

Chemotherapy-induced recruitment of myeloid-derived suppressor cells abrogates efficacy of immune checkpoint blockade



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Background & aims: Immune checkpoint blockade (ICB) has been approved for treatment of hepatocellular carcinoma (HCC). However, many patients with advanced HCC are non-responders to ICB monotherapy. Cytotoxic chemotherapy has been proposed to modulate the tumor microenvironment (TME) and sensitize tumors to ICB. Thus, we aimed to study the combination of cytotoxic chemotherapy and ICB in an orthotopic HCC model.

Methods: Preclinical orthotopic HCC mouse models were used to elucidate the efficacy of 5-fluorouracil (5-FU) and ICB. The mice were intrahepatically injected with RIL-175 or Hepa1-6 cells, followed by treatment with 5-FU and anti-programmed cell death ligand 1 (PD-L1) antibody. Myeloid-derived suppressor cells (MDSCs) were depleted to validate their role in attenuating sensitivity to immunotherapy. Flow cytometry-based immune profiling and immunofluorescence staining were performed in mice and patient samples, respectively.

Results: 5-FU could induce intratumoral MDSC accumulation to counteract the infiltration of T lymphocytes and natural killer cells, thus abrogating the anti-tumor efficacy of PD-L1 blockade. In clinical samples, MDSCs accumulated and CD8⁺ T cell numbers decreased following transarterial chemoembolization.

Conclusion: 5-FU can trigger the accumulation of immunosuppressive MDSCs, impairing the response to PD-L1 blockade in HCC. Our data suggest that the combination of specific chemotherapy and ICB may impair anti-tumor immune responses, warranting further study in preclinical models and consideration in clinical settings.

Lay summary: Our findings suggest that some chemotherapies may impair the anti-tumor efficacy of immunotherapy. Further studies are required to uncover the specific effects of different chemotherapies on the immunological profile of tumors. This data will be critical for the rational design of combination immunotherapy strategies for patients with hepatocellular carcinoma.

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Introduction

An immunosuppressive microenvironment plays a key role in mediating immune tolerance and tumor evasion in cancer, which may limit the benefits of immune checkpoint blockade (ICB).¹ Cytotoxic chemotherapy has been proposed to enhance the efficacy of ICB by exerting immunomodulatory effects on the tumor microenvironment (TME).^{2,3} In particular, several cytotoxic drugs, including gemcitabine, 5-fluorouracil (5-FU), doxorubicin and paclitaxel, were shown to deplete myeloid-derived

suppressor cells (MDSCs) in TME.⁴⁻⁷ In the clinical setting, various approaches combining cytotoxic chemotherapy and ICB have been developed in a number of cancers. For example, pembrolizumab in combination with chemotherapy was approved for lung cancer treatment.⁸ For gastrointestinal cancers, the combination of oxaliplatin and 5-FU is currently being investigated in clinical trials (NCT03626922 and NCT02375672).

For HCC, it is evident that unique and potent hepatic-specific immune responses, including the accumulation and activation of MDSCs, are present in the liver.^{9,10} Therefore, the approach of combining chemotherapy and ICB used in other cancer types is also potentially applicable to HCC and similarly proposed for therapeutic development in HCC.¹¹ In fact, clinical trials have been initiated to test ICB in combination with cytotoxic chemotherapy, administered as systemic (NCT03092895) or regional treatment (NCT03778957; NCT04340193; NCT04246177) in HCC. However, the heterogeneous hepatic TME, arising from different

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etiologies, could lead to different treatment outcomes than those observed in other solid tumors.¹²

To evaluate the therapeutic potential of ICB in combination with a chemotherapeutic agent, we first carried out orthotopic HCC mouse experiments using anti-programmed cell death ligand 1 (PD-L1) antibody and 5-FU. 5-FU was chosen in the current study because of its frequent use in regimens of systemic or transarterial treatment for HCC.

Materials and methods

Cell lines and reagents

Murine hepatoma Hepa1-6 cell line was purchased from the American Type Culture Collection and RIL-175 cell line with stable luciferase expression was established by transduction with retroviral vector carrying pBABE-luc-puro. 5-FU was purchased from Cayman Chemical Company. Anti-mouse PD-L1 antibody (10F.9G2), IgG2b control (LTF-2), anti-mouse Ly6G antibody (1A8) and IgG2a control (2A3) were obtained from Bio-X-Cell.

Cytotoxicity assay

Cells were plated in a 96-well plate and treated with 5-FU at concentrations from 10 nM to 1 mM for 24, 48, and 72 hours. Cytotoxicity was quantified by WST-1 assay (Abcam) according to the manufacturer's protocol.

Apoptosis assay

Apoptotic events upon drug treatment were determined by Annexin-V/7-AAD labelling with subsequent flow cytometry analysis. Both early (Annexin-V⁺/7-AAD⁻) and late (Annexin-V⁺/7-AAD⁺) apoptotic events were included for cell death determination.

Mouse HCC tumor model

An orthotopic HCC tumor model was established by injecting 5×10^5 RIL-175 cells or 5×10^6 Hepa1-6 cells into the liver of C57BL/6 mice. Tumor growth was monitored by *in vivo* imaging every 5 days. Mice were randomized into different groups: vehicle control; 5-FU (20 mg/kg); anti-PD-L1 (10 mg/kg); 5-FU (20 mg/kg) plus anti-PD-L1 (10 mg/kg). Anti-PD-L1 and 5-FU were administered intraperitoneally every 5 days and 3 times per week, respectively. Anti-Ly6G or IgG2a isotype was given every 7 days via i.p. injection. This model has been established by our group to study the efficacy of ICB in HCC and under the approval of the Animal Experimentation Ethics Committee (AEEC) at The Chinese University of Hong Kong.¹³

Flow cytometry analysis

Tumor, liver, spleen and blood of the mice were harvested at endpoint and homogenised into single cell suspensions. Cells were stained with a mixture of fluorescence conjugated antibodies as follows: myeloid markers CD11b, Gr-1, Ly6C, Ly6G; T cell markers CD3, CD4, CD8; leukocyte marker CD45. The cells were then analyzed by flow cytometry using BD Aria Fusion.

Immunofluorescence staining

Sections of formalin-fixed paraffin-embedded tumor tissues were collected from HCC patients at Prince of Wales Hospital, Hong Kong. Written consent was obtained from all patients in this study and approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Antigen retrieval was performed with citrate buffer, followed by blocking and incubation with primary antibodies

against CD11b, CD14, CD15 and CD3, CD8. After washing and incubation with fluorophore-conjugated secondary antibodies, images were captured by Axio Observer Z1 (Carl Zeiss, Germany).

Patients

The clinical part of this study came from established HCC cohorts from the Prince of Wales Hospital in Hong Kong.^{14,15} Approvals by ethics committee were obtained for these 2 cohorts. The inclusion and exclusion criteria for both cohorts were reported previously.^{14,15} To evaluate the impact of chemotherapy in this study, patients with treatment of 5-FU-based transarterial chemoembolization (TACE) and available baseline and post-treatment tumor tissues were identified.

Statistical analysis

An unpaired t-test was used to compare 2 groups. One-way or two-way ANOVA with Tukey's *post hoc* tests were used for comparison between multiple groups. Values were presented as mean \pm SD and were considered as statistically significant when $*p < 0.05$; $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Results

Chemotherapy offsets the anti-tumor effect of ICB by counteracting infiltration of T cells and NK cells

Two murine hepatoma cell lines, RIL-175 and Hepa1-6 were found to be sensitive to 5-FU treatment *in vitro* as indicated by the cell viability and apoptosis assay (Fig. 1A-D). The *in vivo* efficacy of 5-FU in combination with PD-L1 blockade was further examined using an orthotopic HCC model^{13,16} (Fig. 1E). Anti-PD-L1 single treatment exerted the most significant anti-tumor activity, as reflected by the retarded tumor growth rate and decreased endpoint tumor weight. On the contrary, tumors did not respond to 5-FU monotherapy, and strikingly, the significant reduction of tumor growth by PD-L1 blockade was offset in mice given combined treatment (Fig. 1F-H). Beyond tumor burden, tumor apoptotic cells were assessed based on their negative association with tumor size in our model and a similar trend was found in the combined treatment group (Fig. 1I-J). These data illustrated that 5-FU treatment abrogated the effectiveness of PD-L1 blockade in HCC.

We then investigated the underlying immunomodulatory mechanisms in TME. Upon co-treatment, the increase in infiltration of CD8⁺ T cells and natural killer (NK) cells observed with single anti-PD-L1 treatment was reversed (Fig. 1K-L). The number of intratumoral CD8⁺ T cells and NK cells positively correlated with apoptotic tumor cell percentage (Fig. 1M-N) and negatively correlated with tumor weight (Fig. 1O-P), illustrating that their direct killing effect on tumor cells was the prominent factor in eradicating HCC, a benefit which was abolished by 5-FU.

Recruitment of MDSCs by chemotherapy blunts the response to immunotherapy

Next, we found that myeloid cells, CD11b⁺Gr-1⁺Ly6G⁺Ly6C^{int} polymorphonuclear MDSCs (PMN-MDSCs) but not mononuclear MDSCs (M-MDSCs) drastically increased in mice receiving 5-FU in both monotherapy and combined treatment (Fig. 2A-B). Given the well-known T cell suppressive activity of MDSCs, we subsequently examined the association between myeloid cells and immune effector cells and found that the abundance of CD8⁺ T cells was selectively influenced by PMN-MDSCs (Fig. 2C). To functionally verify the involvement of MDSCs in attenuating the

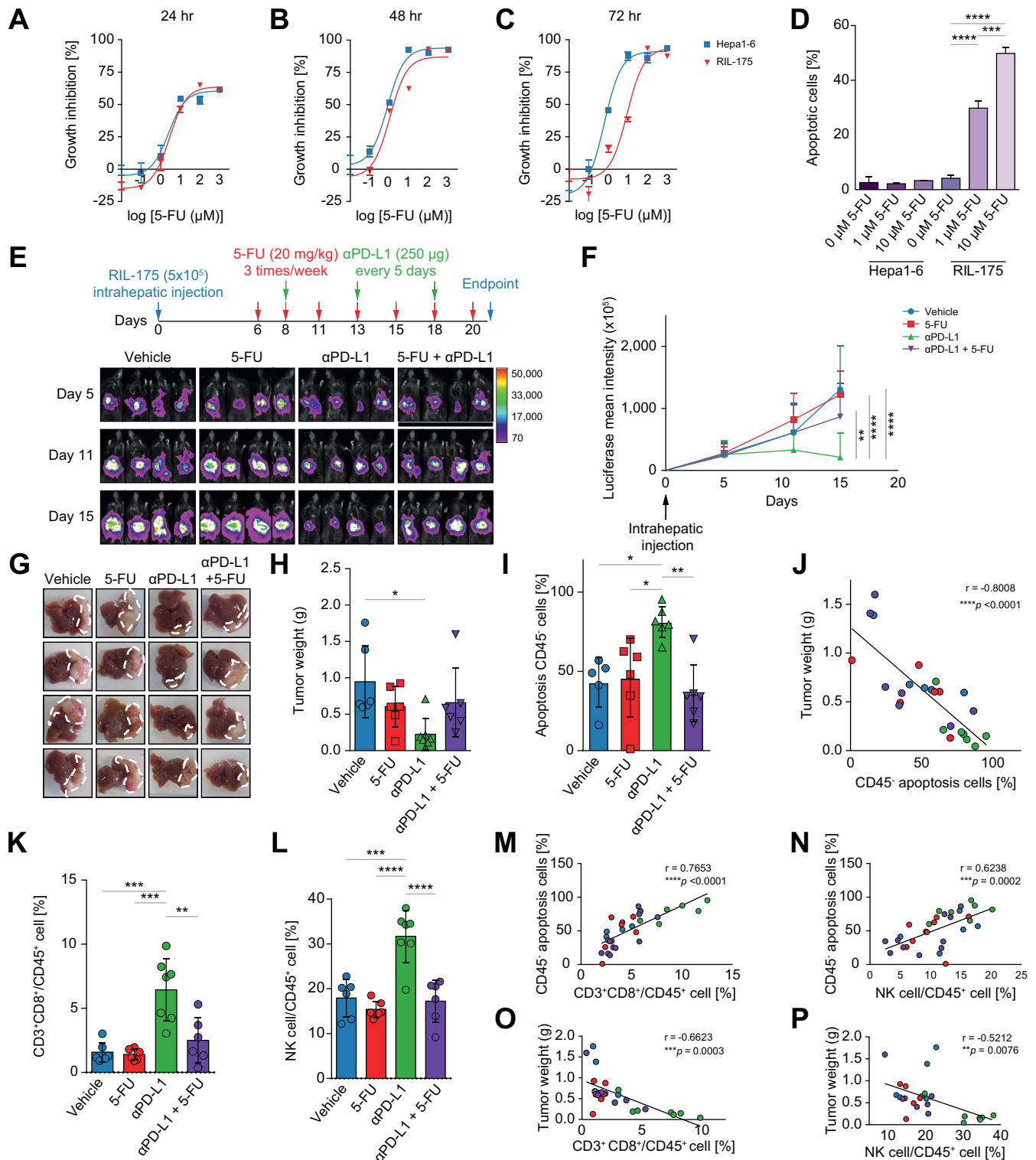


Fig. 1. Chemotherapy hindered anti-PD-L1 efficacy by inhibiting infiltration of immune effector cells. (A-C) Dose-response curves of 5-FU from RIL-175 and Hepa1-6 murine hepatoma cell lines. (D) Apoptotic event was determined by Annexin-V/ 7-AAD co-staining with one-way ANOVA test. (E) Orthotopic HCC model was established by intrahepatic injection of RIL-175 cells, followed by 5-FU (20 mg/kg), anti-PD-L1 (10 mg/kg) or combined treatment. Tumor growth was monitored by *in vivo* imaging as shown. (F) Average luciferase intensity at each time point was calculated (n >6 per group) and analysed by two-way ANOVA. (G, H) Endpoint tumor weight was measured with images displaying tumor morphology. (I) Percentage of apoptotic CD45⁺ tumor cells under drug treatment was determined with one-way ANOVA. (J) Correlation between tumor weight and percentage of apoptotic tumor cells were denoted using Pearson correlation coefficient test. (K, L) Proportions of CD8⁺ T cells and NK cells in overall CD45⁺ leukocytes were measured in tumor. (M-P) Percentages of tumor immune effector cells were positively correlated with the percentage of apoptotic CD45⁺ tumor cells and negatively associated with tumor weight. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. 5-FU, fluorouracil; HCC, hepatocellular carcinoma; NK, natural killer; PD-L1, programmed cell death ligand 1.

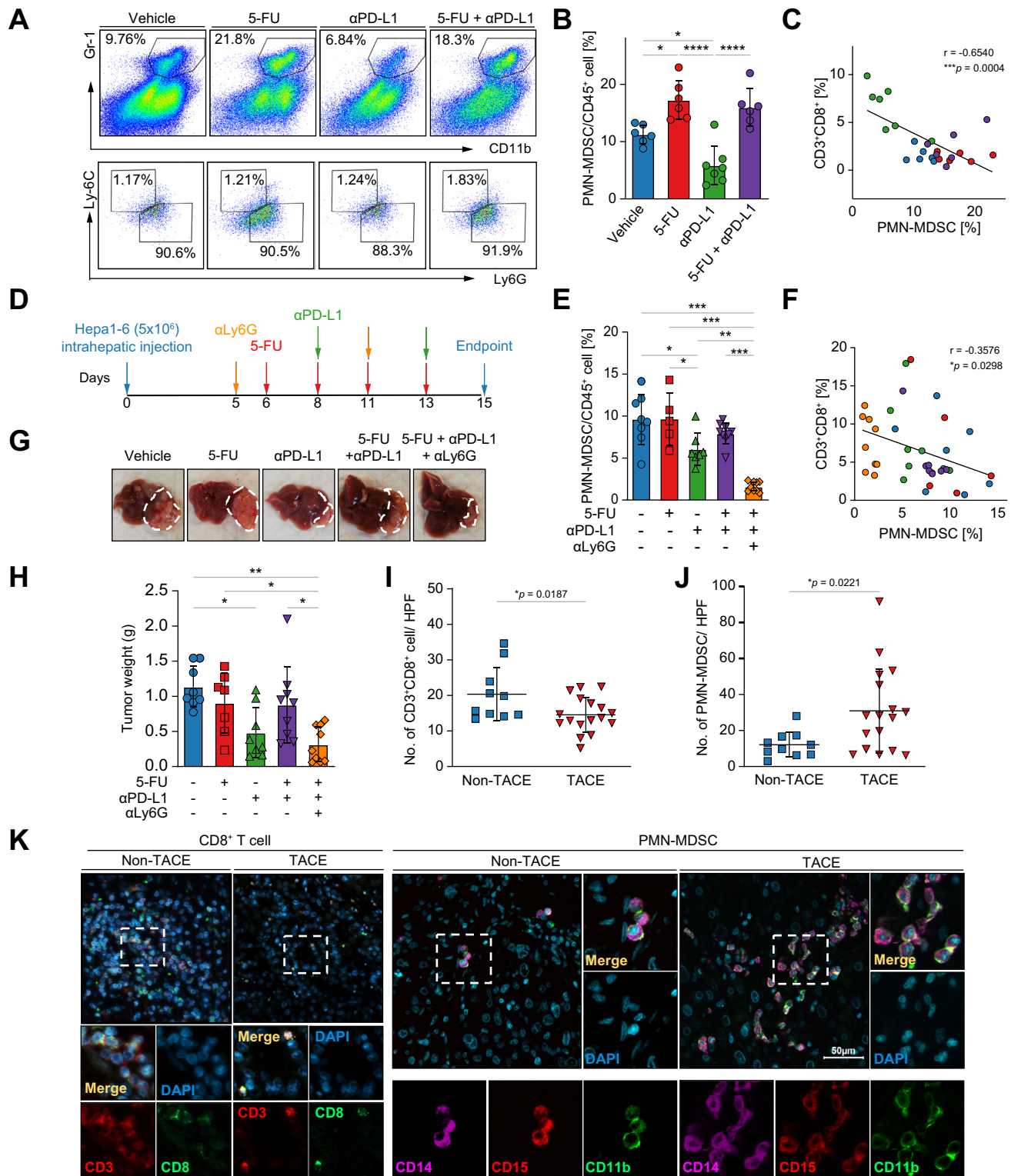


Fig. 2. 5-FU induces accumulation of myeloid cells in tumor. (A) Representative flow cytometry dot plots of myeloid cells from mice tumor were displayed. (B) Proportion of PMN-MDSC among 4 groups of RIL-175 tumor-bearing mice and statistical significance was analysed by one-way ANOVA test. (C) Tumor-infiltrating PMN-MDSCs were negatively associated with intratumoral CD8⁺ T cells using Pearson correlation coefficient test. (D) Orthotopic mouse model using Hepa1-6 cells was established and followed by drug dosing schedule as shown. MDSC depletion was done by i.p injection of anti-Ly6G antibody. (E) Proportion of PMN-MDSCs was determined by flow cytometry analysis with one-way ANOVA test. (F) Correlation between PMN-MDSCs and CD8⁺ T cells in Hepa1-6 tumor site was determined by Pearson correlation coefficient test. (G, H) Representative tumor morphology is displayed and the tumor weights were measured at endpoint. (I, J) Immunofluorescence staining of CD8⁺ T cells and PMN-MDSCs was performed using TACE-treated (n = 17) and non-TACE-treated (n = 10) HCC patient tumor specimens followed by unpaired *t* test for statistical analysis. CD3⁺CD8⁺ T cells, PMN-MDSCs were quantified and averaged from 3 individual fields, represented as the number of cells per high power field. (K) Cell co-stained with CD11b and CD15 is regarded as a PMN-MDSC. Scale bar, 50 μ m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. 5-FU, fluorouracil; HCC, hepatocellular carcinoma; PD-L1, programmed cell death ligand 1; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; TACE, transarterial chemoembolization.

response to immunotherapy, PMN-MDSCs were depleted by anti-Ly6G antibody after tumor implantation in an additional Hepa1-6 orthotopic model (Fig. 2D).¹⁷ Consistent with the RIL-175 tumor model, reduction of PMN-MDSCs by anti-PD-L1 was counteracted by 5-FU in the co-treatment group (Fig. 2E) and the proportion of PMN-MDSCs was negatively associated with CD8⁺ T cell numbers as well (Fig. 2F). Depleting PMN-MDSCs in the mice given combination treatment remarkably restored the anti-tumor effect of PD-L1 blockade compared to the co-treated mice without MDSC depletion (Fig. 2G-H). This result revealed that intratumoral 5-FU induced increased in PMN-MDSCs reduce the sensitivity of HCC tumors to anti-PD-L1.

We validated this immunomodulation in HCC samples from patients treated with TACE. A slight decrease in CD3⁺CD8⁺ T cells was detected in tumor specimens of patients given TACE (n = 17) in comparison to those not receiving TACE (n = 10). Consistently, PMN-MDSCs were enriched in TACE-treated tumors relative to non-TACE-treated tumors (Fig. 2I-K). Taken together, chemotherapy was shown to induce accumulation of immunosuppressive MDSCs and reduce CD8⁺ T cells in the TME.

Discussion

In the current study, we demonstrated that 5-FU induced tumor cell apoptosis *in vitro* but not in an orthotopic mouse model. Such disparity implied that treatment response could be remarkably altered by immune stimulation in the TME. We showed that an elevation in immune effector cells by ICB is critical for anti-tumor immunity, yet this benefit was offset when co-treated with chemotherapy. Detailed analysis of the mechanisms underlying the adverse effects of 5-FU revealed that 5-FU increased PMN-MDSC numbers in the tumor site, leading to suppression of T cells and thereby diminishing the anti-tumor immunity elicited by PD-L1 blockade in combination treatment.

Previously, a number of chemotherapeutic agents have been reported to enhance anti-tumor immunity through elimination of immunosuppressive cells.¹⁸ Unlike our results, a study found that gemcitabine and 5-FU depleted MDSCs in EL4 tumors.⁵ In fact, discrepancies were identified between various studies regarding immunomodulation of 5-FU. For instance, 5-FU depletes MDSCs in the context of FOLFOX (folinic acid, 5-FU, and oxaliplatin), whereas such an effect is reversed in FOLFIRI (folinic

acid, 5-FU and CPT11).³ Besides, it is notable that MDSC depletion by 5-FU in the EL4 tumor model was transient, with MDSC elevation occurring soon after tumor growth. The fact that our results were obtained at a relatively late time point reflecting advanced HCC, may explain why different immunoregulatory effects were obtained with the same chemotherapeutic agent.

Conventional chemotherapy most often causes toxic side effects, whereas low-dose metronomic chemotherapy mediates moderate antineoplastic activity.^{19,20} However, the capability of 5-FU to trigger immunogenic cell death (ICD) remained controversial, as it promoted non-histone chromatin-binding protein high-mobility group box 1 release yet failed to induce endoplasmic reticulum stress and subsequent chaperone calreticulin exposure.²¹ In our study, the dosage of 5-FU, at 20 mg/kg, was well-tolerated with no observable side effects and the equivalent dose in humans is known to be asymptomatic as well.²² In this scenario, though 5-FU was insufficient to induce significant ICD, we may exclude the possibility of ICD in causing TME alteration.

Two caveats should be considered when analyzing these results. First, only 5-FU was used in our experiments. Our findings on MDSCs may not directly apply to other chemotherapeutic agents. More studies are needed to evaluate the impact of other chemotherapeutic agents on the immune environment of HCC. Second, the sample size of the clinical cohort was relatively small due to the difficulty in identifying patients with tumor tissues obtained after TACE procedures. Based on our findings, it is possible that agents that help diminish PMN-MDSC populations in the regimen of combinational 5-FU and ICB may improve anti-tumor efficacy. For example, sunitinib or gemcitabine have been shown to counteract MDSCs.^{23–25} Further studies in HCC are needed to support the feasibility of this additional combination approach.

Taken together, this study uncovered a novel immunosuppressive role of a chemotherapeutic agent in HCC which adversely effected the efficacy of a combination immunotherapy approach. In order to achieve optimal therapeutic efficacy in HCC, it is essential to clarify the precise immunomodulation caused by an individual drug candidate before testing in clinical trials. As we observed similar immune alterations in clinical samples, ICB should be used with caution in chemotherapy-pretreated patients.

Abbreviations

5-FU, fluorouracil; HCC, hepatocellular carcinoma; ICB, immune checkpoint blockade; ICD, immunogenic cell death; NK, natural killer; MDSC(s), myeloid-derived suppressor cell(s); M-MDSC, mononuclear MDSC; PD-L1, programmed cell death ligand 1; PMN-MDSC, polymorphonuclear MDSC; TACE, transarterial chemoembolization; TME, tumor microenvironment.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study design: CHW, JYZ, ASLC and SLC; Data acquisition and analysis: TT; Clinical resources: AWHC, SLC; Writing of manuscript: TT, SLC; Critical review of manuscript: All authors.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2020.100224>.

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