A Subset of Human Uterine Endometrial Macrophages is Alternatively Activated

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Abstract

Problem—Human uterine macrophages must maintain an environment hospitable to implantation and pregnancy and simultaneously provide protection against pathogens. Although macrophages comprise a significant portion of leukocytes within the uterine endometrium, the activation profile and functional response of these cells to endotoxin is unknown.

Method of Study—Flow cytometric analysis of surface receptors and intracellular markers expressed by macrophages isolated from human endometria was performed. Uterine macrophages were stimulated with LPS. Cytokines, chemokines, and growth factors expressed by these cells were analyzed using Bio-Plex analysis.

Results—CD163^{high} human endometrial macrophages constitutively secrete both pro- and anti-inflammatory cytokines as well as pro-angiogenic factors and secretion of these factors is LPS-inducible.

Conclusions—A major population of human uterine macrophages is alternatively activated. These cells secrete factors in response to LPS that are involved in the activation of immune responses and tissue homeostasis.

Keywords

Macrophage; endometrium; CD163; M2

Introduction

The uterine endometrium is an immunologically unique mucosal site, as it must simultaneously protect against pathogens and tolerate allogeneic sperm and a semi-allogeneic fetus. In this regard, uterine macrophages play a key role in ensuring immune defense within the endometrium, as they recognize invading microbes and elaborate a broad range of chemokines and cytokines in response to pathogenic challenge.

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Macrophages function as key effectors of both innate and humoral immunity, as they actively phagocytose foreign molecules and display antigens on their surface for recognition by T lymphocytes. The phenotype and function of tissue macrophages are affected by and uniquely dependent on the cellular milieu to which they are exposed, including local cytokines, chemokines and other biological effector molecules, as well as extracellular matrix and cellular components. Thus, the local microenvironment plays a significant role in macrophage activation and polarization [1]. In this regard, previous studies have demonstrated that macrophages derived from unique anatomical sites, including mucosal tissue (intestines), adipose tissue and alveolar tissue, possess properties and activation states that are distinct (reviewed in [2]). Despite the importance of uterine macrophages in the regulation of immunity within the uterine endometrium, relatively little is known about the influence of the uterine microenvironment on human uterine macrophage polarization and activation.

Polarized macrophages can be broadly classified as either "classically activated" (M1) or "alternatively activated" (M2), which represent the extremes of a continuum of functional states [3]. M1 macrophages are elicited through stimulation with IFN-γ, select cytokines such as GM-CSF and TNF-α, and microbial products such as LPS. These cells are considered microbicidal and pro-inflammatory and have a high capacity for antigen presentation and elevated production of reactive oxygen intermediates (ROI). In contrast, M2 macrophages are cells that have been activated by any means other than those used to generate M1 macrophages. Recent studies have expanded the definition of M2 macrophages to many subsets, including M2a, M2b and M2c. M2 cells are diverse—they are activated by different types of stimuli and elaborate distinct cytokines and chemokines following stimulation. Despite these differences, M2 macrophages share some common features. In general, M2 macrophages are poorly microbicidal and exhibit decreased expression of ROI, MHC Class II and IL-12, but have enhanced expression of anti-inflammatory mediators such as IL-10 (reviewed in [4]). Significantly, M1 macrophages are thought to initiate humoral immunity, while M2 macrophages are considered key players in angiogenesis, the resolution of inflammation and the coordination of tissue repair following an acute inflammatory reaction (reviewed in [3, 5, 6]. M2 polarized macrophages can be further characterized in terms of their activation status. M2a and M2c produce low levels of pro-inflammatory cytokines and high levels of IL-10. In contrast, M2b macrophages, which are activated by TLR agonists, produce high levels of pro-inflammatory IL-1, TNFα and IL-6 in addition to anti-inflammatory IL-10 [3].

In addition to their role in host immune defense, macrophages also have an active part in maintaining endometrial tissue homeostasis. Macrophage density in the human endometrium increases during the secretory stage of the menstrual cycle in preparation for menstruation, where macrophages help mediate tissue breakdown through expression of degrading enzymes like matrix metalloproteinases (MMPs) [7, 8]. As phagocytes, macrophages participate in clearance of the shed endometrial lining [7]. Since macrophages have a well-defined role in wound healing and angiogenesis [9], they may also contribute to regeneration of the endometrial lining and angiogenesis through secretion of growth and angiogenic factors.

Although macrophages comprise approximately 10 percent of the total leukocyte population within the human endometrium [10, 11], the activation profile of these cells is largely unknown. To date, most studies involving endometrial macrophages rely on identification of these cells by expression of CD68 [8, 12, 13] or CD14 [11, 14-16]. Although expression of these molecules is enriched in macrophages, recent studies have shown that CD68 and CD14 are also expressed by other cell types. Indeed, CD68 immunoreactivity has been detected in both myeloid and non-myeloid cell types, including dendritic cells, NK cell,

basophils and endothelial cells and fibroblasts [17-19]. Isolation of CD68^{+} cells from tissue is difficult because this marker is expressed intracellularly. Moreover, although CD14 is expressed largely on monocytes and macrophages, granulocytes also express low levels of CD14 [20].

In contrast, the scavenger receptor CD163 is a surface molecule expressed exclusively on monocytes and macrophages [21-23] and is a marker of M2 macrophages [5, 24-27]. CD163 is an endocytic receptor for hemoglobin-haptoglobin complexes that mediates the clearance of free hemoglobin and minimizes oxidative tissue damage [28]. CD163 is expressed by mature tissue macrophages [29, 30] and CD163^{+} cells are present during the healing phase of acute inflammation [30, 31].

Because of the role that macrophages play in immune defense and the importance of macrophages in angiogenesis and tissue remodeling, we hypothesized that human uterine macrophages would be M2 or alternatively activated. In the present study, we demonstrate the human endometrial macrophages are predominantly CD163^{+}, a marker of M2 macrophages. Flow cytometric analysis of this previously uncharacterized uterine macrophage population demonstrated that these cells also express CD14 and CD68, as well as the co-stimulatory molecules CD40, CD80 and CD86. Because infection within the endometrium has serious negative consequences on reproductive success, we determined the responsiveness of CD163^{+} human endometrial macrophages to TLR stimulation. We now report that human uterine endometrial macrophages produce both pro- and anti-inflammatory mediators as well as high levels of pro-angiogenic factors, indicating that these cells are characteristic of the alternatively activated M2b phenotype.

Methods

Human uterine endometrial tissues and blood

Endometrial tissue was obtained immediately following hysterectomy for non-endometrial pathology from female patients who had given informed consent. A summary of the patient population is provided in Table 1. Menstrual cycle staging of endometrial tissues was determined in accordance with accepted histologic practice using hematoxylin/eosin-stained paraffin sections. Approval to use human tissues was obtained from the Dartmouth Institutional Review Board in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services. Uterine endometrial-donor-matched PBMCs were isolated from heparinized whole blood with Ficoll-Hypaque (d=1.077). Monocytes were purified from mononuclear cell fractions as described by Mentzer et al. [32]. Monocyte purity was >95% as determined by CD14 expression using flow cytometry (data not shown). To generate M1-or M2-polarized macrophages, monocytes were incubated with either 10 ng/ml GM-CSF or 100 ng/ml M-CSF for 7 days [33, 34].

Two-color immunophenotyping and confocal scanning laser microscopy

Six tissue sections were dissected and stained as previously described [35]. Sections were maintained in ice-cold PBS throughout processing to prevent internalization of surface markers and immunofluorescent staining was performed immediately after cutting. Two mg/100 ml each of CD68-FITC (Thermo Scientific, clone KiM7) and CD14-PE (Caltag, clone TUK4) or CD163-PE (Trillium Diagnostics, clone MAC2-158) were used to stain sections. C ytoplasmic staining with antibody specific for CD68 was achieved by fixing cells with paraformaldehyde, followed by treatment with saponin. Fixed and permeabilized tissue was incubated with specific or control antibody. Stained sections were wet-mounted in anti-fade (Molecular Probes, Eugene, OR), sealed with nail varnish, and stored at 4°C in the dark for up to 10 days before confocal imaging.

Immunofluorescently labeled sections were optically sectioned using a Zeiss LSM5-10 Confocal Scanning Laser Microscope System. Two-color fluorescent sections were evaluated for the presence of CD68 and/or CD14 or CD163. Unstained and fluorochrome isotype controls were used to control for auto-fluorescence and non-specific antibody binding, respectively.

Uterine endometrial tissue digestion and macrophage isolation

Endometrial tissue sections were processed as described by White *et al.* [36]. Briefly, tissue sections were minced and incubated in an enzyme cocktail containing final concentrations of 3.4 mg/ml pancreatin (Gibco), 0.1 mg/ml hyaluronidase (Worthington) and 1.6 mg/ml collagenase I (Worthington) in Hank's Buffered Saline Solution (HBSS) containing 2 mg/ml D-glucose (Sigma) at 37°C for 2 hours. Following digestion, cells were dispersed by straining through a 250 μm mesh screen and washed with HBSS. Tissue cells were stained and fixed for flow cytometric analysis. Prior to macrophage isolation, dead cells were removed from the culture using the dead cell removal kit (Miltenyi). Cell viability was assessed by trypan blue exclusion, which ranged between 80% and 95%. Cells were incubated with biotinylated anti-CD163 mAb (Maine Biotechnology) for one hour at room temperature. Following thorough washing, cells were incubated with strepavadin-conjugated magnetic microbeads (Miltenyi) and passed over a magnetic column. The CD163$^+$ cells retained on the column were eluted and used in LPS stimulation assays. In control studies, peripheral blood monocyte-matured macrophages were incubated with the enzyme cocktail used to digest uterine endometrial tissue. No effect was observed on surface expression of MHC Class II, CD80, CD86, CD40, TLR4, CD14, CD16, and CD163, consistent with results reported by White et al [36].

Flow cytometry

To assess surface expression of macrophage markers, endometrial tissue cells were stained for two-color flow cytometry with anti-CD163 and antibodies to CD14, MHC-II (HLA-DR), CD16, TLR4, CD40, CD80 or CD86. Surface marker expression of control blood-derived macrophages was analyzed using single color flow cytometry. To assess CD68 expression in macrophages, cells were initially stained for surface expression of CD14 or CD163, fixed in 1% MFF and permeabilized in saponin, and then stained intracellularly for CD68. Table 2 lists the fluorochrome-conjugated antibodies utilized in flow cytometric analysis of intracellular and surface marker expression. Cells were analyzed using CellQuest software and a FACScan flow cytometer (BD Biosciences).

LPS stimulation, Bio-Plex assay, and ELISA

CD163$^+$ isolated endometrial macrophages or blood-derived monocyte-differentiated macrophages were plated at 2×10^5 cells per well in 24-well tissue culture dishes in complete RPMI. Cells were either unstimulated or treated with 10 ng/ml ultrapure *E. coli* LPS (Invitrogen), for 24 hours. Cell-free culture supernatants were aliquoted and stored at −80°C until further use. The Bio-Plex suspension array system using fluorescently dyed Luminex microspheres (beads) was used (Bio-Rad, Hercules, CA) to determine the responsiveness of endometrial macrophages to LPS stimulation. This assay system is ideally suited to measure multiple cytokines from one sample. Standards were prepared in the same fresh medium that was used for culturing experimental samples and were assayed in triplicate. Spiked controls accurately reflected the added cytokine, chemokine or growth factor concentration. Assays were performed in a 96-well filtration plate (Millipore, Billerica, MA). The fluorescence intensity for each bead was measured using the Bio-Plex array reader, and Bio-Plex Manager software with five-parametric-curve fitting was used for data analysis. The level of detection of each analyte was 7.8 pg/ml. Analysis of IL-10, IL-6, and IL-12p40 in control

blood-derived monocyte-matured macrophages was performed by ELISA (R and D Systems, Minneapolis, MN).

Results

CD163 is widely expressed on uterine endometrial macrophages

Although macrophages have been identified immunohistochemically in the human uterine endometrium on the basis of CD14 and CD68 expression [37], no studies to date have determined the polarization of primary human non-pregnant uterine macrophages. Flow cytometric analysis was used to discern the activation profile of macrophages within the human non-pregnant uterine endometrium. Single cell suspensions of whole uterine endometrial tissue digests were stained for the pan-macrophage marker CD68 and for CD14, which is enriched in M1 macrophages or CD163, which is highly expressed by M2 macrophages. CD163 was expressed on 71.9% of CD68$^+$ cells, indicating that CD163 is widely expressed on human endometrial tissue macrophages (Figure 1A). As demonstrated in Figure 1A, CD14$^+$ cells represented a minor population of CD68$^+$ cells (10.3%). Low expression of CD14 was not attributable to proteolytic cleavage of this molecule from the cell surface during cell isolation, as previous work indicates that the methodology used for tissue dispersal in this study does not cleave surface expression of CD14 from macrophages [36]. Confocal imaging confirmed that uterine endometrial macrophages express CD163 and imaging analysis corroborates flow cytometric findings that indicate more uterine macrophages are CD163^{high} compared with CD14^{high} (Figures 1B and 1C). The high level of CD163 expression on CD68$^+$ macrophages suggests that these cells are polarized toward an alternatively activated or M2 phenotype.

To validate the characterization of M1 and M2 polarization in these cells, we performed control studies in parallel using immunophenotypic analysis of donor-matched peripheral blood monocytes that were differentiated with either GM-CSF or M-CSF. Differentiation of monocytes with these cytokines permits good discrimination of M1 vs. M2 activation [5]. Results of flow cytometric analysis of surface marker expression on blood-derived macrophages are summarized in Table 3. Cells were also stimulated with LPS and expression of IL-6, IL-10, and IL-12p40 was evaluated by ELISA to verify macrophage polarization. As expected, GM-CSF-differentiated cells produced high levels of IL-6 and IL-12p40, while M-CSF-differentiated macrophages secreted enhanced levels of anti-inflammatory IL-10 (data not shown).

Uterine macrophage surface expression

Because human uterine CD163$^+$ macrophages have not been previously characterized, we examined the expression pattern of innate immune receptors present on these cells. Whole tissue human uterine cell suspensions were analyzed by flow cytometry and macrophages were gated based on CD163 expression (Figure 2A). Because of their importance in the recognition of Gram-negative microbes, our initial studies focused on analysis of CD14 and TLR4. CD14 is a co-receptor for LPS with TLR4 and MD-2 and is necessary for macrophage activation in response to bacterial infection [38]. As indicated in Figure 2A, levels of CD14 and TLR4 surface expression are similar on CD163$^+$ macrophages. However, although CD14 is present on a subset of these cells (30%), TLR4 expression is more widely distributed (62%) (Figure 2B). We next examined this cell population for expression of CD16 (FCγRIII), which recognizes the Fc portion of IgG antibodies and stimulates pro-inflammatory cytokine production [39]. Enhanced surface expression of CD16 has also been reported on a subset of alternatively activated CD163$^+$ macrophages [5]. As demonstrated in Figures 2A and 2B, CD16 is expressed at low levels on approximately 12% of CD163$^+$ uterine macrophages. These data imply that human CD163$^+$

uterine macrophages differ from gut-derived mucosal macrophages in terms of TLR4, CD14 and CD16 expression. In contrast to uterine endometrial macrophages, intestinal macrophages express uniformly low levels of CD14, CD16, and TLR4, which may enable them to remain hypo-responsive to environmental antigens and commensals in the gut [40].

Macrophages present antigen to T cells through expression of MHC on the cell surface, and co-stimulatory molecule signaling is necessary for the generation of adaptive immune responses. As shown in Figures 2A and 2B, approximately 30% of all CD163^{+} uterine macrophages express low levels of MHC-II. Notably, these cells express similar levels of the co-stimulatory molecules CD80 and CD86 (Figure 2A). CD86 is expressed on almost 50% and CD80 is expressed by roughly 15% of CD163^{+} uterine macrophages. (Figure 2B). This pattern is similar to that of alveolar and intestinal macrophages, which also express low levels of MHC-II, CD80 and CD86 [40, 41].

CD40 is a co-stimulatory receptor expressed by macrophages and binding of its ligand, CD40L (CD154), results in potent activation. CD40L is expressed mainly by activated T cells and allows for back talk from T cells to antigen presenting cells [42]. In contrast to macrophages derived from other mucosal sites [43, 44], CD40 is highly expressed on most CD163^{+} uterine macrophages (Figures 2A and 2B). This suggests that uterine macrophages are particularly sensitive to activation by CD40L.

Uterine macrophage cytokine expression

Microbial infection is a major cause of pre-term birth, infertility and ectopic pregnancy; therefore, protection from uterine infection is crucial to ensuring reproductive success [45]. Given the important role of the endometrium in the maintenance of fetal implantation and development, it is advantageous to mount a rapid immune response to microbial challenge. To determine the responsiveness of uterine macrophages to endotoxin challenge, CD163^{+} macrophages were isolated from uterine tissue by positive selection. Cell purity ranged between 89-95%, as determined by CD163 staining. Flow cytometric data in Figure 3A are representative of cell isolations from three individual donors. Following isolation, cells were stimulated with 10 ng/ml of ultra pure *E. Coli* LPS for 24 hours and cytokine secretion was measured by Bio-Plex assay. As demonstrated in Figure 3B, uterine macrophages secrete a wide range of pro-inflammatory cytokines in response to LPS including TNFα, IL-12, IL-17 and IL-1β. These data indicate that TLR4 signaling is functional in these cells.

IL-1β and its receptor antagonist, IL-1ra, co-ordinate a wide range of biological activities within the human uterine endometrium, both facilitating embryonic implantation as well as conferring protection from pathogenic challenge [45]. In previous studies, we have demonstrated that human uterine macrophages produce bioactive IL-1β in response to LPS [15]. We now show that in addition to IL-1β, uterine macrophages also express high levels of IL-1ra (Figure 3B). Because the secretion of IL-1ra exceeds that of IL-1β by 6-fold, IL-1β signaling within the human uterine endometrium may be attenuated. Similarly, CD163^{+} uterine macrophages also secrete IL-10 in response to LPS, which may also dampen the effects of pro-inflammatory cytokines (Figure 3B). These data suggest that CD163^{+} endometrial macrophages are most likely M2b polarized because they produce both pro- and anti-inflammatory cytokines in response to LPS stimulation.

Uterine macrophage chemokine expression

Leukocytes are recruited to the uterine endometrium throughout the menstrual cycle and are an important component of tissue turnover and repair [7]. The influx of migratory cells is orchestrated through local chemokine expression within the cycling endometrium [46]. In Figure 4, we demonstrate that CD163^{+} uterine macrophages constitutively express low

levels of MIP-1β and MCP-1, implicating these cells in the active recruitment of neutrophils and monocytes to the endometrium. In addition, recent studies implicate a role for MCP-1 in M2 macrophage polarization [47]. Constitutive expression of MCP-1 may be important in the maintenance of this phenotype in uterine macrophages.

Because tissue resident macrophages produce chemokines in response to microbial challenge as an early step in the recruitment of additional immune effector cells, we next investigated whether LPS activation elicits chemokine secretion from uterine macrophages. As demonstrated in Figure 4, LPS stimulation markedly induces MIP-1α and MIP-1β secretion by uterine macrophages. Similarly, MCP-1, eotaxin, RANTES and IP-10 are LPS-inducible in uterine macrophages. As these chemokines are involved in the recruitment of monocytes, dendritic cells, T cells and eosinophils, these results suggest that macrophages mediate localization of these immune cell subsets to the uterine endometrium in response to microbial challenge.

Uterine macrophage growth factor expression

Macrophages have an active role in tissue turnover and remodeling in the human endometrium [48]. Following shedding of the endometrial lining during menstruation, expression of growth factors and angiogenic molecules promotes tissue growth and vascular repair. As demonstrated in Figure 5, uterine macrophages secrete G-CSF and GM-CSF in response to LPS. In addition to regulating the survival and differentiation of granulocytes and macrophages, GM-CSF is also a chemo-attractant for neutrophils [49].

Angiogenesis occurs during endometrial repair and vascular integrity is imperative for successful embryo implantation (reviewed in [50]). In this regard, uterine macrophages secrete low constitutive levels of the pro-angiogenic factors VEGF, FGF2, and PDGF, which are enhanced by LPS stimulation (Figure 5). Activated platelets are a major source of PDGF within the uterine endometrium [51], and as demonstrated in Figure 5, macrophages provide an additional source of endometrial PDGF. These data demonstrate that CD163^{+} uterine macrophages produce important factors involved in the maintenance of endometrial tissue homeostasis and angiogenesis.

Discussion

The uterine endometrium is an immunologically unique site, as it must simultaneously protect against microbial infection and tolerate allogeneic sperm and a semi-allogeneic fetus. Macrophages within the uterine endometrium have a significant role in mediating host defense in addition to maintaining tissue homeostasis. Although macrophages comprise a significant number of leukocytes within the non-pregnant uterine endometrium, no studies to our knowledge have addressed the functional polarization of these cells. To address this question, we characterized the repertoire of immunoreceptors expressed by human uterine macrophages and the profile of cytokines, chemokines and growth factors produced by these cells in response to LPS.

CD163 expression is restricted to cells of monocytic lineage and is widely expressed by mature tissue macrophages [29, 30], making it an excellent marker for identification and isolation of tissue macrophages. Moreover, high CD163 expression is a bona fide marker of the M2 macrophage subtype [5]. Figure 1 shows that CD163 is expressed at high levels on 71.9% of CD68^{+} endometrial macrophages, whereas CD14 expression is limited to a smaller sub-population of macrophages. These findings demonstrate that the majority of macrophages within the human endometrium express high levels of CD163, consistent with an M2 phenotype. Moreover, our data corroborate results of a recent study in which CD14^{high}CD68^{+} M1 polarized macrophages were shown to constitute a relatively small

population of the total immune cell population in the human non-pregnant myometrium [37]. In our current study, we have identified and characterized for the first time a distinct CD163^{high}CD68^{+} M2 polarized uterine macrophage population.

To further characterize these cells, CD163^{+} macrophages were analyzed for surface expression of other macrophage markers. In Figure 2, we show that a subset (approximately 30%) of CD163^{+} human uterine macrophages also express CD14, a marker of classically activated macrophages. Intriguingly, expression of CD16, which is characteristic of M2 macrophages, is low and limited to only 10% of total CD163^{+} cells. This may be attributable to the diverse nature of alternatively activated macrophages. Down-regulation of CD14 and CD16 is also observed in macrophages derived from other mucosal sites, including the lamina propria of the gut [52, 53] and the vaginal mucosa [54].

However, in contrast to macrophages of the gut mucosa where TLR4 expression is low or undetectable [52, 55-57], a large percentage of uterine macrophages (~60%) is positive for TLR4 expression. Since commensal bacteria colonize the gut, limiting TLR expression may be advantageous for minimizing inappropriate immune activation. Commensal organisms also colonize the lower regions of the female reproductive tract; however, they are absent from the upper tract, including the uterine endometrium and Fallopian tubes [58]. Our previous work has shown that TLR4 expression progressively declines in tissues from the upper to lower reproductive tract, with the highest levels expressed in the Fallopian tube and uterine endometrium [59]. High expression of TLR4 in the uterine endometrium may be critical to ensuring reproductive success, since this tissue is most likely to be challenged by Gram-negative *N. gonorrhoeae* and *C. trachomatis* [58]. Increased innate surveillance at this site (manifested by enhanced TLR4 expression) may provide a means of ensuring sterile conditions while conferring protection from microbial challenge. In this regard, it has recently been reported that in addition to recognizing hemoglobin-haptoglobin complexes, CD163 also functions as an immune receptor for both Gram-negative and Gram-positive bacteria [60]. Therefore, it is notable that uterine macrophages express elevated levels of CD163 in addition to TLR4. High expression of these receptors suggests that these cells are poised to recognize bacterial infection within the uterine endometrium.

As key effector cells of the innate immune system, macrophages interact with CD4^{+} T cells through MHC II and co-stimulatory molecule expression. As demonstrated in Figure 2, MHC II, CD80 and CD86 expression on endometrial macrophages is low, indicating that these cells may have reduced ability to mediate CD4^{+} T cell activation. CD80 and CD86 have overlapping expression patterns and identical function. Both molecules serve as ligands for CD28, the activating receptor expressed on the surface of T cells, as well as CTLA-4, an inhibitory receptor expressed by T cells [61]. Whether CD80 and CD86 provide activating or inhibitory signals depends on the relative expression of CD28 and CTLA-4 on uterine CD4^{+} T cells and is an area of ongoing investigation in our laboratory.

CD40 is a member of the tumor necrosis factor-α family and is expressed on antigen presenting cells including macrophages and B cells (reviewed in [42]). CD40L, the endogenous ligand for CD40, is expressed mainly on activated T cells and is also present in soluble form in the human endometrium [62]. In contrast to monocytes and *in vitro* derived macrophages, which express low levels of CD40 [63], uterine macrophages express high levels of CD40. Macrophage activation through CD40 stimulation leads to the production of both pro- and anti-inflammatory cytokines as well as the up-regulation of MHC II, CD80, CD86 and CD40 itself [64]. Activated platelets serve as a reservoir of sCD40L [65]. Since platelet numbers in the endometrium increase during menstruation [11], sCD40L levels may be an important signal for macrophage involvement in uterine endometrial tissue turnover

and repair. Therefore, high CD40 expression on uterine macrophages is likely important in both the context of infection and in tissue homeostasis.

We also investigated whether CD163^{+} uterine macrophages were responsive to endotoxin challenge. In response to LPS, isolated uterine endometrial macrophages secrete the pro-inflammatory cytokines TNFα, IL-12, IL-17 and IL-1β as well as anti-inflammatory IL-1ra and IL-10. As previously reported, endometrial macrophages express bioactive IL-1β in response to endotoxin challenge, and expression of this cytokine elicits the secretion of HBD2 by the endometrial epithelium [15]. Interestingly, IL-1ra is expressed in excess of IL-1β, a characteristic of alternatively activated macrophages [66]. It is notable that a similar level of recombinant IL-1β induces higher levels of HBD2 than does conditioned media from LPS-stimulated endometrial macrophages [15]. Although IL-1ra levels were not measured in that study, our results suggest that high levels of IL-1ra expression may explain this observation. Therefore, in addition to secreting pro-inflammatory cytokines to combat microbial infection, uterine macrophages also produce anti-inflammatory factors that aid in the resolution of inflammation. These characteristics are consistent with M2b macrophage alternative activation.

Intriguingly, uterine macrophages produce high levels of IL-17 in response to LPS. IL-17 is a pro-inflammatory cytokine that also induces neovascularization and can promote the expression of other angiogenic factors [67]. In humans, T cells are the major source of IL-17; however, monocytes and macrophages have now also been identified as significant producers of IL-17 [68-70]. IL-17 also up-regulates chemokine and MMP expression, which enables recruitment of inflammatory cells to sites of infection (reviewed in [71]). Given that MMPs contribute to the breakdown of tissue during menstruation, this highlights a potential role for IL-17 in regulation of endometrial tissue turnover, and suggests that this cytokine is an important mediator of macrophage maintenance of uterine endometrial tissue homeostasis.

In addition to cytokines, uterine macrophages also produce significant levels of chemokines. As demonstrated in Figure 4, endometrial macrophages express MIP-1α, MIP-1β and MCP-1. Levels of these chemokines in the human endometrium vary throughout the menstrual cycle with highest levels reported during the late secretory phase [72, 73], consistent with the role these factors play in the recruitment of leukocytes for endometrial shedding. Because rapid trafficking of immune effector cells to sites of infection is essential, it is fitting that uterine macrophage production of chemokines is LPS-inducible. In addition to recruiting leukocytes to the endometrium, macrophages also produce factors important in leukocyte maturation and differentiation. In this work, we have shown that endometrial macrophages produce G-CSF and GM-CSF, which are important in the differentiation of granulocytes and macrophages.

Most mature human tissues do not exhibit significant angiogenesis except in response to injury. However, the human uterine endometrium regularly undergoes extensive vascular remodeling in preparation for embryo implantation. The process of endometrial angiogenesis is mediated by local production of growth factors, most prominently VEGF [74]. Our study suggests that macrophages in the uterine endometrium may contribute to the process of angiogenesis through both constitutive and induced expression of angiogenic factors such as VEGF, FGF2 and PDGF. Notably, secretion of pro-angiogenic factors is also characteristic of M2 macrophages.

Notably, uterine macrophages constitutively secrete many chemokines and cytokines, including MCP-1 and IL-6. Intriguingly, recent work demonstrates that MCP-1 skews macrophages towards an alternatively activated phenotype (M2b or M2d) [47]. Furthermore,

IL-6 secretion may also play a role in maintaining M2 polarization, as IL-6 mediates up-regulation of CD163, a validated marker of alternatively activated macrophages [27, 75].

In this regard, the functional plasticity of macrophages has been well-documented. Because macrophages are exquisitely sensitive to micro-environmental signals, they readily adapt functional patterns to respond to changes in the local cellular milieu [76]. In recent studies, we have shown that culture of M2-polarized uterine macrophages with LPS enhances expression of CD14 and MHC-II and inhibits expression of CD163 (data not shown). These results suggest that microbial encounter induces phenotypic and functional changes in M2 uterine macrophages that result in an activation program aimed at inhibition of infection rather than implantation. Further studies are needed to elucidate the mechanism and functional consequences of macrophage repolarization.

Because the local uterine micro-environment plays a role in the modulation of macrophage activation and function, it is possible that differences in hormone levels (such as those observed during the proliferative and secretory phases of the menstrual cycle) influence macrophage polarization. All tissues were staged prior to macrophage isolation and flow analysis. Review of our data failed to establish a statistically significant link between menstrual cycle status and macrophage activation. However, this may be attributable to the relatively limited sample size assessed in our study. Current work in our laboratory may provide greater insight as to the influence of cycle-dependence on macrophage polarization, as this work is focused on determining how estradiol and/or progesterone modulate macrophage activation.

In summary, we have now shown that the major population of human uterine macrophages exhibits characteristics of alternatively activated or M2 macrophages. These CD163$^+$ cells express a repertoire of immunoreceptors similar to that of other mucosal macrophages, but with higher levels of TLR4 and CD40. Elevated expression of TLR4 is likely important in mounting rapid responses to invading pathogens to ensure reproductive success in the face of infection. As endometrial macrophages play a significant role in tissue remodeling, high CD40 expression may permit these cells to respond to sCD40L produced by activated platelets during menstruation. In this study, we have shown that endometrial macrophages are sensitive to endotoxin challenge and respond by producing a profile of cytokines, chemokines, growth and pro-angiogenic factors similar to that of M2b activated macrophages. Collectively, these data suggest that CD163$^+$ endometrial macrophages play an important role in host defense and the regulation of tissue homeostatic functions including tissue breakdown, clearance and angiogenic remodeling.

Acknowledgments

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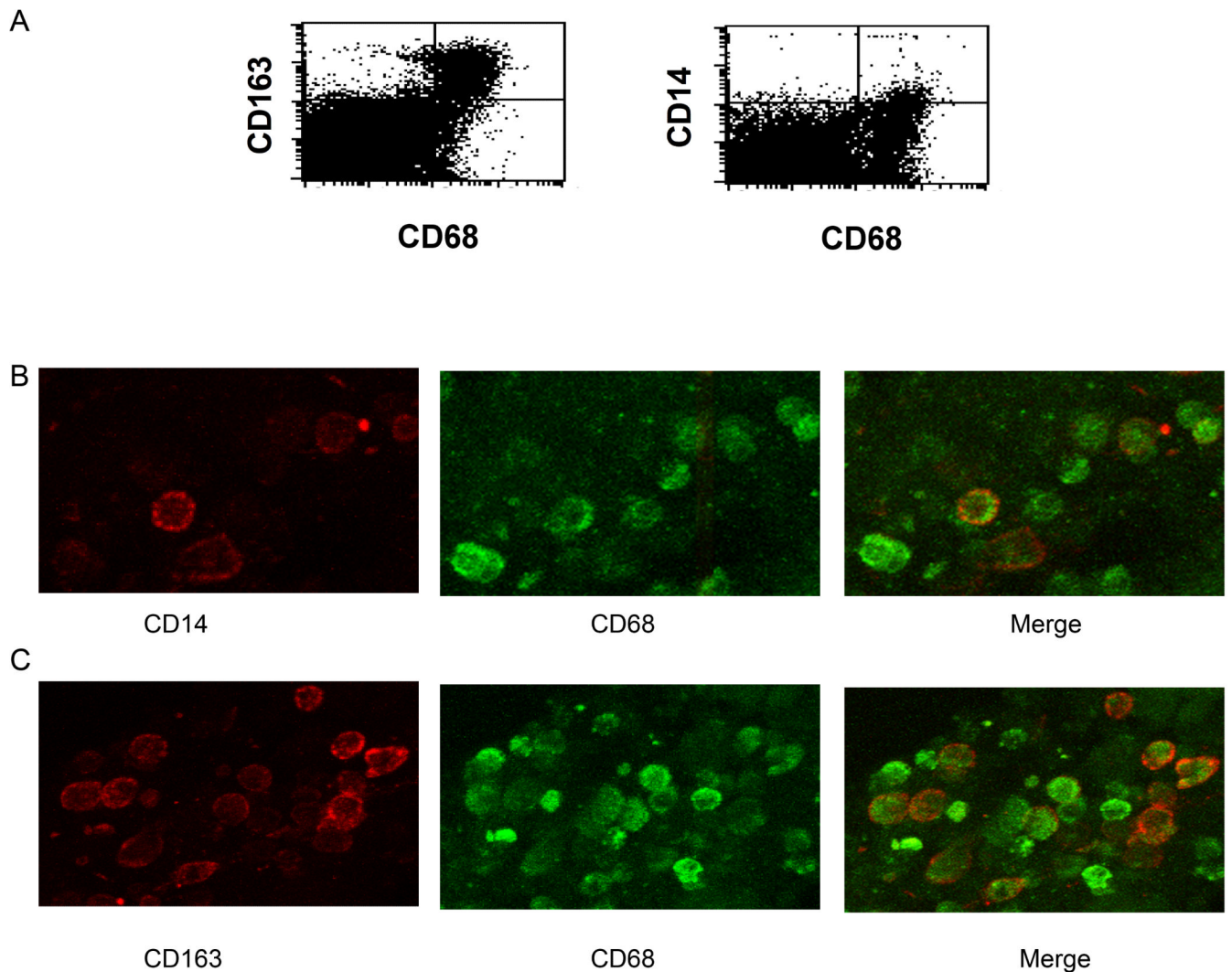
Figure 1. CD163 is highly expressed by uterine macrophages
Freshly obtained uterine endometrial tissue sections were enzymatically digested into single cell suspensions, stained for CD68 and CD163 or CD68 and CD14 (A) and analyzed by flow cytometry. The numbers inset in the histograms are the percentage of CD163$^+$/CD68$^+$ or CD14$^+$/CD68$^+$ cells. (B and C) Confocal imaging of uterine endometrial macrophages. Endometrial tissues were sectioned and stained for either (B) CD14 and CD68 or (C) CD163 and CD68. Images shown are representative of tissue sections from six individuals.
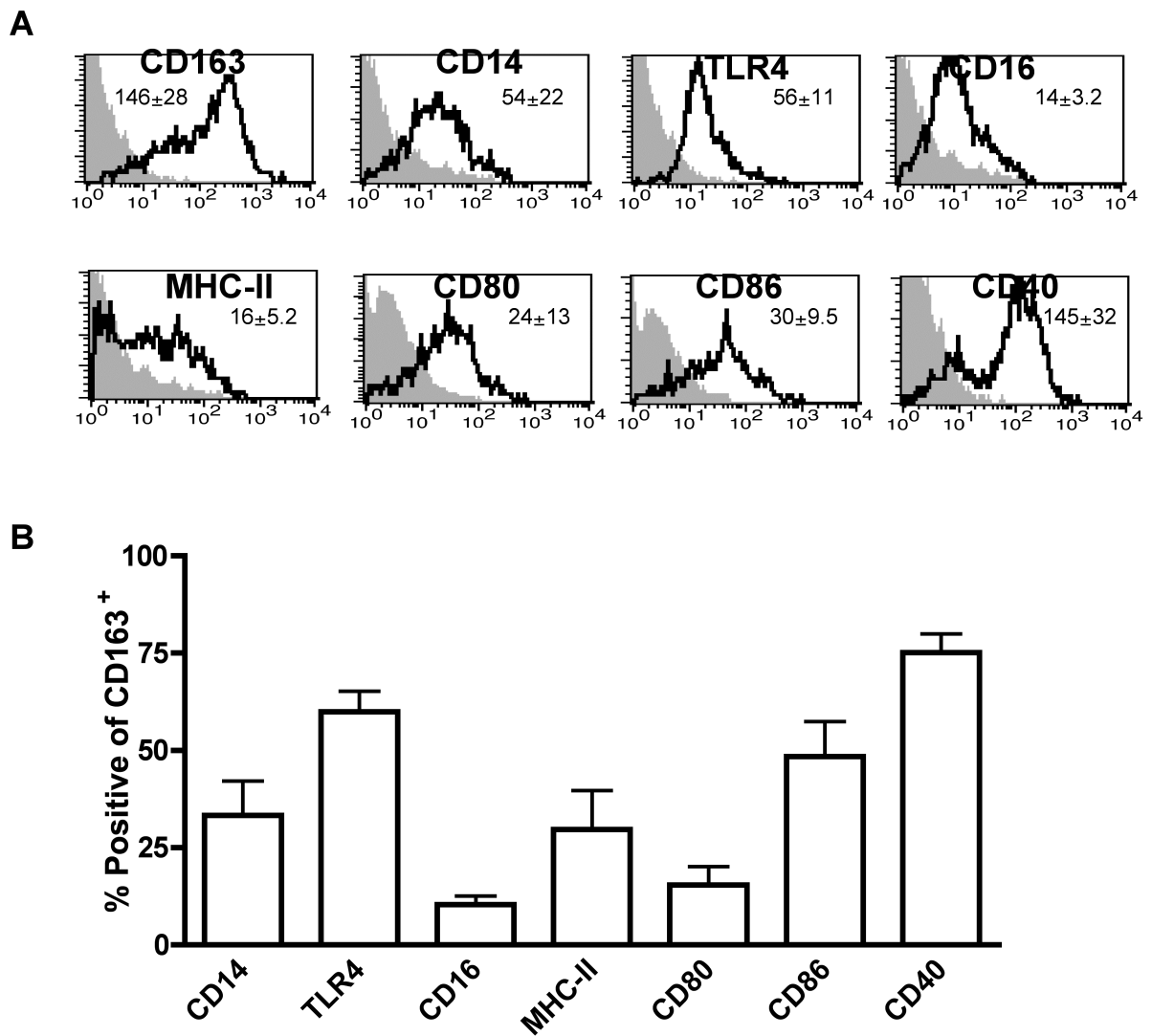
A

B

Figure 2. Uterine macrophage surface expression phenotype

Freshly obtained uterine endometrial tissue sections were enzymatically digested into single cell suspensions. Cells were stained for surface antigens in addition to CD163 and analyzed by flow cytometry. (A) Representative histograms of mean fluorescence intensity of macrophages gated on CD163 expression. Inset numbers indicate mean MFI ± SEM for N = 4-7 patients. (B) Percentage of positive cells gated on CD163 expression.
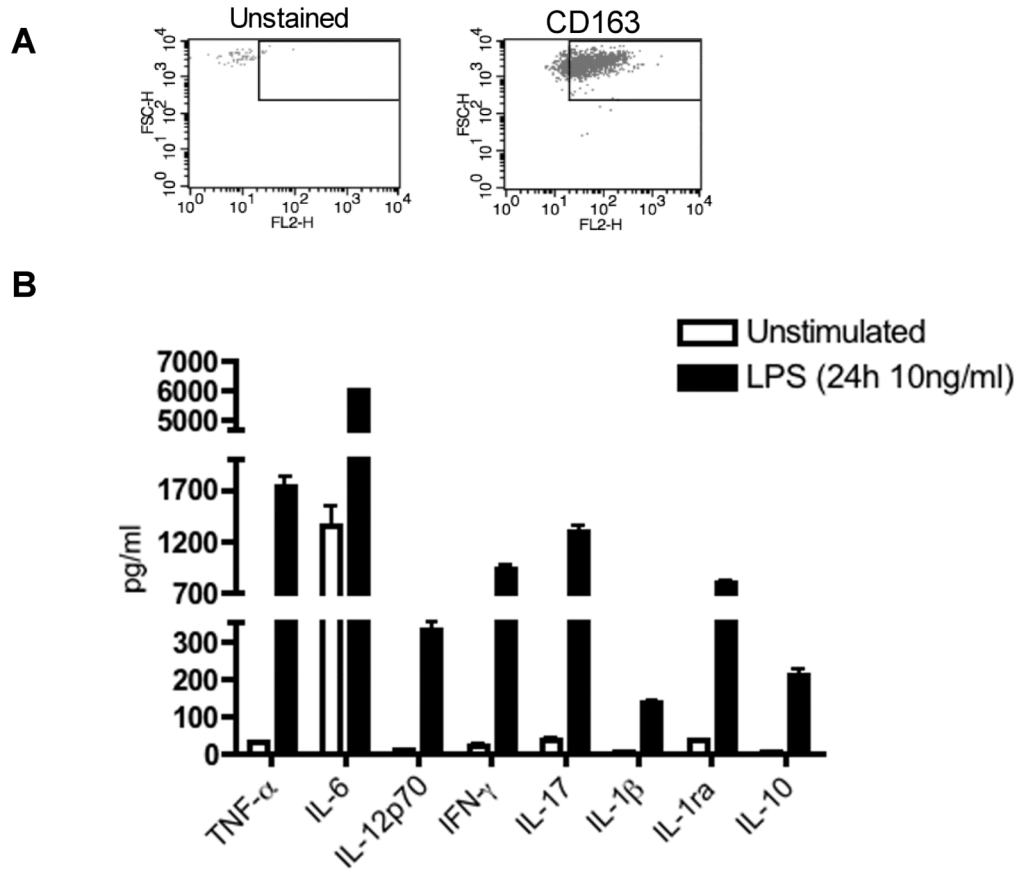
Figure 3. Isolation of CD163^{+} macrophages and cytokine expression
(A) Uterine macrophages were isolated by CD163 positive selection. The purity of the isolated cells typically ranged between 89-95 percent, as determined by staining for CD163 compared to unstained controls. (B) CD163^{+} selected macrophages from 3 individual donors were stimulated with 10 ng/ml LPS for 24 hours and analyzed for cytokine secretion in culture supernatants by Bio-Plex analysis.
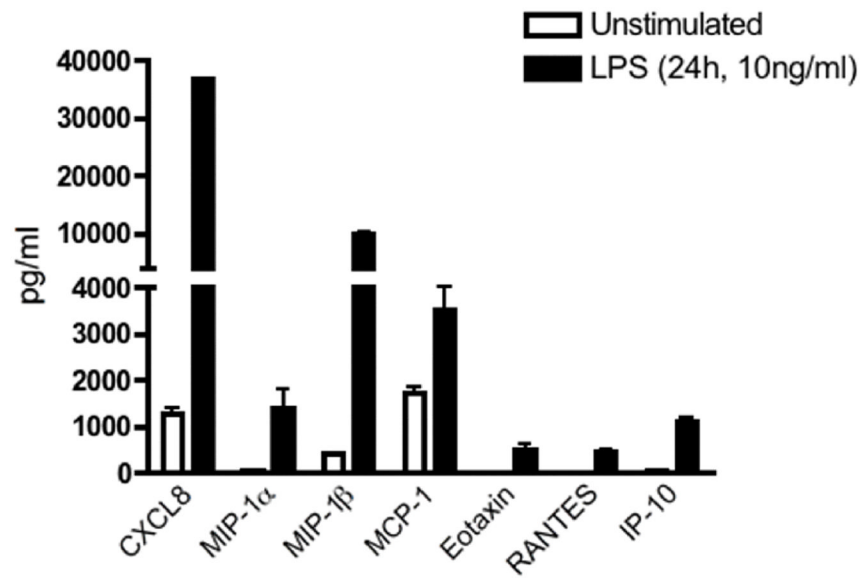
Figure 4. Uterine macrophage chemokine expression
Culture supernatants were analyzed for chemokine expression from uterine macrophages as described in Figure 3.
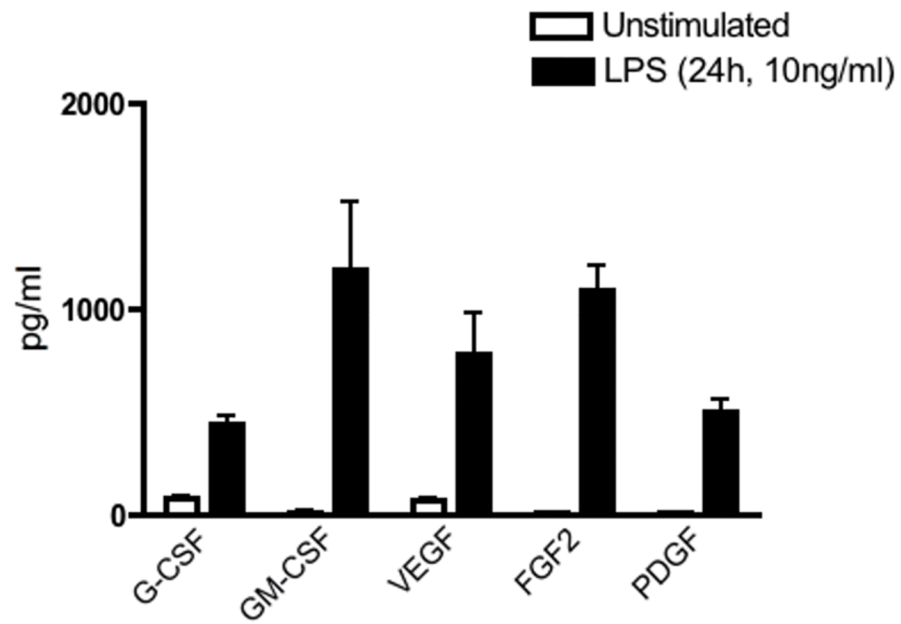
Figure 5. Uterine macrophage growth factor expression
Growth factor expression in response to LPS stimulation was analyzed by Bio-Plex analysis as described in Figure 3.

Table 2

Antigen	Fluorochrome	Source	Antibody Clone
CD14	FITC	Medarex	AML223
CD16	FITC	Medarex	3G8
CD40	PE	R and D Systems	82111
CD68	FITC	Thermo Scientific	Kim7
CD80	FITC	R and D Systems	37711
CD86	PE	Beckman Coulter	HA5.2B7
CD163	PE	Maine Biotechnology	Mac 2-158
CD163	FITC	Maine Biotechnology	Mac 2-158
MHC-II	FITC	R and D Systems	L203
TLR4	PE	R and D Systems	267518

Table 1

Patient #	Age (years)	Diagnosis	Staging
4960	39	Fibroids	Inactive
4970	44	Pelvic Pain	Unknown
4975	42	Prolapse	Proliferative
4983	39	Prolapse	Secretory
5034	40	Colonic Inertia	Secretory
5045	52	Prolapse	Unknown
5815	40	Prolapse	Proliferative
5845	47	Prolapse	Secretory
6048	37	Menorrhagia	Proliferative
6078	41	Fibroids	Proliferative
6084	44	Pelvic Pain	Secretory
6098	44	Fibroids	Secretory

Table 3

Measurement	M1 Mφ	M2 Mφ
CD163	+	++++
CD14	+++	+
CD16	+/-	+++
CD40	++	+++
CD80	+++	++
CD86	+++	+
MHC-II	+++	+
TLR4	+++	++