

3. Chapter 3: Evaluation of antitumor immune response of caffeine and anti-PD1 monoclonal antibody combination against carcinogen and cell line induced tumors in mice

3.1 Introduction

Immunotherapy represented by immune checkpoint blockers (ICBs) such as anti-CTLA4 and anti-PD1 monoclonal antibodies (mAbs) has emerged as a promising treatment option for cancer patients in recent years.(Pardoll, 2012; Zou et al., 2016). ICBs have shown durable clinical responses in multiple cancers of both early and advanced stages (Topalian et al., 2012; Brahmer et al., 2012; Hodi et al., 2010). However, the clinical response rate of ICBs is limited to a subset of patient population (15-30%), while majority of patients are primarily resistant to PD1 blockade (Topalian et al., 2012; Brahmer et al., 2012) (Hodi et al., 2010). The tumor biopsies of patients treated with anti-PD1 mAb revealed that patients who did not respond to the therapy lacked CD8⁺ T cells inside tumor lesions (Tumeh et al., 2014). There is accumulating evidence indicating that resistance to anti-PD1 therapy is largely dependent on tumor microenvironment where tumor cells utilize multiple and non-overlapping immunosuppressive mechanisms to facilitate immune escape (Allard et al., 2013). It can be hypothesized that a combination immunotherapy designed to attract CD8⁺ T cells into tumor microenvironment and to block non-overlapping immunosuppressive mechanisms may improve the antitumor activity of anti-PD1 mAb in resistant patients. This hypothesis is supported by several studies showing that combined blockade of multiple immunosuppressive mechanisms can produce synergistic anti-cancer activity in mice (Allard et al., 2013; Curran et al., 2010; Woo et al., 2012).

One of the immunosuppressive pathways involved in tumor immune escape is adenosine-A2A receptor pathway (Whiteside, 2017). We previously reported that caffeine enhances anti-tumor immune response in mice possibly through blockade of A2A receptor. Because activation of both A2A receptor and PD1 on activated T cells suppresses T cell function, co-targeted blockade of both A2A receptor and PD1 may enhance the anti-tumor activity of anti-PD1 mAb. In the present study, we aimed to investigate whether caffeine can enhance the therapeutic activity of anti-PD1 mAb against carcinogen- and cell line-induced tumor models in mice.

3.2 Materials and methods

3.2.1 Animals

C57BL/6J mice (6-8 weeks old) were purchased from National Institute of Nutrition, Hyderabad, India. Adult female albino mice (6-8 weeks old) were procured from

the central animal house of the university. All experimental procedures were performed in accordance with the principles established by CPCSEA and were approved by the Institutional Animal Ethical Committee of Banaras Hindu University, Varanasi, India (Dean/2019/IAEC/1249).

3.2.2 Cell line, Drugs, and Chemicals

B16F10 melanoma cell line was purchased from National Centre for Cell Science, Pune, India. Purified anti-mouse PD-1 mAb (Clone RMP1-14) and purified control Ig (Cat no. BE0093) were purchased from BioXCell, USA. Caffeine (Cat no. C0750), 3-Methylcholanthrene (Cat no. 213942), DNase (Cat no. 10104159001), Percoll (P1644), and collagenase type IV (Cat no. C5138) were purchased from Sigma Aldrich, India. All the remaining chemicals used in this study were of analytical grade and purchased from local vendors.

3.2.3 Carcinogen-induced tumor model

Carcinogen-induced tumors were established in albino mice through subcutaneous injection of 400 µg of 3-methylcholanthrene (3-MCA) in the hind flank region as described previously. Once tumors were established (> 5 mm diameter), mice were treated with caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 µg, weekly twice) or intraperitoneal injections of control Ig (100 µg, weekly twice) for 6 weeks. The tumors were monitored weekly during the treatment period. The dose of 0.08% w/v caffeine was selected from our previous study which showed greater tumor reduction and maximal tumor lymphocyte infiltration and anti-PD1 mAb was selected based on previous study (Allard et al., 2013).

3.2.4 Cell line-induced tumor model

C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells in 0.1 ml of RPMI1640 medium as described previously (Overwijk and Restifo, 2000). Once tumors were established (> 5 mm diameter), mice were treated with caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 µg, weekly twice) for 3 weeks.

3.2.5 Tumor measurement and survival analysis

The perpendicular diameters of each tumor were measured using digital caliper and the tumor sizes (mm²) were calculated as the product of two perpendicular diameters. Mice with tumor greater than 5 mm diameter and showing progressive growth were considered as tumor positive mice. The survival of mice from each group was monitored from day 1 (3-MCA inoculation) to 200 days. The number of mice dead and day of death from each group on or before 200 days were noted and the percentage of mice that survived upto 200 days in each group was calculated using statistical analysis.

3.2.6 Body, heart, liver and kidney weight measurement

Body weights of all the mice were measured before the start of the experiment and randomized into different groups. After the end of treatment period, body weights of all the mice in each group were measured again. The final body weight of each mice from each group after the end of the treatment was calculated by subtracting the body weight from the weight of the tumor. Mice were then euthanized by CO₂ inhalation and the major organs like heart, liver and kidney were collected and weighed. Body weights and organ weights of dead mice were also taken in case of any deaths, during the middle of the treatment.

3.2.7 Flow cytometric analysis of tumor infiltrating lymphocytes

B16F10 melanoma tumors were excised after the end of the treatment and tumor infiltrating lymphocytes (TILs) were isolated as described previously by Allard B et.al (Allard et al., 2016). Briefly, tumors were finely sliced into small pieces and incubated for 1 hr at 37 °C in a digestion medium containing collagenase type IV and DNase. After complete digestion, tumor cell suspension was passed through a 40-μm cell strainer, washed twice in phosphate buffered saline, and resuspended in 30% Percoll. The cell suspension was gently layered onto 70% Percoll, and centrifuged at 4 °C for 10 min. The TILs located at the interface were collected, rinsed twice with excess FACS buffer, resuspended in FACS buffer, and counted. Then, the Fc receptors were blocked with anti CD-16/32 monoclonal antibody and stained with a specific antibody. The following antibodies (purchased from Thermo Fisher Scientific) were used in this study: rat anti-mouse CD3 FITC (Clone 17A2, Cat no. 11-0032-82), rat anti-mouse CD4 PerCP-Cy 5.5 (Clone RM4-5, Cat no. 45-0042-82), rat anti-mouse CD8a PE (Clone 53-6.7, Cat no. 12-0081-81), rat anti-mouse CD25 PE (Clone PC61.5, Cat no. 12-0251-81), armenian hamster

anti-mouse CD279 APC (Clone J43 , Cat no. 17-9985-80), and rat anti-mouse CD16/32 (Clone 93, Cat no. 14-0161-81). After 30 min of staining, flow cytometry was conducted using BD FACSCALIBUR and analyzed using FlowJo software.

3.2.8 Analysis of intratumoral levels of TNF- α and IFN- γ by ELISA

B16F10 melanoma tumors were excised, homogenized using glass Teflon homogenizer and centrifuged (12000 rpm) at 4 °C for 45 min. The supernatant was collected and tested for intra-tumoral levels of TNF- α and IFN- γ using ELISA, according to the manufacturer's protocol.

3.2.9 Statistical analysis

Results were expressed as mean $\pm$ SEM. The significance of differences between survival curves was performed by Gehan–Breslow–Wilcoxon test. All the other remaining data were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Effects of caffeine, anti-PD1 mAb, or their combination on tumor progression and survival in carcinogen-induced tumor model

Tumor progression and overall survival period reflects the therapeutic activity of anticancer drugs (Pazdur, 2008). In this study, we evaluated the therapeutic activity of caffeine monotherapy, anti-PD1 mAb monotherapy, and their combination therapy by assessing individual tumor size and tumor growth rate in 3-MCA-induced tumor model. The maximal tumor size observed in control group ranged from 238 to 438 mm² (Figure. 3.1A) whereas, caffeine, anti-PD1 mAb, and the combination treated groups showed maximal tumor size ranged from 179 to 286 mm² (Figure. 3.1B), 143 to 192 mm² (Figure. 3.1C), and 0 to 232 mm² (Figure. 3.1D), respectively. Notably, 2 out of 8 mice treated with combination therapy showed complete tumor regression and 2 out of 8 mice showed partial response (Figure. 3.1D). Further, the comparison of tumor growth rates (mm²/day) of all groups showed that monotherapy of caffeine (2.6 mm²/day) and anti-PD1 mAb (1.9 mm²/day) significantly ($P < 0.05$) decreased the tumor growth rate compared with control group (5.3 mm²/day) (Figure. 3.1E). The combination therapy caused significantly ($P < 0.05$) higher reduction in tumor growth rate as compared to control group (0.9 mm²/day

versus 5.3 mm²/day), caffeine monotherapy group (0.9 mm²/day versus 2.6 mm²/day), and anti-PD1 mAb monotherapy group (0.9 mm²/day versus 1.9 mm²/day) (Figure. 3.1E).

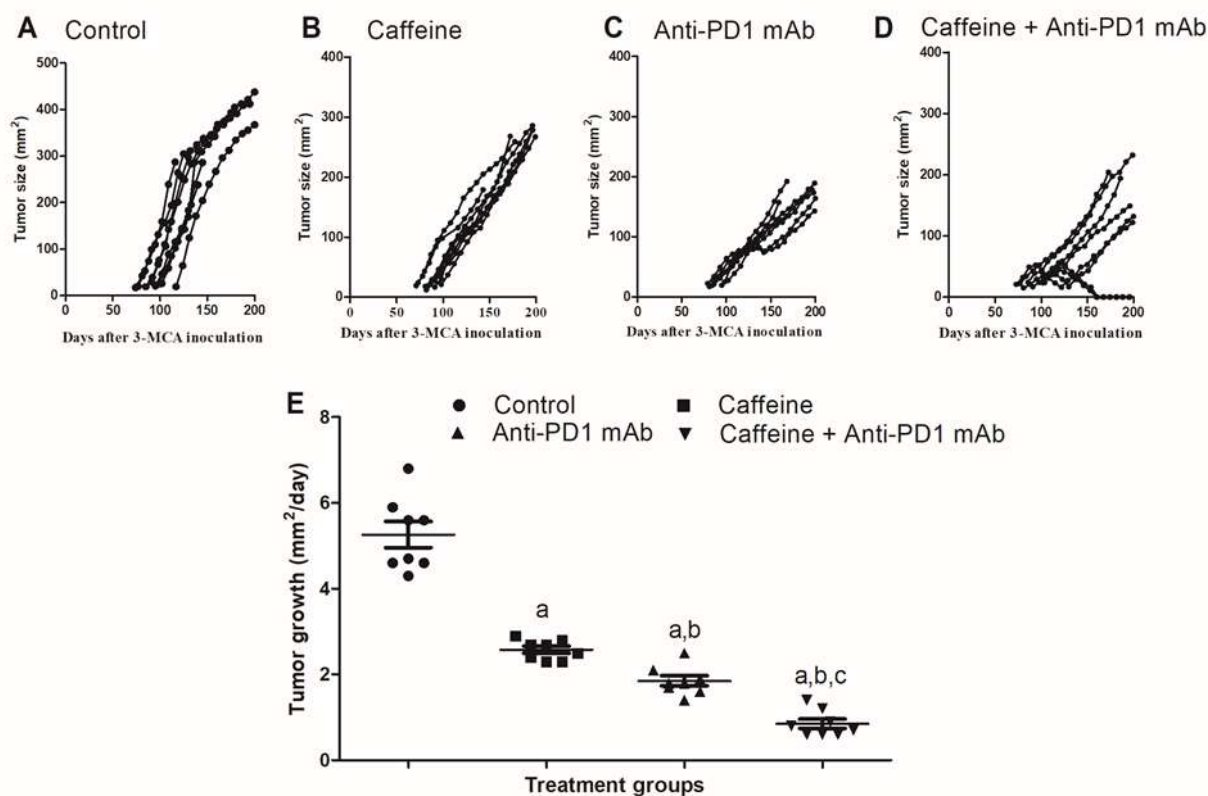


Figure 3.1: Combination of caffeine and anti-PD1 mAb inhibited the carcinogen-induced tumor growth. Carcinogen-induced tumors were established in albino mice through subcutaneous injection of 400 µg of 3-methylcholanthrene (3-MCA) in the hind flank region. Once tumors were established (> 5 mm diameter), mice were randomly allocated into (A) Control group: received intraperitoneal injections of control Ig (100 µg, weekly twice) (B) Caffeine group: received caffeine in drinking water (0.08% w/v, daily) (C) Anti-PD1 mAb group: received intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice) (D) Caffeine + Anti-PD1 mAb group: received combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 µg, weekly twice). (E) Tumor growth rates of individual mice from each group was calculated by dividing tumor size after 6 weeks of treatment with 42 treatment days. Data represents mean ± SEM of 8 mice per group. ^aP < 0.05 versus control, ^bP < 0.05 versus caffeine, ^cP < 0.05 versus anti-PD1 mAb.

Further, the analysis of survival curves revealed that caffeine monotherapy and anti-PD1 mAb monotherapy caused statistically insignificant ($P > 0.05$) prolongation in the overall survival period compared with the control group (Figure 3.2). However, the combination therapy caused statistically significant ($P < 0.05$) prolongation in the overall survival period compared with control group (Figure 3.2).

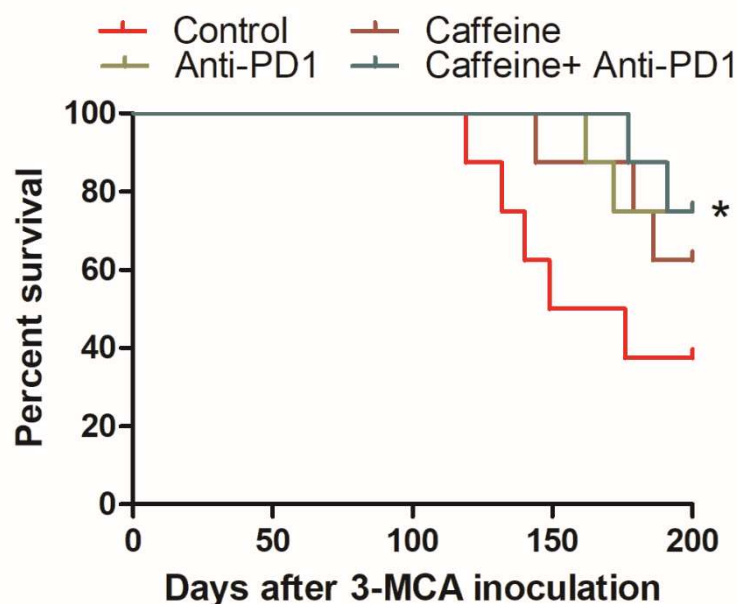


Figure 3.2: Caffeine and anti-PD1 mAb combination therapy increased the overall survival period in carcinogen-induced tumor model. Albino mice were inoculated subcutaneously in the hind flank region with 400 μ g of 3-methylcholanthrene (3-MCA) in 0.1 ml of olive oil. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 μ g, weekly twice) for 6 weeks. Mortality in all groups was monitored daily over the course of 200 days starting from inoculation of 3-MCA. Significance of differences in survival between all groups was estimated by Gehan–Breslow–Wilcoxon test. * $P < 0.05$ versus control.

3.3.2 Effects of caffeine, anti-PD1 mAb or their combination on tumor progression in cell line-induced tumor model

We further evaluated the therapeutic activity of both monotherapies and combination therapy of caffeine and anti-PD1 mAb against B16F10 melanoma tumor bearing mice. The results revealed that caffeine (on day 25, 81.1 mm^2) and anti-PD1 mAb (on day 25, 61.8 mm^2) monotherapy caused statistically significant ($P < 0.05$) reduction in tumor growth compared with control group (on day 25, 121.6 mm^2) (Figure. 3.3). The combination therapy (on day 25, 42.1 mm^2) showed statistically significant ($P < 0.05$) synergistic reduction in tumor growth compared with control group (on day 25, 121.6 mm^2), caffeine monotherapy (on day 25, 81.1 mm^2), and anti-PD1 mAb monotherapy (on day 25, 61.8 mm^2) (Figure. 3.3).

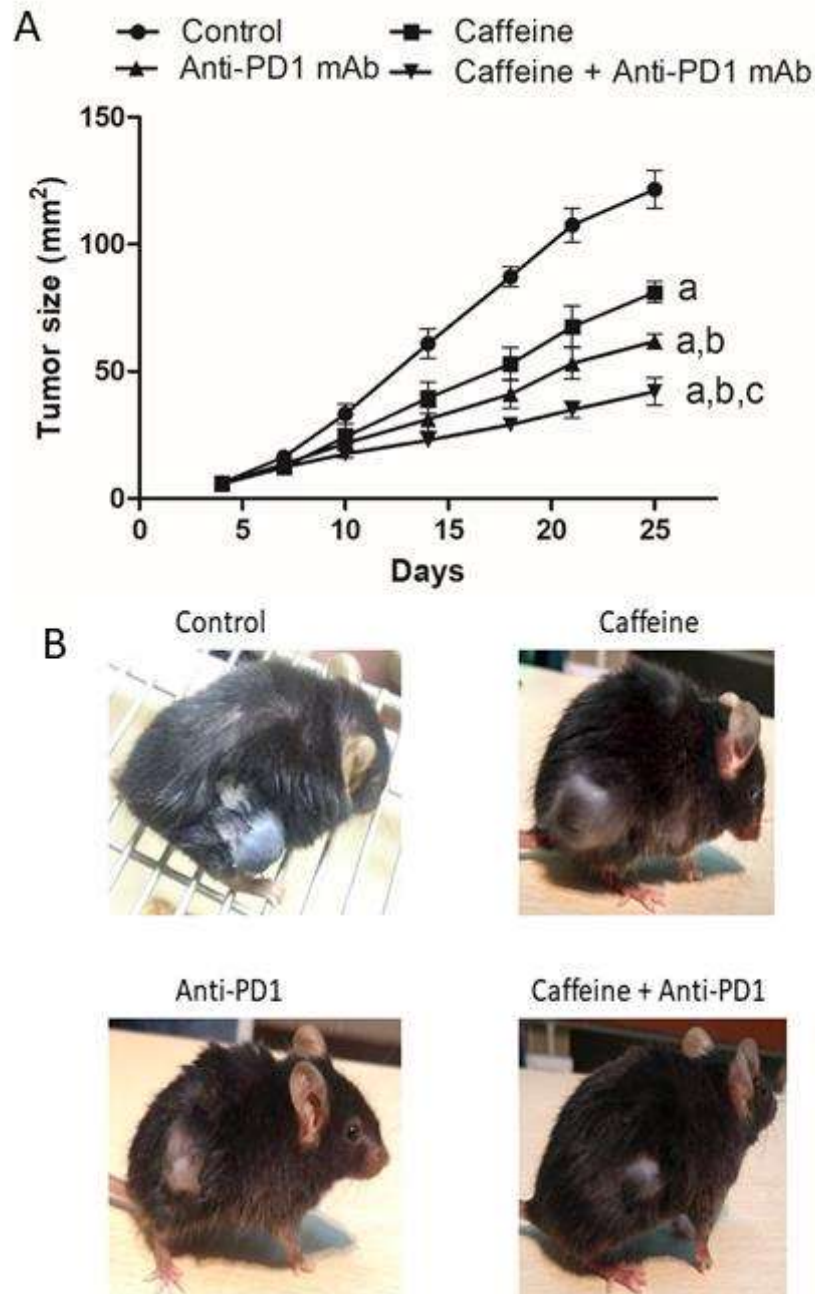


Figure 3.3: Caffeine and anti-PD1 mAb combination therapy inhibited cell line-induced tumor growth. C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 μ g, weekly twice) for 3 weeks. A) On day 25, the mean tumor size in all groups was analyzed by one way ANOVA followed by Tukey's multiple comparison test. B) Mice with B16F10 melanoma tumors from different groups were represented. Data represents mean $\pm$ SEM of 8 mice per group. ^a $P < 0.05$ versus Control, ^b $P < 0.05$ versus caffeine, ^c $P < 0.05$ versus anti-PD1 mAb.

3.3.3 Effects of caffeine, anti-PD1 mAb, or their combination on T-lymphocyte infiltration into cell line-induced tumor model

In order to examine whether the observed anti-tumor effects of caffeine and anti-PD1 mAb combination therapy involved anti-tumor immune response, we isolated TILs from all groups and examined total T-lymphocyte population. The total T-lymphocyte infiltration was significantly ($P < 0.05$) increased in caffeine (25%) and anti-PD1 mAb (32.6 %) treated groups compared with control group (15.9%) (Figure. 3.4). However, the combination therapy (37.5%) caused a statistically significant ($P < 0.05$) increase in T-lymphocyte infiltration compared with control (15.9%), caffeine monotherapy (25%), and anti-PD1 monotherapy (32.6%) (Figure. 3.4).

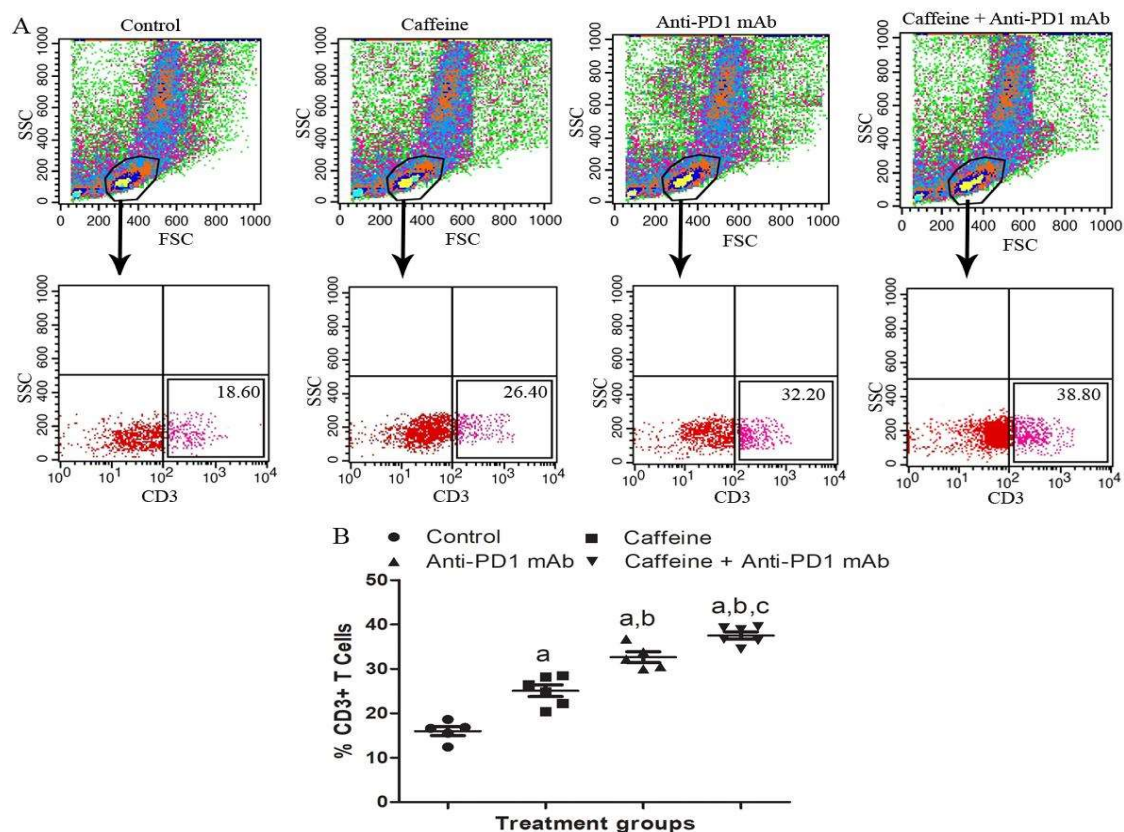


Figure 3.4: Caffeine and anti-PD1 mAb combination therapy increased the T lymphocyte infiltration into cell line-induced tumors. C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD1 mAb (100 μ g, weekly twice) for 3 weeks. On day 25, tumors were isolated and single-cell suspensions of TILs were prepared and analyzed by flow cytometry. (A) Representative flow cytometric images of CD3+ T cells of all groups. (B) Statistical results of percentages of CD3+ T cells. Data represents mean $\pm$ SEM of 5-6 mice per group. ^a $P < 0.05$ versus Control, ^b $P < 0.05$ versus caffeine, ^c $P < 0.05$ versus anti-PD1 mAb.

3.3.4 Effects of caffeine, anti-PD1 mAb or their combination on CD4 T-lymphocyte infiltration into cell line-induced tumors

We next evaluated the effect of caffeine monotherapy, anti-PD1 mAb monotherapy, and their combination therapy on infiltration of CD4 T-lymphocytes into B16F10 melanoma tumors. The CD4 T-lymphocyte infiltration was significantly ($P < 0.05$) increased in caffeine (13.4%) and anti-PD1 mAb (16.4%) treated groups compared with control group (10.3%) (Figure. 3.5). However, the combination therapy (19.1%) caused a statistically significant ($P < 0.05$) increase in CD4 T-lymphocyte infiltration compared with control (10.3%), caffeine monotherapy (13.4%), and anti-PD1 monotherapy (16.4%) (Figure. 3.5).

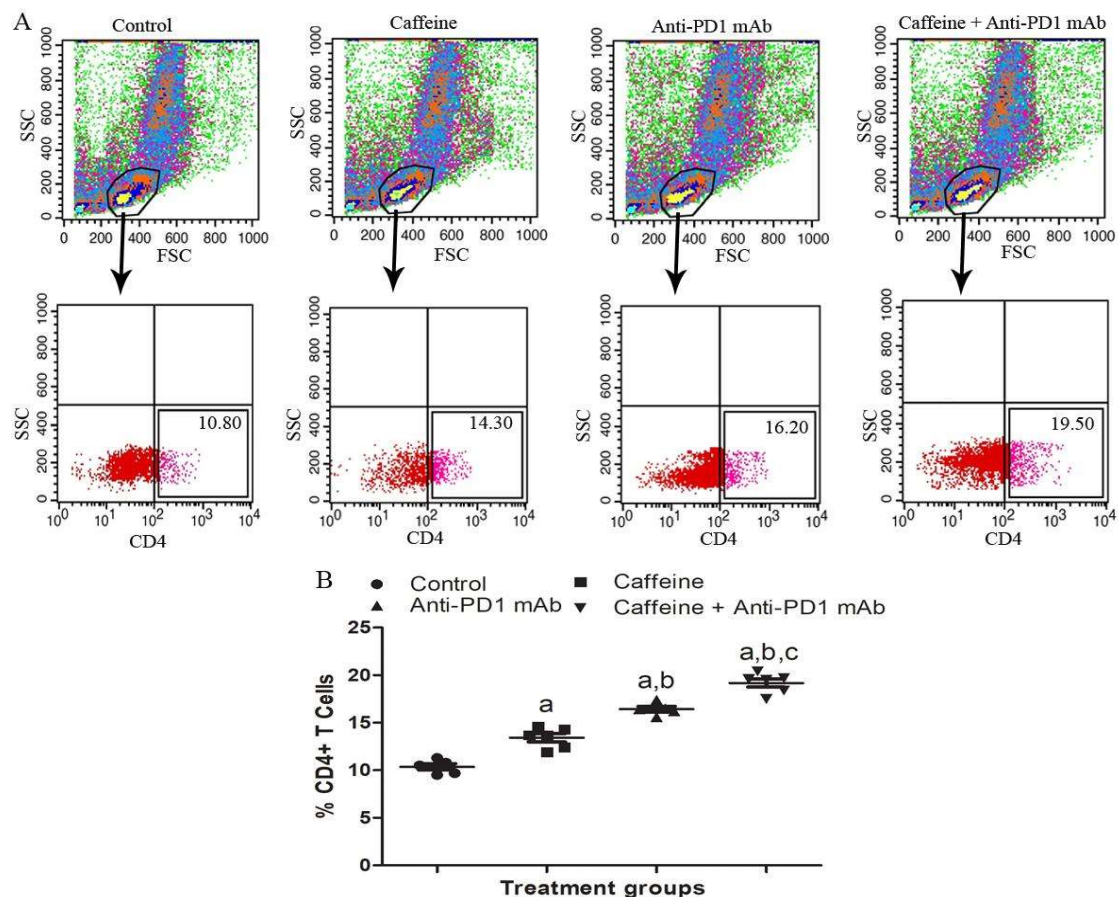


Figure 3.5: Caffeine and anti-PD1 mAb combination therapy increased the CD4 T-lymphocyte infiltration into cell line-induced tumors. C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD-1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD-1 mAb (100 μ g, weekly twice) for 3 weeks. On day 25, tumors were isolated and single-cell suspensions of TILs were prepared and analyzed by flow cytometry. (A) Representative flow cytometric images of CD4 T-lymphocytes of all groups. (B) Statistical results of percentages of

CD4 T-lymphocytes. Data represents mean $\pm$ SEM of 5-6 mice per group. ^aP < 0.05 versus control, ^bP < 0.05 versus caffeine, ^cP < 0.05 versus anti-PD1 mAb.

3.3.5 Effects of caffeine, anti-PD1 mAb or their combination on CD8 T-lymphocyte infiltration into cell line-induced tumors

We next evaluated the effect of caffeine monotherapy, anti-PD1 mAb monotherapy, and their combination therapy on infiltration of CD8 T-lymphocytes into B16F10 melanoma tumors. The CD8 T-lymphocyte infiltration was significantly ($P < 0.05$) increased in caffeine (12.1%) and anti-PD1 mAb (16.9%) treated groups compared with control group (8.2%) (Figure. 3.6). However, the combination therapy (22.4%) caused a statistically significant ($P < 0.05$) increase in CD8 T-lymphocyte infiltration compared with control (8.2%), caffeine monotherapy (12.1%), and anti-PD1 monotherapy (16.9%) (Figure. 3.6).

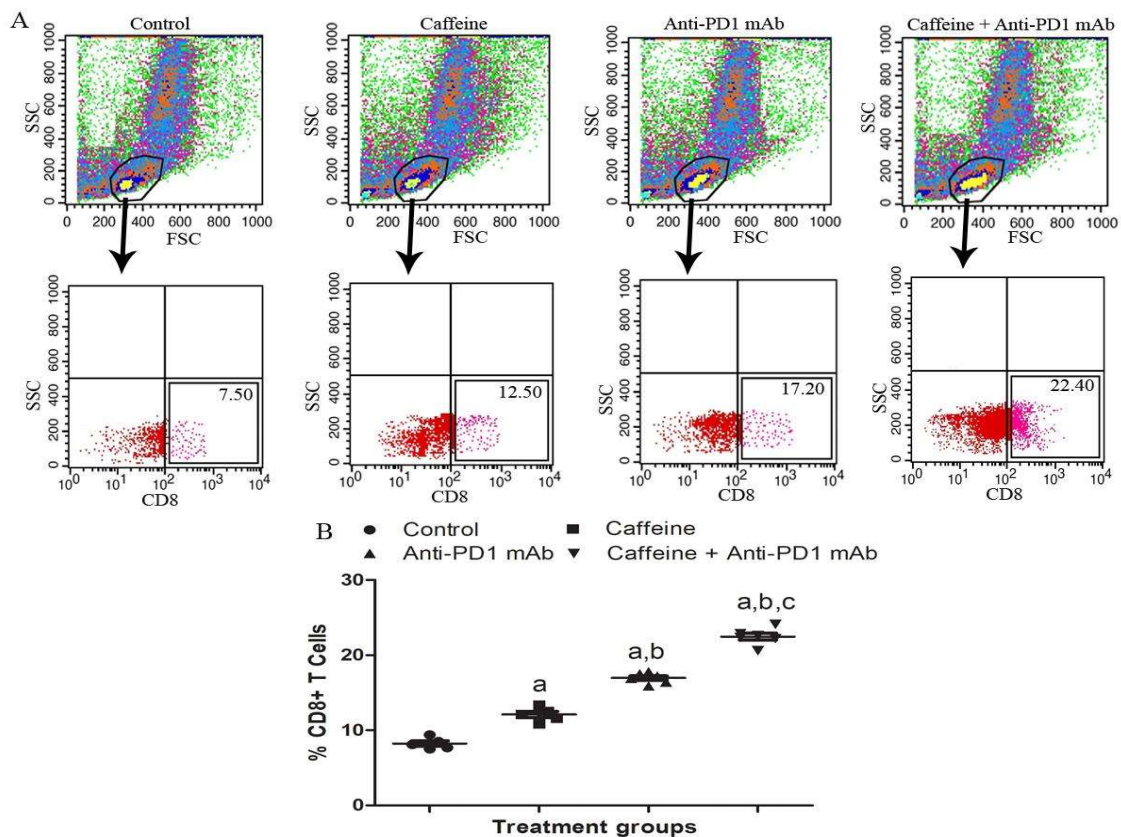


Figure 3.6: Caffeine and anti-PD1 mAb combination therapy increased the CD4 T-lymphocyte infiltration into cell line-induced tumors. C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD-1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD-1 mAb (100 μ g, weekly twice) for 3 weeks. On day 25, tumors were isolated and single-cell suspensions of TILs were prepared and analyzed by flow cytometry. (A) Representative flow cytometric images of CD8 T-lymphocytes of all groups. (B) Statistical results of percentages of

CD8 T-lymphocytes. Data represents mean $\pm$ SEM of 5-6 mice per group. ^aP < 0.05 versus control, ^bP < 0.05 versus caffeine, ^cP < 0.05 versus anti-PD1 mAb.

3.3.6 Effects of caffeine, anti-PD1 mAb or their combination on CD4+ CD25+ T-lymphocyte infiltration into cell-line induced tumors

We further evaluated the effect of caffeine monotherapy, anti-PD1 mAb monotherapy, and their combination therapy on infiltration of CD4+ CD25+ T-lymphocytes into B16F10 melanoma tumors. The CD4+ CD25+ T-lymphocyte infiltration was significantly ($P < 0.05$) decreased in caffeine (53.4%) and anti-PD1 mAb (46.7%) treated groups compared with control group (68.8%) (Figure. 3.7). However, the combination therapy (35.3%) caused a statistically significant ($P < 0.05$) decrease in CD4+ CD25+ T-lymphocyte infiltration compared with control (68.8%), caffeine monotherapy (53.4%), and anti-PD1 monotherapy (46.7%) (Figure. 3.7).

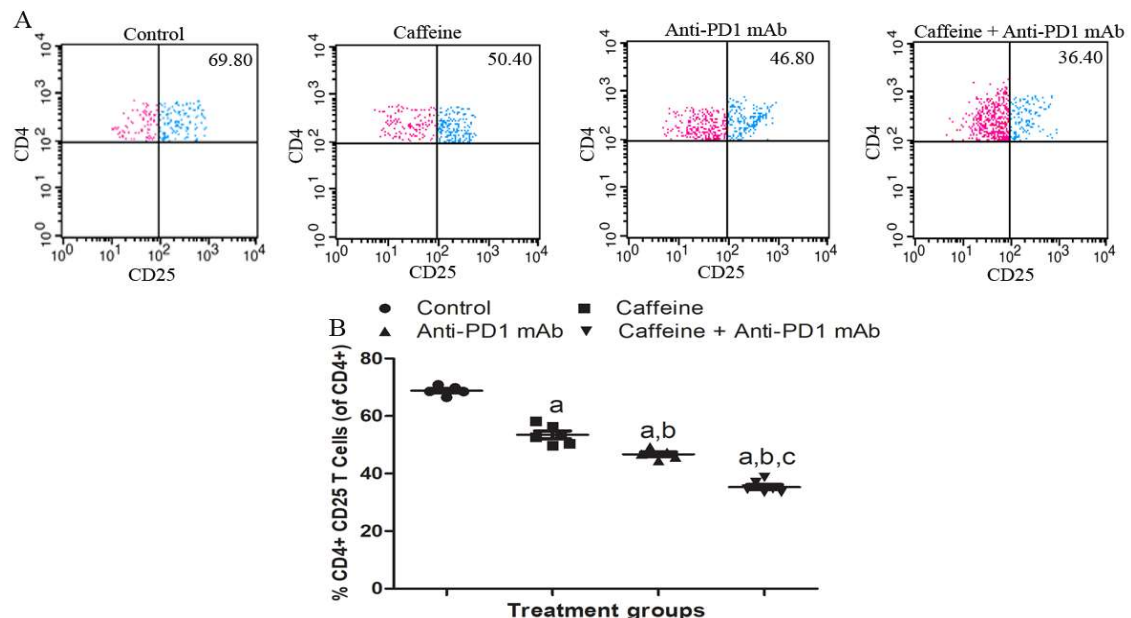


Figure 3.7: Caffeine and anti-PD1 mAb combination therapy decreased the infiltration of CD4+ CD25+ T-lymphocytes in cell line-induced tumor model. C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD-1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD-1 mAb (100 μ g, weekly twice) for 3 weeks. On day 25, tumors were isolated and single-cell suspensions of TILs were prepared and analyzed by flow cytometry. (A) Representative flow cytometric images of CD4+ CD25+ T-lymphocytes gated on CD4+ T cells of all groups. (B) Statistical results of percentages of CD4+ CD25+ T-lymphocytes gated on CD4+ T cells. Data represents mean $\pm$ SEM of 5-6 mice per group. ^aP < 0.05 versus control, ^bP < 0.05 versus caffeine, ^cP < 0.05 versus anti-PD1 mAb.

3.3.7 Effects of caffeine, anti-PD1 mAb or their combination on intra-tumoral levels of TNF- α and IFN- γ

To further investigate whether the observed anti-tumor effect of caffeine and anti-PD1 mAb combination therapy is mediated through the release of cytokines, we estimated the intra-tumoral levels of TNF- α and IFN- γ in all groups by ELISA. The TNF- α levels were significantly ($P < 0.05$) higher in caffeine (1979.8 pg/g of tumor) and anti-PD1 mAb (3390.3 pg/g of tumor) treated groups compared with control group (1111.4 pg/g of tumor) (Figure. 3.8). Furthermore, combination therapy (4622.6 pg/g of tumor) caused a statistically significantly ($P < 0.05$) increase in TNF- α compared with control (1111.4 pg/g of tumor), caffeine monotherapy (1979.8 pg/g of tumor), and anti-PD1 monotherapy (3390.3 pg/g of tumor) (Figure. 3.8).

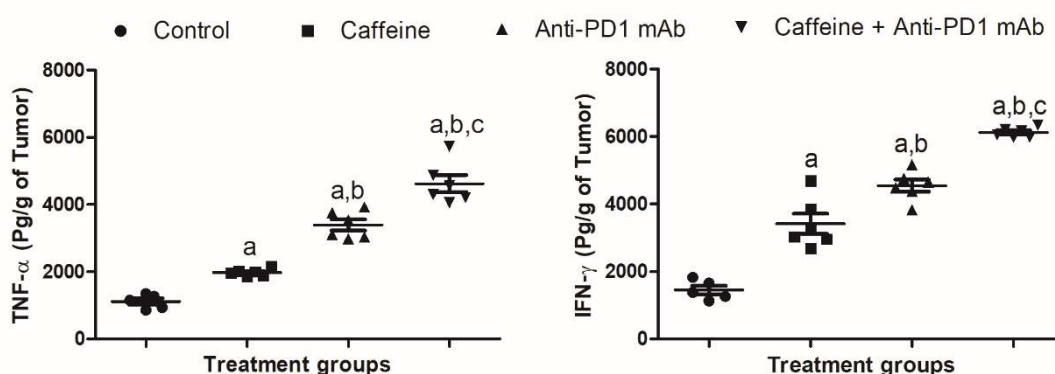


Figure 3.8: Caffeine and anti-PD1 mAb combination therapy increased the intra-tumoral levels of TNF- α and IFN- γ . C57BL/6J mice were injected subcutaneously with 1×10^5 B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 μ g, weekly twice), or caffeine in drinking water (0.08% w/v, daily), or intraperitoneal injections of anti-PD-1 mAb (100 μ g, weekly twice), or combination of caffeine in drinking water (0.08% w/v, daily) and anti-PD-1 mAb (100 μ g, weekly twice) for 3 weeks. On day 25, tumors were isolated and intra-tumoral levels of TNF- α and IFN- γ were estimated by ELISA. Data represents mean $\pm$ SEM of 5-6 mice per group. ^a $P < 0.05$ versus control, ^b $P < 0.05$ versus caffeine, ^c $P < 0.05$ versus anti-PD1 mAb.

Similarly, the IFN- γ levels were significantly ($P < 0.05$) higher in caffeine (3413.3 pg/g of tumor) and anti-PD1 mAb (4543.1 pg/g of tumor) treated groups compared with control group (1448.2 pg/g of tumor) (Figure. 3.8). Furthermore, combination therapy (6123 pg/g of tumor) caused a statistically significantly ($P < 0.05$) increase in IFN- γ compared with control (1448.2 pg/g of tumor), caffeine monotherapy (3413.3 pg/g of tumor) and anti-PD1 monotherapy (4543.1 pg/g of tumor) (Figure. 3.8).

3.3.8 Effect of caffeine, anti-PD1 or their combination on body, heart, liver and kidney weights

In order to investigate the safety of caffeine, anti-PD1 or their combination, body weights of individual mice from each group and weight of major organs were measured. Caffeine, anti-PD1 and their combination treatment appears safe with no major changes in body weights of mice with carcinogen induced tumors (Table 3) and B16F10 melanoma induced tumors (Table 4). In line with that, no major changes in the weights of heart, liver and kidney of mice with carcinogen induced tumors (Table 5) and B16F10 melanoma induced tumors (Table 6).

Table 3: Effect of caffeine, anti-PD1 or their combination on body weight of mice with carcinogen induced tumors

Mice	Control group		0.08% w/v caffeine		Anti-PD1		Combination	
	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)
1	28	33	25	35	28	36	25	37
2	25	38	25	36	26	38	26	38
3	26	34	28	38	26	37	27	40
4	25	36	26	35	27	35	27	39
5	26	37	26	36	27	38	28	37
6	26	36	27	36	28	39	30	41
7	28	37	26	38	25	38	29	40
8	26	38	28	37	25	36	27	38

Table 4: Effect of caffeine, anti-PD1 or their combination on body weight of mice with B16F10 melanoma induced tumors

Mice	Control group		0.08% w/v caffeine		Anti-PD1		Combination	
	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)
1	26	28	28	30	28	30	30	33
2	28	31	27	29	30	33	29	31
3	30	32	26	38	28	31	28	30
4	25	27	28	31	29	31	27	29
5	26	29	29	31	30	32	28	30
6	28	30	28	30	28	31	27	29
7	27	30	30	32	27	29	30	33
8	26	29	30	32	27	29	30	32

Table 5: Effect of caffeine, anti-PD1 or their combination on major organs of mice with carcinogen induced tumors

Mice	Control group			0.02% w/v caffeine			0.04% w/v caffeine			0.08% w/v caffeine		
	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)
1	1.6	2.3	0.15	1.7	2.4	0.18	1.6	2.2	0.18	1.9	2.3	0.17
2	1.7	2.2	0.13	1.8	2.0	0.16	1.8	2.0	0.18	1.7	2.2	0.16
3	1.5	2.1	0.17	1.4	2.1	0.19	1.8	2.5	0.19	1.5	2.0	0.14
4	1.8	2.0	0.19	1.5	2.4	0.24	1.7	2.4	0.2	1.6	2.2	0.13
5	1.4	2.3	0.2	1.4	2.3	0.17	1.4	2.2	0.22	1.9	2.4	0.15
6	1.8	2.5	0.21	1.7	2.4	0.21	1.6	2.0	0.2	1.6	2.1	0.16
7	1.5	2.7	0.2	1.5	2.0	0.18	1.8	2.2	0.23	1.7	2.0	0.17
8	1.4	2.8	0.16	1.8	2.4	0.25	1.9	2.5	0.19	1.7	2.2	0.24

Table 6: Effect of caffeine, anti-PD1 or their combination on major organs of mice with B16F10 melanoma induced tumors

Mice	Control group			0.02% w/v caffeine			0.04% w/v caffeine			0.08% w/v caffeine		
	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)	H (g)	L (g)	K (g)
1	1.7	2.2	0.15	1.8	2.5	0.22	1.9	2.2	0.18	2.0	2.4	0.18
2	1.9	2.5	0.22	1.9	2.0	0.24	1.7	2.4	0.19	2.1	2.5	0.19
3	1.7	2.4	0.24	2.1	2.4	0.19	1.8	2.3	0.17	1.7	2.2	0.18
4	1.9	2.6	0.21	2.2	2.6	0.26	1.9	2.2	0.22	1.8	2.0	0.19
5	2.0	2.8	0.22	2.1	2.5	0.19	1.7	2.1	0.24	1.6	2.1	0.21
6	1.9	2.9	0.24	2.2	2.6	0.23	1.5	2.0	0.2	1.6	2.2	0.2
7	1.8	2.5	0.19	1.9	2.8	0.22	1.8	2.4	0.24	1.7	2.4	0.19
8	1.9	2.6	0.23	1.9	2.7	0.25	1.6	2.3	0.19	1.9	2.1	0.21

3.5 Discussion

The therapeutic potential of immunotherapy, specifically through combination of agents targeting immunosuppressive pathways, is becoming increasingly evident against multiple cancers (Wolchok et al., 2013; Larkin et al., 2015). Antibodies targeting PD1 receptor have emerged as a promising therapeutic strategy against multiple types of solid cancers (Topalian et al., 2012). However, relatively low complete response rates observed with anti-PD1 mAb monotherapy emphasizes the importance of testing new immunotherapeutic combinations (Topalian et al., 2012).

In our previous study, we reported that caffeine can enhance anti-tumor immune response against 3-MCA-induced tumors in mice. In the present study, we investigated the

anti-tumor effect of caffeine and anti-PD1 mAb combination therapy against 3-MCA-induced tumors in mice. We found that the combination therapy showed synergistic anti-tumor activity than caffeine or anti-PD1 mAb monotherapy. Tumor growth inhibition has been shown to promote overall survival period of animals in experimental tumor models (LeBlanc et al., 2002). In this study, the combination therapy caused a significant prolongation in the overall survival period of mice. The anti-PD1 mAbs are primarily approved for the treatment of melanoma. Therefore, we further investigated the therapeutic activity and the possible mechanism of action of combination therapy against B16F10 melanoma tumors. Our results revealed that the combination therapy possess a significant anti-tumor activity against B16F10 melanoma tumors. These results are consistent with the previous findings that simultaneous mAb-mediated blockade of CD73 and PD-1 synergistically enhances tumor control and survival in mice (Allard et al., 2013; Hay, 2016).

To identify the possible mechanism of action, we isolated TILs from the harvested B16F10 melanoma tumors and subjected to flow cytometric analysis. The cellular immune response by T cells plays a major role in generating and regulating the immune response against tumor antigens (Nagorsen et al., 2003). The results revealed a lower T lymphocyte infiltration into the tumors of control mice, indicating tumor cells ability to avoid immune destruction. However, the possible blockade of adenosine-A2A receptor pathway by caffeine and blockade of PD1 pathway by anti-PD1 mAb increased the infiltration of total T lymphocyte population into the melanoma tumors. The combination therapy showed synergistic increase in infiltration of T lymphocytes into the tumors, possibly due to combined blockade of both the pathways. The T lymphocytes are primarily composed of CD4⁺ T helper cells and CD8⁺ T cytotoxic cells. The CD4⁺ T cells further classified into effector T cells and T regulatory cells (Luckheeram et al., 2012). The effector T cells primarily act as immune stimulator, help in activating B lymphocytes and cytotoxic T cells, and also secretes cytokines such as IL-2, TNF- α , and IFN- γ (Luckheeram et al., 2012). The T regulatory cells primarily act as immune suppressor, secrete inhibitory cytokines to down regulate the induction and proliferation of cytotoxic T cells (Luckheeram et al., 2012). Our results demonstrate that caffeine or anti-PD1 mAb monotherapy significantly increased the frequency of CD4⁺ and CD8⁺ T cells than control group. Furthermore, the combination therapy synergistically increased the frequency of CD4⁺ and CD8⁺ T cells. Conversely, the percentage of CD4⁺CD25⁺ T regulatory cells was decreased by caffeine or anti-PD1 mAb monotherapy when compared with the control group. The combination

therapy further decreased the percentage of CD4⁺CD25⁺ T regulatory cells than caffeine or anti-PD1 mAb monotherapy. A decrease in percentage of T regulatory cells allows the induction and proliferation of cytotoxic T cells (Mempel et al., 2006). Once cytotoxic T cells were activated, they destroy the tumor cells by releasing either cytokines like TNF- α and IFN- γ , or perforins and granzymes, or through induction of apoptosis. In our study, we found that caffeine and anti-PD1 mAb combination therapy significantly increased intra-tumoral TNF- α and IFN- γ levels leading to cytotoxic effect on tumor cells. Blockade of adenosine pathway by caffeine demonstrated similar outputs with the previously reported results that showed dual therapy with adenosine blocking agents and anti-PD1 increases intra-tumoral CD8⁺ tumor-specific T cells and IFN γ mRNA levels as compared to single-agent treatments (Allard et al., 2013). Several clinical trials targeting adenosine pathway with anti-CD73 mAb along with anti-PD-1 mAb (NCT03454451, NCT03549000) or anti-PDL-1 mAb (NCT02503774, NCT03773666, NCT03267589, NCT03334617) of advanced solid tumors are recruiting or underway.

3.6 Conclusions

Anti-PD1 mAbs such as nivolumab and pembrolizumab have already been approved for the treatment of melanoma. However, relatively low complete response rates observed with anti-PD1 mAb monotherapy emphasizes the importance of testing new immunotherapeutic combinations that can enhance anti-tumor immunity. The production of extracellular adenosine in solid tumors was recently identified as a major immunosuppressive pathway, targeting this pathway will synergize the therapeutic activity of anti-PD1 mAbs. This view was supported by the previous studies that involves the combined blockade of non-overlapping immunosuppressive pathways like indoleamine 2,3-dioxygenase and CTLA-4, can induce synergistic anti-tumor activity (Holmgaard et al., 2013). In order to achieve maximal therapeutic benefit, increase in frequency of effector T cells and cytokines production in tumor microenvironment was critical. Our work suggests that administration of caffeine and anti-PD1 mAb harness the therapeutic potential of effector T cells in vivo possibly due to combined blockade of PD1 and adenosine-A2A receptor pathway. Our study provides the scientific basis for testing combination regimens of caffeine and anti-PD1 mAbs for sustained tumor control in cancer patients.