2. Chapter 2: Evaluation of antitumor immune response efficacy of caffeine against carcinogen induced tumors in mice

2.1 Introduction

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring, habitually consumed food constituent throughout the world (Graham, 1978). Prospective cohort studies revealed that caffeine consumption reduces the relative risk of various cancers (Holick et al., 2010; Nkondjock, 2009; Oba et al., 2006; Song et al., 2012).

Infiltration of T lymphocytes into the tumor is a major challenge because the tumor cells regulate lymphocytes infiltration through multiple mechanisms (Lanitis et al., 2017). Even after infiltration, tumor-infiltrating lymphocytes (TILs) are still challenged by multiple immunosuppressive pathways orchestrated by tumor cells (Lanitis et al., 2017). One of the major immunosuppressive pathways is the adenosine-A2A receptor pathway (Allard et al., 2012) (Bours et al., 2006). During hypoxic conditions of solid tumors, adenosine concentration is increased locally (Sitkovsky et al., 2004). Following its release, adenosine acts on A2A receptors expressed on stromal cells and downregulates adhesion proteins such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), or P-selectin leading to decreased infiltration of TILs (Figure. 2.1) (Allard et al., 2012). In addition, adenosine inhibits the proliferation of cytotoxic T cells and increases the proliferation of T regulatory cells (Tregs) (Allard et al., 2012). Expression of inhibitory receptors like programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on TILs is the other major contributing immunosuppressive pathway (Pardoll, 2012). It has been reported that adenosine enhances the PD-1 expression on TILs via activation of A2A receptors (Allard et al., 2013). In a recent study, Hadar et al. reported that caffeine enhances the release of proinflammatory cytokines (Eini et al., 2015). However, it is unclear whether caffeine promotes anti-tumor immune response through infiltration of T lymphocytes or decreased expression of PD-1 receptor on T lymphocytes.

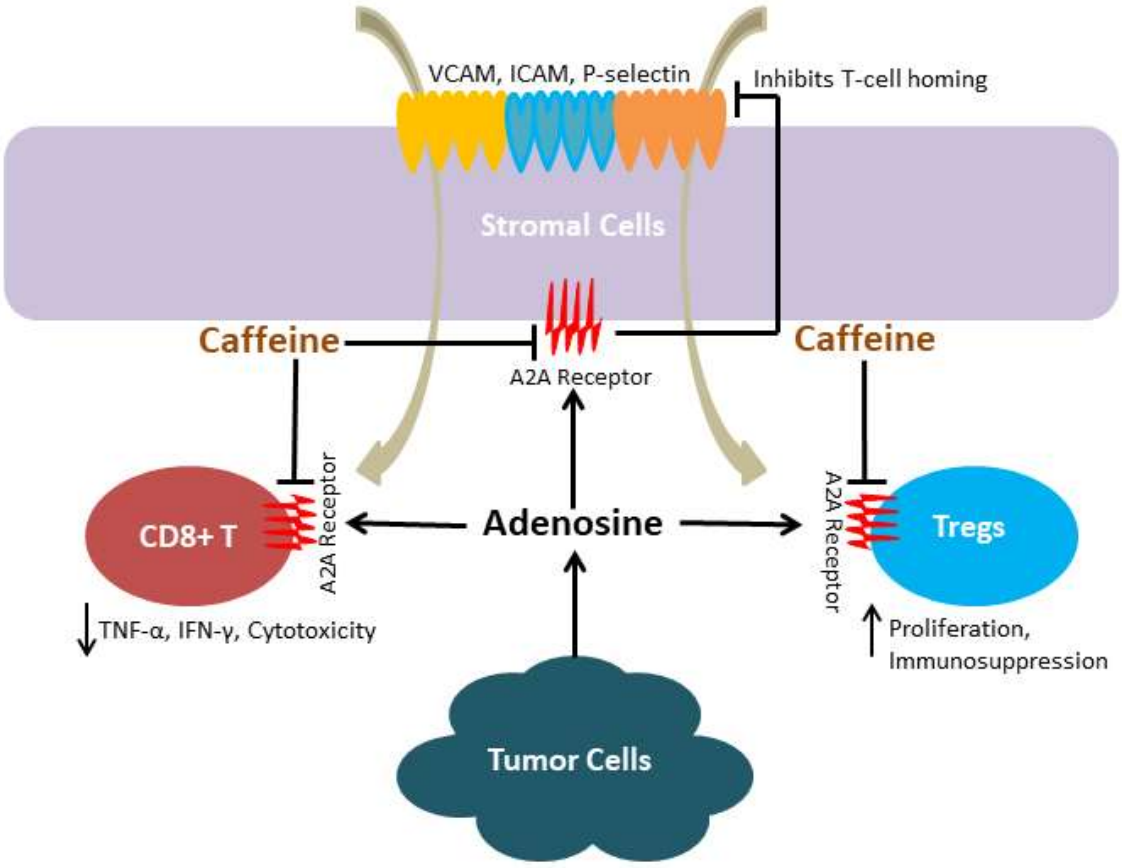

Figure 2.1: Mechanism of caffeine induced antitumor immune response

Based on prospective epidemiology and its predominant antagonistic effect on A2A receptor, we aimed to investigate the effect of caffeine on T cell infiltration and PD-1 expression in a carcinogen-induced tumor model.

2.2 Material and methods

2.2.1 Animals

Adult female albino mice (6-8 weeks old, and 25-30 g) were obtained from central animal house of university. All the experimental procedures were performed in accordance with the principles established by CPCSEA and were approved by the Central Animal Ethical Committee of the University (Banaras Hindu University, Varanasi, India) (Dean/2018/CAEC/631).

2.2.2 Drugs, Chemicals, and Antibodies

Caffeine (Cat no. C0750), 3-Methylcholanthrene (3-MCA) (Cat no. 213942), Collagenase type IV (Cat no. C5138), and DNase (Cat no. 10104159001) were purchased

from Sigma Aldrich, India. For flow cytometric analysis, the following antibodies purchased from Thermo Fisher Scientific (USA) were used: 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). TNF-α (Cat no. KB2145) and IFN-γ (Cat no. KB2011) ELISA kits were purchased from Krishgen Biosystems, India.

2.2.3 Carcinogen-induced tumor model

Mice were injected subcutaneously with 400 µg of 3-MCA in 0.1 mL of olive oil in the hind flank region. Mice were inspected weekly for tumor development of local fibrosarcomas (tumor) over the course of 250 days. Tumors were considered positive after they reach > 5 mm diameter. Once the tumors were positive, measurements were made weekly upto 250 days using digital caliper. The tumor size was calculated using the product of two perpendicular diameters (mm^2).

2.2.4 Caffeine treatment protocol

Mice were randomly allocated into four groups (n = 8) and injected with 3-MCA. One group was allowed to drink water and the other three groups were allowed to drink water containing 0.02%, 0.04%, or 0.08% w/v caffeine, respectively. Caffeine in drinking water was prepared weekly twice. Considering a mouse drinks 5 mL water per day, the daily dose of caffeine equates to 1, 2, and 4 mg/mouse/day with respect to 0.02%, 0.04%, and 0.08% w/v of caffeine, respectively. Considering the average weight of a mouse as 0.025 kg, the doses 1, 2, and 4 mg/mouse/day corresponds to 40, 80, and 160 mg/kg/day, respectively. The human equivalent dose (HED) for mouse doses 40, 80, and 160 mg/kg/day equates to approximately 200, 400, and 800 mg/day, respectively (Nair and Jacob, 2016). Caffeine was given from the starting day i.e., the day that mice received 3-MCA, and continued up to eight weeks from the appearance of tumors. Water consumption of all the groups were measured and it was shown non-significant difference between all treatment groups. Each group (contains 8 mice) consumed 40 ml of drinking water per day on average. The amount of water consumption per mice is in line with the previously assumed mice water consumption of 5 mL per day.

2.2.5 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.

2.2.6 Flow cytometric analysis of tumor infiltrating lymphocytes

3-MCA-induced tumors were excised and tumor infiltrating lymphocytes (TILs) were isolated as described previously (Allard et al., 2016). Briefly, tumors were finely chopped and digested by incubating for 1 h at 37 °C in a medium containing collagenase IV and DNase. After complete digestion, tumor cell suspension was passed through a 40-µm cell strainer, washed twice with PBS, and resuspended in 30% percoll. The resuspended cell suspension was gently layered onto 70% percoll and centrifuged at 4 °C for 10 min. The TILs located at the interface were collected and rinsed twice with large excess of FACS buffer. Then, the TILs were stained with trypan blue and counted using Neubauer chamber (Marienfeld, Germany) before blocking the Fc receptors with anti CD-16/32 monoclonal antibody. Further, the TILs were stained for 30 min with the following antibodies: rat anti-mouse CD3 FITC, rat anti-mouse CD4 PerCP-Cy 5.5, rat anti-mouse CD8a PE, rat anti-mouse CD25 PE, and armenian hamster anti-mouse CD279 APC. Finally, the flow cytometric analyses of TILs were conducted using BD FACSCALIBUR and FlowJo software

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

The intra-tumoral levels of tumor necrosis factor-α (TNF-α) and interferon γ (IFN-γ) were measured by taking approximately 300 mg of tumor tissue. The tumor samples were homogenized (glass Teflon homogenizer) on ice, centrifuged (10,000 g) at 4 °C for 45 min, and the supernatant was collected for assay. The assay was performed according to the manufacturer's protocol.

2.2.8 Histopathological analysis of tumor tissue

Tumor tissues were fixed with 10% formalin for 24 h and embedded in paraffin. Then, 5 µm sections were obtained using a microtome and stained with hematoxylin and eosin (H&E). Histology preparations were examined for leucocyte infiltration under 10X and 40X magnifications.

2.2.9 Statistical analysis

Results were expressed as mean ± SEM. The significance of differences in tumor incidence was performed by log-rank (Mantel-Cox) 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.

2.3 Results

2.3.1 Effect of caffeine on tumor incidence and growth

To determine whether the innate immune system of caffeine-treated mice was capable of tumor immune surveillance, we compared the incidence of carcinogen-induced tumors in water-drinking group with caffeine-treated groups. After 250 days of 3-MCA inoculation, caffeine-treated groups displayed a significantly lower tumor incidence compared with water-drinking group (Figure. 2.2). The water-drinking group showed 100% tumor incidence whereas, caffeine at doses 0.02%, 0.04%, and 0.08% showed 62.5%, 62.5%, and 37.5%. In addition, caffeine-treated groups developed tumors lately than water-drinking group, indicating a possible role of enhanced innate immune system in caffeine-treated mice.

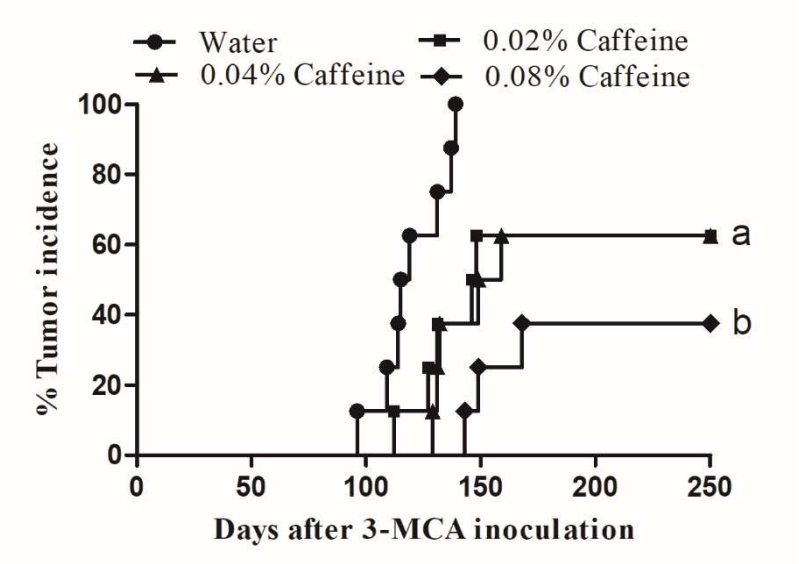

Figure 2.2: Caffeine treatment decreased carcinogen-induced tumor incidence. Mice (n = 8 per group) were injected subcutaneously with 400 µg of 3-MCA and were administered either water or 0.02% or 0.04% or 0.08% of caffeine in drinking water. Development of tumors was monitored weekly over the course of 250 days. Tumors > 5 mm in diameter and showing progressive growth were considered as tumor positive. [a]P < 0.05 versus water, [b]P < 0.001 versus water.

The effect of caffeine on tumor growth was assessed by comparing individual tumor size observed in different treatment groups. The maximal tumor size observed in the water-drinking group ranged from 291–339 mm^2 (Figure. 2.3A) whereas, 0.02%, 0.04%, and 0.08% caffeine-treated groups showed maximal tumor size ranged from 277–307 (Figure. 2.3B), 219–254 (Figure. 2.3C), and 131–156 mm^2 (Figure. 2.3D), respectively. Further, tumor growth rates (mm^2/day) of caffeine-treated groups were compared with water-drinking group. A significant (P < 0.05) and dose-dependent decrease in tumor growth rate (5, 4.1, and 2.5 mm^2/day versus 5.5 mm^2/day) was observed in 0.02%, 0.04%, and 0.08% caffeine-treated groups compared with water-drinking group (Figure. 2.3E).

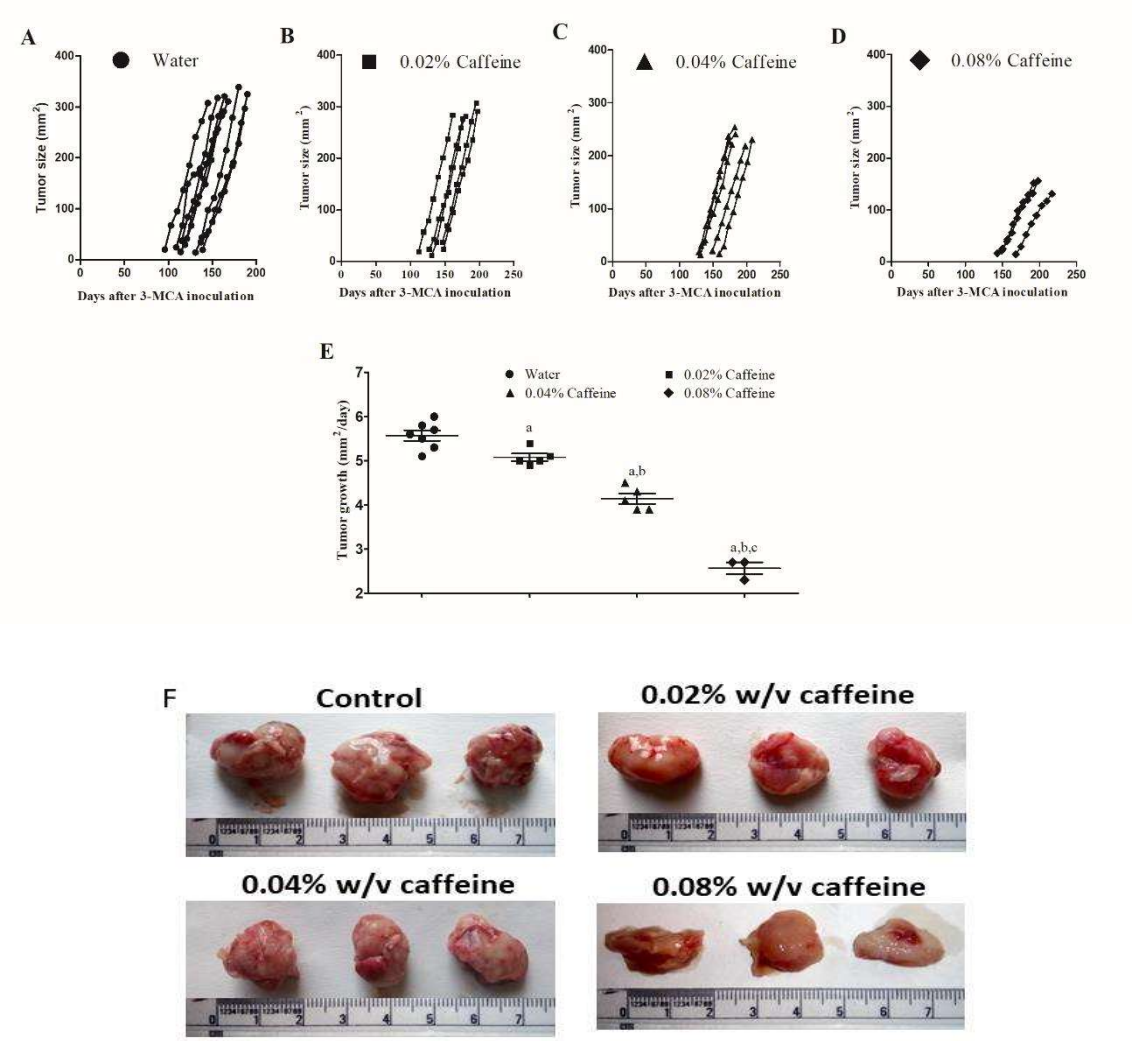

Figure 2.3: Caffeine treatment decreased the rate of carcinogen-induced tumor growth. Tumor growth curves of individual mice administered with (A) water, (B) 0.02% caffeine, (C) 0.04% caffeine, (D) 0.08% caffeine in drinking water were recorded. (E) Tumor growth rates of individual mice from each group was calculated by taking tumor size from first to final day and dividing by number of days. (F) Excised tumors from each group was represented. Data represents mean + SEM of 3-5 mice per group. $^{a}P < 0.05$ versus water, $^{b}P < 0.05$ versus 0.02% caffeine, $^{c}P < 0.05$ versus 0.04% caffeine.

2.3.2 Effect of caffeine on anti-tumor immune response

In order to determine whether the observed anti-tumor effect of caffeine was due to enhanced anti-tumor immune response, we isolated TILs from caffeine-treated and water-drinking groups to estimate the T-lymphocyte population using flow cytometry. Increase in leucocyte infiltration was also evidenced by histopathological observation of tumor samples from caffeine-treated and water-drinking mice (Figure. 2.4).

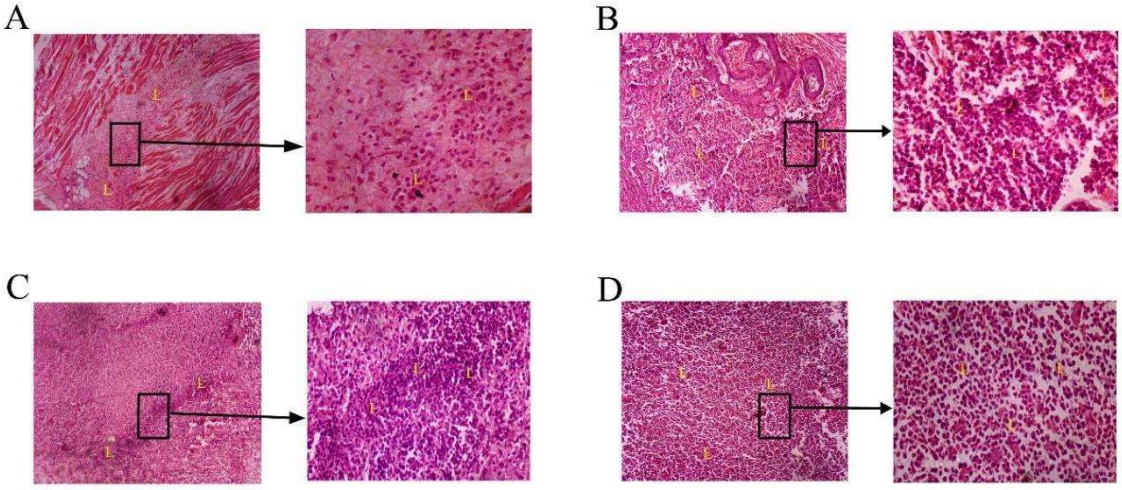

Figure 2.4: Caffeine treatment is associated with increased leukocytes infiltration in carcinogen-induced tumors. Mice were sacrificed and tumors were harvested after eight weeks of treatment from the appearance of tumors. One part of the tumor was stained with haematoxylin and eosin (H&E) and analyzed for leukocyte infiltration. Representative histology images are shown. Typical areas are displayed at 40X and 10X magnifications. L, leucocytes.

2.3.3 Effect of caffeine on infiltration of T-lymphocytes

The total T-lymphocyte infiltration was significantly ($P < 0.05$) increased in 0.04% (38.98%) and 0.08% (49.55%) caffeine-treated groups compared with water-drinking group (27.98%) (Figure. 2.5A). The difference in T-lymphocyte infiltration between 0.02% caffeine-treated group (30.43%) and water-drinking group (27.98%) did not reach statistical ($P > 0.05$) significance (Figure. 2.5A).

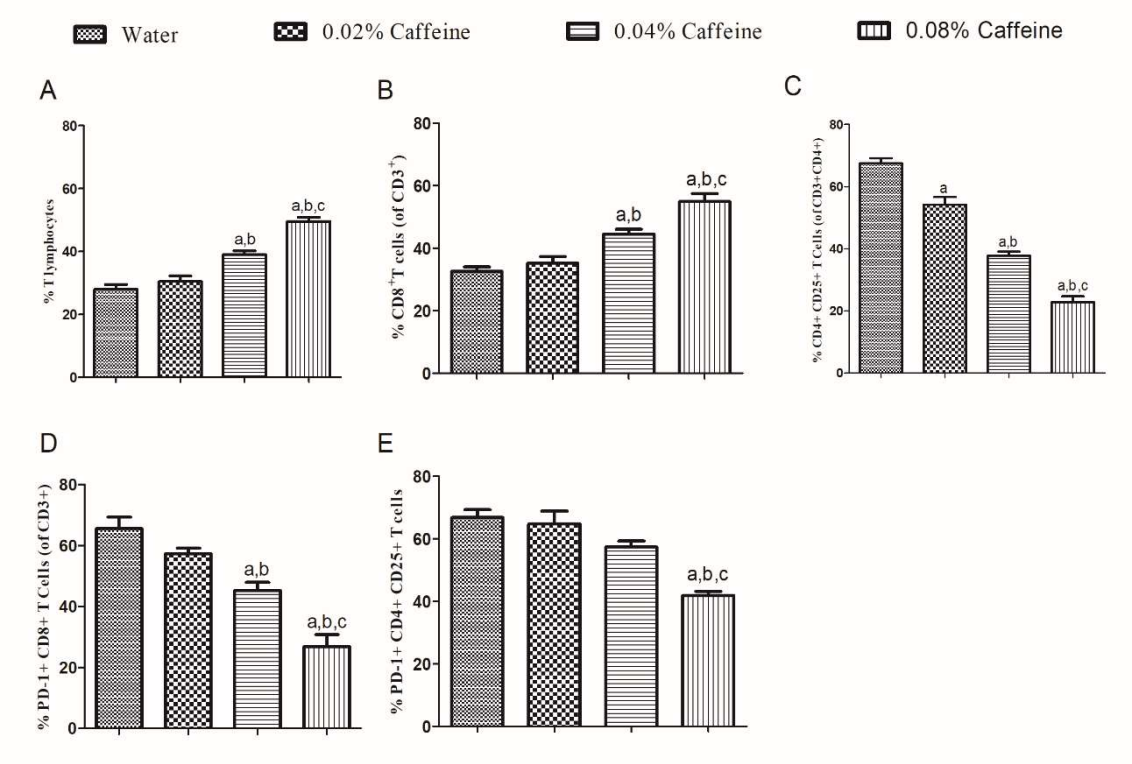

Figure 2.5: Caffeine treatment increased T lymphocyte infiltration in carcinogen-induced tumors. Statistical results of (A) percentages of CD3+ T cells, (B) percentages of CD8+ T cells, (C) percentages of CD4+CD25+ T cells, (D) percentages of PD-1+ CD4+CD25+ T cells, (E) percentages of PD-1+CD8+ T cells of all groups were analyzed. Data represents mean + SEM of 3-5 mice per group. $^{a}P < 0.05$ versus water, $^{b}P < 0.05$ versus 0.02% caffeine, $^{c}P < 0.05$ versus 0.04% caffeine.

The representative flow cytometric images showed both T-lymphocyte infiltration and further gating strategy used to estimate T-lymphocyte subpopulation (Figure. 2.6).

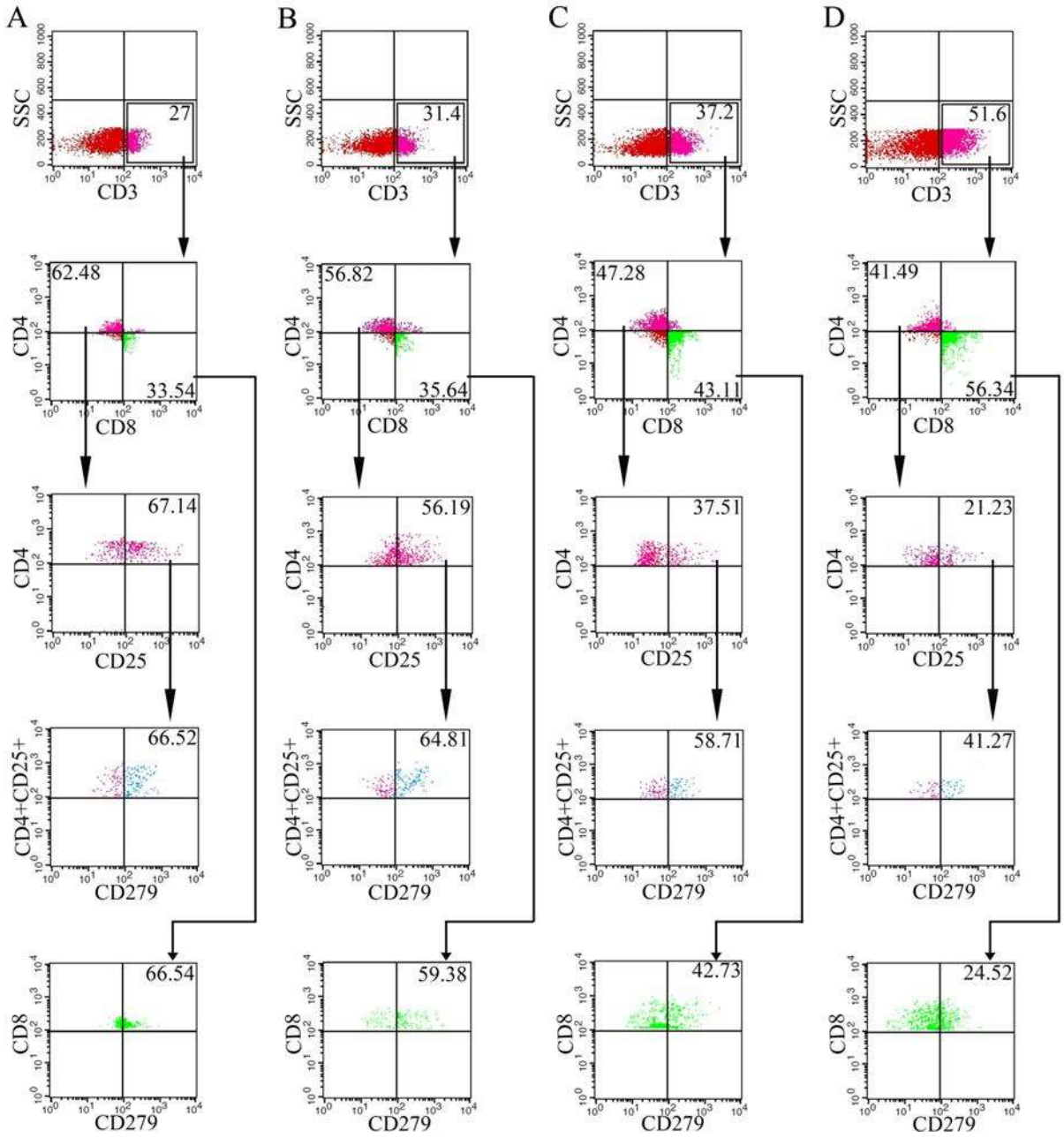

Figure 2.6: Caffeine treatment increased T lymphocyte infiltration in carcinogen-induced tumors-Flow cytometric results. Mice were sacrificed and tumors were harvested after eight weeks of treatment from the appearance of tumors. TILs were isolated and estimated by flow cytometry. Representative results of flow cytometric analysis of TILs of (A) water-drinking mice, (B) 0.02% caffeine-treated mice, (C) 0.04% caffeine-treated mice, (D) 0.08% caffeine-treated mice were showed.

2.3.4 Effect of caffeine on infiltration of CD8+ and CD4+CD25+ regulatory T cells

We next investigated the effect of caffeine treatment on tumor-infiltrating CD8+ T cells and CD4+CD25+ regulatory T cells subpopulation in total T-lymphocyte population. The CD8+ T lymphocyte infiltration was significantly ($P < 0.05$) increased in 0.04%

(44.55%) and 0.08% (54.86%) caffeine-treated groups compared with water-drinking group (32.56%) (Figure. 2.5B). The difference in CD8+ T lymphocyte infiltration between 0.02% caffeine-treated group (35.19%) and water-drinking group (32.56%) did not reach statistical ($P > 0.05$) significance (Figure. 2.5B). The infiltration of CD4+CD25+ regulatory T lymphocyte was significantly ($P < 0.05$) decreased in 0.02% (54.24%), 0.04% (37.77%), and 0.08% (22.89%) compared with water-drinking group (67.43%) (Figure. 2.5C).

2.3.5 Effect of caffeine on expression of PD-1 on CD8+ and CD4+CD25+ regulatory T cells

We further investigated whether caffeine treatment could regulate the expression of PD-1(CD279) on TILs. The PD-1 expression on CD8+ T lymphocytes was significantly ($P < 0.05$) reduced in 0.04% (45.3%) and 0.08% (26.84%) caffeine-treated groups compared with water-drinking group (65.53%) (Figure. 2.5D). The difference in PD-1 expression on CD8+ T lymphocytes between 0.02% caffeine-treated group (57.29%) and water-drinking group (65.53%) did not reach statistical ($P > 0.05$) significance (Figure. 2.5D). The PD-1 expression on CD4+CD25+ regulatory T lymphocytes was significantly ($P < 0.05$) reduced in 0.08% (41.83%) caffeine-treated group compared with water-drinking group (66.8%) (Figure. 2.5E). The difference in PD-1 expression on CD4+CD25+ T lymphocytes between 0.02% (64.74%) and 0.04% (57.39%) caffeine-treated group, and water-drinking group (66.8%) did not reach statistical ($P > 0.05$) significance (Figure. 2.5E)

2.3.6 Effect of caffeine on intratumoral levels of TNF-α and IFN-γ

We further investigated whether the observed anti-tumor effect of caffeine is mediated through the release of cytokines. The intratumoral levels of TNF-α and IFN-γ in all groups were estimated by ELISA. The TNF-α levels were significantly ($P < 0.05$) higher in 0.04% (3256.6 pg/g of tumor) and 0.08% (4570 pg/g of tumor) caffeine-treated groups compared with water-drinking group (347.6 pg/g of tumor) (Figure. 2.7A). The difference in TNF-α level between 0.02% caffeine-treated group (727.2 pg/g of tumor) and water-drinking group (347.6 pg/g of tumor) did not reach statistical ($P > 0.05$) significance (Figure. 2.7A). The IFN-γ levels were significantly ($P < 0.05$) higher in 0.02% (1130.8 pg/g of tumor), 0.04% (2638.2 pg/g of tumor), and 0.08% (7377.67 pg/g of tumor) caffeine-treated groups compared with water-drinking group (477.12 pg/g of tumor) (Figure. 2.7B)

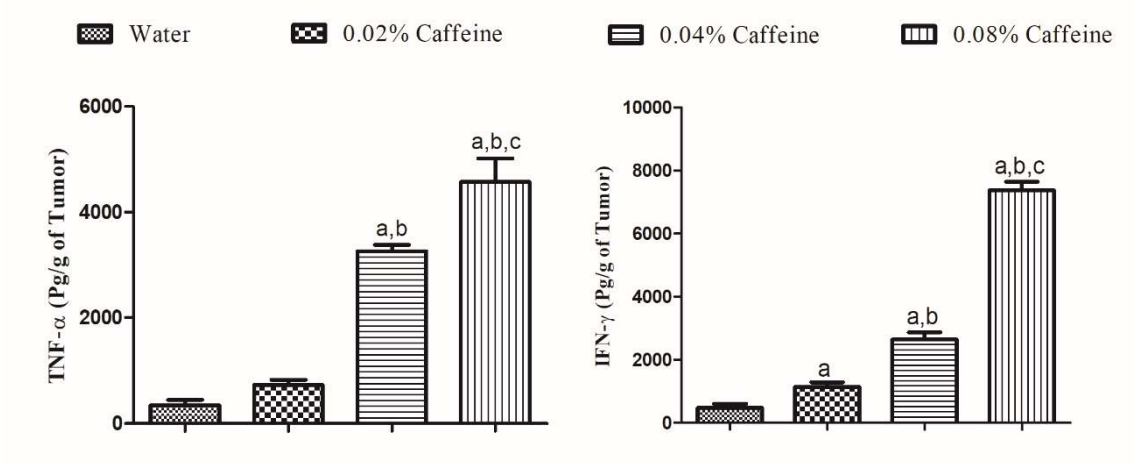

Figure 2.7: Caffeine treatment increased TNF-α and IFN-γ levels in carcinogen-induced tumors. Mice were sacrificed and tumors were harvested after eight weeks of treatment from the appearance of tumors. One part of the tumor was homogenized, supernant was collected, and measured by ELISA. Data represents mean + SEM of 3–8 mice per group. $^{a}P < 0.05$ versus wate**r,** $^{b}P < 0.05$ versus 0.02% caffeine, $^{c}P < 0.05$ versus 0.04% caffeine.

2.3.7 Effect of caffeine on body, heart, liver and kidney weights

In order to investigate the safety of different concentrations of caffeine, body weights of individual mice from each group and weight of major organs were measured. Caffeine treatment appears safe with no major changes in body weights of mice in treated groups (Table 1) and no major changes in the weights of heart, liver and kidney (Table 2) of mice from each group.

Table 1: Effect of caffeine on body weight

Mice	Control group		0.02% w/v caffeine		0.04% w/v caffeine		0.08% w/v caffeine	
	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)	Initial (g)	End (g)
1	26	35	25	38	25	37	26	40
2	28	37	27	39	26	38	27	39
3	28	36	27	37	28	35	28	36
4	25	35	25	34	27	36	25	38
5	26	36	25	36	26	38	26	37
6	26	38	28	37	28	37	27	35
7	25	30	26	38	27	37	28	40
8	27	38	27	38	25	38	26	38

Table 2: Effect of caffeine on major organs

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	1.5	2.4	0.17	1.6	2.3	0.18	1.7	2.2	0.16	1.7	2.2	0.15
2	1.7	2.6	0.2	1.8	2.2	0.21	1.5	2.4	0.17	1.8	2.4	0.18
3	1.6	2.4	0.21	2.0	2.5	0.24	1.4	2.3	0.17	1.4	2.0	0.15
4	1.5	2.5	0.19	1.8	2.7	0.25	1.8	2.2	0.19	1.5	2.1	0.15
5	1.5	2.7	0.17	1.6	2.8	0.18	1.5	2.1	0.2	1.4	2.4	0.13
6	1.5	2.4	0.17	1.7	2.5	0.19	1.4	2.0	0.21	1.6	2.3	0.17
7	1.4	2.3	0.19	1.5	2.5	0.25	1.4	2.4	0.2	1.5	2.2	0.18
8	1.7	2.5	0.22	1.8	2.4	0.23	1.6	2.3	0.16	1.8	2.0	0.2

2.4 Discussion

Several epidemiological studies reported the inverse relationship between caffeinated coffee consumption and risk of multiple cancers in humans. The present study was aimed to identify the molecular mechanism that correlates caffeinated coffee consumption and lower tumor incidence in humans. In order to represent the etiology of tumor initiation, 3-MCA, a carcinogen that mediates both tumor initiation and promotion was used in the present study. Our results demonstrate that caffeine treatment decreases the incidence and growth of carcinogen-induced tumors. These findings are in line with the previous studies that demonstrated knock-out of CD73, an enzyme that coverts ATP to AMP, hindered tumor growth and metastatic spreading and mice inoculated with tumor cells lacking CD73 survive longer than mice inoculated with tumor cells expressing this ecto-enzyme (Stagg et al., 2011 Stagg, 2011). Indeed, administration of anti-CD73 monoclonal antibodies or of a CD73-specific pharmacologic inhibitor impairs tumor growth and metastasis while increasing survival (Vigano et al., 2019). Further, the present study demonstrates that the effect of caffeine on reduction of tumor incidence and tumor growth is primarily through the enhancement of anti-tumor immune response against 3-MCA. The presence of TILs within the tumor microenvironment is a direct reflection of host immune response against tumor antigens (Clemente et al., 1996; Jass, 1986; Naito et al., 1998). In the present study, we observed increased number of total T lymphocytes in tumors of caffeine-treated mice than those of water-drinking mice, indicating caffeine-enhanced anti-tumor immune response. The lack of enough total T lymphocytes in tumors of water-drinking mice reflects one of the emerging hall marks of cancer “avoiding immune destruction” (Hanahan and Weinberg, 2011). T lymphocytes are primarily

composed of three different subpopulations namely, helper T cells, cytotoxic T cells, and regulatory T cells. Helper T cells play a major role in activating adaptive immune system (Mosmann and Coffman, 1989; Zhu and Paul, 2008). They not only activate B cells to secrete antibodies but also help in activation and proliferation of cytotoxic T cells (Zhu and Paul, 2008). Cytotoxic T cells once activated by antigen-presenting cells and helper T cells, destruct the targeted tumor cell by different mechanisms (explained below) (Andersen et al., 2006). Furthermore, it has been reported that infiltration of cytotoxic T cells control the clinical progression of various types of cancer (Fukunaga et al., 2004; Sato et al., 2005). In contrast to cytotoxic T cells, regulatory T cells are immunosuppressive and generally down-regulate the induction and proliferation of cytotoxic T cells and produce a number of inhibitory cytokines (Mempel et al., 2006).

Our results demonstrate that percentage of cytotoxic T cells were significantly more in tumors of caffeine-treated mice than water-drinking mice. Conversely, percentages of regulatory T cells were significantly less in tumors of caffeine-treated mice than water-drinking mice. The decrease in regulatory T cells in turn enhances the proliferation and function of cytotoxic T cells. The findings are in line with the previous findings that the blockade of adenosine A2A receptor enhances CD8+ T cells an decrease Treg cells (Ma et al., 2017). Once infiltrated into the tumors, cytotoxic T cells destruct the target cells either by the release of cytokines (TNF-α and IFN-γ) or perforins and granzymes or through the induction of apoptosis (Andersen et al., 2006). Further, our results indicated that caffeine-treated mice have higher intra-tumoral levels of TNF-α and IFN-γ than water-drinking mice. It has been reported that the cytotoxic T cells are capable to destruct already developed tumor cells and the precancerous cells before they develop into tumors (Swann and Smyth, 2007). This supports our finding that lower tumor incidence and decreased tumor growth was observed in caffeine-treated mice, which may be due to enhanced infiltration of cytotoxic T cells. These findings are further supported by reports that showed administration of caffeine along with cytotoxic T cells destructed the established CMS4 lung metastatic nodules and enhanced the efficacy of adoptive T-cell therapy (Ohta et al., 2006).

In the tumor environment, the anti-tumor functions of T lymphocytes are suppressed due to the interaction between PD-1 receptors on the cytotoxic T cells and programmed cell death ligand 1 released by tumor cells (Zhao et al., 2018). The expression of PD-1 on the cytotoxic T cells and regulatory T cells correlates with a worse survival of various cancers (Zhao et al., 2018). The regulatory T cells interact with programmed cell

death ligand 1 through PD-1 receptor and block cytotoxic T cell function to induce immunosuppression (Kumar et al., 2017). Our results demonstrate that caffeine treatment decreases the expression of PD-1 on cytotoxic T cells and regulatory T cells. Therefore, we suggest that caffeine-enhanced anti-tumor activity involves blockade of immunosuppression induced by PD-1 expression.

The possible mechanism behind anti-tumor immune response of caffeine treatment relates to the antagonism of adenosine A2A receptor. Activation of A2A receptors by adenosine on T cells leads to decreased proliferation of cytotoxic T cells, decreased production of cytokines (TNF-α and IFN-γ), and decreased expression of PD-1 (Whiteside, 2017). The Ki of caffeine against mice A2A receptor is 44 μM. In the present study, lower levels of caffeine 0.02 % w/v (equivalent to 40 mg/kg/day), 0.04 % w/v (equivalent to 80 mg/kg/day), and 0.08 % w/v (equivalent to 160 mg/kg/day) were used (Shi and Daly, 1999). In an earlier study, a serum concentration of 40 μM was observed in mice following a single injection of 20 mg/kg caffeine (Ohta et al., 2006). Therefore, the doses of caffeine used in the present study would have antagonized the A2A receptors. It is also interesting that in humans consuming moderate to heavy caffeine a plasma concentration of 10-50 μM was observed (Lelo et al., 1986; Cook et al., 1996).

2.5 Conclusion

Taken together, we show here for the first time that caffeine treatment enhanced the anti-tumor immune response through increased infiltration of cytotoxic T lymphocytes and decreased expression of PD-1 on cytotoxic T lymphocytes. The findings of the present study unraveled the immune-related mechanisms behind the caffeinated coffee consumption and lower tumor incidence in humans. Finally, we suggest that blockade of adenosine pathway by caffeine may constitute an effective means to enhance anti-tumor immune response.