Ovarian phagocyte subsets and their distinct tissue distribution patterns

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Abstract

Ovarian macrophages, which play critical roles in various ovarian events, are probably derived from multiple lineages. Thus, a systemic classification of their subsets is a necessary first step for determination of their functions. Utilizing antibodies to five phagocyte markers, i.e. IA/IE (major histocompatibility complex class II), F4/80, CD11b (Mac-1), CD11c, and CD68, this study investigated subsets of ovarian phagocytes in mice. Three-color immunofluorescence and flow cytometry, together with morphological observation on isolated ovarian cells, demonstrated complicated phenotypes of ovarian phagocytes. Four macrophage and one dendritic cell subset, in addition to many minor phagocyte subsets, were identified. A dendritic cell-like population with a unique phenotype of CD11chighIA/IEhighF4/80low was also frequently observed. A preliminary age-dependent study showed dramatic increases in IA/IE⁺macrophages and IA/IE⁺dendritic cells after puberty. Furthermore, immunofluorescences on ovarian sections showed that each subset displayed a distinct tissue distribution pattern. The pattern for each subset may hint to their role in an ovarian function. In addition, partial isolation of ovarian macrophage subset using CD11b antibodies was attempted. Establishment of this isolation method may have provided us a tool for more precise investigation of each subset's functions at the cellular and molecular levels.

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Introduction

Macrophages are widely distributed in almost all tissues. As a part of innate immunity, they naturally recognize and further present microbial antigens to initiate and/or enhance acquired immunity. Mounting data have also revealed their functions beyond immune defenses. Macrophages play a critical role in tissue homeostasis and tissue remodeling through active phagocytosis, induction of apoptosis, and by releasing an array of cytokines (Papatriantafyllou 2012). Although all macrophages share several common markers and display a similar morphology, they in fact consist of distinct subsets (Murray & Wynn 2011). Each subset may fulfill one or more specific tasks. Identification of subsets of macrophages in a tissue location will be necessary to elucidate their functions.

Ovaries contain abundant resident macrophages in almost all locations. The first differential analysis of ovarian macrophages with immunohistochemistry was performed five decades ago. Along with macrophages, other phagocytes such as monocytes and dendritic cells were also found in the ovarian tissues (Brannstrom et al. 1993). Ovarian tissue is constantly undergoing remodeling due to follicular atresia, ovulation, and the generation and regression of corpus luteum. Many studies have investigated potential roles of ovarian macrophages in those ovarian events, and significant progress has been achieved during the last two decades. Macrophages have been shown to participate in ovulatory processes and in luteinization (Cohen et al. 1999, Bukulmez & Arici 2000, Wu et al. 2004, Turner et al. 2011). Their involvement in ovarian follicular atresia has also been demonstrated in various mammalian species (Kasuya 1997, Gaytan et al. 1998a, 1998b, Inoue et al. 2000). On the other hand, many studies have reported changes in macrophage density and subsets during ovarian cycles, likely in response to hormone regulation, and/or because of recruitment of a subset(s) with special functions (Petrovska et al. 1996, Katabuchi et al. 1997, Takaya et al. 1997). More recent studies further suggested macrophages’ function as part of the paracrine system, which may affect other hormonal regulation on follicular development (Kirsch et al. 1981, Fukumatsu et al. 1992, Katabuchi et al. 1996, Duda et al. 2011,

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Tingen et al. 2011). As each ovarian event occurs at a very specific location, a macrophage's tissue location may reflect their involvement in such events.

However, like their counterparts in other tissues, ovarian macrophages may be derived from multiple lineages with very different natures and functions (Bukulmez & Arici 2000, Wu et al. 2004, Murray & Wynn 2011). In spite of the abundant data on ovarian macrophages, there has been a lack of studies devoted to systemic classification of ovarian macrophages and determination of their tissue distribution pattern. Such studies will be a necessary step to predict their potential functions. This study aims to provide the first insight regarding macrophage and/or phagocyte subpopulations through simultaneous use of three of five common phagocyte markers: IA/IE, F4/80, Mac-1, CD11c, and CD68. IA/IE, which are major histocompatibility complex (MHC) class II molecules in mice, were exclusively expressed in professional antigen-presenting cells such as macrophages and dendritic cells. F4/80 is a well-known marker for subsets of tissue macrophages and, in some cases, dendritic cells. Mac-1 (CD11b) and CD11c, which are adhesion molecules of the integrin family and critical for phagocyte trafficking, are widely expressed by macrophages and dendritic cells. CD68 is an intracellular adhesion molecule mainly expressed in macrophages. Tissue distribution of those identified subsets was then analyzed.

**Materials and methods**

**Antibodies**

The following antibodies were purchased from BD Biosciences (San Jose, CA, USA): biotin-labeled anti-mouse MHC class II molecules IA/IE (rat IgG2a, 2G9), Alexa-647-labeled anti-mouse CD11b/Mac-1, and FITC-labeled anti-mouse CD11c (hamster IgG1, HL3). Alexa-488-labeled anti-mouse F4/80 (rat IgG2a, BM8) and Alexa-488-labeled anti-mouse CD68 (rat IgG2a, FA-11) were obtained from Biolegend (San Diego, CA, USA). Secondary reagents Alexa-555-labeled (Invitrogen) and PE-labeled (BD Biosciences) streptavidin were used to visualize biotin-labeled antibodies. Biotin/avidin and anti-mouse CD16/32 MAB (D34-485, BD Biosciences) were used for blocking non-specific binding. Various immunoglobulin isotypes as negative controls were from BD Biosciences (Supplementary Figure 1, see section on supplementary data given at the end of this article).

**Animals and ovarian phagocyte isolation and culture**

C57BL/6 (B6) females (4–18 weeks) were purchased from Harlan (Indianapolis, IN, USA). Mice of 12 or 17 weeks were used for experiments if the age of mice is not mentioned. All experiments involving animals have been approved by the Institutional Animal Welfare Committee. The mice is maintained in the animal facility at The University of Texas Health Science Center at Houston and allowed to acclimate for a minimum of 7 days. For analysis of ovarian cells, four pairs of ovaries from same-aged animals were used for isolation of ovarian cells following our previously published method (Zhou et al. 2009). The experiments were repeated at least three times for each age. Briefly, ovaries were removed and immediately placed in a complete DMEM medium (37°C) as described previously. The ovaries were torn by repeated needle punch and digested by initially using dispase (Digestion Kit, Stemcell Technologies, Vancouver, BC, Canada) followed by DNase incubation. The enzymes were removed by washing and cells were placed in DMEM with 5 mM EDTA. Digested tissues were then ground, passed through a cell constrainer (40 μm, BD Falcon, San Jose, CA, USA), and washed with the same medium. Dead cells were further removed by Ficoll gradient (density 1.119) centrifugation. As controls, peripheral blood leukocytes (PBLs) or splenocytes were isolated following our previously published method (Zhou et al. 2009). The isolated cells were used for flow cytometry immediately or for immunofluorescence after an overnight culture. In some cases, cells were used for sorting using magnetic bead-conjugated antibody to CD11b on a semi-automatic cell sorter following the manufacturer's instruction (Miltenyi Biotech, Auburn, CA, USA).

**Immunofluorescence and flow cytometry**

Ovaries, fixed or non-fixed depending on the activity of the antibody to be used, were frozen and 3 μm frozen sections were cut. For ovarian cells, isolated ovarian cells were placed in each well of Chamber slide from Millipore (Billerica, MA, USA). After overnight culture, phagocytes adhered to the bottom of the wells and were used for immunofluorescence. Prior to staining, all sections or cells were blocked in 3% BSA with antibodies to CD16/32. For multi-color staining, antibodies that were directly conjugated to a fluorescent dye were used. If one biotin-labeled antibody was used, this antibody was used for the first staining after blocking with a biotin and avidin blocking kit from Vector BioLab (Philadelphia, PA, USA). Fluorescent dye-labeled streptavidin was then used as a secondary reagent. The ovarian sections or ovarian phagocytes on the slides were observed by a fluorescent microscope (Nikon 90i Eclipse, Tokyo, Japan) and images were analyzed with NIS Elements 3.2 from Nikon. In some cases, fluorescent images were taken by a confocal microscope in M.D. Anderson Cancer Center (Houston, TX, USA). For detection of macrophage subsets in the theca of follicles, follicles were classified to stages I–VIII (Pedersen & Peters 1968). Ten to 20 follicles randomly selected for each stage from three mice (12 weeks) were used for counting macrophages. For flow cytometry, freshly isolated ovarian cells were used for staining on the ice. The stained cells were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA). For three-dimensional (3D) flow cytometry plots, the data were transferred from the flow cytometer to a conventional computer, and a 3D chart was constructed using Teraplot Software (Kylebank, Ayrshire, UK).
Results

Subsets of ovarian phagocytes

Single-cell suspensions were prepared from ovaries and used for the following experiments. First, a combination of antibodies to three common phagocyte markers, i.e. MHC class II (IA/IE), F4/80, and Mac-1 (CD11b), was used to identify ovarian phagocyte subsets. For immunofluorescence, the freshly isolated cells were further cultured for 1 day, allowing phagocytes to adhere to the culture wells. Three-color immunofluorescence revealed highly diverse cell phenotypes and cell morphologies among the isolated ovarian phagocytes (Fig. 1A). However, the majority of the cells could be categorized into three major phenotypes: i) IA/IE<sup>high</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>, ii) IA/IE<sup>dull</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>, or iii) IA/IE<sup>+</sup>CD11b<sup>−/low</sup>F4/80<sup>low</sup> (Fig. 1B, C and D). Cells with the first phenotype were observed in the highest frequency and usually expressed very high levels of IA/IE and mid to low levels of CD11b and F4/80. Upregulation of MHC class II expression is one of the characteristics for activated tissue macrophages (Adams & Johnson 1992). Those IA/IE<sup>high</sup> cells were mostly activated macrophages. These cells commonly displayed flattened cell bodies with limited ‘dendrite’-like cellular projections (Fig. 1B). This morphology was consistent with that of activated macrophages. The cells of the second phenotype showed very low or undetectable expression of IA/IE (termed as IA/IE<sup>dull</sup>) but expressed high levels of CD11b and F4/80 (Fig. 1C). Although these IA/IE<sup>dull</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells exhibited a branched cytoplasm, their bodies usually did not flatten completely or develop ‘dendrite’-like cellular projections. Those observations suggested that they were probably resting macrophages. Finally, phagocytes of the third phenotype expressed relatively high levels of IA/IE but lacked CD11b expression and/or expressed a low level of F4/80 (Fig. 1D). Thus, they were IA/IE<sup>+</sup>CD11b<sup>−/low</sup>F4/80<sup>low</sup>. Those cells morphologically resembled a typical dendritic cell and possessed numerous ‘dendrite’ projections. Thus, they probably belonged to a subset of tissue dendritic cells. In addition to the three described groups, we also observed cells in relatively low frequency with phenotypes of IA/IE<sup>high</sup>CD11b<sup>high</sup>F4/80<sup>dull</sup>, IA/IE<sup>−</sup>CD11b<sup>+</sup>F4/80<sup>−</sup>, and IA/IE<sup>−</sup>CD11b<sup>−</sup>F4/80<sup>high</sup>. Among those, IA/IE<sup>+</sup>CD11b<sup>high</sup>F4/80<sup>dull</sup> cells had a spherical body and kidney-shaped nucleus, suggesting that the cells were monocytes from PBL. We will not further discuss these minor groups in this paper.

Large autofluorescent bodies were often observed within the cytoplasm of IA/IE<sup>high</sup>F4/80<sup>+</sup> cells (probably the first subset) near their nuclei (Fig. 2A). The presence of large autofluorescent bodies within the cell of the first group was confirmed in the unstained cells. Those autofluorescent bodies were not an artifact due to culture, as similar autofluorescent bodies were also detected in the cells on ovarian tissue sections (Fig. 2B). The exact nature of these structures remains undefined.

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**Figure 1** Immunofluorescent identification of major ovarian phagocyte subsets by three phagocyte markers: MHC class II (IA/IE), F4/80, and Mac-1 (CD11b). (A) A group of isolated ovarian cells show diverse expression patterns for IA/IE (red), F4/80 (green), and CD11b (purple) with distinct shapes of cell body and cellular projections, 200×. (B) A phagocyte shows characteristics of activated macrophage with high IA/IE expression and fine cellular projections. (C) A phagocyte resembling a resting macrophage with extremely low IA/IE expression and no cellular projections. (D) A dendritic cell shows IA/IE expression with numerous dendrite-like cellular projections. Nuclei were counterstained with DAPI, 400×.
However, confocal microscope revealed that fine projections extended from IA/IE\textsuperscript{high} cells were in contact with structures that displayed the same pattern of autofluorescence as those within their cytoplasm (Fig. 2 C). Phagosomes often contain the metabolic lipid product lipofuscin (Colucci-Guyon et al. 2011), which is fluorescent and can be detected by Sudan-Black-B (SBB; Schnell et al. 1999). SBB staining showed that autofluorescence was completely overlapped with SBB on the section (Supplementary Figure 2, see section on supplementary data given at the end of this article). This observation suggested that their cytoplasmic autofluorescent bodies were most likely phagosomes, although more experiments are needed to reach the final conclusion. By contrast, IA/IE\textsuperscript{dull} cells (i.e. the second group) did not have such large autofluorescent bodies, although small autofluorescent granules were occasionally observed within their cytoplasm.

Using the same markers, three-color flow cytometry was performed on freshly isolated ovarian cells. Similar to immunofluorescence, flow cytometry also identified three major groups of phagocytes: IA/IE\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+} (green dots), IA/IE\textsuperscript{low}CD11b\textsuperscript{+}F4/80\textsuperscript{+} (red dots), and IA/IE\textsuperscript{+}CD11b\textsuperscript{low}/F4/80\textsuperscript{low} (blue dots) (Fig. 3A, upper left panel). Quantitatively, expression levels of each marker allow further division of each group into subgroups. Based on their IA/IE expression level, the first group (i.e. IA/IE\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+}) could be further divided into major IA/IE\textsuperscript{high} and minor IA/IE\textsuperscript{mid-low} subgroups (gated by solid and dotted green lines respectively in Fig. 3A). The major IA/IE\textsuperscript{high} subgroup is most likely the same IA/IE\textsuperscript{high}CD11b\textsuperscript{+}F4/80\textsuperscript{+} group observed in the above immunofluorescence (Fig. 1B). However, it remains unclear about the minor IA/IE\textsuperscript{mid-low} subgroup. The second group (i.e. IA/IE\textsuperscript{low}CD11b\textsuperscript{+}F4/80\textsuperscript{+}) could also be divided into two subgroups based on F4/80 expression level: F4/80\textsuperscript{low} and F4/80\textsuperscript{high} subgroups (gated by solid and dotted red lines respectively in Fig. 3A, also see the upper-right panel in Fig. 3A). Those two subgroups were probably

Figure 2 Phagocytosis by IA/IE\textsuperscript{+} ovarian macrophages. (A) A cultured ovarian macrophage, identified by its IA/IE expression (red), shows a phagosome with autofluorescent body (green, indicated as Auto-f) within its cytoplasm, 400\texttimes. (B) An ovarian tissue section shows co-localization of autofluorescent bodies (green, arrows) to IA/IE\textsuperscript{+}F4/80\textsuperscript{+} macrophages (red and green), 200\texttimes. (C) Confocal microscope shows a cultured macrophage of IA/IE\textsuperscript{+} (red), F4/80\textsuperscript{+} (green) in contacting an autofluorescent body (green, arrow) with its cellular projection. Notice another autofluorescent body (green, arrow) within its cytoplasm, 1000\texttimes. Nuclei were counterstained with DAPI.
resting macrophages, i.e. IA/IE\textsuperscript{E
dull}CD11b\textsuperscript{+}F4/80\textsuperscript{+} group observed in the immunofluorescence. However, it is also highly possible that those two subgroups (i.e. F4/80\textsuperscript{high} and F4/80\textsuperscript{low}) could belong to two distinct macrophage lineages. The third group (i.e. IA/IE\textsuperscript{+}CD11b\textsuperscript{−}/lowF4/80\textsuperscript{−}/low) was tissue dendritic cells (gated by solid blue line) (also refer to Fig. 1D). In summary, three-color flow cytometry identified at least

five subsets of phagocytes: IA/IE\textsuperscript{E\textsuperscript{high}}CD11b\textsuperscript{+}F4/80\textsuperscript{+}, IA/IE\textsuperscript{E\textsuperscript{mid:low}}CD11b\textsuperscript{+}F4/80\textsuperscript{+}, IA/IE\textsuperscript{E\textsuperscript{dull}}CD11b\textsuperscript{+}F4/80\textsuperscript{+}, IA/IE\textsuperscript{E\textsuperscript{dull}}CD11b\textsuperscript{+}F4/80\textsuperscript{low}, and IA/IE\textsuperscript{+}CD11b\textsuperscript{−}/lowF4/80\textsuperscript{−}/low. Although there were small discrepancies in classifications between flow cytometry and immunofluorescent methods, the subsets of ovarian phagocytes identified by the two methods were similar.

Next, another set of antibodies to three phagocyte markers, i.e. IA/IE, F4/80, and CD11c, was combined for immunofluorescence. Similar to the first set of experiments, three-color immunofluorescence also revealed highly diverse phenotypes and morphologies of ovarian phagocytes. Four major groups were identified. The first group expressed high levels of IA/IE and F4/80 but not CD11c (IA/IE\textsuperscript{E\textsuperscript{high}}F4/80\textsuperscript{+}CD11c\textsuperscript{−}) with a shape typical of macrophages (Fig. 4A). As macrophages usually do not express or express a low level of CD11c, this group was probably the same group of IA/IE\textsuperscript{E\textsuperscript{high}}F4/80\textsuperscript{+}CD11b\textsuperscript{+} described previously. The second group with a phenotype of IA/IE\textsuperscript{−}F4/80\textsuperscript{−}CD11c\textsuperscript{−} displayed a morphology of resting macrophages (Fig. 4A). Thus, this group is most likely the IA/IE\textsuperscript{E\textsuperscript{dull}}CD11b\textsuperscript{+}F4/80\textsuperscript{+} resting macrophages found in the first set of experiments. The third group was IA/IE\textsuperscript{E\textsuperscript{+}}F4/80\textsuperscript{−}CD11c\textsuperscript{+} (Fig. 4B). The cells from this group morphologically resembled dendritic cells, suggesting that they were the same CD11c\textsuperscript{+} tissue dendritic cells as those observed in the first set of experiments. Based on expression patterns of CD11b and CD11c in the first and second set of experiments, we summarized the ovarian phagocyte subsets as follows: i) activated macrophages with a phenotype of IA/IE\textsuperscript{E\textsuperscript{high}}F4/80\textsuperscript{+}CD11b\textsuperscript{+}CD11c\textsuperscript{−}; ii) resting macrophages of IA/IE\textsuperscript{E\textsuperscript{dull}}F4/80\textsuperscript{+}CD11b\textsuperscript{+}CD11c\textsuperscript{−}; and iii) tissue dendritic cells of IA/IE\textsuperscript{E\textsuperscript{+}}F4/80\textsuperscript{−}CD11b\textsuperscript{−}/lowCD11c\textsuperscript{+}.

Interestingly, the second set of antibodies also revealed a group of cells with a phenotype of IA/IE\textsuperscript{E\textsuperscript{−}}F4/80\textsuperscript{−}CD11c\textsuperscript{high} in relatively high frequency (Fig. 4A and B); those cells were morphologically similar to dendritic cells. More experiments may be needed to define whether these cells are myeloid. In summary, two combinations of three antibodies to four phagocyte markers identified at least four subsets of macrophages, one subset of tissue dendritic cells, and one type of dendritic cell-like cells. Their phenotypes and possible cell types are summarized in Table 1.

Partial quantitative analysis on those ovarian phagocytes was also carried out after cell sorting. We focused on CD11b\textsuperscript{+} phagocytes (i.e. macrophages). CD11b antibody-conjugated magnetic beads were used to isolate CD11b\textsuperscript{+} cells to a purity of 64%. Among CD11b\textsuperscript{+} cells, nearly 100% of cells expressed F4/80, but 58% expressed mid–high levels of IA/IE. Based on their IA/IE expression level, the ratio of the cells with high-level, mid-level, and no expression of IA/IE was 34:24:42% respectively.

Figure 3: Flow cytometry identification of ovarian phagocyte subsets by three phagocyte markers: MHC class II (IA/IE), F4/80, and Mac-1 (CD11b). (A) Upper left panel shows 3D dot-plot using three phagocyte markers (IA/IE, CD11b, and F4/80). Colored dots represent different phagocyte populations (see text). Other three panels are 2D plots for three different combinations of two markers as indicated. Phagocyte subsets in those plots are indicated by colored gates. Note the presence of several minor cell populations. Ovarian cells were isolated from 17-week-old mice and pooled. (B) Summary of flow cytometry analysis of ovarian phagocytes from different ages as indicated. Size of a population is expressed as % of all ovarian cells; three samples for each age group were analyzed using pooled ovarian cells from three mice of the same age.
Tissue distribution of phagocyte subsets

Based on the subsets identified by the above in vitro methods, we examined their distribution within the ovarian tissue. We first used a combination of two common markers, IA/IE and F4/80, for the identification of phagocyte subsets on the tissue sections. Because immunofluorescence alone was not able to quantitate the expression level of the markers, it was difficult to precisely classify all five subsets as revealed by flow cytometry on the tissue sections. Therefore, we focused on two major subsets of macrophages, i.e. IA/IE$^{\text{high}}$F4/80$^{\text{low}}$ (activated macrophages or dendritic cells) and IA/IE$^{\text{dim}}$F4/80$^{\text{high}}$ (resting macrophages). For determination of phagocyte tissue locations, ovarian tissue was divided into four locations: normal developing follicles at various stages, atretic follicles, corpus lutea, and interstitial tissue (Fig. 5A). However, phagocytes in the interstitial tissue will not be discussed in this paper.

Atretic follicles

Clustered IA/IE$^{+}$F4/80$^{+/low}$ were observed surrounding or within atretic follicles (Fig. 5B). Those cells expressed extremely high levels of IA/IE. Many macrophages of IA/IE$^{-}$F4/80$^{+}$ were also observed to target atretic follicles. Those two types of macrophages were sometimes seen intermingled with each other. However, the involvement of CD11c$^{+}$ dendritic cells was not ruled out as those cells were IA/IE$^{+}$F4/80$^{\text{low}}$.

Normal developing follicles

Both IA/IE$^{+}$F4/80$^{+}$ and IA/IE$^{-}$F4/80$^{+}$ cells were found in the theca layer of all follicles at different development stages except for stages I and II (Figs 5C and 6). In addition, a smaller number of IA/IE single-positive macrophages were also observed (IA/IE$^{+}$ in Fig. 6). IA/IE expression levels in IA/IE$^{+}$F4/80$^{+}$ cells were much lower than those in atretic follicles as shown in Fig. 5B. The ratio between IA/IE$^{+}$F4/80$^{+}$ and IA/IE$^{-}$F4/80$^{+}$ cells was $\sim$1:1 for antral follicles to 1:1.5 for other developmental follicles (Fig. 6). Although the number of theca macrophages per follicle increased with growth of follicles, it was unclear whether macrophages per theca tissue area remained constant. The number of macrophages in the theca varied among the follicles. It needs to be determined whether it was caused by polarized phagocyte distribution or due to development stages. Occasionally, IA/IE$^{+}$F4/80$^{+}$ cells were found among granulosa cells.

Corpus lutea

Distribution of phagocytes in corpus lutea was complicated (Fig. 5D). A few corpus lutea showed evenly

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<th>Table 1 Summary of major ovarian phagocyte subsets.</th>
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<td><strong>Phenotype</strong></td>
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<td>IA/IE$^{\text{high}}$CD11b$^{+}$F4/80$^{+/low}$CD11c$^{-}$</td>
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<tr>
<td>IA/IE$^{\text{high}}$CD11b$^{+}$F4/80$^{+}$</td>
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<td>IA/IE$^{+}$CD11b$^{+}$F4/80$^{+}$</td>
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AF, atretic follicles; NF, normal follicles; CL, corpus luteum; IN, interstitial tissue.
scattered IA/IE/F4/80+ and IA/IE/F4/80− cells. However, in most corpus lutea, phagocyte distribution tended to be heavily polarized with a majority of IA/IE/F4/80+ cells on one side and much fewer IA/IE/F4/80− cells on the other side. In both distribution patterns (evenly scattered vs polarized), IA/IE/F4/80+ and IA/IE/F4/80− cells never mingled to the extent seen at atretic follicles. Similar to those in the theca of follicles, IA/IE expression levels in IA/IE/F4/80+ cells was much lower than those in atretic follicles.

Finally, the intracellular phagocyte marker CD68 was included for three-color immunofluorescence to investigate phagocytes in different locations. Intracellular CD68 was detected in a portion of IA/IE/F4/80− cells in atretic follicles (Fig. 7A). Thus, atretic follicular IA/IE/F4/80− macrophages could be further divided into two subgroups: CD68+ and CD68−. On the other hand, all IA/IE/F4/80low macrophages, which clustered within atretic follicles, showed no CD68 expression (Fig. 7A). For those macrophages in corpus lutea, almost all IA/IE/F4/80+ and IA/IE/F4/80− cells showed intracellular CD68 (Fig. 7B). This suggested that macrophages with similar IE/IA and F4/80 expression probably did not belong to the same subset. Table 1 summarizes the tissue distribution of ovarian phagocyte subsets.

Age influence of ovarian phagocytes

We examined quantitative and qualitative changes in ovarian phagocytes at different ages. Ovarian cells were collected from 4-week (immature), 8-week (puberty), and 18-week (mature) old mice. Flow cytometry revealed similar phagocyte subsets in all three age groups (Fig. 3B). However, there were significant quantitative differences among the three groups. Generally, the number of phagocytes increased with age, with <2% for 4 weeks, 3.9% for 8 weeks, and 5.6% for 18 weeks. The most significant increases were observed between 4 and 8 weeks. This was largely due to increases in the numbers of IA/IE/F4/80+ and IA/IE/F4/80− subsets, which were located in atretic follicles and corpus lutea respectively. The above data suggested a close relationship between phagocytes and ovarian function. On the other hand, IA/IE/F4/80+ and IA/IE/F4/80− subsets, which were located in the...
least five major types of ovarian phagocytes, which cytometry on the isolated ovarian cells has identified at cated ovarian phagocyte subsets. In this study, 3D flow lack of studies that aim to systemically clarify compli- abundant data on ovarian macrophages, there is still a stages (Gordon 1999, Murray & Wynn 2011). In spite of interstitial tissue and theca of developing follicles, were detected in all three groups. Unexpectedly, a significant increase was also seen in IA/IE^+ F4/80^- dendritic cells in mature ovaries when compared with immature ones.

Discussion

Ovarian macrophages or phagocytes most likely belong to diverse subsets (Wu et al. 2004, Murray & Wynn 2011). They may have differentiated from multiple lineages and/or be at different activation/differentiation stages (Gordon 1999, Murray & Wynn 2011). In spite of abundant data on ovarian macrophages, there is still a lack of studies that aim to systemically clarify complicated ovarian phagocyte subsets. In this study, 3D flow cytometry on the isolated ovarian cells has identified at least five major types of ovarian phagocytes, which include four subsets of macrophages (CD11b^+) and one subset of dendritic cells (CD11b^-). Among those phagocyte subsets, one important characteristic was their MHC class II (IA/Ii) expression level, ranging from very high to virtually absent. Similarly, F4/80 and/or CD11b expression patterns were also distinct from one subset to others. In addition, several minor populations of phagocyte were also observed. Thus, using multiple markers with quantitated levels of their expression is required for a detailed analysis of ovarian phagocytes. Owing to a lack of quantitative analytic capacity, immunofluorescence on isolated ovarian cells identified only three major subsets of macrophages, mainly based on either positivity or negativity for the markers IA/Ii and F4/80. However, immunofluorescence was able to reveal additional minor subsets of phagocytes and also cells’ shapes. Together with morphological observations, flow cytometry and immunofluorescence in this study allowed us to categorize those phagocytes (Table 1). We also used CD68, an intracellular marker for phagocytes, for our study. This marker added another dimension to

The classification. For example, IA/Ii^- F4/80^+ macrophages in atretic follicle could be CD68^+ or CD68^-.

Finally, it is worthwhile to mention the subset of IA/Ii^- F4/80^-CD11c^+ dendritic cell-like ovarian cells. We believe that this novel population has not been described or reported. Unlike other dendritic cells, this population lacks IA/Ii expression. Currently, we are investigating whether those cells are myeloid.

This study has related subsets of macrophages to special tissue locations in the ovaries. Although much more studies are needed to reach the final conclusions, our observations regarding the relationship between macrophage subsets and their special locations may hint at their roles in ovarian functions. First, similar to those in other organs/tissues, ovarian macrophages are involved in tissue homeostasis. Follicular atresia leads to apoptosis in a large number of cells. Rapid clearance of apoptotic cells is important to inhibit inflammation and autoimmune response against intracellular antigens (Itoh et al. 1999). Ovarian macrophages play a critical role in this process through phagocytosis or other mechanisms. autofluorescent bodies probably from apoptotic cells were frequently found in macrophages located in atretic follicles, suggesting active phagocytosis by macrophages during atresia process (Kasuya 1997, Itoh et al. 1999, Inoue et al. 2000). Our study showed at least two subsets of macrophages in the atretic follicles. However, only IA/Ii^high F4/80^low macrophages contained autofluorescent bodies. Because MHC class II is required for induction of immune response or tolerance, this suggests that those IA/Ii^high F4/80^low macrophages not only eliminate the debris but also prevent autoimmunity to the debris (Elliott & Ravichandran 2010). It is equally interesting to ask why IA/Ii^low F4/80^+ macrophages, which were not capable of phagocytosis, were present in the atretic follicle. Secondly, a previous study reported that depletion of macrophages through CD11b disrupted ovarian vasculature (Turner et al. 2011), suggesting a critical role of macrophages in angiogenesis. Remodeling of vasculature is dramatic during formation of a corpus luteum. Since at least two distinct macrophage subsets exist in corpus lutea, it will be interesting to ask which one or both is(are) involved in vasculature remodeling. Thirdly, theca macrophages are involved in follicular growth (Fukumatsu et al. 1992, Cohen et al. 1999, Wu et al. 2004, Tingen et al. 2011). Our study showed two subsets of macrophages in the theca layer, with the majority being IA/Ii^- F4/80^+. It will be interesting to determine which subset of macrophages may have this function. Similarly, there are several subsets of phagocytes in the interstitial tissues. Ovarian macrophages may act as paracrine sources. Expression of cytokines such as TNF and IFNs by ovarian macrophages plays critical roles in multiple ovarian functions (Andreani et al. 1991). It remains to be determined which subset of macrophages released those cytokines. Obviously, each macrophage subset will need to be

Figure 7 Three-color immunofluorescent detection of CD68 expression in ovarian phagocytes in an atretic follicle (A) or in corpus lutea (B). Two adjacent corpus lutea (CL), the boundary of which is indicated by a dashed line, are shown in (B). Macrophages are shown by their expression of IA/Ii (red) and/or F4/80 (green). Note that CD68 (purple, intracellular) was detected in a portion of IA/Ii^- F4/80^+ macrophages in an atretic follicle (A) but in most of either IA/Ii^- or IA/Ii^- macrophages in corpus lutea (B), 200×. Nuclei were counterstained with DAPI.

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isolated and purified for further determination of their nature and function. In this case, our study may have provided useful markers for such purification. We have conducted partial purifications of ovarian macrophages. In this study, CD11b^+ or IA/IE^+ ovarian cells have been successfully enriched to over 35% purity from ~2%. With improved techniques and sufficient ovarian cells, further purification of each subset is highly possible.

Our study on special tissue distribution of ovarian phagocytes may have revealed those cells’ function. However, as a preliminary observation, this study also has many limitations. More investigations are needed to relate a special subset of ovarian macrophages to its potential functions. First, this study did not classify many subsets of phagocytes. However, it is highly possible that those minor subsets may also play critical roles in ovarian functions. Thus, phenotype and tissue location of those cells using multiple markers should be determined. Secondly, fluctuation of the general macrophage population during the estrous cycle has been reported, although those macrophages were not further characterized (Katabuchi et al. 1996, 1997, Takaya et al. 1997). Involvement of macrophages in ovulation has been demonstrated by various methods (Brannstrom et al. 1993, Watanabe et al. 1997, Van der Hoek et al. 2000, Matsuura et al. 2002). It remains to be determined which types of leukocyte(s)/macrophage(s) are more critical players. Similarly, invasion of macrophages into corpus lutea suggests their functions in luteinization or luteolysis (Kirsch et al. 1981, Gaytan et al. 1998a, 1998b, Pate & Landis Keyes 2001). This study did not examine the leukocytes/macrophages during ovulation. We also did not determine the estrous cycle of the experimental mice. Thus, it is possible that some subsets, which may be related to important ovarian functions such as ovulation, may have been missed. For example, we have identified a CD8^+ phagocyte-like population (Zhou et al. 2009). This phagocyte-like population invades mature follicles during super-ovulation. However, our study failed to detect this important population. Thirdly, although our study showed the presence of several subsets of phagocytes in the same location, it remains unclear how they were related: if those macrophages descend from the same precursor at different stage or from a totally different lineage. For example, we have observed mingled macrophages that at different stage or from a totally different lineage. For those macrophages descend from the same precursor location, it remains unclear how they were related: if presence of several subsets of phagocytes in the same population. Thirdly, although our study showed the presence of several subsets of phagocytes in the same population. Finally, this study did not classify many subsets of phagocytes. Although those macrophages were not further characterized (Katabuchi et al. 1996, 1997, Takaya et al. 1997). Involvement of macrophages in ovulation has been demonstrated by various methods (Brannstrom et al. 1993, Watanabe et al. 1997, Van der Hoek et al. 2000, Matsuura et al. 2002). 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Thirdly, although our study showed the presence of several subsets of phagocytes in the same location, it remains unclear how they were related: if those macrophages descend from the same precursor at different stage or from a totally different lineage. For example, we have observed mingled macrophages that expressed either extremely high or low levels of IA/IE in atretic follicles. It is not clear whether the IA/IE^high macrophages were activated ones of IA/IE^low or if they were two unrelated subsets with different functions. This also applies to the macrophages in corpus lutea. On the other hand, macrophages with a similar phenotype could be found in different locations. For example, IA/IE^F4/80+ macrophages were found in theca, corpus lutea, atretic follicles, and interstitial tissues. However, those seemly the same type of macrophages may totally differ in their function and nature. To address the above question, a necessary step is required to understand their roles in ovarian function and related mechanisms.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0134.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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