Expression of the cell adhesion molecules on leukocytes that demarginate during acute maximal exercise

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Van Eeden, Stephan F., John Granton, Jennifer M. Hards, Barbara Moore, and James C. Hogg. Expression of the cell adhesion molecules on leukocytes that demarginate during acute maximal exercise. J. Appl. Physiol. 86(3): 970–976, 1999.—The pulmonary vascular bed is an important reservoir for the marginated pool of leukocytes that can be mobilized by exercise or catecholamines. This study was designed to determine the phenotypic characteristics of leukocytes that are mobilized into the circulation during exercise. Twenty healthy volunteers performed incremental exercise to exhaustion [maximal O2 consumption (Vo2max)] on a cycle ergometer. Blood was collected at baseline, at 3-min intervals during exercise, at Vo2max, and 30 min after exercise. Total white cell, polymorphonuclear leukocyte (PMN), and lymphocyte counts increased with exercise to Vo2max (P < 0.05). Flow cytometric analysis showed that the mean fluorescence intensity of L-selectin on PMN (from 14.9 ± 1 at baseline to 9.5 ± 1.6 at Vo2max, P < 0.05) and lymphocytes (from 11.7 ± 1.2 at baseline to 8 ± 0.8 at Vo2max, P < 0.05) decreased with exercise. Mean fluorescence intensity of CD11b on PMN increased with exercise (from 10.2 ± 0.6 at baseline to 25 ± 2.5 at Vo2max, P < 0.002) but remained unchanged on lymphocytes. Myeloperoxidase levels in PMN did not change with exercise. In vitro studies showed that neither catecholamines nor plasma collected at Vo2max, during exercise changed leukocyte L-selectin or CD11b levels. We conclude that PMN released from the marginated pool during exercise express low levels of L-selectin and high levels of CD11b.

LEUKOCYTES THAT LEAVE the circulating bloodstream are delayed along the walls of microvessels, forming a sequestered or “marginated” pool that can be rapidly mobilized back into the circulation (1, 14, 16, 33). Stress, such as physical exercise and catecholamine infusion, increases the circulating leukocyte counts by mobilizing this marginated pool (11, 25, 28). Several studies have shown that the pulmonary and splanchnic microvascular beds are important reservoirs for this marginated pool (5, 14, 37). The margination of polymorphonuclear leukocytes (PMN) in the pulmonary vascular bed has been studied extensively and has been attributed primarily to their mechanical properties and to the discrepancy between PMN size and the size of pulmonary capillaries (8, 13, 42). Several mechanisms have been proposed to explain how PMN demarginate. Thomassen and colleagues (37) showed that an increase in pulmonary blood flow causes demargination of leukocytes from the pulmonary capillary bed. They suggest that the redistribution of flow to lung regions sequestering leukocytes or changes in the mechanical properties of leukocytes induced by the increased flow releases leukocytes from the lung. Humoral factors, such as catecholamines, that increase during exercise (2, 17) could influence the leukocyte-endothelial cell interactions, resulting in the release of cells into the circulation. Lymphocytes, granulocytes, and endothelial cells have β2-receptors, and stimulation of these receptors diminishes the adherence of PMN to endothelial cells (6). However, blocking these receptors with β-blockers only partially reduces the leukocytosis of exercise (11).

The cell adhesion molecules, which are expressed on the surface of leukocytes and endothelial cells, play a pivotal role in leukocyte-endothelial cell interaction (3, 19, 30, 43). This interaction is an early and requisite event in inflammation, contributes to the margination of PMN in activated vascular beds, and plays a key role in lymphocyte homing and recirculation (3, 30, 43). L-selectin (CD62L) and the β2-integrins on the surface of leukocytes play an important role in this leukocyte-endothelial cell interaction (3). These molecules undergo quantitative and qualitative changes in response to various stimuli and could be involved in the leukocyte-endothelial cell interactions that contribute to margination and demargination of leukocytes. Immature PMN released from the bone marrow have been shown to preferentially marginate in lung capillaries (26, 39). Furthermore, studies from our laboratory have shown that younger PMN released from the bone marrow express higher levels of L-selectin (39, 40) and lower levels of the integrin CD18 on their surface (21).

On the basis of these findings, we reasoned that the marginated PMN should be enriched, with younger cells expressing high levels of L-selectin and low levels of CD18. The present study was designed to test this hypothesis by measuring the surface density of the adhesion molecules L-selectin and CD18/CD11b on leukocytes mobilized into the circulation during exercise. We used a model of short-term intense exercise that has been shown to release leukocytes from the marginated pool into the circulation (11, 25, 28).

METHODS

Subjects. Twenty healthy infection-free volunteers, 25–48 yr of age, were recruited for the study. Informed consent was obtained from all subjects, and the Human Experimentation...
Committee of the University of British Columbia approved the study. All subjects had participated in some form of recreational or competitive sport, and their current exercise activity was graded as equivalent to grade 1 (running or other aerobic exercise for <1 h/wk), grade 2 (1–3 h/wk), grade 3 (4–6 h/wk), or grade 4 (>6 h/wk) (7).

Experimental protocol. All subjects were studied in the morning between 8 and 10 AM and asked to refrain from exercise during the 24 h before the study. All subjects were nonsmokers, used no medication, and consumed a light breakfast in the morning ~2 h before the exercise. An intravenous indwelling 18-gauge catheter was placed in a large vein in the forearm for blood sampling and was maintained open by flushing with normal saline. Baseline samples were taken 15 min after placement of the catheter and every 3 min while cycling, at maximal O2 consumption (V̇O2max), and 30 min after the exercise was discontinued. Blood samples were collected into 5-ml sterile tubes containing potassium EDTA (Becton Dickinson) and analyzed within 1 h of collection. Blood was also collected in separate tubes to obtain plasma (EDTA tubes).

Subjects were tested on a Cardiogenics cycle ergometer with an incremental exercise test protocol. After a warm-up of 2 min at 60 W, the workload was increased by 30 W every 2 min to voluntary exhaustion in ~15 min. The average time to V̇O2max was ~10 min. Criteria for attaining V̇O2max included a plateau in O2 consumption, a heart rate close to the subject’s age-related maximum and no further increase with increasing workload, and signs of exertional intolerance (inability to maintain required pedal rhythm, and fatigue).

Leukocyte counts. The total leukocyte count was determined by a Sysmex cell counter (model E4000, Toa Medical Electronics, Kobe, Japan), and the differential counts and band cells counts were done on Wright-stained blood smears. All counts of circulating leukocytes were adjusted for changes in hematocrit during exercise according to the following equation: Ca = Ctn x Htn/Htn, where Ca is the adjusted count, Ctn is the observed count at time n, Htn is the hematocrit at time 0 or baseline before the start of exercise, and Htn is the hematocrit at time n. The blood smears were evaluated in a blinded fashion by evaluating 100 leukocytes in randomly selected fields of view.

L-selectin and CD11b expression on leukocytes. Blood collected in EDTA was used to immunolabel circulating leukocytes for the presence of surface L-selectin, CD18, and CD11b. A whole blood method was used to prepare cells for flow cytometry using a commercially available kit (Coulter Clone, Coulter Immuno, Hialeah, FL). Briefly, 100 µl of EDTA blood were incubated for 10 min with the anti-CD18/CD11b monoclonal antibody at 0.1 µg/ml final concentration (DAKO Laboratories, Copenhagen, Denmark) or the anti-L-selectin monoclonal antibody Leu-8 at 1.2 µg/ml final concentration (Becton-Dickinson, Mississauga, ON, Canada). All experiments were performed at room temperature and with use of the buffer provided by the manufacturer. Isotype nonimmune mouse IgG (DAKO Laboratories) in concentrations similar to the primary antibody were used as negative controls. After they were labeled with the primary antibody, cells were washed and incubated with a FITC-conjugated goat antimouse secondary antibody at 0.2 µg/ml final concentration for 10 min (DAKO Laboratories). Red blood cells were lysed with Immunolysis (Coulter Immuno), and the remaining leukocytes were washed twice and fixed with 1% paraformaldehyde. As a positive control, cells were incubated with f-Met-Leu-Phe (fMLP; Sigma Chemical, St. Louis, MO; 10 nM final concentration) before they were labeled for L-selectin, CD11b, or CD18, as described above. Flow cytometry was performed using analysis gates for PMN and lymphocytes from typical forward and side-angle light-scattering patterns (Profile Epics XL, Coulter Electronics). A total of 5,000 cells/specimen were evaluated, and the results are expressed as the mean fluorescence intensity (MFI).

Circulating mediators and leukocyte L-selectin and CD11b expression. Baseline blood was exposed to autologous plasma obtained at V̇O2max to determine the possible effect of circulating factors released during exercise on the expression of surface L-selectin and CD11b of leukocytes. Plasma was obtained by centrifugation of EDTA blood (2,000 rpm or 750 g) for 10 min. We incubated 100 µl of baseline whole blood (EDTA) with a similar volume of 1:100, 1:10, 1:5, 1:2, or 1:1 dilution or a full-strength plasma (diluted in PBS, pH 7.4) for 15 min at 37°C. Cells were washed with PBS and centrifuged, the supernatant was removed, and the cell pellet was resuspended in 200 µl of PBS. Immunofluorescent staining of leukocytes for surface expression of L-selectin and CD11b was done as described above.

Catecholamines and leukocyte L-selectin and CD11b expression. Baseline blood was exposed to incremental doses of epinephrine or norepinephrine to determine the effect of these two stress hormones on the surface expression of L-selectin and CD11b. Blood was incubated with 0.25, 0.5, 1, 10, 100, or 1,000 ng/ml (final concentration) of epinephrine and norepinephrine for 15 min at 37°C. Levels of these hormones vary from 0.2 to 3 ng/ml during exercise (2). The cells were then washed with PBS and centrifuged, the supernatant was removed, and the cell pellet was resuspended in 200 µl of PBS. Immunofluorescent staining of leukocytes for surface expression of L-selectin and CD11b was done as described above.

Myeloperoxidase content of PMN. Myeloperoxidase (MPO) content of leukocytes was determined by using the substrate diaminobenzidine (DAB; Sigma Chemical) and stained on slides prepared from leukocyte-rich plasma (LRP). Blood used for the preparation of LRP was collected in acid-citrate-dextrose. The erythrocytes were sedimented by addition of 4% dextran (average relative mol wt 267,000; Sigma Chemical) in PMN buffer (in mM: 138 NaCl, 27 KCl, 8.1 Na2HPO4, 1.5 KH2PO4, and 5.5 glucose, pH 7.4). The resulting LRP was cytospun at 180 g for determination of LRP by LRP Products, Cheshire, UK) to obtain a monolayer of cells on slides precoated with 3-aminopropyltriethoxysilane. Slides were air dried and fixed in 1% paraformaldehyde at 4°C for 30 min. Slides were washed with distilled water, and cells were permeabilized for 15 min at room temperature with N-octyl-β-D-glucopyranoside (Sigma Chemical; 6 µg/ml) diluted in Tris-buffered saline (TBS), pH 7.4. Slides were washed twice with TBS and treated with DAB substrate (1 ml of 5 mg/ml DAB diluted in 9 ml of TBS, pH 7.4, with 0.033 ml of 3% H2O2) in the dark for 60 min at room temperature. Slides were washed twice in TBS, counterstained with Mayer’s hematoxylin for 2 min, dehydrated, and coverslipped. The intensity of staining was evaluated by grading PMN as highly, intermediate, or weakly stained (see Fig. 7). One hundred cells selected in random fields were evaluated from blood samples collected at all time points. This grading system was evaluated for inter- and intraobserver variability.

PMN MPO was also evaluated in a subgroup of five subjects by using flow cytometry and an anti-human MPO monoclonal antibody at 0.5 µg/ml (DAKO Laboratories). A whole blood method was used as described above, and cells were permeabilized before they were labeled with use of a commercially available kit (Fix and Perm, Caltag Laboratory, Burlingame, CA). Isotype nonimmune mouse IgG (DAKO Laboratories) in concentrations similar to the primary anti-
body was used as a negative control. After they were labeled with the primary antibody, cells were washed and incubated with an FITC-conjugated goat anti-mouse secondary antibody (0.2 µg/ml final concentration) for 10 min (DAKO Laboratories) and fixed with 1% paraformaldehyde. Flow cytometry was performed using analysis gates for PMN and lymphocytes from typical forward and side-angle light-scattering patterns (Profile Epics XL, Coulter Electronics). A total of 5,000 cells/specimen were evaluated, and the results are expressed as the MFI.

Data analysis. Unpaired Student’s t-test was used to compare adhesion molecules at baseline and after fMLP stimulation. To assess the effect of exercise on temporal data, an ANOVA for repeated measures was used, and Bonferroni corrections were made for multiple comparisons. Values are means ± SE; P < 0.05 was considered significant.

The inter- and intraobserver variations in the grading of MPO staining were evaluated by calculating the Pearson coefficient of mean-square contingency (R²) for each grade and expressing R² as a fraction of the maximum possible value (R²max), which represents the value of the Pearson χ² coefficient if there is 100% agreement in the grading score between or within observers; thus R²max = ±0.82 (32).

RESULTS

Table 1 shows the demographic characteristics of the study population. The study group was predominantly men (15:5 ratio of men to women) with a fitness level of 2.8 ± 0.2 (grades 1–4, see METHODS) and an average VO₂max of 3.8 ± 0.2 l/min.

Leukocyte counts. Peripheral blood total leukocytes increased from 4.9 ± 0.4 at baseline to 8.7 ± 0.9 × 10⁹/l at VO₂max and decreased to 6.9 ± 0.8 × 10⁹/l at 30 min after exercise. This was due to a rise in PMN and lymphocyte counts, with a more pronounced lymphocyte response (Fig. 1). A small but nonsignificant (P > 0.05) increase in circulating band cells was observed during the exercise protocol (Fig. 1).

CD11b and L-selectin expression on leukocytes. Figure 2 shows a representative flow cytometric profile of PMN and lymphocytes for L-selectin and CD11b at baseline and VO₂max. L-selectin expression on circulating PMN gradually decreased during exercise and was lower than baseline at VO₂max and after exercise (P < 0.05; Fig. 3). CD11b increased during exercise to peak at VO₂max (P < 0.002) and then declined after exercise but did not return to baseline levels (Fig. 3). A similar increase was observed for CD18 (data not shown). The drop in L-selectin and the increase in CD11b were smaller than those achieved by cell activation by use of the chemoattractant fMLP (Fig. 4). CD11b expression on lymphocytes did not change, but L-selectin decreased (P < 0.05) during and after exercise (Fig. 5).

Circulating mediators and leukocyte L-selectin and CD11b expression. Incubating whole blood obtained at baseline with incremental concentrations of epinephrine or norepinephrine did not significantly change the expression of CD11b or L-selectin on PMN (Fig. 6). Similar results were obtained with lymphocytes (data not shown). Baseline blood incubated with increasing dilutions of plasma obtained at VO₂max showed no changes in CD11b or L-selectin on PMN or lymphocytes.

MPO in PMN. The variability of PMN staining for MPO is shown in Fig. 7. This variability allows us to grade the intensity of MPO staining of circulating PMN as high, intermediate, or low. R²max for inter- and intraobserver variability was >0.75. Figure 8 shows the results of this grading method. No significant changes were observed in PMN MPO activity during or after exercise. This finding was confirmed in a subset of subjects (n = 5) by using flow cytometry and monoclonal antibodies against human MPO (MFI = 2.8 ± 0.9, 3.2 ± 1, 2.2 ± 1.1, 2.9 ± 0.8, and 2.5 ± 1.2 at baseline, 3 min of exercise, 6 min of exercise, VO₂max, and after exercise, respectively).

DISCUSSION

Exercise and catecholamine infusion increase blood leukocyte counts (11, 25, 28). This leukocytosis is caused by mobilization of the marginated pool of leukocytes and not bone marrow release of leukocytes. This study shows that exercise mobilized PMN into the circulation without increasing the band cell counts, excluding bone marrow release as a reason for the increase in circulating PMN counts. It also shows that the mobilized PMN express lower levels of L-selectin and higher levels of CD11b on their surface than before exercise. Lymphocytes mobilized into the circulation during exercise also express low levels of L-selectin. In vitro incubation of leukocytes with catecholamines or

Table 1. Demographic data of subjects

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Mean ± SE</th>
<th>Range</th>
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<tbody>
<tr>
<td>Fitness</td>
<td>2.8 ± 0.2</td>
<td></td>
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<tr>
<td>Pulse, beats/min</td>
<td>85.9 ± 2.9</td>
<td>63–105</td>
</tr>
<tr>
<td>Resting</td>
<td>169 ± 2.9</td>
<td>148–190</td>
</tr>
<tr>
<td>VO₂max, l/min</td>
<td>3.8 ± 0.2</td>
<td>1.88–5.02</td>
</tr>
<tr>
<td>Ventilation, l/min</td>
<td>136 ± 9.4</td>
<td>80–190</td>
</tr>
</tbody>
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Values are representative of 20 subjects (15 men and 5 women). Fitness was graded from 1–4 (see METHODS). VO₂max, maximal O₂ consumption.
plasma collected at $\dot{V}O_2\text{max}$ did not change the adhesion molecules on PMN or lymphocytes. This suggests that circulating factors in the blood during acute maximal exercise are not responsible for the changes we observed in leukocyte cell adhesion molecules.

In this study we used exercise to mobilize leukocytes from the marginated pool and an incremental exercise protocol of ≤15 min. This short-term protocol was used, because endurance exercise has been associated with changes in PMN adhesion molecules compatible with release of PMN from the bone marrow (24). Although Kurokawa and colleagues (24) did not measure band...
cell counts, the changes they report in PMN L-selectin with 1 h of endurance exercise are similar to those we previously showed to be associated with active bone marrow release (26, 38, 39). Total white cell, PMN, and lymphocyte counts increased during the exercise (Fig. 1), but there was no accompanying increase in circulating band cell counts during or after exercise compatible with bone marrow release of PMN. These findings are consistent, in that the increase in leukocyte counts is largely due to the mobilization of leukocytes from the marginated pool.

Several studies have shown that the pulmonary circulation is an important reservoir of marginated leukocytes (5, 14, 37). In the present study we showed an increase in CD11b expression on circulating PMN during incremental exercise to VO_{2max} (Figs. 2 and 3), suggesting mobilization of PMN expressing high levels of CD11b from the marginated pool. This is in contrast to findings from Kurokawa and colleagues (24) that showed no changes with endurance exercise. A possible explanation for this discrepancy is that during endurance exercise an equilibrium develops between PMN expressing high levels of CD11b marginating and demarginating in the lung. Because an increase in surface density of CD11b signifies PMN activation (19), mobilization of PMN expressing high levels of CD11b during exercise suggests that activated PMN are preferentially delayed in lung microvessels. In normal infection-free subjects, these could be PMN activated during their intravascular life when they encounter mildly activated vascular beds such as the upper respiratory tracts, bladder, and large bowels. Changes in PMN deformability associated with mild cell activation (8, 7, 13–15, 42) are most likely responsible for the preferential margination of PMN expressing high levels of CD11b in the lung. We suspect that the increase in pulmonary blood flow during exercise (16, 33, 37) mobilizes PMN expressing high levels of CD11b from the lung.

Alternatively, local factors in the pulmonary capillaries could activate PMN in the lung. The process of demargination may also contribute to changes in cell adhesion expression. Recent studies from our laboratory showed that mechanical deformation of PMN increases CD11b expression (20). PMN have to negotiate ~50–60 pulmonary capillary segments from the

![Fig. 5. Changes in CD11b and L-selectin on circulating lymphocytes during incremental exercise to VO_{2max} on a cycle ergometer. Values are means ± SE of 20 subjects and are expressed as mean fluorescence intensity. CD11b did not change, but L-selectin gradually declined during exercise, was significantly lower at VO_{2max}, and remained low in postexercise recovery period. *P < 0.05 vs. baseline.](image1)

![Fig. 6. Changes in L-selectin and CD11b on PMN when stimulated in vitro with epinephrine (A) and norepinephrine (B). Whole blood was incubated for 15 min with increasing doses of catecholamines before immunolabeling. Values are means ± SE of 5 experiments and are expressed as mean fluorescence intensity. No change was observed in L-selectin or CD11b.](image2)

![Fig. 7. Myeloperoxidase (MPO) in PMN at baseline. Cytospins made from leukocyte-rich plasma were stained (brown) for presence of MPO with use of substrate diaminobenzadine. Variability of MPO in PMN allowed us to grade intensity of staining as high, intermediate, or low.](image3)
arterial to the venous side of the pulmonary circulation (8, 13, 14). The average pulmonary capillary segment size is 5–8 µm, which would cause minimal PMN deformation, but 10–15% of capillary segments are <5 µm in diameter, which requires PMN to deform. This deformation is associated with an increase in CD11b expression (20, 41, 43). Furthermore, the increase in pulmonary blood flow during exercise would increase the likelihood that PMN would encounter small capillary segments and also increase shear forces on the PMN. Both factors have been shown to increase PMN CD11b expression (9, 20).

Circulating mediators such as catecholamines have been shown to increase during exercise (2, 17), but incubation of whole blood with exercise-related or pharmacological concentrations of epinephrine and norepinephrine did not alter the expression of CD11b on PMN (Fig. 6). Furthermore, exposing baseline whole blood to plasma collected at VO2max also failed to change CD11b expression on leukocytes. These studies suggest that circulating mediators generated during exercise are unlikely to be responsible for the increase in CD11b.

Gray and colleagues (12) showed that intense intermittent running induced granulocyte activation measured as the release of PMN elastase from the primary granules. This type of exercise has been shown to cause muscle damage and PMN activation (29, 31). We have measured MPO, an enzyme in the secondary granules. This type of exercise has been shown to cause muscle damage and PMN activation (29, 31). We have measured MPO, an enzyme in the primary granules, of PMN and could not demonstrate any change induced by the short-term exercise protocol we used (Fig. 8).

The drop in L-selectin expression on PMN during exercise (Figs. 2 and 3) agrees with the observations of Kurokawa and colleagues (24), who showed a decline in L-selectin on circulating PMN during 1 h of endurance exercise at 60% VO2max (24). L-selectin has been shown to be important in the margination of PMN along the vessel wall in postcapillary venules in the systemic and pulmonary circulations (3, 22, 23, 43). Kuebler and colleagues (22) showed that leukocyte retention in pulmonary arterioles, venules, and capillaries depends on the interaction of leukocytes with capillary endothelium. By blocking selectin-dependent pathways with fucoidan, the transit time of leukocytes through capillaries is reduced by ~62%, suggesting that, in addition to mechanical factors, the selectins contribute significantly to leukocyte retention in the lung (22). The release of these leukocytes from the marginated pool during exercise may be associated with a loss of L-selectin. Alternatively, PMN expressing low levels of L-selectin may tend to marginate preferentially. We previously showed that L-selectin levels on PMN decline as they age in the circulation (38). These older PMN also express higher levels of CD18 (38), produce more oxygen radicals when stimulated (34), and could potentially be more harmful to the host when activated in the lung by stimuli such as cigarette smoke.

Exercise causes a brisk lymphocytosis and a decrease in the expression of L-selectin on circulating lymphocytes (Figs. 2 and 5), suggesting that lymphocytes mobilized from the marginated pool of cells express low levels of L-selectin. The majority of B and virgin T lymphocytes express L-selectin, whereas only a subpopulation of memory T cells and NK cells are L-selectin positive (36). L-selectin is also weakly expressed by the majority of splenic lymphocytes (35). Mobilization of these lymphocytes expressing low levels of L-selectin from the spleen or the lung could result in the drop in L-selectin that we observed during exercise.

The splanchnic circulation receives 20% of the cardiac output, and the spleen and liver are the major organs that contribute to the marginated pool of leukocytes in the splanchnic bed (10, 18). The splenic sinusoids are also the major site for removal of older, activated, and apoptotic PMN from the circulation (4, 10). Mobilization of these older PMN from splenic sinusoids during exercise could also contribute to the drop in L-selectin and the rise in CD11b on PMN in circulating blood during exercise. The relative contribution of these leukocytes released from the splanchnic vascular bed on the adhesion molecule profile of circulating leukocytes during exercise needs to be determined.

We have shown that PMN demarginating during exercise express lower levels of L-selectin and higher levels of CD11b than their circulating counterparts. Lymphocytes that demarginate during exercise express lower levels of L-selectin and may represent the mobilization of a subpopulation of memory T and NK cells. The low levels of L-selectin on demarginated PMN suggest that these cells are older. The high levels of CD11b on demarginated PMN support the hypothesis that these PMN are mildly activated. These findings underline the differences in phenotypic characteristics of circulating and marginated leukocytes. We speculate that these characteristics play an important role in leukocyte-mediated tissue inflammation and injury.

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