

Coffee extracts and caffeine upregulate the expression of the immune checkpoint factors, PD-1 and PD-L1

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Abstract. Coffee is considered to exert preventive effects on malignant tumors; however, the underlying molecular mechanisms are not yet fully elucidated. The immune checkpoint functions as a mechanism of carcinogenesis and its inhibition by an antibody is a promising strategy for the treatment of malignant tumors. In the present study, the association between coffee and the immune checkpoint was analyzed. The present study examined whether extracts of coffee affect the transcriptional regulation of the immune checkpoint factors, programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) and found that the coffee extracts increased the transcriptional activities of these factors in chronic myelogenous leukemia K562 cells. In addition, caffeine, a component of coffee, enhanced the transcriptional activities of PD-1 and PD-L1. Reverse transcription-quantitative qPCR revealed that caffeine induced an increase in the mRNA levels of these factors. Cell cycle analysis revealed that the coffee extracts and caffeine induced the death of K562 cells. On the whole, the findings of the present study provide novel insight into the effects of coffee and caffeine, and provide a novel regulatory mechanisms of the immune checkpoint in malignant tumors.

Introduction

To date, the consumption of coffee has been suggested to prevent malignant tumors. Epidemiological studies have reported that the consumption of coffee is associated with a reduced the risk

of certain types of cancer, such as hepatocellular carcinoma and colorectal cancers (1-4). In addition, *in vivo* studies using animals and *in vitro* studies using cultured cells also indicate the suppressive effects of coffee on malignant tumors (5-10). Chlorogenic acid, a polyphenol contained in coffee, possesses antioxidant functions and it is considered to contribute to the antitumor effects; however, the molecular mechanism underlying the antitumor effects of coffee have not yet been fully elucidated.

The immune checkpoint was discovered as a novel immune system (11-13), which is induced by the binding between programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) (14-16). The immune checkpoint suppresses the excess immune response to prevent auto-immune disease, but functions as a mechanism of carcinogenesis. In normal cells, immune surveillance eliminates newborn tumor cells. On the other hand, malignant tumor cells acquire a means with which escape from immune surveillance by the expression of the immune checkpoint factor, PD-L1, on their cell surface (17-19). PD-L1 suppresses the immune response by binding to PD-1, which is expressed on immune cells. The antibody agents, nivolumab (brand name, Opdivo) (20-22) and pembrolizumab (brand name, Keytruda) (23,24), which inhibit the PD-1 and PD-L1 interaction exhibit a high efficacy in the treatment of malignant tumors. Thus, immune checkpoint inhibitors have been approved for the treatment of melanoma, lung cancer, gastric cancer, renal cell carcinoma, Hodgkin lymphoma, head and neck cancer, liver cancer, B cell lymphoma, breast cancer and colorectal cancer. by the Food and Drug Administration (FDA) (25,26). Tasuku Honjo and James P. Allison were awarded the Nobel prize in physiology or medicine in 2018 for their discovery of the immune checkpoint (<https://www.nobelprize.org/prizes/medicine/2018/summary/>). The immune checkpoint is an attractive research field, however, the regulatory mechanisms of PD-1 and PD-L1 expression remain poorly understood.

Recently, some food components have been reported to modulate immune checkpoint factors (27). Lycopene, a type of carotenoid, downregulates the signaling and expression of PD-L1 in tongue squamous cell carcinoma cells (28) and flavonoids, such as quercetin and apigenin also decrease PD-1 expression in a number of types of malignant tumors (29-32). On the other hand, vitamin D upregulates PD-L1 expression

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Abbreviations: PD-1, programmed death-1; PD-L1, programmed death-ligand 1

Key words: coffee, caffeine, immune checkpoint, PD-1, PD-L1, malignant tumor

through vitamin D response elements of the gene in epithelial and myeloid cells (33). These findings indicate that food factors contribute to controlling immune responses for onsets of inflammation and malignant tumors through regulation of immune checkpoint factors.

The present study analyzed the effects of coffee on the immune checkpoint in an aim to elucidate the molecular mechanisms underlying the preventive effects coffee on malignant tumors.

Materials and methods

Cells and cell culture. The chronic myelogenous leukemia cell line, K562 (cat. no. RCB-0027), was obtained from the RIKEN BioResource Center and maintained in RPMI-1640 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). The cervical cancer cell line, HeLa (cat. no. RCB0007), was also obtained from the RIKEN BioResource Center. The liver cancer cell line, HepG2, (cat. no. HB-8065) and the colon cancer cell line, SW480, (cat. no. CCL-228) were obtained from the American Type Culture Collection (ATCC). These cells were maintained in DMEM supplemented with 10% FBS and 2 mM-glutamine (Gibco; Thermo Fisher Scientific, Inc.). These cells were maintained at 37°C in humidified air with 5% CO₂.

Generation of plasmids. Transcriptional regulation regions, promoters on the 5'-flanking region of the human *PD-1* or *PD-L1* gene were obtained from human genomic DNA (Promega Corporation) by PCR using PrimeStar GXL DNA polymerase (Takara Bio, Inc.) and inserted into a luciferase reporter plasmid pGL3 (Promega Corporation) at the *KpnI* sites. The plasmids, hPD-1-luc and hPD-L1-luc, were generated. DNA sequences were analyzed using a Genetic analyzer 3500 (Thermo Fisher Scientific, Inc.).

Reagents. Coffee extracts were generated by the dissolution of instant coffee powder Nescafe Excella (Nestle) with sterilized water. Caffeine, chlorogenic acid and caffeic acid were purchased from Nacalai Tesque, Inc. Quinic acid was purchased from Alfa Aesar; Thermo Fisher Scientific, Inc. These reagents were dissolved in dimethyl sulfoxide (DMSO) (FUJIFILM Wako Pure Chemical). Caffeine was used at a final concentration 1, 2 or 4 mM and the other reagents were used at final a concentration 10 μM as reoffered to in previous studies (34,35).

Transfection of plasmids. The plasmids were transfected into K562 cells by electroporation with Nucleofector2b (Program T-016) (Lonza Group, Ltd.). Cells and plasmids were applied into 100 μl buffer of the AMAXA Cell Line Nucleofector Kit V (Lonza Group, Ltd.) in a cuvette equipped with aluminium electrodes of the kit and immediately pulsed at room temperature. Following electroporation, the cells were recovered with RPMI-1640 with 10% FBS. Subsequently, 24 h following incubation at 37°C, the cells were treated with the reagents mentioned above for 24 h, followed by analyses. In the HeLa, HepG2 and SW480 cells, the plasmids were transfected by lipofection with Lipofectamine 2000® (Thermo Fisher

Scientific, Inc.). Plasmid hPD-1-luc or hPD-L1-luc (1.25 μg) which were generated by the insertion of the promoter region into pGL3 (Promega Corporation) and phRL-TK (Promega Corporation) (0.0625 μg) in 25 μl of Opti-MEM I (Life Technologies; Thermo Fisher Scientific, Inc.) and 0.5 μl Lipofectamine 2000® in 25 μl Opti-MEM I were incubated for 5 min at room temperature. These solutions were then mixed, incubated for 20 min at room temperature and added to the cells supplemented in 250 μl RPMI-1640 with 10% FBS. At 24 h following transfection, the cells were treated with the reagents mentioned above for 24 h and collected for use in the experiments.

Luciferase assay. The cells were transfected by electroporation [Nucleofector2b (Lonza Group, Ltd.)] for the K562 cells and by lipofection [Lipofectamine 2000® (Thermo Fisher Scientific, Inc.)] for the HeLa, HepG2 and SW480 cells. The cells transfected with the plasmids, hPD-1-luc or hPD-L1-luc, which were generated by the insertion of the promoter region derived from the human *PD-1* or human *PD-L1* gene into pGL3 (Promega Corporation) and phRL-TK (Promega Corporation) were incubated at 37°C for 24 h. The cells were then treated with the reagents mentioned above for 24 h, harvested and lysed with a 1X concentration of Passive lysis buffer (Promega Corporation) and cell lysate were used for luciferase assay with Dual Luciferase Reporter Assay System (Promega Corporation) and a luminometer (Lumat LB9507, Berthold Detection Systems). The *Renilla reniformis* luciferase plasmid phRL-TK was also transfected with the reporter plasmids to standardize the transfection rate.

Reverse transcription-quantitative qPCR (RT-qPCR). Total RNA was prepared from the cultured cells using Isogen II (Nippon Gene Co., Ltd.). Complementary DNA (cDNA) was generated from total RNA using oligo (dT) (Thermo Fisher Scientific, Inc.), dNTP mix (Thermo Fisher Scientific, Inc.) and superscript IV (Thermo Fisher Scientific, Inc.). RT-qPCR was performed with specific primers for human PD-1 and human PD-L1 synthesized by Eurofins Genomics, THUNDERBIRD™ SYBR qPCR mix (Toyobo Life Science), generated cDNA and the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 10 sec, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, then 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The quantification method used was the ΔΔCq method (36). The primer sequences were the following: Human PD-1 forward, 5'-GACAGCGGCACC TACCTCTGTG and reverse, 5'-GACCCAGACTAGCACC ACCAGG; human PD-L1 forward, 5'-GGAGATTAGATC CTGAGGAAAACCA and reverse, 5'-AACGGAAGATGA ATGTCAGTGCTA; and human actin forward, 5'-GCTGTG CTACGTCGCCCTG and reverse, 5'-GGAGGAGCTGGA AGCAGCC.

Cell proliferation assay. The number of viable cells was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). The cells were incubated with the CCK-8 reagent at 37°C, for 4 h. The absorbance (450 nm) was determined using SpectraMax iD3 (Molecular Devices, LLC).

The percentage growth was determined relative to the control (sterilized water for the coffee extracts and DMSO for caffeine).

Cell cycle analysis. The cultured cells were harvested and suspended in propidium iodide (PI) (Nacalai Tesque, Inc.) solution (PBS containing 10 $\mu\text{g/ml}$ PI, 0.1% Triton X-100) for 5 min at room temperature. Cell cycle and cell death were analyzed by flow cytometry using a FACS Canto II flow cytometer (BD Biosciences) and BD FACS Diva software v6.1.3 (BD Biosciences). The sub-G1 population was estimated as dead cells.

Analysis of apoptosis. For the analysis of cell apoptosis, the cells were counterstained using the Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque, Inc.) for 15 min at room temperature, and were analyzed using a BD Accuri C6 Flow Cytometer (BD Biosciences) and BD Accuri C6 Plus Software (BD Biosciences).

Analysis of cell surface expression. The cells were collected following treatment with caffeine at 4 mM and incubated with anti-PD-1 antibody labeled FITC (cat. no. 11-9969-42, Invitrogen; Thermo Fisher Scientific, Inc.) or anti-PD-L1 antibody labeled PE (cat. no. 12-5983-42, Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h on ice. The analysis was performed using flow cytometry with a FACS Canto II flow cytometer (BD Biosciences) and BD FACS Diva software v6.1.3 (BD Biosciences).

Giemsa staining. K562 cells were attached to a glass slide using a cytospin (cytospin 3, Shandon; Thermo Fisher Scientific). The glass slide was first stained with May-Grunwald's eosine-methylene blue solution (Merck KGaA) for 3 min at room temperature and then stained with Giemsa's azur eosine methylene blue solution (Merck KGaA) for 30 min at room temperature. The slide glass was washed with water. Cell morphology was observed under a microscope (Eclipse E800 optical microscope, Nikon Corporation).

Immunostaining. All cells were treated with 4 mM caffeine for 24 h. The HepG2 and SW480 cells were inoculated in a 12-well plate with cover glasses at the bottom of the plate prior to caffeine treatment. The K562 cells were attached to glass slides using a cytospin after caffeine treatment. The cells were fixed with 100% methanol for 2 min at room temperature, washed with PBS and blocked with PBS with 2% of FBS for 30 min at room temperature. Anti-PD-1 rabbit monoclonal antibody (cat. no. 86163, Cell Signaling Technology, Inc.) or anti-PD-L1 rabbit monoclonal antibody (cat. no. 13684, Cell Signaling Technology, Inc.) was reacted with cells at a 1/200 dilution for 1 h at room temperature. The cells were then reacted with AlexaFluor 488 goat anti-rabbit IgG (cat. no. A11008, Life Technologies; Thermo Fisher Scientific, Inc.) at a 1/500 dilution for 1 h at room temperature. The nuclei were then stained using Prolong Gold antifade reagent with DAPI (P36931, Invitrogen; Thermo Fisher Scientific, Inc.). Immunostaining was observed with a BZ-X710 microscope (Keyence Corporation) at a magnification x400 (scale bar, 20 μm). The mean of fluorescence intensity per cell was measured using ImageJ software (<https://imagej.net/ij/>).

Statistical analysis. Data are presented as the mean of three results \pm standard deviation (SD). To confirm the reproducibility, we performed the experiments more than three times. Data were statistically analyzed using a two-tailed Student's t-test with Microsoft Excel (Microsoft Corporation) for comparisons among two groups, or one-way ANOVA followed by Tukey's post hoc test with GraphPad Prism Version 8.4.3 (Dotmatics) for comparisons among multiple groups. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Coffee extracts enhance the transcriptional activity of human PD-1 and PD-L1. To examine whether coffee affects the expression of the immune checkpoint factors, PD-1 and PD-L1, first, the cloning of the PD-1 and PD-L1 promoter regions was performed. A part of human genomic DNA upstream transcription-start site of human PD-1 or human PD-L1 was amplified by PCR and ligated to the luciferase assay vector pGL3, and hPD-1-luc and hPD-L1-luc were generated (Fig. 1A). To confirm that the cloned regions functioned as a promoter, hPD-1-luc and hPD-L1-luc were transfected into HeLa cells and luciferase assay was performed. The values of luciferase assay for hPD-1-luc and hPD-L1-luc were compared to those of the empty vector, pGL3 (Fig. 1B). The luciferase activities of hPD-1-luc and hPD-L1-luc were markedly increased. The results indicated that the cloned regions functioned as promoters of the PD-1 and PD-L1 genes. Subsequently, the transcriptional regulations of PD-1 and PD-L1 by coffee extracts were examined. The coffee extracts were generated by instant coffee powder dissolved in sterilized water. The concentrations of the coffee extracts used in the present study were as referred to in a previous study (6). The HeLa cells transfected with hPD-1-luc or hPD-L1-luc were cultured in medium containing coffee extracts and luciferase assay was performed with the cell lysate. No changes in transcriptional activity were not observed in the cells transfected with hPD-1-luc; however, the coffee extracts increased the promoter activity of hPD-L1-luc-transfected cells compared to the control (Fig. 2A). The same experiment was then performed using the K562 cells. The transcriptional activities of PD-1 and PD-L1 were markedly increased by the coffee extracts in a concentration-dependent manner (Fig. 2B).

Caffeine activates the transcription of human PD-1 and PD-L1. Subsequently, the components contained in coffee were examined. Caffeine, chlorogenic acid, caffeic acid and quinic acid were used. The concentrations used in the present study were as referred to in previous studies (34,35). Luciferase assay was performed with these components in HeLa cells; however, no change was observed (Fig. 3A). When the luciferase assay was performed in K562 cells, caffeine but not the other components increased the PD-1 and PD-L1 transcriptional activities (Fig. 3B). The enhancements of the PD-1 and PD-L1 transcriptional activities by caffeine occurred in a concentration-dependent manner (Fig. 4A). It was then examined whether endogenous mRNA levels were affected by caffeine using RT-qPCR. Treatment

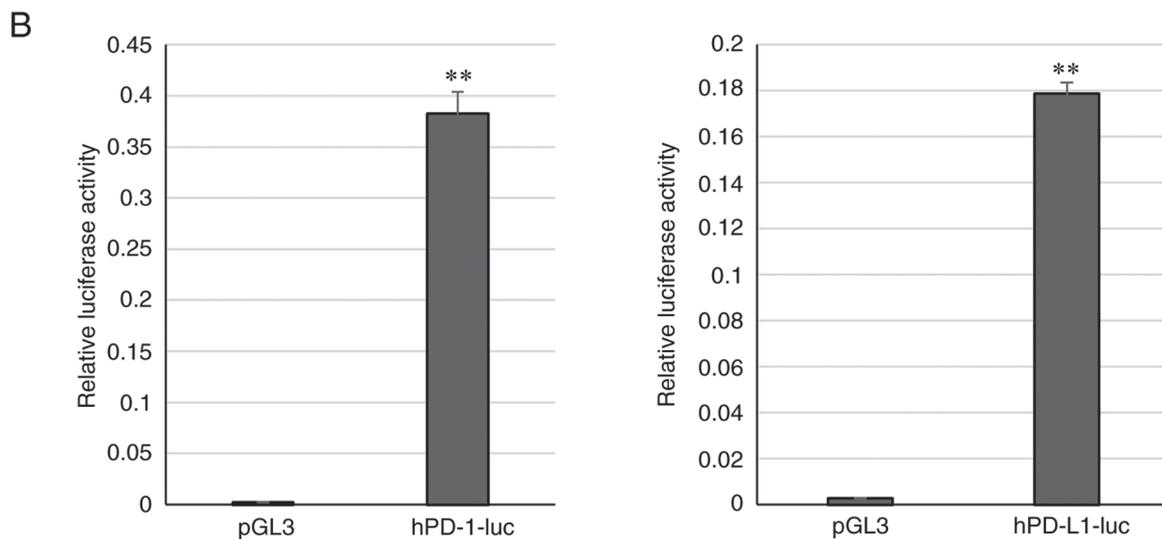
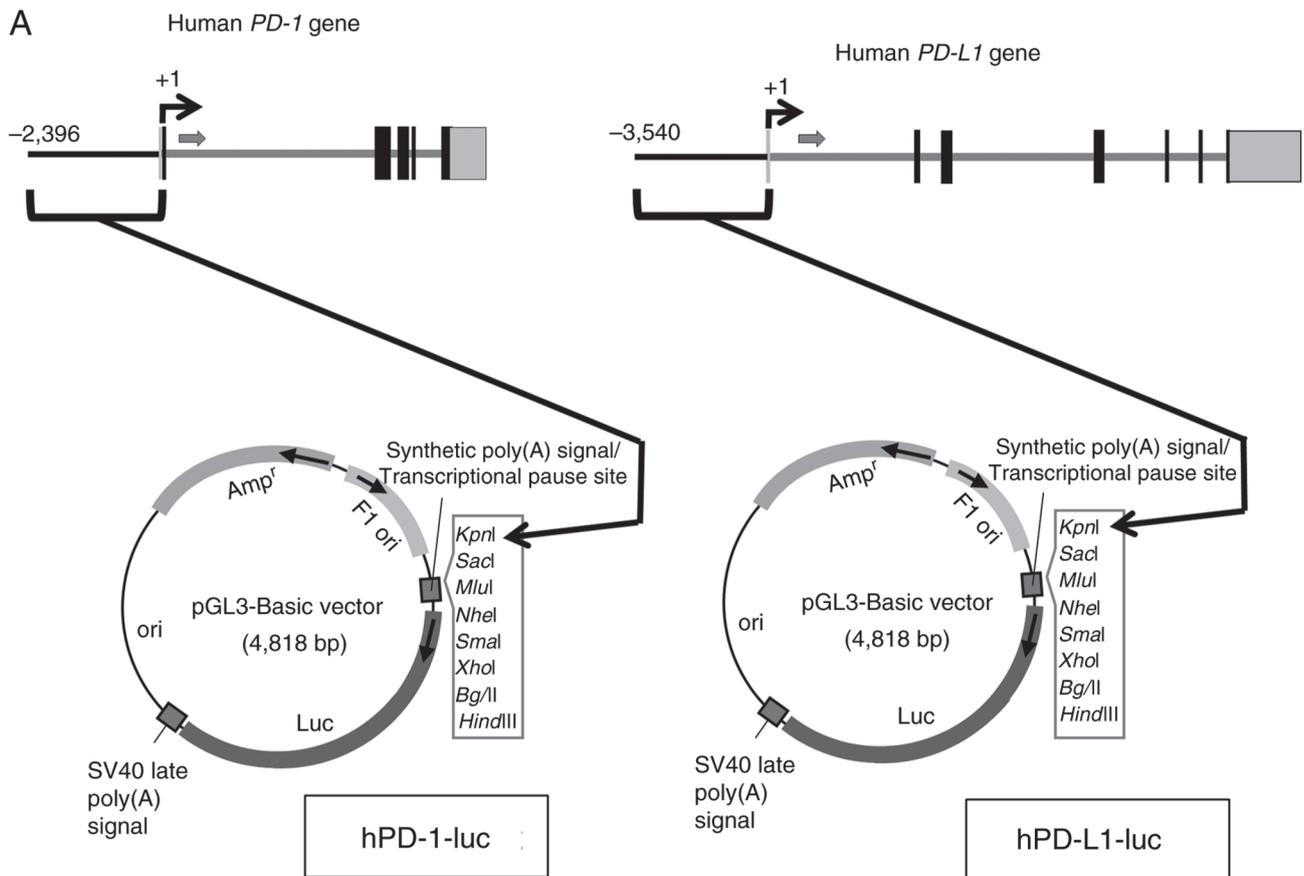


Figure 1. Transcriptional activities of human PD-1 and human PD-L1. (A) Reporter plasmids hPD-1-luc and hPD-L1-luc were generated. The DNA fragment (2,396 bp) upstream human *PD-1* gene or DNA fragment (3,540 bp) upstream human *PD-L1* gene was inserted into multiple cloning sites of a luciferase assay plasmid pGL3 at the *KpnI* site. Black boxes in genes are exons and gray boxes in genes are untranslated regions. (B) Luciferase assay of hPD-1-luc and hPD-L1-luc. hPD-1-luc and the hPD-L1-luc were transfected into HeLa cells and luciferase assay was performed. The empty vector pGL3 was used as a control. The data are presented as the mean of three results \pm standard deviation. ** $P < 0.01$, vs. the control (pGL3). PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

with caffeine increased the mRNA expression levels of PD-1 and PD-L1 (Fig. 4B). Moreover, the cell surface expression of PD-1 and PD-L1 was examined at the protein level, following treatment with caffeine. As shown in Fig. 4C, caffeine increased PD-1 and PD-L1 protein expression on the cell surface.

Effects of coffee extract and caffeine on cell cycle and cell death. The present study then used flow cytometry to reveal whether coffee extracts and caffeine affect the cell cycle and death of K562 cells. When the cells were treated with PI solution, Triton X-100 permeabilized the nucleus and nucleic DNA was stained by PI. Thus, DNA contents

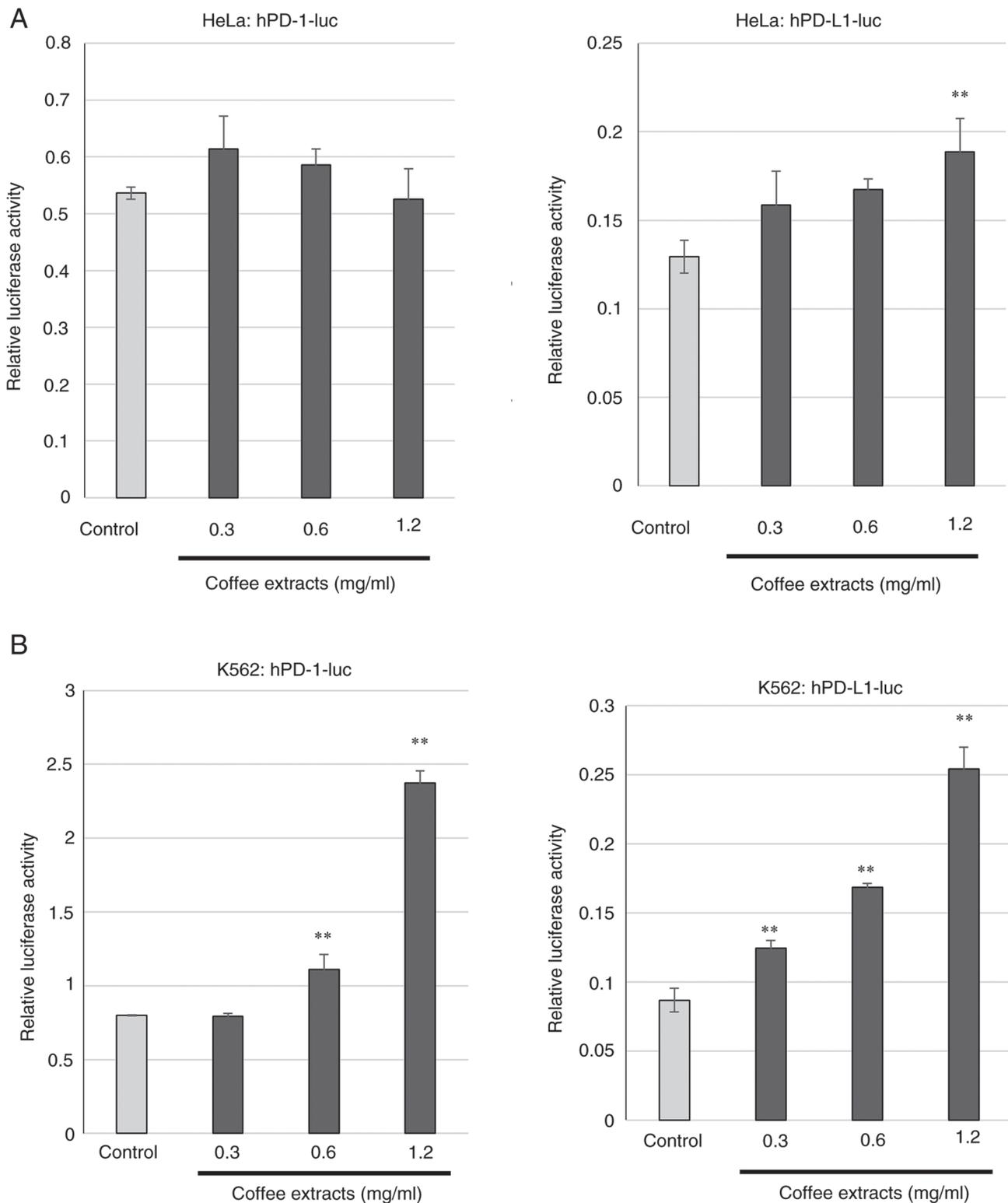


Figure 2. Coffee extracts enhance the transcriptional activity of human PD-1 and PD-L1. The hPD-1-luc and the hPD-L1-luc were transfected into cells and cultured in medium containing coffee extracts for 24 h. Cell lysate was prepared and luciferase assay was performed. (A) HeLa cells and (B) K562 cells. The data are presented as the mean of three results \pm standard deviation. ** $P < 0.01$, vs. the control. PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

were measured. When the cells died, DNA became fragments and the sub-G1 population, which had a lower DNA content than the G1 population, then appeared. Sub-G1 cells were estimated as dead cells. The sub-G1 population of K562 cells treated with the coffee extracts increased in a concentration-dependent manner (Fig. 5A). Treatment with

caffeine also increased the sub-G1 cell population, although the effect was less prominent than that of the coffee extracts (Fig. 5B). In addition, the G2/M cell population was slightly increased following treatment with caffeine. These data indicate that coffee extracts and caffeine induce the death of malignant tumor cells.

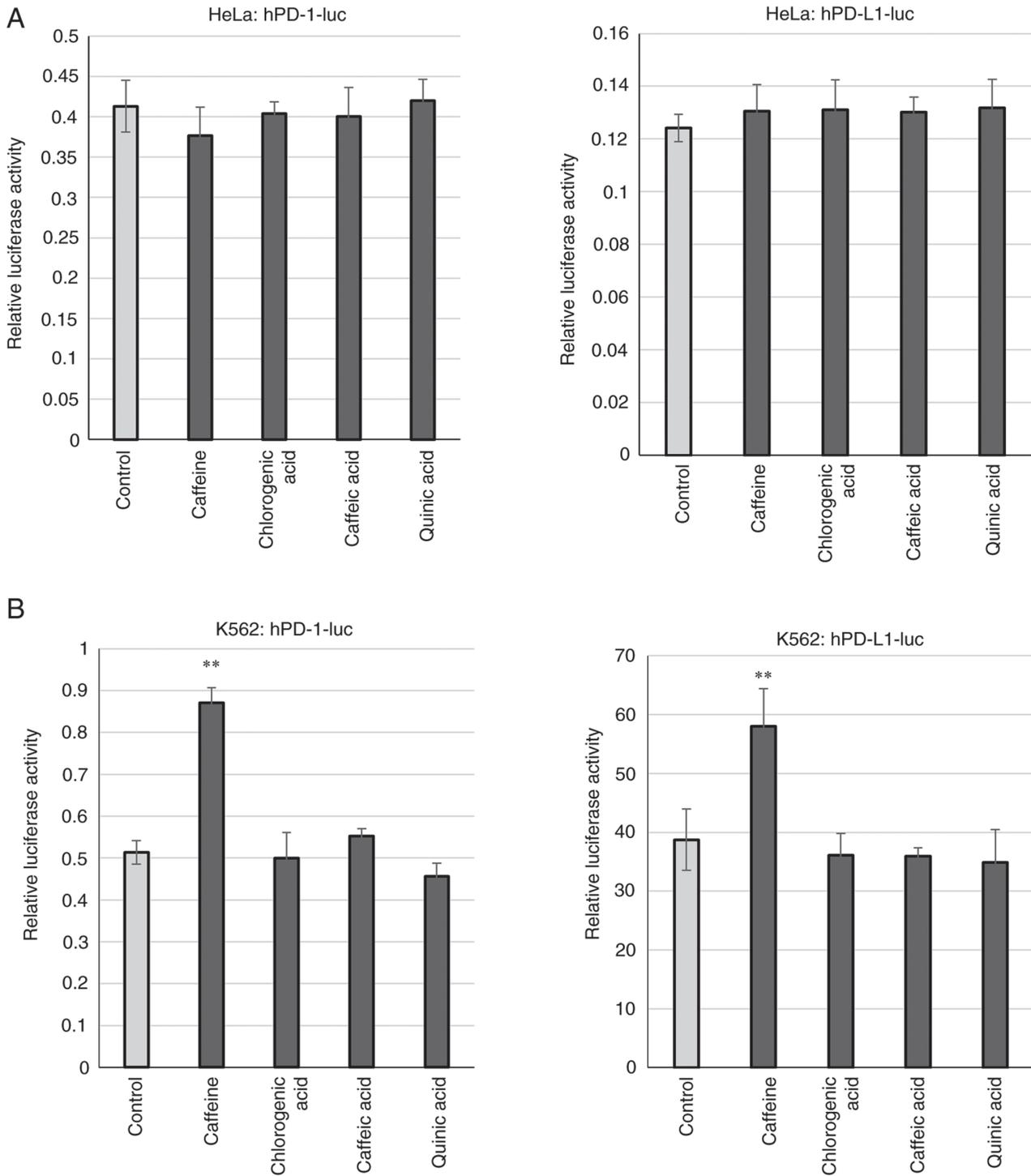


Figure 3. Caffeine activates the transcription of human PD-1 and PD-L1. hPD-1-luc and the hPD-L1-luc were transfected into the cells and the cells were cultured in medium containing components of coffee for 24 h. Cell lysate was prepared and luciferase assay was performed. (A) HeLa cells and (B) K562 cells. The data are presented as the mean of three results \pm standard deviation. ** $P < 0.01$, vs. the control. PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

Subsequently, cell proliferation was examined using CCK-8 assay. The coffee extracts and caffeine inhibited cell proliferation in a concentration-dependent manner (Fig. 5C). The induction of apoptosis was analyzed using Annexin V/PI staining (Figs. 5D and S1). Coffee extracts and caffeine increased the number of Annexin V-positive cells, indicating that apoptosis was induced by treatment with coffee extracts and caffeine. The cell morphologies of the cells treated with the coffee extracts or caffeine were observed (Fig. S2). Neither

coffee extracts nor caffeine induced cell differentiation, such as megakaryocytes, a change which was previously reported in 12-O-tetradecanoyl phorbol 13-acetate-treated K562 cells (37). In the cells treated with the coffee extracts, a number of cells exhibited shrunken shapes and nuclear fragmentation, indicating the induction of apoptosis. The present study examined whether the overexpression of PD-1 and PD-L1 induced cell death; however, a high level of PD-1 and PD-L1 expression did not induce cell death (Fig. S3).

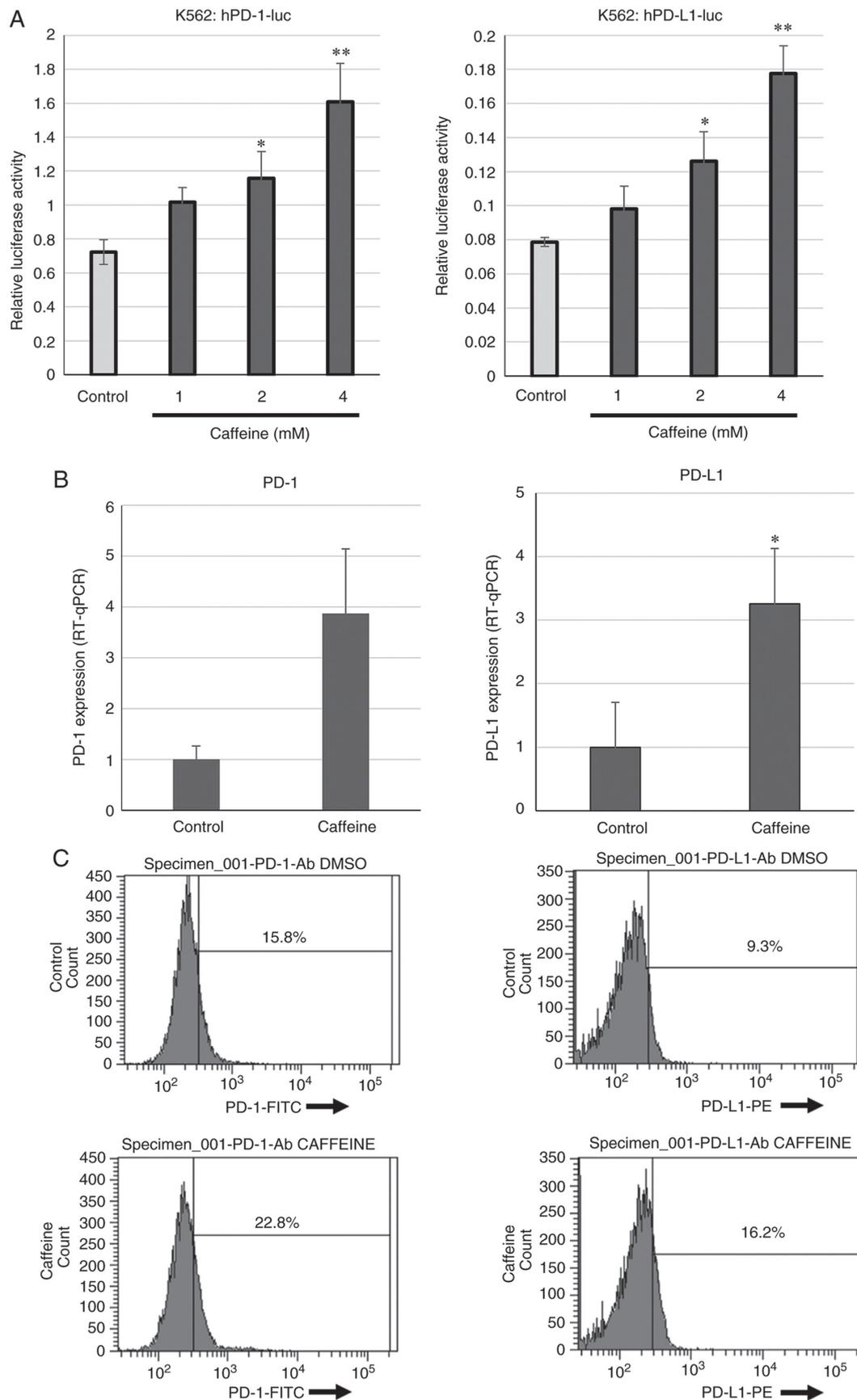


Figure 4. Caffeine increases the mRNA expression of PD-1 and PD-L1. (A) hPD-1-luc and the hPD-L1-luc were transfected into K562 cells and the cells were cultured in medium containing the indicated concentrations of caffeine for 24 h. Cell lysate was prepared and luciferase assay was performed. (B) RT-qPCR of PD-1 and PD-L1 expression in K562 cells treated with caffeine. K562 cells were treated with caffeine for 24 h. Total RNA was extracted and cDNA was synthesized. RT-qPCR was performed with specific primers for PD-1 and PD-L1. The results were normalized to the actin values. The data are presented as the mean of three results \pm standard deviation. (C) Cell surface expression levels of PD-1 and PD-L1 were analyzed by flow cytometry following treatment with caffeine for 24 h. * $P < 0.05$ and ** $P < 0.01$, vs. the control. PD-1, programmed death-1; PD-L1, programmed death-ligand 1; RT-qPCR, reverse transcription-quantitative PCR.

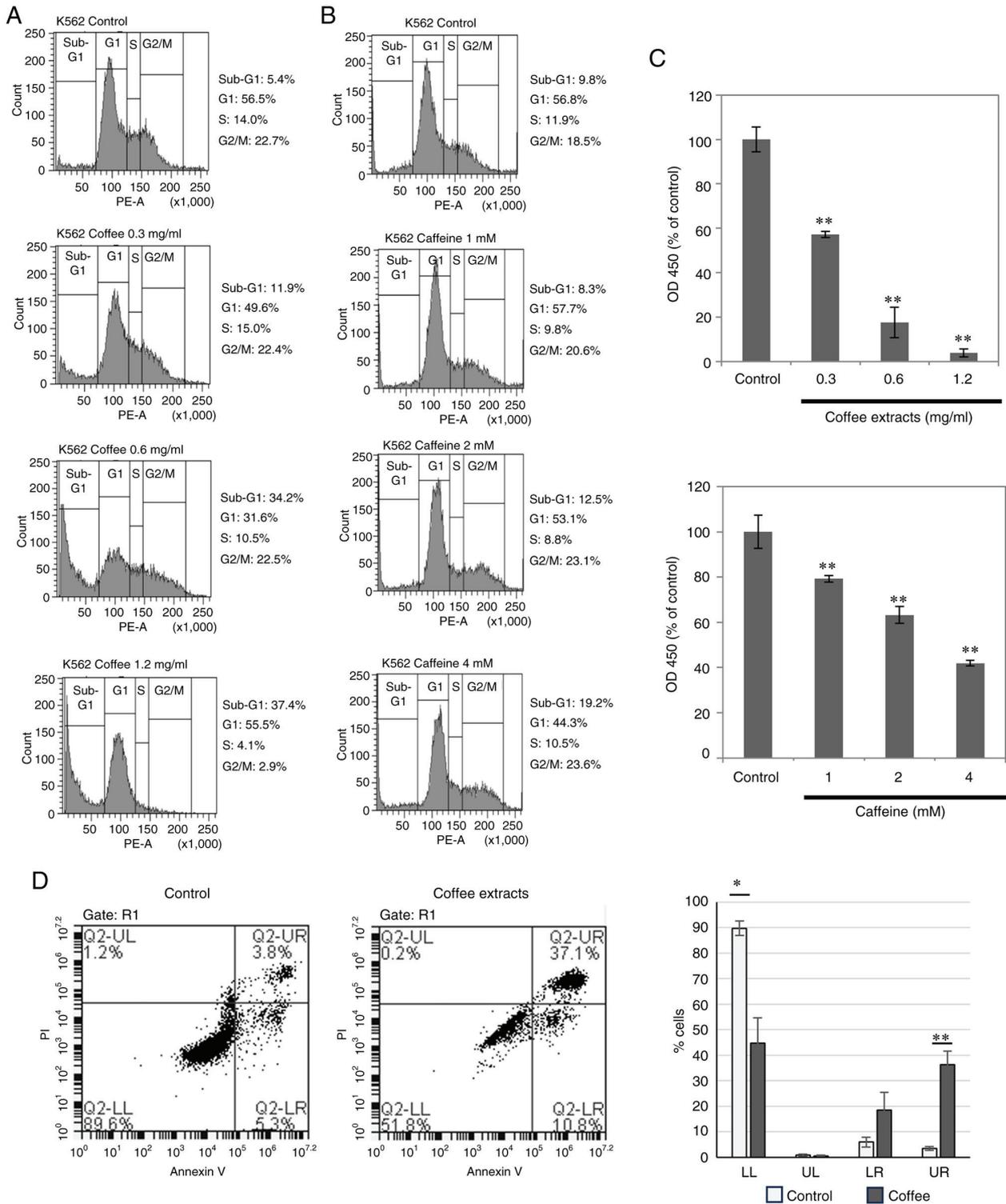


Figure 5. Cell cycle and cell death analyses of coffee extracts and caffeine. K562 cells were treated with (A) coffee extracts or (B) caffeine and cultured for 24 h. Cells were suspended into PI solution. Cell cycle and cell death were analyzed using flow cytometry. The percentage of cells in the sub-G1, G1, S and G2/M phases are shown in the histograms. (C) Cell proliferation was measured using CCK-8 assay. The data are presented as the mean of three results \pm standard deviation. (D) Apoptosis induction was examined by Annexin V/PI staining. A bar graph is shown on the right panel. The data are presented as the mean of three results \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$, vs. the control.

Coffee extracts and caffeine activate the transcription of human PD-1 and PD-L1 in liver cancer and colon cancer cells. The present study demonstrated that PD-1 and PD-L1 expression were increased at a transcriptional level in chronic myeloid leukemia K562 cells. Subsequently, the

present study examined whether this activation also occurs in cell lines derived from other tissues. Liver cancer HepG2 cells and colon cancer SW480 cells were used. As shown in Fig. 6A, coffee extracts and caffeine activated both the PD-1 and PD-L1 promoters in a concentration-dependent manner

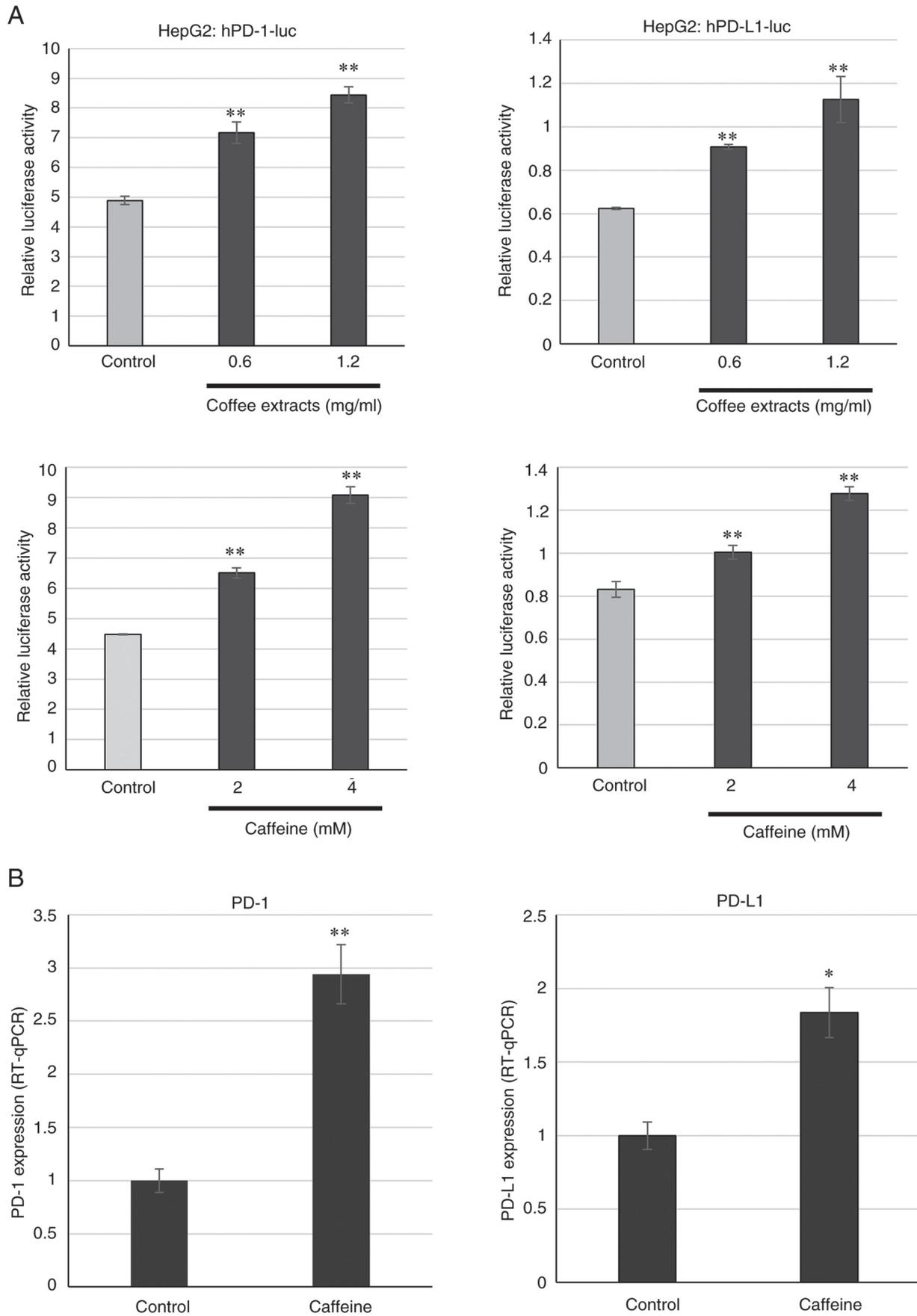


Figure 6. Coffee extracts and caffeine upregulate PD-1 and PD-L1 expression in liver cancer HepG2 cells. (A) hPD-1-luc and the hPD-L1-luc were transfected into HepG2 cells and the cells were cultured in medium containing coffee extracts (upper panels) or caffeine (lower panels) for 24 h. Cell lysate was prepared and luciferase assay was performed. (B) Reverse transcription-quantitative PCR of PD-1 and PD-L1 in HepG2 cells treated with caffeine for 24 h. Those results were normalized to the actin values. The data are presented as the mean of three results \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$, vs. the control. PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

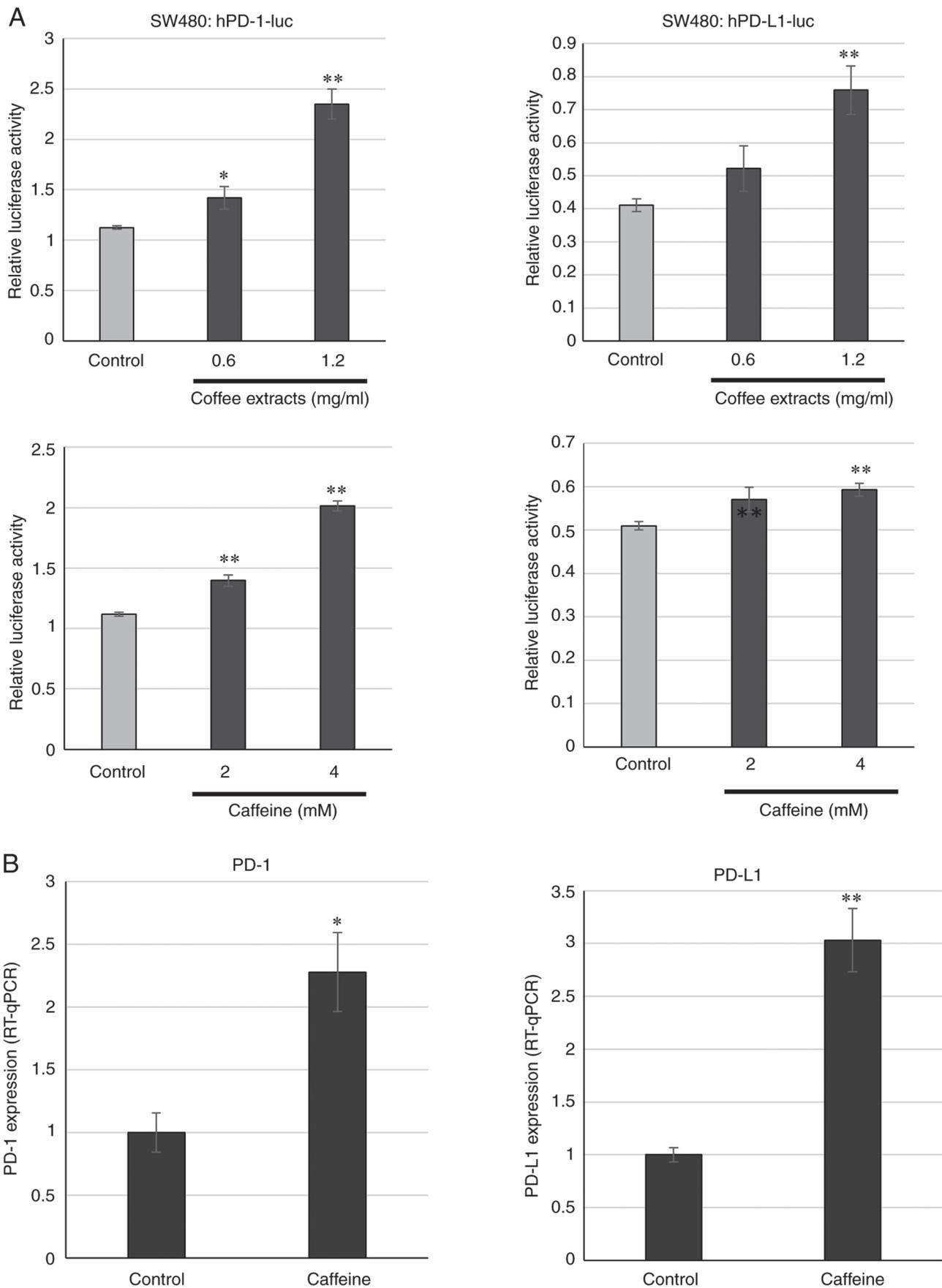


Figure 7. Coffee extracts and caffeine upregulate PD-1 and PD-L1 expression in colon cancer SW480 cells. (A) hPD-1-luc and the hPD-L1-luc were transfected into SW480 cells and the cells were treated with coffee extracts (upper panels) or caffeine (lower panels) for 24 h. Cell lysate was prepared and luciferase assay was performed. (B) Reverse transcription-quantitative PCR of PD-1 and PD-L1 in SW480 cells following treatment with caffeine for 24 h. The results were normalized to the actin values. The data are presented as the mean of three results \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$, vs. the control. PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

in HepG2 cells. Moreover, treatment with caffeine increased the PD-1 and PD-L1 mRNA levels in HepG2 cells (Fig. 6B). In addition, the promoter activities of PD-1 and PD-L1 increased by the coffee extracts and caffeine in colon cancer SW480 cells (Fig. 7A). PD-1 and PD-L1 mRNA expression also increased following treatment with caffeine in SW480 cells (Fig. 7B). In addition, immunostaining revealed the increase in both PD-1 and PD-L1 protein levels in HepG2, SW480 and K562 (Figs. S4 and S5). These results indicate that coffee extracts and caffeine regulate PD-1 and PD-L1 transcription not only K562 cells, but also in HepG2 and SW480 cells.

Discussion

The present study examined the effects of coffee extracts on the transcriptional regulation of PD-1 and PD-L1 in an aim to reveal the association between coffee and the immune checkpoint. These coffee extracts enhanced the transcriptional activity of PD-1 and PD-L1 in K562 cells. Moreover, when the components contained in coffee were analyzed, caffeine increased the transcriptional activity of PD-1 and PD-L1, suggesting that the effect of coffee was exerted via caffeine. This transactivation occurred in liver cancer HepG2 and colon cancer SW480 cells, as well as in a leukemia cell line. These results indicate that the upregulation in the expression of PD-1 and PD-L1 is induced through a common signal transduction mechanism possessed by various tissues. On the other hand, both promoters were not activated by caffeine and the PD-1 promoter was also not activated by coffee extracts in cervical cancer HeLa cells. This discrepancy may have occurred due to the tissue specificity of the cervix or human papilloma virus infection. However, in order to reveal the tissue specificity, it is necessary to perform analyses using other cell lines. It has been reported that caffeine inhibits ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR), which are kinases acting on the DNA damage response (34). The inhibition of ATM and ATR may mediate PD-1 and PD-L1 expression levels regulated by coffee and caffeine. In addition, as caffeine has a structure similar to that adenosine, it can bind to adenosine receptor (38,39). Coffee and caffeine may regulate PD-1 and PD-L1 expression levels through these signals; however, the present study did not analyze the effects of ATM/ATR and adenosine receptor on PD-1 and PD-L1 expression. It has been reported that caffeine inhibits phosphodiesterases, leading to an increase in cytosolic cAMP (40,41). Thus, PD-1 and PD-L1 expression may be also considered to be regulated by cAMP signaling downstream of caffeine treatment. It has been reported that cAMP increases PD-L1 transcription and protein expression in diffuse large B-cell lymphoma cells (42).

The present study also found that coffee extracts and caffeine induced the death of K562 cells. The cell death-inducing effect of caffeine was weak, suggesting that coffee also induces cell death via a route other than caffeine. Caffeine induced G2/M phase arrest, although the coffee extract had no such effect. This is considered to be due to other components in coffee more strongly inducing cell death, rather than G2/M phase arrest. It was found that coffee extracts and caffeine

induced cell death, and increased the expression of PD-1 and PD-L1. The results revealed that the overexpression of PD-1 and PD-L1 did not induce cell death. These results indicate that cell death and the increase in PD-1 and PD-L1 expression occurred in parallel following treatment with the coffee extracts and caffeine. The present study did not examine the effects of coffee and caffeine on cell invasion and migration. Epidemiological studies have demonstrated that the consumption of coffee decreased the risk of developing myelodysplastic syndrome in non-smoking individuals (43). Therefore, coffee and caffeine are suggested to be used for prevention and treatment of leukemia, including chronic myelogenous leukemia analyzed in the present study. On the other hand, the upregulation of PD-1 and PD-L1 expression will protect tumor cells, since PD-L1 suppresses the immune response by binding to PD-1, which is expressed on immune cells. Thus, anti-PD-1 and anti-PD-L1 antibodies are used in clinical practice against various types of malignant tumors. Moreover, cancer not only escapes the body's regulatory mechanisms, including the immune system, but also gains the ability to affect local and systemic homeostasis (44). The immunosuppressive effects of PD-1 and PD-L1 expression cause the abnormal release of immune mediators. Therefore, as PD-1 and PD-L1 exert immune suppressive effects in malignant tumors, the results of the present study suggest that the inhibitor of the immune checkpoint may synergistically enhance the preventive effects of coffee on myeloid disease and malignant tumors. However, the results presented herein are limited to *in vitro* experiments and thus need to be further validated *in vivo* in future studies.

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Availability of data and materials

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

Authors' contributions

TY performed the experiments and wrote the manuscript. KY, MH and KT performed the experiments and edited the manuscript. TY, KY, MH and KT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

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