

Intrauterine Undernutrition in Rats Interferes with Leukocyte Migration, Decreasing Adhesion Molecule Expression in Leukocytes and Endothelial Cells¹

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ABSTRACT Experimental and epidemiologic data have shown that malnutrition predisposes individuals to infections. Immune responses are compromised, particularly in undernourished children. Therefore, we investigated the migratory capacity of leukocytes, using the intravital microscopy technique, in male Wistar rats (8–9 wk of age) that were undernourished in utero after their dams were fed 50% less food than the amount consumed by control dams. The number of leukocytes rolling along the venular endothelium, sticking after stimulation with leukotriene B₄, tumor necrosis factor- α (TNF- α) or zymosan-activated plasma, or migrating after TNF- α stimulation was significantly reduced in the undernourished rat offspring. Compared with nourished rat offspring, undernourished offspring had significantly reduced numbers of circulating leukocytes, higher blood pressure, and higher leukocyte rolling velocity (V_{WBC}), as well as a higher ratio between V_{WBC} and RBC velocity (V_{RBC}). Endothelial P-selectin and intercellular adhesion molecule-1 (ICAM-1) expression, analyzed by immunohistochemistry, and basal leukocyte L-selectin expression, analyzed by flow cytometry, were significantly reduced in the undernourished rat offspring. Because the groups did not differ in leukocyte CD11/18 expression, endothelial expression of platelet-endothelial cell adhesion molecule-1, or venular blood flow velocity and, consequently, venular shear rate, we conclude that intrauterine undernutrition in rats reduces leukocyte migration, downregulates endothelial expression of P-selectin and ICAM-1, as well as leukocyte expression of L-selectin, while reducing leukocyte counts. The higher V_{WBC} and V_{WBC}/V_{RBC} ratio may also play a role in this reduced leukocyte migration. Our data suggest that this phenomenon is involved in the increased predisposition to infections in undernourished subjects. J. Nutr. 135: 1480–1485, 2005.

KEY WORDS: • *intrauterine malnutrition* • *leukocyte migration* • *adhesion molecules*

Reduction in the inflammatory response has been observed in children with nutritional deficiencies or, in particular, malnutrition. Numerous experimental studies have shown that nutritional deficiencies and other influences that reduce growth during critical periods of life can permanently affect the structure and physiology of a variety of organs and tissues (1,2).

Failure of the maternal-placental supply to match fetal nutrient demand results in a range of fetal adaptations and developmental changes. Although these adaptations may be beneficial for short-term survival, they can lead to permanent alterations in body structure and metabolism and thereby to cardiovascular and metabolic diseases in adult life (3,4).

Clinical observations and epidemiologic studies lend sup-

port to the concept that nutritional deficiencies increase the frequency and severity of infection. This may be related to reduced polymorphonuclear leukocyte migration, both random and in response to chemotactic stimuli (5).

In experimental studies of mice and guinea pigs, it was demonstrated that protein-energy malnutrition reduced the granuloma formation and macrophage activation induced by administration of bacillus Calmette-Guerin vaccine and impaired the effector mechanisms, including intracellular pathogen rejection (6,7). In rats, such malnutrition was found to decrease monocyte migration and limit the inflammatory response induced by chemical irritants (8,9).

Most of the experimental studies involving inflammatory responses of immune or nonimmune origin have used models of protein-energy malnutrition. However, intrauterine malnutrition is subject to a more modern approach.

Recently, interest in prenatal conditions and the effect they may have on health status in later life has increased. In particular, there is speculation that prenatal malnutrition is

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related to increased susceptibility to infections in adults. The present study aimed to investigate the effects of intrauterine malnutrition in rats by assessing leukocyte migration, an important component of the inflammatory response. Leukocyte migration, together with expression of the adhesion molecules involved, was studied using intravital microscopy, immunohistochemistry, and flow cytometry.

MATERIALS AND METHODS

Animals. All procedures used in this study were approved and performed in accordance with guidelines established by the Ethics Committee of the University of São Paulo Institute of Biomedical Sciences. Wistar rats from our own colony (Hypertension Laboratory, University of São Paulo Institute of Biomedical Sciences) were housed in a $22 \pm 1^\circ\text{C}$ environment at 60% humidity and were maintained on a 12-h light:dark cycle. A total of 40 adult Wistar rats (30 virgin females and 10 males) were used to generate the offspring used in this study.

Feeding protocol. Timed mating was carried out in age-matched (12- to 16-wk-old) female and male Wistar rats. To assess the stage of estrus of the females, vaginal smears were checked before the introduction of the males. Day 1 of the pregnancy was defined as the day on which spermatozoa were detected in the vaginal smear. After confirmation that mating had occurred, the rats were housed individually in standard rat cages. Female rats were randomly divided into 2 groups: ad libitum (nourished; NR)³ and undernourished rats (UR). Rats in the NR group were fed a standard commercial rat diet (Nuvital) containing protein (minimum 22.0%), carbohydrates (maximum, 54.0%), fat (minimum, 4.5%), cellulose (maximum, 8.0%), minerals (maximum, 10.0%), water (maximum, 12.5%), and vitamins. Rats in the UR group were fed the same diet at 50% of the NR intake, as determined by the amount of food consumed by the control group, from d 1 of pregnancy until d 23 (parturition). All rats were fed daily, in the morning, and consumption was determined 24 h later. The body weight of dams was determined during pregnancy (on d 5, 10, 15, and 20), and the body weight of pups was determined at birth. After parturition, UR dams consumed their food ad libitum. Therefore, UR pups differed from NR pups only in their prenatal dietary experience. To prevent variation in neonatal growth related to the unavailability of milk during suckling, litter size was standardized to 8 pups. After suckling, the undernourished dams were killed. After weaning, the pups were fed the same diet as the dams. At 8–9 wk of age, male offspring were used in experiments to assess leukocyte activity. The rats used in the experiments were killed by an overdose of anesthesia.

Measurement of metabolic variables. At 4 wk of age, the NR and UR offspring were housed individually in metabolic cages to investigate the interference of food and water intake in body weight gain. The rats had access to a known quantity of food and water, and intakes were calculated after 24 h. Urine volume was measured, and urine glucose was determined using a reagent strip (Diasstix, Bayer). The procedure was repeated 3 times over a 3-wk period.

Study of leukocyte behavior and physiologic variables. Blood leukocyte counts and intravital microscopy studies were performed as previously described (10). Under anesthesia, the internal spermatic fascia was exteriorized and observed using a closed-circuit television camera coupled to a microscope. The numbers of leukocytes rolling along the venular endothelium were determined, both for those adherent after stimulation with leukotriene B₄ (LTB₄; Sigma), zymosan-activated plasma (ZAP; Sigma), or tumor necrosis factor- α (TNF- α ; Sigma) stimuli and for those migrating after stimulation with TNF- α . Determination of other variables, such as blood pressure, venular blood flow velocity, wall shear rate, and the leukocyte rolling velocity/RBC velocity ($V_{\text{WBC}}/V_{\text{RBC}}$) ratio was performed as previously described (10,11).

³ Abbreviations used: ICAM-1, intercellular adhesion molecule-1; LTB₄, leukotriene B₄; NR, nourished rat; PECAM-1, platelet-endothelial cell adhesion molecule-1; TNF- α , tumor necrosis factor- α ; UR, undernourished rat; V_{RBC} , red blood cell velocity; V_{WBC} , white blood cell velocity (leukocyte rolling velocity); ZAP, zymosan-activated plasma.

Immunohistochemistry. Sections obtained from the whole testis of the rats were incubated for immunohistochemistry analysis for detection of intercellular adhesion molecule-1 (ICAM-1, Seikagaku), platelet-endothelial cell adhesion molecule-1 (PECAM-1, Pharmingen), and P-selectin (Pharmingen) as previously described (10). Subsequently, cross sections were incubated with streptavidin-peroxidase (Amersham Pharmacia Biotech) for 1 h at room temperature and then immersed in 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Sections were lightly stained with hematoxylin, dehydrated with alcohol and xylene, and mounted with Permount (Fischer). The density of the brown staining in the venular endothelial cells of the internal spermatic fascia was evaluated on a Nikon Eclipse E-800 inverted microscope using a 20X objective at an aperture of 0.45 and analyzed using Image-Pro[®] Plus software (Media Cybernetics). The results are expressed as the mean density of brown staining (arbitrary units \pm SEM).

Flow cytometry. To quantify L-selectin (Seikagaku) or CD18 (Immunotech) expression, leukocytes were isolated from heparinized blood (Liquemine, Roche S/A) collected from the abdominal aorta. Subsequently, erythrocyte lysis was performed using ammonium chloride solution (0.13 mol/L), and leukocytes were recovered after washing with HBSS. Cells (1×10^6) were incubated with or without LTB₄ (10^{-6} mol/L for 45 min at 37°C). After being washed, leukocytes were further incubated for 30 min at 4°C in the dark with antibody against L-selectin (50 mg/L; Seikagaku) or CD18 (50 mg/L; Immunotech). As a negative control, leukocytes were treated in the same manner as those obtained from experimental group rats, except that antibodies and LTB₄ were not added. After incubation, cells were washed and analyzed on an automated fluorescence-associated cell-separation system (FACScan Star and Cell Quest Program, Becton-Dickinson). Data were obtained from 10,000 cells. The results are expressed as mean intensity of fluorescence (arbitrary units \pm SEM, $n = 6$).

Statistical analysis. Repeated-measures ANOVA followed by the Tukey-Kramer multiple comparisons test was used to analyze dam body weights during gestation, male offspring body weights from d 28 to 42, and metabolic variables. To analyze data from immunohistochemistry and flow cytometry, 2-way ANOVA followed by the Tukey-Kramer multiple comparisons test was used, and Student's t test was used to analyze leukocyte behavior, hemodynamic variables, and blood leukocyte counts. The level of significance was set at $P \leq 0.05$. Values in the text are expressed as means \pm SEM.

RESULTS

Characteristics of the pregnant rats and their offspring. Nutritional restriction during pregnancy markedly decreased maternal body weight between conception and d 15 of gestation. Subsequent weight recovery was observed until parturition, at which time body weight was similar to d 0 but significantly lower than that of the nourished females (Fig. 1A). Maternal undernutrition resulted in fetal growth retardation, as evidenced by a marked reduction in the birth weights of rat offspring exposed to intrauterine undernutrition ($5.8 \text{ g} \pm 0.1$ vs. 3.5 ± 0.1 , $n = 21$, $P < 0.0001$). However, litter size did not differ between UR (8.78 ± 0.46) and NR dams (9.89 ± 0.45), indicating that reproductive ability was unaffected. From d 28 to 35, UR offspring had greater body weights than did NR offspring, although the groups did not differ on d 42 (Fig. 1B). At 8 wk of age, UR male rat offspring had a body weight ($236.0 \pm 8.7 \text{ g}$) similar to that of the male rat offspring in the NR group ($230.0 \pm 6.6 \text{ g}$).

Metabolic variables. From d 28 to 42, water and food intakes, urinary volume, and urine glucose concentration did not differ between the NR and UR groups (data not shown).

Leukocyte behavior. The numbers of leukocytes rolling along the venular endothelium, either sticking after stimulation with LTB₄, ZAP, or TNF- α , or migrating after TNF- α stimulation, were reduced in UR offspring compared with NR offspring (Table 1).

Arterial blood pressure, heart rate, blood flow velocity, wall shear rate, and $V_{\text{WBC}}/V_{\text{RBC}}$ ratio. In unanesthetized

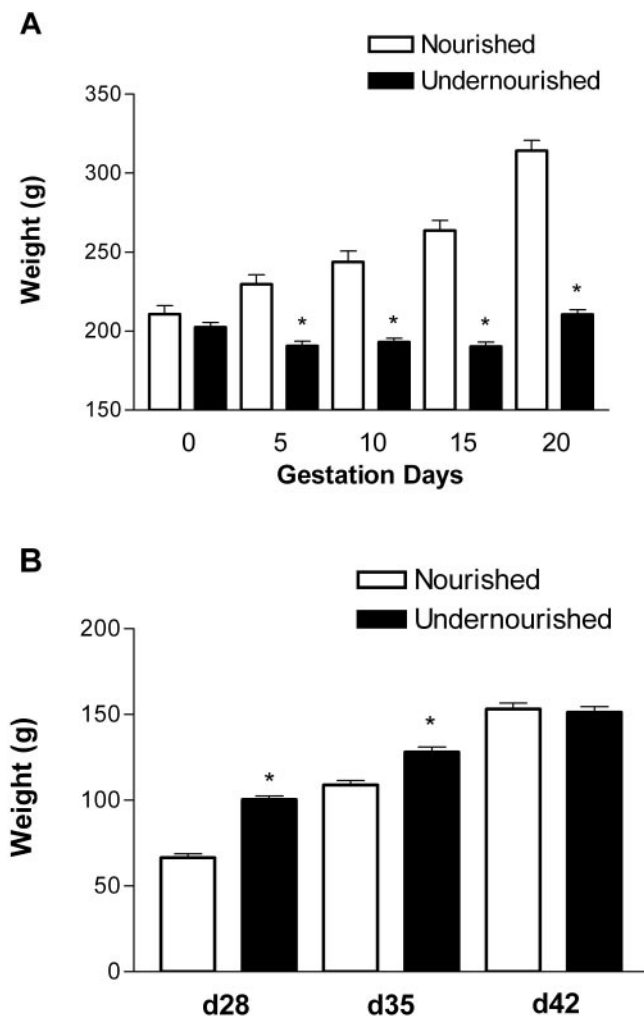


FIGURE 1 Maternal body weight of NR and UR dams during gestation (A) and weight development of the UR and NR offspring after weaning until d 42 (B). Values are means \pm SEM, $n = 16$ dams and 24 pups. *Different from nourished groups, $P < 0.001$.

rats, blood pressure was higher in UR offspring than in NR offspring, whether determined by the tail-cuff method (128.2 ± 1.8 mm Hg, $n = 10$ vs. 120.5 ± 1.5 mm Hg, $n = 13$, $P < 0.005$) or by the direct method (106.3 ± 3.0 mm Hg, $n = 6$ vs. 98.1 ± 2.1 mm Hg, $n = 6$, $P < 0.05$). Arterial blood pressure did not differ in anesthetized UR and NR offspring (94.9 ± 0.4 mm Hg, $n = 9$ vs. 93.9 ± 0.5 mm Hg, $n = 8$,

respectively). Heart rates did not differ between the groups (UR: 360 ± 10 bpm, $n = 9$; NR: 360 ± 16 bpm, $n = 8$). The venular wall shear rate did not differ between the NR and UR groups (Table 2). In UR offspring, the V_{WBC}/V_{RBC} ratio was significantly higher than in NR offspring, a difference that was attributable to the marked increase in V_{WBC} (Table 2).

Immunohistochemistry analysis. The basal expression of P-selectin (Fig. 2A and B) or ICAM-1 (Fig. 3A and B) did not differ between NR and UR groups. However, after stimulation with TNF- α , UR offspring had reduced expression of these adhesion molecules compared with NR offspring (Figures 2A/3B and 3A/4B). The groups did not differ in basal and stimulated expression of PECAM-1 (data not shown).

Flow cytometry analysis. Under basal conditions, L-selectin expression was lower in the leukocytes of UR offspring than in those of NR offspring (Fig. 4). As expected, reduced expression of L-selectin was detected in the leukocytes of NR offspring after stimulation with LTB $_4$ due to cleavage of this adhesion molecule in response to an inflammatory stimulus. The same did not occur in the leukocytes of UR offspring (Fig. 4). Basal and LTB $_4$ -stimulated expression of CD11/CD18 did not differ between the 2 groups (data not shown).

Leukocyte counts. Total leukocyte counts were lower in UR offspring than in NR offspring due to reduced numbers of neutrophils and lymphocytes in the former group (Table 3).

DISCUSSION

In the present study, we demonstrated that restriction of maternal nutrition in pregnant Wistar rats resulted in severe maternal weight loss during gestation and in a significant reduction in birth weights of the offspring, similar to the findings of Chandra and Matsubara (12) and Franco et al. (13). When maternal diet is restricted, the availability of nutrients for transplacental transport is decreased, reducing the nutrient supply and thereby limiting the growth of the fetus (14).

On d 28 and d 35, UR offspring had a higher body weight than NR offspring. This weight gain could be related to accelerated catch-up growth in the UR offspring, which in turn could be attributed to reduced fat oxidation rate and increased carbohydrate metabolism (15). Because body weights did not differ on d 42, it is possible that adaptive mechanisms were effective in correcting the earlier alterations. However, the altered body weights observed during this period were unrelated to any of the evaluated metabolic variables, which were comparable in the 2 groups.

Leukocyte infiltration is a multistep mechanism that requires leukocytes moving at very high speeds in the bloodstream to make initial transient contact with endothelial cells

TABLE 1

Numbers of rollers, stickers (10 min after application of ZAP and LTB $_4$ or 2 h after TNF- α injection) and migrated leukocytes (2 h after TNF- α injection) in venules of NR and UR male offspring¹

Group	Rollers <i>n/10 min</i>	Stickers			Migrated <i>n/2500 μm²</i>
		ZAP	LTB $_4$	TNF- α	
NR	122.2 ± 1.3	17.7 ± 0.8	15.9 ± 0.8	14.5 ± 0.9	15.2 ± 0.8
UR	$79.3 \pm 2.8^*$	$12.4 \pm 0.4^*$	$8.8 \pm 0.4^*$	$7.4 \pm 0.6^*$	$8.1 \pm 0.7^*$

¹ Values are means \pm SEM; $n = 7$ –12. * Different from NR, $P < 0.001$.

TABLE 2

Leukocyte rolling velocity (V_{WBC}), RBC velocity (V_{RBC}), and shear rate determined in venules (<25 μm) of the internal spermatic fascia of NR and UR male offspring¹

Group	V_{WBC} $\mu\text{m/s}$	V_{RBC} mm/s	V_{WBC}/V_{RBC}	Shear rate s^{-1}
NR	7.5 ± 0.5	2.3 ± 0.1	3.3 ± 0.2	1050.5 ± 55.9
UR	$14.1 \pm 1.3^*$	2.2 ± 0.1	$6.4 \pm 0.6^{**}$	982.3 ± 65.3

¹ Values are means \pm SEM; $n = 5-9$. Asterisks indicate different from NR: * $P < 0.05$; ** $P < 0.001$.

lining the vessel wall and roll along at a greatly reduced velocity relative to the RBC. We studied rolling activity without any stimulus except the “exposure trauma” and found lower numbers of rolling leukocytes in UR offspring, possibly hindering leukocyte migration.

A great number of mediators are released during the inflammatory response (16), including $\text{TNF-}\alpha$, LTB_4 , and ZAP, which are widely used to study inflammatory processes. These mediators are highly chemotactic for neutrophils (17,18) and act quickly (2–3 min) for LTB_4 and ZAP or slowly (2 h) for $\text{TNF-}\alpha$ (19–23). We demonstrated that leukocytes sticking to the venular wall, as well as those migrating after these stimuli, were reduced in the UR offspring.

The decreased leukocyte migration in UR offspring might be related to various factors, such as hemodynamic variables and leukocyte counts, as well as expression of endothelial and leukocyte adhesion molecules.

Although unanesthetized UR offspring had higher blood pressures than unanesthetized NR offspring, this difference does not explain the altered leukocyte behavior because leukocyte behavior was examined in anesthetized rats, a condition in which blood pressure did not differ between the 2 groups. The higher blood pressures observed in the UR offspring might be due to endothelial dysfunction, as demonstrated previously in older undernourished rats that were hypertensive (blood pressure >150 mm Hg) (24). However, in our case, the rats used cannot be considered hypertensive because they had blood pressures <130 mm Hg. In addition, venular blood flow velocity, and, consequently, wall shear rate did not differ between NR and UR offspring. Therefore, we excluded these variables as possible causes of the altered leukocyte migration in UR offspring, despite the fact that leukocyte adhesion was shown to be dependent on shear rate, both in vivo (25,26) and in vitro (27).

Leukocyte rolling is a prerequisite for firm adhesion, although slow rolling (<10 $\mu\text{m/s}$) is necessary for efficient conversion from rolling to firm adhesion (28). It was demonstrated that leukocyte recruitment is reduced by increased rolling velocity (29,30). In the present study, UR offspring had 88% higher V_{WBC} than did NR offspring. This difference could have contributed to the reduced leukocyte migration in UR offspring.

Another variable used to assess leukocyte-endothelial interaction is the V_{WBC}/V_{RBC} ratio. This ratio provides a measure of the fracture stress between leukocytes and the endothelium. The higher the V_{WBC}/V_{RBC} ratio, the lower the amount of energy required to peel a leukocyte from a microvessel wall (31). In our study, we demonstrated that UR offspring had higher V_{WBC}/V_{RBC} ratios than did NR offspring. Therefore, increased V_{WBC} , together with weaker adhesion between

leukocytes and endothelial cells, could explain the impaired migration in UR offspring.

Leukocyte migration depends on the availability of circulating leukocytes, and the systemic leukocyte count is as stringent a predictor of rolling and adhesion as are blood flow velocity and wall shear rate (32). Therefore, leukopenia might lead to reduced leukocyte migration. In fact, leukopenia might have contributed to the reduced migration in the UR offspring.

Alterations in endothelial and leukocyte expression of adhesion molecules might also interfere with leukocyte migra-

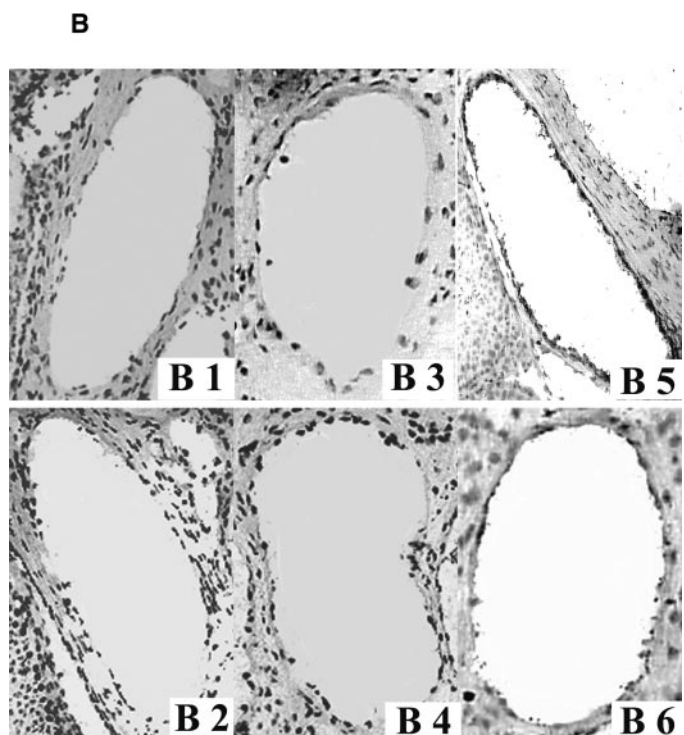
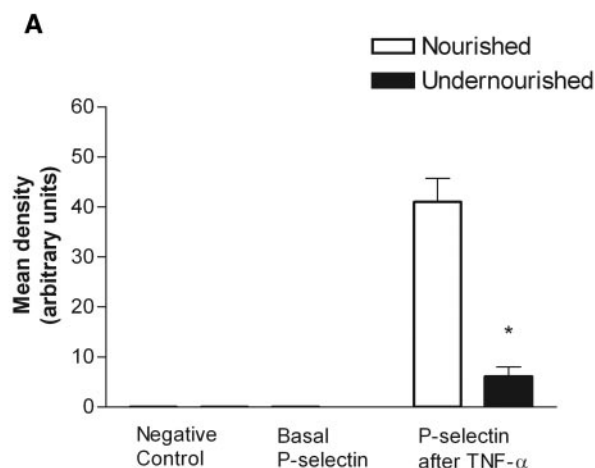


FIGURE 2 Immunohistochemical analysis (A) and representative images of immunohistochemical staining (B) for P-selectin in the venules of the internal spermatic fascia of UR and NR offspring. B1 and B2 = negative controls for the NR and UR, respectively; B3 and B4 = Basal P-selectin expression of the NR and UR, respectively; B5 and B6 = P-selectin expression after $\text{TNF-}\alpha$ stimulation of the NR and UR, respectively. (A) Values are means \pm SEM, $n = 4$. *Different from NR, $P < 0.001$. (B) Magnification, X25.

tion. The molecule L-selectin is constitutively expressed on circulating granulocytes, monocytes, and most lymphocytes (33), whereas P-selectin is found in platelets and is stored in endothelial Weibel-Palade bodies. After endothelial cell activation by stimuli such as cytokines, histamine, thrombin, bradykinin, free radicals, or TNF- α , P-selectin is quickly (within minutes) moved to the cell surface (34–36). Endothelial P-selectin and leukocyte L-selectin act together in the transient tethering and rolling of leukocytes on the endothe-

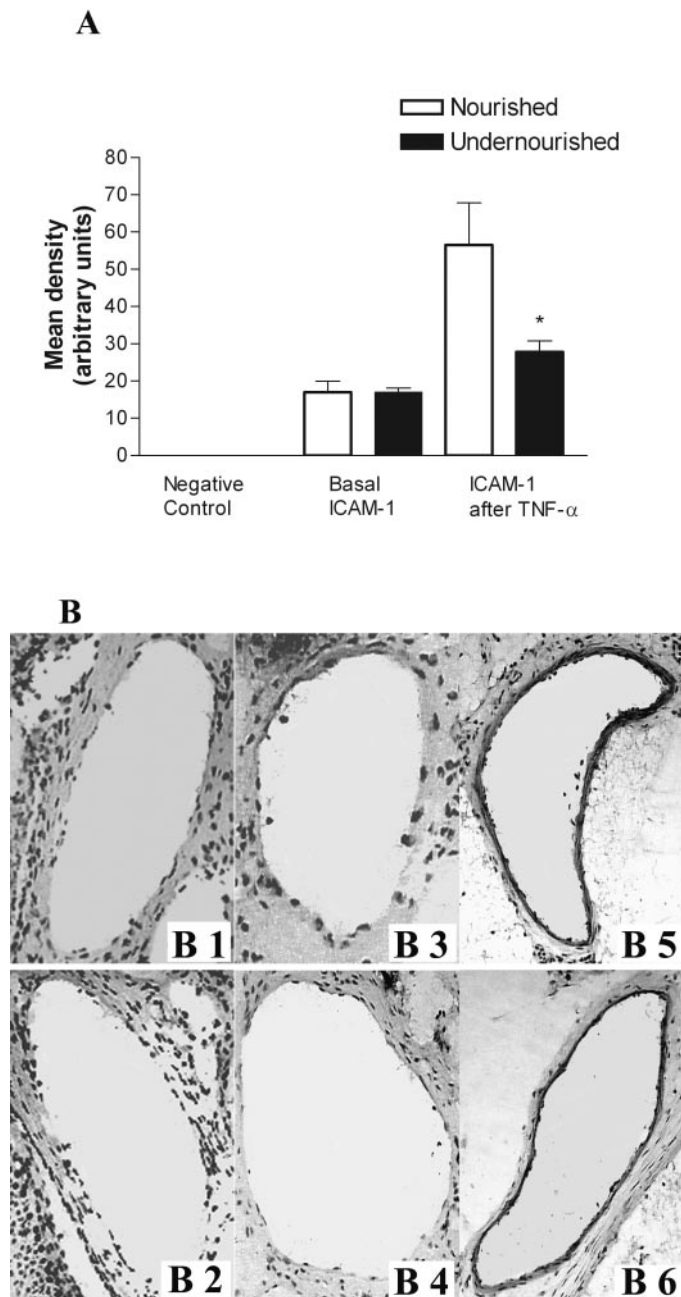


FIGURE 3 Immunohistochemical analysis (A) and representative images of immunohistochemical staining (B) of ICAM-1 in the venules of the internal spermatic fascia of UR and NR offspring. B1 and B2 = negative controls for the NR and UR, respectively; B3 and B4 = Basal ICAM-1 expression of the NR and UR, respectively; B5 and B6 = ICAM-1 expression after TNF- α stimulation of the NR and UR, respectively. (A) Values are means \pm SEM, $n = 4$; *different from NR, $P < 0.05$. (B) Magnification, X25.

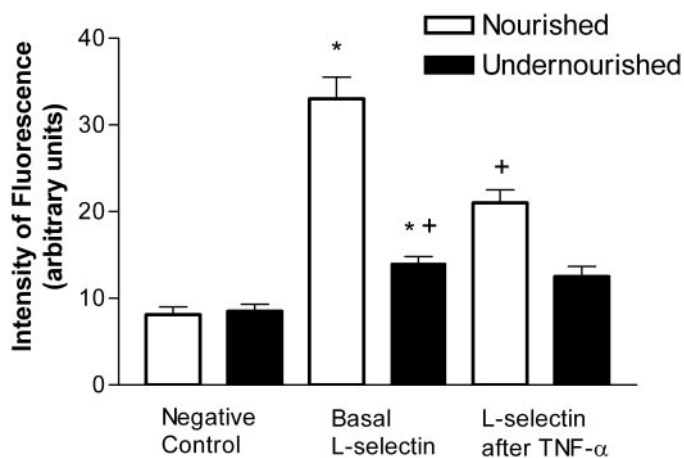


FIGURE 4 Flow cytometry analysis of basal and the LTB₄-stimulated expression of L-selectin in the leukocytes of UR and NR offspring. Values are means \pm SEM, $n = 6$. *Different from negative control, $P < 0.01$; +different from NR basal L-selectin, $P < 0.05$.

lial surface (37). In our study, UR offspring had a marked decrease in the TNF- α -stimulated expression of P-selectin and in the basal expression of L-selectin. Deficiency of P-selectin, L-selectin, or both might attenuate granulocyte migration as demonstrated in mice rendered genetically deficient of these adhesion molecules (33). Therefore, the reduction in rolling activity exhibited by the UR offspring might be explained by reduced basal expression of L-selectin and reduced stimulated expression of P-selectin.

Shedding of L-selectin plays a role in regulating the velocity of leukocyte rolling and thereby leukocyte recruitment (38). Therefore, inhibition of L-selectin shedding might reduce adherence and transmigration (39). In our study, there was no L-selectin shedding after LTB₄ stimulation in UR offspring, and this, together with the decreased basal expression of L-selectin, might have contributed to the reduced leukocyte adherence observed.

Immunoglobulin superfamily members such as ICAM-1 play important roles in the firm adhesion and transendothelial migration of leukocytes (40). Interaction between CD11a/CD18 and ICAM-1 is often required for transmigration across the endothelium (41). Inhibited or eliminated expression of CD11a, CD11b, or ICAM-1 can reduce the ability of neutrophils to adhere to the endothelium and subsequently to migrate (42). Although CD11/CD18 expression was not altered in UR offspring, we can assume that the adherence and transmigration were impaired due to reduced expression of ICAM-1.

TABLE 3

Blood leukocyte counts in NR and UR male offspring¹

Cells	NR	UR
	<i>n/mm³</i>	
Monocytes	10990.0 \pm 630.1	7620.0 \pm 477.6*
Eosinophils	534.1 \pm 75.1	392.9 \pm 21.6
Lymphocytes	80.3 \pm 34.8	152.5 \pm 34.6
Neutrophils	6352.7 \pm 591.9	4170.9 \pm 221.6**
	4022.9 \pm 312.1	2903.7 \pm 294.0***

¹ Values are means \pm SEM; $n = 10$. Asterisks indicate different from NR: * $P < 0.0005$; ** $P < 0.005$; *** $P < 0.05$.

PECAM-1 is a member of the IgG superfamily and plays a key role in the passage of neutrophils and monocytes through endothelial cells (43,44). It is constitutively expressed and concentrated in the lateral borders between endothelial cells and expressed on the surface of neutrophils, monocytes, and some T-cell subsets (45). In our study, endothelial expression of PECAM-1 did not differ between NR and UR offspring. Therefore, altered PECAM-1 expression can be ruled out as a contributing factor in the impaired leukocyte migration observed in UR offspring.

In summary, we demonstrated that various mechanisms, such as higher V_{WBC} , a higher V_{WBC}/V_{RBC} ratio, reduced leukocyte count, and downregulated expression of L-selectin, P-selectin, and ICAM-1, are involved in reducing leukocyte migration in UR offspring. Our data lead us to suggest that reduced leukocyte migration contributes to the increased predisposition to infections in individuals subjected to intrauterine nutritional deprivation.

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