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Roles of C-reactive protein and LOX-1 on cancer and myeloid-derived suppressor cells in the progression of uterine cervical cancer

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We investigated clinical implications of CRP and its receptor, LOX-1, in cervical cancer progression and the underlying mechanisms. Clinical data from 121 patients with cervical cancer administered definitive radiotherapy were analyzed to investigate the relationship among pretreatment serum CRP levels, tumor LOX-1 expression, and treatment outcomes. Clinical samples, cervical cancer cell lines, and mouse xenograft models of cervical cancer were employed to elucidate the mechanisms for CRP-mediated progression of cervical cancer, focusing on LOX-1 expression on cancer and myeloid-derived suppressor (MDSCs) cells. Patients with elevated pretreatment CRP levels showed significantly shorter overall survival when their cervical tumor expressed LOX-1. In contrast, elevated pretreatment CRP levels had no prognostic relevance in patients with cervical tumors not expressing LOX-1. CRP treatment of LOX1-expressing cervical cancer cells induced ERK phosphorylation and stimulated cell proliferation in vitro. In vivo, CRP treatment promoted the progression of LOX-1-expressing cervical cancer. In vitro, it stimulated MDSC survival and augmented their suppressive activity. However, the tumor-promoting effects of CRP were minimal in tumors not expressing LOX-1. In conclusion, CRP facilitates progression of LOX-1-expressing cervical cancer by stimulating LOX-1 and its downstream effectors in cancer cells and MDSCs. Novel treatments targeting CRP or LOX-1 may be against LOX1-expressing cancer.

Keywords Cervical cancer, C-reactive protein, Inflammatory marker, LOX-1, MDSCs, Prognosis

Cervical cancer is the fourth most common malignant disease in women worldwide, with approximately 600,000 new cases and 340,000 deaths reported annually[1]. Although most of these patients can be cured by radical surgery or chemoradiotherapy, or a combination of the two, a significant number of patients experience treatment failures, with the risk of recurrence being 10–20% for FIGO (The International Federation of Gynecology and Obstetrics) stages I-IIA and 50–70% for stages IIB-IVA[2,3]. To improve prognosis, the identification of novel therapeutic targets is urgently needed, especially in locally-advanced (stage IIB-IVA) patients who are mainly treated with definitive concurrent chemoradiotherapy.

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Systemic inflammatory markers, including those related to blood cell counts (thrombocytosis, leukocytosis, and neutrophilia), systemic immune-inflammation index, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and lymphocyte-to-monocyte ratio, have recently gained attention as indicators of increased cancer risk or prognosis in patients with various solid malignancies[4–8] C-reactive protein (CRP) is an acute inflammatory protein synthesized in the liver, the abundance of which is increased at sites of infection or inflammation. CRP has been traditionally utilized as a marker of infection or a prognosticator in patients with cardiovascular or autoimmune diseases. Recent investigations have highlighted the usefulness of elevated CRP levels or an increased CRP-to-albumin ratio as an indicator of poor prognosis or treatment failure in cancer patients[3,9,10]. However, these studies were limited to retrospective examination of clinical data, and mechanistic details of CRP-mediated tumor progression has not been fully investigated.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a 50 kDa transmembrane protein in endothelial cells[11]. It is responsible for recognition of oxidized LDL and triggers downstream pathways that intensify atherosclerosis via endothelial dysfunction. Recent studies have shown that LOX-1 is expressed in certain cancer cells and that its expression is associated with poor prognosis in patients with gastric, colorectal, esophageal, and pancreatic cancers[12–14]. LOX-1 is also expressed on myeloid-derived suppressor cells (MDSCs) and can be used as a marker to distinguish human polymorphonuclear MDSCs (PMN-MDSCs) from mature neutrophils[15]. Moreover, LOX-1 expression on MDSCs is associated with increased suppressive activity of these cells[15,16]. Increased LOX-1 expression on PMN-MDSCs is associated with a poor prognosis in cancer patients[15,16].

Although the interaction between CRP, recognized as a ligand for LOX-1, and LOX-1 has been shown to mediate CRP-induced endothelial dysfunction[17], the role of the CRP-LOX-1 axis in MDSCs and cancer cells has never been fully investigated. In the present study, using clinical data from patients with cervical cancer and in vitro and in vivo cervical cancer models, we investigated the tumor-promoting effects of CRP on cervical cancer progression, with a special focus on the CRP-LOX-1 axis in MDSCs and cancer cells.

Methods

Patients and clinical samples

Permission to proceed with the clinical data acquisition and analysis was obtained from the Osaka International Cancer Institute Institutional Review Board (approval number: 21211). Research involving human research participants have been performed in accordance with the Declaration of Helsinki. Patients treated with definitive radiotherapy for cervical cancer at the Osaka International Cancer Institute between January 2014 and March 2020 (in a retrospective analysis) and between July 2022 and April 2023 (in a prospective analysis) were included in this study. Baseline characteristics are presented in Supplementary Tables 1 and 2. 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 treatment to death or latest observation.

Clinical database analysis

The relative expression levels of *OLR1* (encodes LOX-1) in cervical cancer and normal cervical tissues were validated using GEPIA (http://gepia.cancer-pku.cn), an online analysis software based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression. The prognostic value of *OLR1* expression in ovarian cancer was analyzed using the Kaplan–Meier plotter, which included 304 patients with cervical cancer, as described previously[18]. The prognostic value of *OLR1* expression in cervical cancer deposited in TCGA database was further analyzed. The patients were categorized into two groups (high and low *OLR1* expression) based on a Z score threshold of − 0.4, and the two groups were compared using the Kaplan–Meier method.

Immunohistochemistry

Tumor samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and processed for immunohistochemical staining. Immunohistochemical staining was performed using a VENTANA BenchMark GX (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The primary antibody used was an anti-human LOX-1 rabbit polyclonal antibody (1:200; ab126538, Abcam, Cambridge, UK). Slides were examined under a bright-field microscope. LOX-1 expression on tumor (tumoral LOX-1) and stromal (stromal LOX-1) cells was semiquantitatively assessed, as described previously[14]. Briefly, the intensity of LOX-1 staining was scored as negative/weak (0), intermediate (1), or strong (2). LOX-1-expressing cells were scored as follows: <10% cells expressing LOX-1 (0), 10–25% cells expressing LOX-1 (1), and ≥ 26% cells expressing LOX-1 (2). Final scores were obtained by adding the two scores. Patients were divided into two groups according to their final score: low (score 0–2) and high (score 3–4) tumoral LOX-1 expression groups. The number of stromal LOX-1-expressing immune cells was counted in three selected hotspots using light microscopy (×400 magnification; 0.058 mm^2 field area), and the density was calculated by dividing the number of positive cells by the area (cells/mm^2). Patients were divided into two groups based on the mean density as follows: high (stromal LOX-1-H, > 40.6/mm^2) and low (stromal LOX-1-L, ≤ 40.6/mm^2) LOX-1 expression groups.

Multiplex immunofluorescence (mIF) staining

Multiplex immunofluorescence staining was performed using the Opal 4-Color Automation IHC kit (#NEL820001KT; Akoya Biosciences, Marlborough, MA, USA) on a Leica BOND RX automated immunostainer (Leica Microsystems Ltd., Milton Keynes, UK) according to the manufacturer's instructions. The following combinations of antibodies (dilutions) and Opal reagent were used in the experiment: LOX-1 (1:200, Abcam) for Opal 520 and CD33 (1:200, Abcam, Cambridge, UK) for Opal 570. mIF images were acquired using an Olympus Slide Scanner VS200 (Olympus, Tokyo, Japan).

Cell lines and cell culture

ME-180 cells (human cervical cancer) were purchased from Japanese Collection of Research Bioresources. ID8 cells (mouse ovarian cancer) were purchased from the American Type Culture Collection. The cells were routinely screened for mycoplasma contamination (TaKaRa PCR Mycoplasma Detection Set, Takara Bio, Shiga, Japan) and were regularly authenticated by examining their morphology and growth characteristics. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37 °C.

Plasmid construction

The ORF of *Gcsf* gene was amplified by PCR using PrimeSTAR HS DNA polymerase (R010, TaKaRa Bio, Shiga, Japan), a primer pair, and cDNA from the splenocytes of WT mice as a template. The primer sequence used for PCR are *Gcsf*; sense 5' AGT TAA TTA AGG ATC ACC ATG GCT CAA CTT TCT G-3′; antisense 5′-CTG GCG GCC GCT CGA CTA GGC CAA GTG GTG CAG-3′. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Venlo, The Netherlands) and cloned into the pMXs-puro vector (RTV-012; Cell Biolabs, San Diego, CA, USA) to yield pMXs-G-CSF-puro using the In-Fusion HD Cloning Kit (639648; Clontech, Mountain View, CA, USA), according to the manufacturer's instructions.

cDNA encoding *OLR1* was purchased from R&D Systems (Minneapolis, MN, USA). The cDNA encoding *OLR1* was ligated into the pMXs-puro vector at the BamHI and XhoI sites and named as pMXs-LOX-1-puro. The experiments involving genetically modified organisms were performed in accordance with the Cartagena Protocol on Biosafety.

Retroviral packaging, transduction, and establishment of stable transfectants

The pMXs vectors were transfected into the Plat-A retroviral packaging cell line (RV-102; Cell Biolabs) using the Lipofectamine 3000 reagent (L3000; Thermo Fisher Scientific). After 48 h of culture, supernatants containing retroviral particles were collected. Each supernatant was passed through 0.80 μm syringe filter (SLAA033SS; Merck Millipore, Darmstadt, Germany), and the filtrate was mixed with hexadimethrine bromide (17736-44; Nacalai Tesque, Kyoto, Japan) at a final concentration of 8 μg/mL. The ME-180 and ID8 cells were cultured with 1 mL of retroviral solution for 24 h, and selected with 1 μg/mL of puromycin dihydrochloride (A11138; Thermo Fisher Scientific) for an additional 7 days.

In vitro cell proliferation assay

The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to analyze the effects of CRP and the LOX-1 inhibitor, BI-0115. Recombinant human CRP was purchased from Oriental Yeast (Tokyo, Japan). BI-0115 was kindly provided by Boehringer Ingelheim via its open-innovation platform, opnMe (https://opnme.com). Cervical cancer cells were plated in 96-well plates (5×10^3 cells/well) and exposed to different concentrations of CRP or BI-0115 in the presence of 10% FBS. After 48 h of incubation, the number of surviving cells was assessed by determining the absorbance of the dissolved formazan product at 450 nm, according to the manufacturer's protocol.

Western blotting

Whole cell lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, MA, USA) containing a Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Each cell lysate (10 μg/ lane) was loaded on mPAGE 4–20% Bis-Tris Precast Gel (Merck Millipore, MA, USA) and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto an Immobilon-P transfer membrane (IPVH07850; Merck Millipore), which was then incubated with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA). The membranes were then incubated with using Clarity Western ECL Substrate (1705060; Bio-Rad Laboratories, Hercules, CA, USA) and the bands were detected using a ChemiDoc Touch MP imaging system (Bio-Rad Laboratories).

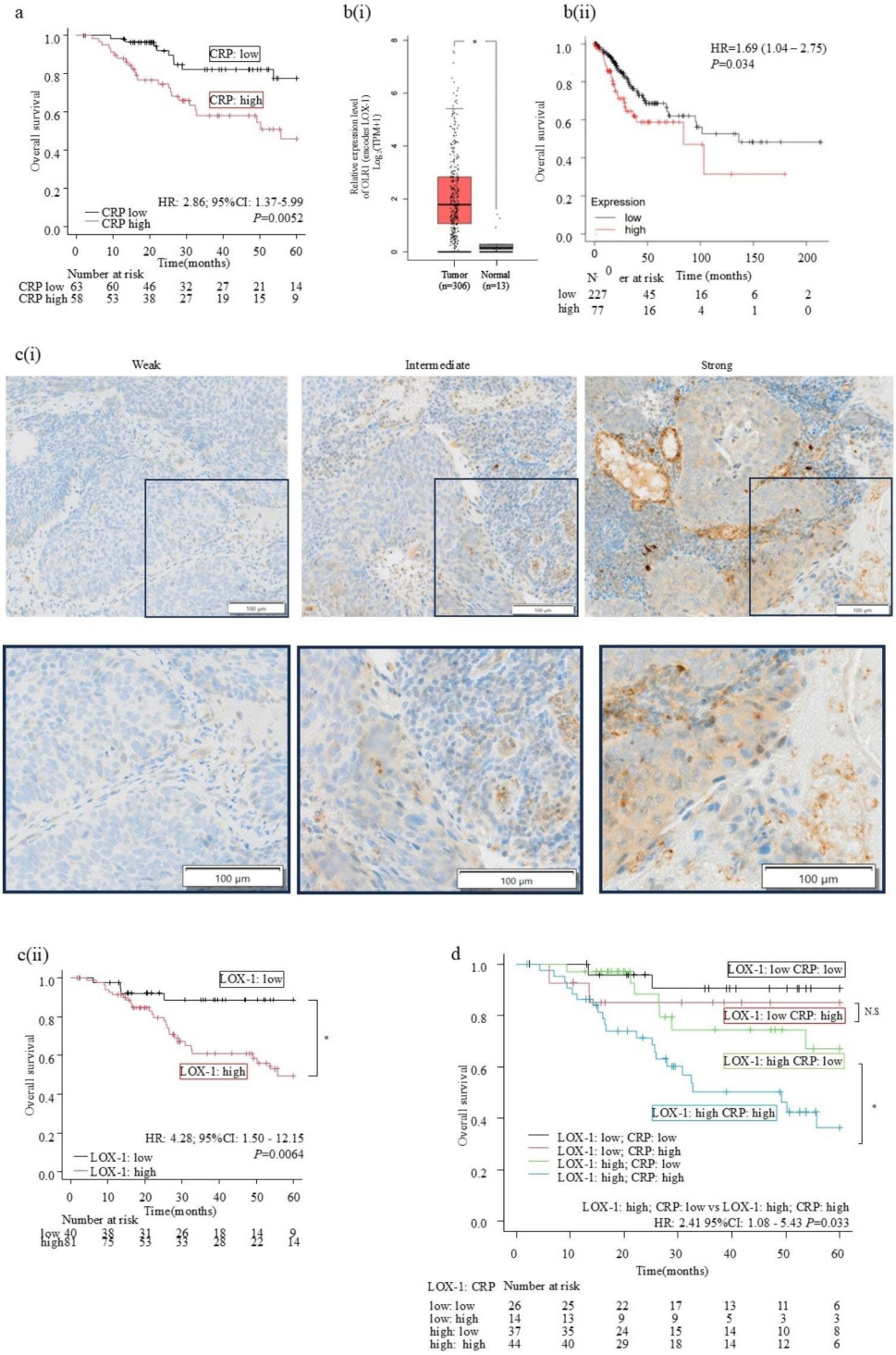
The following antibodies were used for western blotting: p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, MA, USA), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb (Cell Signaling Technology), Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology), Akt Antibody (Cell Signaling Technology), GAPDH (D16H11) XP® Rabbit mAb (Cell Signaling Technology) and β-Actin (13E5) Rabbit mAb (Cell Signaling Technology).

In vivo mouse studies

All procedures involving mice and their care were approved by the Institutional Animal Care and Usage Committee of the Osaka International Cancer Institute in accordance with the institutional and National Institutes of Health guidelines. All mice were purchased by CLEA Japan. Isoflurane anesthesia was used for euthanasia method. the study is reported in accordance with ARRIVE guidelines. To examine the tumor-promoting activity of CRP in vivo, female BALB/c nude mice, aged 5 to 7 weeks, were inoculated with 3×10^6 ME180-control or ME180-LOX1 cells in 100 μL of phosphate-buffered saline (PBS) ($n = 5$ per group). Recombinant human CRP was subcutaneously administered from day 7 of inoculation at a dose of 100 μg per mouse three times a week. Tumor growth was assessed in two dimensions using calipers, and tumor volume was calculated using the formula $V = L \times W \times W \times 1/2$, where V is the volume, L is the length, and W is the width.

Isolation of MDSCs

To obtain mouse MDSCs for analyses, female C57BL/6 mice, aged 5–7 weeks, were inoculated with ID-8-G-CSF cells, based on our previous studies demonstrating that tumor-derived G-CSF increases the number of MDSCs


a
CRP: low
CRP: high
Overall survival
HR: 2.86; 95%CI: 1.37-5.99
P=0.0052
CRP low
CRP high
Time(months)
Number at risk
CRP low 63 60 46 32 27 21 14
CRP high 58 53 38 27 19 15 9
b(i)
Relative expression level of OLR1 (encodes LOX-1) Log2(TPM+1)
Tumor (n=306)
Normal (n=13)
b(ii)
HR=1.69 (1.04 – 2.75)
P=0.034
Overall survival
Expression
low
high
Number at risk
Time (months)
low 227 45 16 6 2
high 77 16 4 1 0
c(i)
Weak
Intermediate
Strong
100 μm
c(ii)
LOX-1: low
LOX-1: high
Overall survival
HR: 4.28; 95%CI: 1.50 - 12.15
P=0.0064
LOX-1: low
LOX-1: high
Time(months)
Number at risk
low 40 38 31 26 18 14 9
high 81 75 53 35 28 22 14
d
LOX-1: low CRP: low
LOX-1: low CRP: high
LOX-1: high CRP: low
LOX-1: high CRP: high
N.S
Overall survival
LOX-1: low; CRP: low
LOX-1: low; CRP: high
LOX-1: high; CRP: low
LOX-1: high; CRP: high
LOX-1: high; CRP: low vs LOX-1: high; CRP: high
HR: 2.41 95%CI: 1.08 - 5.43 P=0.033
Time(months)
LOX-1: CRP Number at risk
low: low 26 25 22 17 13 11 6
low: high 14 13 9 9 5 3 3
high: low 37 35 24 15 14 10 8
high: high 44 40 29 18 14 12 6

◀**Fig. 1**. **a**: Kaplan–Meier estimates of overall survival (OS) according to pretreatment CRP levels [High (≥ 0.3 mg/dL) versus Low (< 0.3 mg/dL); Hazard ratio (HR): 2.86; 95% confidence interval (CI): 1.37–5.99; $P = 0.0052$]. The numbers at risk per group are shown at the bottom of the figure. Log-rank test for *P* values. *$P < 0.05$ **b** (i): LOX-1 mRNA expression in cervical cancer cells. mRNA expression of LOX-1 was significantly upregulated in cervical cancer compared with that in normal cervical tissue (2.45 vs. 0.11; $P < 0.05$). **b** (ii): Kaplan–Meier (KM) estimates of OS using the KM plotter, a platform for performing survival analysis in real time using transcriptomic data from large patient cohorts. High LOX-1 mRNA expression is associated with shorter overall survival (HR: 1.69; 95%CI: 1.04–2.75; $P = 0.034$). **c** (i): Representative image of LOX-1 staining according to tumoral LOX-1 intensity. **c** (ii): KM estimates of OS according to LOX-1 expression [High vs. Low; HR: 4.28; 95%CI: 1.50–12.15; $P = 0.0064$]. The numbers at risk per group are shown at the bottom of the figure. Log-rank test for *P* values. *$P < 0.05$. **d**: KM estimates of OS according to CRP levels and tumor LOX-1 expression. CRP has a prognostic value only in patients with high LOX-1 expression [LOX1-high and CRP-low vs. LOX1-high and CRP-high; HR: 2.41 95%CI: 1.08–5.43 $P = 0.033$]. *$P < 0.05$, N.S indicates not significant.

that can be used for experimental purposes[6,7]. Two to four weeks after inoculation, the mice were sacrificed by isoflurane anesthesia, and their spleens were removed for analyses. MDSCs were isolated from single-cell preparations of mouse splenocytes using the EasySep Mouse MDSC Isolation Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. The purity of the isolated cell populations was determined using flow cytometry, and the frequency of $CD11b^+$ $Gr\text{-}1^+$ cells was > 95% (Supplementary Fig. 1.).

Flow cytometry analysis

Single-cell suspensions prepared from mouse spleen, human peripheral blood, or cervical cancer cells were incubated with antibodies and analyzed using flow cytometry. Flow cytometry data were acquired using a Spectral Cell Analyzer SA3800 (Sony Biotechnology, CA, USA) and analyzed using the FlowJo v10.8.1 software (BD Biosciences, NJ, USA). Cells that had been incubated with irrelevant isotype-matched antibodies and unstained cells served as controls. The Zombie NIR Fixable Viability Kit (BioLegend, CA, USA) was used to eliminate dead cells in the human peripheral blood. The following labeled antibodies were used: anti-human antibodies: Pacific Blue-conjugated anti-HLA-DR antibody (BioLegend, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti-CD15 antibody (BioLegend), phycoerythrin (PE)-conjugated anti-human LOX-1 antibody (BioLegend), Brilliant Violet 605-conjugated anti-human CD14 antibody (BioLegend), allophycocyanin (APC)-conjugated anti-human CD33 antibody (BioLegend) and Alexa Fluor 700-conjugated anti-human CD11b antibody (BioLegend), anti-mouse antibodies; PE-conjugated anti-mouse LOX-1 antibody (R&D systems), FITC anti-mouse/human CD11b antibody (BioLegend), APC anti-mouse Gr-1 antibody (BioLegend), APC anti-mouse CD8a antibody (BioLegend), and APC anti-mouse CD274 (B7-H1, PD-L1) antibody (BioLegend).

T-cell proliferation assay

For T-cell proliferation assay, T cells were seeded in 96-well plates (8×10^4 cells/well) and 2 µL of Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) was added to each well. $CD8^+$ T cells were purified from the spleen of C57BL/6 mice using a $CD8a^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's instructions and labeled with carboxyfluorescein succinimidyl ester (CFSE) (Dojindo Laboratories). To assess the effect of MDSCs on $CD8^+$ T cell proliferation, $CD8^+$ T cells were cocultured with or without MDSCs and CRP for 72 h. T-cell proliferation was evaluated using flow cytometry.

Statistical analysis

Continuous data were compared between groups using Student's *t*-test, Wilcoxon rank-sum test, or median test, as appropriate. Frequency counts and proportions were compared between groups using the χ^2 test or a two-tailed Fisher exact test, as indicated. Comparisons of the means among more than two groups were performed with two-way ANOVA followed by post hoc testing with Tukey's or Dunnett's test. The Spearman's correlation coefficient with a 95% confidence interval (CI) was calculated to assess the relationship between serum CRP levels and the percentage of LOX-1-expressing MDSCs. Univariate analysis was performed by comparing the Kaplan–Meier curves of the subgroups using the log-rank test. Cox proportional hazards regression analysis was performed to identify independent prognostic factors for overall survival. *P*-values < 0.05 were considered statistically significant. All statistical tests were two-sided. EZR (ver. 1.41; Jichi Medical University Saitama Medical Center, Saitama, Japan) was used for all analyses[19].

Results

Prognostic significance of pretreatment serum CRP levels and tumor LOX-1 expression in patients with cervical caner

We first evaluated the survival of patients with cervical cancer according to their pretreatment CRP levels. Baseline characteristics of the patients are presented in Supplementary Table 1. As shown (Fig. 1a), increased pretreatment CRP levels (CRP ≥ 0.3 mg/dL) were associated with significantly shorter overall survival [Hazard ratio (HR): 2.86; 95%CI: 1.37–5.99; $P = 0.0052$]. Next, we examined the prognostic significance of tumor LOX-1 expression in cervical cancer using mRNA expression data from TCGA and GTEx. Tumor LOX-1 mRNA expression was significantly upregulated in cervical cancer tissues [2.45 vs. 0.11; $P < 0.05$; Fig. 1b (i)]. Moreover, in the survival analysis, high LOX-1 mRNA expression was associated with shorter overall survival [HR: 1.69; 95%CI: 1.04–2.75; $P = 0.034$; Fig. 1b (ii)]. We verified these results using biopsy samples and survival data

Patient characteristics		LOX-1 low ($n=40$)	LOX-1 high ($n=81$)	*P*-value
Age	Median (range)	60 (31–73)	58 (28–87)	0.70
BMI kg/m^2	Median (range)	22.3 (17.6–32.9)	21.8 (15.6–29.0)	0.53
Primary treatment	RT	4 (10.0%)	10 (12.3%)	1
	CCRT	36 (90.0%)	71 (87.7%)	
Histology	SCC	36 (90.0%)	71 (87.7%)	1
	Non-SCC	4 (10.0%)	10 (12.3%)	
Stage	I	2 (5.0%)	4 (4.9%)	0.73
	II	11 (27.5%)	15 (18.5%)	
	III	24 (60.0%)	55 (67.9%)	
	IV	3 (7.5%)	7 (8.6%)	
Pretreatment CRP level	Mean (range)	0.14(0.01–16.79)	0.39 (0.01–20.89)	0.073
	≥ 0.3 mg/dL	14 (35.0%)	44 (54.3%)	0.054
	< 0.3 mg/dL	26 (65.0%)	37 (45.7%)	
Recurrence		11 (27.5%)	40 (49.4%)	0.031
Death		4 (10.0%)	30 (37.0%)	0.0023

Table 1. Baseline characteristics according to LOX-1 expression. Abbreviations: LOX-1; lectin-like oxidized low density lipoprotein receptor-1, BMI; body mass index, RT; radiotherapy, CCRT; concurrent chemoradiotherapy SCC; squamous cell carcinoma, CRP; c-reactive protein.

		Univariate analysis		stepwise multivariate analysis	
		HR (95% CI)	*P*	HR (95%CI)	*P*
Age	≤ 50	1	0.28		
	> 50	1.57(0.69–3.58)			
BMI	≤ 25	1	0.39		
	> 25	1.43(0.63–3.27)			
Histology	SCC	1	0.14		
	Non-SCC	2.00(0.79–5.06)			
Treatment	RT	1	0.011	1	0.081
	CCRT	0.55 (0.22–1.37)		0.47 (0.20–1.10)	
FIGO stage	IB-IIB	1	0.091		
	IIIA-IVB	1.14 (0.42–3.04)			
LOX-1	Low	1	0.026	1	0.029
	High	3.39 (1.16–9.92)		3.27 (1.13–9.47)	
CRP	Low	1	0.035	1	0.033
	High	2.35(1.06–5.20)		2.26 (1.07–4.78)	

Table 2. Uni- and Stepwise multivariate analyses of overall survival for patients with cervical cancer. HR; hazard ratio, CI, confidence interval; BMI; body mass index, SCC; squamous cell carcinoma, RT; radiotherapy, CCRT; concurrent chemoradiotherapy, FIGO; international federation of gynecology and obstetrics, LOX-1; lectin-like oxidized low density lipoprotein receptor-1, CRP; c-reactive protein.

obtained from 121 patients with cervical cancer administered definitive radiotherapy at our institution (Table 1; Fig. 1c). Figure 1c (i) shows a representative image of the LOX-1 immunoreactivity. Patients with high tumoral LOX-1 expression (81 of 121) showed significantly shorter overall survival than those with low tumoral LOX-1 expression [HR: 4.28; 95%CI: 1.50–12.15; $P=0.0064$; Fig. 1c (ii)]. In multivariate analyses, high tumoral LOX-1 expression and increased pretreatment CRP levels were found to be independent prognostic factors for overall survival (Table 2). Importantly, when the prognostic significance of pretreatment CRP levels was evaluated in association with tumor LOX-1 expression, a significant difference in survival was observed (Fig. 1d), CRP levels were found to have a prognostic value only in patients with high LOX-1 expression ($P=0.033$). These results strongly suggest that serum CRP affects the survival of patients with cervical cancer via LOX-1 and downstream effectors.

CRP May promote the progression of cervical cancer by stimulating LOX-1 expression on tumor cells

To investigate the mechanism of CRP-mediated progression of cervical cancer, we first established the following experimental models: LOX-1-expressing cervical cancer cells (ME180-LOX1) and LOX-1 non-expressing cells (ME180-control). The expression of LOX-1 in these cells was verified both in vitro and in vivo (Fig. 2a). Next, we investigated the in vitro tumor-promoting effect of CRP on cervical cancer cells using the CCK-8 assay. We first confirmed that the proliferation activities of ME180-LOX1 and ME180-control cells did not differ significantly under the same culture conditions (Supplementary Fig. 2). CRP stimulated the proliferation of ME180-LOX1 cells in a dose-dependent manner [Fig. 2b (i)]. However, the effect was minimal on ME180-control cells [(Fig. 2b (ii)]. Moreover, the CRP-mediated increase in cancer cell proliferation was inhibited by pretreatment with the LOX-1 inhibitor Bl-0115 [Fig. 2b (iii)]. To further investigate the mechanism, we examined the effect of CRP on the activation of ERK and AKT, which are major proteins downstream of LOX-1[20]. ERK phosphorylation was enhanced in response to CRP treatment (Fig. 2c (i)). Moreover, CRP-mediated ERK activation was abrogated by pretreatment with BI-0115 [Fig. 2c (ii)]. AKT phosphorylation was not induced by CRP treatment [Fig. 2c (iii)]. We further evaluated the tumor-promoting effect of CRP on LOX-1-expressing cervical cancer cells in vivo (Fig. 2d). Treatment of mice with CRP significantly promoted the progression of LOX-1-expressing cervical cancer (ME180-LOX1). However, the effect of CRP was not significant on LOX-1 non-expressing cervical cancer (ME180-control). Collectively, these data suggested that CRP promotes the proliferation of cervical cancer cells in association with the stimulation of LOX-1 and downstream ERK signaling.

Prognostic significance of increased pretreatment CRP levels together with stromal LOX-1 expression in cervical cancer patients

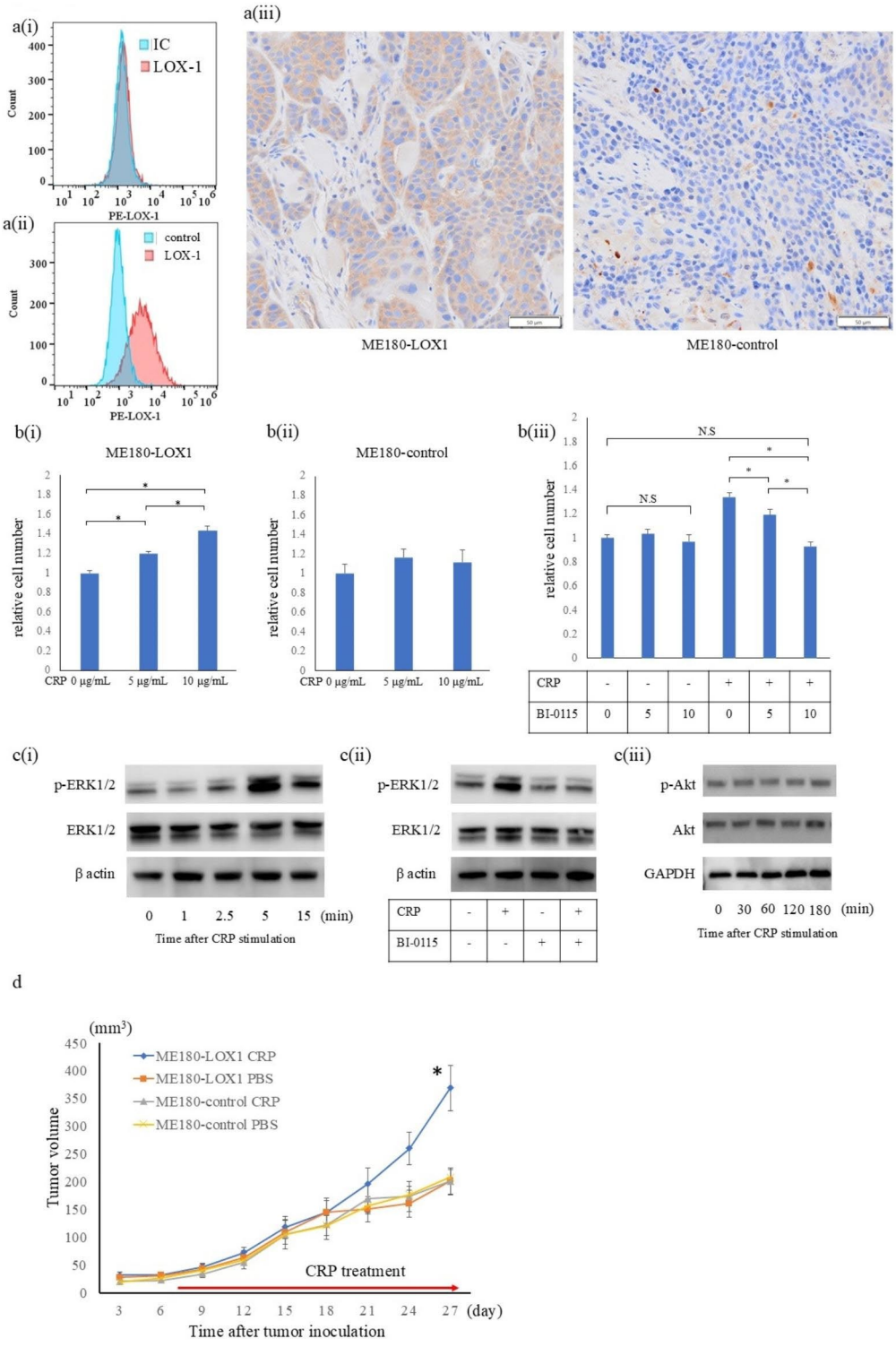
We examined the frequencies of LOX-1-expressing MDSCs in peripheral blood and stromal LOX-1 expression in cervical cancer tissues. Using pretreatment biopsy samples obtained from patients with cervical cancer administered definitive radiotherapy, we investigated the prognostic significance of stromal LOX-1 expression. Immunohistochemical analysis revealed LOX-1-expressing cells in the stroma of cervical cancer [Fig. 3a (i)]: the representative photographs showing high numbers of stromal LOX-1-expressing cells and low numbers of LOX-1-expressing cells are presented. When patients were divided into two groups based on the mean density, patients with cervical tumor exhibiting high numbers of stromal LOX-1-expressing cells (stromal LOX-1-High, $>40.6/mm^2$) had shorter progression-free survival than those with cervical tumor exhibiting low numbers of stromal LOX-1-expressing cells (stromal LOX-1-Low, $\leq 40.6/mm^2$) [HR: 2.36; 95%CI: 1.36–4.10; $P=0.0024$; Fig. 3a (ii)].

We performed mIF staining to examine the biological characteristics of the LOX-1-expressing stromal cells (Fig. 3b). Some stromal cells expressing LOX-1 also expressed CD33, a well-recognized MDSC marker, strongly indicating that some of the LOX1-positive stromal cells exhibit MDSC features. Next, we investigated LOX-1 expression in MDSCs obtained from the peripheral blood of 27 patients with cervical cancer, administered definitive radiotherapy, using flow cytometric analysis. The baseline characteristics of the enrolled patients are presented in Supplementary Table 2. For this purpose, we defined MDSCs as HLA-DR$^-$ CD11b$^+$ CD14$^-$ CD15$^+$ CD33$^+$, and LOX-1 positivity was determined by incubating cells with an irrelevant isotype-matched antibody and confirming LOX-1 expression on the surface of MDSCs. The gating strategy and representative staining results are shown in Fig. 3c and Supplementary Fig. 3.

As LOX-1 expression on the surface of MDSCs in patients with cervical cancer was confirmed, we investigated the relationship between the number of LOX-1-expressing MDSCs and pretreatment serum CRP levels. As shown in Fig. 3d, a positive correlation was observed between LOX-1-expressing MDSCs and pretreatment serum CRP levels ($R=0.50$; 95%CI 0.14–0.74; $P<0.01$; Fig. 3d). LOX-1-expressing MDSCs were more frequently observed in patients with higher pretreatment CRP levels than in those with lower pretreatment CRP levels ($P=0.045$; Fig. 3e). Based on our investigations of stromal LOX-1 expression and its association with CRP and survival of patients with cervical cancer, we surmised that CRP appeared to be associated with tumor progression through the stimulation of LOX-1-expressing MDSCs.

Effects of CRP on LOX-1 expression and suppressive functions of MDSCs

Finally, we explored the mechanisms underlying CRP-mediated tumor progression via LOX-1-expressing MDSCs. Based on previous reports showing that CRP stimulates the expression of LOX-1 in endothelial cells[21] and PD-L1 expression is stimulated via the activation of the ERK-MAPK signaling pathway[22], we evaluated the effect of CRP on the expression of LOX-1 and PD-L1 on MDSCs. We obtained MDSCs, defined as CD11b$^+$ Gr-1$^+$ cells, from mice with G-CSF-expressing cervical cancer, as previously reported[7,8]. The gating strategy is shown in **Supplementary Fig. 4a**. We first confirmed the expression of LOX-1 on MDSCs (Fig. 4a). Using these MDSCs, we investigated the effects of CPR. As shown in Fig. 4b, treatment of MDSCs with CRP increased the expression of both LOX-1 [Fig. 4c **(i)**] and PD-L1 [Fig. 4c **(ii)**]. We further evaluated the effect of CRP on the suppressive function of MDSCs (Fig. 4d and **Supplementary Fig. 4b**). MDSCs significantly suppressed expansion of T cells in a dose-dependent manner and stimulation of MDSCs with CRP further enhanced the suppressive effects of MDSCs on the proliferation of CD8$^+$ T cells (Fig. 4d). Finally, we evaluated the effects of CRP on the viability of MDSCs. The number of viable MDSCs increased in a dose-dependent manner following CRP treatment [Fig. 4e **(i)**]. Moreover, the stimulatory effect of CRP on the viability of MDSCs was abrogated by pretreatment with an anti-LOX-1 neutralizing antibody [Fig. 4e **(ii)**]. To further investigate the mechanism, we examined the effect of CRP on the activation of ERK in MDSCs. Consistent with the findings from cervical cancer cells, ERK phosphorylation was enhanced in response to CRP treatment (Fig. 4f). These results suggest that CRP promotes the survival of MDSCs and enhances their suppressive activity via LOX-1.


a(i)
IC
LOX-1
Count
PE-LOX-1
a(ii)
control
LOX-1
Count
PE-LOX-1
a(iii)
ME180-LOX1
ME180-control
b(i)
ME180-LOX1
relative cell number
CRP 0 μg/mL
5 μg/mL
10 μg/mL
b(ii)
ME180-control
relative cell number
CRP 0 μg/mL
5 μg/mL
10 μg/mL
b(iii)
N.S
relative cell number
CRP - - - + + +
BI-0115 0 5 10 0 5 10
c(i)
p-ERK1/2
ERK1/2
β actin
0 1 2.5 5 15 (min)
Time after CRP stimulation
c(ii)
p-ERK1/2
ERK1/2
β actin
CRP - + - +
BI-0115 - - + +
c(iii)
p-Akt
Akt
GAPDH
0 30 60 120 180 (min)
Time after CRP stimulation
d
(mm³)
ME180-LOX1 CRP
ME180-LOX1 PBS
ME180-control CRP
ME180-control PBS
Tumor volume
CRP treatment
Time after tumor inoculation
(day)

◀**Fig. 2**. **a** (i): Flow cytometry analysis of ME-180 cells. IC indicates isotype-control antibody. **a** (ii): Flow cytometry analysis of ME-180 cells infected with OLR-1 retroviral (ME180-LOX1) and control (ME180-control) particles. Control indicated ME180-control and LOX1 indicated ME180-LOX1. **a** (iii): Representative immunohistochemical staining for LOX-1 in subcutaneous ME180-LOX1- or ME180-control-derived tumors (bar: 50 μm). **b** (i): Effect of CRP on the proliferation of ME180-LOX1. ME180-LOX1 cells were treated with or without recombinant human CRP (0, 5, and 10 μg/mL) for 48 h. Cell proliferation was assessed using the CCK-8 assay ($n = 5$). Bars, SE. $^*P < 0.05$. **b** (ii): Effect of CRP on the proliferation of ME180-control. ME180-control cells were treated with or without recombinant human CRP (0, 5, and 10 μg/mL) for 48 h. Cell proliferation was assessed using the CCK-8 assay ($n = 5$). Bars, SE. $^*P < 0.05$. **b** (iii): Effect of LOX-1 inhibitor, BI-0115, on CRP-induced cell proliferation of ME180-LOX1. ME180-LOX1 cells were treated with or without CRP (10 μg/mL) and with or without BI-0115 (0, 5, and 10 μM) after 48 h. Cell proliferation was assessed using the CCK-8 assay ($n = 5$). Bars, SE. $^*P < 0.05$. **c** (i): Effect of CRP treatment on ERK 1/2 activation in ME180-LOX1. ME180-LOX1 cells were treated with recombinant human CRP (0 and 50 μg/mL). Activation of ERK1/2 in ME180-LOX1 cells was assessed using western blotting. **c** (ii) Effect of BI-0115 on CRP-induced ERK1/2 activation in ME180-LOX1 cells. ME180-LOX1 cells were treated with recombinant human CRP (0 and 50 μg/mL) or BI-0115 (0 and 10 μM) for 5 min. Activation of ERK1/2 in ME180-LOX1 cells was assessed using western blotting. **c** (iii): Effect of CRP treatment on AKT activation in ME180-LOX1 cells. ME180-LOX1 cells were treated with recombinant human CRP (0 and 50 μg/mL). Activation of AKT in ME180-LOX1 cells was assessed using western blotting. **d**: In vivo tumor promoting effects of CRP on ME180-LOX1- or ME180-control-derived tumors. BALB/c nude mice were inoculated with ME180-LOX1 or ME180-control cells. One week after subcutaneous inoculations of ME180-LOX1 or ME180-control cells, the mice were assigned to two treatment groups: PBS (3-times a week, $n = 5$), CRP (recombinant human CRP 100 μg, 3-times a week, $n = 5$). The volumes of the tumors were measured every 3 days for 4 weeks. Bars, SE. $^*P < 0.05$ using Tukey's honestly significant difference tests.

Our results, shown in Figs. 1, 2, 3 and 4, indicate that CRP promotes the progression of cervical cancer by stimulating LOX-1 expression on both cancer cells and MDSCs (Supplementary Fig. 5).

Discussion

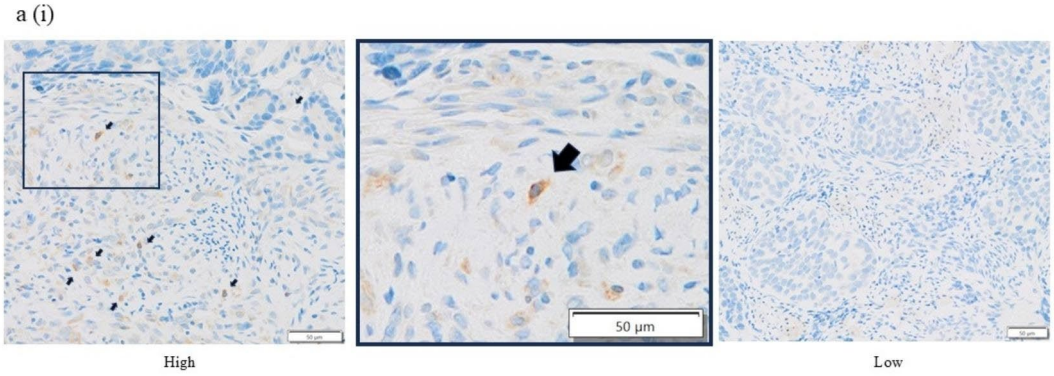
The key findings of the present study are as follows: (i) patients with elevated pretreatment CRP levels showed significantly shorter overall survival when their cervical tumor expressed LOX-1; (ii) CRP promoted the progression of cervical cancer by stimulating LOX-1 expression on cancer cells and its downstream effectors; (iii) by stimulating LOX-1 expression on MDSCs, CRP not only prolonged the survival of MDSCs, but also enhanced PD-L1 expression on MDSCs and directly augmented their suppressive activities, all of which can lead to the progression of LOX-1-expressing cervical cancer. To our knowledge, this study is the first to identify LOX-1 expression in cervical cancer and to demonstrate the interaction between LOX-1 and CRP in human malignancies. Moreover, this is the first study to demonstrate the prognostic significance of increased pretreatment CRP levels in patients with LOX-1-expressing cancer.

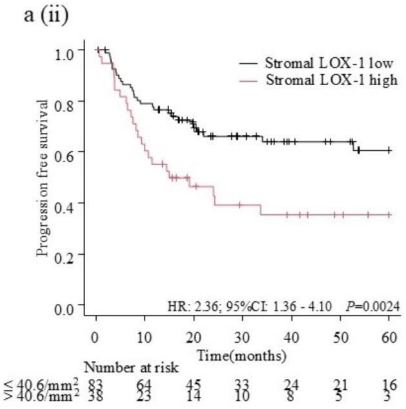
Our results are consistent, at least in part, with those of a previous clinical study showing that elevated serum pretreatment CRP level is an indicator of poor prognosis in patients with cancer, including cervical cancer[9]. However, our findings are unique and scientifically important because we examined the prognostic significance of pretreatment CRP levels in patients with cancer in relation to LOX-1 levels. We confirmed LOX-1 expression in cervical cancer [Fig. 1c (i)], and showed that higher LOX-1 expression is associated with shorter overall survival in patients with cervical cancer [Fig. 1c (ii)]. Importantly, CRP was found to be prognostically important only in patients with high LOX-1 expression (Fig. 1d), strongly indicating the possibility that CRP promotes tumor progression by stimulating LOX-1.

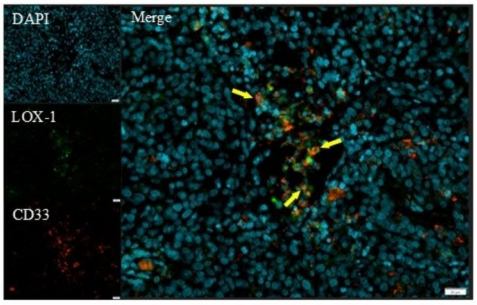
Although our study is the first to demonstrate that the CRP–LOX-1 axis stimulates cancer progression, it is consistent with the following previous findings in endothelial cells: (1) LOX-1 binds to a broad spectrum of ligands with high affinity, such as ox-LDL, HSP70, bacterial products, and CRP[23]; (2) CRP can enhance LOX-1 expression[21]; (3) CRP binds to LOX-1 to enhance vascular permeability and mediate endothelial dysfunction[17,24].

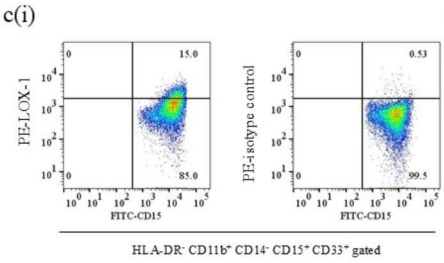
Previous studies have shown that the expression of LOX-1 in tumor cells is associated with poor prognosis in colorectal, esophageal, and pancreatic cancers[12–14]. Mechanistically, LOX-1 has been shown to promote the migration and invasion of gastric cancer cells by driving epithelial–mesenchymal transition through PI3K/Akt/GSK3β activation[25]. Moreover, LOX-1 was shown to modulate the RAS-MEK-ERK signaling pathway to keep the transcription factor EB (TFEB) inactive in the cytosol by interacting with receptor for activated C kinase 1 (RACK1) in esophageal cancer cells[13]. However, the mechanisms underlying the poor prognosis of LOX-1-expressing cancer remain largely unknown. In our mechanistic investigation of the CRP-LOX-1 axis in cervical cancer cells, we found that CRP stimulated the proliferation of LOX-1-expressing cervical cancer cells by inducing the activation of ERK (Fig. 2c), a downstream effector of LOX-1 in endothelial cells[21].

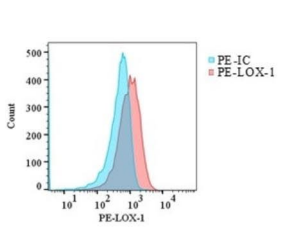
Our results regarding MDSCs presented in Figs. 3 and 4 are also partially consistent with previous reports on cervical cancer showing that an increased number of pretreatment MDSCs is associated with an advanced clinical stage, a high probability of recurrence after definitive radiotherapy, and short survival[7,8]. CRP promotes the expansion of MDSCs, and LOX-1-expressing MDSCs have greater suppressive effect on $CD8^+$ T cells than LOX-1-negative MDSCs[15]. However, we are the first to show that CRP promotes the survival of LOX-1-expressing MDSCs (Fig. 4c and e) and PD-L1 expression (Fig. 4c). Taken together, these results indicate that LOX-1 expressed on cancer cells and MDSCs may be a promising prognostic biomarker for cervical cancer and may offer a novel molecular target for cervical cancer treatment.

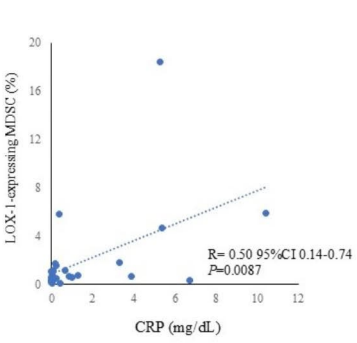

a (i)
50 μm
High
Low

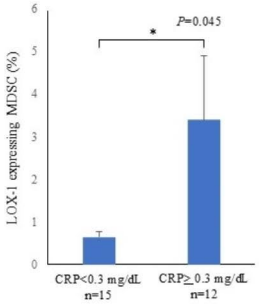

a (ii)
Stromal LOX-1 low
Stromal LOX-1 high
Progression free survival
HR: 2.36; 95%CI: 1.36 - 4.10 P=0.0024
Time(months)
Number at risk
≥ 40.6/mm2 83 64 45 33 24 21 16
< 40.6/mm2 38 23 14 10 8 5 3


b
DAPI
Merge
LOX-1
CD33


c(i)
PE-LOX-1
FITC-CD15
15.0
85.0
PE-isotype control
0.53
99.5
HLA-DR- CD11b+ CD14- CD15+ CD33+ gated


c(ii)
PE-IC
PE-LOX-1
Count


d
LOX-1-expressing MDSC (%)
R= 0.50 95%CI 0.14-0.74
P=0.0087
CRP (mg/dL)

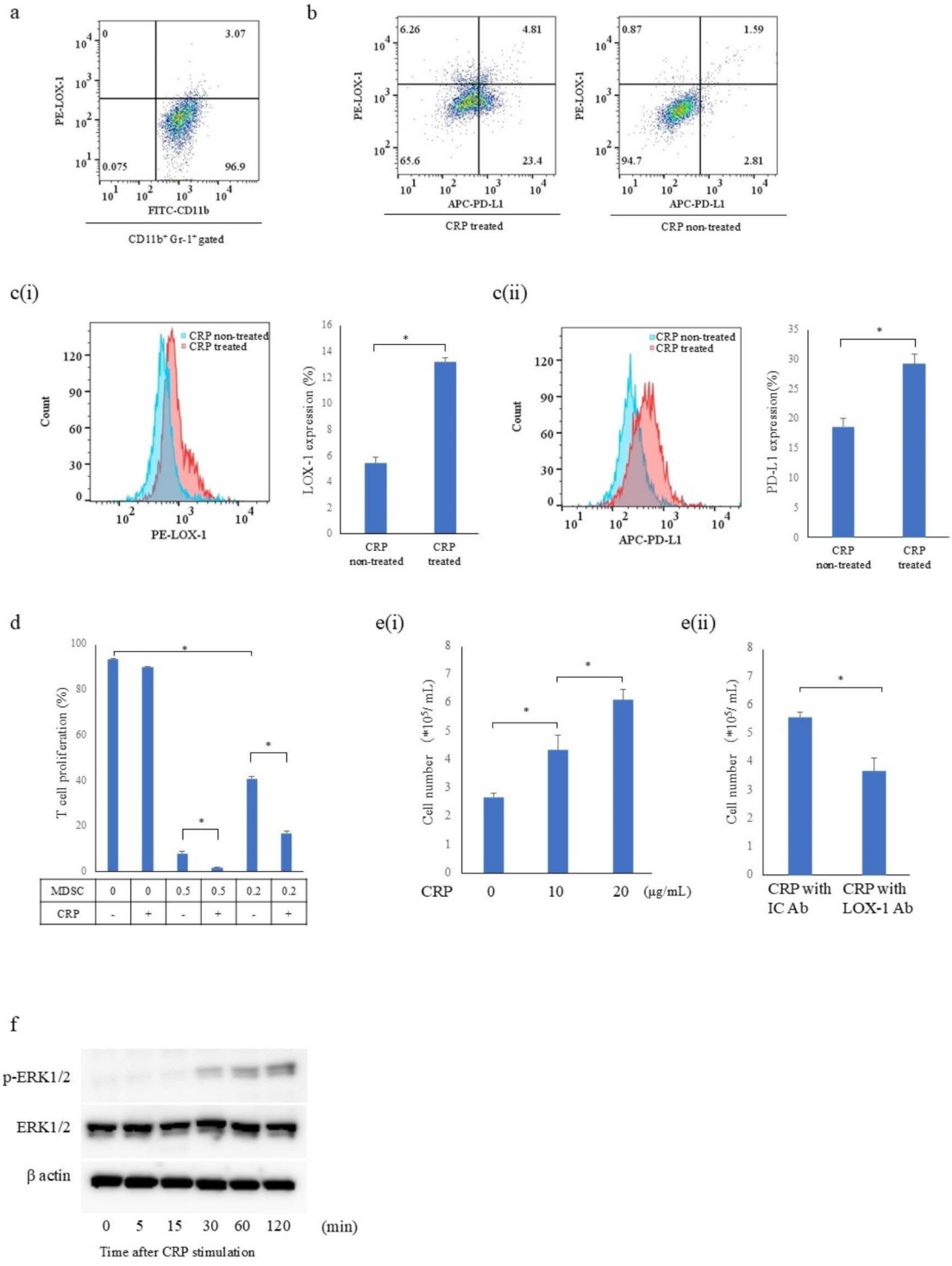

e
P=0.045
LOX-1 expressing MDSC (%)
CRP<0.3 mg/dL
n=15
CRP≥0.3 mg/dL
n=12

◀**Fig. 3**. **a** (i): Representative immunostaining image of LOX-1-expressing cells in stroma (black allows). **a** (ii): Kaplan–Meier estimates of progression-free survival according to the density of LOX-1-expressing stromal cells [high density ($>40.6/mm^2$) versus low density ($\leq 40.6/mm^2$); Hazard ratio (HR): 2.36; 95% confidence interval (CI): 1.36–4.10; $P<0.01$]. **b**: Evaluation of LOX-1-expressing cells using multiplex immunofluorescence in cervical cancer tissues. LOX-1 (green) and CD33 (red). Nuclei were stained with DAPI (blue). Scale bar = 20 μm. Some LOX-1-expressing stromal cells partially express $CD33^+$ cells (yellow arrows).**c**: LOX-1-expressing MDSCs in the peripheral blood cells of patients with cervical cancer assessed using flow cytometry analysis. MDSCs were defined as $HLA\text{-}DR^-$ $CD11b^+$ $CD14^-$ $CD15^+$ $CD33^+$. The LOX-1-positivity was determined by incubating cells with irrelevant isotype-matched antibody. **d**: Correlation between pretreatment CRP levels and number of LOX-1-expressing MDSCs in human peripheral blood. A weak positive correlation was observed between the percentage of LOX-1-positive MDSCs and CRP ($R=0.50$; 95%CI: 0.14–0.74; $P<0.01$). **e**: LOX-1-expressing MDSCs in patients with cervical cancer according to the pretreatment CRP levels. The number of LOX-1-expressing MDSCs in patients exhibiting high pretreatment CRP levels was significantly higher than of those exhibiting low CRP levels ($P=0.045$). Bars, SE.

Our results have important clinical implications. They provide novel insights into the mechanisms underlying inflammation-dependent and CRP-mediated cancer progression. Our mechanistic investigations and clinical studies suggest that prolonged exposure to high CRP levels enhances the growth of LOX-1-expressing cervical cancer. These results clarify as to why elevated CRP levels are a poor prognostic factor in patients with various malignancies[26–28]. Our findings indicate that CRP not only directly stimulates the proliferation of cancer cells via LOX-1 expressed on cancer cells, but also induces immune tolerance by stimulating LOX-1-expressing MDSCs, all of which may ultimately stimulate the progression of cervical cancer. The present results also provide a rationale for new treatment strategies for cancers exhibiting increased CRP levels, targeting CRP, LOX-1, downstream effectors of LOX-1, including MAPK, or MDSCs. To date, no specific inhibitors of LOX-1 or MDSCs that can be used in clinical settings have been developed. However, nonspecific inhibitors of MDSCs, such as chemokine inhibitors targeting the CXCL2-CXCR2 axis, IL-6 inhibitors inhibiting the production of CRP, or MAPK kinase inhibitors are available for human use[29–31]. The clinical activity of such treatments alone or in combination with conventional treatments, including chemotherapy and radiotherapy, needs to be investigated in future studies.

This study had some limitations. First, the clinical investigations in the present study were retrospective and conducted at a single institution. Second, although our study was limited to the patients treated with radiation therapy, as only 10% of our patients received radiotherapy alone (90% received concurrent chemoradiotherapy), we could not evaluate the potential impact of concurrent cisplatin during radiotherapy on the prognosis or CRP-LOX-1 axis in the current study. Third, in cancer patients, CRP can be elevated for a variety of reasons, but we could not examine the extent to which these causes influence our results. Therefore, it is unclear whether the role of CRP-LOX-1 axis differs depending on the cause of high CRP levels in our patients. Fourth is a LOX-1 inhibitor, Bl-0115, used in the current study: Bl-0115 can only be used for in vitro experiments, and no other specific inhibitor of LOX-1 is available for animal or clinical use. Fifth, although the present study focused on MDSCs as a source of stromal LOX-1 expression, we cannot exclude the possibility that other stromal cells, such as tumor-associated neutrophils or tumor-associated macrophages, may also express LOX-1 to play a role in the progression of cervical cancer in a CRP-dependent manner. Sixth, we could not evaluate the role of the CRP-LOX-1 axis in the progression of other cancers, although elevated CRP levels have been found to be a prognostic factor in patients with various human malignancies. In this study, we used BALB/c nude mice because inoculation of human uterine cervical cancer cells into immunocompetent mice did not result in tumor development. Although the tumor-promoting effect of CRP via LOX-1 expression on tumor cells could be evaluated in this experimental model, the suppressive effect of LOX-1-positive MDSCs on $CD8^+$ T cells could not be evaluated because of the immunodeficient nature of the mice. Last, although there are many articles that suggest that patients with high CRP levels have a poor prognosis, the majority of these studies are retrospective investigations of clinical data, and do not examine the underlying mechanism of poor prognosis. Therefore, at present, the reason why C-reactive protein is associated with poor prognosis in cancer patients is largely unknown. Although our results suggested that CRP/LOX-1 mechanism is one of the causes, it is possible that other complementary mechanisms may exist. In fact, a previous preclinical investigations of breast cancer suggested that C-reactive protein binds to integrin α2 and Fcγ receptor I, resulting in the enhanced cell adhesion and progression in vitro[32]. Therefore, further studies on CRP-mediated cancer progression pathways are warranted. Therefore, the clinical implications of the CRP-LOX-1 axis in cancer and the mechanisms by which CRP stimulates cancer progression require further investigation.

In conclusion, increased pretreatment serum CRP levels are associated with shorter overall survival in patients with LOX-1-expressing cervical cancer. CRP may facilitate the progression of LOX-1-expressing cervical cancer by stimulating LOX-1 and its downstream effectors in cancer cells and MDSCs. Novel treatments targeting the CRP-LOX-1 axis may have therapeutic efficacy in patients with LOX-1-expressing cervical cancer who exhibit increased pretreatment serum CRP levels.



MDSC	0	0	0.5	0.5	0.2	0.2
CRP	-	+	-	+	-	+

◀**Fig. 4**. **a**: LOX-1-expressing MDSCs in the spleen of mice inoculated with ID8-G-CSF, assessed using flow cytometry analysis. MDSCs were defined as $CD11b^+$ $Gr\text{-}1^+$. The LOX-1-positivity was determined by incubating cells with irrelevant isotype-matched antibody. **b**: Effect of CRP treatment on the expression of LOX-1 and PD-L1 in MDSCs. MDSCs were treated with or without recombinant human CRP (0 and 10 μg/mL) for 72 h. Thereafter, the LOX-1 or PD-L1 expression was assessed using flow cytometry analysis. **c** (i), (ii): Effect of CRP treatment on the expression of LOX-1 and PD-L1 in MDSCs. MDSCs were treated with or without recombinant human CRP (0 and 10 μg/mL) for 72 h. Thereafter, the LOX-1 or PD-L1 expression was assessed using flow cytometry analysis. Representative histograms of LOX-1 and PD-L1 and the percentage of LOX-1- and PD-L1-positive cells in MDSCs are shown ($n = 4$). Bars, SE. **d**: Effect of CRP on the ability of MDSCs to suppress $CD8^+$ T cells. Mouse $CD11b^+$ $Gr\text{-}1^+$ cells (MDSCs) were isolated from the spleen of ID8-G-CSF-derived tumor-bearing BALB/c nude mice, as described in the Methods. $CD8^+$ T cells (8×10^4 cells/well) were isolated from syngeneic mice. Mouse $CD8^+$ T cells were labeled with CFSE and stimulated with Dynabeads Mouse T-Activator CD3/CD28. Cells were cocultured at the indicated ratio for 72 h and T-cell proliferation was evaluated using flow cytometry ($n = 4$). Bars, SE. * indicates statistical significance, using two-sided Student's *t*-test. **e** (i): Effect of CRP on the survival of MDSCs. MDSCs were seeded in a 96-well plate (8×10^4/wells) and counted using an automated cell counter after 72 h of incubation with or without CRP (0, 10, and 20 μg/mL) ($n = 4$). Dead cells were excluded using typan blue staining. Bars, SE. **e** (ii): Effect of anti-LOX-1 antibody on the survival of MDSCs treated with CRP. MDSCs were treated with recombinant human CRP (10 μg/mL) and anti-mouse LOX-1 antibody (10 μg/mL) or isotype control antibody (10 μg/mL) ($n = 4$). Bars, SE. **f**: Effect of CRP treatment on Erk 1/2 activation in MDSCs. MDSCs were treated with recombinant human CRP (0 μg/mL and 50 μg/mL). The activation of ERK1/2 in MDSCs was assessed using western blotting.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.M., designed and performed the experiments, formal analysis, writing—original draft preparation; Se.M., designed the study, the main conceptual ideas, writing—original draft preparation; T.A., Y.M., H.N., and T.E., data curation, supervision; T.S. and N.K., supervision, writing—review and editing; M.S., T.T., Sh.M., and T.H., supervision, writing—review and editing; S.K., T.K., and H.T., funding acquisition, supervision.

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Additional information

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