Received: 29 March 2024 | Revised: 12 August 2024 | Accepted: 20 September 2024

DOI: 10.1002/cac2.12615

CANCER COMMUNICATIONS Open Access

LETTER TO THE JOURNAL

Tumor derived cell-free nucleic acid upregulates programmed death-ligand 1 expression in neutrophil via intracellular Toll-like receptor signaling

Neutrophils are innate immune cells that function predominantly against pathogens, while recent studies have revealed additional crucial roles in various diseases, including cancers [1–3]. For instance, neutrophils expressing the co-inhibitory molecule programmed death-ligand 1 (PD-L1) were identified as novel immunosuppressive myeloid cells that impair cytotoxic T cell (CTL) activity via programmed cell death protein 1 (PD-1)/PD-L1 interaction [4, 5]. Although some stimuli have been identified, it is still unclear whether the nucleic acid sensing system (NAS) participates in PD-L1 upregulation in neutrophils [6]. Here, we report that increased cell-free nucleic acid (CFNA) upregulates PD-L1 expression via intracellular Toll-like receptor (TLR) activation in neutrophils following tumor expansion.

Flow cytometry analysis showed that the expression of PD-L1 was gradually increased in peripheral blood (PB) neutrophil after inoculating B16-F10 melanoma cells or EO771 breast cancer cells into wildtype (WT) mice (Figure 1A, protocol is shown in the Supplementary Materials and gating strategy of flow cytometry is shown in Supplementary Figure S1). Notably, the expression of PD-L1 was significantly increased in PB neutrophils of B16-F10-inoculated mice as early as day 3 post-injection compared to those of naïve mice. Although EO771-inoculated mice did not show significantly increased PD-L1 expression in PB neutrophil at days 3 and 7 of post tumor inoculation, there was a significant, pronounced upregulation at day 14 (Figure 1A). Intratumor (IT) neutrophils showed the largest increase of PD-L1 expression compared to neutrophils in PB, spleen and bone marrow (BM) 14 days post inoculation in both types of tumors. The PD-L1 expression level in BM neutrophils was lower than that of PB and spleen neutrophils in B16-F10 inoculated mice. In EO771-inoculated mice, the PD-L1 expression levels in BM and spleen neutrophils were similar, but slightly lower than that in PB (Supplementary Figure S2A and B). Interestingly, similar to the observation in PB, spleen and BM neutrophils also showed significant increases in PD-L1 levels in tumor-bearing mice compared to those of naïve mice, implying that neutrophil PD-L1 upregulation occurs systematically in these murine tumor models (Supplementary Figure S2C and D). Given these data, we decided to investigate circulating factors that may induce changes in PD-L1 levels in neutrophils of tumor-bearing mice, and found that the plasma CFNA levels were significantly increased in the tumor-bearing mice compared to the mice before tumor inoculation (Figure 1B). Linear regression analyses showed strong positive correlations between the plasma CFNA and PB neutrophil-associated PD-L1 expression levels in tumor-bearing mice (Figure 1C). Of note, both the plasma CFNA (Figure 1D) and neutrophil PD-L1 expression levels (Supplementary Figure S3) were positively correlated with the tumor volumes in these mice. In vitro experiments revealed that medium supplemented with plasma of tumor-bearing mice (14 days post tumor inoculation) significantly increased PD-L1 expression in naïve mouse BM-derived neutrophils compared to naïve mouse plasma-supplemented medium (Figure 1E). DNase or RNase treatment abolished this effect suggesting that CFNA components are capable of increasing PD-L1 expression in neutrophils, an observation that has important clinical implications given that cancer patients often have increased plasma cell-free DNA (cfDNA) levels compared to healthy individuals [7].

Abbreviations: PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; CTL, cytotoxic T cell; CFNA, cell-free nucleic acid; NAS, Nuclear acid sensing system; TLR, Toll-like receptor; BM, Bone marrow; PB, Peripheral blood; IT, Intratumor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; G-CSF, Granulocyte-colony-stimulating factor; Poly (I:C), Polyinosinic:polycytidylic acid; RT-qPCR, Reverse transcription quantitative polymerase chain rection; WB, Western blot; STAT3, Signal transducer and activator of transcription 3; mAb, monoclonal antibody; IFN-γ, Interferon-gamma; cGAS, cyclic GMP-AMP synthase (cGAS); STING, Stimulator of interferon genes; dsRNA, Double-stranded RNA..

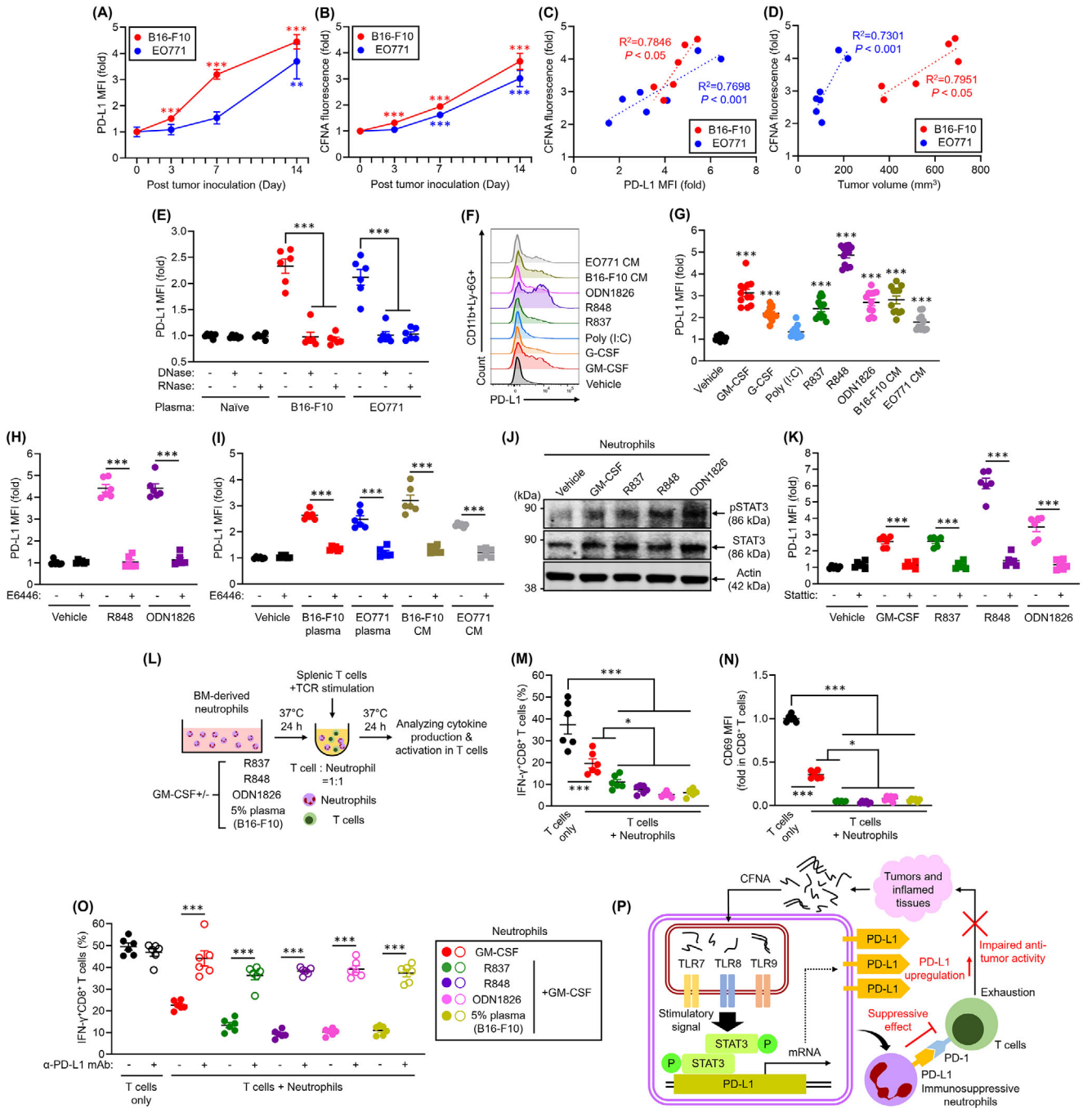

FIGURE 1 Activation of intracellular TLR signaling upregulates PD-L1 expression in neutrophils inhibiting T cell function. The mice (C57BL/6, wild-type (WT)) received either B16-F10 or EO771-inoculation, then PD-L1 expression in PB circulating neutrophils, plasma CFNA levels and tumor volumes were measured on the indicated day after tumor inoculation. (A) PD-L1 expression (fold change of MFI) in PB neutrophils analyzed by flow cytometry. (B) Plasma CFNA levels measured by SYTOX™ Green staining. (C) Linear regression analysis of plasma CFNA levels and PD-L1 expression in PB neutrophils (day 14). (D) Linear regression analysis of plasma CFNA levels and tumor volumes (day 14). R-squared values and *P*-values were calculated by GraphPad Prism 10.0. (E) PD-L1 expression of in vitro cultured neutrophils analyzed by flow cytometry. Plasma samples were prepared from individual naïve or tumor bearing mice (at day 14 of B16-F10 or EO771 inoculation), and BM-derived neutrophils were cultured with 5% plasma supplemented medium at 37°C for 6 h. To degrade CFNA, the plasma supplemented medium was treated with DNase or RNase at 37°C for 16 h prior to use for experiments. (F-G) PD-L1 expression of in vitro stimulated neutrophils analyzed by flow cytometry. BM-derived neutrophils were treated with vehicle, GM-CSF (100 ng/mL), G-CSF (100 ng/mL), Poly (I:C) (25 μg/mL), R837 (25 μg/mL), R848 (10 μg/mL), ODN1826 (5 μg/mL), B16-F10 conditioned medium (CM) or EO771 CM at 37°C for 6 h. Representative histograms and cumulative MFI values (fold change) of PD-L1 expression in neutrophils are shown in (F) and (G), respectively. (H) PD-L1 expression in TLR stimulated neutrophils with TLR7/9 inhibition. BM-derived neutrophils were pre-incubated with vehicle (−) or E6447 (+; TLR7 and 9 inhibitor, 50 μmol/L) at 37°C for 60 min followed by treating with vehicle, R848 (10 μg/mL) or

Next, we investigated whether the activation of intracellular NAS, particularly TLR signaling, triggers PD-L1 upregulation in neutrophils [8]. For this purpose, in vitro stimulation assays were performed using intracellular TLR ligands, such as polyinosinic: polycytidylic acid (Poly (I:C)) for TLR3 stimulation, R837 (Imiquimod) for TLR7/8 stimulation, R848 (Resiquimod) for TLR7/8 stimulation, and ODN1826 (Class B CpG oligonucleotide) for TLR9 stimulation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF) were used as positive controls to increase PD-L1 expression in the neutrophils [4, 5, 9]. Except for Poly (I:C), R837, R848, and ODN1826 significantly increased PD-L1 expressions in BM-derived neutrophils compared to the controls (Figure 1F and G). The conditioned media (CM) of B16-F10 or EO771 cell culture also exhibited similar effects in neutrophil PD-L1 upregulation. The responsibility of intracellular TLR in PD-L1 upregulation was proven by an inhibition assay using E6446 (TLR7/9 inhibitor) in the neutrophils upon R848 or ODN1826 stimulation. PD-L1 expression was suppressed in neutrophils by E6446 treatment (Figure 1H). Intracellular TLR inhibition also suppressed PD-L1 upregulation in neutrophils cultured in tumor-bearing mice plasma supplemented medium or cancer cell CM (Figure 1I). These results further support the essential role of intracellular TLR signaling in neutrophil PD-L1 upregulation. The upregulation of *Pdl1* mRNA expression was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in GM-CSF or R848 treated BM-derived neutrophils (Supplementary Figure S4). In addition, flow cytometry analysis showed that R837 or R848 stimulation increased PD-L1 expression in dimethyl sulfoxide (DMSO)-differentiated HL-60 (dHL-60) cells, a human neutrophil model, suggesting that this intracellular TLR activation-mediated PD-L1 upregulation was mechanistically conserved in both mouse and human cells (Supplementary Figure S5). Western blot (WB) showed that GM-CSF, R837, R848, and ODN1826 treatments increased phosphorylated-signal transducer and activator of transcription 3 (pSTAT3) levels in BM-derived neutrophils, respectively (Figure 1J). PD-L1 upregulation in these treatments was suppressed by inhibition of STAT3 phosphorylation using Stattic in the stimulated neutrophils (Figure 1K). The STAT3 inhibition also suppressed PD-L1 upregulation in neutrophils cultured in cancer cell CM (Supplementary Figure S6). Additionally, STAT3 inhibition significantly suppressed *Pdl1* mRNA upregulation in neutrophils upon GM-CSF or R848 stimulation as compared to control treatment (Supplementary Figure S7). As STAT3 has been known as one of the regulators in PD-L1 expression [6], our results suggest that STAT3 activation may be involved in intracellular TLR-dependent PD-L1 upregulation in neutrophils.

ODN1826 (5 μg/mL) at 37°C for 6 h, then PD-L1 expression in neutrophils was analyzed by flow cytometry. (I) PD-L1 expression in neutrophil cultured in plasma supplemented medium or cancer cell CM with TLR7/9 inhibition. BM-derived neutrophils were pre-incubated with vehicle (−) or E6447 (+; TLR7 and 9 inhibitors, 50 μmol/L) at 37°C for 60 min followed by incubating in tumor bearing mouse plasma supplemented medium or cancer cell CM at 37°C for 6 h. PD-L1 expression in neutrophils was analyzed by flow cytometry. (J) WB images of STAT3 activation in neutrophils. BM-derived neutrophils were treated with vehicle, GM-CSF (100 ng/mL, 15 min), R837 (25 μg/mL, 60 min), R848 (10 μg/mL, 60 min), ODN1826 (5 μg/mL, 60 min). Total and phosphorylated pSTAT3 expressions were detected by WB. (K) PD-L1 expression in neutrophils with STAT3 inhibition. BM-derived neutrophils were pre-incubated with vehicle (−) or Stattic (+; STAT3 inhibitor, 10 μmol/L) at 37°C for 30 min followed by incubating with vehicle, GM-CSF (100 ng/mL), R837 (25 μg/mL), R848 (10 μg/mL), ODN1826 (5 μg/mL) at 37°C for 6 h. PD-L1 expression in neutrophils was analyzed by flow cytometry. (L-N) In vitro T cell suppression assay. BM-derived neutrophils were pre-cultured with GM-CSF (100 ng/mL) with or without R837 (25 μg/mL), R848 (10 μg/mL), ODN1826 (5 μg/mL), 5% plasma (B16-F10-derived tumor bearing mice) at 37°C for 24 h. The neutrophils were washed and co-cultured with splenic $CD3^+$ T cells (at a ratio = 1:1) in the presence of anti-CD3 mAb (10 μg/mL) and anti-CD28 mAb (2 μg/mL) at 37°C for 24 h. The IFN-γ production and CD69 expression in $CD8^+$ T cells were analyzed by flow cytometry (L). Cumulative percentage values of IFN-γ^+CD8^+ T cells and MFIs (fold change) of CD69 expression in $CD8^+$ T cells are shown in (M) and (N), respectively. (O) PD-1/PD-L1 blockade in neutrophil-T cell coculture. The co-cultures established by the protocol indicated in Figure 1K were further treated with isotype antibody (−) or anti-PD-L1 mAb (+; 10 μg/mL). After incubation at 37°C for 24 h, the percentages of IFN-γ^+CD8^+ T cells were analyzed by flow cytometry. (P) Hypothetical diagram of CFNA-mediated generation of PD-L1-upregulated neutrophils and T cell suppression in cancer. The neutrophil and $CD8^+$ T cell populations were determined as $CD45^+CD11b^+Ly\text{-}6G^+$ and $CD45^+CD4^-CD8^+$ in flow cytometry. The gating strategy of flow cytometry analysis and the purities of isolated BM-derived neutrophils and splenic $CD3^+$ T cells are shown in Supplemental Figure S1. Cumulative data are shown as mean ± SEM of six to twelve samples in two or three independent experiments. All MFI values are represented as fold changes (the average value of MFI in control was used for baseline = 1). Student *t*-test (comparison between two groups) or one-way ANOVA (comparison between more than three groups) was used to analyze data for significant differences. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$. ns = not significant. Abbreviations: PB, Peripheral blood; PD-L1, Programmed death-ligand 1; BM, Bone marrow; CFNA, Cell-free nucleic acid; MFI, Mean fluorescence intensity; TLR, Toll-like receptor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; G-CSF, Granulocyte colony-stimulating factor; CM, conditioned medium; WB, Western blot; STAT3, Signal transducer and activator of transcription 3; STAT3; pSTAT3, phosphorylated-STAT3; mAb, monoclonal antibody; IFN-γ, Interferon-gamma.

Since PD-L1$^+$ myeloid cells are characterized as possessing immunosuppressive activity against T cells [4, 5], we investigated whether intracellular TLR-stimulated or CFNA-exposed neutrophils also exhibits a suppressive effect by employing a co-culture system with T cell (Figure 1L). BM-derived neutrophils were first pre-cultured in the medium supplemented with or without R837, R848, ODN1826, or plasma originating from B16-F10 tumor-bearing mice to increase PD-L1 expression as verified by flow cytometry analysis (Supplementary Figure S8). GM-CSF was added to all the cultures to sustain neutrophil survival. The pre-treated neutrophils were washed and then co-cultured with splenic CD3$^+$ T cells in the presence of anti-CD3 and anti-CD28 monoclonal antibodies (mAbs), and the suppressive effect of neutrophils on T cells was assessed by examining the levels of interferon-gamma (IFN-γ) production and activation marker CD69 expression in the T cells. GM-CSF-stimulated neutrophils suppressed IFN-γ production as well as CD69 expression in CD8$^+$ T cells (Figure 1M and N, Supplementary Figure S9). Notably, the TLR ligand or tumor-bearing mouse plasma-exposed neutrophils further decreased IFN-γ production and CD69 expression in CD8$^+$ T cells compared to the neutrophils treated with GM-CSF alone. These pre-cultured neutrophils could also suppress CD4$^+$ T cell function in the co-culture system (Supplementary Figure S10). Nuclease treatments for tumor-bearing mouse plasma supplemented medium suppressed PD-L1 upregulation in the pre-cultured neutrophils (Supplementary Figure S11), and these neutrophils reduced their suppressive effects against CD8$^+$ T cells which resulted in equivalent IFN-γ production to GM-CSF-pre-cultured neutrophils in the co-culture systems (Supplementary Figure S12). Finally, we investigated whether PD-1/PD-L1 blockade can restore CD8$^+$ T cell function in the presence of PD-L1-upregulated neutrophils. The reduced IFN-γ production in CD8$^+$ T cells cocultured with immunosuppressive neutrophils was significantly restored by anti-PD-L1 mAb treatment compared with isotype Ab treatment (Figure 1O).

In summary, this study found that intracellular TLR stimulation upregulated PD-L1 expression in neutrophils. Moreover, we posit that tumor-released CFNA participates in PD-L1 upregulation of neutrophils via intracellular NAS represented by TLR7, 8, and 9. The TLR-mediated PD-L1 upregulation results in neutrophils gaining immunosuppressive activity which dampens T cell function, and thus implicates a potential new target for anti-cancer immunotherapy (Figure 1P). An important remaining question is the mechanism of CFNA uptake in neutrophils, which may be via endocytosis or micropinocytosis. Other NASs, such as cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) and retinoic acid-inducible gene-I (RIG-I)-like receptors, which recognize cytosolic DNA and double-stranded RNA (dsRNA), respectively, may also play roles in sensing tumor-derived CFNA and regulating PD-L1 expression in neutrophils [10]. In addition, specific CFNA sequences triggering intracellular TLR-mediated PD-L1 upregulation in neutrophils also remain to be identified. Our results suggest the need to investigate PD-L1-upregulated immunosuppressive neutrophils in cancer patients and determine whether they may serve as a predictable marker for effectiveness of PD-1/PD-L1 blockade therapy.

AUTHOR CONTRIBUTIONS

Suguru Saito, Duo-Yao Cao, Tomohiro Shibata, Yan Liu, and Aoi Otagiri-Hoshi performed all experiments. Suguru Saito performed data analysis and finalization. Suguru Saito, Xiaojiang Cui and Kenneth E. Bernstein established methodology. Suguru Saito wrote original manuscript. Suguru Saito, Xiaojiang Cui and Kenneth E. Bernstein finalized manuscript. Xiaojiang Cui and Kenneth E. Bernstein supervised this study. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank Ellen A. Bernstein (Cedars-Sinai Medical Center) for maintaining the animal colony.

CONFLICT OF INTERESTS STATEMENT

The authors have no conflict of interests.

FUNDING INFORMATION

This study was supported by the National Institutes of Health grants (R01AI164519, 2R01CA151610, R21CA280458), and American Heart Association's Career Development award (23CDA1052548), U.S. Department of Defense (W81XWH-18-1-0067) and the Glazer Foundation.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All animal experimental protocols were reviewed and approved by the Animal Welfare Committee of Cedars-Sinai Medical Center (#8780, #8109).

DATA AVAILABILITY STATEMENT

All data are available from the author upon reasonable request.

Suguru Saito[1]
Duo-Yao Cao[1]
Tomohiro Shibata[1,2]
Yan Liu[3]
Aoi Otagiri-Hoshi[1,4]
Xiaojiang Cui[3]
Kenneth E. Bernstein[1,5]

[1]*Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California, USA*
[2]*Department of Pharmacology, School of Medicine, Yokohama City Univeristy, Yokohama, Kanagawa, Japan*
[3]*Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, California, USA*
[4]*Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Ibaraki, Japan*
[5]*Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, California, USA*

Correspondence
Kenneth E. Bernstein, M.D., Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
Email: Kenneth.Bernstein@cshs.org

Xiaojiang Cui, Ph D., Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
Email: xiaojiang.cui@cshs.org

ORCID

Suguru Saito https://orcid.org/0000-0003-2235-8866
Xiaojiang Cui https://orcid.org/0000-0002-6843-736X

REFERENCES

1. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018;18(2):134-147.
2. Döring Y, Drechsler M, Soehnlein O, Weber C. Neutrophils in atherosclerosis: from mice to man. Arterioscler Thromb Vasc Biol. 2015;35(2):288-295.
3. Hedrick CC, Malanchi I. Neutrophils in cancer: heterogeneous and multifaceted. Nat Rev Immunol. 2022;22(3):173-187.
4. Tang D, Zhang D, Heng Y, Zhu XK, Lin HQ, Zhou J, et al. Tumor-Infiltrating PD-L1+ Neutrophils Induced by GM-CSF Suppress T Cell Function in Laryngeal Squamous Cell Carcinoma and Predict Unfavorable Prognosis. J Inflamm Res. 2022;15:1079-1097.
5. Pylaeva E, Korschunow G, Spyra I, Bordbari S, Siakaeva E, Ozel I, et al. During early stages of cancer, neutrophils initiate anti-tumor immune responses in tumor-draining lymph nodes. Cell Rep. 2022;40(7):111171.
6. Cheng Y, Li H, Deng Y, Tai Y, Zeng K, Zhang Y, et al. Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. Cell Death Dis. 2018;9(4):422.
7. Cisneros-Villanueva M, Hidalgo-Pérez L, Rios-Romero M, Cedro-Tanda A, Ruiz-Villavicencio CA, Page K, et al. Cell-free DNA analysis in current cancer clinical trials: a review. Br J Cancer. 2022;126(3):391-400.
8. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499-511.
9. Sun R, Xiong Y, Liu H, Gao C, Su L, Weng J, et al. Tumor-associated neutrophils suppress antitumor immunity of NK cells through the PD-L1/PD-1 axis. Transl Oncol. 2020;13(10):100825.
10. Schlee M, Hartmann G. Discriminating self from non-self in nucleic acid sensing. Nat Rev Immunol. 2016;16(9):566-580.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.