Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice

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Abstract

The aim of this study was to investigate the role of intravascular neutrophils in initiating endothelial cell proliferation following oestrogen treatment in ovariectomised mouse endometrium. Uterine tissues were collected from ovariectomised C57/CBA female mice 24 h after oestrogen treatment with or without systemic neutrophil depletion. Neutropenia was achieved with either an in-house anti-neutrophil serum (ANS) or Gr-1 monoclonal antibody. All mice received an i.p. injection of bromodeoxyuridine (BrdU) 4 h prior to dissection to allow visualisation of proliferating cells using immunocytochemistry. Endometrial sections were immunostained for BrdU, vascular endothelial growth factor (VEGF), and neutrophils (using ANS). Oestrogen treatment of ovariectomised mice significantly increased the number of intravascular neutrophils, whereas induction of neutropenia with either ANS or Gr-1 in conjunction with oestrogen treatment prevented this increase. Oestrogen treatment of ovariectomised mice also significantly increased the number of intravascular VEGF-positive cells; however, whereas induction of neutropenia with ANS significantly reduced this increase, Gr-1 did not. In both studies, neutropenia significantly reduced, but did not eliminate, the amount of endometrial endothelial cell proliferation. These results suggest a role for neutrophils in endometrial angiogenesis following acute oestrogen treatment; however, the presence of VEGF-positive cells even after induction of neutropenia suggests that more than one type of leukocyte may be involved.

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Introduction

Growth and regression of the endometrium and its vasculature is ultimately under the control of the steroid hormones oestrogen and progesterone; however, the sex steroids are thought to exert their effects indirectly via a variety of growth factors. In the case of angiogenesis, one of the most important factors is vascular endothelial growth factor (VEGF) (Ferrara & Davis-Smyth 1997, Risau 1997). VEGF is a potent endothelial cell mitogen expressed in a wide variety of cells and tissues, including primate and rodent endometrium (Gordon *et al.* 1995, Torry & Torry 1997, Smith 1998). Various investigators have endeavoured to correlate levels of VEGF mRNA and protein with phases of the human menstrual cycle (Torry & Torry 1997, Rogers & Gargett 1999). Although there have been conflicting results, it appears the majority of endometrial VEGF is glandular in origin (Shifren *et al.* 1996, Zhang *et al.* 1998, Gargett *et al.* 1999). More importantly, the glandular VEGF is secreted apically into

the uterine lumen and is unlikely to have a role in endometrial angiogenesis (Hornung *et al.* 1998).

Although neither epithelial, stromal nor total VEGF expression has been found to correlate with endometrial endothelial cell proliferation (Gargett *et al.* 1999), foci of intense VEGF immunostaining have been observed within the human endometrium; many of these foci were within blood vessels. Using immunocytochemical techniques on full-thickness endometrial sections, the focal VEGF was found to correlate both temporally and spatially with endometrial endothelial cell proliferation (Gargett *et al.* 2001). A significantly greater percentage of focal VEGF-expressing microvessels was found during the proliferative phase in comparison with the secretory phase, with the greatest numbers of immunopositive vessels within the subepithelial capillary plexus. There was also a significant correlation between focal VEGF-expressing microvessels and proliferating vessels in all three regions of the endometrium (subepithelial capillary plexus, functionalis and basalis). The focal VEGF associated with microvessels was

found to correspond to marginating and adherent neutrophils and it was hypothesised that these leukocytes provide a source of VEGF for the endometrial vessels undergoing proliferation (Mueller *et al.* 2000, Gargett & Rogers 2001, Gargett *et al.* 2001).

The aim of this study was to investigate the role of intra-vascular neutrophils in oestrogen-mediated endometrial angiogenesis in mice. Previous research has shown that oestrogen stimulates endometrial endothelial cell proliferation within 24 h of administration in ovariectomised mice (Heryanto & Rogers 2002). Furthermore, the administration of VEGF receptor inhibitors or an anti-VEGF antibody prevented this oestrogen-mediated endometrial endothelial cell proliferation (Heryanto *et al.* 2003). This demonstrated that VEGF is the primary factor mediating the initial endothelial cell proliferation in response to oestrogen within the endometrium. However, the source of this VEGF, or the mechanisms leading to its release, are still unknown. Based on our observation in human endometrium where the appearance of VEGF-containing intra-vascular neutrophils correlated with endothelial cell proliferation, we hypothesised that treatment of ovariectomised mice with exogenous oestrogen would increase the number of marginating and adherent neutrophils present within endometrial blood vessels and that the induction of neutropenia would reduce the number of adherent neutrophils and consequently the amount the endothelial cell proliferation.

Methods and Materials

Animals

Adult female mice (25–30 g, C57BL/6J × CBA) were housed four per cage under controlled environmental conditions (20°C, 16 h light:8 h darkness cycle); food and water were freely available. All mice were ovariectomised after anaesthesia with an i.p. injection of 25 mg/100 g body weight 2,2,2-tribromoethanol (Avertin; Aldrich Chemical Co., Milwaukee, WI, USA). Mice were left for 1 week to allow regression of endometrial tissues. The study was approved by the Monash Medical Centre Animal Ethics Committee A.

Study 1: induction of neutropenia with an in-house anti-rat neutrophil serum (ANS)

On days 6 and 7 following ovariectomy, one group of mice ($n = 5$) was injected i.p. with a previously prepared in-house ANS (0.5 ml/mouse per day). (The serum was collected from rabbits immunised with rat neutrophils (Rogers *et al.* 1992). Serum absorbed against erythrocytes and lymphocytes had a leukoagglutination titre against rat neutrophils of approximately 1/160–1/320 and possessed antibodies cytotoxic against neutrophils in the presence of complement. ANS tested using immunohistochemistry against various purified populations of formalin-fixed, paraffin-embedded leukocytes showed >95% neutrophil

staining and <5% lymphocyte or eosinophil staining. Serum absorbed against neutrophils and tested by immuno histochemistry showed approximately 90% reduced staining (Rogers *et al.* 1992).) On day 7 post-ovariectomy, these mice were also injected s.c. with 100 ng oestradiol (in 100 µl oil) before dissection 24 h later. A group of control mice ($n = 5$) received the oestradiol injection only before dissection 24 h later. A further group of ovariectomised mice was left untreated (no vehicle injections) and dissected on day 7 post-ovariectomy to confirm regression of endometrial tissues ($n = 3$).

Four hours prior to dissection, all mice were injected i.p. with 1 mg bromodeoxyuridine (BrdU) in 0.5 ml 0.9% normal saline (40 mg/kg body weight). At the time of dissection, mice were anaesthetised using Avertin (as for ovariectomy) and perfusion-fixed (via a cannula inserted into the left ventricle of the heart) with 10% buffered formalin after a saline washout at physiological pressure (110–130 mmHg). The uterine tissues were removed and further immersion-fixed in 10% buffered formalin for 4 h before processing into paraffin sections for immunohistochemistry.

A blood sample was also collected and used to confirm neutropenia in treated animals. From the blood sample, a smear was prepared for immunohistochemistry using ANS as the primary antibody. The smears were fixed in absolute methanol for 10–20 min and then stained with haematoxylin for 2 min prior to immunostaining with the same protocol used for paraffin sections (described below). The number of ANS-positive cells within an area of 1 cm² was determined from an even part of the blood smear.

Study 2: induction of neutropenia with anti-neutrophil monoclonal antibody Gr-1

To corroborate data obtained in Study 1, a similar study was performed using an anti-neutrophil monoclonal antibody, Gr-1, purified from hybridoma supernatants, generously donated by Dr M J Hickey and Associate Prof. P Tipping (Centre for Inflammatory Diseases, Monash University, Australia) to induce neutropenia. (The effect of the rodent Gr-1 on the depletion of circulating neutrophils in mice has previously been reported (Thakur *et al.* 1996). Administration of 150 µg of the specific monoclonal antibody RB6-8C5 (Gr-1) to mice caused a >95% reduction in circulating neutrophils; it took 4 days to restore circulating neutrophils to pre-treatment levels (Thakur *et al.* 1996).) A group of ovariectomised mice ($n = 6$) were injected i.p. with 150 µg Gr-1, followed 4 h later by an s.c. injection of 100 ng oestradiol in 100 µl oil. Mice were dissected 24 h after oestradiol treatment. A control group ($n = 6$) received i.p. injections of 0.9% saline in place of Gr-1 prior to oestradiol treatment. As in Study 1, all mice received an i.p. injection of BrdU 4 h prior to perfusion fixation and dissection. A blood sample was collected from these animals and a white blood cell differential count

performed after staining with DiffQuik (performed by Dr M Hickey, Centre for Inflammatory Diseases, Monash University, Australia).

Immunohistochemistry

Neutrophil immunostaining

Neutrophils were identified by immunostaining with ANS. Deparaffinised endometrial sections (5 µm) were treated with 3% H₂O₂ in PBS and then treated with a protein blocking agent (PBA; Immunon Shandon, Pittsburgh, PA, USA). Sections were incubated in ANS (1:100 in 1% BSA/PBS) for 45 min at 37 °C. The sections were then incubated with biotinylated secondary antibody (goat anti-rabbit IgG; Zymed Lab. Inc., San Francisco, CA, USA) before immunostaining was visualised using streptavidin-horseradish peroxidase followed by aminoethyl carbazole (AEC) chromogen. A negative control slide was prepared by substituting the primary antibody with non-immune rabbit serum. A section of mouse spleen was used as a positive control. All the neutrophils present in a single cross-section of uterus were identified using a ×20 objective lens; to confirm whether the neutrophils were within a blood vessel and not just in the stroma, a ×40 or ×100 objective was used. Neutrophils within blood vessels were counted and expressed as a number per endometrial area (mm²) or per section.

VEGF immunostaining

VEGF immunostaining was performed as previously described (Gargett *et al.* 2001). For antigen retrieval, sections (5 µm) were microwaved (500 W microwave, 3 × 5 min on defrost) in 10 mmol/l sodium citrate buffer (pH 6). Endogenous peroxidase was quenched and non-specific binding blocked using PBA. (Sections were incubated with a polyclonal antibody rabbit anti-human VEGF; 2 µg/ml in 1% BSA/PBS) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) for 1 h at 37 °C. Sections were then incubated in biotinylated secondary antibody (goat anti-rabbit IgG), streptavidin-horseradish peroxidase and immunoreactivity visualised with AEC chromogen. A negative control section was obtained by substituting the primary antibody with an rabbit IgG (2 µg/ml). Cells focally expressing VEGF in a single cross-section of uterus were identified using the ×20 objective lens; to confirm

whether these cells were within a blood vessel or in the stroma, a ×40 or ×100 objective was used. VEGF-positive cells within blood vessels were counted and expressed as a number per endometrial area (mm²) or per section.

Cell proliferation detection (BrdU immunostaining)

Sections (5 µm) were microwaved (500 W microwave, 4 × 5 min on defrost) in 10 mmol/l sodium citrate buffer (pH 6) and treated with 0.1 mol/l HCl for 45 min for antigen retrieval. Endogenous peroxidase was quenched and non-specific binding blocked with PBA. The sections were then incubated with a polyclonal antibody sheep anti-BrdU; 10 µg/ml in 1% BSA/PBS) (BioDesign, Saco, ME, USA) for 1 h at room temperature. After incubating sections with biotinylated secondary antibody (donkey anti-sheep IgG) and streptavidin-horseradish peroxidase, BrdU immunostaining was visualised with AEC. A negative control section was obtained by substituting the primary antibody with an sheep IgG (10 µg/ml). A section of mouse intestine was included in each staining run as a positive control. The BrdU immunostaining was used to determine the number of proliferating endothelial cells as a percentage of the total number of endothelial cells.

Statistics

Values are presented as means ± s.e. All statistical tests were performed using SPSS for Windows, Version 11.0.0 (SPSS Inc., Chicago, IL, USA). The effects of treatment were analysed using *t*-tests or one-way ANOVA after log transformation (if necessary) of data to conform to the assumptions of ANOVA. If homogeneity of variance could not be obtained, the equivalent non-parametric test was used. A *P* value < 0.05 was considered significant.

Results

Neutrophil depletion

The number of circulating neutrophils were significantly reduced, although not completely eliminated, in comparison with the control groups following administration of both ANS (Study 1) and Gr-1 (Study 2, see Table 1).

Table 1 Reduction in the number/percentage of circulating neutrophils following induction of neutropenia using ANS (Study 1) or Gr-1 (Study 2) in oestrogen-treated ovariectomised mice.

Experiment	Untreated group (means ± s.e.)	Treated group (means ± s.e.)	Reduction (%)	<i>P</i> value
Study 1. Induction of neutropenia with ANS	44.7 ± 8.9 ¹ (<i>n</i> = 4)	12.0 ± 4.9 ¹ (<i>n</i> = 5)	73.2	<i>t</i> ₍₇₎ = 3.4 ³ , <i>P</i> = 0.011
Study 2. Induction of neutropenia with Gr-1	33.0 ± 6.4 ² (<i>n</i> = 6)	1.8 ± 0.7 ² (<i>n</i> = 6)	94.5	<i>t</i> ₍₁₀₎ = 7.3 ⁴ , <i>P</i> = 0.004

¹ Number of neutrophils per cm² of the blood smear.

² Neutrophils as a percentage of total leukocytes.

³ *t*-test.

⁴ *t*-test on logged data.

Endometrial intravascular neutrophils

Immunostained neutrophils were observed in the stroma and the small capillaries of the endometrium. Perfusion fixation clearly opened the lumen of the capillaries, flushing out the circulating leukocytes and red blood cells. This allowed identification of neutrophils adherent to the microvessel walls (Fig. 1). Slides stained with non-immune rabbit serum did not show any specific staining in individual cells.

In Study 1, ovariectomised mice treated with oestrogen had significantly more intravascular neutrophils than untreated ovariectomised mice, or mice treated with oestrogen and ANS, when counted either per mm² ($F_{(2,10)} = 116.8, P < 0.001$) or per section ($F_{(2,10)} = 120.7, P < 0.001$; Fig. 2). In four out of the five mice treated with oestrogen and ANS, no intravascular neutrophils were observed.

In Study 2, ovariectomised mice treated with oestrogen had significantly more intravascular neutrophils than mice treated with oestrogen and Gr-1 when counted either per mm² ($t_{(10)} = 4.3, P = 0.002$) or per section ($t_{(10)} = 6.0, P < 0.001$; Fig. 2).

Endometrial intravascular focal VEGF

Cells strongly positive for VEGF (focal cells) were observed in the stroma and within endometrial vessels (Fig. 3). No positive staining was observed in sections after the primary antibody was replaced with the rabbit IgG.

In Study 1, ovariectomised mice treated with oestrogen had significantly more intravascular focal VEGF-positive cells than untreated ovariectomised mice or ovariectomised mice treated with oestrogen and ANS when

Figure 1 Mouse endometrium stained with ANS. Inset: endometrial blood vessel. G: gland, LE: luminal epithelium, M: muscle, r: red blood cell, S: stroma. Black arrows: neutrophils within endometrial stroma. White arrow: neutrophil adhered to a blood vessel. Scale bar: main micrograph 100 µm, inset 10 µm.

Figure 2 Mean number of intravascular neutrophils per mm² (black bars) or per section (grey bars) in ovariectomised mice treated with oestrogen (E2) with or without the induction of neutropenia. Study 1: neutropenia induced using ANS. Study 2: neutropenia induced using Gr-1. Error bars represent S.E.M. Numbers above the error bars are sample sizes. Means that do not share a common letter (above error bars) are significantly different (separate results for each experiment).

Figure 3 VEGF-expressing cells within mouse endometrium stained. Inset: endometrial blood vessel. G: gland, LE: luminal epithelium, M: muscle, S: stroma. Black arrows: individual VEGF-positive cells within endometrial stroma. White arrow: VEGF-positive leukocyte adhered to a blood vessel. Scale bar: main micrograph 100 μm , inset 10 μm .

Figure 4 Mean number of intravascular VEGF-positive cells per mm^2 (black bars) or per section (grey bars) in ovariectomised mice treated with oestrogen (E2) with or without the induction of neutropenia. Study 1: neutropenia induced using ANS. Study 2: neutropenia induced using Gr-1. Error bars represent S.E.M. Numbers above the error bars are sample sizes. Means that do not share a common letter (above error bars) are significantly different.

counted either per mm^2 ($F_{(2,10)} = 22.7$, $P < 0.001$) or per section ($F_{(2,10)} = 100.1$, $P < 0.001$; Fig. 4).

In Study 2, there was no significant difference between the number of intravascular focal VEGF-positive cells in ovariectomised mice treated with oestrogen or oestrogen and Gr-1 when counted either per mm^2 ($t_{(10)} = 0.16$, $P = 0.88$) or per section ($t_{(10)} = 1.7$, $P = 0.12$; Fig. 4).

Endometrial endothelial cell proliferation

In Study 1, there was significantly less endothelial cell proliferation in mice treated with oestrogen and ANS than in mice treated with oestrogen only ($t_{(7)} = 3.4$, $P = 0.01$, 42% reduction, Fig. 5). There was no endothelial cell proliferation in untreated ovariectomised mice. There was no evidence of a reduction in luminal epithelial cell proliferation in ANS-treated mice (oestrogen only: $32.8 \pm 2.98\%$, oestrogen + ANS: $38.2 \pm 1.28\%$; Mann-Whitney $U_{(8)} = 6.0$, $P = 0.18$).

In Study 2, there was significantly less endothelial cell proliferation in mice treated with oestrogen and Gr-1 than in mice treated with oestrogen only ($t_{(9)} = 4.1$, $P = 0.003$, 32% reduction, Fig. 5). There was no evidence of less luminal epithelial cell proliferation in ANS-treated mice (oestrogen only: $30.8 \pm 5.81\%$, oestrogen + Gr-1: $36.0 \pm 2.13\%$; $t_{(10)} = 0.85$, $P = 0.41$).

Discussion

Treatment of ovariectomised mice with oestrogen significantly increased the number of marginating and adherent neutrophils within endometrial blood vessels. Concurrent induction of neutropenia in ovariectomised mice significantly reduced, but did not eliminate, the endometrial endothelial cell proliferation stimulated by exogenous oestrogen. These results provide novel data supporting a role for neutrophils in endometrial angiogenesis.

Figure 5 Mean percentage of proliferating endothelial cells in ovariectomised mice treated with oestrogen (E2) with or without the induction of neutropenia. Study 1: neutropenia induced using ANS. Study 2: neutropenia induced using Gr-1. Error bars represent S.E.M. Numbers above the error bars are sample sizes. Means that do not share a common letter (above error bars) are significantly different (separate results for each experiment).

While the current study identifies a role for circulating neutrophils in oestrogen-mediated endometrial angiogenesis, it does not investigate the mechanisms by which neutrophils induce an angiogenic response. Previous research has demonstrated that VEGF is the primary factor mediating oestrogen-driven endothelial cell proliferation in ovariectomised mice (Heryanto *et al.* 2003); however, the source of this VEGF is unknown. Potentially, the intravascular neutrophils identified as having a role in endometrial angiogenesis in the current studies may provide a VEGF source. Current data suggest that VEGF secreted by neutrophils may be essential for control of angiogenesis and vascular permeability during inflammatory processes (Cassatella 1999) and in other models of angiogenesis (e.g. angiogenesis induced in the normally avascular cornea (Taichman *et al.* 1997, Edelman *et al.* 1999)). Neutrophils are known to constitutively express VEGF within their specific (secondary) granules (Gaudry *et al.* 1997); they also express mRNA for two of the most common VEGF splice variants, VEGF 121 and VEGF 165 (Webb *et al.* 1998). This VEGF is released by activated neutrophils in response to inflammatory mediators such as lipopolysaccharide or tumour-necrosis factor- α (Webb *et al.* 1998, McCourt *et al.* 1999, Scapini *et al.* 1999). In the study by Gargett *et al.* (2001), endometrial microvessels that contained a VEGF-positive neutrophil often also stained positively for VEGF. It was hypothesised that the VEGF present in endothelial cells was due to degranulation by neutrophils. However, neutrophils by no means provide the whole explanation; in the present study, neutropenia only partially reduced endometrial endothelial cell proliferation in the mouse. Other mechanisms, including the role and identity of other endometrial intravascular VEGF-positive cells (discussed further below), will have to be explored.

In the current study, although neutropenia in ovariectomised mice significantly reduced endometrial endothelial cell proliferation after an oestrogen stimulus, the data concerning intravascular VEGF immunostaining were equivocal. In Study 1, when neutrophils were depleted

using ANS, neutropenia significantly reduced the number of intravascular VEGF-positive cells. In Study 2, where Gr-1 was used to induce neutropenia, there was no significant change in the number of intravascular VEGF-positive cells. Several hypotheses can be proposed to explain the discrepancy between studies. There was considerable variation within the results concerning intravascular VEGF-positive cells in the study using Gr-1, raising the possibility that one or two outliers have skewed the data. Other VEGF-positive cells may be adhering to endometrial vessels, or the variation may be due to the difference in specificity between the two antibodies used (both discussed further below). Alternatively, the mechanism by which neutrophils reduce oestrogen-induced endothelial cell proliferation may not be due to their VEGF contents. It should be pointed out, however, that the discrepancy between studies concerning VEGF immunostaining does not negate the reduced endothelial cell proliferation observed in both neutropenia studies, even if alternative mechanisms of neutrophil action have to be considered.

The presence of VEGF-positive cells, even after the induction of neutropenia, suggests that other VEGF-positive leukocytes are adhering to endometrial microvessels; several other leukocytes are known to express VEGF and are potential candidates for these alternative cells. VEGF immunoreactivity has been detected in tissue macrophages present in ectopic endometrium (endometriosis) and in activated peritoneal fluid macrophages (McLaren *et al.* 1996); however, VEGF-positive macrophages were not detected in human endometrium through the menstrual cycle (Gargett *et al.* 2001). Murine granulated metrial gland cells (uterine natural killer (uNK) cells) were found to express VEGF, although uNK only appear during pregnancy in mice (Wang *et al.* 2003). In human uNK cells, VEGF-B (Chen *et al.* 2002) and -C (Li *et al.* 2001) are expressed. Eosinophils also constitutively express VEGF (Horiuchi & Weller 1997), but are absent from the human endometrium during most of the menstrual cycle (Salamonsen & Lathbury 2000). To determine the type and temporal distribution of intravascular neutrophils and

other leukocytes in endometrial tissue after oestrogen treatment in ovariectomised mice, it will be necessary to use specific leukocyte markers to examine various time points over the first 24–48 h after oestrogen administration when endothelial cell proliferation reaches maximum levels (Heryanto & Rogers 2002).

Another possible reason for the variation in results between Studies 1 and 2 is the specificity of the antibodies used to induce neutropenia. The ANS used is a polyclonal serum raised against rat neutrophils (Rogers *et al.* 1992). Although the ANS was tested against various purified populations of leukocytes (<5% staining of lymphocytes or eosinophils), it is possible that small populations of mouse leukocytes other than neutrophils have been bound by the ANS. In contrast, Gr-1 is a monoclonal antibody specific to mouse neutrophils. Thus the significant reduction in focal VEGF staining in ANS-treated animals, in contrast to the lack of significant reduction in Gr-1-treated animals, may be due to removal of both neutrophils and some other VEGF-positive leukocytes from circulation. This would support the hypothesis outlined in the previous paragraph that VEGF-positive leukocytes other than neutrophils are also adhering to endometrial microvessels and stimulating proliferation.

In Study 1, the numbers of circulating neutrophils in animals treated with and without ANS were determined by counting the number of neutrophils within a set area of a blood smear. Although this showed that neutropenia had been induced (73.2% reduction), this is not the most accurate method to use. Leukocytes within a blood smear may not be evenly distributed on the slide; in addition, different leukocyte types may not be randomly distributed on the slide (Dacie & Lewis 1995). However, despite the potential lack of accuracy of the actual percentage reduction of circulating neutrophils, the count of neutrophils present within endometrial blood vessels indicates that our induction of neutropenia had been successful and the endometrium was not exposed to neutrophil-derived factors. In Study 2, the appropriate differential white blood cell count was conducted; a 95% reduction in circulating neutrophils was obtained.

To summarise, oestrogen treatment increases the number of intravascular neutrophils and intravascular VEGF-positive cells, and increases endothelial cell proliferation, within the endometrium of ovariectomised mice. Neutropenia significantly reduced endothelial cell proliferation in ovariectomised mice treated with exogenous oestrogen; the effects on numbers of intravascular VEGF-positive cells were less conclusive. These results support a role for neutrophils in endometrial angiogenesis, but also suggest the involvement of other circulating leukocytes.

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