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THE IMMUNOPHENOTYPE OF DECIDUAL MACROPHAGES IN ACUTE ATHEROSIS

Navleen Gill[1,2,*], **Yaozhu Leng**[1,2,*], **DR Roberto Romero**[1,3,4,5], **Yi Xu**[1,2], **Bogdan Panaitescu**[1,2], **Derek Miller**[1,2], **Afrah Arif**[2], **Salma Mumuni**[2], **Faisal Qureshi**[1,6], **Chaur-Dong Hsu**[2,7], **Sonia S. Hassan**[1,2,7], **Anne Cathrine Staff**[8,9], and **DR Nardhy Gomez-Lopez**[1,2,10]

[1]Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services, Bethesda, Maryland, and Detroit, Michigan, USA

[2]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan, USA

[3]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan, USA

[4]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, Michigan, USA

[5]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA

[6]Department of Pathology, Hutzel Women's Hospital, Wayne State University School of Medicine, Detroit, Michigan, USA

[7]Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan, USA

[8]Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Norway

[9]Division of Obstetrics and Gynecology, Oslo University Hospital, Norway

[10]Department of Immunology, Microbiology and Biochemistry, Wayne State University School of Medicine, Detroit, Michigan, USA

Abstract

Problem: Acute atherosis is a uteroplacental arterial lesion that is associated with pregnancy complications such as preeclampsia and preterm birth, the leading cause of perinatal morbidity and mortality worldwide. However, the immunobiology of acute atherosis is poorly understood.

Method of study: Placental basal plate samples were collected from women who delivered with (n = 11) and without (n = 31) decidua basalis lesions of acute atherosis. Multi-color flow cytometry was used to quantify M1- and M2-like macrophage subsets and the expression of iNOS

Address correspondence to: Nardhy Gomez-Lopez, PhD, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Perinatology Research Branch, NICHD/NIH/DHHS, 275 E. Hancock, Detroit, Michigan 48201, USA, Tel (313) 577-8904, nardhy.gomez-lopez@wayne.edu; ngomezlo@med.wayne.edu.

*NG and YL contributed equally to this work.

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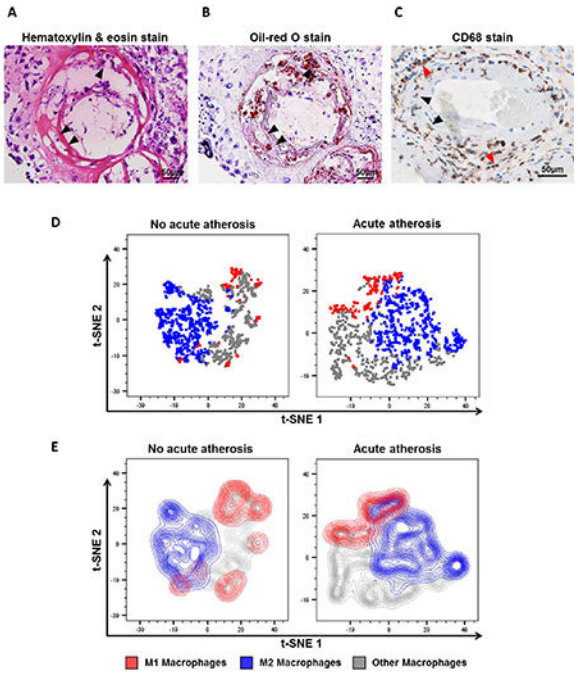
and IL-12 by decidual macrophages. Multiplex fluorescence staining and phenoptics were performed to localize M1-, MOX-, and Mhem-like macrophages in the decidual basalis.

Results: Macrophages displayed diverse phenotypes in the decidua basalis with acute atherosis. M2-like macrophages were the most abundant subset in the decidua; yet, this macrophage subset did not change with the presence of acute atherosis. Decidual M1-like macrophages were increased in acute atherosis, and such macrophages displayed a pro-inflammatory phenotype, as indicated by the expression of iNOS and IL-12. Decidual M1-like pro-inflammatory macrophages were localized near both transformed and non-transformed vessels in the decidua basalis with acute atherosis. MOX and Mhem macrophages were also identified near transformed vessels in the decidua basalis with acute atherosis. Finally, monocyte-like cells were present on the vessel wall of non-transformed decidual vessels, indicating a possible intravascular source for macrophages in acute atherosis.

Conclusions: These findings provide a molecular foundation for future mechanistic inquiries about the role of pro-inflammatory macrophages in the pathogenesis of acute atherosis.

GRAPHICAL ABSTRACT



Keywords

inflammation; innate immunity; monocytes; phenoptics; preeclampsia; preterm labor; spiral artery; pregnancy

INTRODUCTION

During normal pregnancy, the spiral arteries in the placental bed undergo a physiologic transformation into dilated, thinly-walled, less tortuous vessels capable of providing increased blood flow to the placental intervillous space[1–3]. However, in a subset of pregnant

women the spiral arteries fail to undergo complete transformation[4, 5], and such vessels are susceptible to the development of uteroplacental lesions such as acute atherosis[6–12].

Acute atherosis, named due to its histologic similarity to the atheromatous changes observed in atherosclerosis[13], was first described in patients with preeclampsia[14] and is associated with multiple pregnancy complications[4, 12, 15–25] including preterm labor[23]. Acute atherosis has been described in the spiral arteries of the decidua underlying the placenta, termed the decidua basalis, and in the decidua parietalis (underlying the fetal chorioamniotic membranes)[2, 23], as well as in myometrial tissues[26, 27]. Such lesions are characterized by the presence of 1) fibrinoid necrosis of the vessel wall, 2) perivascular lymphocytic infiltration, and 3) accumulation of foam cells[6–11, 13, 14, 21, 28, 29]. The foam cells detected in the decidual tissues from women with acute atherosis have been assumed to be mostly lipid-laden macrophages[30]; however, other non-foamy macrophages are detected in and around these vessels. The phenotype and function of these macrophages have not been described.

Decidual macrophages contribute to local immune tolerance and play a central role throughout pregnancy[31–41]. The classical paradigm described a linear range of macrophage polarization towards either a pro-inflammatory phenotype with high phagocytic/anti-tumor capability (M1 macrophages)[42–48] or a homeostatic phenotype involved in tissue repair and resolution of inflammation (M2 macrophages)[45–47, 49–57]. However, the majority of decidual macrophages do not fit the M1-M2 paradigm[35, 58]. Rather, most of the decidual macrophages display a unique, M2-like phenotype throughout pregnancy[31, 32, 34, 36–40, 58–64]. Decidual macrophages from women who underwent spontaneous labor at term or preterm labor, however, exhibit an M1-like pro-inflammatory phenotype[58]. These findings suggest that the decidual microenvironment alters the phenotype of infiltrating macrophages. Of particular interest, acute atherosis is likely associated with decidual inflammation[65, 66]. Therefore, we hypothesized that macrophages from the decidua basalis with acute atherosis display a pro-inflammatory phenotype that is different from those tissues without this arterial lesion.

The aim of this study was to determine the immunophenotype of macrophages in the decidua basalis with acute atherosis.

MATERIALS AND METHODS

Human subjects, clinical specimens, and definitions

Human placental basal plate samples (maternal side of the placenta, decidua basalis) were obtained at the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services (NICHD/NIH/DHHS), Wayne State University (Detroit, MI, USA), and the Detroit Medical Center (Detroit, MI, USA). The collection and utilization of biological materials for research purposes were approved by the Institutional Review Boards of Wayne State University and NICHD. All participating women provided written informed consent. The study groups included women with (n = 11) and without (n = 31) decidua basalis lesions of acute atherosis. Decidua basalis samples from subsets of these women were used separately in different experiments. The

demographic and clinical characteristics of the study population are shown in Table 1. Among women with acute atherosis, 45.5% (5/11) had preeclampsia compared to 16.1% (5/31) of women without this lesion (Table 1), which is consistent with a previous report[24].

Placental histopathological examination

Placentas were examined histologically by a perinatal pathologist blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols[23, 24, 67]. Briefly, three to nine sections of the placenta were examined, and at least one full-thickness section was taken from the center of the placenta; others were taken randomly from the placental disc. Acute atherosis was diagnosed in the decidua basalis by the presence of fibrinoid necrosis of the spiral artery wall with lipid-laden macrophages in the vessel wall and a perivascular lymphocytic infiltrate[23, 24, 67].

Isolation of decidual leukocytes

Decidual leukocytes were isolated from the decidua basalis, as previously described[68]. Briefly, the decidual tissue was homogenized using a gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA) in StemPro Accutase Cell Dissociation Reagent (Life Technologies, Grand Island, NY, USA). Homogenized tissues were incubated for 45 min at 37°C with gentle agitation. After incubation, tissues were washed in sterile 1X phosphate-buffered saline (PBS) (Life Technologies) and filtered through a 100μm cell strainer (Falcon, Corning Life Sciences, Inc., Durham, NC, USA). The resulting cell suspension was centrifuged at 300 x g for 10 min at 4°C. Decidual leukocytes were then separated by density gradient using the reagent Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden), following the manufacturer's instructions. Cells collected from the mononuclear layer of the density gradient were washed with 1X PBS and immediately used for immunophenotyping.

Immunophenotyping of decidual macrophages

Isolated decidual mononuclear cells were incubated with 20μl of human FcR blocking reagent (Miltenyi Biotec) in 80μl of stain buffer (Cat#554656, BD Biosciences, San Jose, CA, USA) for 10 min at 4°C. The cells were then incubated with fluorochrome-conjugated anti-human monoclonal antibodies (Supplementary Table 1) for 30 min at 4°C in the dark. After extracellular staining, the cells were washed with 1X PBS to remove excess antibody, resuspended in 0.5 mL stain buffer, and acquired using the BD LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva 6.0 software (BD Biosciences). For intracellular immunophenotyping, following extracellular staining, the cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences). Next, the cells were washed with 1X BD Perm/Wash Buffer (BD Biosciences), re-suspended in 50μL of the same buffer, and stained with intracellular antibodies (Supplementary Table 1) for 30 min at 4°C in the dark. Finally, the stained cells were washed with 1X BD Perm/Wash Buffer, re-suspended in 0.5 mL stain buffer, and acquired using the BD LSR II Flow Cytometer and BD FACSDiva 6.0 software. The absolute number of cells was determined using CountBright absolute counting beads (Molecular Probes, Eugene, OR, USA). The analysis and figures were performed using the FlowJo software version 10 (FlowJo, LLC, Ashland, OR, USA). ICAM-3 is downregulated in decidual

macrophages compared with blood monocytes[34, 36]; therefore, we characterized macrophages within the ICAM-3$^-$ gate.

Oil-red O staining

Decidua basalis tissues from the maternal site of the placenta were embedded in Tissue-Tek optimum cutting temperature (OCT) compound (Miles, Elkhart, IN, USA) and snap-frozen in liquid nitrogen. Eight-μm-thick sections of OCT-embedded basal plate were cut, fixed with 10% formalin, and rinsed in distilled water. Following incubation in 100% propylene glycol (American MasterTech Scientific Inc., Lodi, CA, USA) for 2 min, tissue sections were stained with oil-red O staining solution (American MasterTech Scientific Inc.) for 45 min at 37°C. After staining, tissue sections were incubated with 85% propylene glycol (Electron Microscopy Sciences, Hatfield, PA, USA) for 5 min and rinsed with distilled water until the water became clear. Stained tissue sections were then counterstained with modified Mayer's hematoxylin solution (American MasterTech, Lodi, CA, USA), rinsed in distilled water, and mounted with AquaSlip aqueous mounting medium (Cat#MMC0619; American MasterTech). Images were visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan). Pictures were taken using an Olympus DP71 camera and Olympus cellSens Entry software (Olympus).

Multiplex immunofluorescence and phenoptics (i.e. multispectral imaging)

Five-μm-thick sections of formalin-fixed, paraffin-embedded decidua basalis tissues from the maternal site of the placenta were cut and mounted on SuperFrost Plus microscope slides. Multiplex immunofluorescence staining was performed using the Opal 7 kit (Cat#NEL811001KT; PerkinElmer, Waltham, MA, USA), following the manufacturer's instructions. Prior to multiplex immunofluorescence staining, each analyte was individually optimized with single antibody staining combined with different fluorescent TSA reagents (PerkinElmer). After deparaffinization, slides were placed in antigen retrieval (AR) buffer and boiled using a microwave oven. Following blocking to eliminate non-specific binding, slides were incubated with antibodies against human smooth muscle actin (SMA) (CAT#M0851; Dako North America, Carpinteria, CA, USA), cytokeratin-7 (CK7) (CAT# M7018; Dako North America), CD68 (CAT# M0814; Dako North America), B7–1/CD80 (Cat#MAB140; R&D Systems, Inc., Minneapolis, MN, USA), inducible nitric oxide synthase (iNOS) (CAT#PA3–030A; Life Technologies), interleukin (IL)-12A (CAT#HPA001886, Sigma, St. Louis, MO, USA), heme oxygenase-1 (HMOX-1) (CAT#MA1–112, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA), and CD163 (CAT#bs-2527R, Bioss Antibodies, Inc., Woburn, MA, USA) at room temperature. The slides were then washed and incubated with Opal Polymer HRP Ms+Rb (Cat#ARH1001EA; PerkinElmer). Next, the slides were incubated with one of the following fluorescent TSA reagents (PerkinElmer) included in the Opal 7 kit: Opal 520, Opal 540, Opal 570, Opal 620, Opal 650, or Opal 690 (dilution 1:100). After washing, the slides were counterstained with Spectral DAPI (Cat#FP1490; PerkinElmer) and mounted using ProLong Diamond Anti-fade Mountant (Life Technologies). Unstained tissue sections (autofluorescence controls) and tissue sections stained with isotype (negative controls) were also included. Multiplex staining was performed by consecutively staining slide-mounted tissues using the same antibody concentrations and conditions validated through single-plex staining. Each

previous primary and secondary antibody was removed by boiling in AR buffer before the application of the next primary antibody. After multiplex staining, phenoptics was performed in the slides using the Vectra Polaris Multi-spectral Imaging System (PerkinElmer), and images were analyzed and converted to the immunohistochemistry view using the InForm 2.4.1 image analysis software (PerkinElmer).

Statistical analysis

Data were analyzed using IBM SPSS v19.0 (IBM Corporation; Armonk, NY, USA). For patient demographics, the Fisher's exact test was used to compare proportions among groups and the Kruskal-Wallis test was used for comparing continuous variables among groups. Experimental data was compared between study groups using the Mann-Whitney *U*-test. t-SNE plots were generated using the FlowJo v10 software. A p-value of ≤0.05 was considered statistically significant.

RESULTS

The immunophenotype of decidual macrophages in acute atherosis

First, we visualized the accumulation of lipid-laden foam cells in the decidual vessels with acute atherosis using hematoxylin & eosin and oil-red O stains (Figures 1A&B, black arrows). These foam cells were positive for CD68, a marker for macrophage scavenger receptors[69] that is also expressed by histiocytes[70, 71] (Figure 1C, black arrows). Yet, other CD68+ decidual macrophages without lipid accumulation were also localized perivascularly, surrounding the atherotic vessels (Figure 1C, red arrows).

Next, the immunophenotype of decidual macrophages was assessed using multi-color flow cytometry. Figures 1D&E show t-distributed stochastic neighbor embedding (t-SNE) plots of the macrophage phenotypes identified in the decidua basalis with or without acute atherosis. Three unique populations were detected: M1-like ($CD45^+CD14^+ICAM\text{-}3^-CD80^+$ cells), M2-like ($CD45^+CD14^+ICAM\text{-}3^-CD163^+CD209^+$ cells), and others (neither M1- nor M2-like) (Figures 1D&E). The t-SNE plots clearly showed that M2-like macrophages are more abundant than M1-like macrophages (Figures 1D&E). These results illustrate the diversity of macrophage phenotypes in the decidua basalis.

Acute atherosis is characterized by an increase in M1-like decidual macrophages

Next, we investigated whether M1-like macrophages were more abundant in the decidua basalis with acute atherosis. The gating strategy used to identify M1-like macrophages in the decidua basalis is shown in Figure 2A. Both the number and proportion of M1-like macrophages were higher in the decidua basalis with acute atherosis compared to those without this lesion (Figures 2B & C). These results suggest that decidual macrophages undergo an M1-like polarization in the pathologic pro-inflammatory microenvironment of acute atherosis.

Acute atherosis is characterized by an increase in pro-inflammatory decidual macrophages

M1 macrophages release pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS) and interleukin (IL)-12[72–74]. To explore the pro-inflammatory phenotype of decidual macrophages in acute atherosis, we examined the expression of these mediators by flow cytometry (Figure 3A). Consistent with an M1-like phenotype, more macrophages from the decidua basalis with acute atherosis expressed iNOS and IL-12 compared to those without this lesion (Figures 3B&C). These results confirm that macrophages acquire a pro-inflammatory M1-like phenotype in the decidua basalis with acute atherosis.

Acute atherosis does not alter the M2-like decidual macrophage population

An M1 macrophage polarization can be accompanied by a decrease in M2-polarized macrophages[36, 58]. Therefore, we next investigated whether the M1-like polarization in acute atherosis was associated with a reduction in M2-like macrophages. The gating strategy used to identify M2-like macrophages in the decidua basalis is shown in Figure 4A. M2-like macrophages were the most abundant phenotype in the decidua basalis, confirming previous studies[58]. Yet, neither the number nor the proportion of M2-like macrophages was altered in the decidua basalis with acute atherosis (Figures 4B&C). These data indicate that, although M2-like macrophages are abundant, their phenotype does not change during the process of acute atherosis.

Immunolocalization of M1-like macrophages in the decidual vessels with acute atherosis

To localize the M1-like macrophages associated with acute atherosis, we performed phenoptics, a novel technology used to immunophenotype cells in tissue sections[75–79]. Phenoptics allowed us to simultaneously immunolocalize six different markers in nucleated cells (i.e. DAPI-positive cells). The lesions of acute atherosis occur in spiral arteries which fail to fully transform as part of pregnancy adaptations[6–11]; therefore, we first searched for such non-transformed vessels in the decidua basalis.

Non-transformed vessels are characterized by a lack of trophoblast, which invade the spiral arteries and contribute to transformation[80], and the presence of smooth muscle cells, which are normally displaced or destroyed by invading trophoblast [2, 11]. Figure 5A shows a non-transformed vessel, indicated by expression of smooth muscle actin (SMA) around the vessel and a lack of invading cytokeratin-7 (CK7)-positive trophoblast, in the decidua basalis with acute atherosis. Having identified non-transformed decidual vessels, we next determined whether M1-like macrophages were localized in these lesions. Macrophages expressing M1 markers (CD68+CD80+iNOS+IL-12+ cells) were detected in close proximity to non-transformed vessels in the decidua basalis with acute atherosis (Figure 5A, white dotted rectangle). A magnification of the mediators expressed by M1-like macrophages in non-transformed vessels with acute atherosis is shown in Figure 5B.

We then determined whether M1-like macrophages could be localized near transformed vessels in the decidua basalis as well. We identified transformed vessels in the decidua basalis with acute atherosis based on the presence of invading trophoblasts and lack of smooth muscle cells as previously described[2, 11]. We found that M1-like macrophages could

be detected surrounding the transformed vessels in the decidua basalis with acute atherosis (Figures 5C, white dotted rectangle). A magnification of the mediators expressed by M1-like macrophages in transformed vessels with acute atherosis is shown in Figure 5D. These results demonstrate that M1-like macrophages are localized to both transformed and non-transformed vessels in the decidua basalis with acute atherosis.

Monocyte-like cells are present on the endothelium of non-transformed vessels: A possible intravascular origin for decidual macrophages with acute atherosis

Macrophages infiltrating atherosclerotic lesions are recruited from the intravascular space[81]. To complement our study, we addressed whether decidual macrophages in atherotic lesions could be infiltrating from the intravascular space. Interestingly, an accumulation of $CD68^{-}CD80^{+}$ monocyte-like cells was observed on the endothelium of non-transformed decidual vessels with acute atherosis (Figure 6A, white dotted box). A magnification of this image shows monocyte-like cells lining the vessel wall (Figure 6B). This finding suggests an intravascular origin for the decidual M1-like macrophages associated with lesions of acute atherosis.

Acute atherosis is associated with the infiltration of MOX and Mhem macrophages in the decidua basalis

Given that a large proportion of decidual macrophages did not display an M1- or M2-like phenotype, we investigated the non-M1/M2 macrophage subsets present in the decidua basalis. MOX ($CD68^{+}HMOX\text{-}1^{+}CD163^{-}$ cells) and Mhem ($CD68^{+}HMOX\text{-}1^{+}CD163^{+}$ cells) macrophages are present in atherosclerotic plaques[82]; therefore, we explored whether these macrophage populations were present in atherotic lesions using phenoptics. Both MOX and Mhem macrophages were present near transformed vessels ($SMA^{-}CK7^{+}$ vessels) in the decidua basalis with acute atherosis (Figure 7A, white or red dotted rectangles). A magnification of a Mhem macrophage expressing CD68, HMOX-1, and CD163 is shown in Figure 7B. A magnification of a MOX macrophage expressing CD68 and HMOX-1, but not CD163, is shown in Figure 7C. Together, these data indicate that, besides M1-like macrophages, MOX and Mhem macrophages are present in the decidua basalis with acute atherosis.

DISCUSSION

The principle findings of the study are as follows: 1) Macrophages displayed diverse phenotypes in the decidua basalis with acute atherosis; 2) decidual M1-like macrophages were increased in acute atherosis; 3) such decidual macrophages displayed a pro-inflammatory phenotype, as indicated by the expression of iNOS and IL-12; 4) M2-like macrophages were the most abundant subset in the decidua, yet this population did not change with the presence of acute atherosis; 5) decidual M1-like pro-inflammatory macrophages were localized near both transformed and non-transformed vessels with acute atherosis; 6) monocyte-like cells were present on the lumen of non-transformed vessels in the decidua basalis with acute atherosis, indicating a possible intravascular source for decidual macrophages; and 7) MOX and Mhem macrophages were identified near transformed vessels in the decidua basalis with acute atherosis.

M1- and M2-like decidual macrophages in acute atherosis

Macrophages represent a primary immune cell population in the decidua[83–86]. During early and mid-pregnancy, macrophages displaying an alternative or M2-like phenotype[34, 36, 38, 40, 61] are the dominant decidual subset[34, 36, 38, 58], indicating that such cells may play an important role in maternal-fetal tolerance throughout pregnancy[34, 36, 37, 39, 40, 58]. At the end of gestation, during the physiological process of labor, decidual macrophages acquire an M1-like phenotype[58], a phenomenon that also occurs during the pathological process of preterm labor[58] and coincides with the infiltration of other inflammatory cells at the maternal-fetal interface[87–91]. Disruption of the decidual macrophage population has been linked to placental dysfunction implicated in preeclampsia[92, 93]. Of interest, both women with spontaneous preterm labor[94] and those with preeclampsia[4, 95, 96] have a higher incidence of inadequate spiral artery remodeling, a condition often associated with the presence of acute atherosis[6–11].

In the current study, we showed that decidual macrophages are a heterogeneous population comprised of M1- and M2-like macrophages as well as other subsets. M2-like macrophages are the most abundant decidual subset; yet, greater numbers of M1-like macrophages are found in the decidua basalis with acute atherosis compared to decidual tissues without this arterial lesion. Such macrophages expressed greater amounts of the pro-inflammatory mediator iNOS. Inducible NOS-deficient mice have a decreased incidence of diet-induced atherosclerosis and reduced plasma concentrations of lipid peroxides[97]. Together with studies showing that iNOS is expressed in human atherosclerotic plaques[98–100], these findings suggest that decidual macrophages expressing iNOS participate in the pathophysiology of acute atherosis.

Macrophages also expressed high levels of IL-12 in the decidua basalis with acute atherosis. IL-12 is expressed in atherosclerotic plaques and is secreted by monocytes in response to oxidized low-density lipoproteins[101]. Indeed, the blockade of IL-12 signaling in a mouse model of atherosclerosis reduced atherogenesis and improved plaque stability[102]. These data indicate that, similar to atherosclerosis, IL-12 may be implicated in the inflammatory milieu which accompanies acute atherosis.

Interestingly, we showed that M1-like pro-inflammatory macrophages could be found near both non-transformed and transformed vessels in the decidua basalis with acute atherosis. Acute atherosis is a lesion that does not affect all of the decidual spiral arteries or every segment of such vessels[11]. Together, these data imply that the vascular-based inflammatory response characteristic of acute atherosis[11, 21, 30, 103, 104] likely affects the entire decidua, inducing a tissue-wide M1 macrophage polarization.

A putative intravascular origin for decidual macrophages in acute atherosis

An intravascular inflammatory response can result in the activation of endothelial cells[105–110] and accumulation of circulating monocytes[103]. During the pathogenesis of acute atherosis, monocytes seem to infiltrate the smooth muscle layer of affected spiral arteries[30]. As the tissue beneath the vessel endothelium becomes necrotic, it has been proposed that lipids released from the cellular membranes are ingested by scavenging macrophages

leading to the formation of foam cells[30]. Herein, we observed monocyte-like cells on the endothelium of a non-transformed decidual vessel, suggesting that such cells extravasate from the intravascular space as an early step in the formation of acute atherotic lesions. This process resembles a similar mechanism for monocyte recruitment described in vessels with atherosclerosis[81].

MOX and Mhem decidual macrophages in acute atherosis

Besides M1- and M2-like phenotypes, we also found macrophages expressing heme oxygenase-1 (HMOX-1) in the decidual tissues with acute atherosis. HMOX-1 is an enzyme which catalyzes the degradation of heme[111] into carbon monoxide[112], ferrous iron[113], and biliverdin[114]. Such byproducts have antioxidant and anti-inflammatory properties and, therefore, HMOX-1 is considered an atheroprotective molecule[115, 116]. Yet, HMOX-1 expression has been described on two distinct macrophage subsets[82]: 1) “Mhem” macrophages, which are atheroprotective cells induced by CD163-mediated scavenging of hemoglobin in a heme-rich environment characteristic of atherosclerotic lesions[117]; and 2) “MOX” macrophages, which are inflammatory cells that lack CD163 expression and display reduced phagocytic capabilities in a microenvironment rich in oxidized phospholipids[118]. In the study herein, both MOX and Mhem macrophages were identified in the decidua basalis with acute atherosis. An imbalance between atherogenic and atheroprotective macrophage subsets has been implicated in the pathogenesis of atherosclerosis[82]. Moreover, acute atherosis has been linked to increased oxidative stress and lipid content in the decidua of preeclamptic women[65, 119–121]. Oxidative stress is also implicated in the pathophysiology of preeclampsia[122–128] and fetal growth restriction[129–131]. Hence, we proposed that macrophages are implicated in the mechanisms leading to oxidative stress, and therefore, obstetrical disease (i.e. acute atherosis).

Conclusion

The current study shows that different subsets of macrophages are present in the decidua with acute atherosis, namely M1-like, M2-like, MOX, and Mhem subsets. Yet, these decidual lesions are enriched with M1-like macrophages that display a pro-inflammatory phenotype, as indicated by the expression of iNOS and IL-12. The fact that M1-like macrophages were present in both non-transformed and transformed vessels indicates that acute atherosis exerts a decidua-wide effect. Finally, we proposed an intravascular origin for decidual macrophages in acute atherosis. Collectively, these findings provide the first molecular characterization of the immunophenotype of decidual macrophages in acute atherosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

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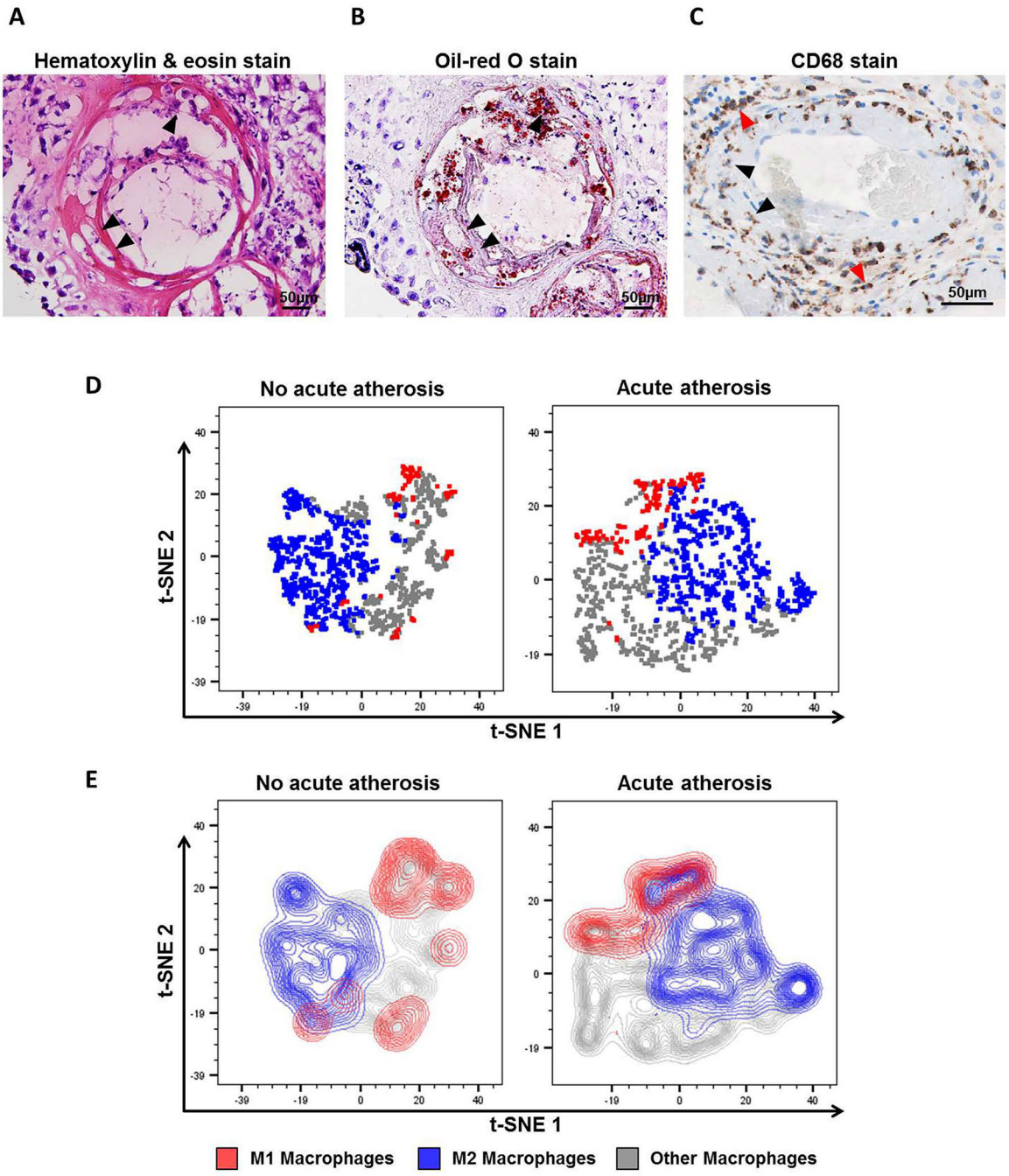

Figure 1. The immunophenotype of decidual macrophages in acute atherosis.

Representative images of histological staining of decidual vessels using (A) hematoxylin & eosin or (B) oil-red O. (C) Representative image of immunohistochemical staining of CD68 to detect macrophages and foam cells. Black arrows indicate foamy macrophages, and red arrows show non-foamy macrophages. Magnification = 200X. Scale bars = 50μm. Flow cytometry analysis of decidual macrophage subsets was performed using t-distributed stochastic neighbor embedding (t-SNE) shown as (D) dot plots or (E) contour plots. Red =

M1-like macrophages ($CD45^+CD14^+ICAM\text{-}3^-CD80^+$ cells), blue = M2-like macrophages ($CD45^+CD14^{+I}CAM\text{-}3^-CD209^+CD163^+$ cells), and grey = non-M1/M2 macrophages.

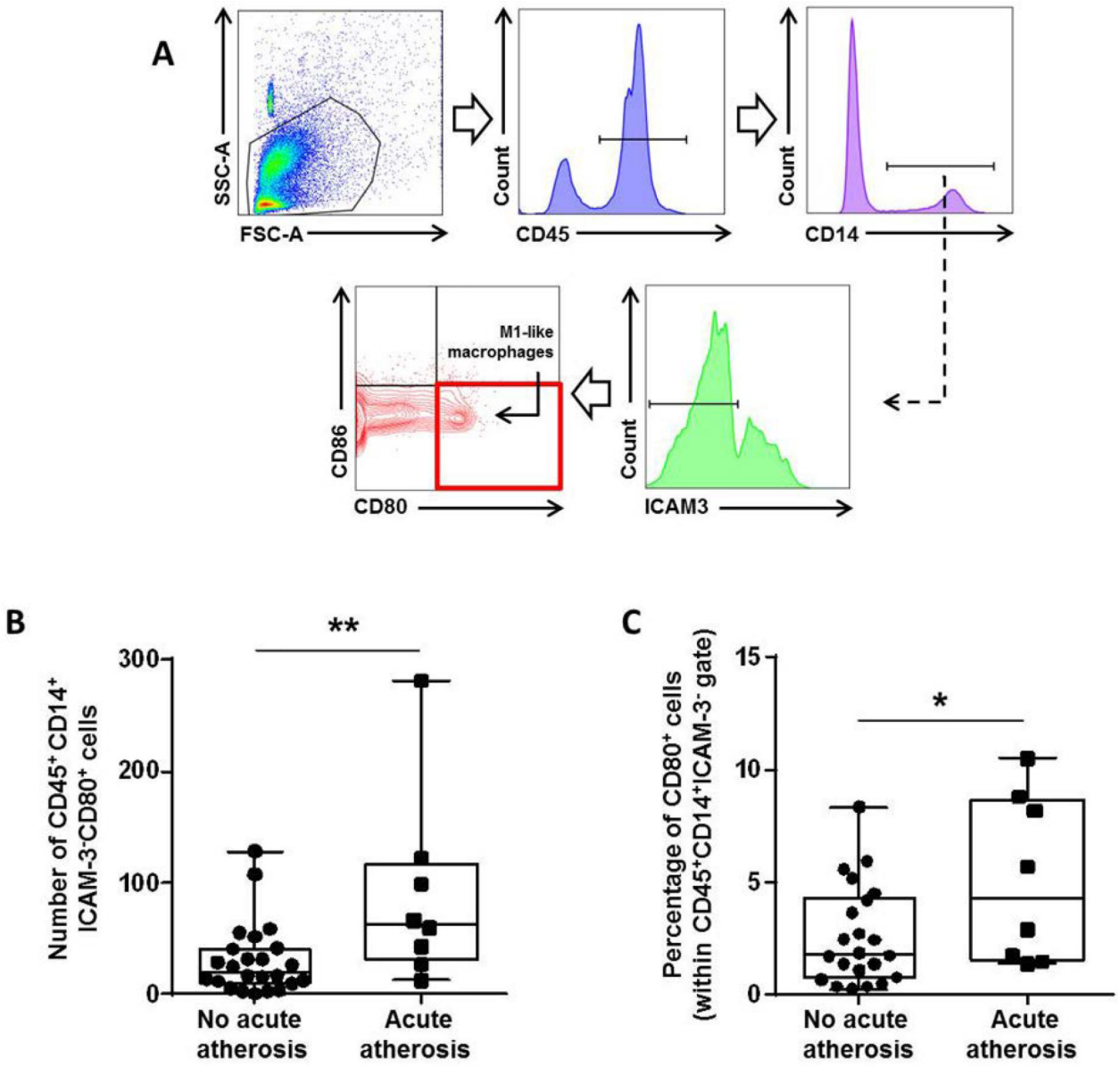

Figure 2. Acute atherosis is characterized by an increase in M1-like decidual macrophages.

(A) Flow cytometry gating strategy used to identify decidual M1-like macrophages ($CD45^+CD14^+ICAM\text{-}3^-CD80^+$ cells). Dot plots illustrate the (B) number and (C) proportion of M1-like macrophages in the decidua basalis with or without acute atherosis. Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. Cases with (n=8) or without (n=24) acute atherosis. *$p<0.05$, **$p\leq0.01$

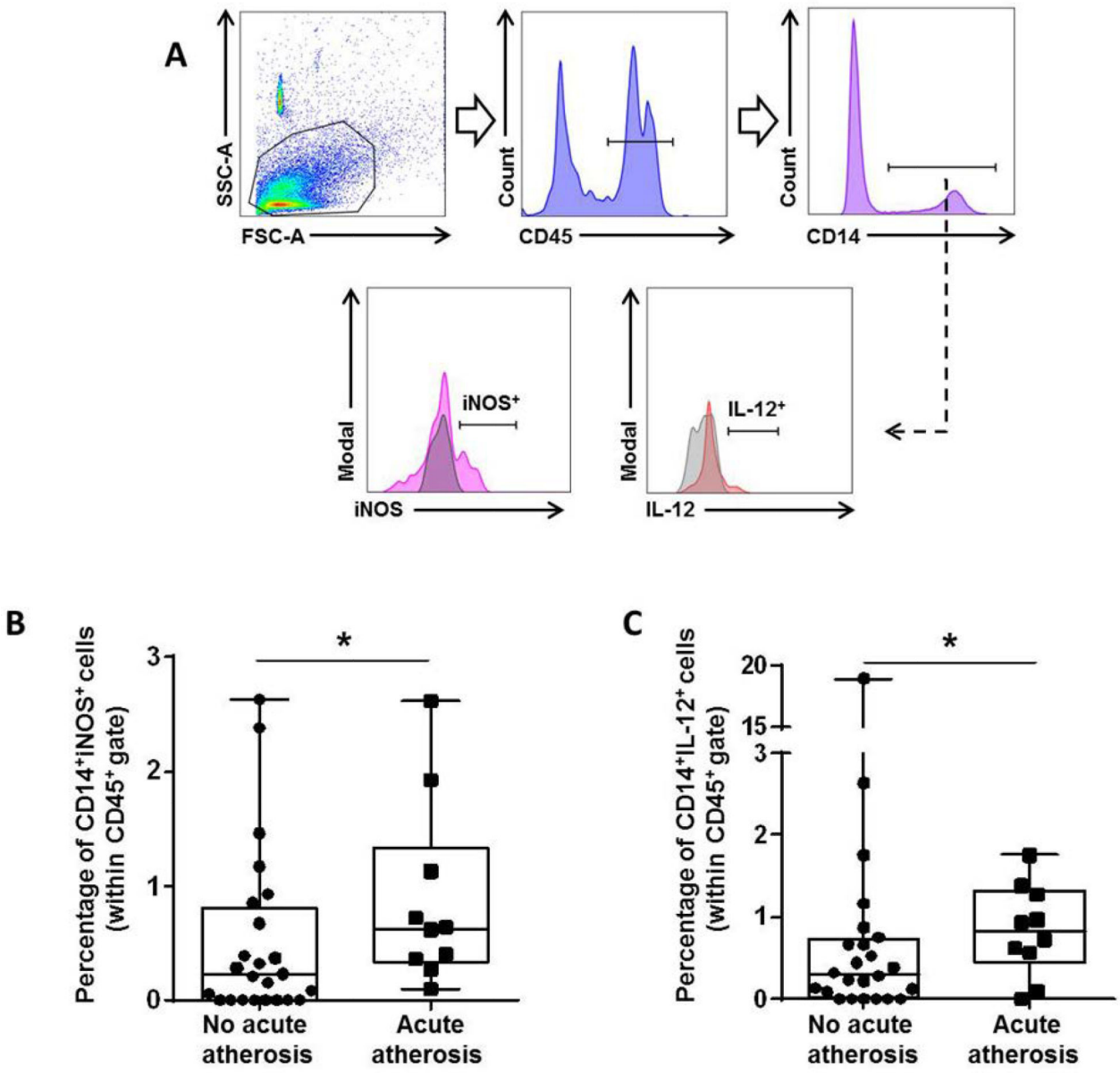

Figure 3. Acute atherosis is characterized by an increase in pro-inflammatory decidual macrophages.

(A) Flow cytometry gating strategy used to identify decidual macrophages expressing the pro-inflammatory mediators inducible nitric oxide synthase (iNOS) or interleukin (IL)-12 ($CD45^+CD14^+iNOS^+$ or $CD45^+CD14^+IL\text{-}12^+$ cells). Dot plots illustrate the percentage of (B) $CD45^+CD14^+iNOS^+$ macrophages and (C) $CD45^+CD14^+IL\text{-}12^+$ macrophages in the decidua basalis with or without acute atherosis. Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. Cases with (n=10) or without (n=24) acute atherosis. *$p<0.05$

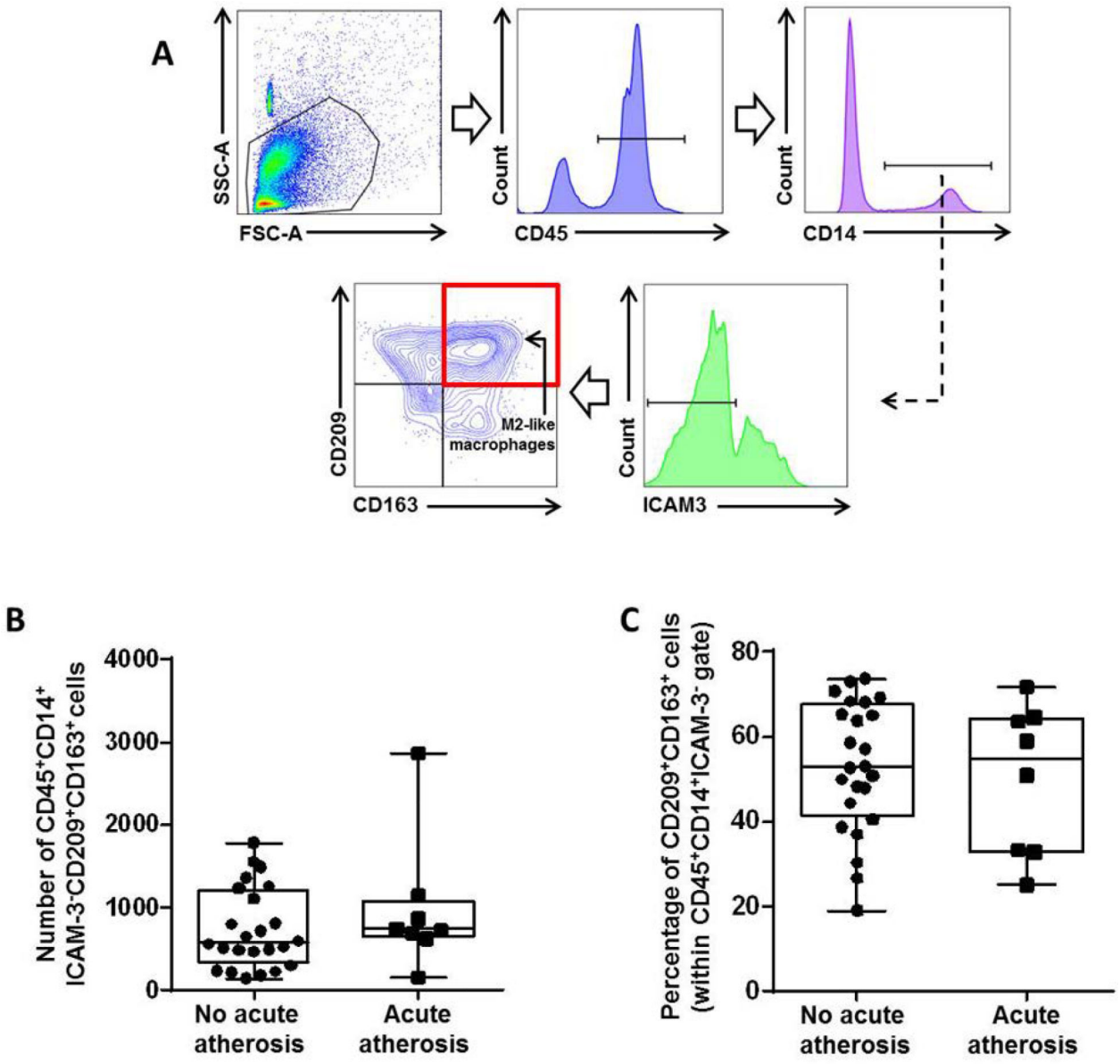

Figure 4. Acute atherosis does not alter the M2-like decidual macrophage population.

(A) Flow cytometry gating strategy used to identify decidual M2-like macrophages ($CD45^+CD14^+ICAM\text{-}3^-CD209^+CD163^+$ cells). Dot plots illustrate the (B) number and (C) proportion of M2-like macrophages in the decidua basalis with or without acute atherosis. Mid-lines indicate medians, boxes indicate interquartile ranges, and whiskers indicate min-max range. Cases with (n=8) or without (n=24) acute atherosis.

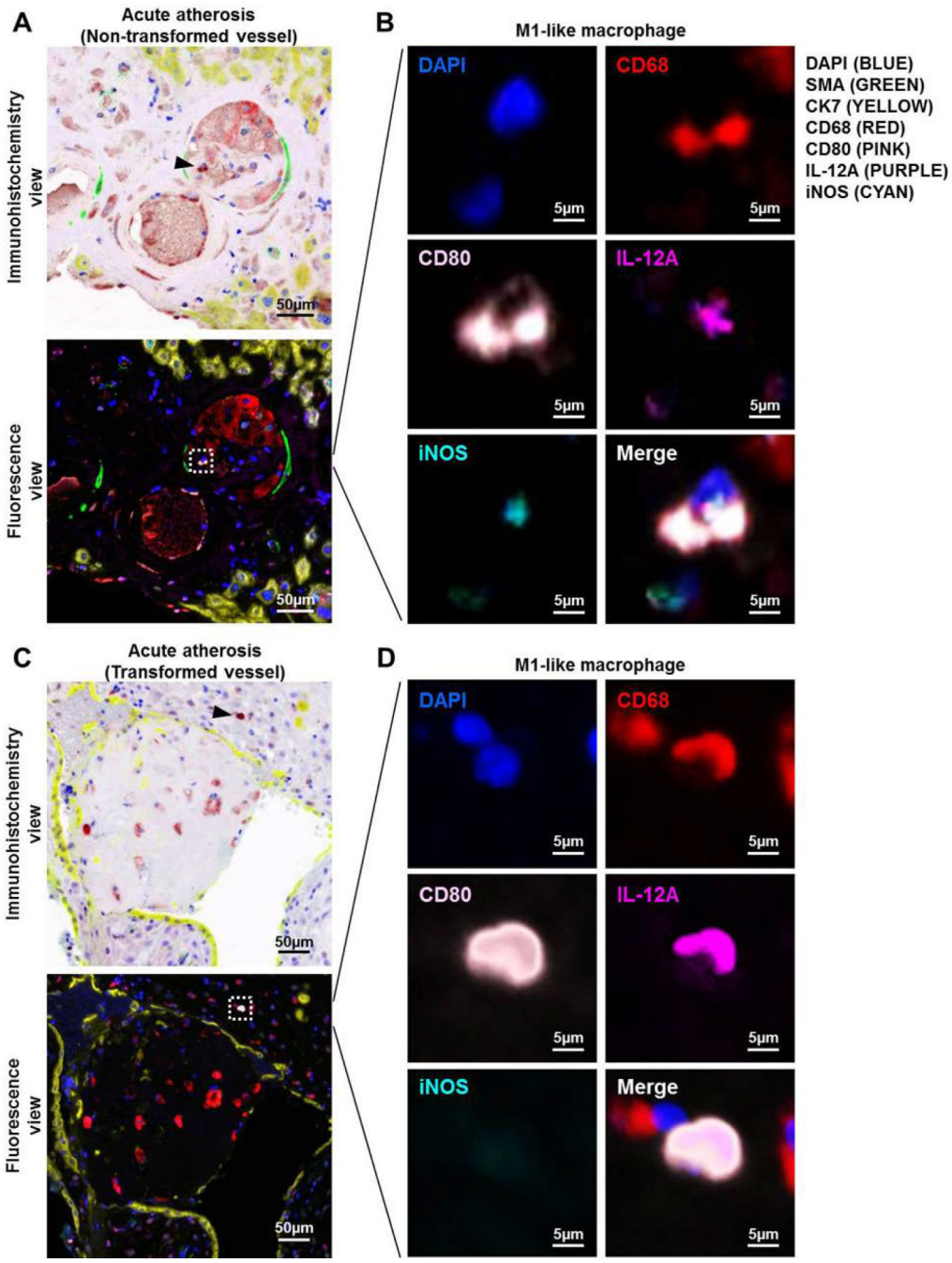

Figure 5. Immunolocalization of M1-like macrophages in the decidual vessels with acute atherosis.

Multiplex immunofluorescence staining showing nuclear staining (4′,6-diamidino-2-phenylindole, DAPI, blue), smooth muscle actin (SMA, green), cytokeratin-7 (CK7, yellow), CD68 (red), CD80 (pink), interleukin (IL)-12A (purple), and inducible nitric oxide synthase (iNOS, cyan) in the decidua basalis with acute atherosis. Representative images showing (A) the immunohistochemistry and fluorescence views and (B) magnification of an M1-like macrophage in the vessel wall of a non-transformed decidual vessel with acute atherosis. Representative images showing (C) the immunohistochemistry and fluorescence views and

(D) magnification of an M1-like macrophage near a transformed decidual vessel with acute atherosis. Phenoptics was performed to generate separate and merged immunofluorescence images (B&D), and to convert fluorescence images to the immunohistochemistry view (A&C). Black arrows in the immunohistochemistry view and dotted boxes in the fluorescence view indicate an M1-like macrophage. Images are representative of 3 experiments per group. Images were taken at 200X magnification, and a close-up of an M1-like macrophage is shown. Scale bars = 50μm (original image) or 5μm (close-up image).

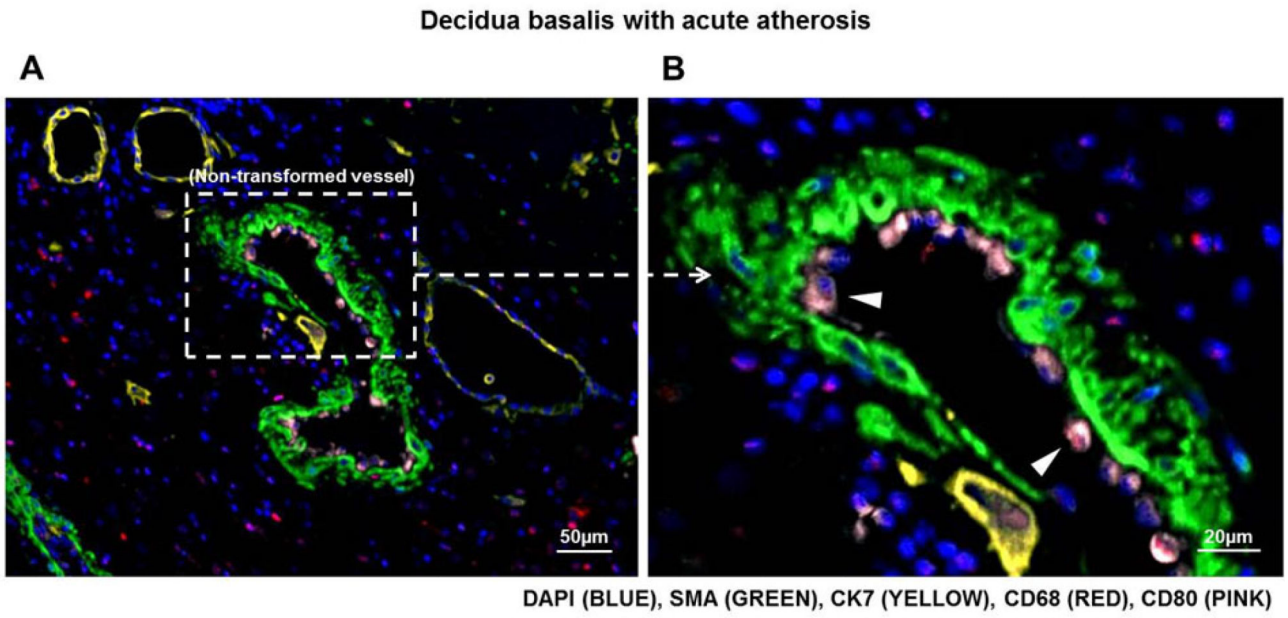

Figure 6. Monocyte-like cells are present on the endothelium of non-transformed vessels with acute atherosis.

Multiplex immunofluorescence staining showing nuclear staining (4′,6-diamidino-2-phenylindole, DAPI, blue), smooth muscle actin (SMA, green), cytokeratin-7 (CK7, yellow), CD68 (red), and CD80 (pink) in the decidua basalis with acute atherosis. Representative and magnified images showing monocyte-like cells ($CD68^{-}CD80^{+}$ cells) localized on the endothelium of a non-transformed decidual vessel with acute atherosis. White arrows indicate monocyte-like cells. Phenoptics was performed to generate immunofluorescence images. Images are representative of 3 experiments per group. Images were taken at 200X magnification, and a close-up of monocyte-like cells on the vessel endothelium is shown. Scale bars = 50μm (original image) or 20μm (close-up image).

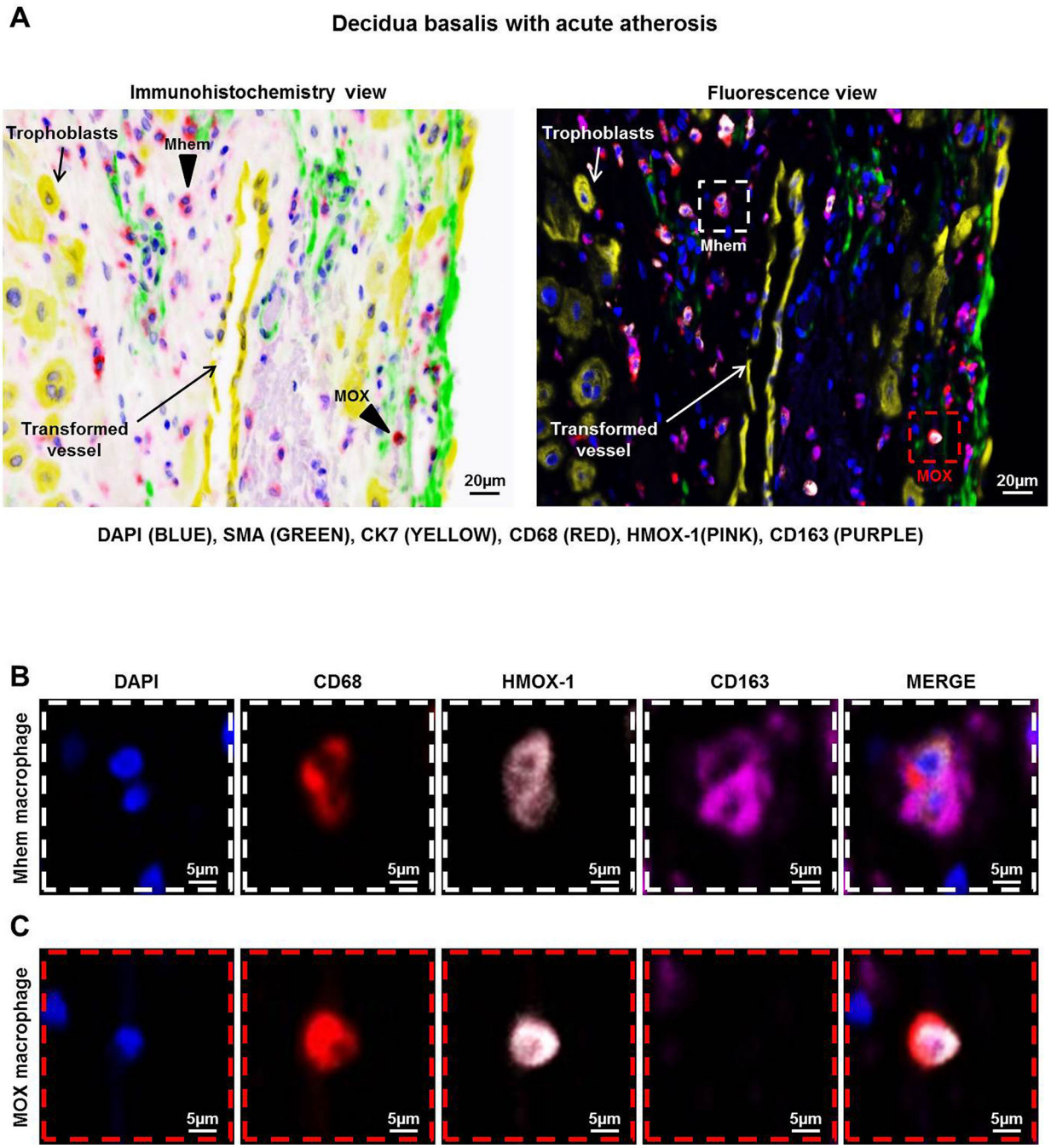

Figure 7. Acute atherosis is associated with HMOX-1-expressing decidual macrophage subsets.

Multiplex immunofluorescence staining showing nuclear staining (4′,6-diamidino-2-phenylindole, DAPI, blue), smooth muscle actin (SMA, green), cytokeratin-7 (CK7, yellow), CD68 (red), heme oxygenase-1 (HMOX-1, pink), and CD163 (purple) in the decidua basalis with acute atherosis. (A) Representative images showing the immunohistochemistry and fluorescence views of Mhem ($CD68^+HMOX\text{-}1^+CD163^+$) (white dotted box) and MOX ($CD68^+HMOX\text{-}1^+CD163^-$) (red dotted box) macrophages localized near a transformed decidual vessel with acute atherosis. Magnification of (B) a Mhem macrophage and (C) a

MOX macrophage. Phenoptics was performed to generate separate and merged immunofluorescence images. Images are representative of 3 experiments per group. Images were taken at 200X magnification, and a close-up of a Mhem and MOX macrophage is shown. Scale bars = 20μm (original image) or 5μm (close-up image).

Table 1.

Demographic and clinical characteristics of the study population

	No acute atherosis (n=31)	Acute atherosis(n=11)	p-value
Acute atherosis of decidua basalis spiral arteries[b]	0 (0/31)	100 (11/11)	<0.001
Maternal age (years)[a]	28 (24.5–31.5)	30 (23.5–35.5)	0.7
Pre-pregnancy body mass index (kg/m^2)[a]	25 (22–29)[c]	28.1 (25.3–34.2)	0.2
Race[b]			0.1
African-American	54.8 (17/31)	90.9 (10/11)	
Caucasian	22.6 (7/31)	0 (0/11)	
Hispanic	9.7 (3/31)	0 (0/11)	
Other	12.9 (4/31)	9.1 (1/11)	
Delivery route[b]			1
Vaginal	41.9 (13/31)	45.5 (5/11)	
Cesarean section	58.1 (18/31)	54.5 (6/11)	
Preeclampsia[b]	16.1 (5/31)	45.5 (5/11)	0.09
Gestational age at delivery (weeks)[a]	38.4 (36.5–39.1)	33.7 (31.7–36.2)	0.001
Birth weight[a]	3030 (2385–3445)	1675 (1427.5–2530)	0.001

Data are given as median (interquartile range) and percentage (n/N)

[a]Kruskal-Wallis test

[b]Fisher's exact test

[c]Three missing data