IMMUNOGENIC CHEMOTHERAPY WITH OXALIPLATIN AND CAFFEINE COMBINATION ENHANCED ICD INDUCTION AND T-CELL INFILTRATION IN MICE TUMOR MODELS

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ABSTRACT

Majority of chemotherapeutic agents can elicit antitumor immunity and modulate the composition, density, function, and distribution of tumor infiltrating lymphocytes, to influence differential therapeutic responses and prognosis in cancer patients. The clinical success of these agents, particularly platinum based agents like oxaliplatin, not only depends on their cytotoxic activity but also by the enhancement of pre-existing immunity primarily through induction of immunogenic cell death. However, resistance for the induction of immunogenic cell death either intrinsic or acquired is a major hurdle for most of these drugs. In order to enhance immunogenic cell death by these agents, it has become clear that blockade of adenosine production or its signaling need to be specifically targeted as they represent highly resistant mechanisms. Given the prominent role of adenosine mediated immunosuppression and resistance to immunogenic cell death induction in tumor micro environment, combination strategies that involve immunogenic cell death induction and adenosine signaling blockade are further warranted. The purpose of the present study is to investigate the combination therapy of oxaliplatin and caffeine against carcinogen and cell-line induced tumor models in mice. Study results demonstrated significant tumor growth inhibition by the oxaliplatin and caffeine combination therapy against both the tumor models. Additionally, significant T-cell infiltration and enhanced immunogenic cell death induction evidenced by increased intra-tumoral calreticulin and HMGB1 levels, was observed in B16F10 melanoma mice.

Conclusion: The possible mechanism behind the observed anti-tumor activity might be due to the enhanced immunogenic cell death induction and subsequent T-cell infiltration by the combination therapy. In order to prevent the development of resistance and to enhance the anti-tumor activity of Immunogenic cell death inducing drugs like oxaliplatin, combination with adenosine-A2A receptor pathway inhibitors like caffeine might be a potential strategy.

Keywords: Immunogenic cell death; Adenosine A2A receptor; Anti-tumor immune response; Cancer immunotherapy; Chemotherapy.

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INTRODUCTION

Cytotoxic chemotherapy is a highly recognized and widely used treatment option for many patients with cancer. However, as a monotherapy for late-stage patients, the response rates for cytotoxic chemotherapeutic agents were very low (7.4%), regardless of the type of cancer or drug regimen used.(Abbott et al. 2015) Platinum-based chemotherapeutic drugs, a class of cytotoxic chemotherapeutics,

cause cytotoxicity by disrupting DNA replication through cross-linking DNA strands. Despite their cytotoxic properties, they also promote anti-tumor immunity through the induction of immunogenic cell death (ICD).(Kepp and Kroemer 2020) The clinical success of these agents not only depends on their cytotoxic activity but also by the enhancement of pre-existing immunity. However, resistance for the induction of ICD either intrinsic or acquired is a major hurdle for most of these drugs.

It has been reported that CD39 or CD73 enzymes overexpression limits the ICD caused by chemotherapeutic agents like anthracyclines and oxaliplatin.(Michaud et al. 2012) ATP secretion is one of the hallmarks of ICD, and the released ATP stimulates dendritic cells (DCs) recruitment and activation and ultimately the ICD induction. Over expressed CD39 and CD73 on tumor cells converts the chemotherapy induced ATP into adenosine and dampens its capacity to attract DCs into the proximity of dying and dead tumor cells.(Michaud et al. 2012) In addition, the converted adenosine exhibits potent immunosuppressive activities in the tumor microenvironment (TME) on multiple immune effector cells by acting on A2A receptors and contributes to the resistance against chemotherapy.(Tej and Nayak 2018) In order to enhance ICD by these agents, it has become clear that blockade of adenosine production or its signaling need to be specifically targeted as they represent highly resistant mechanisms. Given the prominent role of adenosine mediated immunosuppression and resistance to ICD induction in TME, combination strategies that involve ICD induction and adenosine signaling blockade are further warranted.

Oxaliplatin is one of the platinum based chemotherapeutic agents that can effectively augment antitumor immune response by inducing immunogenic cell death and also facilitates the activation of dendritic cells.(Galluzzi et al. 2012) It has been reported that adenosine A2A receptor inhibition by caffeine, increased cytotoxic T cell infiltration, decreased Tregs infiltration and decreased PD-1 expression on infiltrated T lymphocytes in carcinogen induced tumors.(GN et al.) In the present study, we aimed to investigate the therapeutic role of oxaliplatin in the murine model of melanoma and evaluate the therapeutic efficacy of oxaliplatin and caffeine co-administration.

MATERIALS AND METHODS

Mice

Adult female C57BL/6J mice (20-30 g) and 6-8 weeks old female albino mice were purchased from National Institute of Nutrition, Hyderabad, India. 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%). All experimental procedures were approved by the Institutional Animal Ethical Committee of Hindu college of Pharmacy and conducted according to CPCSEA guidelines (1253/PO/Re/S/09/CPCSEA).

Drugs, chemicals, and cell line

B16F10 melanoma cell line was obtained from NCCS, Pune, India. Oxaliplatin (61825-94-3), caffeine (58-08-2), 3-methylcholanthrene (Cat no. 213942), collagenase type IV (Cat no. C5138), percoll (P1644) and DNase (Cat no. 10104159001) were procured from Sigma Aldrich. All the other chemicals were analytical grade and procured from local vendors.

3-methylcholanthrene (3-MCA) induced tumor model

Tumors in the hind flank region of albino mice were generated by SC administration of 400 µg of 3-MCA.(Tej et al. 2019) After tumor establishment (> 5 mm diameter), mice were treated with oxaliplatin (5 mg/kg, IP, weekly once), or caffeine (0.8% w/v in drinking water, daily), or combination of oxaliplatin (5 mg/kg, IP, weekly once) and caffeine (0.8% w/v in drinking water, daily) for 6 weeks. During the treatment period, mice were monitored for tumor development, weekly. The dose of oxaliplatin(Grasselly et al. 2018) and caffeine(GN et al.) were determined from previous studies.

B16F10 melanoma tumor model

Tumors were generated in C57BL/6J mice by SC administration of $1x10^5$ B16F10 melanoma cells in 100 µl of RPMI1640 medium.(Overwijk and Restifo 2000) After tumor establishment (> 5 mm diameter), mice were treated with oxaliplatin (5 mg/kg, IP, weekly once), or caffeine (0.8% w/v in drinking water, daily), or

combination of oxaliplatin (5 mg/kg, weekly once) and caffeine (0.8% w/v in drinking water, daily) for 3 weeks.

Flow cytometry (BD FACSCALIBUR; Flow Jo software)

B16F10 melanoma tumors were surgically removed and tumor infiltrating lymphocytes (TILs) were extracted as described previously.(Allard et al. 2016) Rat anti-mouse flow cytometry antibodies including CD3 FITC (Cat no. 11-0032-82), CD8a PE (Cat no. 12-0081-81) and CD4 Per CP-Cy 5.5 (Cat no. 45-0042-82), were procured from Thermo Fisher Scientific, USA.

ELISA

Supernatant was collected from B16F10 melanoma tumors by homogenization and centrifugation (4 °C, 12000 rpm, 45 min). Calreticulin and HMGB1 levels were quantified according to the manufacturer's protocol.

Statistical analysis

Results were analyzed using one-way ANOVA followed by Tukey's multiple comparison test and expressed as mean ± SEM. Survival curves were analyzed by Gehan–Breslow–Wilcoxon test. P value < 0.05 was considered statistically significant.

RESULTS & DISCUSSION

Oxaliplatin and caffeine combination decreased tumor growth progression and increased survival of mice with 3-MCA induced tumors

Therapeutic efficacy of anticancer drugs can be evaluated by their ability to control tumor growth progression and survival.(Pazdur 2008) The progression of tumor growth from the mice in the control group has ranged 298 to 463 mm^2 (Fig. 1A). However, caffeine monotherapy, oxaliplatin monotherapy and the combination therapydecreased the tumor growth progression and has ranged from 293 to 374 mm^2 (Fig 1B), 221 to 295 mm^2 (Fig. 1C), and 153 to 251 mm^2 (Fig. 1D), respectively.Of the 8 mice treated with combination therapy, 5 showed partial tumor regression (Fig. 1D). Additionally, it has been found that the tumor growth rate per day of mice from oxaliplatin monotherapy (2.0 mm^2/day) and caffeine monotherapy (2.9 mm^2/day) were significantly ($P < 0.05$) lower when compared with the growth rates of mice from control group (5.4 mm^2/day) (Fig. 1E). Furtherly, the growth rates of mice from combination therapy were significantly ($P < 0.05$) lowerwhen compared to all other groups.

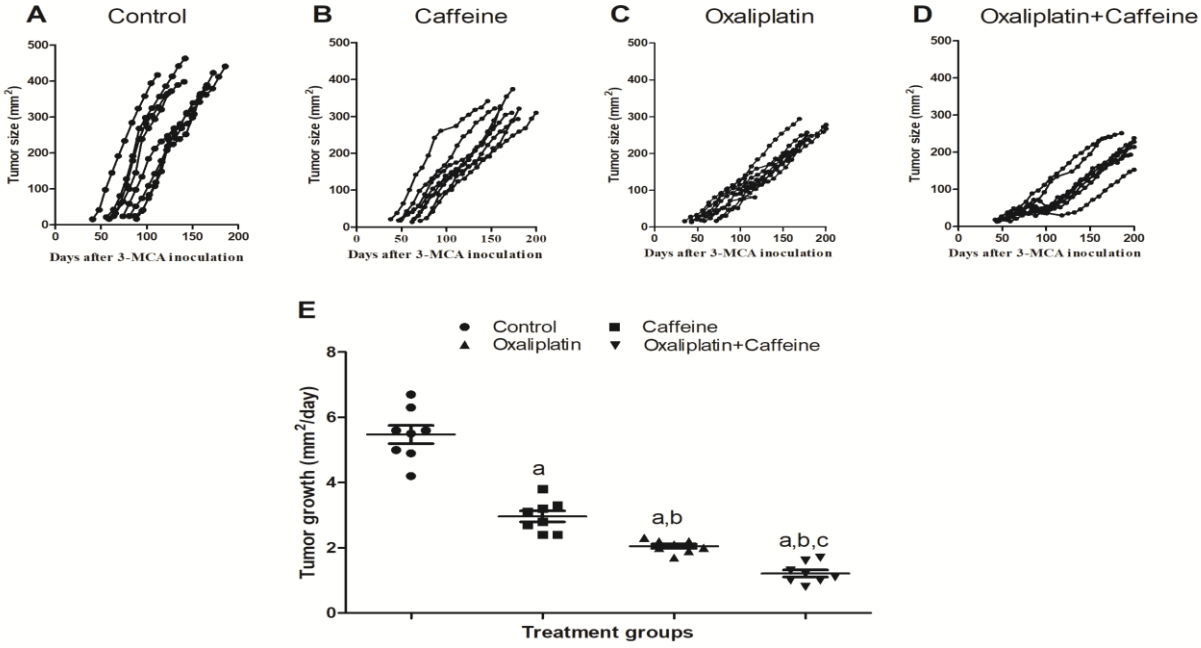

Fig. 1: Oxaliplatin and caffeine combination decreased 3-MCA tumor growth progression.

Tumors in the hind flank region of albino mice were generated by SC administration of 400 µg of 3-MCA. Mice were randomized to (A) Control group: received drinking water (B) Caffeine group: received caffeine 0.8% w/v in drinking water, daily (C) Oxaliplatin group: received oxaliplatin 5 mg/kg, IP, weekly (D) Oxaliplatin + Caffeine: received oxaliplatin 5 mg/kg, IP, weekly and caffeine 0.8% w/v in drinking water, daily. (E) Individual tumor growth rates of mice per day from all the groups. Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin.

Analysis of survival curves demonstrated that caffeine (median survival 173 days) and oxaliplatin monotherapy (median survival 173.5 days) increased the survival of mice as compared to control group (median survival 142.5 days) (Fig. 2). However, the difference is statistically insignificant ($P > 0.05$). But the combination therapy (median survival 186 days) significantly ($P < 0.05$) increased the survival of mice (median survival 142.5 days) (Fig. 2) as compared to all other groups.

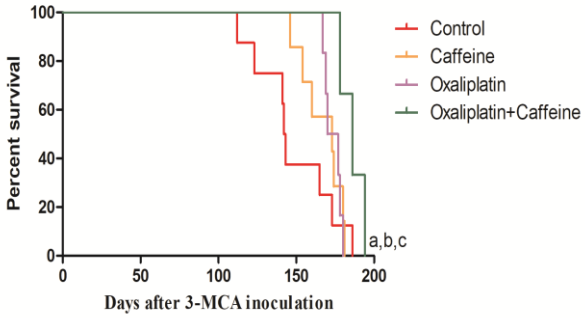

Fig. 2: Oxaliplatin and caffeine combination increased survival of mice with 3-MCA induced tumors. Tumors in the hind flank region of albino mice were generated by SC administration of 400 µg of 3-MCA. Mice were randomized to (A) Control group: received drinking water (B) Caffeine group: received caffeine 0.8% w/v in drinking water, daily (C) Oxaliplatin group: received oxaliplatin 5 mg/kg, IP, weekly (D) Oxaliplatin + Caffeine: received oxaliplatin 5 mg/kg, IP, weekly and caffeine 0.8% w/v in drinking water, daily. (E) Individual tumor growth rates of mice per day from all the groups. Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin

Oxaliplatin and caffeine combination decreased tumor growth progression in mice with B16F10 melanoma tumors

Therapeutic activity of caffeine and oxaliplatin monotherapy and combination therapy was further evaluated against B16F10 melanoma tumors. It was found that mice from caffeine (on day 25, 123 mm^2) and oxaliplatin (on day 25, 72.3 mm^2) monotherapy groups have significantly ($P > 0.05$) decreased tumor growth as compared to control group (on day 25, 203.1 mm^2) (Fig. 3). Further, the combination therapy demonstrated significantly ($P < 0.05$) lower tumor growth (on day 25, 36.4 mm^2) as compared to all other (Fig. 3).

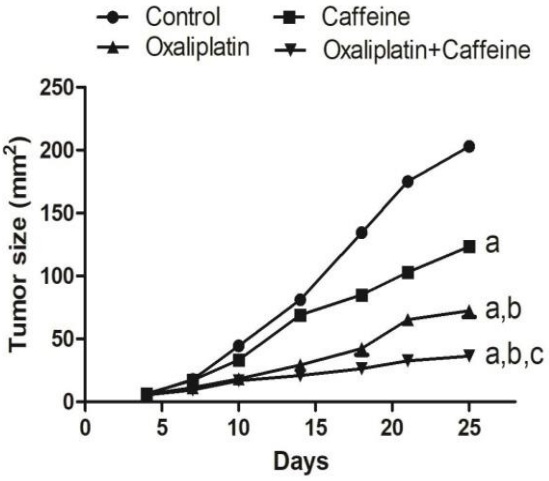

Fig. 3: Oxaliplatin and caffeine combination decreased tumor growth progression in mice with B16F10 melanoma tumors. Tumors were generated in C57BL/6J mice by SC administration of 1x105 B16F10 melanoma cells in 100 µl of RPMI1640 medium. Mice were treated with oxaliplatin (5 mg/kg, IP, weekly once), or caffeine (0.8% w/v in drinking water, daily), or combination of oxaliplatin (5 mg/kg, weekly once) and caffeine (0.8% w/v in drinking water, daily) for 3 weeks. Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin.

Oxaliplatin and caffeine combination increased CD4 T-lymphocyte infiltration into B16F10 melanoma tumors

To further evaluate the observed anti-tumor activity of combination therapy is through enhanced immune infiltration, we analyzed CD4

T-lymphocyte population in B16F10 melanoma tumors by flow cytometry. It was found that CD4 T-lymphocyte infiltration was significantly ($P < 0.05$) high from the tumors of mice treated with caffeine (13.68%) and oxaliplatin (18.17%) monotherapy as compared to control group (10.20%) (Fig.4). Further, the infiltration in tumors of mice treated with combination (27.82%) was significantly ($P < 0.05$) higher compared to all other groups (Fig. 4).

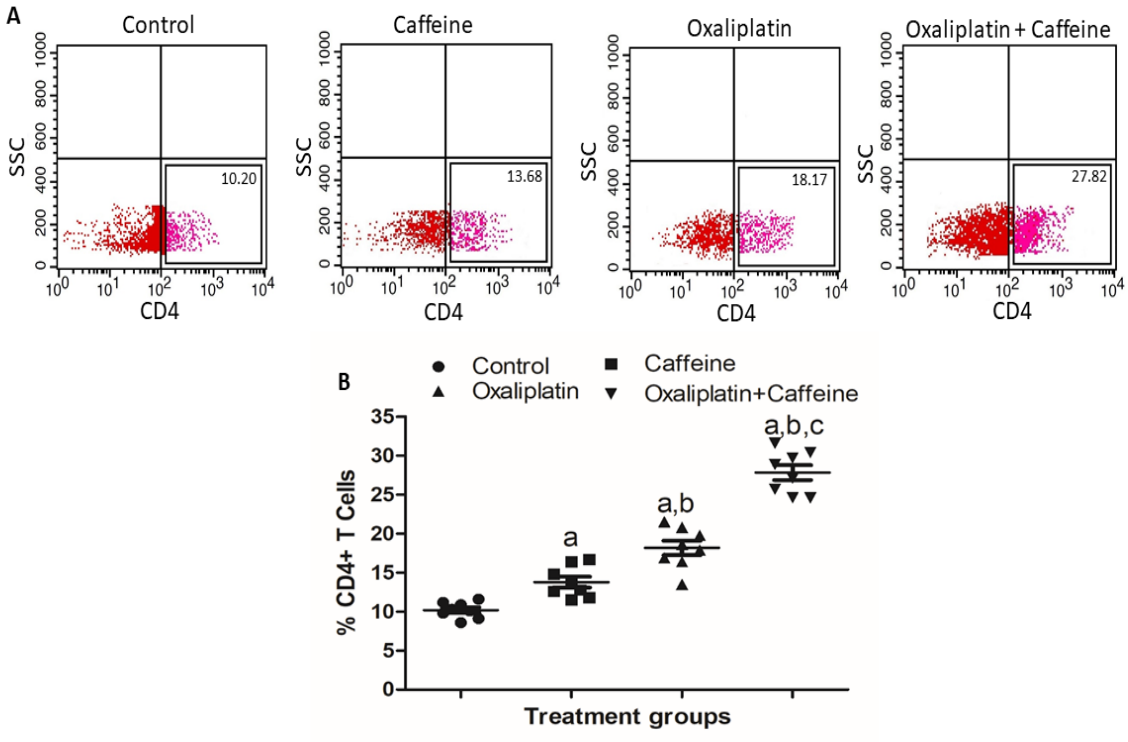

Fig. 4: Oxaliplatin and caffeine combination increased CD4 T-lymphocyte infiltration into B16F10 melanoma tumors.

Tumors were generated in C57BL/6J mice by SC administration of 1x105 B16F10 melanoma cells in 100 µl of RPMI1640 medium. On day 25, B16F10 melanoma tumors were surgically removed and tumor TILs) were extracted and analyzed by flow cytometry. (A) Representative flow cytometric images of CD4T-lymphocytesof all groups. (B) Statistical results of percentages of CD4T-lymphocytes. Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin.

Oxaliplatin and caffeine combination increased CD8 T-lymphocyte infiltration into B16F10 melanoma tumors

We next evaluated the infiltration of CD8 T-lymphocytes and it has been observed that tumors treated caffeine (13.42%) and oxaliplatin (18.33%) monotherapy have significantly ($P < 0.05$) higher infiltration compared with control group (8.55%) (Fig.5). Further, the infiltration in tumors of mice treated with combination (23.45%) was significantly ($P < 0.05$) higher compared to all other groups (Fig. 5).

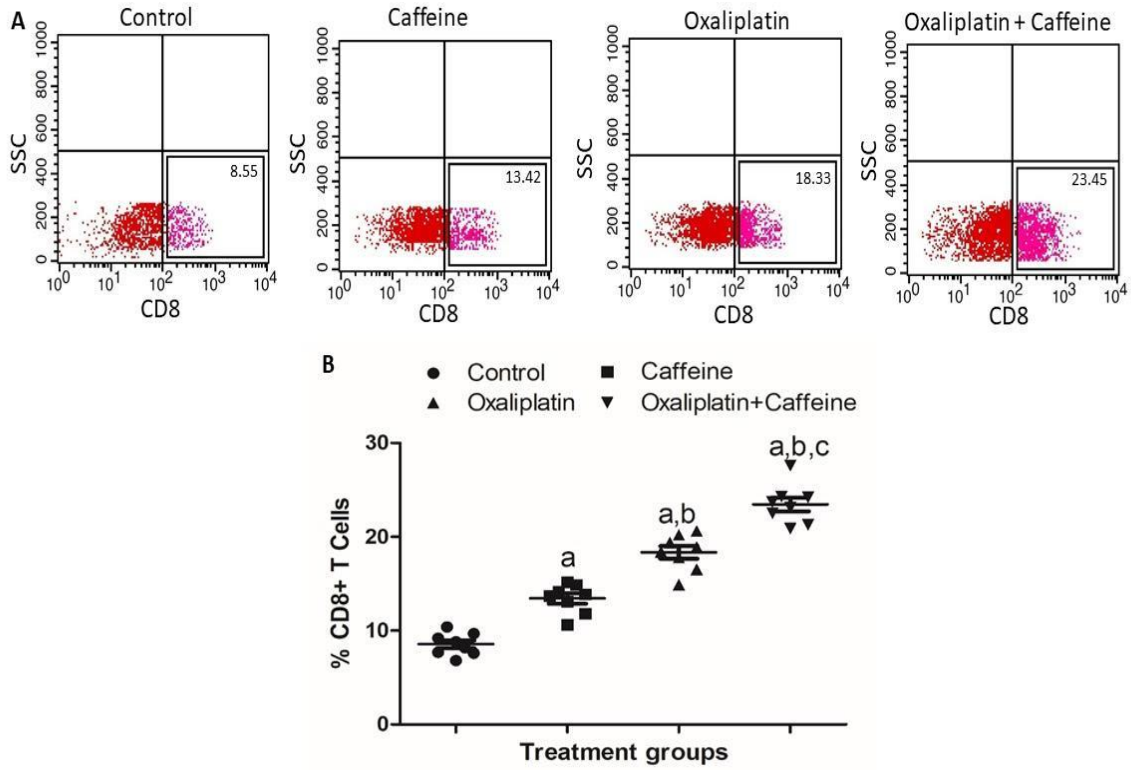

Fig. 5: Oxaliplatin and caffeine combination increased CD8 T-lymphocyte infiltration into B16F10 melanoma tumors.

Tumors were generated in C57BL/6J mice by SC administration of 1x105 B16F10 melanoma cells in 100 µl of RPMI1640 medium. On day 25, B16F10 melanoma tumors were surgically removed and tumor TILs) were extracted and analyzed by flow cytometry. (A) Representative flow cytometric images of CD8T-lymphocytesof all groups. (B) Statistical results of percentages of CD8T-lymphocytes. Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin.

Oxaliplatin and caffeine combination increased calreticulin and HMGB1 release

In order to investigate whether the enhanced immune infiltration is mediated through the induction of ICD, quantification of calreticulin and HMGB1 was carried out using ELISA. It has been observed that the calreticulin levels in the tumors of caffeine (1483.6 ng/g of tumor) and oxaliplatin (2801 ng/g of tumor) treated mice were significantly ($P < 0.05$) higher compared to control group (820 ng/g of tumor) (Fig. 6). Furthermore, a significant increase in calreticulin was observed in tumors of mice treated with combination therapy (5306.7 ng/g of tumor) compared to all other groups (Fig. 6).

Similarly, the HMGB1 levels in the tumors of caffeine (3155.7 ng/g of tumor) and oxaliplatin (6084.5 ng/g of tumor) treated mice were significantly ($P < 0.05$) higher compared to control group (1839.1 ng/g of tumor) (Fig. 6). Furthermore, a significant increase in HMGB1 was observed in tumors of mice treated with combination therapy (8003.3 ng/g of tumor) compared to all other groups (Fig. 6).

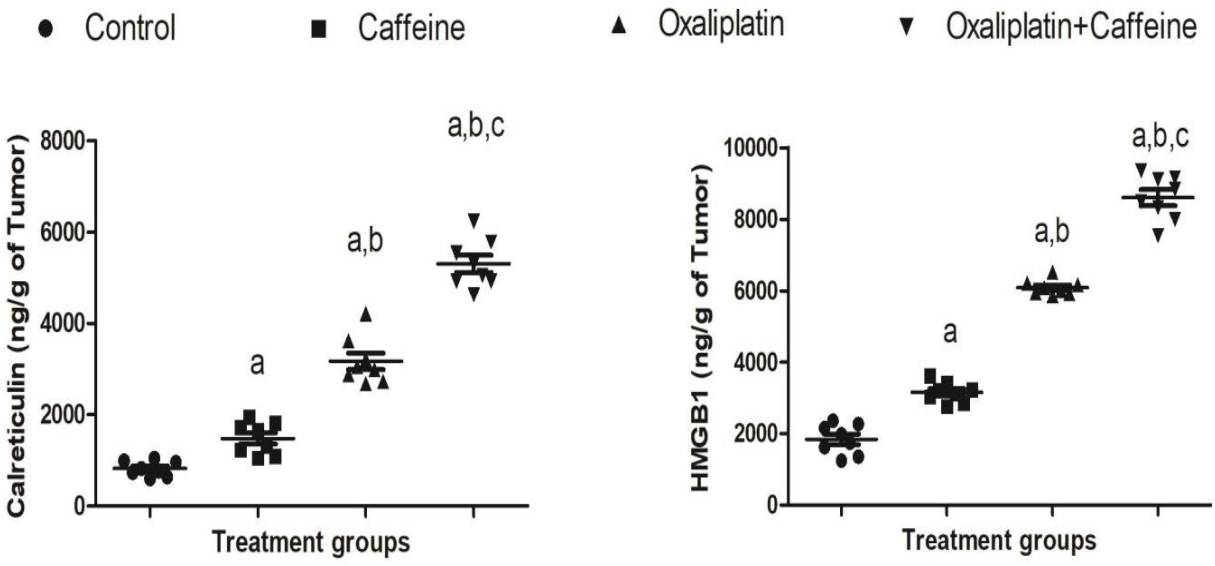

Fig. 6: Oxaliplatin and caffeine combination increased calreticulin and HMGB1 release.

Tumors were generated in C57BL/6J mice by SC administration of 1x105 B16F10 melanoma cells in 100 µl of RPMI1640 medium. On day 25, B16F10 melanoma tumors were surgically removed and intra-tumoral levels of calreticulin and HMGB1 were estimated by ELISA.Data represents mean ± SEM of 8 mice per group. $^{a}P< 0.05$ versus control, $^{b}P< 0.05$ versus caffeine, $^{c}P< 0.05$ versus oxaliplatin.

DISCUSSION

Majority of chemotherapeutic agents can elicit antitumor immunity and modulate the composition, density, function, and distribution of tumor infiltrating lymphocytes (TILs), to influence differential therapeutic responses and prognosis in cancer patients. The clinical success of these agents, particularly platinum based agents like oxaliplatin, not only depends on their cytotoxic activity but also by the enhancement of pre-existing immunity primarily through induction of ICD. However, resistance for the induction of ICD either intrinsic or acquired is a major hurdle for most of these drugs.

The purpose of the present study is to investigate the combination therapy of oxaliplatin and caffeine against 3-MCA and B16F10 melanoma tumor models in mice. Tumor growth inhibition correlates positively with survival period of mice.(LeBlanc et al. 2002) Our results demonstrated that oxaliplatin and caffeine combination enhanced anti-tumor activity by significantly decreasing the 3-MCA-induced tumor growth rate in mice which inturn increased the survival rate.

To further evaluate the anti-tumor immune enhancing potential, we further investigated the effects of combination therapy against poorly immunogenic and highly aggressive(Baird JR et al.) B16F10 melanoma tumors. Treatment with the combination of caffeine and oxaliplatin significantly inhibited the growth of B16F10 melanoma tumors. To identify whether the observed anti-tumor activity of combination therapy is mediated through the enhanced immune infiltration, TILs from these tumors were isolated and subjected to flow cytometric analysis. Our results revealed that the tumors of control mice had very low T-cell infiltration. However, treatment with combination significantly increased the infiltration of effector T cells and cytotoxic T cells. In addition, the release of damage associated with molecular patterns like calreticulin and HMGB1 levels were found to be significantly high in combination treated mice.

CONCLUSION

The possible mechanism behind the observed anti-tumor activity might be due to the enhanced ICD induction and subsequent T-cell infiltration by the combination therapy. The findings of the present study proved our hypothesis that combining ICD inducing

chemotherapy with adenosine-A2A pathway inhibitors can enhance antitumor immune response. In order to prevent the development of resistance and to enhance the anti-tumor activity of these ICD inducing drugs like oxaliplatin, combination with adenosine-A2A receptor pathway inhibitors like caffeine might be a potential strategy.

Conflicts of interest

The authors declared no conflict of interest

Acknowledgements

None

References

Abbott D, Ashdown ML, Robinson AP, et al (2015) Chemotherapy for Late-Stage Cancer Patients: Meta-Analysis of Complete Response Rates. F1000Research 4:1. https://doi.org/10.12688/f1000research.6760.1

Allard B, Allard D, Stagg J (2016) Methods to evaluate the antitumor activity of immune checkpoint inhibitors in preclinical studies. Methods Mol Biol 1458:159–177. https://doi.org/10.1007/978-1-4939-3801-8_12

Baird JR B, KT L, P.H. Immune-Mediated Regression of Established B16F10 Melanoma by Intratumoral Injection of Attenuated Toxoplasma gondii Protects against Rechallenge. J Immunol 190:469–478. https://doi.org/10.4049/jimmunol.1201209

Galluzzi L, Senovilla L, Zitvogel L, Kroemer G (2012) The secret ally: Immunostimulation by anticancer drugs. Nat Rev Drug Discov 11:215–233. https://doi.org/10.1038/nrd3626

GN VCT, K N, SS V, et al Caffeine-enhanced anti-tumor immune response through decreased expression of PD1 on infiltrated cytotoxic T lymphocytes. Eur J Pharmacol 859:. https://doi.org/10.1016/j.ejphar.2019.172538

Grasselly C, Denis M, Bourguignon A, et al (2018) The antitumor activity of combinations of cytotoxic chemotherapy and immune checkpoint inhibitors is model-dependent. Front Immunol 9:. https://doi.org/10.3389/fimmu.2018.02100

Kepp O, Kroemer G (2020) A novel platinum-based chemotherapeutic inducing immunogenic cell death. Taylor & Francis

LeBlanc R, Catley LP, Hideshima T, et al (2002) Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. Cancer Res 62:4996–5000

Michaud M, Sukkurwala AQ, Martins I, et al (2012) Subversion of the chemotherapy-induced anticancer immune response by the ecto-ATPase CD39. Oncoimmunology 1:393–395. https://doi.org/10.4161/onci.19070

Overwijk WW, Restifo NP (2000) B16 as a Mouse Model for Human Melanoma. Curr Protoc Immunol 39:1– 1 9. https://doi.org/10.1002/0471142735.im2001s39

Pazdur R (2008) Endpoints for Assessing Drug Activity in Clinical Trials. Oncologist 13:19–21. https://doi.org/10.1634/theoncologist.13-s2-19

Tej GNVC, Nayak PK (2018) Mechanistic considerations in chemotherapeutic activity of caffeine. Biomed Pharmacother 105:312–319. https://doi.org/10.1016/j.biopha.2018.05.144

Tej GNVC, Neogi K, Nayak PK (2019) Caffeine-enhanced anti-tumor activity of anti-PD1 monoclonal antibody. Int Immunopharmacol 77:. https://doi.org/10.1016/j.intimp.2019.106002