Uterine Cervical Cancer Displaying Tumor-Related Leukocytosis: A Distinct Clinical Entity With Radioresistant Feature

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Background Tumor-related leukocytosis (TRL) is occasionally found in patients with nonhematopoietic malignancies. We investigated the clinical implication of TRL and individualized treatment for TRL-positive cervical cancer, as well as the underlying biological mechanism.

Methods Clinical data from 258 cervical cancer patients treated with definitive radiotherapy were analyzed to investigate the association between TRL and treatment outcome. Clinical samples, cervical cancer cell lines, and a mouse model of cervical cancer were used to examine the mechanisms responsible for TRL in cervical cancer, focusing on the role of tumor-derived granulocyte colony-stimulating factor (G-CSF) and myeloid-derived suppressor cells (MDSCs). All statistical tests were two-sided.

Results TRL was statistically significantly associated with younger age (Wilcoxon rank sum test, \(P = .03\)), larger tumor size (Wilcoxon rank sum test, \(P = .006\)), advanced clinical stage (\(\chi^2\) test, \(P = .01\)) and shorter overall survival (Cox proportional hazard modeling and Wald tests, \(P < .001\)). Among cervical cancer patients, TRL was associated with upregulated tumor G-CSF expression (\(\chi^2\) test, \(P < .001\)), elevated serum G-CSF levels (Student t test, \(P = .03\)), larger spleens (Student t test, \(P = .045\)), and increased MDSC frequencies in the blood (Student t test, \(P < .001\)) compared with the TRL-negative patients. In vitro and in vivo experiments revealed that tumor-derived G-CSF was involved in the underlying causative mechanism of TRL and MDSCs induced by tumor-derived G-CSF are responsible for the rapidly progressive and radioresistant nature of TRL-positive cervical cancer. The administration of anti-Gr-1 neutralizing antibody or the depletion of MDSCs by splenectomy (\(n = 6\) per group) inhibited tumor growth and enhanced radiosensitivity in TRL-positive cervical cancer xenografts (Wilcoxon rank sum test, \(P = .008\) and \(P = .02\), respectively).

Conclusions TRL is associated with resistance to radiotherapy among cervical cancer patients, and MDSC-targeting treatments may have therapeutic potential in these patients.


Cervical cancer is the second most common type of cancer affecting women worldwide, with an incidence of 530,000 new cases per year (1). In Japan alone, roughly 7000 new cases of the disease are reported annually (2).

Although concurrent chemoradiation, the current standard treatment for invasive cervical cancer, is potentially curative, a number of patients still develop recurrent disease: the risk of recurrence is 10% to 20% for International Federation of Gynecology and Obstetrics stages Ib to Ia and 50% to 70% for stages Iib to IVa (3). Thus, identifying prognostic factors that can be used to predict the treatment outcome and the development of novel treatments for patients who are likely to exhibit resistance to the standard treatment are of great importance.

It has been reported that tumor-related leukocytosis (TRL) occurs in 1% to 10% of patients with nonhematopoietic malignancies (4–7). Leukocytosis can be caused by the upregulated expression of hematological growth factors, including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor, interleukin 1, interleukin 6, or tumor necrosis factor alpha (5). In previous studies of uterine cervical cancer, approximately 10% of patients were incidentally found to have TRL at the initial diagnosis (8) or at the time of recurrence (9). However, the precise causative mechanism of TRL and the clinical implication of TRL in cervical cancer remain unknown.

Myeloid-derived suppressor cells (MDSCs), a subpopulation of myeloid cells, have been shown to enhance tumor progression by stimulating tumor angiogenesis, as well as metastasis and immune suppression, in experimental mouse models (10). In mice, MDSCs are characterized by the coexpression of the myeloid cell lineage...
differentiation antigen Gr1 and CD11b. In humans, MDSCs are most commonly defined as CD11b+HLA-DR−/low cells that express the common myeloid antigen CD33 (11). Because an increased level of circulating MDSCs has recently been correlated with clinical stage, tumor burden, and survival in patients with breast or pancreatic cancer (12,13), MDSCs represent an attractive therapeutic target. However, the role of MDSCs in the progression and radiosensitivity of cervical cancer has not been reported to date.

In this study, we investigated the prognostic significance of TRL in cervical cancer patients that were treated with definitive radiotherapy in both retrospective and prospective settings. Moreover, by investigating the underlying causative mechanism of TRL in vitro and in vivo experimental models, we investigated treatment strategies for TRL-positive cervical cancer.

Methods

Patients and Clinical Samples

Permission to proceed with the data acquisition and analysis was obtained from Osaka University Hospital’s Institutional Review Board. Patients who were treated with definitive radiotherapy for cervical cancer at Osaka University Hospital from April 1996 to March 2007 (a retrospective analysis) and from April 2007 to March 2011 (a prospective analysis) were included in this study. Appropriate written informed consent was obtained from each patient, and their clinical data, biopsied tissue specimens, and blood samples were analyzed. Survival was defined as the time from the primary diagnosis to death or the latest observation.

Mouse Studies

All of the procedures involving mice and their care were approved by the Institutional Animal Care and Usage Committee of Osaka University in accordance with institutional and National Institutes of Health guidelines. To examine the antitumor activity of radiotherapy or anti-Gr-1 neutralizing antibody in vivo, female BALB/c nude mice aged 5 to 7 weeks were inoculated with 5×10⁶ ME180-control or ME180-G-CSF cells in 100 µL of phosphate-buffered saline (n = 6 per group). Anti-Gr-1 antibody was intraperitoneally administered from the first day of inoculation at a dose of 200 µg per mouse every 48 hours. External beam irradiation was delivered to the inoculated tumors using a 4 MV x-ray at a dose rate of 2 Gy per minute (EXL-6SP; Varian Medical Systems, Palo Alto, CA). The tumors were irradiated from the fifth day of inoculation, with a dose of 5 Gy per fraction every other day for a total of four fractions (total dose = 20 Gy). Mice were killed by carbon dioxide asphyxiation, and the tumors were collected for analysis.

Splenectomy was performed under isoflurane anesthesia as described previously (15). Ten days after undergoing splenectomy or a sham operation, female BALB/c nude mice aged 5 to 7 weeks were inoculated with ME180-G-CSF cells (n = 6 per group). Then the roles played by the spleen in regulating the number of MDSCs, tumor progression, and the radiosensitivity of cervical cancer were investigated.

Statistical Analysis

Continuous data were compared between groups by Student t test, Wilcoxon rank sum test, or median test, as appropriate. Frequency counts and proportions were compared between groups by χ² test or a two-tailed Fisher exact test, as indicated. Spearman’s correlation coefficient with the 95% confidence interval (CI) was calculated to assess the relationship among serum G-CSF concentrations, white blood cell (WBC) count, and neutrophil count. The Fisher z transformation was used to derive its confidence limits and a P value under a null hypothesis that correlation is equal to zero. We performed univariate analysis by comparing Kaplan–Meier curves of subgroups with the log-rank test. Cox proportional hazards regression analysis was performed to identify independent prognostic factors for overall survival. The proportional hazards assumption for the Cox regression model was verified graphically using complementary log–log plots of survival, with a test based on Schoenfeld residuals. P values of less than .05 were considered statistically significant. All statistical tests were two-sided. All analyses were done with SAS version 9.3 for Windows (SAS Institute, Cary, NC).

All other materials and methods can be found in the Supplementary Materials (available online).

Results

Clinical Implications of TRL

Among a total of 258 patients included in this study, TRL (WBC ≥ 10000/µL) was observed in 37 (14.3%) at the initial diagnosis. The TRL-positive patients presented with a statistically significantly younger age (P = .03), larger tumors (P = .006), and more advanced clinical stage (P = .01) than the patients without TRL (Supplementary Tables 1–3, available online).

Statistically significant differences in tumor response to radiotherapy (retrospective analysis [n = 191]: P < .001; prospective analysis [n = 67]: P = .005) (Table 1) and overall survival (P < .001) (Figure 1A) were observed between the TRL-positive and TRL-negative patients. In the multivariable analysis (Table 2; Supplementary Table 4, available online), TRL remained an independent predictor of compromised survival (hazard ratio = 4.89; 95% CI = 2.76 to 8.67; P < .001).

Because all of the 23 TRL-positive patients in the retrospective analysis had elevated neutrophil counts (data not shown) and a statistically significantly positive correlation was demonstrated between the WBC count and neutrophil count in these patients both in retrospective and prospective analyses (Spearman’s correlation coefficient, r = 0.94 and r = 0.96; P < .001 and P < .001, respectively) (Figure 1B; Supplementary Figure 1A, available online), we next examined the prognostic significance of neutrophilia in a separate multivariable analysis in which WBC count was not included as a prognostic variable. As shown, a neutrophil count of more than 7500/µL was found to be a statistically significant prognostic indicator in terms of overall survival (P < .001) (Figure 1C; Supplementary Figure 1B and Supplementary Tables 5 and 6, available online).

Cancer Cell–Derived G-CSF as a Possible Mediator of TRL in Cervical Cancer Patients

As shown in Figure 1D, statistically significantly strong G-CSF immunoreactivity was observed in the tumors obtained from the TRL-positive patients compared with those obtained from the TRL-negative patients (91.2% of TRL-positive tumors vs 3.6% of TRL-negative tumors had strong G-CSF immunoreactivity; P < .0001).
.001). Strong correlations were observed between the WBC count and G-CSF immunoreactivity (Spearman’s correlation coefficient, \( r = 0.54; P < .001 \)) and between the neutrophil count and G-CSF immunoreactivity (Spearman’s correlation coefficient, \( r = 0.53; P < .001 \)). Moreover, the mean G-CSF levels of the TRL-positive patients were statistically significantly higher than those of the TRL-negative patients (TRL group: mean G-CSF level = 215 pg/mL, 95% CI = 63.2 to 367 pg/mL; non-TRL group: mean G-CSF level = 39.8 pg/mL, 95% CI = 30.5 to 49.2 pg/mL; \( P = .03 \) (Figure 1E)). In the survival analyses, the patients that exhibited higher G-CSF expression demonstrated decreased overall survival compared with the patients that displayed lower G-CSF expression (\( P < .001 \)) (Figure 1F; Supplementary Figure 1C and Supplementary Tables 7 and 8, available online). Collectively, these results strongly indicate that the induction of increased granulopoiesis by tumor-derived G-CSF may be the underlying mechanism behind TRL in cervical cancer patients.

### Experimental Model of TRL-Positive Cervical Cancer

We next established a G-CSF–producing cervical cancer cell line, ME180-G-CSF. The expression of G-CSF in these cells was verified in vitro and in vivo (Figure 2, A and B; Supplementary Figure 2A, available online). The ME180-G-CSF–derived tumors grew statistically significantly faster than the ME180-control–derived tumors (\( P = .005 \) at 20 and 28 days after inoculation; \( P = .008 \) at 24 days after inoculation) (Figure 2B).

Consistent with our findings from the analysis of clinical data, the ME180-G-CSF–derived tumors were less sensitive to radiotherapy and grew statistically significantly faster vs the ME180-control–derived tumors during the course of external beam radiotherapy (\( P = .008 \) at 20 and 36 days after inoculation; \( P = .02 \) at 24 days after inoculation; \( P = .04 \) at 28 days after inoculation; \( P = .01 \) at 32 days after inoculation) (Figure 2C; Supplementary Figure 2, B and C, available online), resulting in the development of systemic metastasis (Supplementary Figure 2D, available online) and a non-statistically significant shorter survival (\( P = .06 \)) (Figure 2C).

### Role of G-CSF–Stimulated MDSCs in the Aggressive and Radioresistant Nature of TRL-Positive Cervical Cancer

As shown in Supplementary Figure 3 (available online), neither the G-CSF receptor expression nor the direct stimulatory effect of G-CSF was observed in cervical cancer cells, suggesting that cancer cell–derived G-CSF does not have a direct growth-stimulating effect on cervical cancer cells.

We then examined the effect of G-CSF treatment on the frequency of MDSCs (CD11b+Gr1+ cells) in mice. As shown, treatment of mice with recombinant human G-CSF protein statistically significantly increased the frequencies of MDSCs in their bone marrow, blood, and spleen (two-sided \( t \) test, \( P < .05 \)) (Supplementary Figure 4, A and B, available online). Moreover, the ME180-G-CSF–derived tumor-bearing mice displayed markedly increased MDSC frequencies in their bone marrow (ME180-control = 66.5%, 95% CI = 56.3 to 76.7%; ME180-G-CSF = 88.4%, 95% CI = 86.1% to 90.7%; \( P = .002 \)), blood (ME180-control = 53.4%, 95% CI = 42.0% to 64.9%; ME180-G-CSF = 98.6%, 95% CI = 98.4% to 98.9%; \( P < .001 \)), and tumor (ME180-control = 2.44%, 95% CI = 1.58% to 3.30%; ME180-G-CSF = 6.65%, 95% CI = 4.33% to 8.96%; \( P = .004 \)) compared with the ME180-control–derived tumor-bearing mice (Figure 3, A and B; Supplementary Figure 4C, available online).

Because the anti-Gr1 antibody recognizes two antigens, Ly6G and Ly6C, CD11b+Gr1+ cells represent a heterogeneous population that includes granulocytic and monocytic MDSCs. Thus, we further investigated whether G-CSF mainly affects granulocytic or monocytic MDSCs in cervical cancer (Supplementary Figure 4D, available online). We observed a marked enrichment in Ly6G+Gr1+ granulocytic MDSCs in the ME180-G-CSF–derived tumor-bearing mice, indicating that granulocytic MDSCs represent the dominant subset that is expanded by the G-CSF.

To gain insights into the tumor-promoting effect of MDSCs, we investigated the role of MDSCs in the expression of Bv8, a potent proangiogenic factor that stimulates the proliferation, survival, and migration of endothelial cells (15). As shown in Figure 3C, the ME180-G-CSF–derived tumors contained statistically significantly higher levels of Bv8 than ME180-control–derived tumors (ME180-control–derived tumor = 0.002, 95% CI = 0 to 0.002; ME180-G-CSF–derived tumor = 0.43, 95% CI = 0.42 to 0.44; \( P < .001 \)). However, Bv8 was not detected in the ME180-G-CSF cells, indicating that stromal cells such as MDSCs are the most likely source
Figure 1. Clinical implications of tumor-related leukocytosis (TRL). A) Kaplan–Meier estimates of survival according to the pretreatment white blood cell (WBC) count in retrospective analysis (n = 191) (i) and in prospective analysis (n = 67) (ii) are shown. B) Correlation between the WBC count and neutrophil count; retrospective analysis is shown. Spearman’s correlation coefficient, \( r = 0.94; P < .001 \). C) Kaplan–Meier estimates of survival according to the pretreatment neutrophil count; retrospective analysis is shown. D) Granulocyte colony-stimulating factor (G-CSF) expression in cervical cancer is shown. Cervical cancer biopsy samples obtained from TRL-positive (TRL group) and TRL-negative patients (non-TRL group) were stained with anti-G-CSF antibody. i) Representative photographs are shown. Scale bar = 50 μm. ii) Histograms indicate the samples’ immunoreactivity profiles. Values indicate the proportions of weakly/strongly stained tumors. \( \chi^2 \) test was used to determine statistical significance. E) Mean serum G-CSF levels of newly diagnosed cervical cancer patients (n = 12 patients with leukocytosis and 12 with normal WBC counts) are shown. Bars = standard deviation. *P < .05, two-sided Student t test. F) Kaplan–Meier estimates of survival according to G-CSF immunoreactivity; retrospective analysis is shown. Log-rank test was used to determine statistical significance in (A, C, and F). All statistical tests were two-sided.
Table 2. Prognostic significance of leukocytosis at the initial diagnosis (retrospective analysis)*

<table>
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<tr>
<th>Characteristic</th>
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<th>Multivariable analysis</th>
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<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P†</td>
</tr>
<tr>
<td>Age, y</td>
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<tr>
<td>≤50</td>
<td>0.45 (0.28 to 0.72)</td>
<td>&lt;.001</td>
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<tr>
<td>≥51</td>
<td>2.36 (1.42 to 3.92)</td>
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<tr>
<td>Clinical stage</td>
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<tr>
<td>Pretreatment hemoglobin level, mg/dL</td>
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<td>.09</td>
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<tr>
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<tr>
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<tr>
<td>Histology</td>
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<tr>
<td>SCC</td>
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<tr>
<td>Non-SCC</td>
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</tr>
<tr>
<td>Duration of RT, days</td>
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</tr>
<tr>
<td>≤55</td>
<td>0.48 (0.28 to 0.82)</td>
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<tr>
<td>≥56</td>
<td>1.49 (0.72 to 3.08)</td>
<td>.29</td>
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<tr>
<td>WBC, /μL</td>
<td>1.00 (referent)</td>
<td>&lt;.001</td>
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<tr>
<td>&lt;10000</td>
<td>5.49 (3.31 to 9.09)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

* CCRT = concurrent chemoradiotherapy; CI = confidence interval; RT = radiotherapy; SCC = squamous cell carcinoma; WBC = white blood cell.
† P values were calculated using the two-sided Wald test in the Cox proportional hazard model.

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of Bv8. In agreement with this hypothesis, we detected statistically significant Bv8 expression in the MDSCs isolated from the ME180-G-CSF–derived tumors. Because the mouse and human Bv8 genes share a common structure, we next examined the proangiogenic activity of MDSC-derived Bv8 in vitro and in vivo. As expected, the coinoculation of human umbilical vein endothelial cells with MDSCs statistically significantly enhanced the tube formation activity of the human umbilical vein endothelial cells (control = 1.0, 95% CI = 0.84 to 1.16; MDSCs = 1.30, 95% CI = 1.17 to 1.42; P = .004) (Figure 3C). Moreover, large CD31-immunopositive vessels were observed in ME180-G-CSF–derived tumors compared with ME180-control–derived tumors (ME180-control = 1.0, 95% CI = 0.27 to 1.74; ME180-G-CSF = 2.75, 95% CI = 1.24 to 4.26; P = .02) (Figure 3C). Because unregulated tumor angiogenesis increases the interstitial fluid pressure and reduces the radiosensitivity of uterine cervical cancer (16), these results indicate that G-CSF–induced MDSCs may promote tumor progression and radioresistance, at least in part, by the stimulation of angiogenesis.

Role of Spleen as a Supplier of MDSCs

Given that ME180-G-CSF–derived tumor-bearing mice exhibited splenomegaly (Figure 4A), we next investigated the role of the spleen in the production of MDSCs in our mouse model. As shown, the removal of the spleen statistically significantly reduced the number of MDSCs in the ME180-G-CSF–derived tumor (sham surgery = 4.87%, 95% CI = 3.13 to 6.60%; splenectomy = 2.65%, 95% CI = 1.77% to 3.53%; P = .02) (Figure 4B). This result indicates that the spleen acts, at least in part, as a supplier of MDSCs (Figure 4B), which is consistent with the previous notion of a “splenic reservoir” (14). Importantly, the reduction in the number of MDSCs in the spleenectomized mice was associated with impaired ME180-G-CSF–derived tumor progression (P = .005 at 15 and 20 days after inoculation) (Figure 4B). In contrast, the growth-inhibitory effect of splenectomy was minimal in ME180-control–derived tumor-bearing mice (Figure 4B), indicating that splenectomy might have therapeutic potential only in TRL-positive cervical cancer patients.

To determine whether the findings obtained in mice are representative of the clinical status of cervical cancer patients, we investigated the numbers of MDSCs in the blood and the volume of the spleen among these patients, as estimated from computed tomography scans, to define human MDSCs according to previous studies (11). Consistent with the findings obtained in mice, the peripheral blood of the TRL-positive patients contained statistically significantly higher numbers of MDSCs (non-TRL group = 3.10%, 95% CI = 2.02% to 4.18%; TRL group = 7.86%, 95% CI = 7.41% to 8.30%; P < .001) (Figure 4C). Moreover, the mean spleen volume among these patients, as estimated from computed tomography scans, was statistically significantly greater in the TRL-positive patients than in the TRL-negative patients (non-TRL group = 98.3 mm; 95% CI = 89.9 to 107 mm; TRL group = 136 mm; 95% CI = 99.8 to 172 mm; P = .045) (Figure 4D). In addition, on 2-deoxy-2-[¹⁸F] fluoro-D-glucose positron emission tomography/computed tomography, homogenous bone marrow fluorodeoxy-D-glucose uptake, which is indicative of increased metabolic activity in the bone marrow, was frequently observed in the TRL-positive patients (Figure 4E, 4F). Therefore, these results indicate that the spleen acts, at least in part, as a supplier of MDSCs.
observed in the patients that displayed TRL (Supplementary Figure 4E and Supplementary Table 9, available online).

**Effect of MDSC Depletion on Tumor Growth and the Therapeutic Efficacy of Radiotherapy**

Treating the mice with anti-Gr1 neutralizing antibody resulted in marked decreases in the tumor burden ($P = .005$ at 12 and 16 days after inoculation; $P = .008$ at 20 days after inoculation) and the frequencies of MDSCs in tumors compared with the mice treated with control immunoglobulin G in ME180-G-CSF-derived tumor-bearing mice (control = 4.63%, 95% CI = 2.94% to 6.33%; anti-Gr1 = 3.03%, 95% CI = 2.33% to 3.73%; $P = .05$) (Figure 5A). In contrast, the effect of anti-Gr1 neutralizing antibody was minimal in ME180-control-derived tumor-bearing mice (Figure 5A).

When combined with radiotherapy (Figure 5B; Supplementary Figure 5A, available online), treatment with anti-Gr1 neutralizing antibody increased the sensitivity of the ME180-G-CSF–derived tumors to radiotherapy ($P = .008$ at 16 and 20 days after inoculation; radiotherapy = 0.79 g, 95% CI = 0.44 to 1.14 g; radiotherapy + anti-Gr1 = 0.40 g, 95% CI = 0.23 to 0.56 g; $P = .02$) (Figure 5B).

We also found that the ME180-G-CSF–derived tumors that developed in the splenectomized mice were statistically significantly
Figure 3. Mechanism responsible for the rapid progression and radioresistance of tumor-related leukocytosis (TRL-positive cervical cancer). A and B) CD11b+Gr1+ cell populations of the bone marrow, blood, spleen, and tumor are shown. Mice were inoculated with ME180-G-CSF (n = 6) or ME180-control cells (n = 6). Four weeks after inoculation, bone marrow, blood, spleen, and tumor were collected and assayed by flow cytometry for myeloid-derived suppressor cells (MDSCs). Bars = standard deviation. *P < .05, two-sided Student t test. C) i) Expression of Bv8 mRNA in tumors, MDSCs isolated from a ME180-G-CSF-derived tumor-bearing mouse, and cervical cancer cells were assessed by real-time reverse-transcriptase polymerase chain reaction analysis. The expression level of Bv8 mRNA was normalized to that of GAPDH mRNA. The hepatic cells, which are known to express Bv8, were used as a positive control. Bars = standard deviation. *P < .05, two-sided Student t test. ii) Proangiogenic activity of MDSCs in vitro. Human umbilical vein endothelial cells (HUVEC) were cultured with or without MDSCs for 8 hours (50:1 ratio of HUVEC/MDSC). Proangiogenic activity was determined using the tube formation assay. Three random fields per sample were recorded, and the tube length of every field was measured. Each experiment was performed at least three times, and data from one representative experiment are shown. Bars = standard deviation. *P < .05, two-sided Student t test. Scale bar = 200 μm. iii) Histograms indicate microvesel area (MVA) of ME180-control– or ME180-G-CSF–derived tumors 4 weeks after inoculation (left). Microvesel staining (dark brown) in the representative ME180-G-CSF–derived tumor is increased compared with representative ME180-control–derived tumor (right). Bars = standard deviation. *P < .05, two-sided Student t test. Scale bar = 100 μm.
**Figure 4.** Role of spleen in myeloid-derived suppressor cell (MDSC) accumulation and tumor progression. 

A) Spleens of mice carrying ME180-control– or ME180-G-CSF–derived tumors that underwent sham surgery are shown.

B) i and ii) MDSC accumulation in ME180-G-CSF–derived tumors are shown. Bars = standard deviation. *P < .05, two-sided Student t test.

iii) Proposed paracrine mechanism responsible for tumor-related leukocytosis (TRL) is shown.

iv and v) Tumor growth curves are shown. Bars = standard deviation. *P < .05, two-sided Student t test. Mice that underwent splenectomy (n = 6) or sham surgery (n = 6) were inoculated with ME180-G-CSF or ME180-control cells. Three weeks after inoculation, the spleens and subcutaneous ME180-G-CSF–derived tumors were collected for evaluation.

C) Circulating MDSC levels of cervical cancer patients. Peripheral blood (PB) samples were obtained from healthy donors (n = 10), TRL-positive cervical cancer patients (n = 6), and TRL-negative cervical cancer patients (n = 9). Human MDSCs, which were defined as CD11b+CD33+HLA-DR− cells, were counted using flow cytometry. Bars = standard deviation. *P < .05, two-sided Student t test.

D) i) Representative computed tomography (CT) scans showing the spleens of patients with or without TRL are shown. ii) The volumes of the spleens of patients with (n = 14) or without (n = 76) TRL, which were calculated based on pretreatment CT images, are shown. Bars = standard deviation. *P < .05, two-sided Student t test. All statistical tests were two-sided.
Figure 5. Effect of myeloid-derived suppressor cell (MDSC) depletion on tumor growth and the radiosensitivity of cervical tumors. A) i and ii) Effects of anti-Gr-1 neutralizing antibody on MDSC accumulation in ME180-G-CSF–derived tumors. Mice that had been inoculated with ME180-G-CSF or ME180-control cells were treated intraperitoneally with anti-Gr-1 neutralizing antibody (n = 6) or control immunoglobulin G (IgG; n = 6) every 2 days. Three weeks after inoculation, the subcutaneous tumors were collected for evaluation. Bars = standard deviation. *P < .05, two-sided Student t test. iii and iv) Tumor growth curves are shown. Bars = standard deviation. *P < .05, Wilcoxon rank sum test.

B) Effects of anti-Gr-1 neutralizing antibody on the radiosensitivity of cervical cancer. Mice carrying ME180-G-CSF–derived tumors were treated with radiotherapy in combination with anti-Gr-1 neutralizing antibody (n = 6) or control IgG (n = 6). i) Tumor growth curves are shown. Bars = standard deviation. *P < .05, Wilcoxon rank sum test. ii) Tumor weights at 3 weeks after inoculation are shown. Bars = standard deviation. *P < .05, Wilcoxon rank sum test.

C) Effects of spleen removal on MDSC accumulation and the radiosensitivity of cervical cancer. Mice that underwent splenectomy (n = 6) or sham surgery (n = 6) were inoculated with ME180-G-CSF cells. Five days after inoculation, the mice were treated with local radiotherapy. i) Tumor growth. Bars = standard deviation. *P < .05, Wilcoxon rank sum test. ii) Tumor weight at 3 weeks after inoculation. Bars = standard deviation. *P < .05, Wilcoxon rank sum test.

All statistical tests were two-sided.
more sensitive to radiotherapy than the ME180-G-CSF–derived tumors that developed in the mice that underwent sham surgery ($P = .03$ at 16 days after inoculation; $P = .02$ at 20 days after inoculation; sham surgery + radiotherapy = 0.74 g, 95% CI = 0.44 to 1.05 g; splenectomy + radiotherapy = 0.44 g, 95% CI = 0.30 to 0.57 g; $P = .045$) (Figure 5C; Supplementary Figure 5B, available online).

**Discussion**

According to previous investigations, the majority of MDSCs reside in the bone marrow, and only small numbers of these cells can be found in the blood and spleen in healthy individuals. MDSCs markedly expand systemically in response to acute inflammation or cancer development as part of the host immune response (14). During this process, the spleen has recently been shown to act as an extramedullary reservoir of MDSCs, which can be mobilized to contribute to the host response (14). Consistent with this report, we found that the removal of the spleen almost completely abrogated G-CSF–induced MDSC accumulation in tumors and the tumor-promoting effect of G-CSF and enhanced the efficacy of radiotherapy in mice. These results indicate that the spleen acts as an MDSC reservoir in our mouse model and, hence, is a potential therapeutic target aimed at enhancing the effectiveness of radiotherapy in TRL-positive cervical cancer preclinical models. Importantly, we confirmed the statistically significantly enlarged spleen and the increased metabolic activity in the bone marrow in the TRL-positive patients compared with the TRL-negative patients. Splenectomy and spleen irradiation have been clinically used as palliative treatments for patients with hematological malignancies (17). Moreover, pharmacological approaches, including the use of all-trans-retinoic-acid, multiple tyrosine kinase inhibitor sunitinib, or gemcitabine, that have been clinically used to treat human malignancies are also shown to reduce the number of MDSCs (18–21). Thus, the efficacy of combining these MDSC-targeting therapies with conventional definitive radiotherapy is worth investigating in future clinical trials for TRL-positive cervical cancer patients.

The limitations of our study need to be addressed. Our data from mice and human investigations suggest the radio-resistant nature of TRL-positive cervical cancer; however, we have to recognize that we cannot completely eliminate the influence of the rapidly progressive nature of this tumor when we assess the response to radiotherapy. Although this study focused on the tumor-derived G-CSF–MDSC axis, it is important to note that our data do not exclude the possibility that other tumor-derived factors also play roles in the expansion of MDSCs in a G-CSF–dependent or G-CSF–independent manner. Moreover, other stromal cells in the tumor microenvironment might be stimulated by tumor-derived G-CSF to enhance tumor progression. Accordingly, radiosensitivity of TRL-positive cervical cancer, the mechanism by which MDSCs are regulated, and the mechanism by which G-CSF promotes tumor progression should be investigated further. Another potential limitation is that our clinical studies in retrospective and prospective settings were conducted in a single institution. To validate our clinical findings and our mechanistic hypothesis, a collaborative multi-institutional investigation, especially in a prospective setting, should be conducted.

In conclusion, this study is the first to demonstrate that cervical cancer displaying TRL is a distinct clinical entity that has high probability to show resistance to current standard radiotherapy. The aberrant paracrine mechanism found in our study might have important clinical implications because TRL arises in a substantial proportion of cervical cancer patients: more than 10% of cervical cancer patients display TRL at the initial diagnosis. Thus, therapeutic approaches targeting G-CSF-induced MDSCs might be valuable.

**References**


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