ELMER PRESS

Original Article

World J Oncol. 2026;17(6):811-819

Fusobacterium nucleatum-Mediated Neutrophil Extracellular Trap Formation Promotes Programmed Cell Death Ligand 1 Expression in Colorectal Cancer Cells via the Toll-Like Receptor 9/Interleukin-1 Receptor-Associated Kinase 4/Nuclear Factor Kappa-B Signaling Pathway

Hong Lei Shen[a, b, c, d, g], Yu Xuan Xie[e, g], Yu Bin Lu[e, g], Yan Yan Lin[e], Jin Lu Wu[e], Xian Long Xie[f], Hao Tao[e], Meng Ling Ye[b, c, d, e, h]

Abstract

Background: Colorectal cancer (CRC) remains a major cause of cancer-related morbidity and mortality worldwide. Immune checkpoint blockade targeting the programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis has shown clinical benefit in selected CRC patients. However, the mechanisms regulating PD-L1 expression in the tumor microenvironment remain incompletely understood. *Fusobacterium nucleatum* (*F. nucleatum*) has been implicated in CRC progression and immune modulation; however, its potential role in regulating PD-L1 expression through neutrophil extracellular traps (NETs) remains to be further elucidated. This study aimed to further confirm whether *F. nucleatum*-induced NET formation promotes PD-L1 expression in CRC cells and to further elucidate the underlying signaling mechanism.

Methods: Human peripheral blood neutrophils were isolated from healthy volunteers and stimulated with *F. nucleatum* to induce NET formation. NET formation was evaluated by immunofluorescence staining of myeloperoxidase (MPO) and citrullinated histone H3 (Cit-H3), together with quantification of extracellular DNA. HCT116 CRC cells were co-cultured with stimulated neutrophils or treated with isolated NETs. PD-L1 expression was assessed by quantitative real-time PCR and Western blotting. DNase I, Toll-like receptor 9 (TLR9) inhibitor, interleukin-1 receptor-associated kinase 4 (IRAK4) inhibitor, and nuclear factor kappa-B (NF-κB) inhibitor were used to examine the involvement of NET-derived DNA and the TLR9/IRAK4/NF-κB pathway.

Results: *F. nucleatum* stimulation promoted NET formation in human neutrophils, as indicated by increased MPO. *F. nucleatum*-stimulated neutrophils upregulated PD-L1 expression in HCT116 cells. Consistently, isolated NETs directly increased PD-L1 expression, whereas DNase I treatment attenuated this effect. Mechanistically, NET exposure was associated with activation of the TLR9/IRAK4/NF-κB signaling pathway, and pharmacological inhibition of TLR9, IRAK4, or NF-κB reduced NET-induced PD-L1 upregulation.

Conclusion: These findings suggest that *F. nucleatum*-induced NET formation enhances PD-L1 expression in CRC cells, at least partly through NET-derived DNA-mediated activation of the TLR9/IRAK4/NF-κB signaling pathway. This microbiota–neutrophil–tumor axis may contribute to immune evasion in CRC and provide a potential therapeutic target for improving immunotherapy efficacy.

Keywords: Colorectal cancer; *Fusobacterium nucleatum*; Neutrophil extracellular traps; PD-L1; TLR9; IRAK4; NF-κB

Manuscript submitted June 11, 2026, accepted August 27, 2026
Published online September 15, 2026

[a]Department of Anesthesiology, Guangxi Medical University Cancer Hospital, Guangxi Medical University, Nanning, Guangxi, China
[b]Guangxi Engineering Research Center for Tissue and Organ Injury and Repair Medicine, Guangxi Medical University, Nanning, Guangxi, China
[c]Guangxi Health Commission Key Laboratory of Basic Science and Prevention of Perioperative Organ Dysfunction, Guangxi Medical University, Nanning, Guangxi, China
[d]Guangxi Clinical Research Center for Anesthesiology, Guangxi Medical University Cancer Hospital, Guangxi Medical University, Nanning, Guangxi, China
[e]Department of Research, Guangxi Medical University Cancer Hospital, Guangxi Medical University, Nanning, Guangxi, China
[f]Intensive Care Unit, Guangxi Medical University Cancer Hospital, Guangxi Medical University, Nanning, Guangxi, China
[g]These authors contributed equally to this work.
[h]Corresponding Author: Meng Ling Ye, Department of Research, Guangxi Medical University Cancer Hospital, Nanning, Guangxi 530021, China.
Email: yemling@gxmu.edu.cn

doi: https://doi.org/10.14740/wjon2827
World Journal of Oncology
1920-4531 (print), 1920-454X (online)

Introduction

Colorectal cancer (CRC) continues to impose a substantial global health burden. Despite continuous improvements in surgery, systemic chemotherapy, and targeted treatment, advanced CRC is still associated with limited therapeutic ben-

Articles © The authors | Journal compilation © World J Oncol and Elmer Press Inc™ | https://wjon.elmerpub.com
This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, including commercial use, provided the original work is properly cited

efit and poor long-term survival [1]. In recent years, immune checkpoint blockade targeting the programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis has emerged as a promising therapeutic strategy, most notably in microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) tumors [1]. However, immunotherapy provides clinical benefit to only a proportion of patients with CRC, underscoring the importance of further investigating the mechanisms that enable tumor immune escape.

PD-L1 expressed on tumor cells is an important mediator of immune suppression, as it can inhibit T-cell activation and help tumor cells evade antitumor immune responses. Although PD-L1 has been evaluated as a potential biomarker for immunotherapy, the mechanisms governing its expression within the tumor microenvironment remain incompletely defined. Increasing evidence suggests that PD-L1 expression is dynamically regulated by both tumor-intrinsic signaling pathways and extrinsic microenvironmental cues [2].

The gut microbiota has recently emerged as a key regulator of tumor progression and therapeutic response in CRC [3]. Among gut microbial species implicated in CRC, *Fusobacterium nucleatum* (*F. nucleatum*) has received considerable attention because of its enrichment in CRC tissues and its association with poor prognosis, increased metastatic potential, and therapeutic resistance [4]. Mechanistically, *F. nucleatum* promotes tumor progression by activating inflammatory signaling pathways and suppressing antitumor immune responses [5, 6], thereby contributing to the establishment of an immunosuppressive tumor microenvironment.

Emerging evidence suggests that the gut microbiota can modulate neutrophil recruitment and activation, leading to the formation of neutrophil extracellular traps (NETs), web-like chromatin structures decorated with antimicrobial proteins. Although NETs are originally recognized as innate immune defense mechanisms, accumulating studies have demonstrated their pro-tumorigenic roles in promoting cancer progression, metastasis, and immune suppression by facilitating tumor cell dissemination and impairing cytotoxic lymphocyte function. In CRC, *F. nucleatum* has been reported to promote NET formation through pathways involving Toll-like receptor 4 (TLR4), reactive oxygen species (ROS), and nucleotide-binding oligomerization domain-containing protein (NOD)1/2 [7], suggesting a potential link between microbial signals and NET-mediated tumor regulation. Furthermore, TLR signaling and its downstream effector nuclear factor kappa-B (NF-κB) constitute a central inflammatory axis that not only mediates innate immune responses but also contributes to the transcriptional regulation of PD-L1 expression in cancer cells [8]. However, whether microbiota-induced NET formation contributes to PD-L1-mediated immune evasion in CRC remains largely unclear.

In this study, we examined how *F. nucleatum* contributes to immune-related changes in the CRC tumor microenvironment. We demonstrate that *F. nucleatum* induces NET formation in neutrophils, and that NET-derived DNA activates the TLR9/IRAK4/NF-κB signaling axis in CRC cells, thereby increasing PD-L1 expression. Our findings reveal a previously unrecognized mechanistic link between the gut microbiota, neutrophil-mediated innate immunity, and immune checkpoint regulation, suggesting that targeting the *F. nucleatum*–NETs–PD-L1 axis may represent a potential strategy to enhance immunotherapy efficacy in CRC.

Materials and Methods

Institutional Review Board Approval

This study was approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, Nanning, China (Approval No. LW202612), and was conducted in accordance with the Declaration of Helsinki. All samples and clinical information were anonymized prior to analysis. No animal experiments were performed in this study.

Clinical tissue samples and *F. nucleatum in situ* hybridization

Human CRC tissue and peripheral blood samples were collected from patients who underwent surgical resection at Guangxi Medical University Cancer Hospital (Nanning, China). The clinicopathological characteristics of these patients are summarized in Supplementary Material 1 (wjon.elmerpub.com). For the detection of *F. nucleatum* in tumor tissues, fluorescence *in situ* hybridization was performed using an *F. nucleatum*-specific probe. The probe sequence was 5′-CGCAATACAGAGTTGAGCCCTGC-3′. The probe was designed, and the *in situ* hybridization experiments were performed by Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) according to a standardized protocol. *F. nucleatum* signals were visualized by fluorescence microscopy, and nuclei were counterstained with DAPI.

Cell culture

The human CRC cell line HCT116 was purchased from Procell Life Science & Technology Co., Ltd. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cell cultures were incubated at 37 °C in a humidified incubator with 5% CO_2. Cells were regularly examined for mycoplasma contamination and were used during the logarithmic growth phase.

Pharmacological inhibition

To investigate the involvement of the TLR9/IRAK4/NF-κB signaling pathway in NET-mediated PD-L1 regulation, pharmacological inhibitors were used as indicated. E6446 dihydrochloride (Selleck, Cat. No. S6719) was used as the TLR9 inhibitor, IRAK4-IN-7 (MedChemExpress, Cat. No. HY-109585) was used as the IRAK4 inhibitor, and JSH-23 (MedChemExpress, Cat. No. HY-13982) was used as the NF-κB inhibitor. Vehicle-treated cells received an equivalent concentration of dimethyl sulfoxide (DMSO). Following inhibitor

 Articles © The authors | Journal compilation © World J Oncol and Elmer Press Inc™ | https://wjon.elmerpub.com

treatment, HCT116 cells were exposed to NETs or co-cultured with activated neutrophils as indicated, and PD-L1 expression was subsequently evaluated by quantitative real-time PCR or Western blot analysis.

Neutrophil isolation and culture

Peripheral blood samples from healthy volunteers were collected into EDTA-anticoagulated tubes and processed within 1 h after collection to minimize spontaneous neutrophil activation and NET formation. Human neutrophils were isolated using a human peripheral blood neutrophil isolation kit (Solarbio, P9040) according to the manufacturer's instructions. After density-gradient separation and removal of residual erythrocytes, the isolated neutrophils were washed and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum. The cell density was adjusted to approximately 5×10^6 cells/mL. Neutrophil purity was evaluated by Wright–Giemsa staining, and cell viability was assessed by trypan blue exclusion. Freshly isolated neutrophils were used for subsequent experiments.

F. nucleatum stimulation and induction of NET formation

Fusobacterium nucleatum strain BNCC337404 was cultured in fluid thioglycollate medium under anaerobic conditions at 37 °C. Bacteria in the mid-logarithmic growth phase were collected, washed with phosphate-buffered saline (PBS), and resuspended before use. Freshly isolated neutrophils were exposed to *F. nucleatum* at a multiplicity of infection (MOI) of 100:1 to induce NET formation. PBS-treated neutrophils served as the negative control, whereas neutrophils treated with phorbol 12-myristate 13-acetate (PMA, 1.62 μM) served as the positive control.

Isolation of *F. nucleatum*-induced NETs

Following *F. nucleatum* stimulation, the culture supernatant was carefully aspirated without disturbing the adherent neutrophil/NET layer. The cell layer was gently washed twice with ice-cold calcium- and magnesium-free PBS to remove residual bacteria and culture medium. Subsequently, 1 mL of ice-cold calcium- and magnesium-free PBS was added to each well, and the culture plate was placed in a water-bath sonicator at room temperature for 1–3 min to mechanically fragment and release NET structures. The surface of each well was then gently rinsed three to five times with a pipette to recover the released NET material. The resulting suspension was transferred to low-protein-binding centrifuge tubes and centrifuged at 300 × g for 10 min at 4 °C to remove intact cells and cellular debris. The supernatant containing soluble NET fragments was carefully collected. DNA concentration in the NET preparations was determined using a NanoDrop spectrophotometer before subsequent treatment of CRC cells. Where indicated, NET preparations were treated with DNase I (Roche, Cat. No. 11284932001) at 200 U/mL for 30 min at 37 °C before being added to HCT116 cells.

Immunofluorescence detection of NET formation

Poly-L-lysine-coated coverslips were placed in 24-well plates, and freshly isolated neutrophils were seeded onto the coverslips and allowed to adhere for 2 h. Cells were subsequently treated with PBS, PMA, or *F. nucleatum* for 4–6 h to induce NET formation. After stimulation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with PBST. Cells were permeabilized with 0.1% Triton X-100 for 20 min, washed three times with PBS, and blocked with 5% bovine serum albumin for 30 min. The cells were incubated at 4 °C with primary antibodies against myeloperoxidase (MPO; Santa Cruz Biotechnology, sc-52707; 1:50) and citrullinated histone H3 (Cit-H3; Abcam, ab5103; 1:2,000). After three washes with PBST, cells were incubated for 60 min at room temperature in the dark with donkey anti-rabbit IgG conjugated to Alexa Fluor 594 (Abcam, ab150076) and goat anti-mouse IgG conjugated to Alexa Fluor 488 (Abcam, ab150113). After three additional washes with PBS, the coverslips were mounted using an antifade mounting medium containing DAPI. Fluorescence images were acquired using a fluorescence microscope. NET formation was evaluated based on extracellular chromatin structures associated with MPO and Cit-H3 staining.

Neutrophil–CRC cell co-culture

A Transwell co-culture system with 0.4-μm pore inserts was used to examine the effects of activated neutrophils on CRC cells. HCT116 cells were seeded in the lower chamber and allowed to reach approximately 50% confluence. Freshly isolated neutrophils were placed in the upper chamber and treated with PBS, *Escherichia coli*, *F. nucleatum*, or PMA, as indicated. After 8 h of co-culture, HCT116 cells in the lower chamber were collected for subsequent quantitative PCR or Western blot analysis. In separate experiments, HCT116 cells were directly treated with isolated *F. nucleatum*-induced NETs, DNase I-treated NETs, or NETs in combination with the indicated pathway inhibitors to determine the contribution of NET-derived DNA and downstream signaling pathways to PD-L1 regulation.

Quantification of extracellular DNA

Extracellular DNA released during *F. nucleatum*-induced NET formation was quantified using culture supernatants from the Transwell co-culture system. HCT116 cells were cultured in the lower chamber, while *F. nucleatum* and/or neutrophils were added to the upper chamber as indicated. The experimental groups included control, *F. nucleatum* (Fn), Fn plus neutrophils (Fn + Neut), and Fn + Neut plus DNase I. After 8 h of co-culture, 1 mL of culture supernatant from each group was collected and centrifuged at 250 × g for 5 min to remove residual cells and cellular debris. DNA was subsequently purified

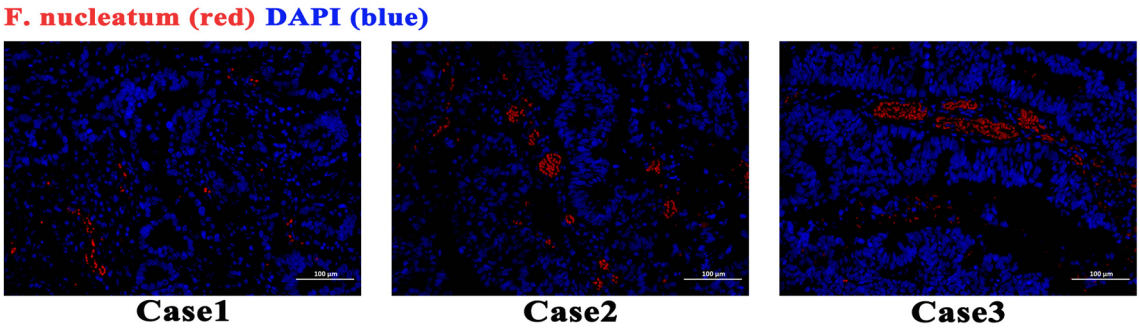


Figure 1. *Fusobacterium nucleatum* detection in human colorectal cancer tissues. Representative images of *F. nucleatum* detected by *in situ* hybridization in human colorectal cancer tissues. Positive *F. nucleatum* signals are shown in red, and nuclei were counterstained with DAPI (blue). Scale bar, 100 μm.

from the clarified supernatant using a commercial DNA purification kit (TransGen Biotech) according to the manufacturer's instructions. The concentration of purified extracellular DNA was measured using a NanoDrop spectrophotometer.

Serum MPO measurement

Peripheral blood samples were obtained from patients with CRC and healthy volunteers. Serum MPO concentrations were measured using a human MPO enzyme-linked immunosorbent assay (ELISA) kit (Multi Sciences, Cat. No. EK1133-96) according to the manufacturer's instructions. MPO concentrations were calculated based on the corresponding standard curve.

Western blot

Total cellular proteins were extracted using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit. After normalization, equal amounts of protein from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies against PD-L1 (Cell Signaling Technology, #13684), phospho-NF-κB p65 (Abcam, ab76302), and GAPDH (Servicebio, GB15004). After washing, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein signals were visualized using enhanced chemiluminescence (ECL) and quantified by densitometric analysis.

Quantitative real-time PCR

Total RNA was isolated from cultured cells with TRIzol reagent according to the manufacturer's instructions. Complementary DNA was synthesized using a commercial reverse transcription kit according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green Master Mix on a qTower3/G Touch system (Analytik Jena, Jena, Germany). GAPDH was used as the internal control. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: PD-L1 forward, 5′-GACTGCCACCCACTGTCCTTT-3′ and reverse, 5′-TTCCCCTCGCATCATCCTTAT-3′; GAPDH forward, 5′-CTCTGATTTGGTCGTATTGGGC-3′ and reverse, 5′-CCTGGAAGATGGTGATGGGATT-3′.

Statistical analysis

All experiments were independently repeated at least three times. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test for comparisons between two groups and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test for comparisons among multiple groups, as appropriate. A P value < 0.05 was considered statistically significant.

Results

F. nucleatum is detected in human CRC tissues

In situ hybridization was performed to evaluate the presence and distribution of *F. nucleatum* in human CRC tissues. *F. nucleatum* signals were readily detected within the tumor tissues, with relatively abundant bacterial signals observed in tumor regions (Fig. 1a). These findings confirm the presence of *F. nucleatum* in human CRC tissues and provide a clinical basis for further investigating its potential role in the CRC tumor microenvironment.

F. nucleatum induces NET formation in neutrophils

Human neutrophils were isolated from peripheral blood samples collected from healthy volunteers. The isolated cells exhibited typical morphological characteristics, including high transparency, strong refractive index, and well-defined cellular contours (Fig. 2a). Wright–Giemsa staining further confirmed the high purity of the neutrophil population (Fig. 2b). To investigate the

Articles © The authors | Journal compilation © World J Oncol and Elmer Press Inc™ | https://wjon.elmerpub.com

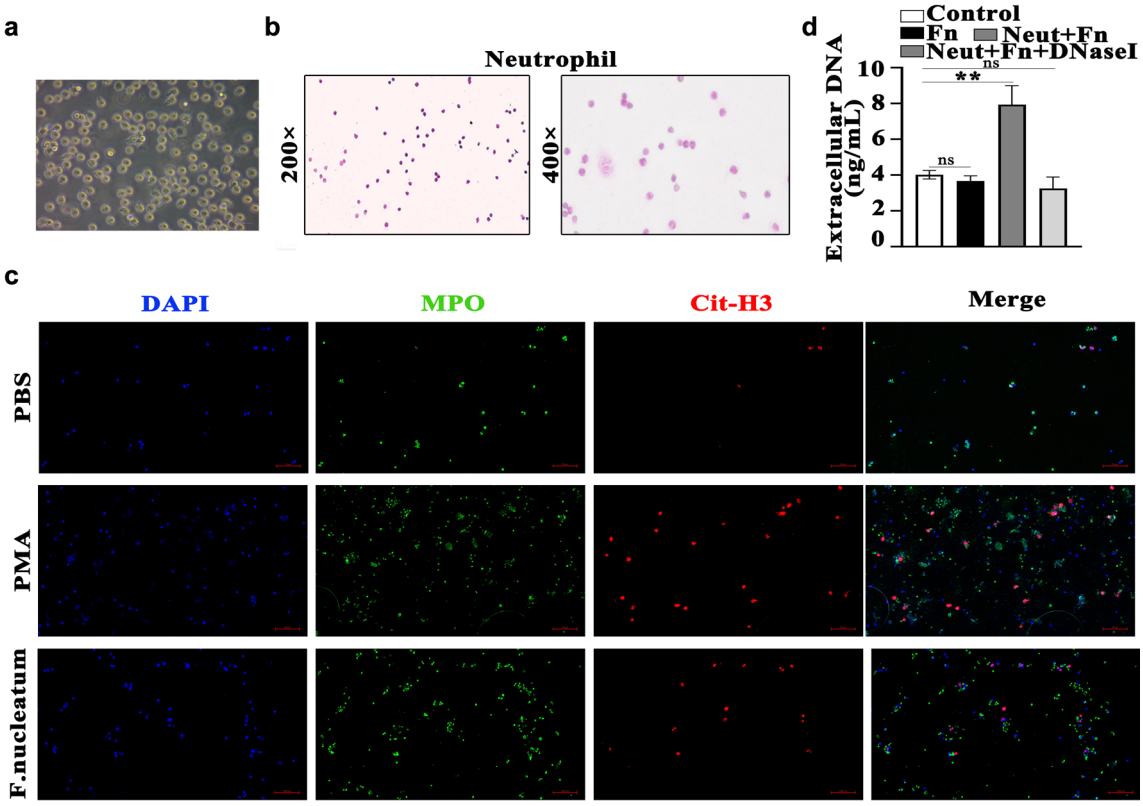


Figure 2. Characterization of neutrophils and induction of neutrophil extracellular trap (NET) formation by *Fusobacterium nucleatum*. (a) Representative morphology of isolated neutrophils. Scale bar, 10 μm. (b) Wright–Giemsa staining of isolated neutrophils. (c) Immunofluorescence staining of myeloperoxidase (MPO, green) and citrullinated histone H3 (Cit-H3, red) in neutrophils treated with phosphate-buffered saline (PBS), *F. nucleatum*, or phorbol 12-myristate 13-acetate (PMA). Scale bar, 50 μm. (d) Extracellular DNA levels in the control, *F. nucleatum* (Fn), Fn plus neutrophils (Fn + Neut), and Fn + Neut plus DNase I groups. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. ns: not significant; **P < 0.01.

effect of *F. nucleatum* on NET formation, isolated neutrophils were infected with *F. nucleatum.* PBS served as the negative control, while PMA was used as a positive control for NET induction. *F. nucleatum* stimulation resulted in stronger MPO and Cit-H3 staining compared with the PBS control (Fig. 2c). Consistently, extracellular DNA levels were markedly increased following *F. nucleatum* stimulation in the presence of neutrophils, whereas DNase I treatment significantly reduced extracellular DNA levels (Fig. 2d). Together, these findings indicate that *F. nucleatum* promotes NET formation in human neutrophils.

F. nucleatum-stimulated neutrophils promote PD-L1 expression in CRC cells

Peripheral blood samples were collected from eight patients with CRC and eight healthy volunteers. Serum levels of MPO were measured by ELISA, which revealed a significant elevation in CRC patients compared with healthy controls (Fig. 3a). A Transwell co-culture system was then established to model the interaction between neutrophils and CRC cells (Fig. 3b). Neutrophils isolated from the peripheral blood of healthy donors were treated with *Escherichia coli*, *F. nucleatum*, or PMA, and subsequently co-cultured with HCT116 cells. Quantitative PCR analysis demonstrated that, compared with the *E. coli*–treated group, *F. nucleatum* treatment markedly upregulated PD-L1 mRNA expression in HCT116 cells (Fig. 3c).

NETs directly upregulate PD-L1 expression in CRC cells

Peripheral blood samples were collected from three healthy volunteers, and neutrophils were isolated. Neutrophils were stimulated with *F. nucleatum* to induce the formation of NETs, which were subsequently used to treat HCT116 cells. NET treatment significantly increased PD-L1 mRNA expression in HCT116 cells, whereas direct *F. nucleatum* stimulation did not significantly affect PD-L1 expression (Fig. 4a), suggesting that the effect of *F. nucleatum* on PD-L1 expression is primarily mediated through NET formation.

NET-derived DNA mediates PD-L1 upregulation

Neutrophils were stimulated with PMA and subsequently co-cultured with HCT116 cells. Quantitative PCR showed that PMA-

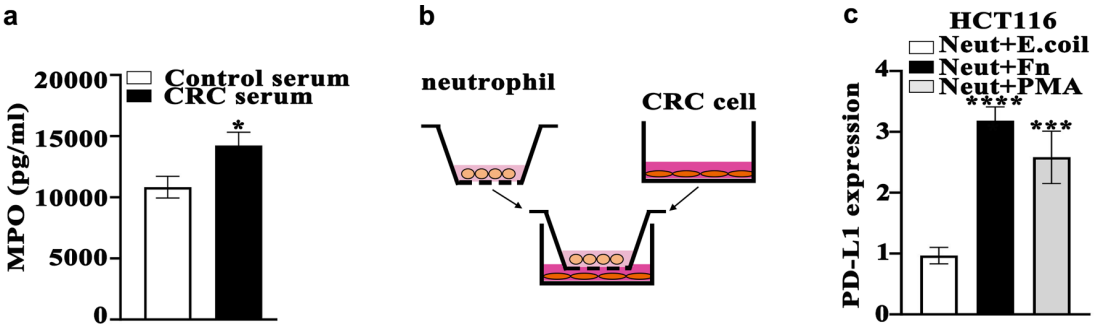


Figure 3. (a) Serum myeloperoxidase (MPO) levels in healthy volunteers and patients with colorectal cancer (CRC) were measured by enzyme-linked immunosorbent assay (ELISA). (b) Schematic illustration of the co-culture system between neutrophils and CRC cells. (c) Neutrophils were treated with *Escherichia coli*, *Fusobacterium nucleatum*, or phorbol 12-myristate 13-acetate (PMA) and subsequently co-cultured with HCT116 cells; programmed cell death ligand 1 (PD-L1) mRNA expression was quantified by quantitative PCR. Data are presented as mean ± standard error of the mean (SEM). *P < 0.05 versus the healthy control group in (a); **P < 0.01 and ***P < 0.001 versus the Neut + *E. coli* group in (c).

activated neutrophils significantly increased PD-L1 expression in HCT116 cells compared with untreated neutrophils (Fig. 5a). DNase I treatment markedly attenuated this effect (Fig. 5b). More importantly, *F. nucleatum*-induced NETs directly increased PD-L1 expression in HCT116 cells, whereas DNase I treatment significantly reduced this increase (Fig. 5c, d), further supporting an important role for NET-derived DNA in PD-L1 regulation.

NETs activate the TLR9/IRAK4/NF-κB signaling pathway in CRC cells

We next examined the signaling mechanisms underlying NET-induced PD-L1 upregulation in CRC cells. Quantitative PCR analysis showed that, in the neutrophil–HCT116 co-culture system, inhibition of either TLR9 or IRAK4 significantly suppressed the PMA-induced increase in PD-L1 expression in HCT116 cells (Fig. 6a). More importantly, DNase I treatment, as well as TLR9 or IRAK4 inhibition, markedly attenuated the upregulation of PD-L1 induced by *F. nucleatum*-derived NETs (Fig. 6b, c), providing more direct evidence that NET-derived DNA promotes PD-L1 expression through the TLR9/IRAK4 pathway. We next investigated the involvement of NF-κB signaling. Treatment with an NF-κB inhibitor significantly reduced NET-induced PD-L1 mRNA expression in HCT116 cells (Fig. 6d). Consistently, Western blot analysis showed that NET treatment increased phosphorylation of the NF-κB subunit p65 compared with untreated controls, indicating activation of the NF-κB pathway (Fig. 6e). Together, these findings support the involvement of the TLR9/IRAK4/NF-κB signaling pathway in NET-mediated PD-L1 upregulation in CRC cells.

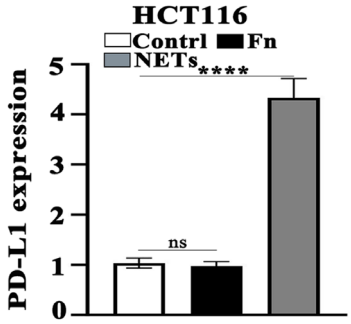


Figure 4. Programmed cell death ligand 1 (PD-L1) mRNA expression in HCT116 cells treated with *F. nucleatum* or *F. nucleatum*-induced neutrophil extracellular traps (NETs), as determined by quantitative real-time PCR. Data in are presented as mean ± standard error of the mean (SEM). ns: not significant; ****P < 0.0001.

Discussion

Immune checkpoint blockade has improved the therapeutic landscape for patients with dMMR or MSI-H CRC [9]. However, only a limited proportion of patients with CRC experience durable clinical benefit from immunotherapy, highlighting the urgent need to better understand the regulatory mechanisms governing immune checkpoint expression, particularly PD-L1, in the tumor microenvironment. PD-L1 is a key immune checkpoint molecule that mediates tumor immune evasion and exhibits marked heterogeneity in CRC [10]. Its expression is tightly regulated by tumor-intrinsic genetic alterations as well as extrinsic factors, including inflammatory signaling, immune cell infiltration, and the gut microbiota [11]. Emerging evidence suggests that the tumor microenvironment plays a critical role in shaping PD-L1 expression, thereby influencing therapeutic response. In this study, we identify a novel mechanism by which *F. nucleatum* promotes immune evasion in CRC by inducing NETs, thereby activating the TLR9/IRAK4/NF-κB signaling pathway and subsequently upregulating PD-L1 in tumor cells. These findings suggest a mechanistic connection among gut microbiota-associated signals, innate immune activation, and immune checkpoint regulation in CRC.

NETs have emerged as critical regulators of tumor progression rather than merely antimicrobial defense structures [12]. In the tumor microenvironment, NETs can facilitate immune evasion by forming extracellular DNA-protein networks around tumor cells and compromising cytotoxic lymphocyte-mediated antitumor responses [13]. Although NET-associated PD-L1-dependent T-cell exhaustion has been reported in several malig-

 Articles © The authors | Journal compilation © World J Oncol and Elmer Press Inc™ | https://wjon.elmerpub.com

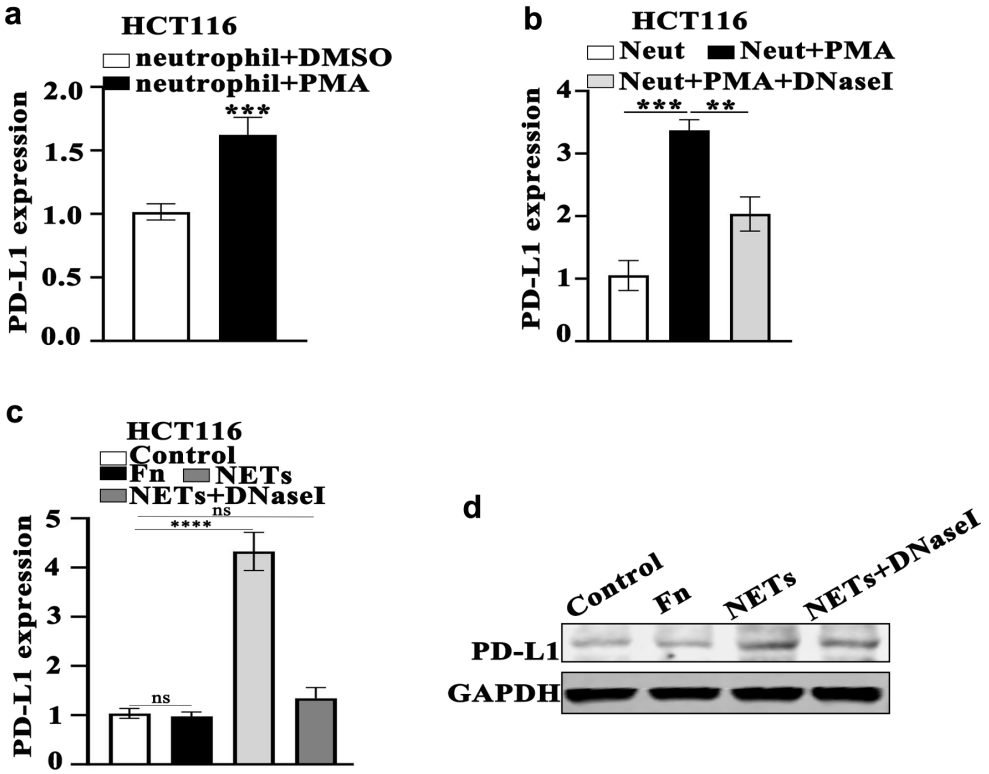


Figure 5. (a) Neutrophils were treated with or without phorbol 12-myristate 13-acetate (PMA) and subsequently co-cultured with HCT116 cells. Programmed cell death ligand 1 (PD-L1) mRNA expression in HCT116 cells was assessed by quantitative PCR. ***P < 0.001 versus the Neut + dimethyl sulfoxide (DMSO) group. (b) Neutrophils were treated with PMA in the presence or absence of DNase I prior to co-culture with HCT116 cells, and PD-L1 mRNA expression was assessed by quantitative PCR. **P < 0.01; ***P < 0.001 for the indicated comparisons. (c, d) HCT116 cells were treated with phosphate-buffered saline (PBS), *F. nucleatum*, *F. nucleatum*-induced neutrophil extracellular traps (NETs), or DNase I-treated NETs. PD-L1 expression was assessed by quantitative PCR (c) and Western blot analysis (d), respectively. Data are presented as mean ± standard error of the mean (SEM). ****P < 0.0001 versus the control group in (c).

nancies, the contribution of NETs to PD-L1 regulation in CRC cells [14], particularly in the context of *F. nucleatum*-induced NET formation, remains to be further characterized. In this study, we found that NET exposure markedly increased PD-L1 expression in CRC cells, suggesting a direct link between NET formation and immune checkpoint regulation. Furthermore, the suppressive effect of DNase I on PD-L1 upregulation supports the essential contribution of NET-derived DNA to this process.

At the mechanistic level, NET-derived extracellular DNA has been implicated in the activation of TLR9-dependent innate immune signaling [13]. NF-κB is also known to participate in the transcriptional control of PD-L1, providing a possible connection between inflammatory signaling and immune checkpoint regulation in cancer cells [15]. In line with this concept, our results showed that NET exposure was associated with activation of the TLR9/IRAK4/NF-κB pathway in CRC cells. Moreover, inhibition of TLR9, IRAK4, or NF-κB reduced NET-induced PD-L1 upregulation, supporting the involvement of this signaling cascade in the observed response. These findings suggest that CRC cells may respond to NET-associated extracellular DNA through innate immune sensing pathways, potentially contributing to an immunosuppressive tumor microenvironment.

PD-L1 has important biological and clinical relevance in CRC. On the one hand, increased PD-L1 expression may contribute to suppression of antitumor immune responses through the PD-1/PD-L1 axis. On the other hand, PD-L1 expression is also considered a clinically relevant marker in the context of immune checkpoint blockade, although its predictive value may vary depending on the immune status and molecular features of the tumor. In this regard, NET-induced PD-L1 upregulation may represent a potential mechanism linking microbial and inflammatory signals to immune checkpoint regulation in CRC cells. These findings further emphasize the complexity of PD-L1 regulation within the tumor microenvironment and suggest that upstream factors such as *F. nucleatum*-induced NET formation may influence tumor immune signaling.

Several limitations should be noted. First, although our *in vitro* experiments provide mechanistic evidence supporting the involvement of NETs and the TLR9/IRAK4/NF-κB pathway in PD-L1 regulation, most mechanistic experiments were performed in HCT116 cells. Therefore, validation in additional CRC cell lines with different molecular backgrounds, as well as patient-derived models, will be important to establish the generalizability of these findings. Second, although NET formation was assessed using Cit-H3, MPO, and extracellular DNA, more comprehensive characterization using additional

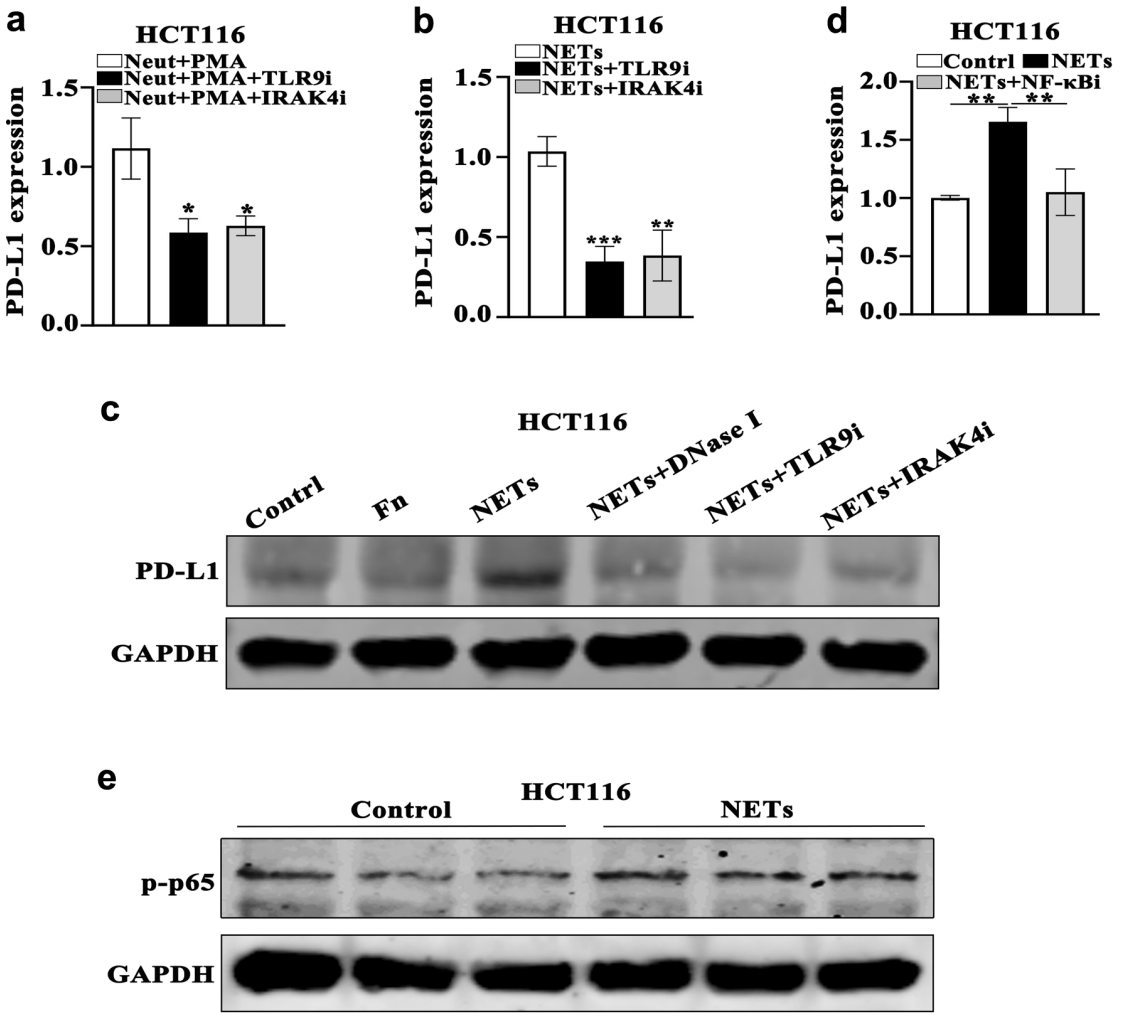


Figure 6. (a) Neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA) in the presence or absence of Toll-like receptor 9 (TLR9) or interleukin-1 receptor-associated kinase 4 (IRAK4) inhibitors and subsequently co-cultured with HCT116 cells. Programmed cell death ligand 1 (PD-L1) mRNA expression in HCT116 cells was assessed by quantitative PCR. *P < 0.05 versus the Neut + PMA group. (b) HCT116 cells were treated with *F. nucleatum*-induced neutrophil extracellular traps (NETs) with or without TLR9 or IRAK4 inhibitors, and PD-L1 mRNA expression was assessed by quantitative PCR. **P < 0.01 and ***P < 0.001 versus the NETs group. (c) PD-L1 protein expression under the indicated treatments was assessed by Western blot analysis. (d) HCT116 cells were treated with NETs in the presence or absence of a nuclear factor kappa-B (NF-κB) inhibitor, and PD-L1 mRNA expression was assessed by quantitative PCR. **P < 0.01 for the indicated comparisons. (e) Phosphorylated p65 (p-p65) levels in HCT116 cells following NET treatment were assessed by Western blot analysis. Data are presented as mean ± standard error of the mean (SEM).

NET-associated markers, such as neutrophil elastase, would further strengthen the evidence for NET involvement. Third, further *in vivo* studies are needed to confirm the physiological relevance of this mechanism. In addition, the clinical sample size was limited, and validation in larger patient cohorts is required. Finally, the broader interactions among *F. nucleatum*, NETs, CRC cells, and other immune cell populations within the tumor microenvironment remain to be further investigated.

Supplementary Material

Suppl 1. Clinicopathological characteristics of patients with colorectal cancer.

Acknowledgments

None to declare.

Financial Disclosure

The research was supported by the Joint Project on Regional High-Incidence Diseases Research of Guangxi Natural Science Foundation under Grant (Grant No. 2023GXNSFBA026202), and the Key R&D Program of Scientific Research and Technology Development Project of Guangxi (Grant No. GUI KE AB23026078).

Articles © The authors | Journal compilation © World J Oncol and Elmer Press Inc™ | https://wjon.elmerpub.com

Conflict of Interest

The authors declare that there are no conflicts of interest.

Informed Consent

Written informed consent was obtained from all participants before sample collection.

Author Contributions

Hong Lei Shen performed the experiments, collected and analyzed the data, and validated the results. Yu Xuan Xie, Yu Bin Lu, and Yan Yan Lin contributed to cell culture experiments and Western blot analyses. Xian Long Xie was responsible for data organization and statistical analysis. Yu Xuan Xie, Jin Lu Wu, and Hao Tao assisted with experiments and critically reviewed the manuscript. Meng Ling Ye conceived and coordinated the study, secured funding support, supervised the project, and wrote and revised the manuscript.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

AI Use Declaration

The authors used ChatGPT (OpenAI) solely for English language editing and polishing. All AI-assisted text was reviewed and revised by the authors, who take full responsibility for the content of the manuscript.

References

1. Ranjan R, Kumar A, Kumar V, Kumar Mahato B, Sarkar A, Kumar Yadav R, Gond V, et al. Emerging immunotherapeutic approaches in colorectal cancer: from checkpoint inhibitors to CAR-T cell and viral vector vaccines. Immunol Invest. 2026;55(3):602-637. doi pubmed
2. Yamaguchi H, Hsu JM, Yang WH, Hung MC. Mechanisms regulating PD-L1 expression in cancers and associated opportunities for novel small-molecule therapeutics. Nat Rev Clin Oncol. 2022;19(5):287-305. doi pubmed
3. Li Z, Xiong W, Liang Z, Wang J, Zeng Z, Kolat D, Li X, et al. Critical role of the gut microbiota in immune responses and cancer immunotherapy. J Hematol Oncol. 2024;17(1):33. doi pubmed
4. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207-215. doi pubmed
5. Gao Y, Bi D, Xie R, Li M, Guo J, Liu H, Guo X, et al. Fusobacterium nucleatum enhances the efficacy of PD-L1 blockade in colorectal cancer. Signal Transduct Target Ther. 2021;6(1):398. doi pubmed
6. Jiang SS, Xie YL, Xiao XY, Kang ZR, Lin XL, Zhang L, Li CS, et al. Fusobacterium nucleatum-derived succinic acid induces tumor resistance to immunotherapy in colorectal cancer. Cell Host Microbe. 2023;31(5):781-797.e789. doi pubmed
7. Kong X, Zhang Y, Xiang L, You Y, Duan Y, Zhao Y, Li S, et al. Fusobacterium nucleatum-triggered neutrophil extracellular traps facilitate colorectal carcinoma progression. J Exp Clin Cancer Res. 2023;42(1):236. doi pubmed
8. Antonangeli F, Natalini A, Garassino MC, Sica A, Santoni A, Di Rosa F. Regulation of PD-L1 expression by NF-kappaB in cancer. Front Immunol. 2020;11:584626. doi pubmed
9. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017;357(6349):409-413. doi pubmed
10. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-264. doi pubmed
11. Wuyi W, Tao P. Microbe-driven immune suppression in colorectal cancer: the Fusobacterium nucleatum playbook. Front Immunol. 2026;17:1795703. doi pubmed
12. Hu W, Lee SML, Bazhin AV, Guba M, Werner J, Niess H. Neutrophil extracellular traps facilitate cancer metastasis: cellular mechanisms and therapeutic strategies. J Cancer Res Clin Oncol. 2023;149(5):2191-2210. doi pubmed
13. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018;18(2):134-147. doi pubmed
14. Kaltenmeier C, Yazdani HO, Morder K, Geller DA, Simmons RL, Tohme S. Neutrophil extracellular traps promote T cell exhaustion in the tumor microenvironment. Front Immunol. 2021;12:785222. doi pubmed
15. Peng S, Wang R, Zhang X, Ma Y, Zhong L, Li K, Nishiyama A, et al. EGFR-TKI resistance promotes immune escape in lung cancer via increased PD-L1 expression. Mol Cancer. 2019;18(1):165. doi pubmed