5. Chapter 5: Discussion and conclusions

The main outcome of the current study is that caffeine dose-dependently enhanced anti-tumor activity, anti-tumor immune response and cytokines against carcinogen-induced tumor model. In addition, caffeine-anti-PD1 combination and oxlaplatin-anti-PD1 combination caused significant improvement in anti-tumor immune response and anti-tumor activity against carcinogen and cell line-induced tumor models in mice. The combination therapies were able to enhance the anti-tumor immune response by increasing the infiltration of T lymphocytes like cytotoxic T cells and helper T cells, decreased infiltration of immunosuppressive regulatory T cells, enhanced release of cytokines.

Several epidemiological studies reported the inverse relationship between caffeinated coffee consumption and risk of multiple cancers in humans. In the present study we aimed to identify the molecular mechanism that correlates caffeinated coffee consumption and lower tumor incidence in humans. 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). We found 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). 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. 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. 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. In the tumor environment, the anti-tumor functions of T lymphocytes is 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). 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). The observed caffeine-enhanced anti-tumor immune response in the present study at 0.04% and 0.08% suggests that consuming 4 and 8 cups of coffee per day such effects can be achieved in humans.

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 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. 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 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. 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.

In order to increase to response rates of these immune checkpoint inhibitors, combination studies that can boost the anti-tumor immune response of immune checkpoint inhibitors are further warrented. In the present study, we also investigated the anti-tumor effect of oxaliplatin 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 oxaliplatin 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 the present study, the combination therapy of oxaliplatin and anti-PD1 caused a significant prolongation in the overall survival period of mice with carcinogen-induced tumors. In order to evaluate the anti-tumor efficacy in specifc tumor type and the possible mechanism of action, we further investigated the effect of combination terapy against B16F10 melanoma tumors. Our results revealed that the combination therapy of oxaliplatin and anti-PD1 caused a significant anti-tumor activity against B16F10 melanoma tumors. In order to identify the possible mechanism of action, we isolated TILs from the harvested B16F10 melanoma tumors and subjected to flow cytometric analysis. T cells particulary, cytotoxic (CD8) and helper T (CD4) cells plays a major role in generating and regulating the immune response against tumor antigens (Nagorsen et al., 2003). In the present study, the observed lower CD4 and CD8 T lymphocyte infiltration into the tumors of control mice, indicates the ability of tumor cells in avoiding immune destruction. Our results demonstrate that oxaliplatin 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. In the present study, it has been also observed that combination therapy demonstrated significant increase in the release of DAMPs like calreticulin and HMGB1 and cytokines like TNF-α, and IFN-γ. Based on the observed results, the possible mechanism action behind the increased antitumor activity of the combination therapy might be due to the release of DAMPs by oxaliplatin and simultaneous inhibition of PD1 blockade by anti-PD1 antibody. Release of DAMPs by oxaliplatin has been implicated in the induction of ICD and the maturation of dendritic cells which further increases the activation, expansion and infiltration of tumor specific cytotoxic T cells. 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 oxaliplatin and anti-PD1 mAb combination therapy significantly increased intra-tumoral TNF-α and IFN-γ levels leading to cytotoxic effect on tumor cells. Our study

provides the scientific basis for testing combination regimens of oxaliplatin and anti-PD1 mAbs for sustained tumor control in cancer patients.

5.1 Summary of major findings

Immunotherapy with PD-1/PD-L1 blockers is a promising cancer treatment strategy, which has revolutionized the treatment landscape of various malignancies. Over the last decade, immunotherapy with PD-1/PD-L1 has achieved clinical success against a broad range of cancers. Currently checkpoint inhibitors targeting PD-1 or PD-L1 and is at the forefront of cancer immunotherapy with sustained survival benefits in multiple malignancies (Egen et al., 2020). However, clinical evidence indicated that more than 50% of patients might not respond to PD-1/PD-L1 blockade even for patients with tumors highly positive for PD-L1 (Reck et al., 2016). Clinical responses of these checkpoint inhibitors vary across different tumors due to their heterogeneity. For example, the objective response rate was 15–20% in NSCLC (Borghaei et al., 2015), 30–45% in melanoma (Robert et al., 2015), 22–25% in kidney cancer (Motzer et al., 2015) and 13% in head and neck carcinoma (Ferris et al., 2019). Besides, acquired resistance remains another problem for most of the patients experiencing initial clinical response (Hamid et al., 2019; Bai et al., 2017). In order to increase response rates of these immune checkpoint inhibitors, combination studies that can boost the anti-tumor immune response of immune checkpoint inhibitors are further warranted. The present research work was designed to evaluate the combination therapy of caffeine with anti-PD1 and oxaliplatin with anti-PD1 in carcinogen and cell line-induced tumor models. Major findings of the present research work are as follows:

- Caffeine at dose of 0.08% was found highly efficacious in enhancing anti-tumor activity through anti-tumor immune response
- Caffeine monotherapy enhanced the anti-tumor immune response through increased infiltration of cytotoxic T lymphocytes and decreased expression of PD-1 on cytotoxic T lymphocytes
- Caffeine and anti-PD1 combination therapy effectively reduced tumor growth and increased the overall survival period possibly due to combined blockade of PD1 and adenosine-A2A receptor pathway

- Oxaliplatin and anti-PD1 combination therapy effectively reduced tumor growth and increased the overall survival period possibly due to ICD-driven anti-tumor immunity and simultaneous blocking of PD-1

5.2 Scope for further work

The combination strategies for anti-PD1 followed in this thesis was based on the potential of immune enhancement effects of caffeine and oxaliplatin through adenosine-A2A pathway and ICD induction, respectively. The present study revealed the antitumor potential of these combination therapies in the syngeneic tumor model. In the future, evaluation of these combinations in patient derived xenograft models are further warranted to translate into clinical studies. Since, metastasis is the greatest contributor to deaths from cancer, the efficacy of these combinations in metastatic models of mice are further warranted. In vitro studies that evaluates the expression of PD1 in the presence of caffeine or the expression of A2A on TILs in the presence of anti-PD1 and pharmacologic inhibition studies that evaluates the inhibitory activity of these combinations on multiple T cell types are further warranted