4. Chapter 4: Evaluation of antitumor immune response of oxaliplatin and anti-PD1 monoclonal antibody combination against carcinogen and cell line induced tumors in mice

4.1 Introduction

Monoclonal antibodies targeting inhibitory programmed cell death pathway have proved to be highly efficacious and showed durable clinical responses in various cancer patients. However, despite the remarkable clinical efficacy of these agents in a number of malignancies, it has become clear that they are not sufficiently active for many patients. Majority of patients still show reduced or no clinical benefit and evidence suggests that the tumors of those patients might have favorable mutational landscapes, express the PD-1 ligand (PD-L1), and lack of T-cell infiltration (Rizvi et al., 2015; Herbst et al., 2014). In order to broaden the number of responding patients, extend the duration of response, and to overcome the resistance of single agent therapy, it appears important to employ the strategies that convert tumors lacking T cell infiltration to ones displaying antitumor T cells and then to determine whether this approach sensitizes tumors to checkpoint therapy.

One approach to attain this goal involves the induction of immunogenic conditions in the tumor microenvironment. For example, some chemotherapies can stimulate T cell immune surveillance by influencing tumor-host interactions (Zitvogel et al., 2013; Shalapour et al., 2015). Oxaliplatin is one of the platinum based chemotherapeutic agent that can effectively augment antitumor immune response by inducing ICD and also facilitates the activation of dendritic cells, which inturn causes the activation, generation and proliferation of cytotoxic T cells (Galluzzi et al., 2012). Recently it has been also reported that oxaliplatin enhances the therapeutic efficacy of immune checkpoint inhibitors in a murine model of colorectal and lung carcinoma (Wang et al., 2017; Sun et al., 2019). Hence we hypothesized that simultaneous blocking of PD-1 with anti-PD1 and induction of ICD with oxaliplatin may improve the anti-tumor immune response of anti-PD1 antibody. In the present study, we aimed to investigate the therapeutic role oxaliplatin in murine model of melanoma and evaluate the therapeutic efficacy of oxaliplatin and anti-PD1 monoclonal antibody co-administration.

4.2 Materials and methods

4.2.1 Animals

Adult female C57BL/6J mice (20-30 g) were procured from National Institute of Nutrition, Hyderabad, India. Adult female albino mice (6-8 weeks old) were obtained from central animal house of the university. All animals were acclimatized for 7 days in an animal room (12:12 h light/dark cycle, temperature 25 ± 2°C, relative humidity of 30 to

70%) in the Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University). All experimental procedures were approved by the Institutional Animal Ethical Committee of Banaras Hindu University and conducted in accordance with the principles established by CPCSEA (Dean/2019/IAEC/1249).

4.2.2 Cell line, Drugs, and Chemicals

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

4.2.3 Carcinogen-induced tumor model

Carcinogen-induced tumors in albino mice were generated in the hind flank region with subcutaneous injection of 400 µg of 3-methylcholanthrene (3-MCA) as described previously. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, weekly twice) or intraperitoneal injections of control Ig (100 µg, weekly twice) for 6 weeks. Mice were weekly monitored for tumor development during the treatment period. The dose of oxaliplatin (Cubas et al., 2018) and anti-PD1 mAb (Allard et al., 2013) was selected based on previous studies.

4.2.4 Cell line-induced tumor model

C57BL/6J mice were subcutaneously injected with $1x10^5$ B16F10 melanoma cells in 100 µl of RPMI1640 medium as described previously. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, weekly twice) or intraperitoneal injections of control Ig (100 µg, weekly twice) for 3 weeks.

4.2.5 Tumor measurement and survival analysis

The diameter of tumor were measured using digital caliper and the tumor sizes (mm^2) were calculated as the product of two perpendicular diameters. Mice with tumor diameter greater than 5 mm and showing progressive growth were considered as tumor positive. 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.

4.2.6 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.

4.2.7 Flow cytometric analysis of tumor infiltrating lymphocytes

After the end of the treatment period, B16F10 melanoma tumors were excised and tumor infiltrating lymphocytes (TILs) were isolated as described previously by Allard B et.al (Allard et al., 2016). Briefly, tumors were sliced into small pieces and incubated in a digestion medium containing DNase and collagenase type IV at 37 °C for 1 hr. After complete digestion, tumor cells were passed through a 40-µm cell strainer, rinsed twice in phosphate buffered saline, centrifuged at 1200 rpm, and the pellet was resuspended in 30% percoll. The cell suspension in 30% percoll was gently layered onto 70% percoll, and centrifuged at 1200 rpm, at 4 °C for 30 min with no brake. The TILs located at the interface were collected, washed twice with excess FACS buffer, and resuspended in FACS buffer. Then, Fc receptors of TILs were blocked with anti CD-16/32 monoclonal antibody before staining with specific antibodies. The following antibodies (purchased from Thermo Fisher Scientific) were used in this study: rat anti-mouse CD4 PerCP-Cy 5.5 (Clone RM4-5, Cat no. 45-0042-82), rat anti-mouse CD3 FITC (Clone 17A2, Cat no. 11-0032-82), rat anti-mouse CD8a PE (Clone 53-6.7, Cat no. 12-0081-81), armenian hamster anti-mouse

CD279 APC (Clone J43 , Cat no. 17-9985-80), rat anti-mouse CD25 PE (Clone PC61.5, Cat no. 12-0251-81). After 30 min of staining, flow cytometry was conducted using BD FACSCALIBUR and analyzed using FlowJo software.

4.2.8 Analysis of intratumoral levels of calreticulin, HMGB1, 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 calreticulin, HMGB1, TNF-α and IFN-γ using ELISA, according to manufacturer's protocol

4.2.9 Statistical analysis

Results were expressed as mean ± 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.

4.2 Results

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

Therapeutic activity of anticancer drugs was evaluated by their effects on tumor progression and overall survival period (Pazdur, 2008). In this study, the therapeutic activity of oxaliplatin monotherapy, anti-PD1 mAb monotherapy, and their combination therapy was demonstrated in 3-MCA-induced tumor model by assessing individual tumor size and tumor growth rate. The tumor size in control group ranged from 284 to 468 mm^2 (Figure. 4.1A) whereas, oxaliplatin, anti-PD1 mAb, and the combination treated groups showed tumor sizes that ranged from 197 to 315 mm^2 (Figure. 4.1B), 184 to 249 mm^2 (Figure. 4.1C), and 0 to 152 mm^2 (Figure. 4.1D), respectively. Especially, 4 out of 8 mice in combination therapy have complete tumor regression and 2 out of 8 mice showed partial response (Figure. 4.1D). In addition, mean tumor growth rates (mm^2/day) of oxaliplatin monotherapy (2.2 mm^2/day) and anti-PD1 mAb monotherapy (1.5 mm^2/day) were significantly ($P < 0.05$) lower when compared with control group (5.1 mm^2/day) (Figure. 4.1E). Further, the combination therapy caused significant ($P < 0.05$) reduction in tumor growth rate as compared to control group (0.7 mm^2/day versus 5.3 mm^2/day), oxaliplatin

monotherapy (0.7 mm^2/day versus 2.2 mm^2/day), and anti-PD1 mAb monotherapy (0.7 mm^2/day versus 1.5 mm^2/day) (Figure. 4.1E).

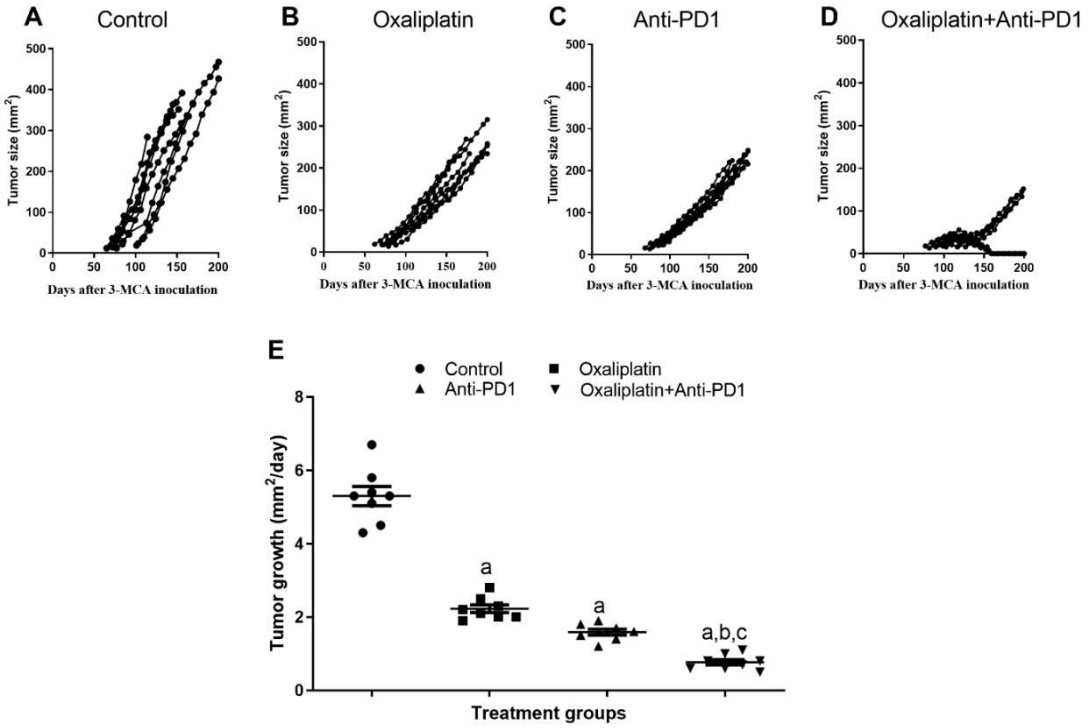

Figure 4.1: Combination of oxaliplatin and anti-PD1 mAb inhibited the carcinogen-induced tumor growth. Carcinogen-induced tumors in albino mice were generated in the hind flank region with subcutaneous injection of 400 µg of 3-methylcholanthrene (3-MCA). Once tumors were established (> 5 mm diameter), mice were randomly allocated into (A) Control group: received intraperitoneal injections of control Ig (100 µg, weekly twice) (B) Oxaliplatin group: received intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) (C) Anti-PD1 mAb group: received intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice) (D) Oxaliplatin + Anti-PD1: received intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) 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. $^{a}P < 0.05$ versus control, $^{b}P < 0.05$ versus oxaliplatin, $^{c}P < 0.05$ versus anti-PD1 mAb.

Further, the analysis of survival curves revealed that oxaliplatin treatment increased the survival of mice but was statistically insignificant ($P > 0.05$) when compared with control treatment (Figure. 4.2). However, anti-PD1 monotherapy and combination therapy significantly ($P < 0.05$) prolonged the overall survival period compared with the control group (Figure. 4.2).

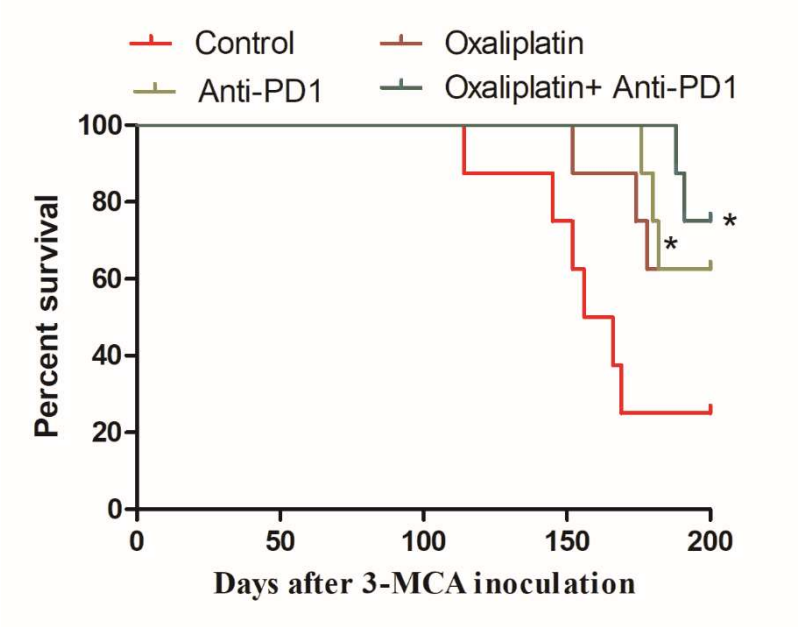

Figure 4.2: Combination of oxaliplatin and anti-PD1 mAb increased the overall survival period in carcinogen-induced tumor model. Carcinogen-induced tumors in albino mice were generated in the hind flank region with subcutaneous injection of 400 µg of 3-methylcholanthrene (3-MCA). Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, 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 Log-rank (Mantel-Cox) test. $^{*}P < 0.05$ versus control.

4.2.2 Effects of oxaliplatin, 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 oxaliplatin and anti-PD1 mAb against B16F10 melanoma tumor bearing mice. The results revealed that oxaliplatin (on day 25, 74.8 mm^2) and anti-PD1 mAb (on day 25, 50.6 mm^2) monotherapy caused statistically significant ($P < 0.05$) reduction in tumor growth compared with control group (on day 25, 146.6 mm^2) (Figure. 4.3). The combination therapy (on day 25, 30.1 mm^2) showed statistically significant ($P < 0.05$) reduction in tumor growth compared with control group (on day 25, 146.6 mm^2), oxaliplatin monotherapy (on day 25, 74.8 mm^2), and anti-PD1 mAb monotherapy (on day 25, 50.6 mm^2) (Figure. 4.3).

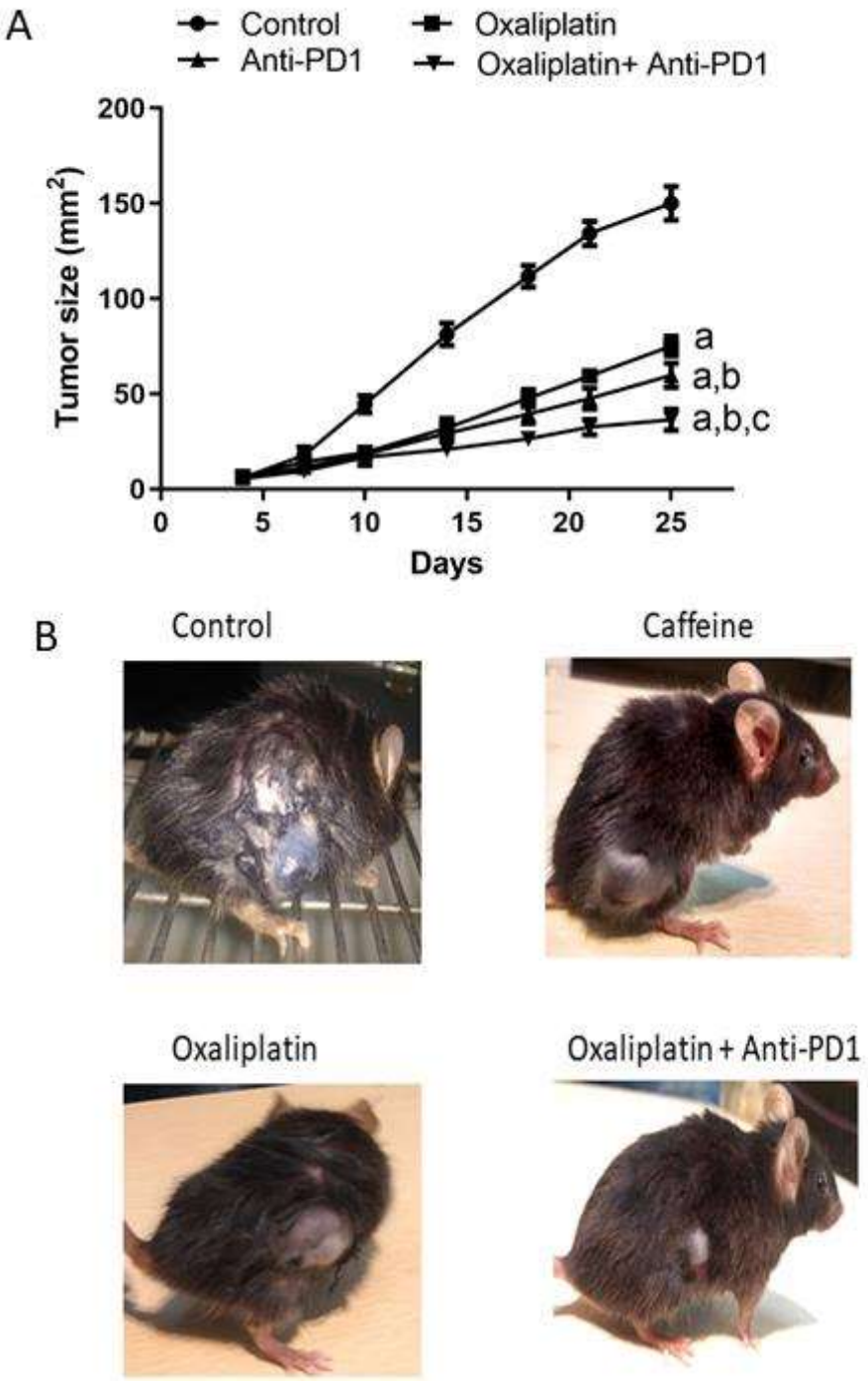

Figure 4.3: Oxaliplatin and anti-PD1 mAb combination therapy inhibited cell line-induced tumor growth. C57BL/6J mice were injected subcutaneously with $1x10^5$ B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, 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 melnoma tumors from different groups were represented. Data represents mean ± SEM of 8 mice per group. $^{a}P < 0.05$ versus Control, $^{b}P < 0.05$ versus oxaliplatin, $^{c}P < 0.05$ versus anti-PD1 mAb.

4.2.3 Effects of oxaliplatin, anti-PD1 mAb or their combination on CD4 T lymphocytes into cell line-induced tumor model

We next evaluated the effect of oxaliplatin 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 oxaliplatin (13.6%) and anti-PD1 mAb (18.7%) treated groups compared with control group (10.2%) (Figure. 4.4). However, the combination therapy (27.8%) caused a statistically significant ($P < 0.05$) increase in CD4 T-lymphocyte infiltration compared with control (10.2%), oxaliplatin monotherapy (13.6%), and anti-PD1 monotherapy (27.8%) (Figure. 4.4)

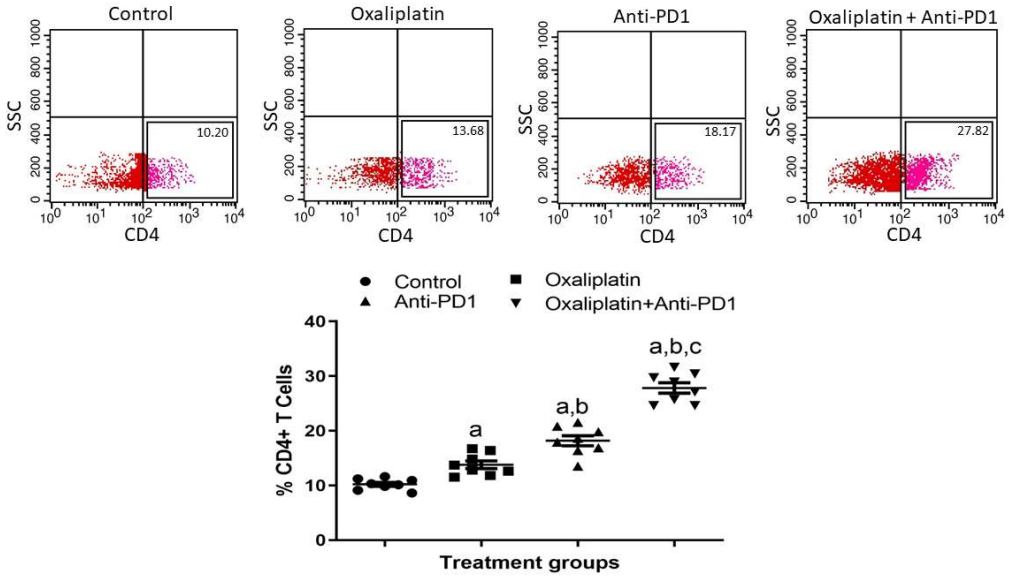

Figure 4.4: Effects of oxaliplatin, anti-PD1 mAb or their combination on CD4 T lymphocytes into cell line-induced tumor model. C57BL/6J mice were injected subcutaneously with $1x10^5$ B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, weekly twice) for 3 weeks. On day 25, the mean tumor size in all groups was analyzed by one way ANOVA followed by Tukey's multiple comparison test. Data represents mean ± SEM of 8 mice per group. $^aP < 0.05$ versus Control, $^bP < 0.05$ versus oxaliplatin, $^cP < 0.05$ versus anti-PD1 mAb.

4.2.4 Effects of oxaliplatin, anti-PD1 mAb or their combination on CD8 T lymphocytes into cellline-induced tumor model

We next evaluated the effect of oxaliplatin 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 oxaliplatin (13.4%) and anti-PD1 mAb (18.3%) treated groups compared with control group (8.7%) (Figure. 4.5). However, the combination therapy (23.4%) caused a statistically significant ($P < 0.05$) increase in CD4 T-lymphocyte infiltration compared with control (8.7%), oxaliplatin monotherapy (13.4%), and anti-PD1 monotherapy (18.3%) (Figure. 4.5)

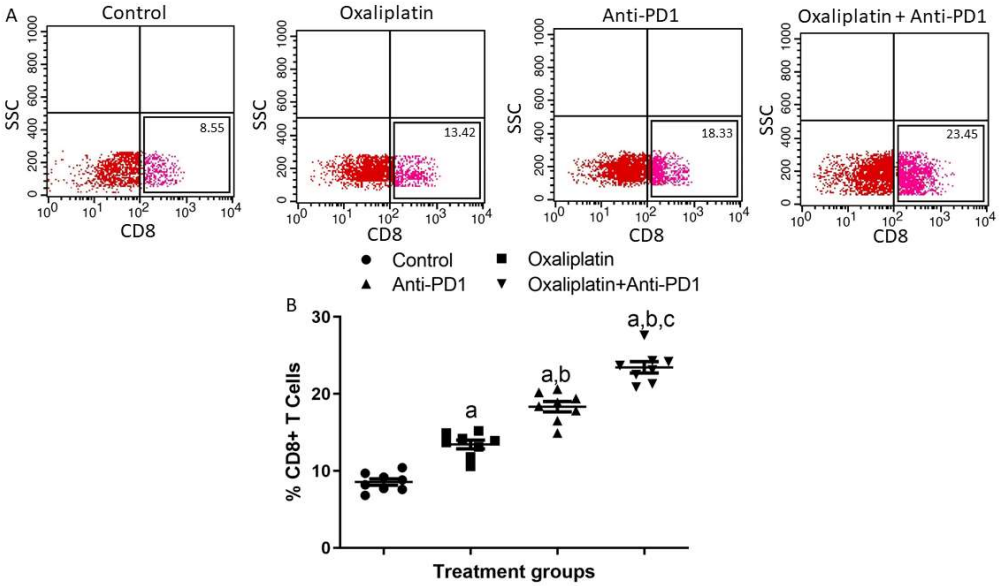

Figure 4.5: Effects of oxaliplatin, anti-PD1 mAb or their combination on CD8 T lymphocytes into cell line-induced tumor model. C57BL/6J mice were injected subcutaneously with $1x10^5$ B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, weekly twice) for 3 weeks. On day 25, the mean tumor size in all groups was analyzed by one way ANOVA followed by Tukey's multiple comparison test. Data represents mean ± SEM of 8 mice per group. $^aP < 0.05$ versus Control, $^bP < 0.05$ versus oxaliplatin, $^cP < 0.05$ versus anti-PD1 mAb.

4.2.5 Effects of oxaliplatin, anti-PD1 mAb or their combination on damage associated molecular patterns (DAMPs)

To further investigate whether the observed anti-tumor effect of oxaliplatin and anti-PD1 mAb combination therapy is mediated through the release of DAMPs, we estimated the intra-tumoral levels of calreticulin and HMGB1 in all groups by ELISA. The calreticulin levels were significantly ($P < 0.05$) higher in oxaliplatin (1683.8 ng/g of tumor) and anti-PD1 mAb (1875.4 ng/g of tumor) treated groups compared with control group (478.4 ng/g of tumor) (Figure. 4.6). Furthermore, combination therapy (5678.6 pg/g of tumor) caused a statistically significantly ($P < 0.05$) increase in calreticulin compared with

control (478.4 ng/g of tumor), oxaliplatin monotherapy (1683.8 ng/g of tumor), and anti-PD1 monotherapy (1875.4 ng/g of tumor) (Figure. 4.6).

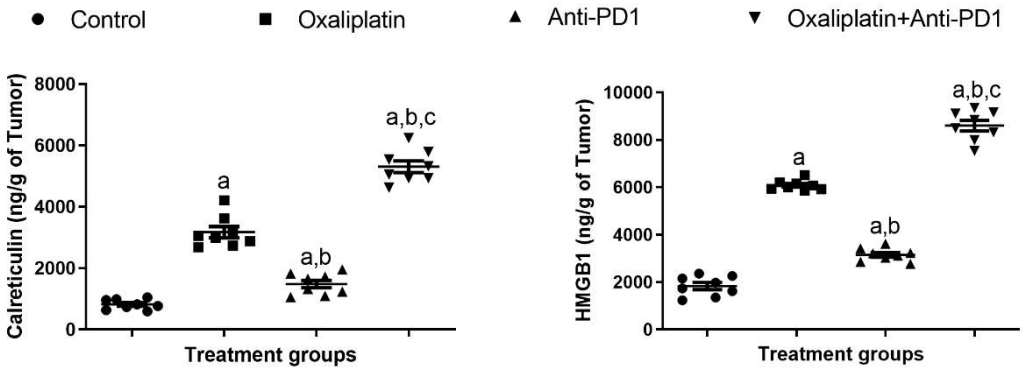

Figure 4.6: Effects of oxaliplatin, anti-PD1 mAb or their combination on DAMPs. C57BL/6J mice were injected subcutaneously with $1x10^5$ B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, weekly twice) for 3 weeks. On day 25, tumors were isolated and intra-tumoral levels of calreticulin and HMGB1 were estimated by ELISA. Data represents mean ± SEM of 5-6 mice per group. $^aP < 0.05$ versus control, $^bP < 0.05$ versus caffeine, $^cP < 0.05$ versus anti-PD1 mAb.

Similarly, the HMGB1 levels were significantly ($P < 0.05$) higher in oxaliplatin (6013.3 ng/g of tumor) and anti-PD1 mAb (3345.1 ng/g of tumor) treated groups compared with control group (1897.2 ng/g of tumor) (Figure. 4.6). Furthermore, combination therapy (8345.6 ng/g of tumor) caused a statistically significantly ($P < 0.05$) increase in HMGB1 compared with control (1897.2 ng/g of tumor), oxaliplatin monotherapy (6013.3 ng/g of tumor) and anti-PD1 monotherapy (3345.1 ng/g of tumor) (Figure. 4.6)

4.2.6 Effects of oxaliplatin, 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 oxaliplatin and anti-PD1 mAb combination therapy is mediated 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 oxaliplatin (3018.7 pg/g of tumor) and anti-PD1 mAb (3652.1 pg/g of tumor) treated groups compared with control group (1868.1 pg/g of tumor) (Figure. 4.7). Furthermore, combination therapy (7493.6 pg/g of tumor) caused a statistically significantly ($P < 0.05$) increase in TNF-α compared with

control (1868.1 pg/g of tumor), oxaliplatin monotherapy (3018.7 pg/g of tumor), and anti-PD1 monotherapy (3652.1 pg/g of tumor) (Figure. 4.7).

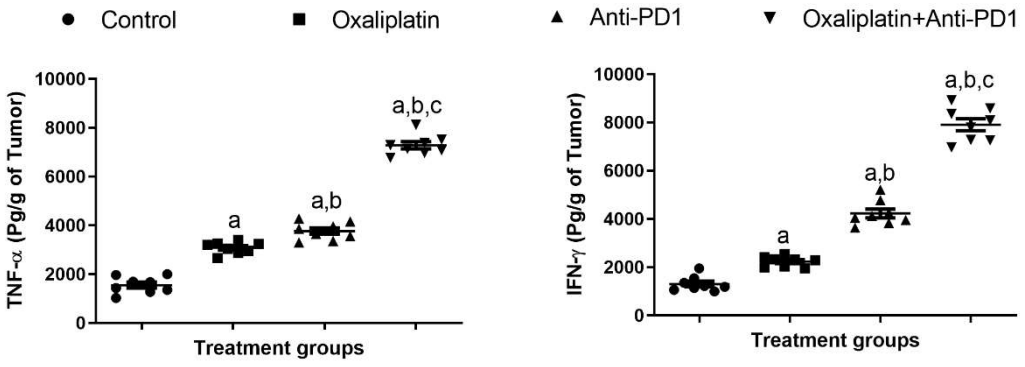

Figure 4.7: Effects of oxaliplatin, anti-PD1 mAb or their combination on intra-tumoral levels of TNF-α and IFN-γ. C57BL/6J mice were injected subcutaneously with $1x10^5$ B16F10 melanoma cells. Once tumors were established (> 5 mm diameter), mice were treated with intraperitoneal injections of control Ig (100 µg, weekly twice), or intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once), or intraperitoneal injections of anti-PD1 mAb (100 µg, weekly twice), or combination of intraperitoneal injections of oxaliplatin (5 mg/kg, weekly once) and anti-PD1 mAb (100 µg, 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 ± SEM of 5-6 mice per group. $^aP < 0.05$ versus control, $^bP < 0.05$ versus caffeine, $^cP < 0.05$ versus anti-PD1 mAb.

Similarly, the IFN-γ levels were significantly ($P < 0.05$) higher in oxaliplatin (2234.3 pg/g of tumor) and anti-PD1 mAb (3959.1 pg/g of tumor) treated groups compared with control group (1767.2 pg/g of tumor) (Figure. 4.7). Furthermore, combination therapy (7896.6 pg/g of tumor) caused a statistically significantly ($P < 0.05$) increase in IFN-γ compared with control (1767.2 pg/g of tumor), oxaliplatin monotherapy (2234.3 pg/g of tumor) and anti-PD1 monotherapy (3959.1 pg/g of tumor) (Figure. 4.7).

4.2.7 Effects of oxaliplatin, anti-PD1 mAb 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. Oxaliplatin, anti-PD1 and their combination treatment appears safe with no major changes in body weights of mice with carcinogen induced tumors (Table 7) and B16F10 melanoma induced tumors (Table 8). In line with that, no major changes in the weights of heart, liver and kidney of mice with carcinogen induced tumors (Table 9) and B16F10 melanoma induced tumors (Table 10).

Table 7: Effect of oxaliplatin, 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	26	36	27	40	28	40	26	39
2	27	35	29	41	27	39	28	38
3	30	41	25	38	25	38	29	40
4	25	38	26	37	27	39	29	42
5	28	34	27	39	29	40	27	39
6	27	36	27	38	28	42	30	41
7	27	37	28	42	30	40	30	42
8	28	40	28	40	30	42	29	41

Table 8: Effect of oxaliplatin, 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	28	30	28	31	29	31	27	30
2	29	31	29	29	29	31	27	29
3	30	32	28	31	27	29	28	30
4	30	32	28	31	28	29	26	28
5	30	32	30	32	30	32	30	32
6	29	30	29	31	27	29	29	30
7	27	30	30	32	27	30	28	31
8	29	31	29	32	26	29	29	32

Table 9: Effect of oxaliplatin, 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	1.8	2.1	0.16	1.8	2.3	0.19	1.4	2.3	0.20	1.8	2.2	0.18
2	1.8	2.2	0.16	1.5	2.2	0.17	1.9	2.4	0.21	1.8	2.4	0.19
3	1.7	2.3	0.18	1.3	2.0	0.18	1.7	2.2	0.22	1.6	2.1	0.15
4	1.6	2.1	0.2	1.4	2.2	0.22	1.6	2.0	0.21	1.5	2.0	0.15
5	1.5	2.2	0.21	1.7	2.4	0.20	1.5	2.1	0.19	1.9	2.1	0.16
6	1.9	2.0	0.2	1.7	2.4	0.19	1.6	2.0	0.21	1.6	2.4	0.18
7	1.4	2.4	0.22	1.8	2.1	0.19	1.8	2.3	0.22	1.8	2.	0.19
8	1.6	2.2	0.18	1.9	2.3	0.24	1.9	2.4	0.18	1.9	2.3	0.21

Table 10: Effect of oxaliplatin, 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	2.3	0.18	1.9	2.6	0.26	2.0	2.2	0.18	2.2	2.5	0.21
2	1.8	2.2	0.19	1.7	2.5	0.19	2.1	2.1	0.19	2.0	2.2	0.20
3	1.6	2.1	0.17	1.5	2.6	0.23	1.7	2.2	0.20	1.9	2.1	0.19
4	1.6	2.0	0.19	1.8	2.4	0.22	1.8	2.0	0.19	1.9	2.2	0.21
5	1.9	2.5	0.26	1.6	2.5	0.25	1.6	2.2	0.19	1.8	2.4	0.21
6	1.7	2.6	0.19	1.7	2.6	0.19	2.0	2.1	0.24	1.6	2.1	0.24
7	1.8	2.8	0.23	1.5	2.7	0.23	1.6	2.0	0.20	1.7	2.0	0.17
8	1.9	2.6	0.22	1.8	2.5	0.22	1.6	2.0	0.19	2.0	2.2	0.22

4.3 Discussion

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 varies 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 to response rates of these immune checkpoint inhibitors, combination studies that can boost the anti-tumor immune response of immune checkpoint inhibitors are further warranted.

In the present study, we 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 specific tumor type and the possible mechanism of action, we further investigated the effect of combination therapy 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 data confirm previous reports from the group of Christina that demonstrated combination of oxaliplatin with ant-PD1 elicited immunogenic phenotypes on KP tumor cells and also increased CD8+ T cell infiltration into KP tumors and delayed cancer progression (Pfirschke et al., 2016).

4.4 Conclusions

Anti-PD1 mAbs such as pembrolizumab and nivolumab 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 induction of ICD by conventional chemotherapeutic agents like oxaliplatin in solid tumors has been recently identified as a major immunostimulatory pathway, targeting this pathway will synergize the therapeutic activity of anti-PD1 mAbs. Our work suggests that administration of oxaliplatin and anti-PD1 mAb enhances the anti-tumor immune response in vivo possibly due to induction of ICD and simultaneous blockade of PD1. Our study provides the scientific basis for testing combination regimens of oxaliplatin and anti-PD1 mAbs for sustained tumor control in cancer patients.