Surgical Stress Increases Circulating Low-Density Neutrophils Which May Promote Tumor Recurrence

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**Abstract**

**Background:** Low-density neutrophils (LDN) have been shown to be increased in peripheral blood in patients with various diseases and closely related to immune-mediated pathology. However, the frequency and function of LDN in circulating blood of the patients following abdominal surgery have not been well understood.

**Methods:** LDN were determined by CD66b(+) cells, which were copurified with mononuclear cells by density gradient preparations of peripheral blood of surgical patients. The effects of the purified LDN on T cell proliferation and tumor cell lysis were examined in vitro. Neutrophil extracellular traps (NETs) production was examined by extracellular nuclear staining.

**Results:** The number of LDN with an immature phenotype is markedly increased in peripheral blood samples in patients after abdominal surgery. The frequency of LDN correlated positively with operative time and intraoperative blood loss. The purified LDN significantly suppressed the proliferation of autologous T cells stimulated with anti-CD3 mAb coated on plate and partially inhibited the cytotoxicity of lymphocytes activated with recombinant interleukin-2 against a human gastric cancer cell, OCUM-1. The LDN also produced NETs after short-term culture in vitro, which efficiently trap many OCUM-1.

**Conclusions:** The LDN may support the lodging of circulating tumor cells via NETs formation and inhibit T cell–mediated antitumor response in target organs, which may promote postoperative cancer metastases. Functional blockade of LDN might be an effective strategy to reduce tumor recurrence after abdominal surgery.

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Introduction

Surgery still remains the mainstay of treatment for patients with gastrointestinal malignancies. However, surgical-induced physiological stress often suppresses cell-mediated immunity, which may promote postoperative organ failure and tumor recurrence. Although elevation of immunosuppressive hormone levels preoperatively, such as cortisol, catecholamines, and prostaglandins, have been proposed to be related to this phenomenon, the detailed mechanisms of inducing impaired cell-mediated immunity have not been fully elucidated.

Neutrophils are the most abundant leukocyte population and known as the critical effector in innate immunity. However, recent studies have demonstrated that neutrophils are functionally and phenotypically divided into various subtypes, which exert different regulatory properties on the adaptive immune response. Low-density neutrophils (LDN), which are co-segregated with mononuclear cells by Ficoll-Paque density gradient preparations, have been shown to have distinct biological characteristics from conventional high-density neutrophils (HDN). Morphologically, LDN are divided into two distinct subpopulations, mature granulocytes with multilobular nuclei and relatively immature neutrophils with less segmented nuclei, and exert either proinflammatory or immunosuppressive properties, which are often described as low-density granulocytes and granulocytic myeloid-derived suppressor cells (G-MDSC), respectively.

Previous reports have shown that the frequency of LDN is significantly increased in the peripheral blood of patients with various diseases, such as systemic lupus erythematosus, asthma, sepsis, cancer, and HIV infection. However, the impact of LDN in each disease state has not been fully characterized. In this study, we examined the frequency of LDN in the peripheral blood of patients who underwent abdominal surgery and asked how LDN regulate cell-mediated immunity, which might affect the postoperative outcome.

Materials and methods

Reagents, antibodies, and cells

Fluorescein isothiocyanate (FITC)-conjugated mAb against CD66b and phycoerythrin (PE)-conjugated monoclonal mAb against CD11b, CD16, CD45, CD62L, and CXCR2 were purchased from Becton–Dickinson (San Jose, CA). PE-conjugated mAb against PD-L1 (29E.2A3) and control IgG2b were obtained from BioLegend (San Diego, CA) and anti-CD66b mAb conjugated with microbeads, and magnetic cell separation columns were purchased from Miltenyi Biotec (Auburn, CA). Carboxyfluorescein Nhydroxysuccinimide ester (CFSE) and 7-aminoactinomycin D (AAD) were purchased from Thermo Fisher Scientific (Waltham, MA), and FITC-conjugated Annexin V was from BioVision (Milpitas, CA). Anti-CD3 mAb (OKT-3) for T cell stimulation was purchased from Bio-X cell (Stanford, CA). SYTOX orange nucleic acid stain and DNAse I were also purchased from Thermo Fisher Scientific, and PKH67 was from Sigma–Aldrich (St Louis, MO).

Human gastric cancer cell line MKN45, NUGC-4, and colon cancer cell line SW480 were obtained from RIKEN (Tsukuba, Japan), and another human gastric cancer cell line, OCUM-1, was obtained from Dr M. Yashiro (Osaka City University). The cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Grand Island, NY) in our laboratory and stored in liquid nitrogen to ensure that cells used for experiments were passaged for less than 3 wk.

Neutrophil purification

Peripheral blood (5 mL) was obtained from patients who underwent abdominal surgery in the Department of Gastrointestinal Surgery, Jichi Medical University, with written informed consent. Blood was obtained before and after surgical procedures. After dextran sedimentation, leukocyte-enriched plasma was overlaid on Ficoll-Paque solution (Pharmacia Biotech, Piscataway, NJ) and centrifuged at 3000 rpm for 15 min. The intermediate and bottom layers were taken and washed twice with phosphate-buffered saline (PBS) + 0.02% ethylenediaminetetraacetic acid. CD66b(+) granulocytes contained in the former and latter layers were considered as LDN and HDN, respectively.

In functional experiments, LDNs were positively purified from cells recovered from the intermediated layer using MACS method using anti-CD66b microbeads according to the manufacturers’ recommendation, and the remaining CD66(−) cell population was used for proliferation assays. HDNs were also obtained using the same method after hypotonic lysis of red blood cells of the bottom layers. Usually, the positively selected fraction contains more than 96% purity both for LDNs and HDNs.

This study was approved by the Institutional Review Board of Jichi Medical University and carried out in accord with the ethical standards of the Helsinki Declaration of 1975.

Morphologic observations

LDN and HDN were placed on coverslips, fixed in 2.5% glutaraldehyde and postfixed by repeated incubation with 1% osmium tetroxide 1% tannic acid. Samples were then dehydrated using a graded series of ethanol and propylene oxide, after which the samples were observed using a transmission electron microscope (H7600; Hitachi, Tokyo, Japan).

Flow cytometry

LDN recovered from the mononuclear cell layer were suspended in 100 μL of PBS + 0.02% ethylenediaminetetraacetic acid, incubated with 10 μL of Fc-blocker for 20 min and then incubated with FITC-conjugated anti-CD66b and PE-conjugated mAbs to CD45, CD11b, CD16, CD62L, CXCR2, and PD-L1 as well as mouse IgG for 30 min at 4°C. After washing, 7-AAD was added at a final concentration of 0.25 μg/mL. CD66b(+) CD45(+) 7-AAD(−) cells were determined as living
LDNs, and expression of each antigen on LDN was analyzed in CD66b(+) 7-AAD(−)-gated area using FACSCaliber (BD Biosciences, NJ).

Aptosis assay

Purified LDN and HDN were cultured in RPMI-1640 + 10% FBS on 24-well plates. After an appropriate time interval, the cells were recovered and stained with FITC-conjugated Annexin V and 7-AAD for 10 min according to the manufacturers’ recommendation. Ratios of Annexin V(+) 7-AAD(−) and Annexin V(+) 7-AAD(+) cells were then calculated as early and late apoptotic cells, respectively.

Proliferation assay

T cell proliferation induced by anti-CD3 mAb was assessed using standard CFSE dilution methods. Briefly, anti-CD3 mAb were incubated in 96-well plates at a concentration of 5 µg/mL overnight. LDN was positively purified as described previously, and the remaining CD66b-negative fraction was used as a peripheral blood mononuclear cell (PBMC) and stained with CFSE. After washing the plate with PBS three times, labeled PBMC (1 × 10⁵) were seeded in anti-CD3 mAb-coated plate, and purified autologous LDN (3 × 10²–1 × 10⁵) were added to the culture. After 4 d of culture, cells were harvested and stained with PE-conjugated anti-CD4mAb and APC-conjugated anti-CD8 mAbs, and CFSE signal was analyzed by flow cytometry on gated either CD4(+) or CD8(+) T lymphocytes.

Cytotoxic assay

Cytotoxicity by lymphokine-activated killer (LAK) cells was evaluated using flow cytometry as described previously. PBMCs derived from healthy volunteers were cultured in RPMI-1640 + 10% FBS with 20 U/mL recombinant interleukin-2 (r-IL-2) for 10~14 d. OCUM-1 (1 × 10⁵) were added to the culture. After 4 d of culture, LAK cells were harvested and stained with CFSE. After washing the plate with PBS three times, labeled PBMC (1 × 10⁵) were seeded in anti-CD3 mAb-coated plate, and purified autologous LDN (3 × 10²–1 × 10⁵) were added to the culture. After 4 h, the cells were collected, washed, and incubated with PE-conjugated anti-CD4 and APC-conjugated anti-CD8 mAbs, and CFSE signal was analyzed by flow cytometry on gated either CD4(+) or CD8(+) T lymphocytes.

Detection of neutrophil extracellular traps with fluorescence microscopy

Adhesion of tumor cells to neutrophil extracellular traps (NETs) was examined as described previously. LDN (5 × 10⁵) were cultured on poly-l-lysine–coated six-well plate for 2 h, and extracellular DNA was visualized with 50 nM SYTOX orange under a fluorescence microscope (BZ8000; Keyence, Osaka, Japan). OCUM-1 stained green by PKH67 and 1 × 10⁶ cells resuspended in 1 mL of RPMI-1640/1 mL were added on LDN monolayer. In blocking experiment, the LDN were pretreated with 100 U/mL DNase (Thermo Fisher Scientific, Waltham, MA) for 5 min before the addition of labeled OCUM-1. After 5 min of coincubation, the wells were gently washed with warmed media three times, and NETs and attached tumor cells were observed using appropriate wavelength filters for SYTOX orange and PKH67, respectively, and the number of the attached cells were counted at three randomly selected fields.

Statistical analysis

Ratios of LDN were expressed as median (minimum to maximum) and compared using Wilcoxon’s rank-sum test or Kruskal–Wallis rank-sum test, and other continuous normal variables were given as mean ± standard deviation (SD) and compared using Student’s t-test. Correlation was examined using Spearman’s test. P values < 0.05 were considered to be statistically significant.

Results

LDN with an immature phenotype are increased in the peripheral blood of patients after surgery.

Cells were recovered from the peripheral blood of 97 patients before and after undergoing abdominal surgery. Among the cells recovered from the intermediate mononuclear cell layer by Ficoll-Hypaque centrifugation, CD66b(+) neutrophils were determined to be LDN. In most cases, only a few LDN were found in preoperative specimens with a median ratio of CD66b(+) LDN in CD45(+) leukocytes of 0.56% (0.04%–12.9%; Fig. 1A). The ratios were not different among patients with benign and malignant diseases and with various clinical stages of disease (Table 1).

However, the ratio of LDNs was markedly elevated in samples obtained postoperatively (median = 5.7%, 0.12–86.2%; P < 0.001; Fig. 1A). As shown in Table 2, the ratios of LDN after open chest esophagectomy were higher than those gastrectomy and colectomy (P < 0.05). Although, no significant difference was detected between open and laparoscopic gastrectomy or colectomy (Table 2). Electron scanning microscopy revealed that most of the LDN in postoperative samples showed less segmented nuclei with various types of granules compared with HDN, suggesting band cells (Fig. 1B). Flow cytometric analysis revealed that LDN expressed CD11b, CD16, and CD66b less than HDN, whereas the expression of CD62L and CXCR2 was higher in LDN compared with HDN (Fig. 2). These findings suggest that LDN in postoperative peripheral blood samples consisted mainly of the immature type of neutrophils. In addition, PD-L1 was slightly but significantly expressed on LDN although scarcely on HDN (Fig. 2).

The ratio of LDNs in postoperative blood samples positively correlated with operative time and intraoperative blood loss.

We next examined the association between the ratio of LDN in blood obtained postoperatively and operative time or intraoperative blood loss. As shown in Figure 3, the frequency of LDN in postoperative blood samples showed positive correlation with operative time (r = 0.5362, 0.3599–0.6601; P < 0.001). The ratio also tended to be higher after operations with greater intraoperative blood loss (r = 0.2279, 0.238–0.4137; P < 0.025). As both factors correlate with the amount of
surgical stress, this suggests that LDN are recruited into the peripheral blood because of a surgically induced stress response.

**LDN were less apoptotic than HDN**

LDN were purified from postoperative blood PBMC fraction using anti-CD66b conjugated with microbeads, and their function examined compared with similarly manipulated HDN. First, LDN and HDN were cultured in RPMI-1640 with 10% fetal calf serum (FCS), and apoptosis was examined with Annexin V staining. After 16 and 24 h in culture, 25.7\% and 48\% of HDN were positively stained with Annexin V, respectively (Fig. 4). In comparison, 6.6\% and 18.4\% of LDN were positive for Annexin V, significantly less than HDN \( (P < 0.05, n = 4) \), indicating that LDN can live longer than HDN (Fig. 4).

### Table 1 – Frequency of LDN in preoperative circulating blood in patients with various diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number</th>
<th>Median (minimum to maximum)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal cancer</td>
<td>10</td>
<td>1.01 (0.15-10.18)</td>
<td>0.45</td>
</tr>
<tr>
<td>Stomach cancer</td>
<td>39</td>
<td>0.54 (0.04-6.26)</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>44</td>
<td>0.54 (0.04-12.85)</td>
<td></td>
</tr>
<tr>
<td>Benign disease</td>
<td>4</td>
<td>2.19 (0.14-4.66)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage in malignant disease</th>
<th>Number</th>
<th>Median (minimum to maximum)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>36</td>
<td>0.59 (0.04-12.85)</td>
<td>0.63</td>
</tr>
<tr>
<td>II</td>
<td>22</td>
<td>0.43 (0.04-6.77)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>22</td>
<td>0.43 (0.08-7.30)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>0.69 (0.06-10.18)</td>
<td></td>
</tr>
</tbody>
</table>

The ratio of LDN in preoperative blood samples from 97 patients with various diseases and different clinical stages in malignant disease. Cells recovered from the mononuclear cell layer after Ficoll-Hypaque centrifugation were immunostained with FITC-conjugated anti-CD66b and PE-conjugated anti-CD45 mAbs and the ratios of CD66b\( (+) \) LDN in CD45\( (+) \) leukocytes were calculated using flow cytometry.

\( P \) value was evaluated with Kruskal–Wallis rank sum test.

### Table 2 – Frequency of one LDN in postoperative circulating blood in patients with malignant diseases who underwent open or laparoscopic surgery.

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Number</th>
<th>Median (minimum to maximum)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagectomy</td>
<td>10</td>
<td>9.45 (2.98-86.16)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Gastrectomy</td>
<td>39</td>
<td>3.56 (0.18-39.48)</td>
<td></td>
</tr>
<tr>
<td>Colectomy</td>
<td>44</td>
<td>4.55 (0.12-40.53)</td>
<td></td>
</tr>
<tr>
<td>Gastrectomy Open</td>
<td>25</td>
<td>3.10 (0.18-39.48)</td>
<td>0.23</td>
</tr>
<tr>
<td>Laparoscopic Open</td>
<td>14</td>
<td>6.43 (0.97-25.72)</td>
<td></td>
</tr>
<tr>
<td>Colectomy Open</td>
<td>26</td>
<td>4.13 (0.12-33.87)</td>
<td>0.71</td>
</tr>
<tr>
<td>Laparoscopic Open</td>
<td>18</td>
<td>4.55 (0.21-40.53)</td>
<td></td>
</tr>
</tbody>
</table>

The ratio of LDN in postoperative blood samples from 93 patients with esophageal, gastric, and colorectal cancers who received open or laparoscopic surgery. All the patients with esophageal cancer underwent thoracotomy. Frequency of LDN was examined as Table 1.

\*\( P \)-value was evaluated among three procedures using Kruskal–Wallis rank sum test.

\|\( P \)-value was evaluated using Wilcoxon’s rank-sum test.
LDN suppress autologous T cell proliferation

The PBMC fraction was divided into CD66b(+) LDN and CD66b(-) mononuclear cells. The latter cells were stained with CFSE and stimulated with coated anti-CD3 mAb with or without autologous CD66b(+) LDN. As shown in Figure 5, the addition of the purified LDN significantly inhibited CFSE dilution both in CD4(+) and CD8(+) fractions. The inhibition was significant at the number of LDN of 3 x 10^3 cells against 1 x 10^5 PBMC (CD4; 72.3 ± 2.5% versus 64.2 ± 3.7%, P < 0.05, CD8; 48.1 ± 9.7% versus 39.8 ± 10.4%, P < 0.05), and the rate of inhibition became more prominent in a dose-dependent manner.

LDN suppress cytotoxicity of IL-2-activated LAK cells

Peripheral blood lymphocytes were cultured with r-IL-2 and examined for cytotoxicity against a human gastric cancer cell line, OCUM-1, for 4 h. In flow cytometry, OCUM-1 with early and late apoptosis were determined as Annexing(+) 7-AAD(-) and Annexin(+) 7-AAD(+) cells in CD45(-) CD326(+) gated population, respectively. As shown in Figure 6, OCUM-1 if cultured alone, showed 5%–6% early and late apoptosis. Although when cultured together with LAK cells, those values increased to 32.5 ± 0.3% and 24.5 ± 1.8%, respectively. However, the presence of autologous LDN reduced the cytotoxicity of LAK cells. When same number of LDN was added to the LAK cells, the rates of early and late apoptosis of OCUM-1 was significantly decreased (26.1 ± 0.3%, P < 0.01 and 17.3 ± 0.4%, P < 0.01).

The LDN form NETs and trapped tumor cells

Finally, the purified LDN were cultured in RPMI1640 with 10% FCS for 2 h, and NETs formation was examined for the staining of SYTOX green, membrane-impermeable dye to bind DNA. As shown in Figure 6, many thread-like structures were observed under the fluorescein microscope (Fig. 7A), which was eliminated by pretreatment with DNAse I for 5 min (Fig. 7B). When PKH67-stained OCUM-1 was recovered from intermediate (LDN) and bottom (HDN) layers after density gradient centrifugation of postoperative blood samples were immunostained with PE-conjugated mAbs to each antigen and FITC-conjugated CD66b mAb, and mean fluorescein channel (MFC) was analyzed in CD66b(+)7-AAD(−) gated area. Data show a representative FACS profile. (Color version of figure is available online.)
added on the NETs forming culture plate and co-incubated for 5 min, many OCUM-1 was observed to attach to the NETs structure (Fig. 7C). Same findings were obtained in human gastric and colon cancer cells (Supplementary Data). No adhesion was observed to poly-l-lysine substrate, and the adhesion was almost totally abrogated when LDN were pretreated with DNAse I (Fig. 7D and E). This suggests that the LDN-derived NETs can effectively trap circulating tumor cells.

Discussion

There is increasing evidence suggesting the presence of multiple neutrophil phenotypes with proinflammatory or immunosuppressive functions, which actively participate in the regulation of adaptive immunity in inflammation and cancer.11,12 Among the neutrophil subpopulations, LDN have recently attracted much attention because of their unique
**Fig. 6** — LDN in postoperative blood samples inhibit the cytotoxicity of r-IL-2–stimulated lymphocytes against tumor cells. OCUM-1 cells (1 × 10^5) were cocultured with activated lymphocytes by 20 U/mL r-IL-2 for 10–14 d in a 24-well plate. After 4 h, the cells were harvested and stained with PE-conjugated anti-CD45 and APC-conjugated anti-CD326 mAbs, followed by staining with FITC-conjugated Annexin V and 7-AAD, and Annexin V(+)7-AAD(−) and Annexin V(+)/7-AAD(+) cells were calculated in CD45(−) CD326(+) gated areas as early and late apoptotic cells (A). Data show mean ± SD in triplicates in one of the four different experiments (B). *P value < 0.05, **P value < 0.01. (Color version of figure is available online.)

**Fig. 7** — (A) LDN produce NETs which trap tumor cells. LDN (5 × 10^5) purified from postoperative blood samples were suspended in 1 mL RPMI-1640 supplemented with 10% FCS were cultured on poly-L-lysine coated six-well plates. After 2 h, SYTOX orange was added and observed with a fluorescence microscope. (B) The same culture was pretreated with DNase (100 U/mL) for 5 min before the addition SYTOX. (C) PKH67-stained OCUM-1 (1 × 10^6) cells were added on the NETs, and the wells were gently washed after 5 min, and NETs and attached tumor cells were observed using appropriate wavelength filters for SYTOX orange and PKH67, respectively, and two photos merged. (D) The LDN were pretreated with 100 U/mL DNase I for 5 min before the addition of OCUM-1, and attachment was evaluated as described above. (E) The number of the attached cells to LDN monolayer cultured on poly-L-lysine–coated plate was counted at three randomly selected fields. Data show mean ± SD in a representative experiment.
characteristics. Previous reports have shown that the frequency of LDN is significantly increased in the peripheral blood of patients with various malignancies\(^{18-20}\) as well as severe sepsis\(^{18,20}\) and their frequency correlates with disease severity.

In this study, we found that relatively few LDN are in the peripheral blood of patients before undergoing abdominal surgery. No significant difference was found in patients with benign or malignant diseases at various clinical stages, which may result in part from the sample size. Interestingly, however, the ratio of LDN was markedly increased in blood obtained postoperatively, especially after open chest esophagectomy. More importantly, the ratio had positive correlation with operating time and intraoperative blood loss. The morphology of the LDN was similar to that of immature type neutrophils. Flow cytometric analysis revealed that the LDN strongly expressed L-selectin and IL-8 receptor and live longer than HDN ex vivo. These findings are consistent with those of immature LDN, which are often detected in the peripheral blood of patients with sepsis\(^{18,20}\) or lupus erythematosus.\(^{13,29}\)

It is well known that major surgical procedures induce a systemic inflammatory response, which causes not only the activation of circulating mature neutrophils but also enhances mobilization of immature neutrophils in the peripheral circulation.\(^{30}\) These data suggest that LDN in postoperative blood samples consists mainly of immature neutrophils recruited to the periphery as “emergency granulopoiesis” induced by an inflammatory signal triggered by surgical stress.

These results clearly show that LDN suppress T cell–mediated tumor immunity. Addition of LDN inhibits autologous T cell proliferation against TCR–CD3–mediated stimulation. LDN suppress the cytotoxicity of r-IL-2–activated lymphocytes against OCUM-1. Although many studies have shown that MDSC suppresses the T cell response in mice,\(^{31}\) it remains controversial based on the definition of MDSC in humans. However, previous studies have indicated that neutrophils recovered from PBMC layers suppress T cell activation and proliferation in patients following traumatic injuries\(^{12}\) and septic shock\(^{19}\) and in healthy volunteers challenged with endotoxin.\(^{33}\) Our data suggest that the immature-type LDN have the capability to suppress the T cell response.

Flow cytometric analysis demonstrated that LDN in postoperative blood samples significantly expressed PD-L1. Neutrophils have been shown to express PD-L1 by stimulation with interferon-γ\(^{34-36}\) and granulocyte-macrophage colony-stimulating factor.\(^{34,37}\) As surgical stress can increase the circulating level of inflammatory cytokines including interferon-γ,\(^{38}\) it may induce PD-L1 on LDN in postoperative blood samples causing suppression of T cell response. In fact, PD-L1 has been shown to play a pivotal role in immunosuppression in sepsis\(^{39,40}\) or tumor\(^{37,38}\) models.

Another important finding is that LDN in postoperative blood samples results in the formation of NETs, which can efficiently trap tumor cells in vivo. NETs are complex structures composed of chromatin decorated with histones, proteases, and granular and cytosolic proteins and were recognized as an important antimicrobial mechanism to immobilize and kill pathogens.\(^{42}\) More recently, however, NETs in hepatic sinuses have been shown to capture circulating tumor cells, which results in the augmentation of hepatic metastases in murine models.\(^{33,44}\) The results of the present study are consistent with these previous results and suggests that NETs are produced by LDN in the vasculature of target organs in humans and capture shed circulating tumor cells during surgery.

In summary, many immature-type LDN are recruited to the circulation early in the postoperative period probably through surgical stress. These LDN can support the lodging of circulating tumor cells through NETs formation in target organs and locally suppress the T cell–mediated antitumor response, which may facilitate the survival and growth of tumor cells in the postoperative period. It is well known that the surgical stress response may promote the development of metastases although the mechanisms have not been fully elucidated.\(^{35,46}\) The results of this study suggest that the phenomenon may be, at least partly, dependent on immunosuppressive LDN, and that functional blockade of LDN in the postoperative period might be an effective approach to reduce the growth of minimal residual disease and improve the outcomes of patients who underwent resection of their primary tumor.

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Authors’ contributions: Y.K., H.O., and J.K. conceived and designed the experiments. Y.K., H.O., and H.M. performed the experiments and analyzed the data. H.H., Y.H., and N.S. contributed reagents/materials/analysis tools. A.K.L., N.S., and J.K. contributed to the writing of the article.

**Disclosure**

All authors report no conflicts of interest in this study.

**Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jss.2019.08.022.

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