Macrophage contributions to ovarian function

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Macrophages are multifunctional cells that play key roles in the immune response and are abundant throughout female reproductive tissues. Macrophages are identified in tissues by their expression of cell surface receptors and can execute diverse functional activities, including phagocytosis and degradation of foreign antigens, matrix dissolution and tissue remodelling, and production and secretion of cytokines, chemokines and growth factors. Their specific localization and variations in distribution in the ovary during different stages of the cycle, as well as their presence in peri-ovulatory human follicular fluid, suggest that macrophages play diverse roles in intra-ovarian events including folliculogenesis, tissue restructuring at ovulation and corpus luteum formation and regression. This review presents the existing evidence for the regulation of ovarian function by macrophages and macrophage-derived products, highlighting the implications of these cells in ovarian diseases, particularly polycystic ovary syndrome, endometriosis and premature ovarian failure.

Key words: cytokine/macrophage/ovary/ovulation/polycystic ovary syndrome

Introduction

Macrophages are ubiquitous immune cells that play key roles in both innate and acquired immunity. However, in addition to protecting the body from foreign organisms and antigens, they maintain homeostasis in many tissues through their cytokine production and remodelling capabilities. With regard to female reproduction, macrophages contribute to the regulation of the pituitary–gonadal axis and are found throughout female reproductive tissues including the ovary, uterus, oviduct and mammary gland. In the ovary, macrophages have been detected in fluctuating numbers at various stages of the menstrual cycle (Brannstrom et al., 1993a, 1994b), and have had many different functions ascribed to them. The purpose of this review is to present the existing evidence for the regulation of ovarian function by macrophages and macrophage-derived products, highlighting the implications of these cells in ovarian diseases.

General characteristics and functions of macrophages

Macrophages are immune cells derived from bone-marrow precursors, which when mature, enter the bloodstream as monocytes. The adhesion of immune cells to endothelial cells and their subsequent migration into tissue is a three-step process (Butcher, 1992). Initial tethering via interactions between selectins on leukocytes (L-selectin) and endothelial cells (E- and P-selectins) mediates the slow rolling of leukocytes on endothelial cells. Exposure to cytokines and chemokines during this time activates the monocytes and stimulates the production of cell surface β2 integrins, such as Mac-1 and LFA-1. Interactions between these leukocyte integrins and endothelial cell adhesion molecules, particularly those in the ICAM family, mediate firm adhesion of the monocytes and enable their migration between endothelial cells into tissues. Within tissues, differentiation of monocytes into macrophages occurs in response to the surrounding microenvironmental context, which directs the acquisition of tissue-specific phenotypes. For example, the initial cytokine exposure received by infiltrating monocytes can determine the functional phenotype of the maturing macrophage (Erwig et al., 1998) while the structure of the extracellular matrix (ECM) in vitro can also modulate macrophage function (Newman and Tucci, 1990; Gudewicz et al., 1994).

Within most organs, macrophages are involved in tissue homeostasis via their ability to execute diverse functional activities, including (i) phagocytosis and degradation of foreign antigens, (ii) matrix dissolution and tissue remodelling, and (iii) production and secretion of growth factors, cytokines and chemokines (Gordon, 1999a). These effector functions allow the macrophage to regulate local immune and inflammatory responses as well as influence normal tissue function. Each of these major macrophage effector functions and the various phases of the ovarian cycle that they are thought to impact are shown.
schematically in Figure 1 and will be described in greater detail below.

**Identification of ovarian macrophages using macrophage-specific markers**

Macrophages are identified in tissues by their expression of specific protein markers (Table I), which are predominantly cell surface receptors. The proteins considered most exclusively restricted to macrophages are F4/80 (the human homologue is EMR-1) and CD68 (also known as macrosialin in humans). F4/80, although its function is not fully understood, has been used extensively as a marker to identify macrophages in many tissues. In mouse ovaries, F4/80 positive cells exhibit marked changes in their distribution from neonatal to postpartum development.

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### Table I. Localization and function of macrophage-specific markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Location</th>
<th>Understood functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80 (EMR-1)$^a$</td>
<td>Pan murine macrophage marker</td>
<td>Cell–cell adhesion following adhesion to the extracellular matrix as well as some signalling capacity</td>
</tr>
<tr>
<td>Macrosialin (CD68)$^a$</td>
<td>Predominantly intracellular in tissue macrophages.</td>
<td>Binds oxidized lipoprotein</td>
</tr>
<tr>
<td>Complement receptor 3 (CD11b/Mac-1)$^a$</td>
<td>Transmembrane</td>
<td>Binds complement, participates in phagocytosis of particles, and mediates leukocyte migration out of vasculature</td>
</tr>
<tr>
<td>Class II MHC (Ia)$^a$</td>
<td>Found on antigen-presenting macrophages, i.e. dendritic cells</td>
<td>Binds exogenously derived peptides for recognition by T cells</td>
</tr>
<tr>
<td>Fc receptors (CD16/CD32/CD23)</td>
<td>Transmembrane/soluble</td>
<td>Binds constant portion of IgG molecule initiating either transport of IgG across the epithelial cell surface or the initiation of an immune response</td>
</tr>
<tr>
<td>Mannose receptor (CD206)</td>
<td>Type I transmembrane molecule, found on mature tissue macrophages</td>
<td>Binds bacterial and fungal glycoproteins and mediates uptake during host defence</td>
</tr>
<tr>
<td>Scavenger receptors (CD204)</td>
<td>Transmembrane glycoprotein on mature tissue macrophages</td>
<td>Binds lipoproteins and participates in uptake of apoptotic cells and pathogens</td>
</tr>
<tr>
<td>Sialoadhesin (SER-4/3D6)</td>
<td>Transmembrane on stromal macrophages in bone marrow, and lymphoid organs</td>
<td>Adhesion molecule which mediates the binding but not uptake of attached cells</td>
</tr>
<tr>
<td>Lipopolysaccharide receptor (CD14)$^a$</td>
<td>Glycosylphosphatidylinositol-linked plasma-membrane glycoprotein, on all macrophages</td>
<td>Binds bacterial lipopolysaccharide, triggers inflammatory responses</td>
</tr>
</tbody>
</table>

$^a$Indicates markers which have been detected in the ovary. (Compiled from Austyn and Gordon, 1981; Ross et al., 1985; Lachapelle et al., 1996; McKnight et al., 1996; Daeron, 1997; Devitt et al., 1998; McKnight and Gordon, 1998; Linehan et al., 1999; Platt et al., 1999.)

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**Figure 1.** Macrophage involvement in ovarian function. As summarized in the text and cited references, macrophages: (1) are phagocytic and involved in the removal of apoptotic cellular debris; (2) present peptide antigens and activate T cells; (3) release cytokines and growth factors that are known to regulate many functional aspects of granulosa and theca cells; (4) release chemokines that attract and activate additional monocytes, neutrophils and T cells into the ovary from circulation; (5) secrete an array of proteases that degrade matrix as well as release/alter matrix- or membrane-bound proteins. Thus, the tissue specific localization and diverse functions of macrophages enable them to potentially impact multiple aspects of ovarian function.
Within the corpus luteum (Simon et al., 1999; Gordon, 1999b). Among them, complement receptor 3 (CR3) and various scavenger receptors (reviewed by Aderem and Underhill, 1999) are also used as macrophage markers and include Fc receptors, mannose receptor, sialoadhesin, and scavenger receptors (reviewed by Aderem and Underhill, 1999; Gordon, 1999b). Receptors that are involved in phagocytosis are also used as macrophage markers and include Fc receptors, complement receptors, mannose receptor, sialoadhesin, and scavenger receptors (reviewed by Aderem and Underhill, 1999; Gordon, 1999b). Among them, complement receptor 3 (Mac-1/CD11b) is involved in cell–cell and cell–matrix adhesion and CD11b positive cells have been shown in the mouse ovary within the theca layer and stroma immediately after ovulation and within the corpus luteum (Simon et al., 1994a; Tamura et al., 1998).

It is important to consider that the marker used to identify macrophages reveals specific information about the changing functional characteristics of the cells; for instance, in the rabbit, luteolysis was associated with an initial increase in scavenger receptor positive macrophages followed by recruitment of CD68 positive macrophages (Krusche et al., 2002). Table I highlights that multiple markers are used to identify macrophages in the ovary, as well as give insight into the various functions these macrophages may be exerting during the ovarian cycle.

**Phagocytosis and antigen presentation**

Macrophages are considered ‘professional phagocytes’ and can internalize particles much more rapidly and efficiently than other cells due to their expression of specific cell surface receptors (see Table I). Phagocytosis is a complex process involving recognition of an antigen by macrophage cell surface receptors, which initiates actin polymerization and internalization of the foreign molecule or organism into a phagosome (Allen and Aderem, 1996). Phagocytosis is followed by fusion of the phagosome with lysosomes and degradation of the particle. Macrophages phagocytose endogenous and exogenous substances, such as cell debris, bacteria and viruses (Miller et al., 1983; Wei et al., 1988) and in culture will even phagocytize latex beads (Kirsch et al., 1981). When administration of exogenous particles was used to visualize phagocytosis, however, ovarian macrophages were reported to be less phagocytic than macrophages from other tissues (Itoh et al., 1999). Therefore ovarian macrophages may use phagocytosis primarily to remove apoptotic cells during specific phases of tissue remodelling as opposed to removal of foreign debris. Macrophages in vivo recognize and internalize apoptotic and necrotic cells, bringing about their efficient removal, and it has been shown that ovarian macrophages phagocytose atretic granulosa cells and apoptotic luteal cells in guinea-pigs, mice and humans (Paavola, 1979; Kuryszko and Adamski, 1987; Kasuya, 1997) thereby contributing to follicular atresia and luteolysis.

Following the phagocytosis of a foreign antigen, the macrophage is capable of intracellular degradation and presentation of the peptide fragments on the cell surface in association with the class II MHC. These class II MHC–antigen complexes are recognized by helper T cells via the T cell receptors CD3 and CD4. This results in the activation of both the T cell and the antigen-presenting macrophage and the initiation of a rapid immune response. Class II MHC-positive cells have been identified in the rat and human ovary (Hill et al., 1990; Bowen and Keyes, 2000) and equine corpora lutea (Lawler et al., 1999). In the human corpus luteum, abundant class II MHC positive cells within the granulosa-luteal layer, as well as helper and suppressor T cells within the thecal trabeculae, suggest a possible role for these immune cells in the ovary (Petrovska et al., 1992). Interestingly, granulosa-lutein cells exhibit increased staining for class II MHC antigens during the late luteal phase, concurrent with T cell invasion (Bukovsky et al., 1995).

**Secretion of cytokines, chemokines and growth factors**

Macrophages are major secretory cells capable of releasing cytokines, chemokines and growth factors that function in normal, inflammatory and disease processes of most tissues. A selection of macrophage products and their established functions is given in Figure 2. In general, the production and release of these secretory proteins is tightly regulated in temporal and tissue-specific manners by paracrine and autocrine regulatory mechanisms. Cytokines are small proteins (15–60 kDa) that act locally through specific cell surface receptors to coordinate interactions of the immune system and surrounding tissues. Macrophages secrete a diverse repertoire of cytokines, including interleukin (IL)-1, -6, -10, and -12, interferon α (IFNα), tumour necrosis factor α (TNFα), and granulocyte macrophage-colony stimulating factor (GM-CSF). These cytokines have been identified in the ovary of many species and are known to impact many aspects of ovarian function (reviewed by Brannstrom and Norman, 1993; Terranova and Rice, 1997; Bukulmez and Arici, 2000) including follicle growth and differentiation, ovulation, and corpus luteum formation and function, as detailed in subsequent sections.

Macrophages also produce and secrete chemokines, small (60–70 amino acids) chemotactic cytokines containing conserved cysteine residues, which are important mediators of leukocyte recruitment and activation. Chemokines consist of two families: the C-C family, potent chemoattractants for monocytes/macrophages, includes monocyte chemoattractant protein (MCP)-1 and MCP-3, and RANTES (regulated upon activation, normal T cell expressed and secreted), and the C-X-C family, which selectively recruits neutrophils, includes IL-8, epithelial-derived neutrophil attractant-78 (ENA-78), and growth-regulated oncogene α (GROα). Several chemokines have been identified in the rat ovary (Wong et al., 2002), some of which are hormonally regulated.

Growth factors secreted from macrophages include epidermal growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF) α and β, each of which is important for normal ovarian function. These growth factors are also expressed by granulosa and theca cells, but macrophages have been largely neglected as...
another potential source in the ovary. In the rat ovary, however, EGF was detected in macrophages surrounding developing follicles (Katabuchi et al., 1996) and TGFβ was detected in macrophages within functional corpora lutea (Matsuyama and Takahashi, 1995), indicating that during specific phases of the ovarian cycle macrophage-derived growth factors are likely to influence neighbouring cells in a paracrine manner. In the case of IGF-I, although it is produced by some macrophages (Nagaoka et al., 1990; Sunderkotter et al., 1994), it has not yet been localized to these cells in the ovary. However, since IGF has been found to modulate monocyte proliferation (Long et al., 1998) and macrophage cytokine production in vitro (Renier et al., 1996), IGF (as well as other growth factors) produced by granulosa and theca cells are likely to reciprocally regulate macrophage effector functions.

Definitive experiments identifying which cytokines and growth factors derived from macrophages are important for ovarian function are greatly lacking, due to the ubiquitous production of many of these factors by multiple ovarian cell types: granulosa cells, theca cells and macrophages. This knowledge gap, however, can be resolved by emerging techniques that specifically isolate ovarian macrophages, in order to better characterize their patterns of gene expression and protein production. For example, ongoing experiments from our own group have revealed that in isolated ovarian macrophages, the mRNA and protein expression of several cytokines is hormonally controlled (K.H.Van der Hoek, unpublished data), providing evidence that macrophage-derived factors are influencing follicular function at precise stages in the ovarian cycle.

**Secretion of proteolytic enzymes**

Macrophages have the capacity to produce and release a highly diverse group of proteolytic enzymes (Owen and Campbell, 1999a) which are able to degrade ECM, and activate or inhibit...
downstream protease cascades, as well as alter bioactive proteins such as leukocyte adhesion molecules, matrix-bound growth factors and membrane-bound cytokines (Owen and Campbell, 1999b; Bauvois, 2001). Highlighted here will be proteases that can be produced by macrophages and that have also been shown to be relevant in the ovary.

The cysteine proteases, cathepsins S, L, B, H and D are produced by and localized to the lysosomes of most cells, including macrophages and some macrophages also have the specialized ability to secrete these enzymes for pericellular matrix degradation (Owen and Campbell, 1999b). Each of these cathepsins has been detected in the mouse ovary and exhibits distinct patterns of cellular localization and hormonal regulation (Oksjoki et al., 2001). Although not definitively attributable to macrophages, some cathepsin proteins are preferentially expressed in macrophage-rich regions of the ovary such as the theca layer, corpus luteum and atretic follicles (Dhanasekaran and Moudgal, 1986; Oksjoki et al., 2001).

Serine proteases produced by macrophages include human leukocyte elastase, proteinase 3, and cathepsin G. Another member, urokinase-type plasminogen activator (uPA), cleaves plasminogen to proteolytically active plasmin and has been extensively studied in macrophages (Saksela et al., 1985; Dewerchin et al., 1996) as well as the ovary (reviewed by Ny et al., 2002). In the mouse ovary, in response to ovulatory gonadotrophin (LH/hCG), there is induced expression of uPA, as well as tissue-type plasminogen activator (tPA) in cells throughout the ovarian stroma which are reminiscent of ovarian macrophages, in addition to the high levels in granulosa cells (Hagglund et al., 1996).

Macrophages primarily produce matrix metalloproteinases (MMP), including: MMP-1 (collagenase); MMP-2 and MMP-9 (gelatinases); MMP-3, MMP-10 and MMP-11 (stromelysins); and MMP-7 (matrilysin) (Owen and Campbell, 1999a). Each of these MMP has also been detected in the ovary of several species (Hagglund et al., 1999; Ricke et al., 2002) and in general MMP are thought to regulate multiple aspects of ovarian function (reviewed by Curry and Osteen, 2001). Although studies have not specifically addressed production by ovarian macrophages, many MMP are expressed in the macrophage-rich regions of the ovary. MMP-2, MMP-9 and MMP-1 (MMP-13 in the rodent), for example, are expressed and active in the theca cell layers concurrent with ovulation (Hagglund et al., 1999; Curry and Osteen, 2001; Curry et al., 2001). Both MMP-14 (also known as membrane-type (MT)1-MMP) and MMP-19 are also rapidly expressed in response to ovulatory LH/hCG in scattered cells surrounding preovulatory follicles (a macrophage-like pattern) prior to expression in granulosa cells (Hagglund et al., 1999). MMP-3, MMP-7 and MMP-11 are up-regulated in ovarian regions undergoing apoptosis, concurrent with macrophage infiltration into these sites (Hagglund et al., 2001; Ricke et al., 2002). Also of interest, there are a number of MMP that are targeted for transmembrane expression on the cell surface, allowing for even greater control of protease localization within tissues (Bauvois, 2001). These proteases include members of the ADAM (a disintegrin and metalloprotease) family, such as TACE (TNFα convertase), and members of the MT-MMP family whose expression in the ovary is only beginning to be addressed.

Remodelling of ECM and release/activation of bound effector molecules occurs during many phases of ovarian function and requires precise regulation and appropriate localization of proteolytic enzymes. Thus, by virtue of their ability to be specifically recruited into particular ovarian regions, macrophages represent a possible mechanism by which to tightly control ovarian proteolysis.

Cumulatively, the above studies demonstrate that macrophages can be identified using multiple marker proteins and are present in the ovary of many species. Macrophages are able to: (i) phagocytose cellular debris and present antigens, (ii) secrete cytokines, chemokines and growth factors, and (iii) produce proteases that mediate matrix dissolution; thereby enabling them to influence multiple aspects of ovarian function (Figure 1). The following sections will focus on stages of ovarian follicle and corpus luteum development and describe the functional contributions of macrophages to each phase.

Roles of macrophages and macrophage-derived molecules in ovarian function

The ovary is composed of growing and atretic follicles, developing and regressing corpora lutea, and stromal/interstitial tissue. All components are present simultaneously in the adult ovary, with varying proportions of each dependent on the ovarian cycle stage. Interactions between the ovarian steroid hormones and the gonadotrophins from the pituitary are primary regulators of the ovarian cycle; however, many data suggest that macrophages, through their trophic functions in reproductive tissues, are essential accessory cells for optimal fertility (Norman and Brannstrom, 1994; Cohen et al., 1999).

The presence of macrophages in the ovary has been established for many years. Their identification was the result of studies carried out in 1964 examining the distribution of the macrophage enzymes alkaline phosphatase, esterase and β-glucuronidase in the rat ovary (Bulmer, 1964). Their specifically localized distribution and temporal variations during the cycle (Figure 3) suggest that macrophages play multiple roles in intraovarian events. Their ability to regulate ovarian cellular proliferation, inflammation and steroidogenesis further implicate these cells as regulators of ovarian function.

Regulation of follicular growth and atresia

Follicle growth occurs when small follicles are recruited to undergo granulosa and theca cell proliferation and antrum formation in response to FSH, estrogens and other locally produced factors. Only a fraction of growing follicles, however, reach the preovulatory stage and ultimately ovulate. The majority succumb to follicular atresia whereby granulosa cells undergo apoptosis and the follicle regresses and is resorbed by the ovary.

Direct interactions between macrophages and primordial follicles have not been observed, thus the earliest phases of follicle growth appear to be independent of macrophage influences. During follicle growth, however, the distribution of ovarian macrophages changes and they increase in number and localize to the theca cell layer of healthy follicles (see Figure 3B and C).

Two interesting rodent models exhibit reduced numbers of ovarian macrophages as well as reduced follicle growth and impaired fertility. The first, osteopetrotic (op/op) mice, have
severely reduced numbers of mature macrophages due to a natural mutation in the CSF-1 gene. These mice have a hypothalamic lesion that impacts estrus cyclicity (Cohen et al., 2002) but also exhibit reduced numbers of ovarian macrophages, which may be either a cause or effect of the decreased follicle growth. Secondly, feed restriction in rats also reduces the numbers of macrophages surrounding preovulatory follicles (Duggal et al., 2002), providing an interesting link between nutrition and ovarian macrophage populations.

The activation status of ovarian macrophages is also likely to be regulated during follicle growth. During early stages of rodent follicular development, CSF-1 both increases the number of ovarian macrophages and up-regulates macrophage scavenger receptor activity (Nishimura et al., 1995). Also concurrent with follicle growth, there is increased synthesis and release of the cytokine GM-CSF from ovarian macrophages, as well as theca-interstitial cells (Tamura et al., 1998).

It is thought that macrophages located in the theca of growing follicles, by secreting growth factors and/or cytokines, play a synergistic role in stimulating cellular proliferation and follicle growth, and in suppressing follicular apoptosis. Indeed, co-culture of rat granulosa cells with peritoneal macrophages results in proliferation of the granulosa cells (Fukumatsu et al., 1992). Some of the macrophage-derived factors that are known to impact follicular growth are hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), EGF, TGFα/β, and IGF. There are many studies that demonstrate the influences of these factors on ovarian cell types (reviewed by Geva and Jaffe, 2000; Monget and Bondy, 2000; Ingman and Robertson, 2002; Richards et al., 2002) with a subset of examples detailed below. Although macrophages are not the only source of these factors in the ovary, their localization to specific follicle types makes it likely that they exert paracrine influences. HGF can be produced by activated macrophages (Kodelja et al., 1997), receptors for this factor can be found in growing follicles (Yang and Park, 1995) and HGF stimulates granulosa cell proliferation and prevents granulosa cell apoptosis in vitro (Parrott et al., 1994). bFGF is a major regulator of angiogenesis that is also produced by macrophages (Sunderkotter et al., 1994). bFGF plays a role in the regulation of granulosa cell mitosis and differentiation, thecal cell differentiation and can prevent the spontaneous apoptosis of granulosa cells that occurs in culture (Oury et al., 1992; Tilly et al., 1992). EGF and TGFα have been demonstrated to be present in cells of the thecal layer in the rat (Kudlow et al., 1987; Skinner et al., 1987), bovine (Skinner and Coffey, 1988) and human (Scurr et al., 1994) ovary. EGF stimulates follicle DNA synthesis to a degree equivalent to that of FSH in the hamster (Roy and Greenwald, 1991), and in gonadotrophin-primed immature rats can stimulate granulosa cell proliferation and modulate follicular development through a paracrine mechanism (Fukumatsu et al., 1995; Katabuchi et al., 1996). TGFβ is also well known to potentiate the effects of FSH on granulosa cell DNA synthesis and follicle growth (Ingman and Robertson, 2002). Similarly, IGF amplify gonadotrophin-stimulated proliferation and steroidogenesis in ovarian cell types (Monget et al., 1996).

Macrophages are only found in the granulosa cell layer at advanced stages of atresia (Petrovska et al., 1996) and because they are known to phagocytose apoptotic cells it is thought that they participate in the removal of cell debris created during granulosa cell apoptosis (Takaya et al., 1997; Gaytan et al., 1998a). However, they may also have an active role in the production of factors promoting follicular atresia. Cytokines produced by macrophages in vitro, particularly TNFα (Kaipia et al., 1996), induce apoptosis in ovarian cell types and follicles.
which supports this theory. In contrast, in vitro experiments show that both EGF and TGFβ can prevent spontaneous apoptosis of follicles or isolated granulosa cells (Tilly et al., 1992). Thus whether macrophages are strictly involved in removal of apoptotic cells or whether they are involved in the initiation of apoptosis and atresia is not clear and must be addressed in future studies.

In short, macrophages and/or macrophage-derived products are important mediators of follicle development via regulating the balance between cellular proliferation and apoptosis, as well as angiogenesis and steroidogenesis. Thereby, they have the ability to influence processes that either commit or rescue follicles from atresia.

**Presence in human follicular fluid**

A study examining periovulatory human follicular fluid from IVF cycles found considerable numbers of macrophages and monocytes in this fluid, and documented their presence in normal human ovary tissue sections (Loukides et al., 1990). Although granulosa-luteal cells predominate in follicular fluid aspirates, it was found that 5–15% of the cells are macrophages and monocytes. Baranao et al. (1995) evaluated macrophage and HLA positive cells in the follicular fluid and found that human follicular fluid contained ~10% macrophages but that only 7.85% were ovarian-derived and the rest attributed to contamination with peripheral blood monocytes.

Because macrophages are a significant component of the intrafollicular compartment, many studies have been undertaken to evaluate whether macrophage-derived cytokines are also present in human follicular fluid. Levels of IL-1β, IL-6, IL-10 and GM-CSF in follicular fluids have been measured in multiple studies (Wang and Norman, 1992; Calogero et al., 1998). IVF patients with infertility due to immunological causes had higher levels of TNFα and IL-6 and lower concentrations of GM-CSF in their periovulatory follicular fluid compared to patients with tubal factor infertility (Cianci et al., 1996; Calogero et al., 1998). Follicular fluid-derived cells express IL-1β mRNA which may be a product of macrophages, granulosa cells, or both (Loukides et al., 1990; Baranao et al., 1995). Moreover, co-culture of follicular fluid-derived macrophages and granulosa cells from ovulatory follicles resulted in increased numbers of IL-1β-producing granulosa cells (Machelon et al., 1995).

Macrophages are a prevalent cell type in human follicular fluid aspirates and represent an important source of follicular fluid cytokines that are likely to be involved in follicle growth and ovulation. Much work is necessary, however, to determine how alterations in macrophage numbers, activation status or cytokine production correlate with follicular dysfunction in infertile women from whom the follicular fluid is obtained.

**Roles in ovulation**

Ovulation involves the rupture of preovulatory follicles at the surface of the ovary and extrusion of the oocyte into the oviduct. The hypothesis that mammalian ovulation is comparable to an inflammatory reaction, sharing the characteristics of edema, vasodilation, heat and pain, was first proposed by Espey (1980). In gonadotrophin-primed immature rats, there is an increase in ovarian blood volume, edema due to vasodilation and increased vascular permeability within a few hours of hCG treatment, which persists to the time of follicular rupture (Tanaka et al., 1989).

Macrophages are a prevalent cell type in human follicular fluid aspirates and represent an important source of follicular fluid cytokines that are likely to be involved in follicle growth and ovulation. Much work is necessary, however, to determine how alterations in macrophage numbers, activation status or cytokine production correlate with follicular dysfunction in infertile women from whom the follicular fluid is obtained.
Macrophages have the capacity to release numerous cytokines demonstrated to be important in the ovulatory process, particularly IL-1β (Adashi, 1998) and TNFα (Brannstrom et al., 1995). IL-1β levels increase in the ovary as ovulation approaches (Brannstrom et al., 1994a), addition of IL-1β to the media of in vitro-perfused ovaries stimulates ovulation in the rat (Brannstrom et al., 1993c) and rabbit (Takehara et al., 1994), and injection of the IL-1 receptor antagonist (IL-1RA) inhibits ovulation *in vivo* (Peterson et al., 1993; Simon et al., 1994b). Furthermore, nitric oxide (NO), an important mediator of the pro-inflammatory actions of IL-1β, is essential for optimal ovulation rate as demonstrated by mutant mouse models (reviewed by Dixit and Parvizi, 2001), and suppression of NO production with the pharmacological inhibitor L-NAME reduces both the number of ovarian leukocytes and the IL-1β-stimulated ovulation rate (Bonello et al., 1996). Expression of TNFα mRNA and protein has been localized to macrophage-like cells within the ovarian interstitium (as well as other ovarian cell types) (Chen et al., 1993) and this cytokine has also been shown to stimulate LH-induced ovulation in the perfused rat ovary (Brannstrom et al., 1995) as well as induce production of ovulatory mediators in pre-ovulatory rat follicles cultured *in vitro* (Brannstrom et al., 1993b). It has also been proposed that the release of TNFα by cells within the thecal layer stimulates local cellular apoptosis, thus facilitating follicular rupture (Murdoch et al., 1997).

The production of proteinases at the apex of the preovulatory follicle is essential for degradation of the basement membrane and the follicular wall at ovulation. Inhibition of collagenase/proteinase activity results in the inhibition of ovulation (Brannstrom et al., 1988; Butler et al., 1991); however, the identity of the critical ovulatory protease(s) has not yet been elucidated. As described above, macrophages and monocytes produce a diverse array of proteinases cumulatively capable of degrading all types of matrix proteins (Werb et al., 1980; Owen and Campbell, 1999a). It has therefore been proposed that macrophages in the theca of the preovulatory follicle produce proteolytic factors under the influence of locally produced paracrine or autocrine regulators. For instance, macrophages are known to produce plasminogen activator which, when perfused into the rabbit ovary, rapidly triggers ovulation (Yoshimura et al., 1987). Also, mice which are doubly deficient in the plasminogen activators tPA and uPA exhibit impaired ovulation (Leonardsson et al., 1995). The protease proprotein convertase 4 (PC4) has been localized to macrophage-like cells in the mouse ovary and shown to regulate cytokine production by these cells (Tadros et al., 2001). Interestingly, mice null for PC4 protease exhibit decreased ovarian weight, decreased progesterone production and decreased litter size (Mbikay et al., 1997), providing a compelling link between ovarian macrophage proteases and female fertility.

Macrophages have been shown to be important potentiators of ovulation, but the essential secondary messengers remain to be conclusively identified. To date, proteases and cytokines are the most likely candidates for the macrophage-derived products that facilitate ovulation.

**Regulation of corpus luteum formation and regression**

After ovulation, a complete reorganization of the ruptured follicle is required to produce a corpus luteum. This is characterized by terminal differentiation (luteinization) of granulosa cells, migration of leukocytes, including macrophages, into the luteinizing follicle and neo-vascularization of the developing corpus luteum. Class II MHC positive macrophages are the most prominent immune cells within the human corpus luteum throughout its lifespan (Petrovska et al., 1992; see also Figure 3D). Several studies have analysed macrophages during corpus luteum lifespan, but the utilization of different macrophage markers has complicated interpretation of the results. Macrophages have been described predominantly in the theca-lutein layer (Lei et al., 1991; Wang et al., 1992) at a maximum in the late luteal phase (Duncan et al., 1998). Another study found that in the human ovary the number of CD68 positive macrophages increased up to the end of the early luteal phase, remained relatively unchanged during the midluteal phase, and decreased at the late luteal phase (Gaytan et al., 1998b), paralleling the functional activity of the corpus luteum. Another study, however, found that the proportion of macrophages in the human corpora lutea did not differ between different stages of the luteal phase (Castro et al., 1998).

The activation status of macrophages is likely to be important for their effector functions within the corpus luteum. Macrophages showed round or elongated cytoplasm during the early and late luteal phases and displayed dendritic features in the mid-luteal phase, morphological changes typically related to their activation state (Gaytan et al., 1998b). Gaytan et al. (1997) also reported both round and spindle-shaped macrophages within the corpus luteum depending on the luteal stage (Gaytan et al., 1997). GM-CSF regulates many aspects of macrophage differentiation and activation (Inaba et al., 1992) and ovarian macrophages from mice lacking GM-CSF exhibit decreased expression of activation markers, including class II MHC and CD11b/Mac-1 (Jasper et al., 2000). These mice also have decreased ovarian weight and decreased progesterone production at day 4 of pregnancy, indicating that GM-CSF has a role in modulating the activation status of ovarian macrophages, which has consequences for appropriate progesterone production (Jasper et al., 2000).

Macrophages may facilitate the establishment of the vasculature in the corpus luteum via secretion of VEGF, EGF and bFGF, which would influence aspects of angiogenesis (Sunderkotter et al., 1994). VEGF, for instance, stimulates proliferation in vitro of microvascular endothelial cells obtained from the primate corpus luteum (Christenson and Stouffer, 1996). Macrophages may also increase progesterone production in the forming corpus luteum. The culture of peritoneal macrophages with luteinizing granulosa cells resulted in increased levels of progesterone production (Chen et al., 1992), and several macrophage-derived factors such as IL-1β, EGF and TNFα (Serta and Seibel, 1993; Yan et al., 1993; Chen et al., 2000) have also been shown to stimulate progesterone production. In contrast, there are also reports that progesterone secretion is markedly inhibited in co-cultures of granulosa cells with peritoneal macrophages. Here the degree of inhibition was dependent on both the number and activation status of the cocultured macrophages (Shakil and Whitehead, 1994).

The number of macrophages in the corpus luteum is highest during regression, implicating these cells in luteolysis (Paavola and Boyd, 1979; Lei et al., 1991; Brannstrom et al., 1994b; Hameed et al., 1995; Best et al., 1996; Senturk et al., 1999). Inflammatory cell infiltration begins in the theca lutein and gradually invades the granulosa lutein, but this infiltration can be prevented by inhibition of luteolysis with hCG to mimic.
pregnancy (Duncan et al., 1998). In the human, the progressive infiltration of lymphocytes and macrophages during luteal regression may occur in response to MCP-1 expression in blood vessels within the corpus luteum (Hameed et al., 1995). In the rat, increased endothelial cell expression of the intercellular adhesion molecule (ICAM)-1 occurs concurrently with macrophage infiltration, suggesting that ICAM-1 mediates macrophage migration into the regressing corpus luteum (Olson et al., 2001). The influx of macrophages also coincides with increased MMP activity in luteal cells (Duncan, 2000) and increased macrophage phagocytosis (Takaya et al., 1997), both of which are thought to be important for ingestion of cellular remnants that result from luteal cell apoptosis (Pate and Landis Keyes, 2001).

The process of luteolysis and regression is known to involve increased synthesis of prostaglandin F$_2$å (PGF$_2$å), decreased progesterone production and luteal cell apoptosis, and macrophages may impact each of these stages. Macrophages may be one source of PGF$_2$å or, via TNF$_a$ secretion, may stimulate PGF$_2$å production by luteal cells (Benyo and Pate, 1992; Wang et al., 1992). Macrophages may also inhibit progesterone production directly, since removal of leukocytes from luteal cell cultures has shown to increase progesterone levels (Kohen et al., 1999). TNF$_a$ has also been shown to inhibit progesterone secretion by mouse luteal cells (Adashi et al., 1990) and TNF$_a$ and IL-1$\beta$ both decrease progesterone production and inhibit survival of bovine luteal cells (Benyo and Pate, 1992). Macrophages also have the capacity to secrete factors that may stimulate apoptosis in the corpus luteum, such as reactive oxygen intermediates, and particularly TNF$_a$. TNF$_a$ mRNA has been observed in the corpus luteum of the mouse (Chen et al., 1993), human (Kondo et al., 1995) and rat (Marcinkiewicz et al., 1994), and in the rabbit, where it was associated with macrophage numbers (Bagavandoss et al., 1988; Bagavandoss et al., 1990). In the bovine corpus luteum, TNF$_a$ induces apoptosis, via the TNF$_a$ receptor type I, specifically in endothelial but not steroidogenic cells (Friedman et al., 2000). In the porcine corpus luteum, macrophages appear to be the primary source of TNF$_a$ (Zhao et al., 1998).

In summary, there are multiple aspects of luteal function that are influenced by ovarian macrophages and/or their secreted products: progesterone secretion, vascularization, prostaglandin production, and apoptosis. In addition, there is evidence that T cell-mediated responses are involved in luteal regression (Pate and Landis Keyes, 2001; Komatsu et al., 2003). Thus macrophage activation of T cells may be an additional, indirect means of influencing luteal regression that needs to be investigated in greater detail.

**Macrophages in ovarian dysfunction and disease**

Many diseases have an immune component, demonstrating that leukocytes exert important effects on tissues which, when misregulated, can lead to dysfunction and pathology. Macrophage effector functions are precisely regulated by temporal and tissue-specific mechanisms. Furthermore, macrophage-mediated effects on ovarian function are complex: they impact multiple aspects of ovarian function and are precisely regulated by hormone, cytokine and matrix signals. There is emerging evidence that macrophages and macrophage-derived products are involved in some ovarian dysfunctions, particularly polycystic ovary syndrome (PCOS), endometriosis and premature ovarian failure (POF).

**Macrophage contributions to ovarian function**

**PCOS ovary syndrome**

PCOS is the most common hormonal disorder of young women, estimated to affect 5–10% of women through and beyond their reproductive years. The prevalence of affected individuals and the wide range of related phenotypes are thought to be due to both genetic and environmental factors (Crosignani and Nicolosi, 2001). PCOS results in anovulation and therefore impaired fertility, but is a multisystem disorder which is also associated with hyperinsulinaemia, hyperlipidaemia, diabetes mellitus, obesity, hyperandrogenemia, hirsutism, acne and increased incidence of endometrial cancer (Norman and McVeigh, 1999; Norman, 2001). It has also been reported that PCOS patients exhibit chronic low-grade inflammation, manifested as elevated levels of C-reactive protein (Kelly et al., 2001). The fact that immune-modulating treatments, such as dexamethasone, are often used to alleviate PCOS symptoms provides further evidence of the involvement of leukocytes in this syndrome. Another treatment option for PCOS is laparoscopic laser drilling which involves piercing the ovarian surface. This method is thought to work by inducing a pro-inflammatory response in the ovary which would facilitate ovulation; indeed studies in the sheep have shown that laser drilling results in the influx of macrophages and lymphocytes (Tozawa et al., 1995). More recently, however, the literature has focused on experiments and clinical trials involving the treatment of PCOS-associated obesity and insulin-resistance. Interestingly, the most effective agents used in the treatment of PCOS are anti-diabetic drugs metformin and troglitazone (an agonist of the PPAR$\gamma$ receptor). PPAR$\gamma$ ligands are well studied for their insulin-sensitising abilities in adipocytes; however, they also exert potent anti-inflammatory effects in macrophages (Tontonoz and Nagy, 1999; Lee and Evans, 2002). In PCOS patients these pathways may be causally linked as metformin treatment not only improves insulin sensitivity in PCOS patients but also reduces inflammation, measured as C-reactive protein levels (Morin-Papunen et al., 2003). Future studies are therefore likely to show an important link between macrophage activation status, obesity/insulin resistance and severity of PCOS symptoms.

Proper follicle development is dependent on the appropriate cytokine and growth factor milieu, as detailed above, and macrophages represent an important source of these factors. Numerous studies have therefore compared serum and follicular fluid cytokine levels in PCOS patients to non-PCOS patients, with conflicting results such that no clear picture has emerged. In a group of PCOS patients that had received no ovarian stimulation, TNF$_a$ in follicular fluid was reported to be no different than normal levels (nor were IL-1$\beta$ levels different) (Jasper and Norman, 1995). Yet, in a study of non-obese/non-diabetic PCOS patients treated with gonadotrophins, serum and follicular fluid TNF$_a$ and IL-6 levels were elevated compared to normal controls (Amato et al., 2003). In a group of obese PCOS patients, dexamethasone treatment was seen to alter follicular fluid cytokine levels indicating that immune cells are likely to be at least partially involved in the production of follicular fluid cytokines. In this study IL-6 levels were unchanged but CSF-1 levels were increased, and TNF$_a$ levels were reduced and associated with increased follicular estradiol compared to untreated PCOS patients (Zolli et al., 1992). Cumulatively these reports suggest that in at least some instances women with PCOS exhibit elevated follicular
and/or serum cytokine levels which are likely to be involved in the observed alterations in follicular maturation and ovarian function.

Studies in sheep and rhesus monkey models have shown that when pregnant animals are administered testosterone, the female offspring exhibit PCOS-like ovaries with characteristic cysts. In these models it is thought that elevated prenatal androgens developmentally program LH hypersecretion and ovarian steroid production, which can further augment androgen action (Abbott et al., 2002). Studies in mice have also shown that neonatal exposure to estradiol results in cystic ovaries that produce elevated levels of TNFα and IL-6, similar to many cases of PCOS. Interestingly, peritoneal macrophages from these mice also produce elevated levels of TNFα and IL-6, indicating that cytokine production by macrophages is altered systemically (Deshpande et al., 2000). Although these animal models have made some inroads into our understanding of immune involvement in PCOS, more work is clearly necessary. In particular, genetically targeted mouse models that exhibit PCOS-like ovaries are lacking and represent an important goal for future research that would begin to shed light on the molecular mechanisms of this complicated disease.

**Endometriosis**

Endometriosis, a condition that is characterized by the presence and growth of endometrial cells outside the uterus, is estimated to affect 3–10% of women of reproductive age and 25–35% of infertile women (Olive and Schwartz, 1993). Endometriosis is frequently found in the ovaries as well as the anterior and posterior cul-de-sac, the uterosacral ligaments and broad ligament of the uterus, and is often associated with pelvic pain and infertility. Although the pathogenesis of endometriosis and the mechanism of endometriosis-associated infertility remains unclear, endometriosis has been implicated as a factor in disordered and/or retarded follicle growth and ovulatory dysfunction as immune confirmed by ultrasonography, as well as in luteal phase defects (Doody et al., 1988).

Recent evidence suggests that leukocytes, including ovarian macrophages and their numerous products, may be involved in the onset and development of this disease (Vinatier et al., 1996). Macrophages infiltrating endometriotic stromal cells exhibit intense immunostaining and are a major source of soluble factors, particularly TGFβ, and may therefore be important regulators of cell proliferation in endometriotic cysts through their paracrine and autocrine actions (Tamura et al., 1999). Flow cytometric evaluation of leukocyte subpopulations present in follicular fluid of infertile women undergoing IVF has shown that the number of CD14 positive cells (macrophages/monocytes) is significantly increased in patients with endometriosis compared to those with tubal factor or idiopathic infertility, and it is suggested that they may adversely affect folliculogenesis or oocyte maturation (Lachapelle et al., 1996). It is suggested that CD14+ cells may affect folliculogenesis or oocyte maturation and that their dysregulation in the ovary could be one of the factors impacting fertility.

Positive immunostaining for IL-6, a macrophage-derived cytokine, has been shown in the theca of antral follicles and in corpora lutea, areas coincident with the main locations of macrophages (Loret de Mola et al., 1996). Endometriotic stromal cells from ‘chocolate cyst linings’ of the ovary produced increased amounts of IL-6 compared with normal endometrial stromal cells (Tsudo et al., 2000). Other studies have shown that the follicular fluid of patients with endometriosis contains increased IL-6, which may be related to the inhibition of follicular development (Pellicer et al., 1998; Garrido et al., 2000; Pellicer et al., 2000). These data suggest that altered gene expression and protein secretion of IL-6, possibly by infiltrating macrophages in the ovarian endometriotic tissue, may contribute to the pathogenesis of this disease and/or to endometriosis-associated infertility (Tsudo et al., 2000). Bergqvist et al. (2001) showed that ovarian endometriotic tissue produces significantly higher concentrations of IL-6 and IL-1β than endometrium from healthy controls (Bergqvist et al., 2001). Another study, however, reported that IL-1β levels in follicular fluid were no different in these patients compared to normal controls, but confirmed increased levels of IL-6 (Pellicer et al., 1998).

VEGF and IL-8 concentrations were also found to be higher in the fluids of the ovarian endometriomas than in those of the follicular cysts of controls, indicating that angiogenesis could play an important role in the progression and maintenance of the ovarian endometriomas (Fasciani et al., 2000). VEGF concentrations in the follicular fluid from patients with endometriosis were lower than in the controls, but elevated VEGF levels have been correlated in IVF with good follicular vascularization and the health of the follicle (Van Blerkom et al., 1997).

The expression of IGF-I and its receptor has been detected in the stroma and epithelium in human ovarian endometriotic tissues. The expression of mRNA and immunohistochemical staining for TGFβ1 has also been detected in the epithelial lining and cellular stroma of ovarian endometriomas, which indicates that TGFβ1, IGF-I and their receptors might play an important role in the pathogenesis of endometriosis (Lovegrove et al., 2001). Tamura et al. (1999) confirmed the expression of TGFβ isoforms and receptors in endometriotic cysts, especially in the epithelial cells, in the human ovary (Tamura et al., 1999). A study of the localization of matrix metalloproteinases in ovarian endometriomas has shown that MMP-3 is mainly expressed in macrophages, which suggests that the destruction of the surrounding matrix by endometriosis might be caused by macrophage-derived MMP (Mizumoto et al., 2002).

The data reviewed here indicate that endometriosis is associated with altered levels of several specific cytokines and chemokines, many of which are known products and/or activators of macrophages. This, along with increased infiltration of leukocytes, is likely to contribute significantly to the pathology observed in this disease.

**Premature ovarian failure**

POF is defined as a syndrome characterized by cessation of ovarian function and menopause before the age of 40 years and is estimated to affect ~1% of the female population (Coulam et al., 1986). POF patients suffer from hypogonadism, anovulation and, in some cases, a variety of cellular immunity defects (Mignot et al., 1989). In particular, several autoimmune aetiologies have been identified. Using HLA class I molecules as markers for macrophages, some patients with autoantibodies were identified as having evidence of a defect in self-antigen presentation similar to that of type I diabetics (Hoek et al., 1997). In addition, there were significantly increased numbers of CD8 positive T cells in
autoimmune POF patients. Hill et al. (1990) found that while in normal human ovaries only occasional cells of macrophage morphology were class II MHC positive and granulosa cells were negative, class II MHC antigen expression was prevalent on granulosa cells from POF patients, suggesting that POF might be associated with inappropriate expression of class II MHC antigen expression by granulosa cells, a phenomenon that can be mimicked by the in vitro culture of granulosa cells with the cytokine IFNγ (Hill et al., 1990). The cumulative data demonstrating autoimmune aetiologies and abnormal expression of leukocyte cell surface markers may assist in the development of tests which could result in early diagnosis, prior to complete ovarian failure (Yan et al., 2000).

The precise function of macrophages and their secretory products in ovarian disorders is still unclear, and more research is required to characterize the specific functions of ovarian macrophages, define their roles in vivo and determine how they may be dysregulated in disease.

Summary

Ovarian macrophages are important regulators of the complex communication between the immune and reproductive systems. They exhibit changes in numbers and phenotype depending on the stage of the estrus cycle, and, via secretion of a number of bioactive molecules, impact many ovarian processes. Macrophages are able to regulate cellular proliferation, differentiation and apoptosis, as well as influence steroid production, vasculatization and tissue remodelling during follicle growth, ovulation and luteinization. It is hypothesized that alterations in the macrophage-mediated regulation of some of these processes contribute to the pathogenesis of ovarian disorders. Importantly, further studies on ovarian macrophages and their secretory products are needed in order to generate diagnostics, and eventually therapeutics, for ovarian diseases such as PCOS, endometriosis and POF.

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