNs in these alymphoid mice may be a previously unsuspected contributor to the absence of their spiral artery remodeling (fewer and smaller vessels and retention of a vascular smooth muscle cell wall) (34). These findings suggest an interaction between dNK cells and neutrophils in uterine vascular remodeling.

CXCL8 (IL-8) is a known potent stimulus of PMN invasion and activation (42). As reported by others and confirmed in our study, PMNs, even those of women at midgestation, express high levels of the CXCL8 receptors CD181 and CD182, and do not express appreciable levels of other chemokine receptors (43). In contrast, only 20–30% of the CD45<sup>+</sup>CD66<sup>b</sup> dN population expresses CD181 and CD182. Flow cytometry analysis of neutrophils found in nonpregnant fallopian tube tissue has similarly revealed decreased expression of CXCR1 and CXCR2 in comparison with PMNs (31). This downregulation of CD181 and CD182 may occur as a result of the internalization of the receptor after CXCL8 ligand stimulation (44) or because of the emergence of a unique dN population that dilutes the CD181/182-expressing cell population. In this study, our functional assays utilizing a neutralizing anti-CXCL8 Ab demonstrated a critical role for decidual derived CXCL8 in PMN transendothelial invasion and differentiation to a proangiogenic dN phenotype. Treatment of isolated PMNs with DCM upregulated the mRNA expression of ICAM1, VEGFA, ARG1, and CCL2 and was blocked by addition of the anti-CXCL8 Ab. We confirmed that these proteins were expressed in vivo by dNs in decidua basalis. The increase in ICAM1 supports the role of the decidual CXCL8 in stimulating the activation and recruitment of the PMNs. These results also correlate well with the upregulation of VEGF and ARG1 that was reported in TAN in mice (30) and demonstrate the plasticity of the pregnant PMN population. Interestingly, ARG1 produced by myeloid-derived suppressor cells is known to inhibit T cell cytotoxicity by depleting intercellular arginine levels (45). Hence the dNs may also contribute to the maternal immune tolerance toward the semiallogeneic fetus.

We further demonstrated that PMNs stimulated by DCM promoted robust in vitro angiogenesis of UtMECs. Interestingly, in the in vitro angiogenesis assays, the PMNs accumulated on and around the forming endothelial tubes, and the DCM-treated PMNs were particularly dense at the branch points. A similar interaction of nonpregnant human PMNs with bovine endothelial cells has been reported to promote endothelial attachment to extracellular matrix (46). When the anti-CXCL8 Ab was added to the DCM pre-treatment, we observed a significant decrease in endothelial tube length and in total network length, indicating a more immature network. CXCL8 is expressed at high levels by numerous cell types in the human decidua including dNK, decidual stromal cells, and CD8 T cells (47). However, we have recently shown that only 5% of all second-trimester human dNK cells express CXCL8 (7). This suggests that decreased CXCL8 via either defective decidual stromal cell function or the absence of T cells in the Rag2<sup>–/–</sup> Il2rg<sup>–/–</sup> mice may underlie the observed lack of dNs. In summary, these results show that decidual-derived CXCL8 promotes the differentiation of PMNs to angiogenic tissue-infiltrated dNs.

Through neutrophil depletion studies in mice, TANs are known to promote tumor growth, increase metastasis, and increase endothelial recruitment to tumors (48–50). Although trophoblast invasion and transformation of the maternal spiral arteries shares some commonality with tumor genesis, it is a tightly regulated process that limits the invasive trophoblast to the superficial decidua and the first 3 mm of the myometrial vessels (14). We contend that the dNs may play a physiological role in the late stages of second-trimester decidual vascular transformation. They may also contribute to the reported re-endothelialization of the vessel (51). Similar processes have been suggested in the stabilization of murine endometrial blood vessels after menstruation. Neutrophils expressing VEGF are observed in association with angiogenic endothelial cells, and depletion of neutrophils retards endometrial repair (52).

Interestingly, the downregulation in CD181 and CD182 on dNs was inversely correlated with an increase in the numbers of dNs expressing CD183, CD184, CD191, and CD195. This chemokine receptor profile is similar to that reported for disease-associated infiltrated neutrophil populations in chronic obstructive pulmonary disease and rheumatoid arthritis (20). Thus, some disease states may mimic the physiological inflammation associated with pregnancy. Numerous cytokines and chemokines, including GM-CSF, IFN-γ, and TNF-α, are produced by the decidua (53, 54). In tumours, these cytokines and growth factors are known to up-regulate neutrophil expression of CD191 and CD195, and to prime neutrophil migration toward CC chemokines, such as CCL2-5 (55–57). Because decidua, dNK cells, and ETV also produce CCL2/4/5 (58, 59), in addition to the CD183 ligand CXCL10 (5) and the CD184 ligand CXCL12 (60), we suggest that these factors additionally contribute to the recruitment of specific neutrophils and to their functions in the second-trimester decidua basalis when the decidua contains EVT and transmigration of murine dNK cells, and thus the absence of recruiting stimuli for dNs. Alternatively, dNK cells may be required for differentiation of mature decidual stromal cells, which are required for the recruitment of PMNs to the decidua. The lack of dNs in these alymphoid mice may be a previously unsuspected contributor to the absence of their spiral artery remodeling (fewer and smaller vessels and retention of a vascular smooth muscle cell wall) (34). These findings suggest an interaction between dNK cells and neutrophils in uterine vascular remodeling.

Table II. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence Accession no.</th>
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<tr>
<td>VEGFA forward</td>
<td>5′-CCGGCCTCCGAAACCATGAACTT-3′</td>
</tr>
<tr>
<td>VEGFA reverse</td>
<td>5′-CCCCCTCTCTCTTTGGACTGTT-3′</td>
</tr>
<tr>
<td>ARG1 forward</td>
<td>5′-ATTGGGCTCCCTCAGCATTT-3′</td>
</tr>
<tr>
<td>ARG1 reverse</td>
<td>5′-AGTGTGATGCTGAAAGTTATG-3′</td>
</tr>
<tr>
<td>CCL2 forward</td>
<td>5′-TTCATGCCCAAGGGGCTGTCGA-3′</td>
</tr>
<tr>
<td>CCL2 reverse</td>
<td>5′-ACTTTTTGTACATTGGCCCTCAAA-3′</td>
</tr>
<tr>
<td>ICAM1 forward</td>
<td>5′-GACCAGAGGAGACAGGGAAC-3′</td>
</tr>
<tr>
<td>ICAM1 reverse</td>
<td>5′-TGGGGCGGCGGGAAGCTCAG-3′</td>
</tr>
<tr>
<td>YWAH reverse</td>
<td>5′-CGGACCAGGACAAACAGATAT-3′</td>
</tr>
<tr>
<td>SDHA forward</td>
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</tr>
<tr>
<td>SDHA reverse</td>
<td>5′-CCACCCGATGCATAATCGAG-3′</td>
</tr>
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8 CXCL8 MEDIATES DECIDUAL NEUTROPHIL DIFFERENTIATION
In conclusion, we have shown that a novel population of angiogenic N2-like neutrophils is recruited to the second-trimester decidua. This is the first comprehensive description of a physiologically, rather than immunological or pathological, role for neutrophils. Our data also show that decidua secretes CXCL8 and other factors that can drive PMN recruitment and differentiation to a proangiogenic phenotype. These results suggest that the dNs may be a good candidate for further studies investigating their potential contribution to the failed uterine vascular transformation in aberrant placentation leading to pre-eclampsia and intrauterine growth restriction.

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Disclosures
The authors have no financial conflicts of interest.

References

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