

Non-classical Monocytes Enhance the Efficacy of Immune Checkpoint Inhibitors on Colon Cancer in a Syngeneic Mouse Model

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Abstract. Background/Aim: The response rate to immune checkpoint inhibitors (ICIs) is approximately 10%-30% and only in a few cancer types. In the present study, we determined whether non-classical monocytes (NCMs) could enhance ICI efficacy in colon cancer using a syngeneic mouse model. Materials and Methods: The MC38 C57BL/6 mouse colon cancer model was used. Cells collected from the bone marrow of C57BL/6 mice were cultured, and NCMs were fractionated by cell sorting and administered via the tail veins to the mice implanted with MC38 cells. The anti-mouse PD-L1 antibody was administered three times, and

tumor volume and overall survival were observed. Results: More tumors were eradicated and more complete response occurred, after cotreatment with ICIs and NCMs than after treatment with ICIs alone. Moreover, no efficacy was observed when NCMs were administered alone. Conclusion: NCMs enhance ICI efficacy. The underlying mechanisms and clinical applications will be studied in the future.

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Key Words: Immune checkpoint inhibitor, non-classical monocyte, flow cytometry, overall survival.

The efficacy of immune checkpoint inhibitors (ICIs) used in cancer immunotherapy is approximately 10-30% and only in certain cancers (1). The efficacy rate of ICIs in cases of microsatellite instability (MSI)-high is approximately 30%-40% (2, 3). Combination treatments are necessary to improve the efficacy of ICIs (4).

In our previous study, we analyzed pretreatment peripheral blood samples from ICI-treated patients with various cancer types and found a correlation between monocyte subsets and overall survival (OS). Specifically, OS was significantly shortened in patients with increased proportions of CD14-positive monocytes expressing high levels of PD-L1. Lung cancer patients characterized by PD-L1 expression $\geq 20\%$, with classical monocytes (CMs; CD14+, CD16-) in the blood have a short OS (5). Furthermore, correlation analysis has shown that OS is significantly shortened in patients with a



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Table I. Treatment efficacy of ICI, NCM, ICI + CM, ICI + NCM on the MC38 colon cancer growing in C57BL/6 mice.

	Response type			% of CR
	CR	PR	PD	
ICI	2	5	8	13.30%
NCM	0	3	6	0.00%
CM + ICI	2	3	3	25.00%
NCM + ICI	6	2	1	66.70%

Anti-mouse PD-L1 antibody was administered intraperitoneally after 7, 10, and 14 days. NCMs were isolated as described in the materials and methods. CMs were isolated from peripheral blood. NCM and CM were administered by tail vein on day 5. CR: Complete response; PR: partial response; PD: progressive disease; CM: classical monocytes; NCM: non-classical monocytes; ICI: immune checkpoint inhibitor.

high proportion of CM in the peripheral blood before treatments. OS is significantly prolonged in patients with high proportions of non-classical monocyte (NCMs; CD14+, CD16+). These findings suggest that the monocyte fraction in peripheral blood is a prognostic biomarker for ICI treatment efficacy and can be used in combinations with ICIs to increase OS (6).

Based on the results observed in clinical blood samples obtained from patients, we set to determine whether the administration of NCMs could enhance the efficacy of ICI treatment. We used the MC38 with a MSI-high that responds to ICI in the syngeneic mouse model C57BL/6. In the present study, we determined whether the administration of NCMs in combination with ICI had increased efficacy.

Materials and Methods

Reagents. Anti-mouse PD-L1 antibody (clone: 10F.9G2, BioXCell, Lebanon, NH, USA, catalog number: #BE0101), Fc block (clone: 93, BioLegend, San Diego, CA, USA, catalog number: #101320), anti-mouse CD115 antibody (clone: AFS98, BioLegend, catalog number: #135512), anti-mouse 11b antibody (clone: M1/70, BioLegend, catalog number: #101226), anti-mouse Ly6C antibody (clone: HK1.4, BioLegend, catalog number: #128015), anti-mouse CCR2 antibody (clone: SA203G11, BioLegend, catalog number: #150605), and anti-mouse CX3CR1 antibody (clone: SA011F11, BioLegend, catalog number: #149025) were used in this study.

Cell culture. C57BL6 murine colon cancer MC-38 cells (Kerafast, Boston, MA, USA, catalog number: # ENH204-FP) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and streptomycin. The cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Experimental animals. Female C57BL/6 mice at 6 weeks of age were used in the present study. The mice were housed under a 12-hour light/12-hour dark cycle and provided unrestricted access to

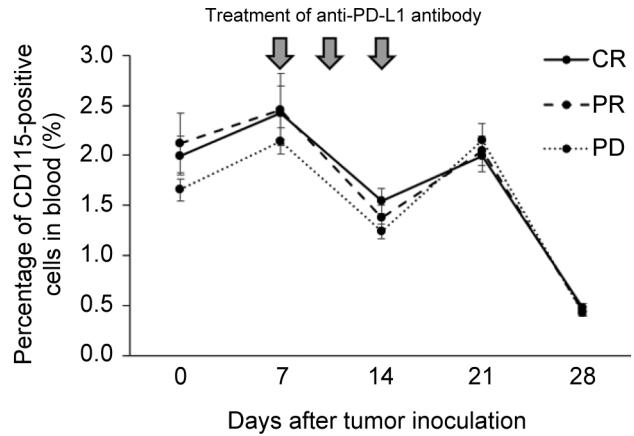


Figure 1. Percentage of CD115-positive cells in the peripheral blood in mice with complete response (CR), partial response (PR), and progressive disease (PD) with immune checkpoint inhibitors (ICIs) treatment.

food. At the time of intervention experiments, there were 9 mice in each group. All animal experiments were conducted in accordance with the Showa University Animal Experimentation Manual and approved by the Showa University Animal Experimentation Committee (approval number 54010).

In all cases, suspensions of exponentially growing MC38 cells (5×10⁵), diluted in 40 µl of PBS, were injected subcutaneously into the right flank of C57BL/6 mice.

Primary culture of mouse monocytes. To obtain NCMs we isolated the femurs and tibias from 6-8-week-old female C57BL/6 mice and washed the bone marrow in 5 mL of cold medium (RPMI 1640 + 10% FBS + 1% penicillin/streptomycin). The bone marrow was resuspended in a homogeneous solution by repeated pipetting and passed through a 70 µm filter to remove debris. After centrifugation at 250 × g for 10 min, the bone marrow was suspended in RBC lysis buffer (Invitrogen, Waltham, MA, USA) on ice for 5 min. The RBC lysis buffer was diluted 10-fold with sterile water, and the solution was centrifuged again at 250 × g for 5 min. Cells at a density of 10⁶ cells/ml were seeded in 6-well ultra-low adherent surface plates with 6 ml of medium per well to prevent permanent adhesion to the bottom of the plate. The suspension was added with 20 ng/ml M-CSF to promote cell differentiation. The cells were maintained in a 37°C humidified 5% CO₂ incubator for 5 days (7), after which adherent cells (macrophages) were discarded and non-adherent cells were collected.

Sorting of NCMs and CMs. For flow cytometry, the forward scatterer (FSC) and side scatterer (SSC) were adjusted based on bead or cell size, and gating was performed using FSC/SSC plots to select live cells or single cells. Debris was removed by excluding regions with high or low FSC values. Duplicate cells were excluded using FSC and SSC, and only single cells were selected. Dead cells were excluded by Propidium Iodide (PI) staining during sorting and 7-Amino-Actinomycin D (7AAD) staining during analysis only; only viable cells were analyzed. CD115- and CD11b-positive cell populations were gated and analyzed based on the expression of Ly-6c, CCR2, and CX3CR1. Ly-6c-negative to weakly positive, CCR2-, and

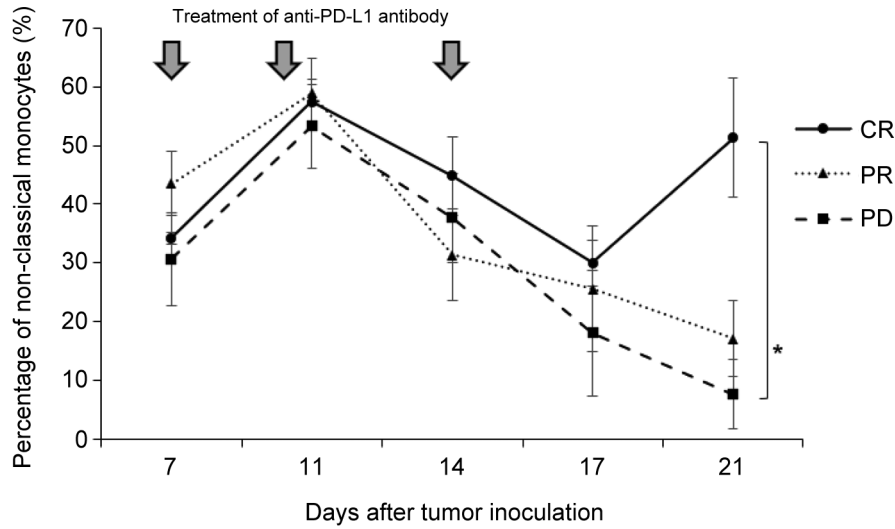


Figure 2. Percentage of non-classical monocytes in the peripheral blood in mice with complete response (CR), partial response (PR), and progressive disease (PD) after immune checkpoint inhibitors (ICIs). * $p=0.044$ (CR vs. PD).

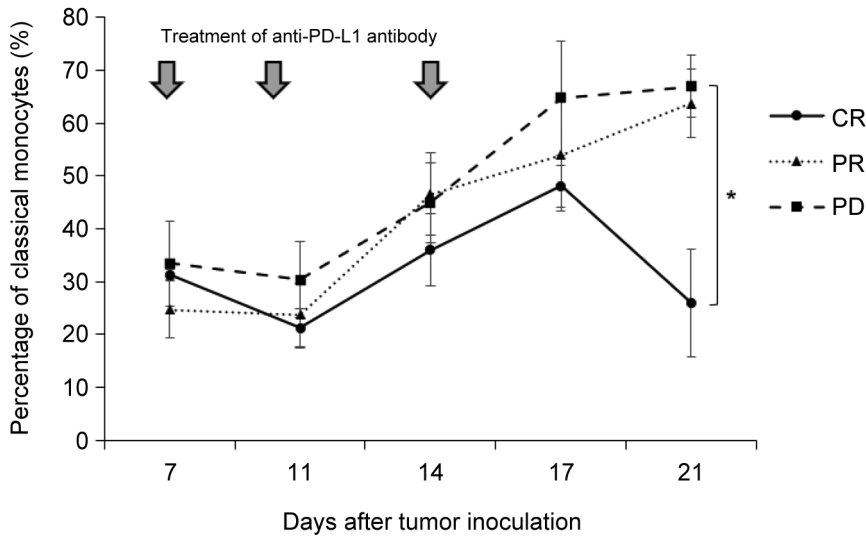


Figure 3. Percentage of classical monocytes among peripheral blood in mice with complete response (CR), partial response (PR), and progressive disease (PD) after immune checkpoint inhibitors (ICIs) treatment. * $p=0.044$ (CR vs. PD).

CX3CR1^{high} cell populations were classified as NCMs, whereas Ly-6c-positive, CCR2⁺, and CX3CR1⁻ to low cell populations were classified as CMs (8, 9). For temporal analysis and sorting, CD115- and CD11b-positive cell populations were gated, and NCMs and CMs were sorted based on Ly-6c expression. The accuracy of sorting was determined by the “purify” setting of the instrument used for sorting. After sorting, the expression levels of CCR2 and CX3CR1 in the cells were measured. NCMs were negative to weakly positive for CCR2 expression and positive for CX3CR1 expression, whereas CMs were positive for CCR2 and negative for CX3CR1 expression.

Flow cytometry for peripheral blood. Blood was collected from the mice by cutting the tail vein with scissors and using capillary blood collection tubes. A total of 75 μ l of blood per mouse was collected and then processed to obtain serum. Red blood cells (RBCs) were lysed using RBC lysis buffer (Invitrogen), and the serum was separated *via* centrifugation at $440 \times g$ for 5 min twice. The resulting serum was adjusted to a concentration of 1×10^6 cells/100 ml using 0.5% bovine serum albumin. The adjusted samples were blocked for the Fc receptors and stained with anti-mouse CD115, anti-mouse 11b, anti-mouse Ly6C, anti-mouse CCR2, and anti-mouse CX3CR1

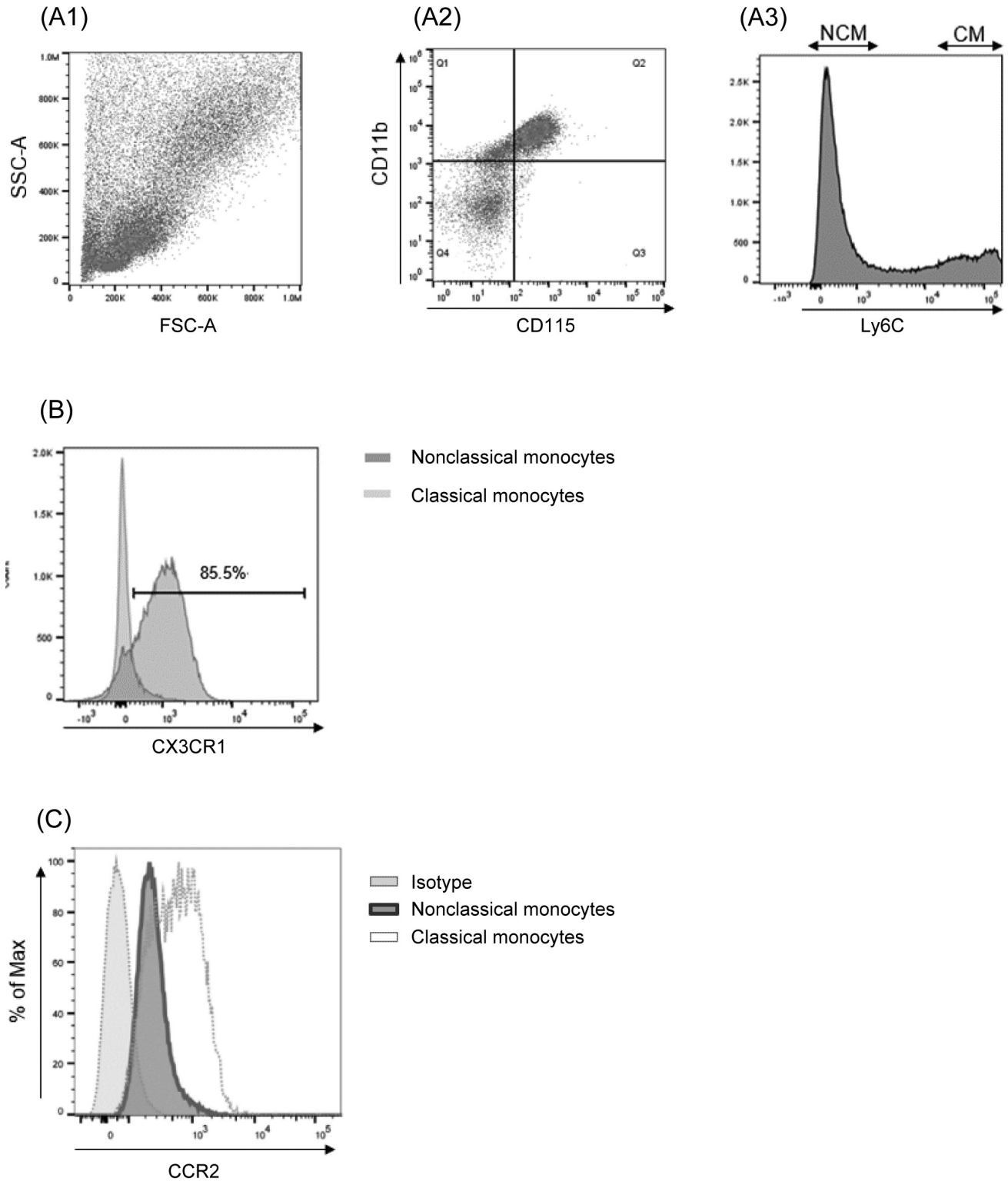


Figure 4. Flow cytometric analysis of monocytes after five days of culture of cells obtained from the bone marrow using the method described in the Materials and Methods. The ratio of Non-classical monocytes (NCMs) was higher than that isolated from peripheral blood. (A1-3) NCM sorting strategies. (B) CX3CR1 expression in each monocyte fraction. Darker areas in the graph indicate NCMs. The numbers indicate the percentage of CX3CR1 expression in NCMs relative to isotype. (C) CCR2 expression in monocyte fractions. Histograms showing CCR2 expression in mouse NCMs, CMs, and isotypes. Thick lines indicate NCMs.

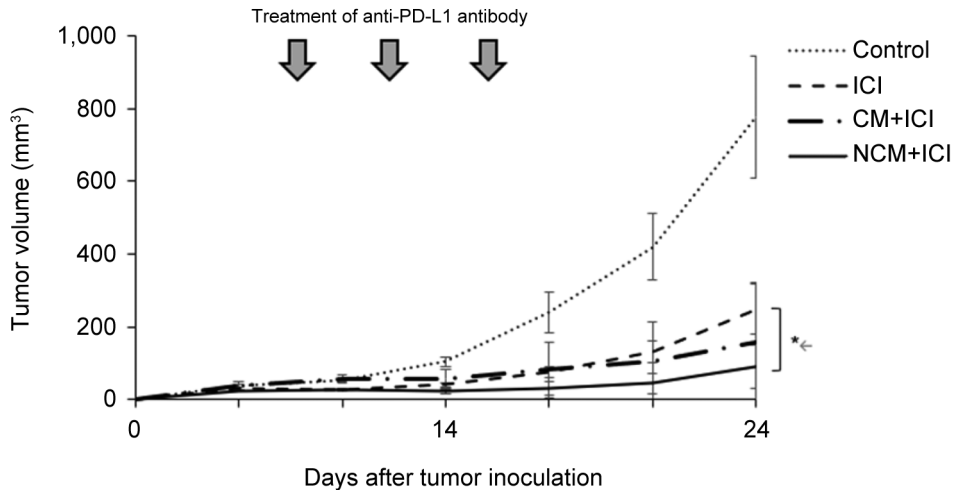


Figure 5. Efficacy of immune checkpoint inhibitors (ICIs), ICIs + classical monocytes (CMs) or ICIs + non-classical monocytes (NCMs) on the MC38 C57BL/6 mice model. Tumor volume after administration of non-classical monocytes was obtained by cell sorting. None of the tumors in the control group was eradicated, one tumor was eradicated in the group treated with ICIs alone. Four tumors were eradicated in the group treated with CMs and ICIs, and six tumors were eradicated in the group treated with NCMs and ICIs. * $p < 0.001$ (ICI vs. NCM + ICI).

antibodies and analyzed by flow cytometry. Flow cytometry was performed using BD FACSLyric flow cytometer (BD Biosciences), and cell sorting was performed using Cell Sorter SH800S (Sony Biotechnology, San Jose, CA, USA). Data were analyzed using FlowJo software (version 10.8.1; Tree Star, Ashland, OR, USA) and gated based on FSC and SSC characteristics. A minimum of 10,000 events per sample were analyzed. Subsequent analyses were performed using the gating strategy described in the text.

Treatment with NCMs and anti-mouse PD-L1 antibody administration in vivo. Six-week-old female C57BL/6 mice were inoculated with MC38 cells (5×10^5) subcutaneously on their backs, and anti-mouse PD-L1 antibody was injected intraperitoneally (200 mg/individual) on days 7, 11, and 14. In the combined group, 1×10^5 NCM or CM obtained by cell sorting were injected through the tail veins 2 days before the first ICI injection.

Statistical analysis. Continuous variables are expressed as means \pm standard deviation. Statistical analyses were performed using JMP Pro v.16.0 (SAS Institute, Cary, NC, USA) and EZR (10). EZR is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) to perform statistical analysis using Student t tests, Mann-Whitney *U*-tests, and linear mixed effects models. Survival curves were analyzed using the log-rank test. Statistical significance was set at $p < 0.05$.

Results

Efficacy of ICI alone on the MC38 tumor. Of the 15 treated mice, two showed complete response (CR), five partial response (PR), and eight progressive disease (PD) (Table I). CR was defined as complete tumor disappearance; PD group was defined as tumor diameter increase of more than +10% after ICI administration; and PR was defined as all other cases.

Correlation of monocyte types and response to ICIs on the MC38 tumor. Monocytes were defined as cells that were positive for both CD115 and CD11b (9, 11). The percentage of monocytes in the peripheral blood was not significantly different among the mice with CR, PR, and PD (Figure 1). The monocytes were further classified into two subpopulations based on Ly-6c expression, NCMs were Ly-6c negative to low, whereas CMs were Ly-6c high. The NCM percentage in the different responders did not differ on day 7 after cancer cell transplantation just before ICIs treatment. However, the NCM percentage increased in the CR group and decreased in the PD group from day 7 to day 14. On day 21 after the ICI treatment, the CR group had a significantly higher percentage of NCMs than the PD group ($p = 0.044$) (Figure 2). Similarly, the CM percentages in the different groups did not differ on day 7. From day 7 to day 14, the CM percentage became lower in the CR group than in the PD group, and this difference became significant on day 21 ($p = 0.044$) (Figure 3). Considering that these results suggest a similar correlation in the peripheral blood of mice as in humans, we next determined whether pre-administration of NCM enhances the effect of ICI treatment.

Analysis of NCMs obtained in culture. Monocytes obtained from bone marrow and cultured for 5 days were analyzed using flow cytometry; CD11b and CD115 positive cells were gated and sorted into NCMs and CMs according to Ly-6c expression. The sorted NCMs were analyzed for expression of CX3CR1, another known expression marker of NCMs, for confirmation, and 85.5% were positive. CCR2 expression was lower than that of CMs (Figure 4A-C).

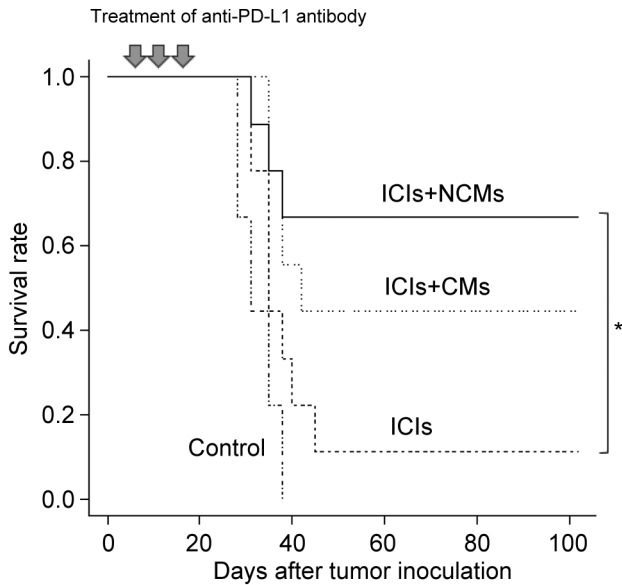


Figure 6. Survival analyzed using the Kaplan–Meier method. CMs: Classical monocytes; NCMs: non-classical monocytes; ICIs: immune checkpoint inhibitors.

Synergistic efficacy of NCMs administration and anti-mouse PD-L1 antibody treatment on the MC38 tumor. A significant difference in tumor growth change was found between the NCM + ICI and ICI groups (Figure 5). Tumor eradication was observed in 6 of 9 animals in the NCM + ICI group. In the CM + ICI group four tumors were eradicated, whereas tumor eradication was observed in only 1 of 9 animals in the ICI group. Mice treated with NCM + ICI had a 50% survive time of more than 100 days. Mice treated with CMs + ICIs had a 50% survive time of 42 days. Mice treated with ICIs had a 50% survive time of 35 days.

Log-rank *p*-value of the comparison between NCMs + ICIs and ICIs was 0.0274, indicating a significant difference in survival rate between the NCMs+ICIs and ICIs groups during the 100-day follow-up period (Figure 6).

The efficacy and CR rates of ICI, NCM, ICI + CM, and ICI + NCM groups were compared (Table I). CR was defined as complete tumor disappearance; PD was defined as tumor diameter increase of more than +10% after ICI administration; and PR was defined as all other cases. In the NCM group, CR was 0 (0.00%), PR was 3, and PD was 6. In the CM+ICI group, CR was 2 (25.00%), PR was 3, and PD was 3. In the NCM+ICI group, CR was 6 (66.70%), PR was 2, and PD was 1. The comparison of the CR rates resulted *p*=0.0215 for ICI vs. NCM+ICI and *p*=0.0091 for NCM vs. NCM+ICI, but the significance level with Bonferroni's correction between the four groups was less than 0.0083, so the difference was not significant. However, there was a trend toward more CR cases in the NCM+ICI group.

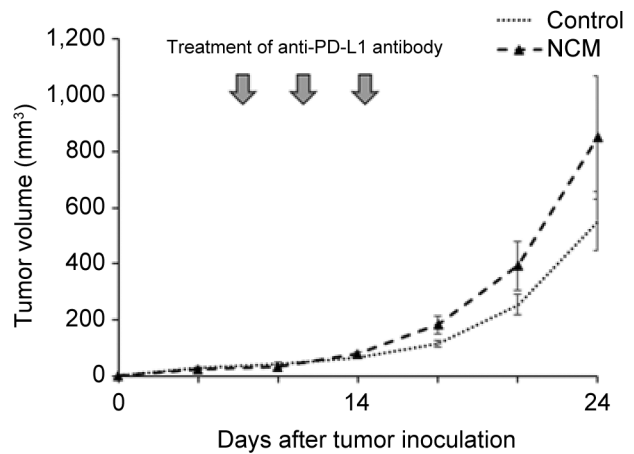


Figure 7. Efficacy of non-classical monocytes (NCMs) alone. NCMs were isolated as described in the Materials and Methods. NCMs were administered by tail vein on day 5.

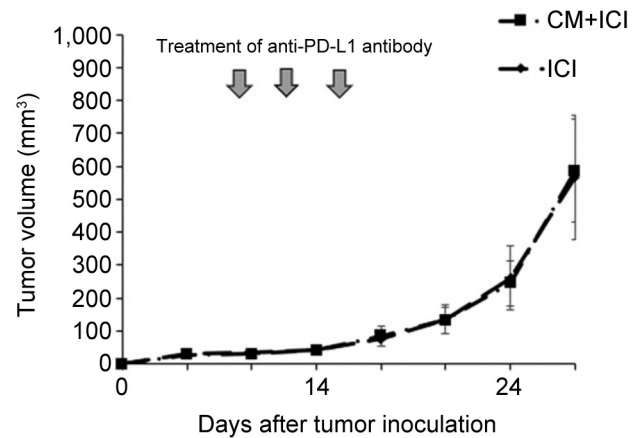


Figure 8. Combined effects of immune checkpoint inhibitor with classical monocytes (CMs) derived from peripheral blood. Tumor volume measured after administration of CM. CM was sorted from peripheral blood on the day of administration. No antitumor effects were observed in the CM group.

To exclude the possibility that the efficacy observed in the NCM group was a direct antitumor effect of NCM, we analyzed the efficacy of NCM treatment alone. Results showed no significant differences in tumor volume growth between the NCM group and control group (Figure 7).

Since CM is also abundant in peripheral blood, CM was then sorted directly from peripheral blood. The efficacy of the combination of CM and ICI was analyzed. The results showed no significant difference in tumor volume between the CM+ICI group and the control group (Figure 8).

Discussion

Our previous study showed that a high proportion of NCMs prior to ICI administration in the peripheral blood of patients

with lung cancer is linked to prolonged OS (6). Therefore, in the present study, we investigated the efficacy of NCMs in combination with ICI therapy on the MC38 C57BL/6 syngeneic colon cancer mouse model.

In mice, the ratio of NCM in the CR group with ICI treatment increased after ICI treatment, similar to that observed in humans. After ICI treatment, the ratio of NCM increased and the ratio of CM decreased in the CR group, whereas the ratio of NCM decreased in the PD group. Notably, the ratio of CM increased in the PD group. The results of the mouse experiments reflect the clinical results.

Based on the results of our previous human study (6), we investigated whether the efficacy of ICI could be enhanced by administering NCM in a syngeneic mouse model of colon cancer. The results showed that the tumor eradication rate was higher in the NCM + ICI group than in the ICI group. The number of mice with tumor eradication was also higher in the CM + ICI group than in the ICI group.

However, CM were obtained by sorting using a special culture method, and no additional effect on ICI was observed when CM were directly sorted and administered from the peripheral blood (Figure 8). Thus, the CMs obtained in culture have a function different from that of CM in the peripheral blood. Considering the previous finding that NCMs exert no direct antitumor effect *in vitro* (12), and the present results that NCM alone demonstrates no efficacy (Figure 7). It can be concluded that NCM enhances ICI treatment.

The present study is the first to demonstrate synergistic anti-tumor efficacy using an anti-PD-L1 antibody combined with NCM. Future research will elucidate the mechanism of enhancement by NCMs and determine whether administration of NCMs in the clinic enhances the effects of ICI.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

TG: Conception, experimental design, data collection and analysis, interpretation, and writing. KI: experimental design, data collection, analysis, and interpretation. TT: Interpretation. SW: conception, experimental design, interpretation, writing, final approval of manuscript, agreement to accountability. SW supervised the conduct of this study. RMH re-wrote the manuscript. All Authors reviewed the manuscript draft and revised it critically on intellectual content. All Authors approved the final version of the manuscript to be published.

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Received October 19, 2023
Revised November 16, 2023
Accepted November 17, 2023