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IMMUNOHISTOCHEMICAL LOCALIZATION OF MACROPHAGE CD68+, HLA-DR+, L1+ AND CD44+ SUBSETS IN UTERINE ENDOMETRIUM DURING DIFFERENT PHASES OF MENSTRUAL CYCLE

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Abstract : Different tissue macrophage subsets were immunohistochemically examined in normal endometrial samples collected from proliferative (n=4), peri-ovulatory (n=6) and secretory (n=8) phases of menstrual cycles in women. The different macrophage subsets, namely CD68 (pan macrophage marker), CD44 (transmembrane adhesion molecule), HLA-DR (trans-membrane heterodimeric protein involved in antigen presentation) and L1 (calprotectin)-positive cells, as well as, CD45 (common leucocytic antigen)-positive cells were examined on the basis of immunohistochemical staining, and areas of immunoprecipitation were analyzed morphometrically using computer-assisted video imaging system. The stage-specific distribution of receptors for estrogen (ER) and progesterone (PR) in endometrial cells were examined and morphometrically analyzed. There was an increase in the number of CD45+ cells ($P<0.01$) and CD68+ cells ($P<0.05$) in secretory phase endometrium compared with proliferative and peri-ovulatory phases. There was no remarkable cycle dependent pattern in HLA-DR+ and L1+ cells. However, there was an increase in CD44 immunopositive area in peri-ovulatory ($P<0.05$) and in secretory ($P<0.01$) phases of endometrium compared with proliferative phase endometrium. A higher ($P<0.01$) degree of immunopositivity for ER was observed during peri-ovulatory phase, and for PR, during peri-ovulatory ($P<0.05$) and secretory ($P<0.01$) phases compared with proliferative phase of cycle. Positive correlations between areas occupied by (i) CD68+ cells and PR ($P<0.01$), (ii) HLA-DR+ and L1+ cells ($P<0.05$), (iii) CD45+ and CD68+ cells ($P<0.01$), (iv) CD45+ and L1+ cells ($P<0.05$), and (v) PR and L1+ cells ($P<0.05$) were obtained. It appears that the recruitment of different macrophage subsets in human endometrium involves a complex set of endocrine and paracrine factors.

Key words : endometrium, immunohistochemistry, macrophage, morphometry, peri-ovulatory phase, proliferative phase, secretory phase

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INTRODUCTION

The cells of mononuclear phagocyte system (MPS) or so-called tissue macrophage system (TMS) are widely distributed throughout the body. A tissue-specific distribution of macrophages has earlier been reported correlating well with tissue functions (1). Macrophage subsets have been found in bone marrow (2), skin (3), spleen (4), central nervous system (5), gut, liver (6), and lung (7). However, our knowledge about different subsets of macrophage population in human endometrium is thin. MPS contributes about 20% of total leucocyte population in the human endometrium (8). They are distributed diffusely in stroma and as aggregates in peri-glandular region (9). A premenstrual increase in macrophage population in human endometrium may functionally relate to endometrial disintegration and its subsequent regeneration (8, 9). It is suggested that recruitment and proliferation of macrophages in endometrium are indirectly regulated by ovarian steroid hormones (10, 11). It has been observed that number of CD68+ macrophages is higher in progesterone-dominated endometrium (12) and they suggestively play a role in the process of focal necrosis not only by releasing cytolytic and cytotoxic molecules but also interacting with other migratory cells and endometrial stromal and epithelial cells which also produce both chemokines and matrix metalloproteinases (13). Estradiol-17β also controls endometrial macrophage migration by inhibiting monocyte chemotactic protein-1 (MCP-1) expression by endometrial stromal cells (14).

Besides CD68+ macrophages, there are other macrophage subsets in different tissue systems; for example, L1 (calprotectin, a calcium-binding protein)-positive macrophages constitute a subset of recently emigrated macrophages and are found in gut mucosa of inflammatory bowel disease, while CD44 (transmembrane hyaluronate receptor)-positive macrophages play a role in lymphocyte homing process, monocyte activation, and cell migration in liver and lymph nodes (15, 16). But there is lack of report regarding their spatio-temporal distribution in human endometrium. In short, although HLA-DR+ macrophages have been observed in endometrial stroma and in peri-glandular aggregates (8), and CD68+ macrophages in progesterone-dominated endometrium (14), there is no report of systematic study regarding the pattern of distribution of different macrophage subsets in human endometrium during different phases of menstrual cycle. In the present study, the pattern of distribution of different subsets of endometrial macrophages, i.e., macrophages expressing CD68 (pan macrophage marker), CD44 (transmembrane adhesion molecule), HLA-DR (transmembrane heterodimeric protein involved in antigen presentation), and L1 (calprotectin) were immunohistometrically analyzed during proliferative, peri-ovulatory and secretory phases of menstrual cycles in women. Furthermore, the association between the cellular distribution of receptors for estrogen (ER) and progesterone (PR) and the pattern of distribution of macrophage subsets in human endometrium was investigated.

METHODS

Tissues

Endometrial biopsy samples (n=26) were obtained during days 8 to 26 of menstrual cycles from non-pregnant, pre-menopausal patients undergoing elective hysterectomy for various reasons such as ovarian neoplasm, prolapse, endometriosis, and fibroid, but not for endometrial malignancy. This study was conducted after receiving permission from the Ethics Committee of All India Institute of Medical Sciences.

Histology

18 human endometrial samples were found to be normal based on the endometrial dating criteria described by Noyes et al (17) and were classified into three groups: proliferative (n=4), peri-ovulatory (n=6) and secretory (n=8) phases of menstrual cycle. All 18 samples showed no pathognomonic features.

Immunohistochemistry

Immunohistochemical studies were performed with neutral buffered formaldehyde fixed and paraffin-embedded tissue samples as described earlier (18,19). Briefly, paraffin blocks were sectioned at 5 µm using a Supercut microtome (Leica, Germany) and sections were collected on poly-l-lysine coated glass slides. Tissue sections were deparaffinised and endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes. Mouse monoclonal antibodies against seven antigens namely CD45, CD44, L1, HLA-DR, CD68, ER and PR were used for immunohistochemistry. Table I provides the details of the primary monoclonal antibodies used in the present study. For immunohistochemical (IHC) staining of CD45, CD68 and HLA-DR, tissue sections were subjected to microwave heating till boiling in 0.1 M sodium citrate buffer (pH 6.0) for 3, 8 and 5 cycles, respectively for antigen retrieval. For ER and PR same procedure was done but for 5 minutes and for 3 and 5 cycles, respectively. It was followed by quenching of endogenous peroxidase activity. For IHC staining of L1, tissue sections were subjected to trypsinization (0.05% w/v, Trypsin Histo-kit, Zymed Laboratories Inc., CA, USA) for 20 minutes at room temperature in a humidified chamber and it was followed by quenching of endogenous peroxidase

TABLE I: Characteristics of primary antibodies used.

Antigen	*Primary antibody*	*Dilution*[1]
CD44	Monoclonal mouse antiserum (Clone DF1485)*	1:100
CD45	Monoclonal mouse antiserum (Clone 2B11+PD7/26)*	1:50
CD68	Monoclonal mouse antiserum (Clone PG-M1)*	1:50
L1	Monoclonal mouse antiserum (Clone MAC387)*	1:50
HLA-DR	Monoclonal mouse antiserum (Clone TAL.1B5)*	1:50
ER	Monoclonal mouse antiserum (Clone 1D5)*	1:50
PR	Monoclonal mouse antiserum (Clone PR10A9)**	1:35

*Dako A/S, Denmark. **Immunotech, Cedex, France. [1]Working dilutions of stock (1 mg/ml) were precalibrated based on 4-5 titration points and the information provided by the manufacturer.

activity. Nonspecific binding was blocked by using normal non-immune blocking serum (1:20, Vector laboratory, Burlingame, CA, USA) for 1 h. Sections were then incubated in primary antibody at pre-titrated dilutions (Table I) at 4°C overnight, followed by incubation with biotinylated secondary antibody, and final visualization was achieved by using the ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (0.05%, w/v, Sigma Chemical Co.) and hydrogen peroxide according to the protocol described earlier (18,19). Duplicate sections were lightly counterstained with Gill's haematoxylin to facilitate the identification of cellular elements except for ER and PR since immunopositive staining for ER and PR were detected primarily in the nuclear compartment (20). For control, only primary antibody was excluded and rest of the procedure was the same as above.

Morphometry

The immunohistochemically stained sections were analyzed microscopically to estimate morphometrically the areas of immunoprecipitation in macrophage cells and endometrial cells in the functionalis zone using a Leica microscope and a precalibrated computer-assisted video image analysis system (QWIN-Quantimate 500+, Cambridge, UK) as described elsewhere (18, 19). It was assumed that the morphometric values of immunoprecipitation in sections express the quantitative estimates of antigen (21). Additionally, numerical evaluations of immunopositive cells in the immunostained sections were performed and expressed as per cent positive cells.

Statistical analysis

Log-transferred morphometric data were subjected to Kruskal-Wallis test which is the non-parametric equivalent of one-way analysis of variance (22). Inter-dependence among different parameters was examined by computing Kendall rank-order correlation coefficient (22). The probability level of $P=0.05$ was taken as the limit of significance.

RESULTS

A total of 26 hysterectomy samples of human endometrium were collected. Of these, 4 samples yielded inadequate tissue materials, and another 4 had abnormal pathology. Finally, 18 samples were selected with normal histology, of which 4 showed the characteristics of proliferative stage endometrium, 6 showed characteristics of peri-ovulatory stage endometrium, and 8 showed characteristics of secretory stage endometrium. The above staging of endometrial samples corroborated well with the menstrual history of the individual subjects.

Table II shows the morphometric data for areas of immunoprecipitation. Figures 1 and 2 show representative immunolocalization for CD45+, CD68+, HLA-DR+, L1+, ER+ and PR+ cells in the endometrial samples collected at three different phases of the menstrual cycles.

Figures 1A, B and C represent the immunolocalization of CD45+ cells in endometrial compartments in proliferative, peri-ovulatory and secretory phases, respectively. CD45+ cells were present

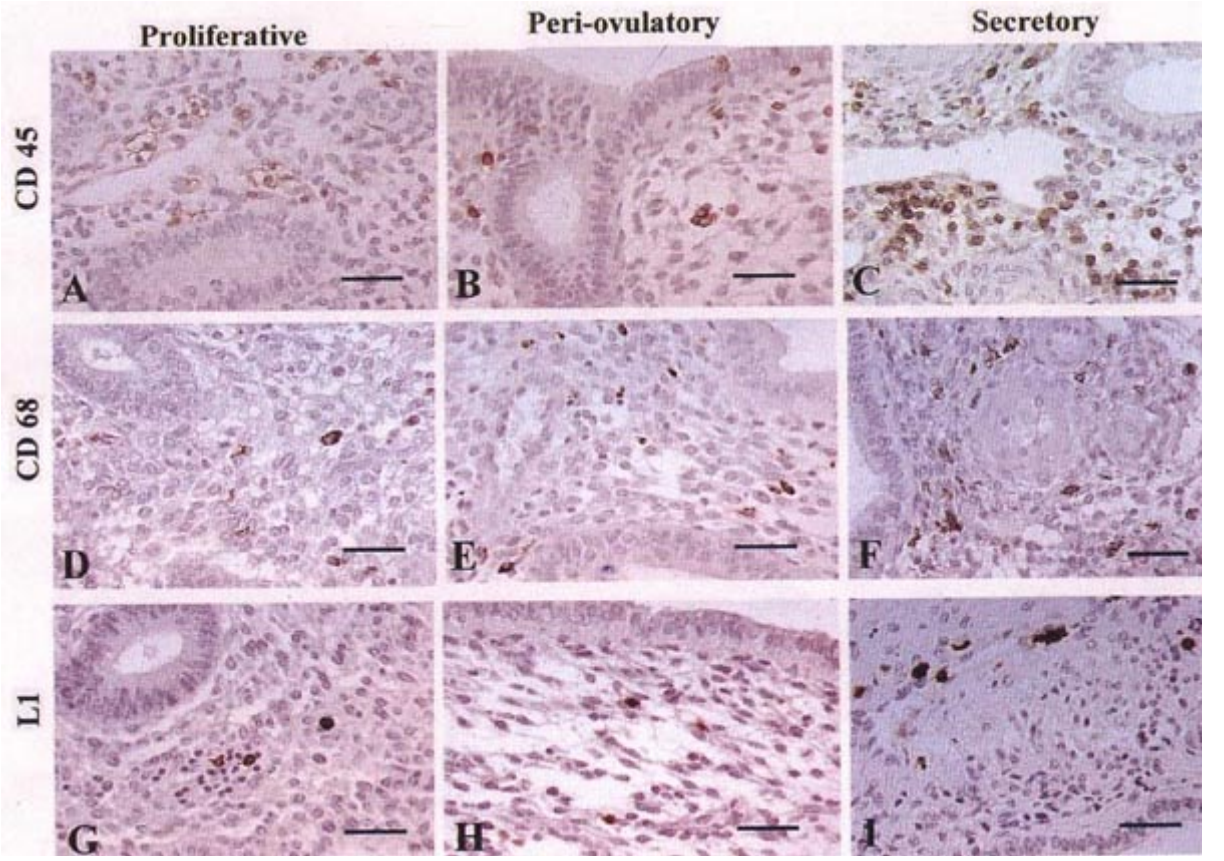

Fig. 1: Immunohistochemical localization of CD45+ cells (A, B, C), CD68+ cells (D, E, F) and L1+ cells (G, H, I) in human endometrial samples collected during proliferative (A, D, G), peri-ovulatory (B, E, H) and secretory (C, F, I) phases, respectively. Bar = 25 µm.

TABLE II: Morphometry of per cent areas of immunoprecipitation.

Antigen	*Median value (ranges)*		
	Proliferative (n=4)	*Peri-ovulatory (n=6)*	*Secretory (n=8)*
CD45	5.9 (2.4-10.4)	6.4 (3.2-11.4)	12.6 (4.3-13.7)**
CD68	1.7 (1.4-2.6)	2.3 (1.3-4.7)	4.9 (2.9-9.9)*
L1	0.9 (0.8-1.2)	0.9 (0.4-2.1)	0.9 (0.6-2.8)
HLA-DR	1.1 (0.5-2.3)	3.6 (0.7-6.9)	1.6 (0.8-2.7)
CD44	3.3 (3.0-3.9)	10.7 (4.5-17.9)[a]	15.1 (8.1-23.2)[b]
ER	4.1 (3.6-4.2)	15.6 (8.3-23.6)**	2.9 (0.6-11.1)
PR	4.5 (1.8-6.3)	10.1 (7.3-11.4)[a]	16.1 (7.8-21.3)[b]

*P<0.05, **P<0.01 compared with other groups. [a]P<0.05, [b]P<0.01 compared with proliferative phase.

mainly as 'clusters' in blood vessels, stroma and occasionally in glands. They were also observed to be polarized towards glands with high degree of heterogeneity in their distribution. There was a gradual increase in the number of CD45+ cells from proliferative (9%) to peri-ovulatory (15%), and to secretory (21%) phases of cycles. Table II provides data on the morphometric analysis of CD45+ cells showing a significant (P<0.01) increase in the area of immunoprecipitation in secretory phase endometrium compared with proliferative and peri-ovulatory phases of the menstrual cycle.

Figures 1D, E and F show representative CD68+ cells immunolocalized in endometrial compartment in proliferative, peri-ovulatory and secretory phases, respectively. Generally, CD68+ cells were diffusely distributed in the stroma with a mean of 10, 11 and 17 cells per 100 cells in endometrial functionalis during proliferative, peri-ovulatory and secretory phases of cycle respectively. Immunohistometric analysis revealed that there was an increase (P<0.05) in CD68+ immunopositive area in endometrium during the secretory phase compared with proliferative and peri-ovulatory phases of the menstrual cycle (Table II).

Figures 1G, H and I show immuno-positive L1+ cell in endometrial compartment in proliferative, peri-ovulatory and secretory phases, respectively. Except for a few L1+ cells seen diffusely distributed in the stroma, L1+ cells were seen mainly within capillaries, post-capillary collecting venules and in small veins of the functionalis zone. Occasionally, they were also seen adjacent to blood vessels. As shown in Table II, no phase-specific distribution pattern of L1+ cells were observed, a mean of 1% of endometrial cells were L1+. However, a higher (P<0.01) number of L1+ cells were found in the vascular compartment of endometrial samples collected during the secretory phase as compared to those seen in the vascular compartment of the endometrial samples collected during other phases of cycles in normal women.

Figures 2A, B and C represent immuno-localization of HLA-DR+ cells in endometrial compartment in proliferative, peri-ovulatory and secretory phases, respectively. Generally, HLA-DR+ cells were found in

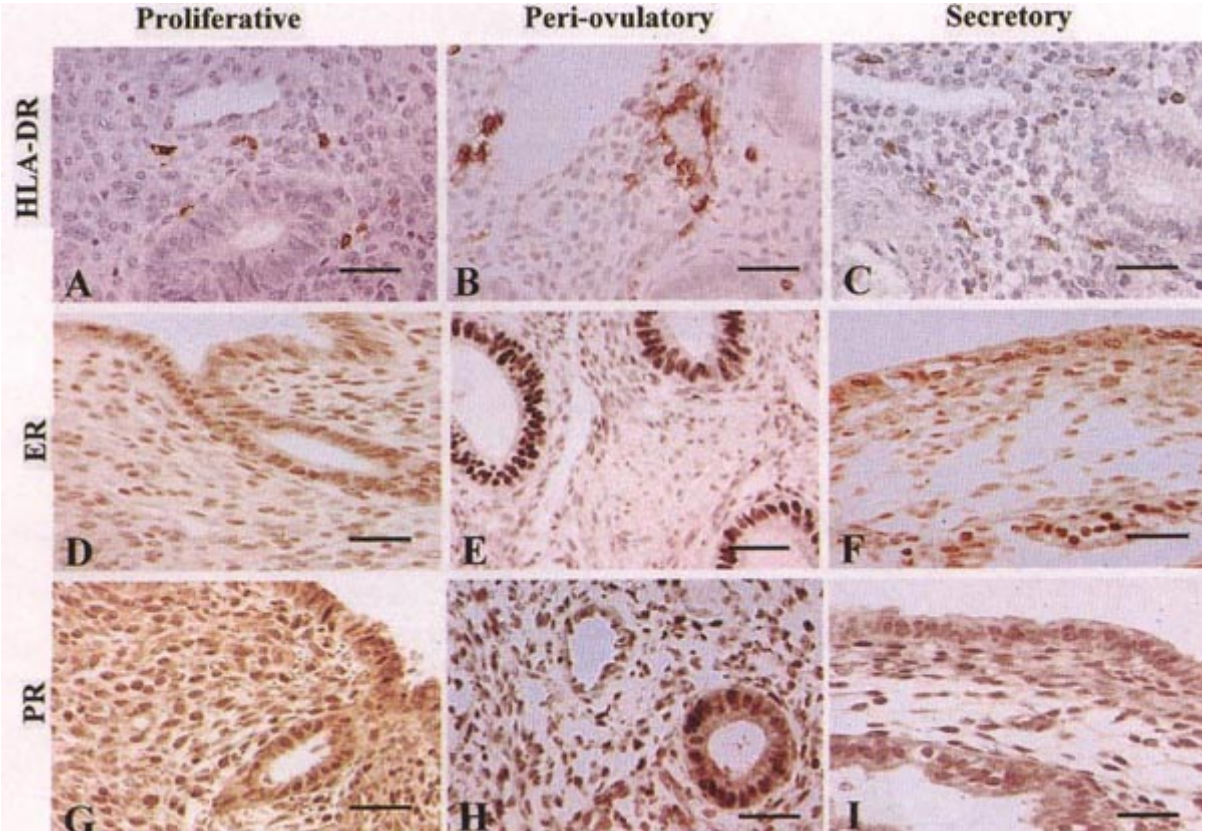

Fig. 2: Immunohistochemical localization of HLA-DR+ cells (A, B, C), ER+ cells (D, E, F) and PR+ cells (G, H, I) in human endometrial samples collected during proliferative (A, D, G), peri-ovulatory (B, E, H) and secretory (C, F, I) phases, respectively. Bar = 25 µm.

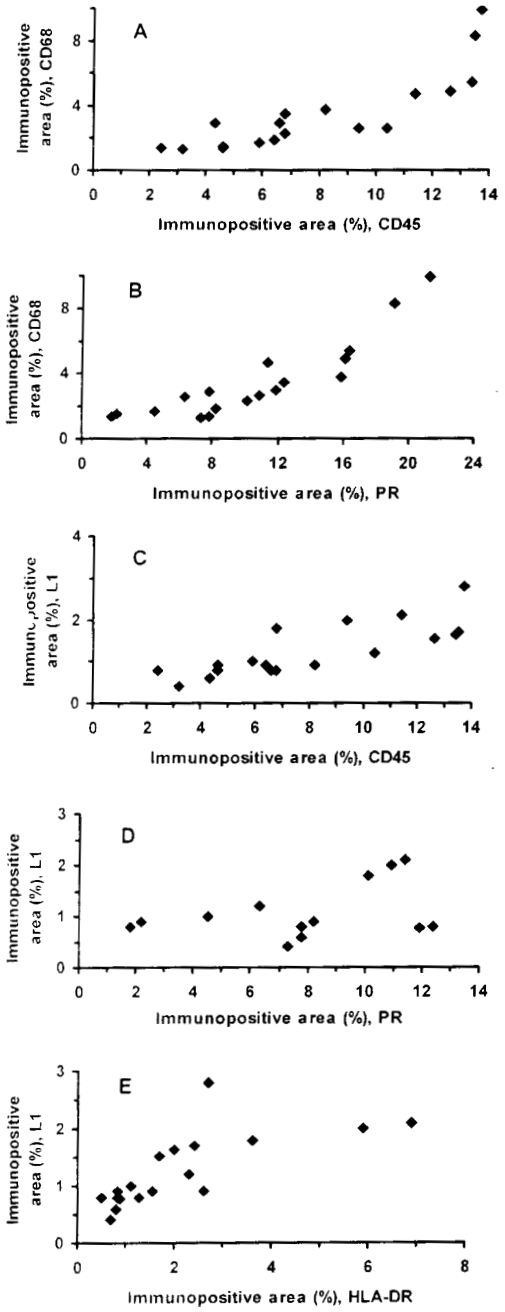

Fig. 3: Correlation between immunopositive areas for (A) CD45 and CD68 (y = 0.5432x + 0.852, r = 0.8386, P<0.01), (B) PR and CD68 (y = 0.3828x + 0.5715, r = 0.8790, P<0.01), (C) CD45 and L1 (y = 0.1351x + 0.1761, r = 0.7932, P<0.05), (D) PR and L1 (y = 0.0709x + 0.5042, r = 0.6193, P<0.05), and (E) HLA-DR and L1 (y = 0.2548x + 0.7122, r = 0.7133, P<0.05).

clusters in the vicinity of glands. Despite an increase in HLA-DR+ cells in peri-ovulatory stage endometrium (12%) compared with proliferative (4%) and secretory (7%) phases of cycle, immunohistometric analysis failed to detect any significant change in different phases of menstrual cycle (Table II).

There was a significant increase in CD44+ area in endometrial samples from peri-ovulatory ($P<0.05$) and secretory ($P<0.01$) phases compared with proliferative phase of cycle (Table II). In basalis zone, CD44+ cells appeared diffusely scattered, while in functionalis zone, they appeared in packs or arranged in rows near to glands.

Figures 2D, E and F show immunolocalization of ER in endometrium during proliferative, peri-ovulatory and secretory phases, respectively. Immunostaining for ER was chiefly seen in nuclei of glandular and luminal epithelial cells and stromal cells of endometrium. As shown in Table II, the per cent area of immunoprecipitation for ER was higher ($P<0.01$) in endometrium during peri-ovulatory phase compared with proliferative and secretory phases of cycle.

Figures 2G, H and I represent immunolocalization of PR in endometrium during proliferative, peri-ovulatory and secretory phases, respectively. Immunostaining for PR was mainly observed in nuclei of glandular and luminal epithelial cells and stromal cells of endometrium. The area of immunostaining showed an increase in PR expression between proliferative and peri-ovulatory ($P<0.05$) and between proliferative and secretory ($P<0.01$) phases, however, no statistically significant

change between samples from peri-ovulatory and secretory phases of cycle was observed (Table II).

As shown in Figure 3, positive correlations were observed between the per cent of immunopositive areas for CD45+ cells and CD68+ cells ($P<0.01$), CD45+ cells and L1+ cells ($P<0.05$), HLA-DR+ cells and L1+ cells ($P<0.05$), PR and CD68+ cells ($P<0.01$), and PR and L1+ cells ($P<0.05$).

DISCUSSION

In the present study, monoclonal antibodies against macrophage cell markers such as HLA-DR, CD68 and L1 were employed to immunolocalize macrophage subsets in human endometrium, and total leucocyte population in endometrial compartment was monitored from the presence of leucocyte common antigen (LCA), CD45. Additionally, CD44 which is a transmembrane adhesion molecule and plays an important rate-limiting role in lymphocyte homing in tissues has been examined.

The present study reports a gradual increase of CD45+ leucocytes in the human endometrium from proliferative to peri-ovulatory and to secretory phase. CD45+ leucocytes were found polarized towards glands with high degree of heterogeneity in their distribution. This report is in concordance with previous report (8).

It was observed in the present study that CD68+ and L1+ macrophages correlated well with CD45+ cells in human endometrium, and there exists a positive correlation between immunopositive areas for PR and CD68, as well as, between immunopositive areas for PR and L1. However, there was no correlation between areas of PR+ cells and CD45+ cells, albeit CD45+ cells were higher in secretory phase endometrium. Thus, we conclude that the accumulation of CD45+ leucocytes in human endometrium is regulated by multifactorial process with higher occupancy in progesterone dominated endometrium, while accumulation of CD68+ and L1+ macrophages in human endometrium is progesterone dependent. It has been reported earlier that the numbers of CD45+ and CD68+ cells increased in progesterone exposed human endometrium (12). The possibility that CD68+ macrophages play important role in pre-decidualization events under progesterone dominance in the secretory phase of the menstrual cycle however remains to be examined.

In addition to being expressed by most circulating and emigrated neutrophils and monocytes, L1 which is a calcium-binding protein has been detected in a subset of reactive macrophages and is considered to be a marker molecule for tissue histiocytes (15). It has anti-bacterial activity *in vitro* and its antiproliferative activity suggestively plays a role in immunosuppression (15). In the present study, highly specific immunolocalization of L1+ macrophages were observed within the endometrial blood vessels, while they were only marginally present in endometrial compartment with however more positive cells in the secretory phase samples. Interestingly, besides having positive correlation with CD45+ and with PR+ areas, L1+ cells showed positive correlation with areas occupied by HLA-DR+ macrophages in human endometrium. HLA-DR+ cells were found mainly in clusters in the vicinity of glands. The significant positive

correlation between L1+ and HLA-DR+ cells as seen in the present study suggests that HLA-DR+ macrophages and L1+ macrophages may be recruited in an associative manner in human endometrium. It is being suggested that the associative recruitment of macrophages bearing HLA-DR and L1 antigen represents a local immune defense mechanism in human endometrium.

CD44 is a transmembrane glycoprotein, which plays an important role in lymphocyte homing process, monocyte activation, cell migration and metastasis (16). There are two isoforms of CD44 in human endometrium: a CD44E, 130 kD isoform which was found in a stage-specific manner in epithelial cells, and a 80 kD CD44H isoform found primarily on cells of hematopoietic origin and on fibroblasts which failed to show any such stage specific distribution (23). On the contrary, we have observed in the present study that there is a rising trend of 85-90 kD isoform of CD44+ cells in the stroma of human endometrium during peri-ovulatory and secretory phases of the cycle.

The use of monoclonal antibodies against receptors for estradiol (ER) and progesterone (PR) permits cell-specific localization of steroid receptors. Through such studies, it has been reported earlier that ER and PR show cyclical variations in endometrial glandular and stromal cells during the menstrual cycle (24,25). The present study documents that the distribution of ER and PR changes in a stage specific manner in human endometrium as has been earlier reported (26).

In conclusion, it appears from the present study that a stage specific distribution of distinct subsets of macrophages in human endometrium takes place in a correlated manner. However, it remains to be deciphered whether different antigens, namely HLA-DR, L1 and CD68 are expressed on the same or different subsets of macrophages. Based on their localization and their stage-specific distribution as documented in the present study, it appears that there are indeed sub-types of macrophages in the human endometrium. Furthermore, the results obtained in the present study indicate that while there are positive correlations between a few parameters, there is no such correlation between other parameters in human endometrium during different phases of menstrual cycle. Thus, we suggest that the recruitment of different subsets of tissue macrophage system is regulated through a complex multifactorial process involving endocrine and paracrine factors at the endometrial level in the human as also suggested by other groups based on different study models (27-29).

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