The Application and Prospect of Immune Checkpoints based on PD1 and CTLA4

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Abstract: Malignant tumors are currently one of the greatest health challenges the world is facing, ranking first among the various lethal factors that cause death each year. There is a high probability of relapse after treatments once treated with traditional approaches. Immunotherapy typifies a promising treatment method to increase survival rates among patients at advanced stages. So far, however, patients can only receive limited benefits from this novel therapy. In this article, we explore two important immune checkpoints, PD-1 and CTLA-4, and discuss different factors influencing the functions of the immune checkpoint inhibitors. We also review the recent developments in the field from the point of view of combinatory therapies. Research proposals aimed to improve immunotherapies are also included to open new perspectives in enhancing the efficacy and safety of the treatment of malignant tumors.

1 INTRODUCTION

Chemotherapy, radiation, and surgery are considered the cornerstones of conventional cancer treatment. However, it remains difficult for conventional treatment programs to completely remove tumor cells largely due to tumors' ability to grow rapidly, and the tendency of developing metastasis, and resistance to radiotherapy and chemotherapy (Hanahan, Weinberg 2011). They also impose lethal effects on normal cells, greatly harming the patients' overall health. Therefore, highly specific treatments with long-lasting effects have become the primary target in treating malignant tumors.

The success of immunotherapy is based on both cancer destruction through the initiation of the host immune system and the regulation of the cancer-immune environment (Robert 2020). Scientists have confirmed that immune checkpoints such as CTLA-4 and PD-1/PD-L1 signaling pathways play an important role in regulating T cell immune response. The immune checkpoints blockade can effectively destroy tumor cells without compromising CTL function, strengthen the outcome of anti-tumor immunity. The finding of immune checkpoints opened up a new path in treating malignant tumors and greatly promote the development of checkpoint inhibitor drugs (Freeman 2000). Since 2011, the FDA has successively approved multiple PD-1/PD-L1 inhibitors for the treatment of various tumors such as metastatic or unresectable melanoma. Immune checkpoint inhibitor therapy provides novel and effectual treatment methods for tumor treatment. It is believed that more breakthroughs can be obtained in the near future.

However, the research related to checkpoint inhibitors is in the early stage, and we have not yet fully understood its biological characteristics and related signal pathways. In addition, checkpoint inhibitors have some potentially serious side effects, many of which are due to an overactive immune response, which is related to inflammation of the intestines, lungs, heart, skin, and other organs. Approximately half of the advanced patients have no obvious response to checkpoint inhibitors or no response at all. Some people live longer without

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treatment or live longer before their condition worsens.

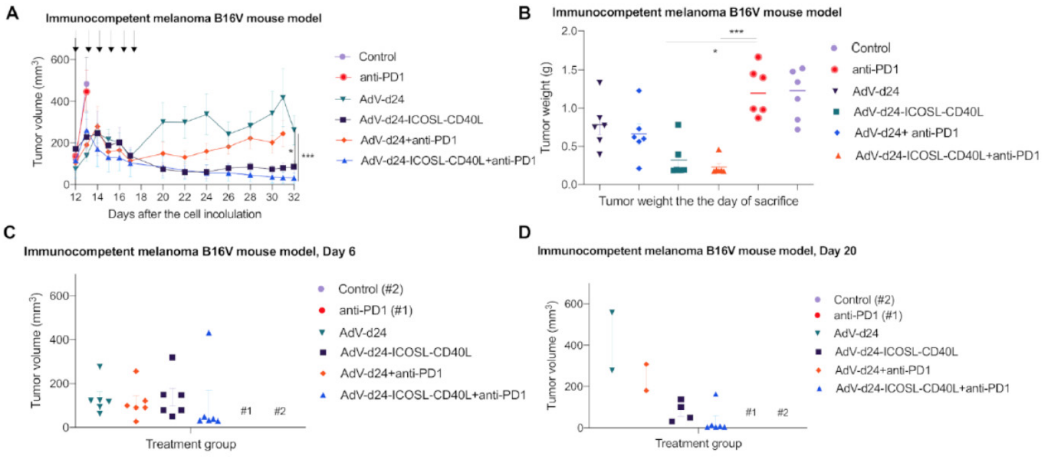
The immune check point-therapy and combination strategies can provide advanced cancer patients with superior treatment which can control or even cure the disease. The specificity, adaptability, and great capacity allow multiple biomarkers to work inside the body. Despite the overall survival for patients have improved, there are limitations and challenges inherent in immune checkpoints therapy. Emerging data and observation suggest that we need to better understand the reason why there are certain cancer types that refuse to respond and find the sealed answer. Motivated by these developments, we now revisit the critical mechanisms and recent findings that associated with immune checkpoint therapy, consider the limitation as well as challenges appear during the clinical application, and expand upon the functional roles to find possible approaches to tackle the corresponding problems of immunotherapy.

2 APPLICATIONS AND IMPROVEMENTS

2.1 Combination Therapy of Novel Oncolytic Adenovirus with PD-L1 Inhibitors Resulted in Strengthened Anti-cancer Effect

Malignant melanoma (MM) is a type of malignant tumor derived from melanocytes. Early-stage malignant melanoma is often treated by surgical resection and a longer survival can be obtained. However, in the middle and late stages, surgical resection alone is not good for patients with melanoma. Chemotherapy, radiotherapy, and targeted therapy are the main therapies for melanoma, but the efficacy is still not optimistic due to relapse and drug resistance.

The emergence of PD-L1 antibodies has completely altered the treatment strategy for advanced and metastatic melanoma. However, it has been reported 40%–60% of melanoma patients do not gain any notable recovery, and a great portion of recipients relapses within two years of the treatment. The low efficiency of the immune checkpoint inhibitors is largely due to their low response rate and potent immunosuppressive effect that creates a "cold" immune tumor microenvironment (TME) (Imbert et al. 2020). Recent researches have shown that through infecting the tumor lesion with engineered Oncolytic adenoviruses, the resulting inflammatory response can trigger the release of a series of immunoglobulins and signaling molecules including proinflammatory cytokines and an influx of NK cells, T cells, and antigen-presenting cells (APC), consequently generating the desired “hot” tumor microenvironment (LaRocca, Warner 2018). Garofalo and Bertinato treat mice models with melanoma tumors using the anti-PD-1 antibody combined with a newly designed oncolytic adenovirus containing modified AdV-D24-inducible costimulator ligand AdV-D24-ICOSL-CD40L that only targets and replicates in cancer cells by intratumor injection (Garofalo et al. 2021), resulting in a strengthened anti-cancer ability and immunogenic cell death in vitro and a significant decrease in tumor volume while ensuring a 100% survival rate in vivo. The findings demonstrate that oncolytic adenovirus-expressing potent immune modulators can drive the systemic efficacy of PD-1 blockade, enhancing anti-cancer effectiveness and survival (see Figure 1).



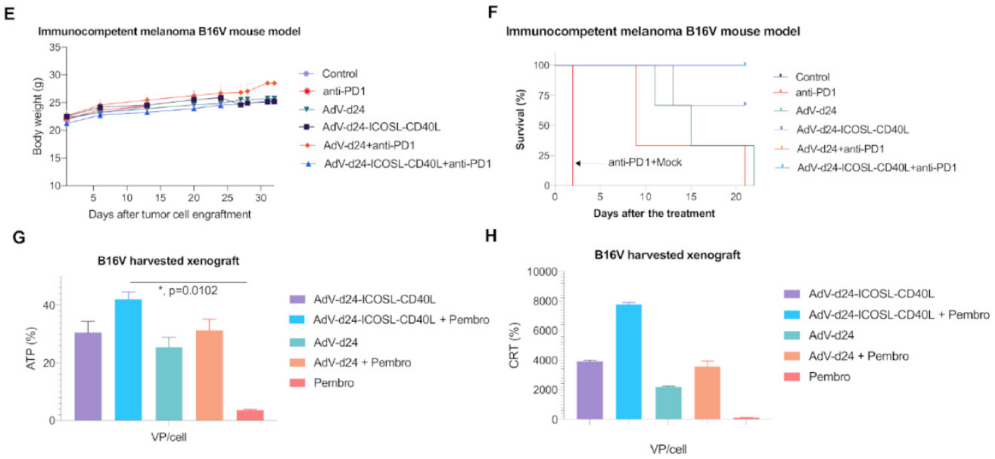


Fig 1. (Garofalo et al. 2021) (A) Tumor volume (mm3) measured through the study. The treatment was performed once per day on days 1–6. The mice were treated according to the scheme (Table 1) with viruses (i.t.) and anti PD-1 antibody (i.v.) (B) At the end of the study, mice were sacrificed and tumors harvested for weight assessment. (C, D) Tumor volume measurement on days 6 and 20, respectively. (E) Body weight measurements throughout the study. (F) Survival profile was calculated by Kaplan–Meier test. (H) Evaluation of CRT exposure after the treatment with oncolytic adenoviruses AdV-24-ICOSL-CD40L and AdV-D24, and in combination with anti PD-1. CRT exposure was measured in the end of the study (after mice sacrifice) with anti-calreticulin antibody staining and subsequent flow cytometry analysis (Beckman-Coulter Cytomics FC500). (G) Assessment of ATP release after the treatment

However, there still contains two major concerns, the potential risk of causing strong immune side-effects and the lack of efficacy on tumors that are undergoing metastasis.

Nanoparticle as a kind of delivery vector is one of the good strategies to solve these issues (Kosmides 2017). Rather than the intratumor injection, a nanoparticle delivery system that combines blockade of PD-1 with the engineer oncolytic adenovirus AdV-D24-ICOSL-CD40L can be hypothesized in order to investigate the efficacy on tumor cells in vivo. Optimistically, this nanoparticle delivery system may increase the targeting and efficacy of oncolytic viruses and anti-PD-1 antibodies, while reducing the immune response of nontarget organs.

2.2 Restricting Glycolysis Preserves T Cell Effector Functions and Augments Checkpoint Therapy (Renner et al. 2019)

The glycolytic activity of tumor cells is enhanced, and lactic acid is accumulated and acidified in the tumor. T and NK cells absorb lactic acid and impair effector functions. But diclofenac can reduce the secretion of lactic acid by inhibiting the lactate transporters MCT1 and MCT4 and enhance T cell function (figure2). So, inhibition of glycolysis improves treatment at checkpoints. In addition, the reduction of lactic acid by diclofenac has nothing to do with changes in glycolysis-related proteins and MCT expression profiles. And MCT inhibition does not impair the in vitro function of T cells.

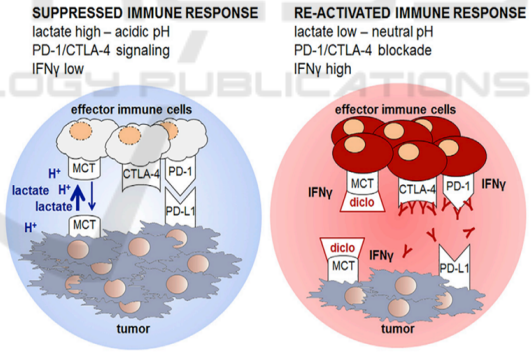


Figure 2: Explains a negative correlation between glycolytic activity in tumors and response to check point therapy.

However, the study mentioned above only has an experimental group and does research on several kinds of objects at the same time. Then a new clinical trial is designed. The purpose is to study whether reducing lactic acid secretion can improve T cell-mediated killing of melanoma cells. Patients over 18 years of age, with good nutritional status, and tumor stages in stage I and stage II will be included. And patients with other tumors, such as pancreatic cancer, gastric cancer, and patients undergoing anti-PD-1 therapy will be excluded.

100 melanoma patients from five medical centers will be divided into a control group and an experimental group. The experimental group will be treated with diclofenac, while the control group will be treated with an equal dose of normal saline. The researchers will follow up 100 patients for six months and test the anti-tumor immunity of cells in two groups. Compared with control cells, more lactic acid and stronger anti-tumor immunity are expected to be detected in the experimental group. Because reducing lactic acid secretion by diclofenac may enhance the T cell-mediated killing effect in melanoma cells. Continue to add lactic acid to the experimental group to a high concentration, which will reverse the positive effect of diclofenac, resulting in suppression of anti-tumor immunity as T cells may die when the concentration of lactic acid increases. Overall, reducing the tumor efflux of lactate can enhance the immune response to checkpoint suppression.

2.3 Caffeine-Enhanced Anti-tumor Activity of Anti PD-1 Monoclonal Antibody

In this study, the authors evaluated the anti-tumor activity of caffeine and anti-PD1 mAb combination therapy against B16F10 melanoma tumors (Tej, Neogi, Nayak 2019). They found that combination therapy showed a decrease in the infiltration of CD4+CD25+ T regulatory cells, an increase in infiltration of CD4 T lymphocytes, CD8 T lymphocytes, intra-tumoral TNF-α, and IFN-γ levels. Experiments have confirmed that the combination of anti-PD1 mAb and caffeine for tumor treatment produced good results due to the blocking of the a2a receptor and PD1.

The immunosuppressive environment in the tumor microenvironment (TME) has always been an obstacle to immune checkpoint inhibitors, of which the CD39-CD73 adenosine pathway is an important participant in the immunosuppressive environment. The immunostimulating molecule ATP is converted to AMP through CD39, AMP is converted to immunosuppressive adenosine through CD73. Once adenosine combines with its receptors, it will promote tumor immune escape. To sum up, there are two primary aspects of immunosuppressive adenosine: one is targeting CD73 and/or CD39 to inhibit the production of adenosine, and the other is targeting the A2a receptors to block adenosine signaling (Leone, Emens 2018). Antibodies that target CD73 or CD39 block adenosine production and relieve immunosuppression, and they can also be used as both a single drug treatment and a synergistic anti-tumor effect in combination with immune checkpoint inhibitors. Referring to the previous article (Tej, Neogi, Nayak 2019), some experiments were designed in this paper to study the effects on tumors by comparing caffeine as an antagonist of A2aR and TTX-030 (BMS-986179) as an anti-CD39 antibody (anti-CD73 antibody) combine with anti-PD1 monoclonal antibodies, respectively. The following design experiments help to further explore the effectiveness of combination therapies.

(The design ideas of this experiment refer to the previous article (Tej, Neogi, Nayak 2019).)

Mice were randomly divided into

(A) Anti-PD1 mAb + caffeine group: receive a combination of injection of anti-PD1 mAb and caffeine in drinking water

(B) Anti-PD1 mAb + TTX-030（BMS-986179）group: receive an injection of anti-PD1 mAb and TTX-030（BMS-986179）

(C) Control group: receive injections of control Ig

After 6 weeks of treatment, calculate tumor growth rates of individual mice from each group through dividing tumor size. The efficacy of different combination therapies is then evaluated in the following areas as shown in Figure 3.

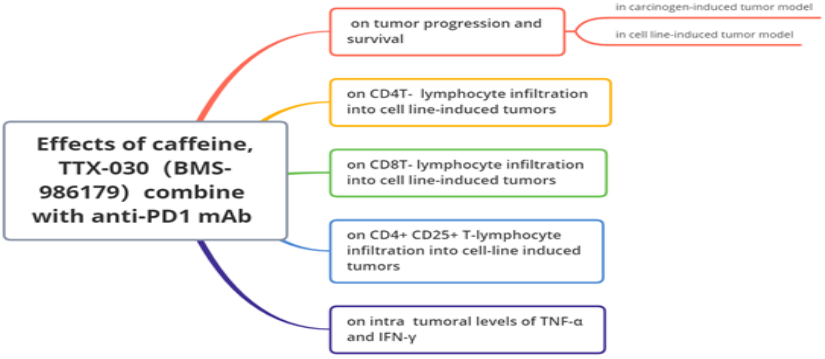


Figure 3: Evaluation of combination therapy in different aspects.

2.4 MHC Protein Confer Differential Sensitivity to CTLA-4 and PD-1 Blockade in Untreated Metastatic Melanoma

Although immune checkpoint blockades (ICB) are treatments for cancer, the mechanism to cure the immune system but not directly treating cancer and the antitumor immune response is highly dependent on the T-cell cognition of surface-expressed antigen which is the major histocompatibility complex (MHC), therefore the reduction of, or the loss proteins associated with MHC protein can potentially be a mechanism which tumor escape antitumor response and induce resistance to the antibodies during ICB course.

When they examined the overall survival, low average tumor MHC class I expression (smaller and equal to 50%) (S. J. Rodig D.G 2018) was characterized with lower overall survival for the patients that started with blockade IPI; better overall survival for the patients that started with NIVO attributed to unaffected tumor MHC class II expression. The authors concluded the anti-CTLA-4 response primarily relied on the melanoma surface MHC class I expression. In comparison, the primary response to anti-PD-1 is associated with existing interferon-gamma-mediated

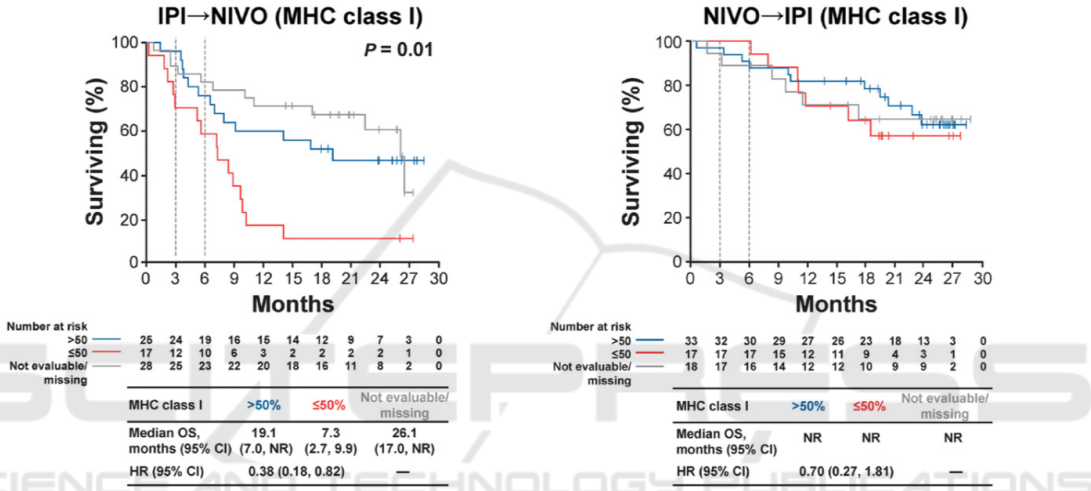


Figure 4: Shows the difference in survival for two group which started with different check-point inhibitors, along with the MHC class I surface expression.

There are limitations inherent in this investigation, the association and responses to single blockade IPI and NIVO lack of experimental results to confirm the observation, etc.

According to the main findings of the articles, the downregulation of the major histocompatibility complex (MHC) is a mechanism of evading antitumor immune response after the ICB, critically, the primary resistance to CTLA-4 blockade was attributed to the reduced melanomaMHC class I expression. Down-regulation of MHC protein surface expression is common in melanoma (seliger 2000) before any approaches have been applied. Whereas the primary response to anti-PD-1 blockade is associated with pre-existing interferon-gamma mediated immune activation.

There are hypotheses stated that interferon-gamma is a profound molecule in manipulating the MHC class I surface expression (Delgado, Ganea 2000, B. Seliger S.H et al 1997). the down-regulation of MHC class I expression is due to the lack of essential components in the expression pathway. interferon-gamma is a significant factor there in terms of modulating the transduction pathway and induce the production of the new proteasome to replace the old one or renovate it (B. Seliger S.H et al 1997).

2.5 Compensatory Upregulation of PD-1, LAG-3, And CTLA-4 Limits the Efficacy of Single-agent Checkpoint Blockade in Metastatic Ovarian Cancer

It is not clear whether the multiple receptors work together in the process of specific immunity can serve a better result for combining the ligands on the cancer cell to the surface of T cells. Therefore, the co-inhabit method is worth researching.

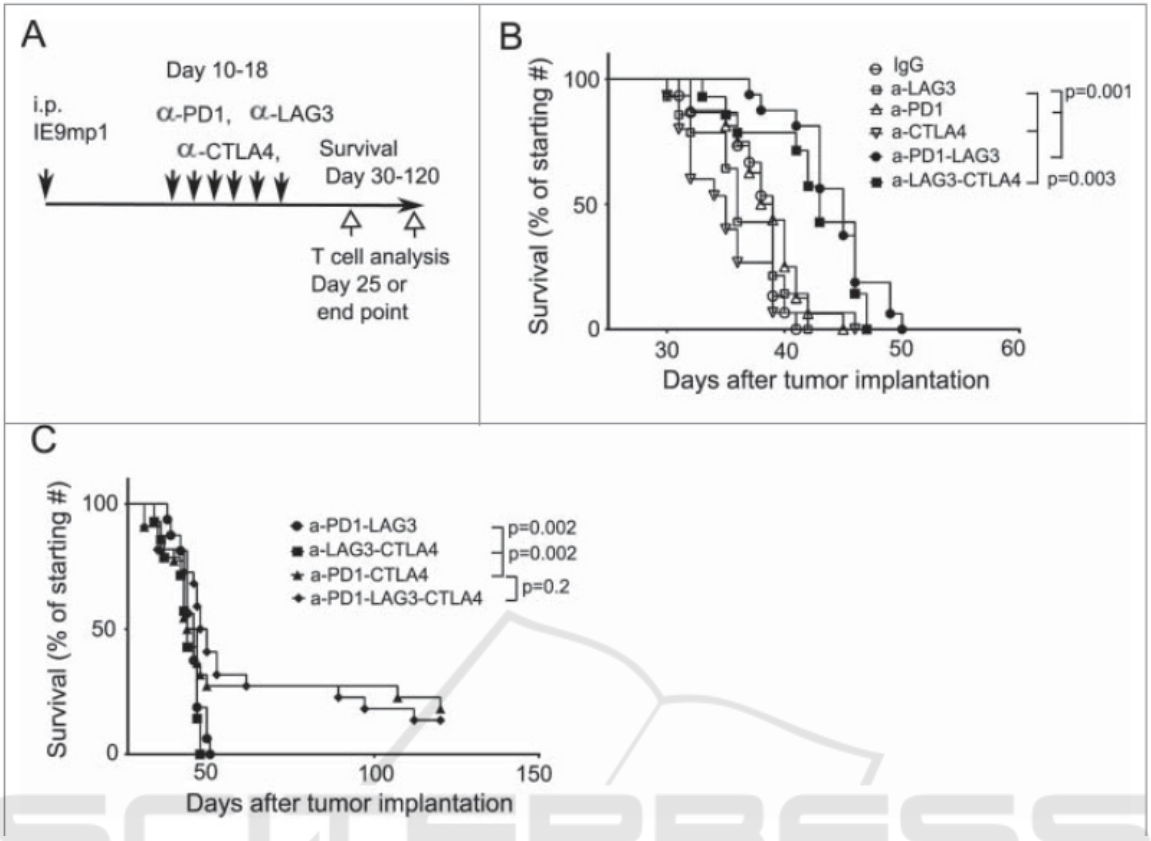


Figure 5: Shows the different survival rate of mice with different receptors.

According to figure 4, researchers have selected three receptors, PD-1, LAG-3, and CTLA-4, which are the immune checkpoints of T cells to regulate the replication of metastatic ovarian cancer cells (Huang et al 2016). The researchers test the survival rate of mice with PD-1, PD-1 and LAG-3, PD-1 and CTLA-4 blocking group, and triple blocking group. The experiment result shows that the mice(C57BL/6) that have two or three inhabitation pathways are slightly more effective in dealing with the repelling of cancer cells. The PD-1KO mice have little cytotoxic materials when they have three inhabit pathways compare to the normal mice.

Researchers established an ovarian tumor model for collecting the data. Also, they splited TALs (Tumor-associated lymphocytes) and TILs (Tumor-infiltrating lymphocytes) from lymphocytes to analyze. By reviewing the result, they knew that several receptors are responsible for inhibiting metastatic ovarian cancer, and the blocking of a single receptor causes the upregulation of the co-inhibitory system. The mice combining four antigens can secret more cytotoxic materials to attack normal cells than the mice with triple checkpoint pathways. The mice with LAG-3, CTLA-4, and PD-1 blockage or the dual inhibition pathways mice are more effective to block the replication of metastatic ovarian cancer than single pathway since knocking out of PD-1 or LAG-3 can enhance the ability of the other receptors to combine with the antigen.

Huang et al. provide evidence suggesting that the block of LAG-3 and CTLA-4 can increase the survival rate of PD-1KO mice. Similarly, the block of PD-1 and CTLA-4 can enhance the survival rate of LAG-3KO mice significantly (Huang et al 2016). A single checkpoint pathway is not efficient compare to dual checkpoint pathways. However, more than three receptors' mice do not serve an excellent response to the wild-type mice. Researchers should test the relation between survival rate and cytotoxic materials for finding the regulation. Researchers can analyze 300 mice in the research and divide them into three groups. The first group of mice (PD-1KO) have LAG-3, CTLA-4, and IgG; the second group will contain the same receptors while they have no treatment; the third group (PD-1KO) will be knocked out the other receptors and only left CTLA-4. After separate the groups, the researchers follow up these

mice for 6 months to compare the overall survival with three different groups, which can find out whether more than three receptors can enhance the secret of cytotoxic materials.

2.6 Immune Microenvironment Modulation Unmasks Therapeutic Benefit of Radiotherapy and Checkpoint Inhibition

Although the clinical effect of ICIs in the treatment of solid tumors is exciting, many patients haven't achieved a sustained response. One of the main barriers to treatment is the immunosuppressive tumor immune microenvironment (TIME). Therefore, researchers made a hypothesis of which the combination of targeted radiotherapy and time-dependent immunomodulation was able to improve the ICI response rate of solid tumors. To test their hypothesis, head and neck tumor was used on C57BL/6J male mice that are 8-10 weeks of age to explore the tumor characteristics limiting the efficacy of immune checkpoint inhibitors in solid tumors and to develop combination therapy strategies to maximize their advantages. After the tumor became established, mice received combinatory treatments involving immune checkpoint inhibitions, tumor-directed radiation, and immunomodulation of cyclophosphamide (CTX) and L-n6-(1-iminoethyl)-lysine (L-NIL). The result is that they found that modulation of TIME using CTX and L-NIL, combined with two checkpoint inhibitors and radiotherapy, had better effects than these treatments alone. It resulted in more than 70% of established mEER tumor rejection, doubling the median survival rate of the B16 melanoma model (Newton J.M et al. 2019).

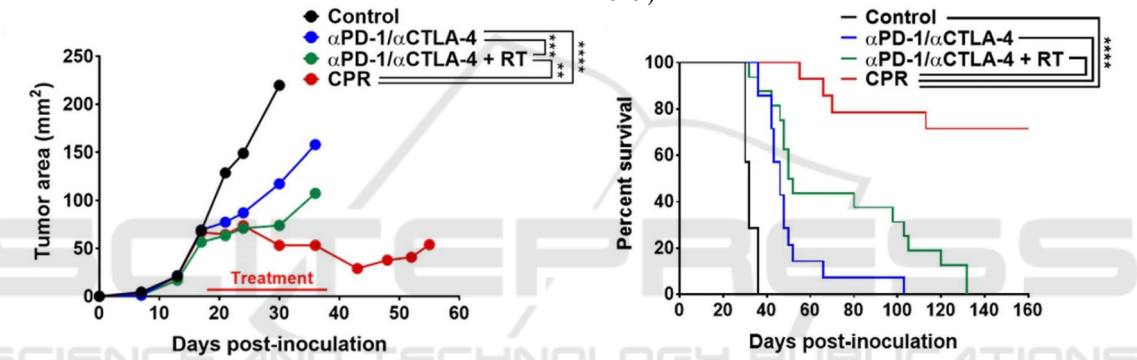


Figure 6: (Newton J.M et al. 2019) The average tumor area and percent survival of mice before the first euthanasia under different treatment combinations (CTX/L-NIL immunomodulation combined with α PD-1/ α CTLA-4 and tumor directed radiation are collectively called the "CPR" regimen).

The previous study (Newton J.M et al. 2019) has shown that regulating the immune microenvironment can release the efficacies of ICIs and radiotherapy to activate immune rejection in the therapy of refractory tumors. However, it only made experiments on head and neck cancer. Therefore, the purpose of the new experiment is to see if the same regimen works on lung cancer and gastric cancer, since these two cancers are solid tumor which will respond to ICIs used well, and they are very common. This study will also use C57BL/6 male mice aged 8-10 weeks. After tumors become established, treatments will start. There will have two sets of experiments, one for lung cancer, and one for gastric cancer. Each set of the experiments will have two groups. One group with modulation of TIME uses combination therapy of PD-1 and CTLA-4 inhibitors, radiotherapy, and immunomodulatory drugs: CTX and L-NIL. Another group is the control experiment without modulation of TIME, combining dual inhibition and radiotherapy. All experiments will be replicated for at least twice, and each experiment will have 5-10 samples in average. For the result, the hypothesis is that the strategy of combining tumor-targeted radiation with tumor immune microenvironment regulation can improve the ICI response rate of lung and gastric tumors.

3 CONCLUSION

This paper first explores the monoclonal antibody immunotherapy of PD-1 in tumors. The novel combination therapy of Oncolytic Adenovirus with Anti-PD1 inhibitors presents an alternative treatment therapy in treating Malignant melanoma. In addition, the inhibition of glycolysis preserved T cell and NK cell function, and enhanced anti-PD-1 treatment

response is observed. Checkpoint immunity also plays an important antitumor role in combination therapy. The combined blockade of the a2a pathway and pd1 pathway showed more effective anti-tumor activity than monotherapy. Another study concluded the primary response to anti-CTLA-4 is rely on the melanoma surface MHC class II expression and that anti-PD-1 is associated with existing interferon-gamma-mediated immune activation. Besides, knocking out of PD-1 and keeping the other co-inhibitory workers can enhance the repelling of metastatic ovarian cancer cells. Finally, a therapy of combining modulation of tumor immune microenvironment with dual checkpoint inhibition (PD-1 and CTLA-4), and radiotherapy has shown a good effect.

This research is of great significance because the immune checkpoint is widely studied in medicine. It is widely used in the treatment of cancer, such as hepatocellular carcinoma (HCC), urinary system malignant tumors, breast cancer, recurrent/metastatic nasopharyngeal cancer and lung cancer. In addition, the Immune checkpoint plays an important role in acute pancreatitis (AP). Besides, scientists have done a lot of research on the interaction between intestinal flora and immune checkpoint inhibitors.

Although ICIs have played an important role in cancer treatment and shown great promise in so many different diseases, much more research on long-term toxicity and survivorship issues is needed since new side effects were found (Kottschade 2019). They were often referred to as immune-related adverse events (irAEs) or immune-mediated adverse reactions. Moreover, as these strategies are used in more and more malignant tumors, more side effects are constantly observed, including but not limited to endocrine toxicity, rheumatologic toxicity. These side effects may be life-threatening, so in the future, researchers should pay attention to acute toxicity, long-term toxicity, and other treatments to improve the life of patients after cure.

Recently, there is a pronounced increase in the number of articles listed in PubMed that associated with bispecific antibodies (biAbs) in the immunotherapy of cancer, apparently, this innovation has become a crucial part of immunotherapy for the next generation. However, in cancer immunotherapy, the competition never stops, another candidate includes chimeric antigen receptor T cells, NK cell-engaging biAbs, or macrophage-engaging biAbs. More importantly, they all have the capacity to prove they can potentially control, or even cure the malignant tumor.

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