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TRIPLE IMMUNOTHERAPY TO OVERCOME IMMUNE EVASION BY TUMOR IN A MELANOMA MOUSE MODEL

by
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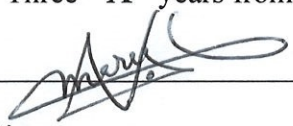

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AN ABSTRACT OF THE DISSERTATION OF

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Title: Triple Immunotherapy to Overcome Immune Evasion by Tumor in a Melanoma Mouse Model

Background: This study devises a triple immunotherapy to treat melanoma in a mouse model. The combination includes anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibodies, monophosphoryl-lipid-A (MPLA), and an indolamine-dioxygenase-1 (IDO1) inhibitor. The aim of the study is, first, to rule out any major toxic effects related to this therapy and, second, to assess its antitumor effects.

Methods: Cancer-free C57BL/6 mice were randomized into control groups and groups receiving single, dual, or triple therapies of the defined treatments. Clinical signs, weight gain, and histological sections from their main organs were assessed. Then, melanoma-bearing mice were segregated into similar groups, monitored for survival, and their tumor size was measured repeatedly. Flow cytometry was used to analyze immune cell populations in the tumor masses including CD4+, CD8+, and regulatory T cells in addition to natural killer cells. Finally, serum levels of interleukin-12 (IL-12), vascular endothelial growth factor (VEGF) and S100 calcium-binding protein B (S100B) were quantified using enzyme-linked immunosorbent assay (ELISA).

Results: No adverse effects were detected in any of the treated groups. Survival analysis indicated that the groups receiving dual or triple therapies had prolonged survival compared to the controls. However, the group receiving triple therapy was the only group to show statistically significant increase in survival compared to the controls. Tumor size progression paralleled the survival outcome. The group receiving the triple therapy showed statistically significant smaller tumor sizes compared to all the other groups throughout the whole monitoring period. Flow cytometry used to analyze immune cell populations in the tumor mass indicated that the triple immune therapy was capable of significantly enhancing the natural killer cell counts as well as the CD3+CD4+/Treg and CD3+CD8+/Treg ratios possibly enhancing the anti-tumorigenic environment. While serum levels of the tumor-suppressive IL-12 came opposed to the expected by being lowest in the group with the most favorable outcome, circulating VEGF and S100B levels were below detection level in the triple immunotherapy group through all detection time points and hence were in accordance with the survival and tumor progression results.

Conclusion: Generated data rule out any major adverse events pertaining to the triple immunotherapy and reveal its enhanced effectiveness in thwarting tumor progression over all other tested treatments. This outcome is mainly achieved through the enhancement of natural killer cells and the ratios of CD4+ and CD8+ T-cells to regulatory T-cells.

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LIST OF ABBREVIATIONS

FDA	Food and drug administration
PD-1	Programmed-death-1
PD-L1	Programmed-death-ligand-1
CTLA-4	Cytotoxic-T-lymphocyte-antigen-4
LAG-3	Lymphocyte-activation-gene-3
TIM-3	T-cell immunoglobulin and mucin domain containing-3
irAEs	Immune-related adverse events
IDO	Indoleamine-2,3-dioxygenase
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-12	Interleukin-12
IFN-γ	Interferon-γ
TNF	Tumor necrosis factor
BCG	Bacillus-Calmette Guerin
HSV-1	Herpes Simplex Virus-1
CAR	Chimeric antigen receptor
GMCSF	Granulocyte macrophage colony-stimulating factor
MAPKs	Mitogen-activated protein kinases
TNFRSF	tumor necrosis factor receptor super family

TGF-β	Transforming-growth-factor-β
T-reg	Regulatory T cells
MDSCs	Myeloid derived suppressor cells
DCs	Dendritic cells
NKT	Natural killer T cells
MPLA	Monophosphoryl-lipid-A
LPS	Lipopolysaccharide
PAMP	Pathogen-associated-molecular-pattern
TLR4	Toll-like receptor 4
MHC	Major histocompatibility complex
DMSO	Dimethylsulfoxide
APC	Antigen presenting cell
D-1MT	D-1 methyl tryptophan
SC	Subcutaneously
IT	Intratumoral
IP	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
ELISA	Enzyme-linked immunosorbent assay
VEGF	Vascular-Endothelial-Growth-Factor

CHAPTER I

INTRODUCTION

A. Immunotherapy

1. *Definition*

Immunotherapy is an approach to therapy that aims at harnessing the innate and adaptive immune responses of a patient to achieve long-term elimination of diseased/defective cells. Although, currently, the word “immunotherapy” is mostly associated to the treatment of cancer, it should be noted that, with the versatility of its applications, the immunotherapeutic approach may be used to treat/prevent a larger spectrum of illnesses including infectious diseases. A very well-known instance of such an application is vaccination which represents the first model of host-directed immunotherapy (Naran, Nundalall, Chetty, & Barth, 2018).

Immunotherapeutic strategies can be broadly classified into active and passive therapies where the former induces the host’s immune response to generate the specific immune effectors needed to abolish the disease, while the latter consists of the administration of immune elements that are generated *ex vivo* to specifically target the diseased cells without stimulating the patient’s immune response (Tur & Barth, 2017). The active immunotherapies can be further categorized at large into (1) drugs that stimulate the immunogenic pathways directly such as agonists of co-stimulatory receptors and antigen presentation enhancers, and (2) agents that target tumor immune evasion by blocking

negative regulatory signals such as immunosuppressive enzymes and co-inhibitory checkpoints. (Velcheti & Schalper, 2016)

In a nutshell, these types of targeted therapies are meant to improve the host's cellular reactions to disease by inducing immune responses, attenuating virulence factors and enhancing immunological memory. They mainly act by targeting the regulatory biochemical pathways and/or mutant proteins that are critical for tumor maintenance and progression. More importantly, immunotherapies are usually designed to act precisely on the diseased cells therefore reducing the extent of collateral tissue damage and the other toxic adverse effects that are frequently seen in other types cancer treatments namely chemotherapy (Wykes & Lewin, 2018).

However, since immunotherapy is based on harnessing the natural powers of the immune system to fight disease, it is essential to have a thorough knowledge of the concepts that underlie the principles of action of the different cancer immunotherapies and that explain the rationale behind their design and application. These concepts are immunosurveillance and immunoediting.

2. *Immunosurveillance*

The first person ever to suggest that the immune system may have control over neoplastic diseases was Paul Ehrlich (Ehrlich, 1909). However, for lack of the possibility to get vigorous evidence at the time, this idea was shortly aborted. It wasn't until the mid-

twentieth century, after the overall development of the immunology field, that Ehrlich's suggestion was revisited. In fact, after Medawar and his team demonstrated the role of the immune system in mediating allograft rejection, it was then clarified that, to accurately test the ability of the immune system to repress tumor, inbred mouse strains should be used in the experiments (Billingham, Brent, & Medawar, 2010). Only by doing so, it would be possible to distinguish between the immune system's ability to properly recognize and reject the tumor itself, therefore providing evidence in favor of the cancer immunosurveillance hypothesis, as opposed to what would have been a simple allograft rejection mechanism whenever non-inbred strains of mice were used. Consequently, many studies showed that immunization against syngeneic transplants can be achieved therefore shedding light on the presence of "tumor-specific antigens" which can be recognized by the immune system (Klein, 1966; Old & Boyse, 1964). Based on all the emerging data, Sir Macfarlane Burnet and Lewis Thomas formulated the "cancer immunosurveillance" formal hypothesis in 1957 stating that: 'it is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence' (M. Burnet, 1957). Concomitantly, Thomas suggested that the cellular immunity is first and foremost aimed at protecting multicellular organisms from neoplastic disease, therefore maintaining their tissue homeostasis, much more than it is to induce allograft rejection (Thomas & Lawrence, 1959).

The immunosurveillance hypothesis along with Thomas speculations contributed to the formation of an evolutionary framework which ended up in the development of the

cancer immunosurveillance concept defined by Burnet as follows: ‘In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character’ (F. Burnet, 1970; M. BURNET, 1964). In the following few decades, however, the several studies that were conducted to scrutinize this concept failed to provide solid experimental evidence to support it. What is more, the presented data offered false indications disapproving the immunosurveillance notion and even suggesting an opposite role of the immune system promoting tumor growth which resulted in the abandonment of this hypothesis and relegating it to the historical scientific dust bin. In hindsight, it was the limited knowledge, at that time, of the immunologic defects in the mouse models used in those studies that led to the misleading interpretation of the results (Hanahan & Weinberg, 2000; H. S. Kaplan, 1971; Stutman, 1976; Thomas, 1982).

Despite the efforts made between the 1970s and 1990s to revive the immunosurveillance concept, after the limitations of the past experiments were disclosed, it is not until the mid-1990s that a genuine interest in immunonosurveillance was renewed based on two major findings (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). First, endogenous interferon γ (IFN γ) demonstrated a substantial host protection against the growth/formation of transplanted, chemically induced and spontaneous tumors (Dighe, Richards, Old, & Schreiber, 1994; D. H. Kaplan et al., 1998). Second, it was shown that

when mice lacked perforin, an important component that mediates lymphocyte-dependent killing, they were significantly more susceptible to the formation of chemically induced tumors when compared to perforin-sufficient counterparts (Street, Cretney, & Smyth, 2001; van den Broek et al., 1996). Therefore, at that point, there was solid evidence that the immune system components played a role in controlling the development of primary tumors. In the following years, a new model of mice entirely lacking lymphocyte antigen receptors, due to a defined gene mutations, was available for the first time. This model allowed scientists to carry out experiments that could be indisputably interpreted and which showed that lymphocytes did not only protect the mice against development of chemically induced primary sarcomas but also inhibit the formation of spontaneous epithelial tumors (Shankaran et al., 2001). Later studies, that used different mouse models with targeted genetic disruptions affecting key components of the immune system, emphasized the importance the immune system's control of tumor development. More interestingly, these studies advocated the contribution of both the innate and adaptive compartments of the immune system in cancer immunosurveillance (Dunn et al., 2002; Girardi et al., 2001).

In the studies that followed, immunosurveillance was shown to be a heterogeneous process that requires the contribution of various immune effectors. It was also demonstrated that the actions of these effectors are dependent on a number of characteristics including the tumor's cell type of origin, transformation mechanism, localization and immunologic recognition mechanism. These findings were reinforced by clinical studies where immunocompromised transplant patients and individuals with inherent immunodeficiencies had a significantly increased relative risk for developing various types of cancer with no

known viral etiology (Gatti & Good, 1971; Penn & Starzl, 1972). Moreover, it was shown that lymphocytes' presence in a tumor is positively correlated to an increased survival rate (Clark Jr et al., 1989; Clemente et al., 1996; Mihm Jr, Clemente, & Cascinelli, 1996). In some instances, this correlation was made specifically with CD8+ T cells suggesting that it is the main population affecting survival (Naito et al., 1998). Therefore, based on the extensive data obtained recently from the work of numerous independent groups on both mice and humans, cancer immunosurveillance was not only proven to exist but also to be highly relevant physiologically. The overall conclusion unequivocally supported the principles of cancer immunosurveillance originally proposed by Burnet and Thomas; that is, the immune system is sufficiently able to recognize and eliminate primary tumors through a process where lymphocytes and cytokines play a major role. Consequently, the first question that arises is why immune-competent individuals still develop cancer despite the demonstrated proficiency of the immunosurveillance. To clarify this paradox, a concept that is broader than cancer immunosurveillance needs to be addressed in the study of the interaction between malignancies and the immune system. A recently proposed term to such a concept is "cancer immunoediting".

3. *Cancer immunoediting*

While the concept of cancer immunosurveillance focuses mainly on the ability of the immune system to recognize and abolish malignant cells therefore hampering the development and/or progression of cancers, it neglects another essential aspect of the

immune system which is immune-selection. In fact, just like it selects for the bacterial and viral strains that resist elimination by immunological reactions, the immune system is thought to exert the same selection for tumor variants that are most capable of surviving in an immunologically intact setting during tumor formation. Additionally, given the intrinsic genetic instability of malignant tumors, the immune responses targeted against them are likely to cause alterations in the cancer cells such as mutations disrupting the tumor antigens encoding genes. Such an immunologic sculpting probably results in the development of tumors that can better resist the tumor-suppressing functions of the immune system by selecting for the cancerous cells that could acquire mechanisms to suppress or evade the immune responses.

Accordingly, it is important to take notice of both the host-protecting and the tumor-sculpting effects of the immune system on developing cancers. In this context, it is not appropriate to define the process occurring between immunity and tumors by “cancer immunosurveillance”, since this term tends to describe merely the protective effect of the immune system against cancer. Whereas, “cancer immunoediting” is a broader term that designates the dual effects of the immune responses that prevent but may also shape the neoplastic disease (Dunn et al., 2002). The scope of the interplay between the immune system and malignant growth thus encompasses three processes that define cancer immunoediting. The first is elimination and it describes the original cancer immunosurveillance concept. When the immune system is successful in obliterating the developing tumor, the immunoediting is completed at this phase. However, if cancer cells escaped elimination by the immune attack, the interaction enters into the second process

which is the dynamic equilibrium. During this phase, the pressure exerted by the immune components is sufficient to contain tumor cells but not enough to fully eradicate them. Many variant cancer cells which initially escaped the elimination phase would be killed through this process, however new ones with more evolved evasion mechanisms arise as a result of the selection pressure. Finally, the third and last phase of this process is escape during which the tumor cell variants which survived the immune attack and developed insensitivity to immunologic recognition and/or elimination start to expand uncontrollably. This is often when the malignancy is clinically detected and if left untreated leads to the host's death.

Based on this broader understanding of the interaction between the neoplastic growth and the immune system, the concept of immunotherapy has been evolving through time. The preliminary notion of this therapy is to harness the responses of the immune system against cancer. An immunotherapeutic treatment is considered successful when it is capable of making the malignant growth retreat to the elimination phase of the immunoediting process after having attained the escape phase. This can be accomplished by targeting the immunonological components that play a role in cancer immunosurveillance and enhancing their properties towards an efficient eradication of the tumor. Although the immunotherapeutic approach in cancer treatment is not new, only recently it has been a very active area of research and yielded quite promising results. In fact, the first attempts at immunotherapy, when the term was yet to be pinned, date back to the 19th century and has since gotten quite alternating attitudes towards it until recent breakthroughs in the field have unquestionably skewed research interest in its direction.

4. *Historical timeline of immunotherapy*

If we are to take immunotherapy with all its facets, the preliminary event to be recorded in its historical timeline would be the use of the first "serum therapy" in Robert Koch's laboratory, where Emil von Behring and Shibasaburo Kitasato found that injecting animals with diphtheria toxin produces an anti-toxin containing serum that has the ability to provide passive anti-diptheria immunity to people (Taylor, 2014). This introduced the use of serum as a treatment and showed that immunity did not necessarily require to arise internally but can be transferred. However, for the purpose of this thesis manuscript, the historical timeline will focus on the events pertaining to the progression of cancer immunotherapy specifically. In this context, the first event to be noted took place in 1891, when William Coley, a New York surgeon, was presented with a patient in a very poor health condition who had recurrent sarcomas in his neck and tonsils that was now the size of hen's egg and deemed inoperable. Based on the literature review where multiple observations of unexplained cancer remissions subsequent to contracting infections were recorded throughout the 18^{th} century, Coley decided to inoculate his sarcoma patient with streptococcal cultures. When the bacterial injections effectively elicited erysipelas around the patient's neck, a remarkable shrinkage of the tumor was noticed and it totally disappeared after 2 weeks. Following remission, this patient who was expected to live a few weeks at best, lived for eight more years before the cancer reoccurred and he died of it (Hall & Rosen, 1997; Tontonoz, 2015).

Inspired by this case, Coley was determined to optimize his treatment. However, it was difficult to induce a complete erysipelas attack using *Streptococci* alone; and, when successfully elicited, erysipelas is a life-threatening disease. To overcome these challenges, Coley resorted to heat or filter sterilized cultures, which produced minor effect. So he mixed them with toxins of *Serratia marcescens*, a gram-negative bacterium, thus creating the mixed bacterial vaccine (Coley, 1893). The first sarcoma patient treated with this mix was a 16-year-old German in a very bad condition. Following the treatment, his inoperable tumor disappeared, he regained a good state and remained healthy, until he died of myocarditis in a subway station 26 years later. Over the next years, Coley injected more than 1000 cancer patients with either bacteria or microbial products, which became known as Coley's Toxins. Of the 1,000 sarcoma patients treated with his mix, 80% experienced up to 5 years survival increase which was, then, an impressive outcome for an illness with no existing effective treatment (Nelson, Diven, Huff, & Paulos, 2015). The rationale behind this finding was that the immune response elicited by infection reinforced the system's ability to attack tumor. Coley's bacterial mix is therefore the first official immune therapy for cancer This marked the birth of cancer immunotherapy which would be defined, later on, by the Cancer Research Institute as "treatments that harness and enhance innate powers of the immune system to fight cancer"(Ledford, 2014). At that time, however, Coley's Toxins were subject of criticism to a great extent because many practitioners did not trust his results. Concomitantly with the development of chemotherapy and radiation therapy, this criticism led to the gradual disappearance of Coley's Toxins from use. Nevertheless, Coley's principles were proven correct by modern immunology and William B. Coley is now considered "Father of Immunotherapy" (Kienle, 2012; E. F. McCarthy, 2006).

In between the 1930s and early 1960s bacterial cultures were again used to treat cancer by causing necrosis and resulted in intermittent success (O'Malley, Achinstein, & Shear, 1962). The next identified cancer immunotherapy was tumor necrosis factor (TNF) in the 1970s and was thought to be a main breakthrough but the outcome was disappointing. The systemic infusion of TNF resulted in severe toxicities which radically limited its use in favor of other available therapies, namely chemotherapy (Balkwill, 2009). Meanwhile, another type of immunotherapy, which was first introduced in the 1920s, are oncolytic vaccines. But they were discarded shortly after because of the fatalities they caused, to be re-introduced in 1976 (Herr & Morales, 2008; Morales, Eidinger, & Bruce, 1976). This time, evidence was presented that Bacillus Calmette-Guerin (BCG) vaccine can be safely and effectively used for the treatment of superficial bladder cancer and was approved by the FDA in 1990 (Olszanski, 2015). Interleukin 2 (IL-2) represents yet another immunotherapeutic which was identified in 1976 and FDA approved, first, in 1991 to treat metastatic kidney cancer, then, in 1998 for metastatic melanoma. High dose IL-12 were proven to be clinically effective by enhancement of T-cell production. However, due to its significant toxicity and with the availability of other therapeutic options, IL-2 therapy was no longer favored (Oiseth & Aziz, 2017). A different type of immunotherapy are monoclonal antibodies which were successfully developed and produced in laboratories during the 1970s, but did not get the FDA approval until 1997 when rituximab, a monoclonal antibody with demonstrated clinically relevant results, was approved to treat non-Hodgkin’s lymphoma. Rituximab binds to CD20 on immature B cells marking them for elimination by Natural Killer (NK) cells which abrogates the effects of the lymphatic malignancy. After Rituximab, the approval of several more monoclonal antibodies

followed for the treatment of Hodgkin lymphoma, and breast, lung, and colorectal cancers among others (Ribatti, 2014).

Also in the realm of immunotherapy are oncolytic viruses. By definition, these viruses are either naturally occurring or genetically modified and they selectively infect, replicate in and kill cancer cells while sparing normal tissues (S. J. Russell, Peng, & Bell, 2012). Apart from their cancer-selective killing feature or the ability to be genetically modified to do so, oncolytic viruses are required to be nonpathogenic (L. Russell & Peng, 2018). The theory about using viruses in cancer treatment has been floating around since the early 1900s and the first targeted use of a virus was that of rabies virus to treat cervical cancer back in 1912 (Fukuhara, Ino, & Todo, 2016). Nevertheless, it is in 1991 that a remarkable milestone was attained in the context of this approach, when herpes simplex virus-1 (HSV-1) was successfully engineered to selectively replicate and target brain tumor cells (Martuza, Malick, Markert, Ruffner, & Coen, 1991). In spite of the well-known limitations of the oncolytic virus therapy, namely toxicity and reduced efficacy because of the circulating antibodies, they still had approvals in China followed by the FDA in the U.S. for the treatment of neck and head cancers in 2005 (Fukuhara et al., 2016). A different kind of immunotherapy are cell therapies. In the early 2000s, several studies investigated the use of T cells in cancer treatment starting with the adoptive cell transfer technique to the more advanced chimeric antigen receptor (CAR) T-cell therapy (Sadelain, Brentjens, & Rivière, 2013). In fact, cancer immunotherapy reached a remarkable milestone through this treatment technology in 2013, when clinical trials testing for CAR T-cell therapy showed impressive results. Patients with aggressive non-Hodgkin's lymphoma, B-cell acute

lymphoblastic leukemia (ALL) and children with ALL exhibited 90% response rates and therapeutic efficacy (Grupp et al., 2013; Kochenderfer et al., 2015). Based on these results, the FDA approved the first CAR T-cell therapy, tisagenlecleucel, in 2017 (Abbott & Ustoyev, 2019).

Last but not least are checkpoint inhibitors. When a French researchers' group discovered a singular cell protein called cytotoxic T lymphocyte antigen 4 (CTLA-4) in the 1980s, little did they know how far-reaching their discovery will be and how it will forever change the landscape of cancer therapies. It was Dr James Allison who demonstrated that CTLA-4 acts as a brake on the responses of the immune system by stopping T cells activity. So he proposed that a blockade of CTLA-4 molecules would release the brake allowing T cells to mount a full immune response and take their attack on cancer cells to further limit and he confirmed his hypothesis using a murine model in 1996 (Leach, Krummel, & Allison, 1996). Then, in 2010, evidence was presented that metastatic melanoma patients treated with CTLA-4 checkpoint inhibitor had significantly prolonged survival and it was the first drug with the ability to improve overall survival of patients with this disease. In 2011 the first CTLA-4 checkpoint inhibitor, ipilimumab, was FDA approved to treat metastatic melanoma (Oiseth & Aziz, 2017). Another brake on T-cells and the immune system are the programmed death-1 and its ligand (PD-1 and PD-L1 respectively). Therapies targeting PD-1 or PD-L1 have also been FDA approved for the treatment of lung cancers and melanoma among others, however the possibility to use these therapies is often determined by the level of PD-L1 expression (D. Pardoll, 2015). In sum, checkpoint inhibitors revolutionized cancer immunotherapy and represented a fundamental

shift in the use of immunity to fight cancer. They remain to this day an active area of research with impressively promising results (Abbott & Ustoyev, 2019).

5. *Immunotherapies of the present-day*

Currently, the two leading immunotherapies used are immune checkpoint inhibitors and CAR T-cell therapies (L. A. Kottschade, 2019). For checkpoint inhibitors, the latest novelties are the 2015 approval of the use of ipilimumab in the adjuvant setting, instead of only at the metastatic setting, and the use of a dual therapy of CTLA-4 and PD-1 inhibitors in 2016 (Eggermont et al., 2015; Hammers et al., 2017; Larkin et al., 2015; Postow et al., 2015). Since then, two more agents were approved also in the adjuvant setting for treating melanoma: pembrolizumab and nivolumab (Eggermont, Robert, & Suciu, 2018; Weber et al., 2017); and several other immune checkpoint inhibitors for the treatment of other cancer types including non-small cell lung cancer and urothelial, Merkel and squamous cell carcinomas (Vaddepally, Kharel, Pandey, Garje, & Chandra, 2020). Present work in the context of immune checkpoint therapies includes the investigation of different combinations of checkpoint inhibitors, as well as their combination with distinct therapeutic agents such as chemotherapy. Researchers are also looking to identify specific biomarkers that would help select the patients who are prone to benefit from checkpoint therapies while trying to find the immunotherapy resistance mechanisms (L. A. Kottschade, 2019). In addition, a new checkpoint inhibitor is being studied: relatlimab which inhibits the lymphocyte activation gene-3 (LAG-3). By inhibiting the LAG-3 protein expressed by

Tcells, relatlimab releases the brakes of the immune system similarly to the other immune checkpoint inhibitors. Studies examining a combination of nivolumab (a PD-1 inhibitor) and relatlimab have demonstrated promising results in metastatic melanoma patients while ongoing studies are investigating the effect of this drug as single agent or in combination with nivolumab as a treatment for several types of cancer including lung, colon, renal and hematologic malignancies (Ascierto et al., 2017). Concomitantly, research in this area also involves seeking ways to limit the unfortunate rise in the autoimmune toxicity resulting from the immune checkpoint therapies. These side effects are usually referred to as immune-related adverse events (irAEs) and can range from mild to life-threatening with a possibility to have chronic consequences. Therefore, researchers are looking to optimize ways for harnessing the body's immune system to its maximal efficacy while controlling the unpredictable irAEs that may accompany treatment (Corsello et al., 2013; L. Kottschade et al., 2016; Richter et al., 2018).

As for CAR T-cell therapy, to date, two distinct therapies which are both anti CD-19, are approved to treat a selection of hematologic malignancies (Bouchkouj et al., 2019; O'Leary et al., 2019; Vaccines). Although CAR T-cell therapy is quite effective for the treatment of this type of malignancies, it is not for solid tumors. In fact, while this area of research is now very active, it is admittedly more difficult to achieve results that are as successful in solid tumors. This is partly because, unlike immune checkpoint inhibitors, CAR T cell targets are tumor specific with many that are possibly not even identified yet (L. A. Kottschade, 2019). Other avenues in cancer immunotherapy include oncolytic viruses. At present, talimogene laherparevec (T-vec) is the only oncolytic virus that is FDA

approved. It is a herpes simplex virus 1-expressing granulocyte macrophage colony-stimulating factor (GM-CSF) and it is used for the treatment of metastatic melanoma through intratumor injections. Other oncolytic viruses are in clinical development however none has been approved so far (Kaufman, Kohlhapp, & Zloza, 2015; Velcheti & Schalper, 2016). Yet another distinct approach to cancer immunotherapy are cancer vaccines. Simply put, the way cancer vaccines work is by enhancing the anti-tumor adaptive immune response through the increase of tumor antigen presentation. The first therapeutic cancer vaccine that was approved by the FDA is called Sipuleucel-T and consists of a recombinant prostate acid phosphatase and requires a prior ex-vivo incubation with antigen presenting cells (APCs) isolated from the patient. This requirement is likely the main reason that this vaccine producing company filed bankruptcy, considering the cost and difficulty to continuously develop clinical-grade preparations (Kantoff et al., 2010). That said, several other therapeutic cancer vaccines are presently in clinical trials.

Finally, it is worth noting that there is an extensive list of potential immunotherapeutic agents that are being studied at the moment for their efficacy and safety in the treatment of various cancer types. They range from novel immune checkpoint inhibitors and immunosuppressive pathways blockers to co-stimulatory checkpoint pathway agonists and cytokines. These potential therapies are at different research stages with some of them still in preclinical studies while others in advanced phases of clinical trials. Two key concepts have been recently highlighted in the domain of cancer therapy and are expected to be the cornerstone for the development of promising treatment strategies. The first concept is bypassing cancer's resistance to current immunotherapies

and the second one is accounting for the multiple evasion mechanisms that tumors use to escape the host's immune system and which generally consist of a reduced immune recognition of cancer cells and the development of an immunosuppressive tumor microenvironment (Muenst et al., 2016). Using combination therapy is one of the strategies that takes into consideration both of these concepts and is therefore suspected to lead the field of cancer immunotherapy considerably further. Examples of combination therapies include but are not limited to the concomitant use of 2 immune checkpoint blockers, the use of immune stimulants as adjuvant therapies as well as the use of immunotherapy in addition to other treatment approaches such as chemotherapy or radiotherapy. (L. A. Kottschade, 2019; Li, Song, Rubinstein, & Liu, 2018; Sambi, Bagheri, & Szewczuk, 2019; Velcheti & Schalper, 2016)

B. Immunotherapeutic treatments for Melanoma

Until the late 19th century, management of melanoma consisted of tumor removal. An upgrade of this protocol was the anticipatory excision of adjacent glands; a procedure that is nowadays known as prophylactic dissection of the lymph nodes. In the present version of the American Joint Committee on Cancer, Melanoma Staging System, Clark levels and Breslow thickness remain the key prognostic factors for melanoma (Rebecca, Sondak, & Smalley, 2012). To date, for early stage melanomas, surgical excision is still the treatment of choice as it can be highly curable. However, for more advanced stages with non-resectable and/or metastasized melanomas, systemic therapy is needed. Hence, two

chemotherapeutic drugs were developed but they were associated with only partial responses, median survival range of 5-11 months and 1-year survival of no more than 27% (Yang & Chapman, 2009). Thus, efforts had to be invested in developing other treatment approaches, most prominent of which is immunotherapy (Figure 1).

Melanoma stood out among the most studied cancer types that can potentially be treated with immunotherapeutic drugs. The first advantage for using immunotherapy in the treatment of melanoma is that it can be considered as a therapeutic option regardless of the mutational status of the malignancy. This does not apply to the second group of melanoma treatment available in the clinic, which are mitogen-activated protein kinases (MAPKs) inhibitors. The latter can only be used when the melanoma patient carries a BRAFV600 mutation. Only 40% to 50% of melanoma patients are found to have this mutation making this treatment only suitable for less than half of the population diagnosed with melanoma (Colombino et al., 2012). Despite the remarkable results associated with the use of this targeted therapy, immunotherapy still gained undeniable momentum in the treatment of melanoma because immunotherapeutic agents are not restricted to a group of patients according to their genetic status and thus are likely to work on a much larger number of melanoma patients.

Melanoma has also been first among other cancer types to get FDA approvals for treatment with immunotherapeutic agents. From cytokines to immune checkpoint inhibitors, melanoma had been and still is one of the malignancies with the most promising outcomes in terms of immunotherapy trials (Franklin, Livingstone, Roesch, Schilling, & Schadendorf, 2017). This might be partly due to the fact that neither radiotherapy was ever

recommended for melanoma treatment nor did chemotherapy ever have encouraging results with this disease. So, while it was acceptable to resort to these kind of therapies for the treatment of other cancer types, it was imperative to find an alternative therapy that would yield equally acceptable results for advanced melanoma treatment. This might be one of the reasons why research efforts in immunotherapy have been concentrated on melanoma more than other malignancies, which yielded quite a long history of melanoma treatment by immunotherapy.

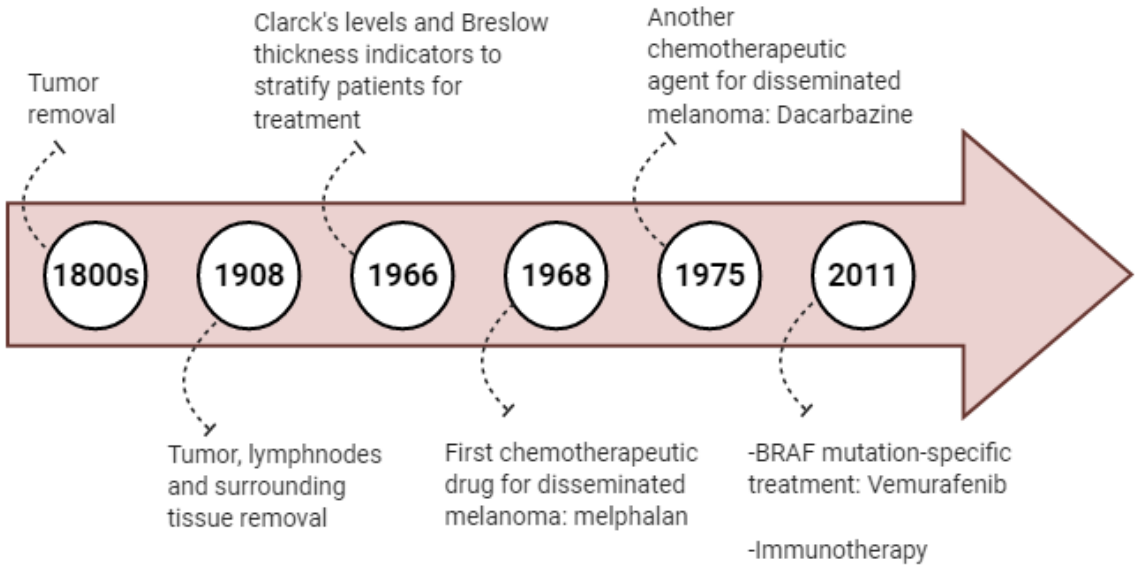

Figure 1: Melanoma treatment timeline. Melanoma treatment progression with time starting with mere tumor removal, followed by the introduction of systemic therapy to treat disseminated melanoma, starting with chemotherapy then targeted therapy and immunotherapy. Years indicated refer to the introduction of the drug for use by patients.

1. *Types of immunotherapies for melanoma*

a. Anti-CTLA4 antibodies

The immunotherapy that revolutionized the metastatic melanoma treatment are checkpoint inhibitors. The first immune-checkpoint blocker to be developed and approved by the FDA is the anti-CTLA4 monoclonal antibody Ipilimumab. The follow-up of patients treated with anti-CTLA4 antibody over extended time periods showed that this agent has the remarkable ability to induce a long-term protection against cancer relapse (Larkin et al., 2015). CTLA-4 (CD152) is a member of the B7/CD28 family that impedes T cell functions. It is expressed constitutively by Tregs but can be upregulated also by other T cell subsets upon their activation. CTLA-4 is usually located in intracellular vesicles and only expressed transiently upon activation in the immunological synapse (Seidel, Otsuka, & Kabashima, 2018). To understand the speculated mode of action that underlies the therapeutic effect of this immune-checkpoint blocker, it is imperative to describe the role of CTLA-4.

i. Physiological role of CTLA-4

CTLA-4 is known as a negative regulator of the activation of naïve T cells. In fact, for an adaptive immune response to take place, two signals are required. The first signal is the interaction between the T-cell receptor and the major histocompatibility complex (MHC) present on the antigen presenting cell (APC). The second needed signal is the interaction between the co-stimulatory molecule CD-28 and CD-80/CD-86 expressed by the T-cell and the APC respectively. Binding of CD28 to CD80 (B7-1) or CD86 (B7-2) on

APCs confers the essential co-mitogenic and anti-apoptotic signals needed for T-cell function (Acuto & Michel, 2003). Nevertheless, once the T-cell is activated, it will start expressing CTLA-4. CTLA-4 is transcribed only after T-cell activation and expressed only upon T cell recognition of antigen, with the exception of regulatory T lymphocytes (Tregs) which express CTLA-4 constitutively and in abundance (Linsley et al., 1996; Linsley et al., 1991; Pentcheva-Hoang, Egen, Wojnoonski, & Allison, 2004; Takahashi et al., 2000). The key for the co-inhibitory nature of CTLA-4 is that it shares great homology with CD28 and outcompetes its binding to CD80 or CD86 with 10 to 100 folds higher affinity (van der Merwe, Bodian, Daenke, Linsley, & Davis, 1997). In addition, CTLA4 on Tregs internalize CD80 and CD86, stripping APCs from their co-stimulatory ligands (Qureshi et al., 2011). This impedes the co-stimulatory effect of CD28 and substantially suppresses T-cell function. CTLA-4 functions are therefore crucial to protect the organism against autoimmune and inflammatory diseases but they thwart the effector T-cell response that is much needed to fight cancer cells.

ii. Mode of action of anti-CTLA4 antibodies

The presented description of the physiological role of CTLA-4 explains how the use of anti-CTLA4 antibodies stimulates T cell activation, and how the global inactivation of CTLA-4 in both mice and humans induces lymphoproliferative autoimmune diseases (Kuehn et al., 2014; Walunas et al., 1994; Waterhouse et al., 1995). It is specifically this concept that provided a hypothetical basis for developing anti-CTLA4 antibodies as

immunotherapeutic agents against cancer. Checkpoint therapy is based on the blockade of the immune-inhibitory pathways that are activated by cancer (D. M. Pardoll & Topalian, 1998). For instance, the use of a CTLA-4 monoclonal antibody will block CTLA-4 leaving the CD-80/CD-86 free to interact with CD-28, forming the second signal and sustaining the T-cell activity (Figure 2).

Interestingly, based on the findings of subsequent studies, another concept arose potentially providing a more accurate explanation of the anti-CTLA4 antibodies therapeutic effect. These studies stated that naïve T cells do not express detectable CTLA-4, and that the CTLA-4 gene is a target of the transcription factor Foxp3 which is inherent specifically to Tregs (C. Chen, Rowell, Thomas, Hancock, & Wells, 2006). Therefore, CTLA-4 is predominantly expressed on Tregs. Also in support of this concept, is a study demonstrating that a lineage-specific deletion of the CTLA-4 gene in Tregs alone was sufficient to largely reverse the morbidity and mortality of lymphoproliferative diseases associated with germline mutations in the CTLA-4 gene (Wing et al., 2008). These results argue that CTLA-4 mainly functions in Tregs. From this perspective, CTLA-4 can be viewed as a cell-intrinsic positive regulator of Treg function subsequently leading to an overall negative regulation of the immune response (Liu & Zheng, 2018). In the light of these findings, the anti-CTLA4 antibodies immunotherapeutic effect can be otherwise interpreted by the inhibition of Tregs suppressive functions primarily, rather than the direct maintenance of the effector T-cells activity.

Also in this context, a study that investigated 2 types of anti-human CTLA4 antibodies, ipilimumab and tremelimumab, showed that both of these agents increased the

intratumoral infiltration of CD4+ and CD8+ T-cells in melanoma tissues. However, ipilimumab demonstrated higher efficiency in depleting Tregs activity in the tumor resulting in a reduced Treg frequency among the total tumor-infiltrating T cells number. It is speculated that this reduction of Treg frequency is the basis for Ipilimumab's reported superior therapeutic effect compared to tremelimumab (A. Sharma et al., 2019). Other studies have taken this concept to an extreme by suggesting that Treg depletion is the main, if not the sole, driving force for tumor rejection by anti-CTLA4 antibodies and that blocking the B7-CTLA4 interactions may be neither essential nor enough by itself for tumor rejection (Du et al., 2018; Liu & Zheng, 2018).

Accordingly, more research is obviously needed to unravel the mechanisms that underlie the anti-tumor effect of anti-CTLA4 antibodies and to accurately describe their mode of action. Meanwhile, this agent remains a leading immunotherapeutic drug which revolutionized the approach to treating melanoma among other cancers. In this context, it is worth noting that although the improvement in survival outcomes for melanoma patients is of undeniable clinical significance, there is still room to achieve tremendous progress in this field. In fact, the maintenance of the anti-tumor adaptive immunity, which is inflicted by the immune checkpoint blockade is interrupted by other immune evasion mechanisms namely the production of the immune-suppressive enzyme such as indolamine-dioxygenase-1 (IDO-1) which is upregulated as a result of anti-CTLA-4 antibodies administration. Hence, a promising strategy is the use of combination therapies that employ two or more immunotherapeutic agents targeting several tumor evasion mechanism.

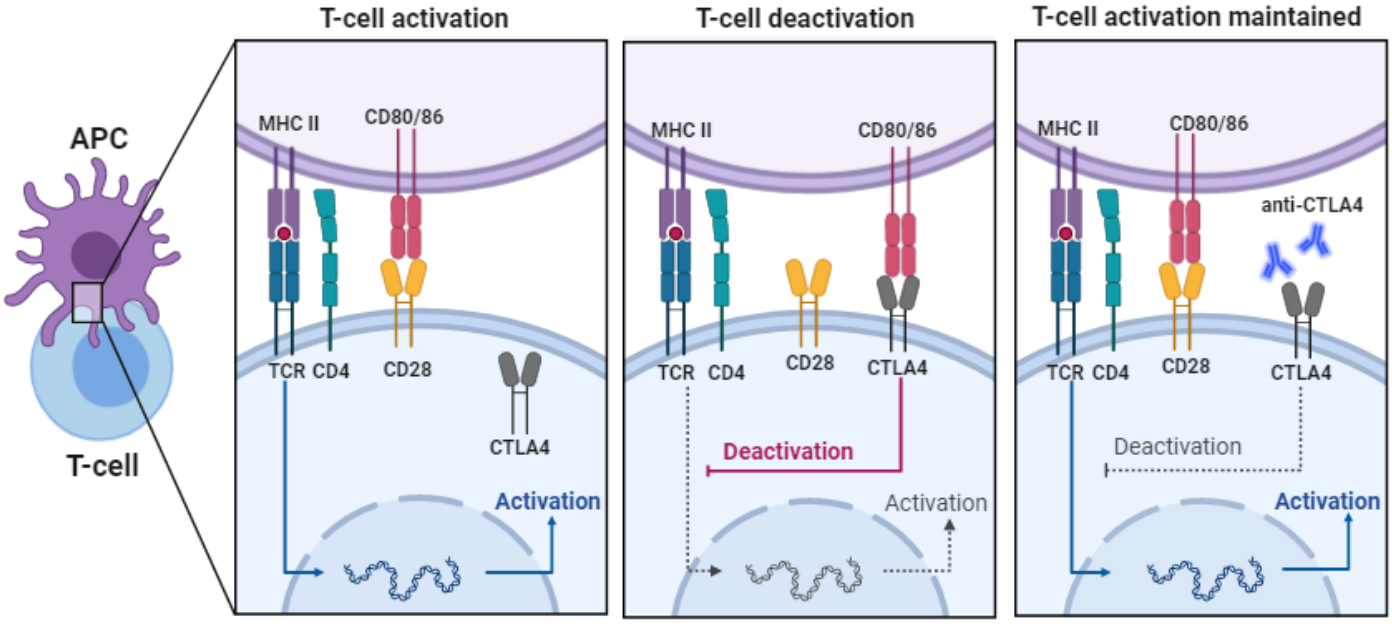

Figure 2: Schematic representation of the mode of action of anti-CTLA4. Upon activation of effector T-cell, CTLA-4 is expressed and competes with CD28 for binding CD80/86 on the antigen presenting cell (APC) which leads to the T-cell deactivation. The monoclonal anti-CTLA4 antibodies neutralize CTLA4 allowing the CD28 and CD80/86 binding and therefore maintaining the effector T-cell activation.

b. Other immunotherapeutic treatments for melanoma

Other immune checkpoint blockers include PD1 and PD-L1. PD1 is a membrane receptor expressed on activated B cells, T cells, and NK cells. It shares homology with CD28 and CTLA4 and binds to PD-L1 or to PD-L2, which also share homology with CD80 making the latter an additional possible PD1 ligand. PD-L1 is expressed on a broad range of cells while PD-L2 is expressed solely on dendritic cells (Dong et al., 2002; Lee et al., 2006; Mühlbauer et al., 2006). Binding of PD1 to any of its ligands blocks signaling

downstream of the CD3-TCR complex and CD28 and leads to T-cell deactivation as well as inhibition of apoptosis in PD-L1 expressing tumor cells (Butte, Keir, Phamduy, Sharpe, & Freeman, 2007; Hirano et al., 2005). Tumor cells can abuse these pathways by overexpressing CTLA4, PD1 and PD-L1 and, as such, evade destruction by the immune system (Mellman, Coukos, & Dranoff, 2011). So far, two anti-PD-1 antibodies (nivolumab and pembrolizumab) have been FDA approved to treat melanoma. In addition, ongoing trials are investigating anti-PD-L1 agents, as well as combinations of different checkpoint inhibitors together or with different cancer therapies including T-VEC, cancer vaccines and other (Abbott & Ustoyev, 2019; Glitza Oliva & Alqusairi, 2018).

In addition, on the basis of the durable objective responses recorded in patients with metastatic melanoma when treated with IL-2, this therapy was approved for the treatment of this disease in 1998 to become the first immunotherapy to obtain regulatory approval (Jiang, Zhou, & Ren, 2016). The therapy consists of high dose IL-2 that requires thorough monitoring due to its pertaining severe toxicities. Most of the side effects seen, including renal impairment, hypotension and edema, are likely caused by what is called capillary leak syndrome and the resulting lymphoid infiltration. These side effects restricted the use of IL-2 therapy to specialized centers but, on a more positive note, these toxicities usually resolved after treatment discontinuation (Schwartzentruber, 2001).

Also approved by the FDA, is the use of T-VEC, the genetically modified oncolytic virus, in the treatment of melanoma. This virotherapy is administered intralesionally but it exerts both a local and a systemic antitumor effect. The mode of action consists of a selective intratumoral replication and the expression of GM-CSF within

melanoma cells. T-VEC injections are usually well tolerated with common side effects including fatigue, nausea, chills and flu-like symptoms. However, being a live virus and therefore risks to cause a herpetic disseminated infection, T-VEC is contraindicated in severely compromised and pregnant women. In fact, T-VEC treated patients were found to shed the virus which entails stringent precautionary guidelines. Finally, a number of current clinical trials are examining whether the efficacy of T-VEC can be enhanced in combination with checkpoint inhibitors or targeted therapies (Andtbacka et al., 2015; Conry, Westbrook, McKee, & Norwood, 2018).

Beside the three immunotherapeutic approaches listed above and which are FDA approved for the treatment of advanced melanoma, another immunotherapeutic approach to treat melanoma patients is adoptive cell therapy. This approach is a patient tailored treatment that uses the patient's derived autologous T cells and is still in clinical trials as of yet. Typically, every patient is pre-treated for lymphodepletion before the T cell infusion and the therapy is usually given in conjunction with high dose IL-2. This approach has been implemented for decades but limited by the necessity of specialized laboratories and hospital units that can manage the IL-2 toxicities (Glitza Oliva & Alqusairi, 2018; Lotze & Rosenberg, 1986).

Moreover, several vaccine approaches were investigated in the advanced melanoma treatment. These vaccines used the identified melanoma tumor associated antigens, such as gp 100, melanoma antigen A1 (MAGE-A1), or the melanoma antigen recognized by T cells (MART-1/Melan-A), to elicit an immune reaction specifically against melanoma cells (Hirayama & Nishimura, 2016). Unfortunately, these vaccines did not

show impressive results when used as single agents and are therefore tested in combinatorial settings.

Rose Bengal, also called PV-10, was also studied in melanoma treatment. It is an iodinated fluorescein derivative that is water-soluble and therefore easily injectable into the malignant lesions. PV-10 works by accumulating into the tumor lysosomes leading to the rapid lysis of cancer cells. In addition, when exposed to ionizing radiation, this agent produces reactive oxygen species that is cytotoxic. Interestingly, even though PV-10 is administered through intralesional injection, it may also elicit an immune antitumor response in distant lesions (Thompson, Hersey, & Wachter, 2008). PV-10 is not yet FDA approved, however clinical trials investigating this therapy are showing promising results so far with favorable toxicity profiles (Read et al., 2018).

2. *Future perspectives in melanoma immunotherapies*

a. 1-Methyl-Tryptophan: an inhibitor of Indolamine 2,3-Dioxygenase

An exciting group of agents that are suspected to play a substantial role in the future immunotherapeutic strategies against melanoma are indolamine 2,3-dioxygenase (IDO) inhibitors. IDO1, IDO2 and TDO are enzymes that catalyze the conversion of the essential amino acid tryptophan into kynurenine. So far, the specific roles of each one of these enzymes is still debatable in the literature. However, current data suggests that mainly IDO-1 and IDO-2 are mainly responsible for catalyzing this pathway. While many studies

are conducted to elucidate the difference between the two, the results are not yet quite conclusive in the literature. Hereafter, IDO-1 and IDO-2 will unanimously be referred to as IDO unless a function specific to one of the two enzymes is to be described.

IDO was found to be expressed by dendritic cells, macrophages, endothelial cells, fibroblasts and tumor cells. It is an enzyme responsible for the regulation of the tryptophan metabolism, along the kynurenine pathway, through catalyzing its rate limiting reaction (Platten, von Knebel Doeberitz, Oezen, Wick, & Ochs, 2014). In fact, following the identification of IDO as a key player in mediating maternal-fetal tolerance, the kynurenin pathway of tryptophan metabolism emerged as a main metabolic pathway that contributes to immune escape. It has also been found that this pathway is over-activated in a number of cancer types and that elevated IDO is correlated with poor prognosis (Heng et al., 2016).

i. Physiological role of IDO

Being the catalyzer of the rate limiting step in tryptophan catabolism, IDO plays a major role in determining the extent of tryptophan breakdown. An increase in IDO leads to the depletion of tryptophan which is an essential component for the function of effector T cells. Tryptophan depletion leads to T cell anergy through decreasing its proliferation and increasing its apoptosis rate. In fact, studies have shown that tryptophan breakdown controls T-cell response. Hence, IDO activity presents a negative feedback loop which controls immune activation through regulating the differentiation, proliferation and

activation of T cells (H Munn, 2011). IDO is also a major inducer of CD4(+)CD25(+) Tregs and therefore induces and amplifies tolerogenic responses (F Fallarino & Grohmann, 2011). In addition, IDO has an ability to block the reprogramming Tregs into TH17-like effector cells (Baban et al., 2009).

Likewise, by catalyzing the tryptophan catabolism, an elevation in the level of IDO results in the abundance of kynurenine which is the main metabolite of the tryptophan pathway. Kynurenine contributes to the activation of T regs which also suppresses the adaptive T cells immunity leading to further weakening of the antitumor immune response (Moon, Hajjar, Hwu, & Naing, 2015). In fact, kynurenine was identified to be an endogenous ligand of the arylhydrocarbon receptor (AhR) (Quintana et al., 2008). AhR receptor is induced by binding to several other ligands too and in response to toxins. This signaling pathway is implicated in embryogenesis, transformation as well as tumorigenesis. It has been shown that AhR activation is involved in regulating the differentiation of Tregs and TH17 cells (Mezrich et al., 2010; Veldhoen et al., 2008).

In general, this immunosuppressive role played by IDO is crucial to protect the organism against autoimmune disease and exaggerated inflammatory responses. However, in case of cancer, this suppression of T-cell responses promotes immune escape thereby favoring tumor cell growth (Gostner, Becker, Uberall, & Fuchs, 2015). Interestingly, studies have shown that IDO levels are upregulated in tumors leading to the suppression of cytotoxic T cells functions and the enhancement of regulatory T cells activity, ultimately resulting in the overall suppression of the anti-tumor immunity. Hence, the inhibition of

IDO will impede the immune-suppression enhancing the likelihood of an adequate anti-tumor immune response. The IDO immunotolerance mechanisms are portrayed in Figure 3.

ii. Mode of action of 1 methyl-tryptophan

The compound 1-methyl tryptophan (1-MT) is one of the first reported inhibitors of IDO activity (Gostner et al., 2015). It is a mixture of two racemic isoforms, the 1-methyl-L-tryptophan (L-1MT) and the 1-methyl-D-tryptophan (D-1MT). Although D-1MT was suggested less active in IDO1 inhibition, it showed higher potency than L-1MT in reversing the T cell suppression mediated by IDO. Thus, D-1MT is being clinically developed as an IDO-inhibitor called indoximod for the treatment of several cancers (Platten et al., 2014). Some preclinical studies reported that the D-1MT reversal of the immunesuppression associated to tumors depends on host expression of IDO1 (Hou et al., 2007). In addition to direct inhibition of IDO, D-1MT can hinder transcellular transport of tryptophan therefore providing through mTOR a tryptophan sufficiency signal to the cell. As L-1MT is mostly capable of producing the same effects, it is not entirely clear yet why D-1MT is more effective in restoring the activity of T cells under physiological conditions (Metz et al., 2012).

Initially, 1-MT was used to block the placental immune privilege. Treating pregnant mice with 1-MT induces the allogeneic fetus rejection by breaking maternal T lymphocytes tolerance for the fetus. The maternal T cell tolerance seemed to rely on the cells expressing

IDO at the maternal-fetal interface which leads to the deprivation of the local microenvironment in tryptophan therefore inhibiting T cell proliferation. In that context, 1-MT restored the local concentration of tryptophan in the placenta allowing activation of T cell which resulted in fetal immune rejection. Accordingly, it was assumed that tolerance for tumor cells can similarly be broken by 1-MT (Agaugué, Perrin-Cocon, Coutant, André, & Lotteau, 2006). Apart from inhibiting the depletion of tryptophan, by blocking IDO, 1-MT hinders the production of the tryptophan catabolites such as kynurenine which were shown to reduce NK cell and T cell proliferation.

Despite the aforementioned mechanisms and the reported safety of 1-MT in phase I clinical trials. This compound did not demonstrate anti-tumor effectiveness when administered by itself (Soliman, Antonia, Sullivan, Vanahanian, & Link, 2009). Therefore, the main interest is to find appropriate combination partners that will synergize the therapeutic effect of this compound. Respectively, some initial experiments applied chemotherapy in combination with 1-MT (Muller, DuHadaway, Donover, Sutanto-Ward, & Prendergast, 2005). More interestingly, based on previous observations that IDO is induced in dendritic cells following ligation of B7 molecules by CTLA4, IDO is suggested to be an essential resistance mechanism weakening the effectiveness of anti-CTLA4 antibodies in cancer treatment (David H Munn, Sharma, & Mellor, 2004). Accordingly, the use of 1-MT to inhibit IDO is a very promising combinatorial approach to amplify the efficacy of anti-CTLA4 immunotherapy by breaking the cancer-induced tolerance. In addition, this approach is particularly interesting since some studies have shown that the use of immune checkpoint blockers contribute to a further upregulation of IDO1 expression by the tumor

as mechanism of resistance to the anti-cancer treatment (F. Fallarino et al., 2003; Platten et al., 2014).

Other IDO inhibitors include epacadostat, an IDO-1 selective inhibitor that has been evaluated as monotherapy and in combinations. While, it failed to demonstrate an independent activity against tumor, it showed great efficacy when used with other checkpoint inhibitors. It is therefore being evaluated in several clinical trials in combination with either pembrolizumab or nivolumab for the treatment of advanced melanoma patients (Yue et al., 2017). BMS-986205 is a different selective inhibitor of IDO1 and is also being tested in combination with ipilimumab and nivolumab in clinical trials (Glitza Oliva & Alqusairi, 2018).

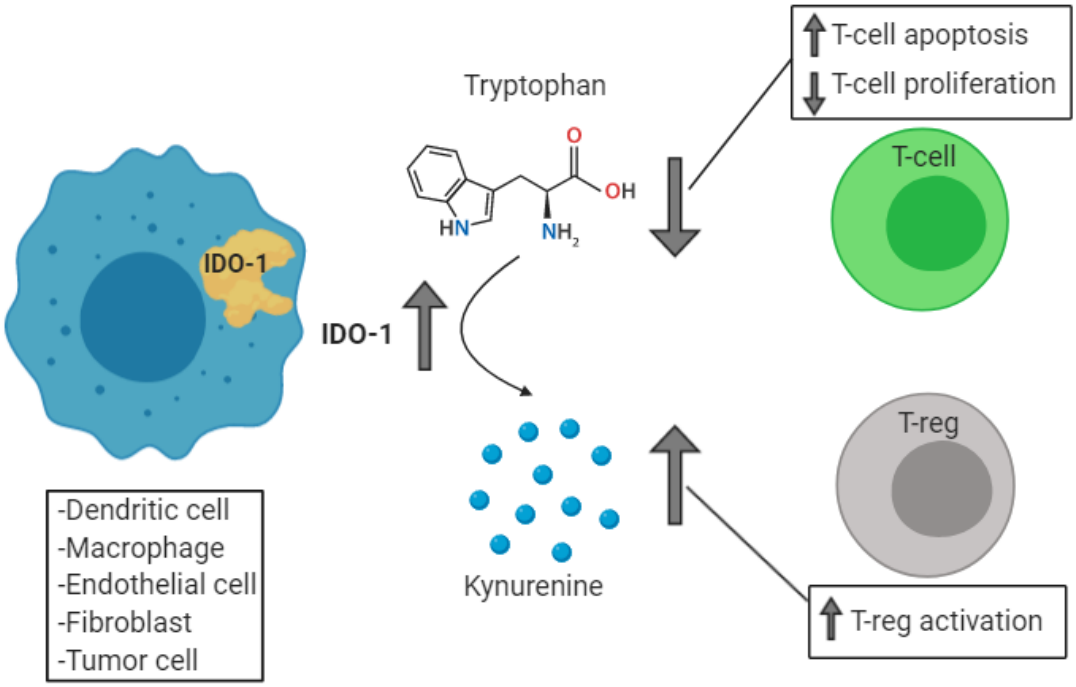

Figure 3: IDO-1 mechanisms of immune tolerance. IDO-1 catalyzes the rate-limiting reaction in the tryptophan catabolism along the kynurenine pathway. Consequently, an enhanced IDO-1 expression leads to shortage in tryptophan, which results in anergy of effector T cells. Also, degradation of tryptophan leads to kynurenine pathway compounds production, which results in promoting Treg differentiation.

b. Monophosphoryl-Lipid-A: a Toll-like receptor 4 ligands

Another interesting approach in the treatment of melanoma is to target toll-like receptors (TLRs) which are members of the pattern recognition receptor family. These receptors are known for their role in the innate and adaptive immune response and many tumor types have been found to express them (B. Huang, Zhao, Unkeless, Feng, & Xiong, 2008). Different TLRs activate different signaling pathways. In 1978, it was reported that post-lipopolysaccharide mouse sera conferred resistance to the TA3-Ha mouse tumor. Moreover, it was shown that the polysaccharide segment (PS now named Monophosphoryl-Lipid-A (MPLA)) of lipopolysaccharide, which activates TLR-4, possessed antitumor activity (Butler, Abdelnoor, & Nowotny, 1978).

MPLA is a derivative of an endotoxin. It is produced by hydrolyzing the native diphosphoryl lipid A which is the component of lipopolysaccharide (LPS) recognized by TLR4. Hydrolysis results in the removal of all but one phosphate group in addition to different degrees of deacylation. These alterations in the compound structure decrease its systemic toxicity by as much as 99% compared to its native lipid A form while retaining a significant immunomodulatory activity. This yields an agent with a significantly increased potential for clinical use (Astiz, Saha, Brooks, Carpati, & Rackow, 1993). Prior treatment with MPLA was shown to increase survival following otherwise lethal exposure to LPS in animal models and has been safely used as an adjuvant in various vaccine trials in humans (Casella & Mitchell, 2008; Thoelen et al., 1998; Wy, Goto, Young, Myers, & Muraskas, 2000). Like its parent compound, MPLA binds and activates TLR-4 and is therefore recognized as a TLR-4 agonist (Romero et al., 2011).

i. Physiological role of TLR-4 agonists

The first described function for TLR4 was recognizing exogenous molecules that originate from pathogens and which are defined as pathogen-associated molecular pattern molecules (PAMPs), particularly the molecules from the outer-membrane of gram-negative bacteria such as LPS. Later, it was extensively shown that TLR4 is also implicated in recognizing the endogenous molecules that are released by necrotic cells and injured tissues. These molecules are known as damage-associated molecular pattern molecules (DAMPs) and they induce the activation of strong proinflammatory responses by interacting with TLR4. Usually, this inflammation has a protective role since it consists of a complex yet coordinated process that is followed by resolution pathways induction which are responsible for the restoration of tissue integrity and function. However, in some cases, excessive and/or poorly regulated inflammatory response can be harmful for the organism (Molteni, Gemma, & Rossetti, 2016).

Also, beyond the transcriptional level induction of proinflammatory mediators, TLR4 interaction with its agonistic ligands also coordinates the induction of certain mediators like microRNAs. These mediators play a major post-transcriptional role in the regulation of the proinflammatory response shutdown and the induction of a temporary state of refractoriness to additional LPS stimulation. Hence, a strict regulation of TLR4 signaling is very important in the homeostasis of tissues through avoiding excessive inflammation and inducing tissue repair after infection or injury (Nahid, Pauley, Satoh, & Chan, 2009; O'neill, Sheedy, & McCoy, 2011).

ii. Mode of action of MPLA

Being a TLR-4 agonist, MPLA binds to TLR-4 leading to the activation of 2 signaling cascades. One cascade is a TRIF-dependent pathway leading to the production of type-I interferons, while the other is a MyD88 dependent pathway leading to the secretion of pro-inflammatory cytokines among which is interleukin 12 (IL-12) (Figure 4) (G. M. McCarthy, Bridges, Blednov, & Harris, 2017). On the one hand, type-I interferons have been recently shown to have an anti-tumor effect and to improve the clinical outcome of cancer patients treated with immunotherapies, namely checkpoint inhibitors (Brockwell & Parker, 2019). On the other hand, IL-12 does not only activate the innate immunity mainly through the activation and proliferation of NK cells but it also contributes to the activation of the adaptive immunity through the maturation and proliferation of T-cells. This is accompanied by the secretion of interferon-γ which is an anti-angiogenic, anti-tumor factor. In addition, IL-12 leads to the inhibition of T-regs therefore contributing to the maintenance of the adaptive immune responses (Lasek, Zagożdżon, & Jakobisiak, 2014). However, these responses cannot be sustained in the presence of immune-checkpoints. From this perspective, multiple studies are underway to examine whether a TLR-based therapy may improve the efficacy of immunotherapies against cancer (Glitza Oliva & Alqusairi, 2018).

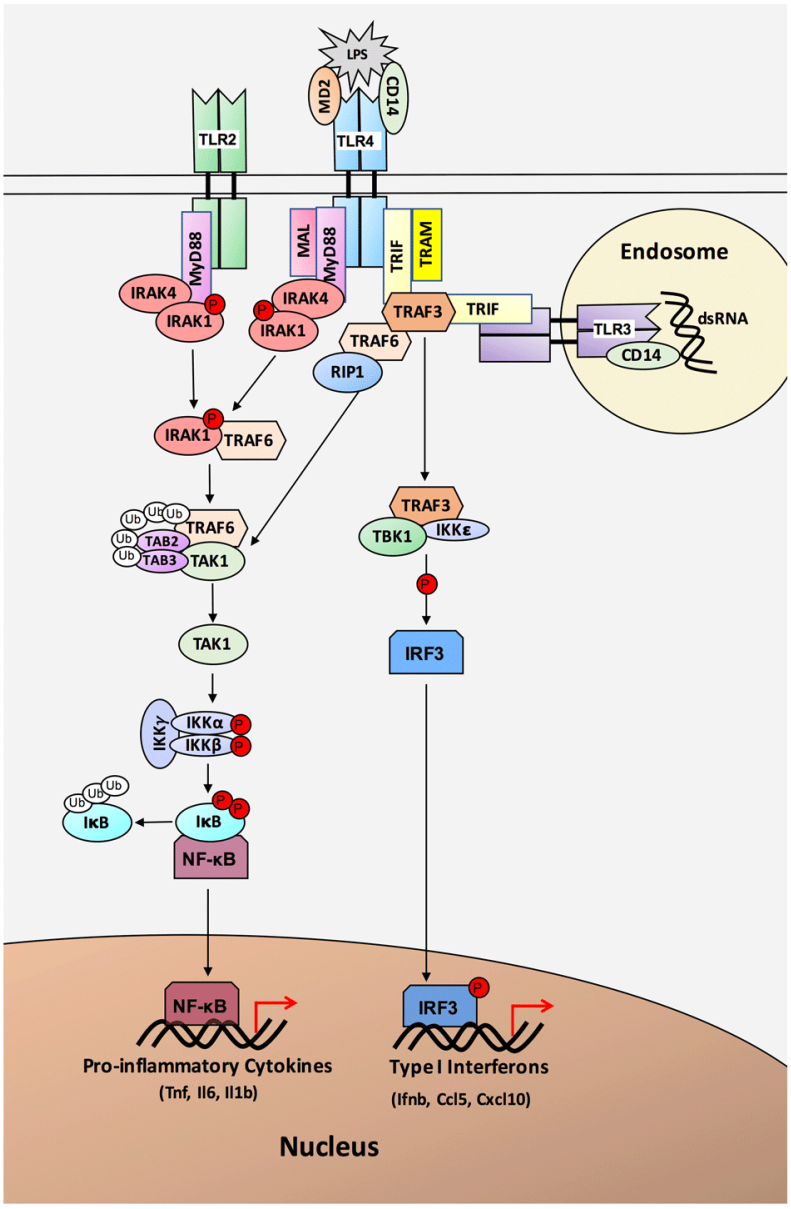

Figure 4: Signaling pathways downstream of TLR4. LPS binds to TLR4 and its co-receptors activating two different downstream pathways, the TRIF-dependent pathway and the MyD88-dependent pathway. The TRIF-dependent pathway, utilizes the TRIF adapter protein which recruits TRAF6 and TRAF3. The former activates NF-κB, while the latter leads to IRF3 activation. Activated IRF3 is then translocated to the nucleus leading to the transcription of Type I interferons. The MyD88-dependent pathway uses the MyD88 adapter protein, which recruits IRAK1, IRAK4, and TRAF6. Following a series of phosphorylation and ubiquitination of IRAK1 and TRAF6, respectively, IKKs and NF-κB are activated. Activated NF-κB then undergoes translocation to the nucleus to promote the transcription of pro-inflammatory cytokines (G. M. McCarthy et al., 2017).

c. Other prospective immunotherapeutic agents

Finally, there is great interest nowadays to study more agents that are expected to guide advancement in the melanoma clinic. For instance, lymphocyte-activation gene 3 (LAG-3) is an immune-checkpoint receptor with multiple effects on the function of T cells. It mainly exerts negative regulation of activation, proliferation and homeostasis of T-cells. Interestingly, LAG-3 is found to be upregulated during exhaustion of T cells which is often the case in cancer. Hence, the efficacy of anti-LAG-3 is currently being evaluated in the treatment of melanoma and other cancer types (Catakovic, Klieser, Neureiter, & Geisberger, 2017; C.-T. Huang et al., 2004). Another example of promising agents are T cell immunoglobulin-3 (TIM-3) antagonists. TIM-3 is expressed on the surface of specific CD4+ and CD8+ cell subtypes that produce IFN-γ as well as other cells such as NK cells. It had been found that, in advanced melanoma patients, the expression of TIM-3 is upregulated in a subset of T cells and these cells seem to be dysfunctional. Several anti-TIM-3 agents are currently being tested as single treatments or in combination with checkpoint inhibitors. It has already been shown that concurrent inhibition of TIM-3 and PD-1 has a synergistic effect in reversing the exhaustion and dysfunction of T cells in tumors (Fourcade et al., 2010; Sakuishi et al., 2010).

Also a promising avenue in the treatment of melanoma is targeting members of the tumor necrosis factor receptor super family (TNFRSF) one of which is OX40. A stimulation of the OX40 ligand in vitro showed prolonged survival, increased proliferation and enhanced effector function of T cells. Treatment with OX40 agonists has also been shown to improve immunity against tumors. The results of several ongoing early phase

trials using agonistic OX40 monoclonal antibodies are quite promising with a noted metastatic lesions regression and more or less tolerable adverse events (Buchan, Rogel, & Al-Shamkhani, 2018; Oberst et al., 2018). One more member of the TNFRSF is 4-1BB which is a costimulatory receptor on T cells among other immune cells. When induced, 4-1BB is responsible for restoring the effector function of these cells. In fact, the interaction between 4-1BB and its ligand leads to the secretion of cytokines and increased CD8+ T cells survival. Although the results of the study using 4-1BB agonistic monoclonal antibodies as a monotherapy were not very promising, a synergistic activity has been demonstrated when this agent was used concurrently with nivolumab in preclinical studies. Therefore, several clinical trials are currently studying this combination as well as other combinations involving 4-1BB and pembrolizumab or 4-1BB and OX40 in the treatment of solid tumors and metastatic carcinomas (Bartkowiak & Curran, 2015; S. Chen et al., 2015; Chester, Sanmamed, Wang, & Melero, 2018).

CHAPTER II

SPECIFIC AIMS

The unprecedented pace at which cancer immunotherapy is progressing has provided extremely valuable insights that drew the roadmap for the future studies needed to advance this field further. First, it has been confirmed that the immune system in its innate and adaptive compartments is capable of recognizing and eliminating tumors in a process called immunosurveillance. Then it has been shown that this process only covers one facet of the interaction between immunity and cancer. Other facets include equilibrium which is when the immune responses are efficient enough to contain the tumor from progressing but not to eradicate it. The final facet is the escape phase where cancer cells overcome the containment by the immune system and are unleashed to their malignant potential. Further investigations of tumor immune evasion delineated the specific mechanisms that are developed by cancer cells and which allow their escape from the host's immune attacks. These mechanisms include impairing antigen presentation, activating inhibitory immune-checkpoints, and elaborating numerous immunosuppressive elements. Beside these mechanisms, tumors abuses the regulatory role of several cell populations to weaken the anti-cancer T-cell mediated immunity (Vinay et al., 2015).

In spite of the revolutionary progress achieved by the recently developed immunotherapies against melanoma, the current prognostic data of this disease underlines, still, the need for more research to discover innovative treatments and/or optimize the ones

lately developed. In this context, it is important to note that one of the major challenges hindering the effectiveness of immunotherapies are the aforementioned immune evasion mechanisms that are inherent to or acquired by tumors (Klener, Otahal, Lateckova, & Klener, 2015). Recent studies showed that a promising endeavor to overcome this challenge is to use combination immunotherapies. Yet, substantial work lies ahead to determine therapeutic combinations that are more effective and which would improve the prognosis of advanced melanoma patients (Holmgaard, Zamarin, Munn, Wolchok, & Allison, 2013; Spranger et al., 2014; Van De Voort, Felder, Yang, Sondel, & Rakhmilevich, 2012; Zaretsky et al., 2016).

Hence, **the overall aim of the experimental work described in this dissertation is to develop a triple immunotherapy that harnesses the innate and acquired immune responses to overcome tumor immune evasion in a B16F10 melanoma mouse model**

While anti-CTLA-4 monoclonal antibody (anti-CTLA-4) is an approved and effective immune-checkpoint blocker for the treatment of advanced melanoma, it mainly works through maintaining the anti-tumor adaptive immunity, yet does not include any enhancement of the innate immune responses which could largely improve the therapeutic outcome. (Mellman et al., 2011; D. Pardoll, 2015) Consequently, the second selected component of the combination is monophosphoryl-lipid-A (MPLA) which is a potent activator of the innate immunity. MPLA is the safe form derived from lipopolysaccharide (LPS), which is a component of the outer-membrane in Gram negative bacteria. It has the same antigenicity of LPS but with reduced toxicity so that it does not cause a toxic shock and hence can be used in the clinic. Like LPS, MPLA is a pathogen-associated-molecular-

pattern (PAMP) that binds to Toll-like receptor 4 (TLR4), which is its pattern recognition receptor leading to the production of type-I interferons and the secretion of anti-tumor cytokines. This treatment not only activates the innate immune responses but also promotes the adaptive immunity while inhibiting regulatory T-cells (Van De Voort et al., 2012; Wang, Zhou, Tang, & Guo, 2012). However, treatments with anti-CTLA-4, MPLA or both lead to the upregulated production of the immune-suppressive enzyme Indolamine-Dioxygenase-1 (IDO-1) (David H. Munn, 2006; Qin et al., 2017). Therefore, the third component of the proposed combination is 1-methyl tryptophan (1MT) which is an IDO-1 inhibitor that will impede the immune-suppression thus enhancing the likelihood of an adequate anti-tumor immune response (Platten et al., 2014). Accordingly, this combination is expected to enclose cancer and impede its ability of immune evasion.

Specific aim 1: Demonstrate lack of systemic adverse effects for administration of the proposed immunotherapies.

Since the main concept of cancer immunotherapy is to unleash the powers of the immune system in an attempt to hinder cancer progression, adverse events related to auto-immune reactions and/or exaggerated inflammation are often a cause of concern whenever an immunotherapeutic approach is used. More so, when a combination of immunotherapies are used concomitantly. The combination we employed not only causes immune-stimulation, which is the case with MPLA, but also suppresses immune-regulatory pathways, which is achieved by both anti-CTLA-4 and 1-MT. It is therefore crucial to ensure the safety of such combination before implementing it.\

Objectives of specific aim 1:

a. **Identification** of the optimal preparation and administration methods of the selected treatments in single, dual and triple therapies to C57BL/6 wild type, cancer-free, mice.
b. **Observation** of the rate of weight gain among the different experimental groups and compare it to untreated controls.
c. **Checking** for the development of any aberrant clinical signs in the mice throughout a three month monitoring period.
d. **Assessment** of the aspect of the mice major organs for any chronic histological abnormalities at the end of the monitoring period.

Specific aim 2: Evaluate the anti-tumor efficacy of the different treatment combinations.

After making sure that it is safe to use the different agents alone and in combinations on cancer free mice with no recorded adverse events nor abnormalities, we could then examine whether these therapies have a therapeutic effect on a melanoma model of tumor-bearing mice. This informs us if the proposed triple combination fulfilled the expected result of overcoming the tumors ability of evading the immune system.

Objectives of specific aim 2:

a. **Generation** of a melanoma mouse model by injecting B16F10 melanoma cells into congeneic C57BL/6 mice.

b. **Assessment** of the therapeutic effect of each treatment and combination by comparing mouse survival and tumor growth rates across the different control and experimental groups.

Specific aim 3: Provide mechanistic insights behind the observed anti-tumor effects of the immunotherapies.

Once the increased therapeutic efficacy is demonstrated for the triple immunotherapy, further testing was needed to try to depict the mechanisms responsible for the enhanced anti-tumor effect. The disclosed data on the cellular and molecular level serves to optimize the current combination and guides the development of new therapeutic strategies based on the analyses of the immune components that made a difference in the anti-tumor response.

Objectives of specific aim 3:

a. **Identification** of the levels of different cytokines including IL-12 and VEGF across the different treatment groups.
b. **Evaluation** of the tumor-infiltrating immune-cell populations in all experimental groups.

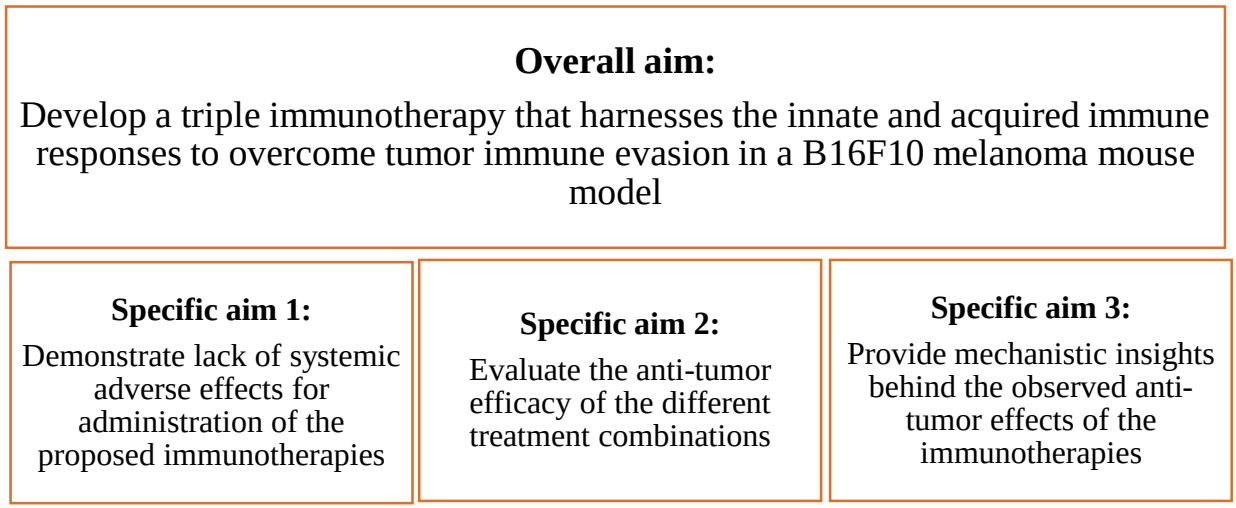

Figure 5: Schematic diagram summarizing the aims of the dissertation. The overall aim is to develop an immunotherapeutic combination that overcomes tumor immune evasion in a melanoma mouse model.

CHAPTER III

MATERIALS AND METHODS

A. Mice

The experiments on mice were conducted according to the regulations of the Institutional Animal Care and Use Committee at the American University of Beirut with the approval number: 18-5RN-408/482. All mice used were female C57BL/6 mice aged 8–10 weeks old, weighing 20–22 g each.

B. Treatment Agents

1. *Anti-CTLA-4 monoclonal antibodies (anti-CTLA4)*

Anti-CTLA4 was obtained from Bioxcell, West Lebanon, NH and stored in the dark at 2-8°C. The needed dilution was prepared using sterile saline.

2. *Monophosphoryl lipid A (MPLA)*

MPLA-SM VacciGrade was obtained from In VivoGen, Toulouse, France and stored at -20°C. It was reconstituted using DMSO according to the manufacturer's recommendation and diluted to the needed concentration using sterile saline.

3. *1-Methyl Tryptophan (1-MT)*

1-MT was obtained from Sigma Aldrich/Merck, Darmstadt, Germany. A stock solution of the desired concentration was prepared by dissolving 1-MT in sterile saline and 2N NaOH. The stock solution was stored at 2-8°C. Directly before each administration, 2 M HCl were sequentially added to reach physiological pH and the treatment was injected immediately.(Jia et al., 2008)

C. Monitoring Adverse Effects

To ascertain that none of the three agents alone or in combination were toxic to C57BL/6 mice, nine groups of three mice each were used and treated as follows: group 1 was an untreated control; group 2 was a saline-treated control (saline being the vehicle of all used treatments); groups 3, 4, and 5 were treated with single therapies of either MPLA, anti- CTLA4-antibodies, or 1-MT; groups 6, 7, and 8 were treated with dual therapies of these treatments; and group 9 was treated with all three immunotherapeutic agents (Table 1). Doses were as follows: 10 μg MPLA was administered subcutaneously into the upper right flank on day 8 and then on day 15. As for anti-CTLA4, 200 μg was given intraperitoneally at day 3 and 100 μg was given on days 6, 9, 12, and 15. 1-MT was given in daily intraperitoneal doses of 2.25mg.

Table 1. Immunotherapeutic regimens administered to different groups of C57BL/6 mice

Treatments	Saline[1]	MPLA	Anti-CTLA4	1-MT
Group 1				
Group 2	×			
Group 3		×		
Group 4			×	
Group 5				×
Group 6		×	×	
Group 7		×		×
Group 8			×	×
Group 9		×	×	×

[1]Vehicle of all treatments

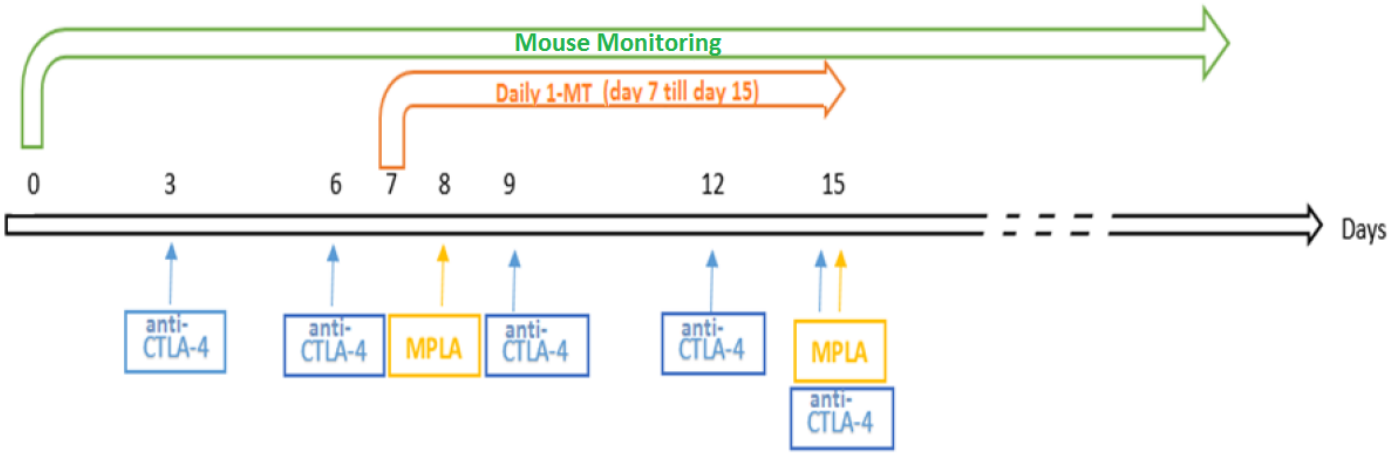

Figure 6: Administration timeline of the three immunotherapeutic agents. Anti-CTLA4 was administered intraperitoneally on days 3, 6, 9, 12 and 15; while 1-MT was administered daily, using the same route, starting day 7 and until day 15. MPLA was injected subcutaneously on the mice upper right flank in cancer free mice and intratumorally in tumor-bearing mice, on days 8 and 15.

1. ***Clinical signs:***

All mice were monitored throughout the treatment period and for the following 3 months. The monitoring included observation of clinical signs such as the grooming of the fur, mobility, hunched posture, respiratory distress, presence/consistency of stools and failure to eat.

2. ***Weight monitoring:***

Weekly weight measurements were performed on all mice to compare weight gain rate among the different groups.

3. ***Histological evaluation:***

At the end of the monitoring period, the mice were sacrificed and histological evaluation of the liver, heart, kidneys, and lungs was performed. The procedure consisted of staining several sections from each organ followed by microscopic examination

D. B16F10 Melanoma Mouse Model

1. ***Cells***

The cells used for the tumor challenge were B16F10 melanoma cells, which are congeneic to the C57BL/6 mice. These cells were cultured in RPMI medium (Lonza, Basel,

Switzerland) supplemented with 10% fetal bovine serum (Sigma-Aldrich/Merk, Darmstadt, Germany), 1% glutamine (Lonza, Basel, Switzerland), and 1% Pen-Strep (Lonza, Basel, Switzerland). All cell cultures and preparations were done under aseptic conditions in level 2 biosafety cabinets.

2.

3. ***The tumor model***

The tumor model was generated by injecting 10^6 melanoma cells subcutaneously into the shaved upper right flank of mice at Day 0 of each experiment. The cells used were taken from culture plates with a maximal 50% confluency to ensure the establishment of tumor growth in the mice. The concentration of the cell suspension was adjusted to 10^6 cells/0.15mL. (Figure 7)

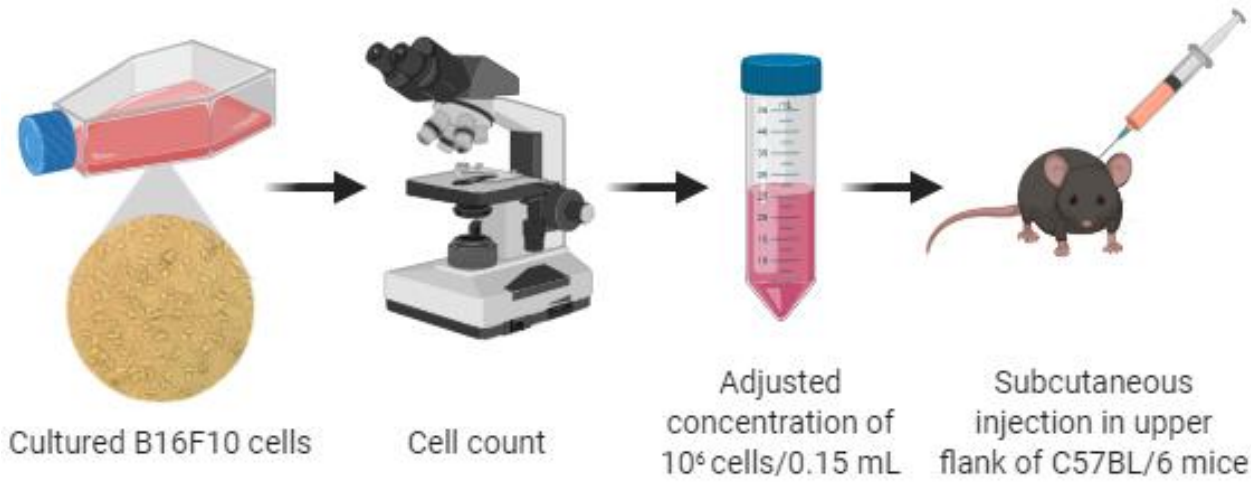

Figure 7: Schematic representation of the mouse tumor model protocol. B16F10 melanoma cells are cultures in complete, RPMI based, medium. Following cell count, the cell suspension concentration is adjusted to 10^6 cells/0.15 mL and injected subcutaneously on the upper right flank of C57BL/6 mice.

E. Evaluating the Antitumor Effect

Mice were segregated into nine groups (containing 12 to 13 mice each) and treated as described above for monitoring adverse events but with MPLA being injected intratumorally.

1. *Survival*

Survival was recorded and mice were monitored. Monitoring included daily observation of clinical signs. Survival was assessed in two independent experiments.

2. *Tumor volumes*

Tumor measurements were performed using a caliper every 3–4 days starting on day 10 and tumor volumes were determined using the following formula: Volume = p/6 (LWW), where L is the longest side measured and W is the shortest side measured. This procedure was conducted in two independent experiments.

3. *Cytokine analysis*

The levels of IL-12, VEGF and S100B in the blood drawn from mice at days 9, 13 and 16 post-tumor injection were measured using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' recommendations. Mouse ELISA kits for IL-12

and VEGF were obtained from Abcam, Cambridge, United Kingdom while the Mouse S100B kit was obtained from Biomatik, Ontario, Canada (Figure 8).

4. *Assessment of Tumor-Infiltrating Immune Cells*

Examining the tumor-infiltrating immune cells was performed as described previously by Pachynski et al. (Pachynski, Scholz, Monnier, Butcher, & Zabel, 2015). Briefly, three mice from each of the nine groups were sacrificed on days 9, 13 and 16 post-tumor inductions. Their tumor masses were excised and mechanically homogenized into cell suspensions using cell strainers. Cells were counted, fixed, and stained for detection of the CD4+ T cell population (using anti-CD3 and anti-CD4), the CD8+ T cell population (using anti-CD3 and anti-CD8), the Tregs (using anti-CD3, anti-CD4, and anti-CD25), and the NK cells (using anti-NK1.1), and analyzed by flow cytometry (BD FACSAria). Antibodies used were purchased from (Biolegend, San Diego, CA) (Figure 8).

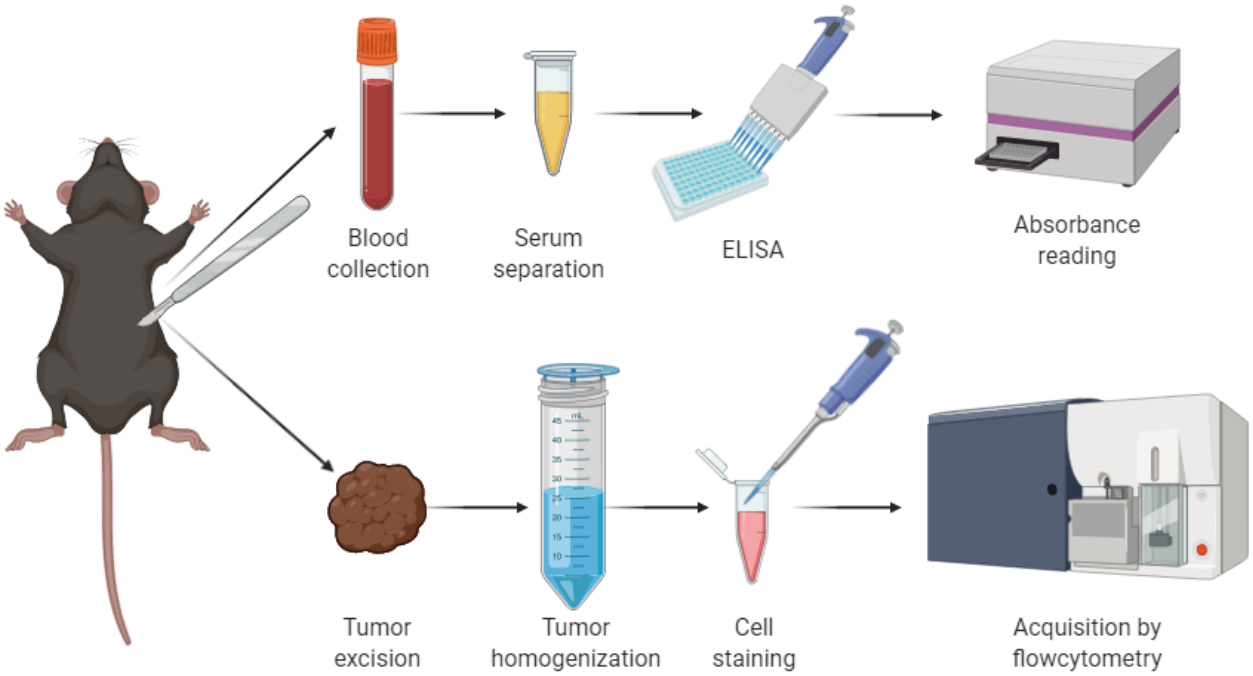

Figure 8: Schematic representation of the experimental protocol for cytokines and tumor infiltrating immune cells analysis. For cytokine analysis, blood was collected and separated. ELISA was performed on serum samples and absorbance was quantified using a multi-reader. Tumors were excised and mechanically homogenized using cell strainers. Then tumor cell suspensions were fixed and stained. The tumor infiltrating CD4+, CD8+ and regulatory T-cells as well as NK cells were quantified using a flow cytometer.

F. Statistical Tests

Data was analyzed using GraphPad Prism. Two-way ANOVA was used to compare more than two groups. Tukey's and Dunnett's post hoc tests were used for multiple comparisons within groups. Kaplan–Meier was used for survival analysis, the outcomes were assessed by the Mantel–Cox log-rank test, and Bonferroni correction was

used to determine significance. P-values less than 0.05 were considered statistically significant unless stated otherwise.

CHAPTER IV

RESULTS

Immunotherapeutic treatments of different cancer free and melanoma-bearing mouse groups are summarized in Table 1 while the timeline of the therapies is described in Figure 6.

A. Ruling Out Adverse Events of the Used Treatments and their Combinations

Prior to testing the efficacy of the different proposed treatments, the presence of any explicitly detrimental effects that might be caused by these combinations was assessed.

1. *Clinical monitoring*

Mice were monitored throughout the treatment period and for the following 3 months during which none of the mice showed abnormal clinical signs. Grooming of the fur, posture, respiration and mobility in all experimental and control groups were comparable. Accordingly, weight measurement indicated similar weight gain patterns among all groups during the treatment period and for the following 3 months. By week 12, the untreated group reached 122% of its original weight at week 0 while the treated groups presented weight gains that range between 119.3% and 124.7% of their original

weights. None of the groups displayed a statistically significant weight difference in comparison with the untreated control group at any time point of the monitoring period (Figure 9).

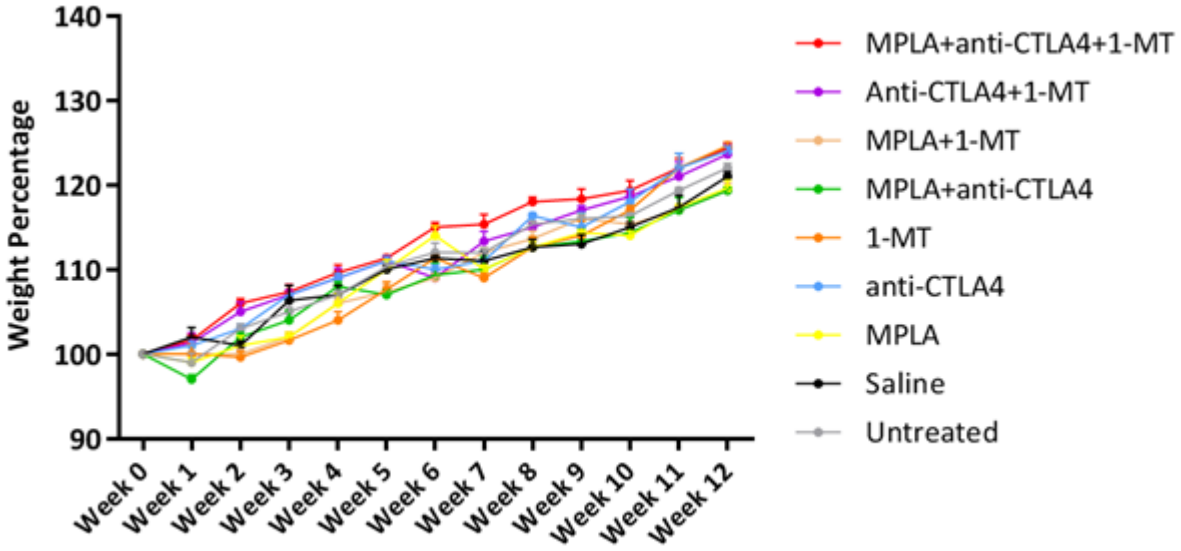

Figure 9: Weight monitoring. Average mouse weight per group of tumor-free C57BL/6 mice treated with MPLA, CTLA-4ab, 1-MT or their combinations (n = 3).

2. *Histological testing*

Microscopic examination of the histological sections performed at the end of the monitoring period showed that there was no pathological change in the lungs, kidneys, heart, and liver tissues of all experimental and control groups. All examined tissues maintained their normal structural details. The absence of necrosis, inflammation and

inflammatory cells infiltrate was noted and no other alterations were encountered. The lungs showed normal alveolar lining with no peculiarities as well as bronchus associated lymphoid tissues (BALTs) of normal size. In the kidneys, the renal tubules, glomerular tuft and renal blood vessels were intact. In the heart, the striations were clear and there was no necrosis, no cellular fibrosis nor an accumulation of fibrotic tissues. Finally, in the liver the nuclei were preserved, the cords and sinusoids were normal and no picnotic cells or inflammatory aggregates were seen. Representative images of histopathological sections taken from the hearts, livers, lungs and kidneys of one untreated mouse and one from the group receiving the triple therapy are shown in Figure 10.

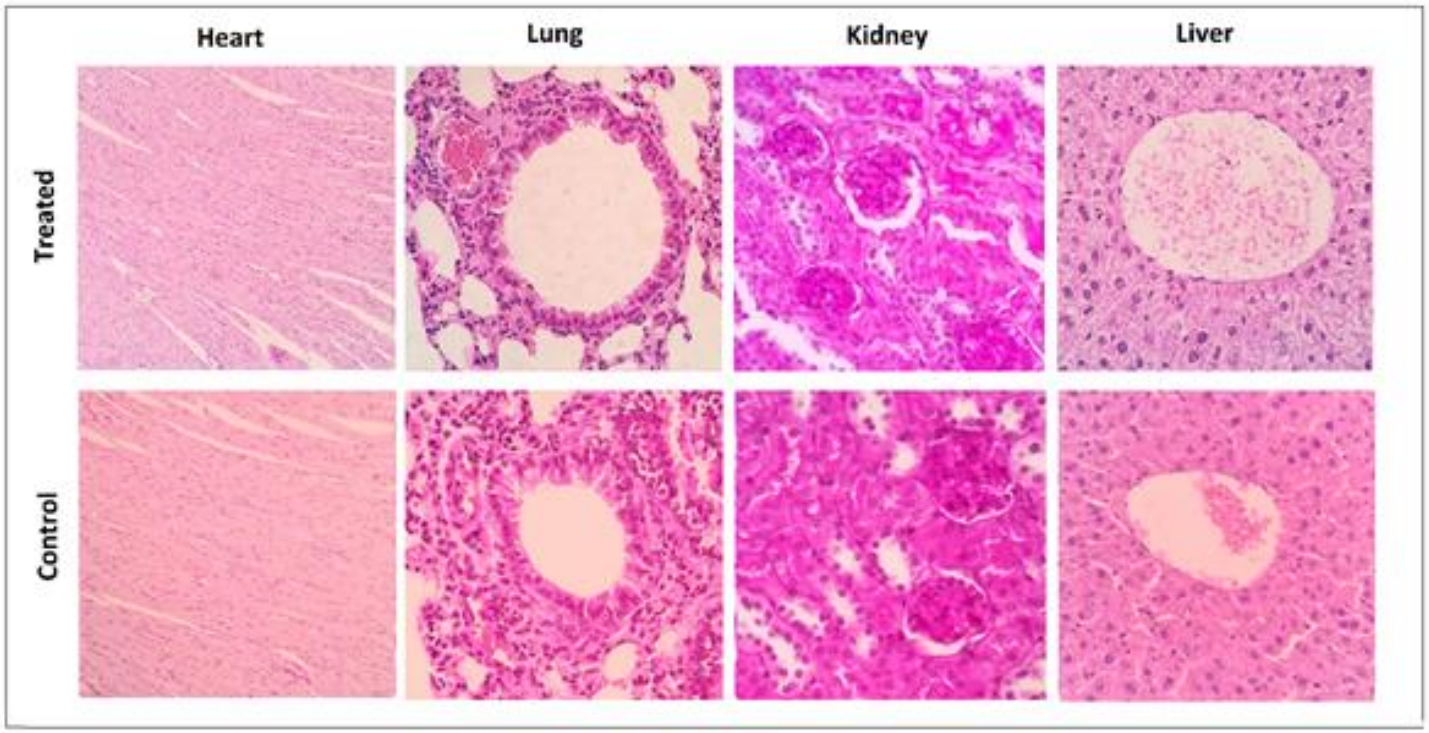

Figure 10: Histology of main organs. Representative histological sections taken at 3 months after completion of the treatment from tumor-free C57BL6 mice in the control group and the group receiving triple therapy (MPLA, CTLA-4ab, and 1-MT). Heart sections are presented at a 100× magnification while lung, kidney, and liver sections are presented at a 400× magnification (n = 3).

B. Assessment of the Therapeutic Effect of the Used Treatments and Their Combinations

Since no adverse events were noted as a result of the various treatment regimens employed, it was possible to investigate the anti-tumor effect of all treatments and their different combinations. Mice were challenged with B16F10 melanoma cells at day 0. They

were segregated into the same 9 groups, administered the same treatments as in the experiment for ruling out adverse events, and their tumor volumes were measured regularly. (Table 1, Figure 6)

1. *Tumor Progression Analysis*

Tumor sizes were decreased in the groups receiving dual and triple therapies at every time point, while the groups receiving single therapies showed tumor progression patterns that are quite similar to the two control groups (untreated and saline-injected groups). Moreover, a statistically significant decrease in tumor size was seen in the group receiving the triple immunotherapy in comparison with all other groups at all 5 measurement time points ($P<0.0001$). At day 10, the average tumor size in the triple therapy group was as low as 0.05 cm^3, the closest average size was that of the group receiving MPLA and 1 MT which was 0.38 cm^3 (almost eight folds larger). At day 24, the average tumor size in the triple therapy group was 4.98 cm^3, the closest average size was that of the group receiving anti-CTLA4 and 1 MT which was 12.44 cm^3 (almost three folds larger). In summary, tumor size progression assessment showed that although some combinations resulted in smaller tumor sizes compared to controls, the group of mice treated with the triple combination was the one to cause the greatest statistically significant reduction in tumor size; this reduction was by about 70% by day 24 post-tumor induction. (Table 2, Figure 11)

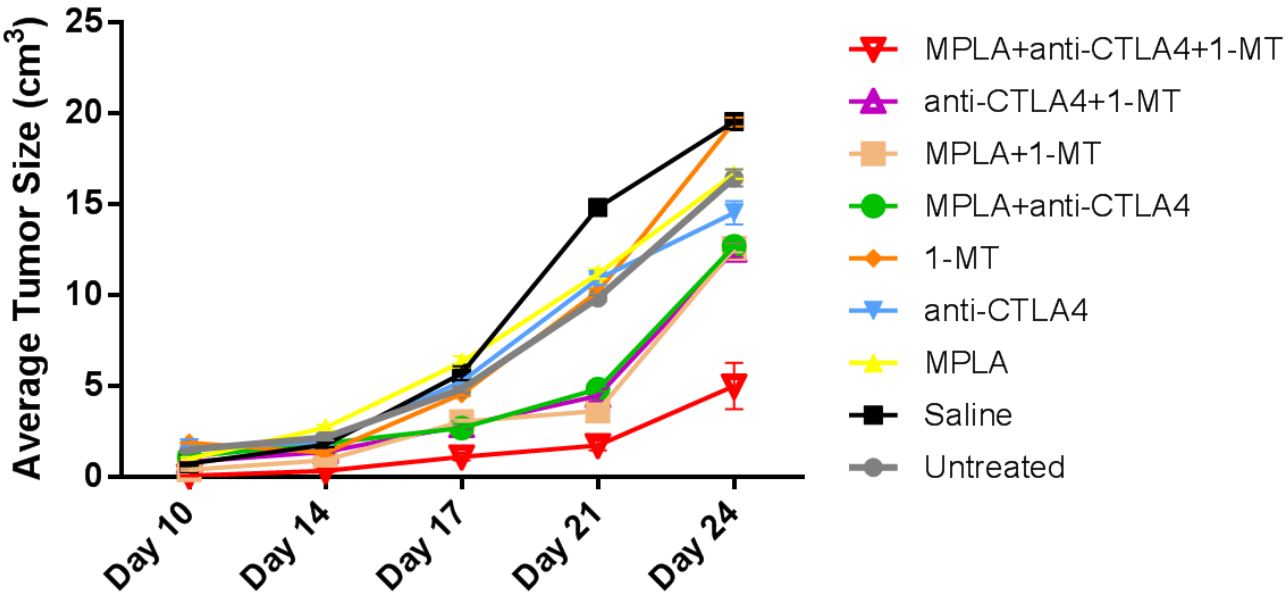

Figure 11: Tumor size progression. Average tumor sizes in C57BL/6 mice following tumor induction with B16F10 melanoma cells and treatment with MPLA, CTLA-4ab, 1-MT, or their combinations (n = 10). Numerical representations and statistical significance of tumor size variations are indicated in Table 3.

Table 2: Values of tumor volumes. Average tumor sizes in C57BL/6 mice following tumor induction with B16F10 melanoma cells and treatment with MPLA, CTLA-4ab, 1-MT, or their combinations (n = 10). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group.

Treatment	Size of tumor (cm^3) on day				
	10	**14**	**17**	**21**	**24**
Untreated	1.49*	2.15*	4.81*	9.82*	16.43*
Saline	0.73*	1.75*	5.68*	14.79*	19.53*
MPLA	1.00*	2.70*	6.31*	11.15*	16.63*
CTLA-4ab	1.59*	1.92*	5.23*	10.86*	14.51*
MT	1.85*	1.33*	4.56*	10.17*	19.51*
MPLA + CTLA-4ab	1.11*	1.85*	2.68*	4.83*	12.68*
MPLA + MT	0.39*	0.91*	3.02*	3.62*	12.56*
CTLA-4ab + MT	0.82*	1.36*	2.81*	4.45*	12.44*
MPLA + CTLA-4ab+ MT	0.06	0.33	1.10	1.72	4.98

2. *Survival Analysis*

Survival was also recorded and the results indicated that although some enhanced survival was observed with the anti-CTLA4 single agent treatment and with some combinations, the enhanced survival was not statistically significant compared to the untreated or saline treated groups. The only combination to cause a statistically significant

difference in survival when compared to both the untreated and the saline control groups ($P<0.005$) and after applying the Bonferoni correction was when mice were treated with all 3 agents. Mice in the control groups were all dead by day 27 while mice in the triple immunotherapy group survived until day 37. Dual therapies prolonged survival but did not reach statistical significance when compared to control groups. Finally, single MPLA or 1-MT therapies showed a survival rate comparable to the control groups. Notably, the group receiving a single anti-CTLA4 therapy presented survival results that are close to those of the triple combination group. However, the increase in survival rate of this anti-CTLA4 group, in comparison to that of the control groups, did not reach statistical significance as opposed to the group receiving the triple therapy. (Figure 12)

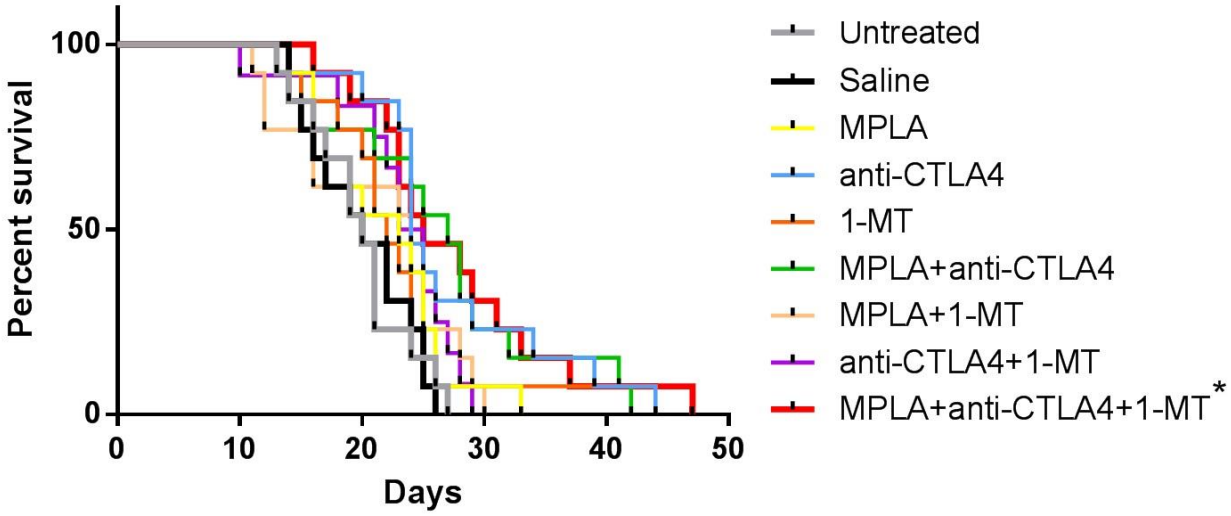

Figure 12: Mouse survival. Percent survival of C57BL/6 mice following tumor induction with B16F10 melanoma cells and treatment with MPLA, anti-CTLA4, 1-MT or their combinations. Data represent two independent experiments (n = 12–13). *$p < 0.005$ compared to the untreated or saline-treated group.

C. Analysis of mechanisms underlying the therapeutic effects

1. *Assessment of cytokine levels*

a. Interleukin-12 (IL-12*)*

IL-12 is generally considered an anti-tumor cytokine that is secreted by macrophages and B cells. Its primary functions involve the NK cells proliferation, IFN production, and promoting cell-mediated immune responses (Zhang & An, 2007). Contrary to what we expected, our ELISA results show that the group treated with the triple combination, which is the group with the best phenotypic outcome in terms of tumor growth and survival, mostly has the lowest levels of IL-12 throughout all the time points of testing. This finding is especially highlighted at the third time point when the whole treatment course was achieved. Moreover, the highest IL-12 levels were found in the groups with the poorest clinical outcome such us the groups treated with single MPLA therapy or a single 1-MT therapy. (Figure 13)

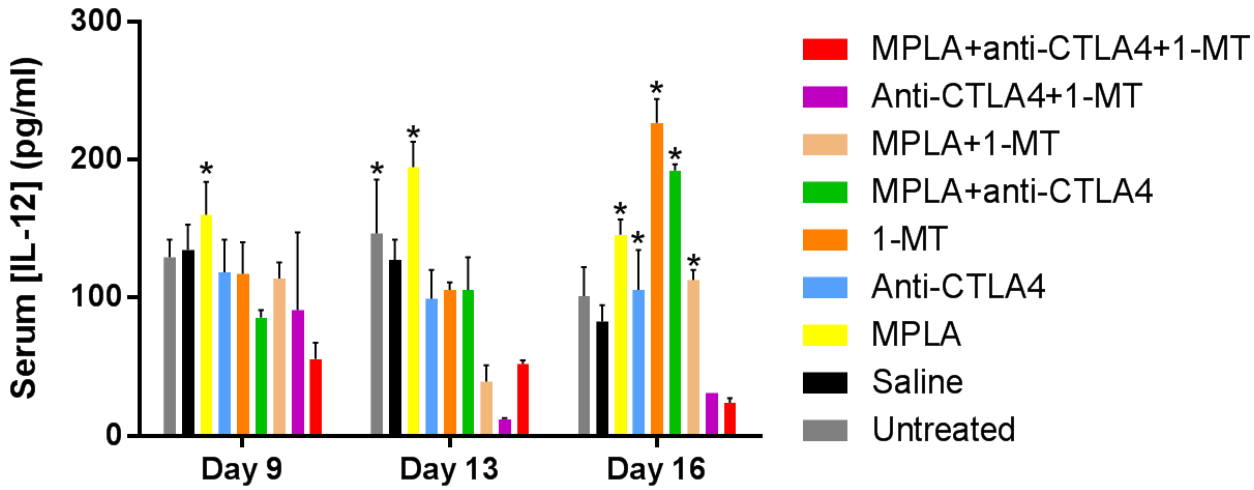

Figure 13: Levels of IL-12. Serum IL-12 levels in the B16F10 melanoma mouse model treated with various immunotherapeutic regimens at days 9, 13 and 16 post tumor challenge (n = 3). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group.

b. VEGF

Vascular endothelial growth factor (VEGF), also termed vascular permeability factor, was initially described as an endothelial mitogen that is cell-specific. It is produced by several types of cells including macrophages, tumor cells, platelets, renal mesangial cells and keratinocytes. VEGF functions are not limited to the vascular system but is also involved in normal physiological activities such as development, hematopoiesis, bone formation and wound healing (Harmey, David, & Judith, 2013). More importantly, VEGF is identified as the most potent angiogenic factor to date. It is, therefore, associated with the

progression and metastasis of solid tumors, including melanoma. It was also found that melanoma cells produce raised concentrations of VEGF (Redondo, Bandrés, Solano, Okroujnov, & García-Foncillas, 2000). The ELISA results of our study show that only 2 groups had VEGF levels below the detection threshold, the group treated with anti-CTLA4 + 1-MT and the group treated with all three agents, MPLA + anti-CTLA4 + 1-MT. These outcomes are in accordance with the phenotypic results of survival and tumor growth. (Figure 14)

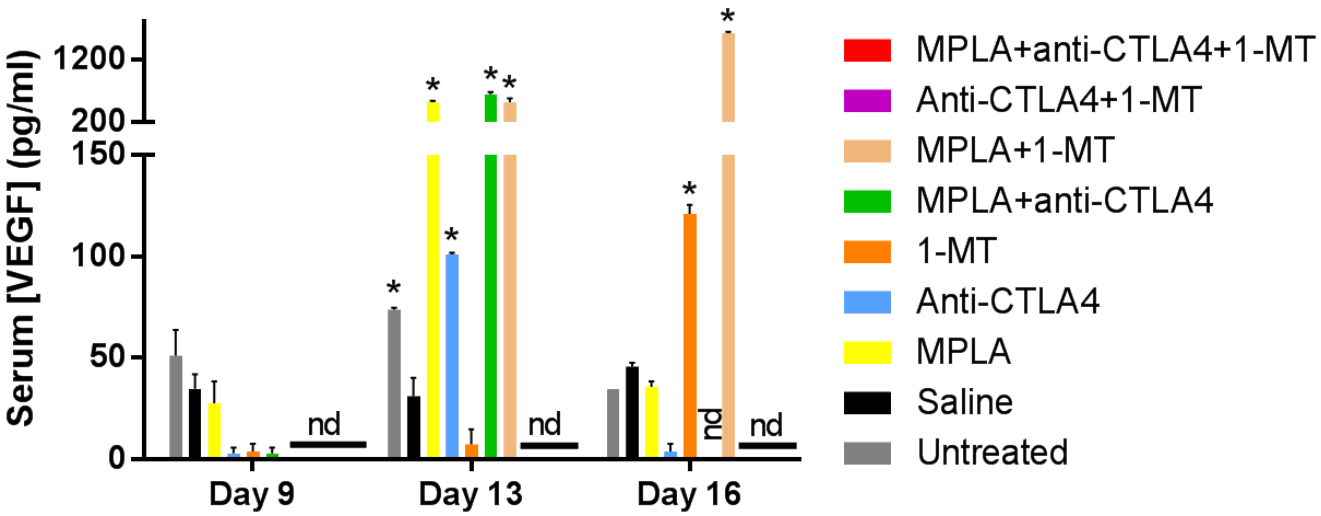

Figure 14: Levels of VEGF. Serum VEGF levels in the B16F10 melanoma mouse model treated with various immunotherapeutic regimens at days 9, 13 and 16 post tumor challenge (n = 3). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group. (nd: not detectable)

c. S100B

The S100 term refers to all the members of a low-molecular-weight multigene family. To date, 20 proteins that belong to the S100 protein family were identified. These proteins are mostly calcium sensor proteins which use calcium binding to modulate biological functions. Melanoma cells were shown to secrete the S100 protein in its soluble form. Particularly, S100B was found to be most abundant in glial cells and also in adipocytes, chondrocytes, and melanocytes. High S100B intratumoral levels were detected in melanoma and some carcinomas. The level of S100B expression was shown to correlate directly with the degree of malignancy with an inverse correlation between expression of S100B and the survival duration. Accordingly, we chose to measure this marker, using ELISA, as an indicator of the effectiveness of the tested treatment combinations. The results found were mostly expected. While none of the groups had an S100B level that is high enough to be detected at day 9, day 13 shows an increase of this marker in control groups as well as in the groups receiving single therapies of MPLA or anti-CTLA 4 and the group receiving a dual therapy of MPLA + 1-MT. Finally, at day 16, all the groups treated with single or dual therapies show elevated levels of S100B indicating a remarkable progression of the disease, while the triple immunotherapy group S100B levels were still not detectable. The only unpredicted result was the drop of S100B serum levels of both control groups below detection level at day 16. (Figure 15)

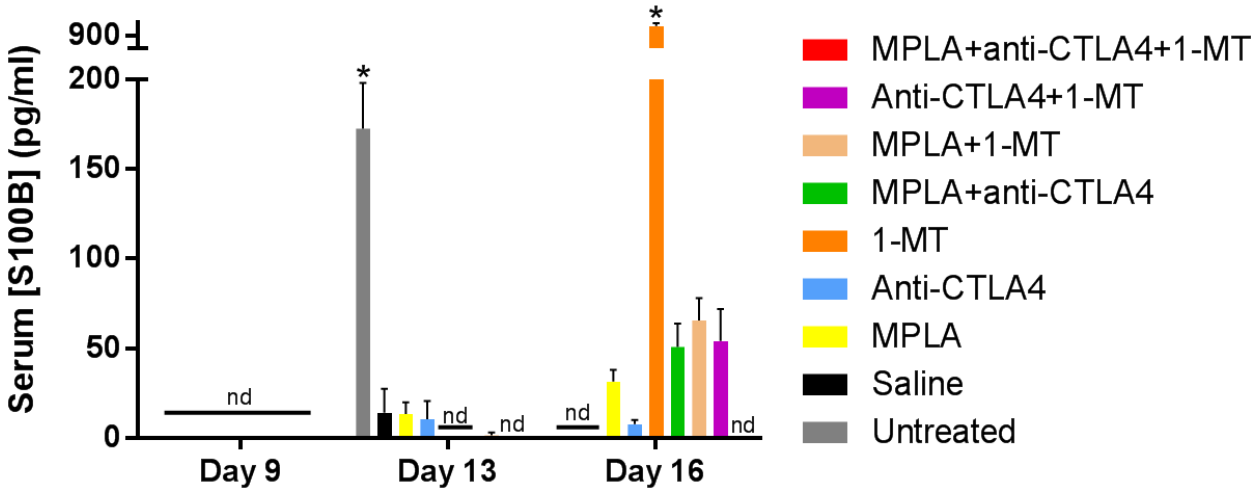

Figure 15: S100B levels. Serum S100B levels in the B16F10 melanoma mouse model treated with various immunotherapeutic regimens at days 9, 13 and 16 post tumor challenge (n = 3). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group. (nd: not detectable)

2. *Assessment of the tumor infiltrating immune cells*

To identify the mechanisms that possibly underlie the favorable survival and tumor progression outcome in the group receiving the triple immunotherapy, we looked at the tumor infiltrating immune cell populations including CD4+, CD8+ and regulatory T cells (Tregs) as well as natural killer (NK) cells. Tregs were mostly found to have the lowest level in the group treated with the triple combination among all the tested group. Concomitantly, this group also had mainly the highest levels of NK cells among all groups. Moreover, while the absolute numbers of CD3+CD4+ and CD3+CD8+ T cells showed little significant changes among the various groups, the ratios of the numbers of these cells to Tregs did. The ratios of CD3+CD8+ cells to Tregs and CD3+CD4+ cells to Tregs were

highest in the group receiving the triple immunotherapy compared to most of the other groups across the three testing time points. (Figure 16 and Figure 17)

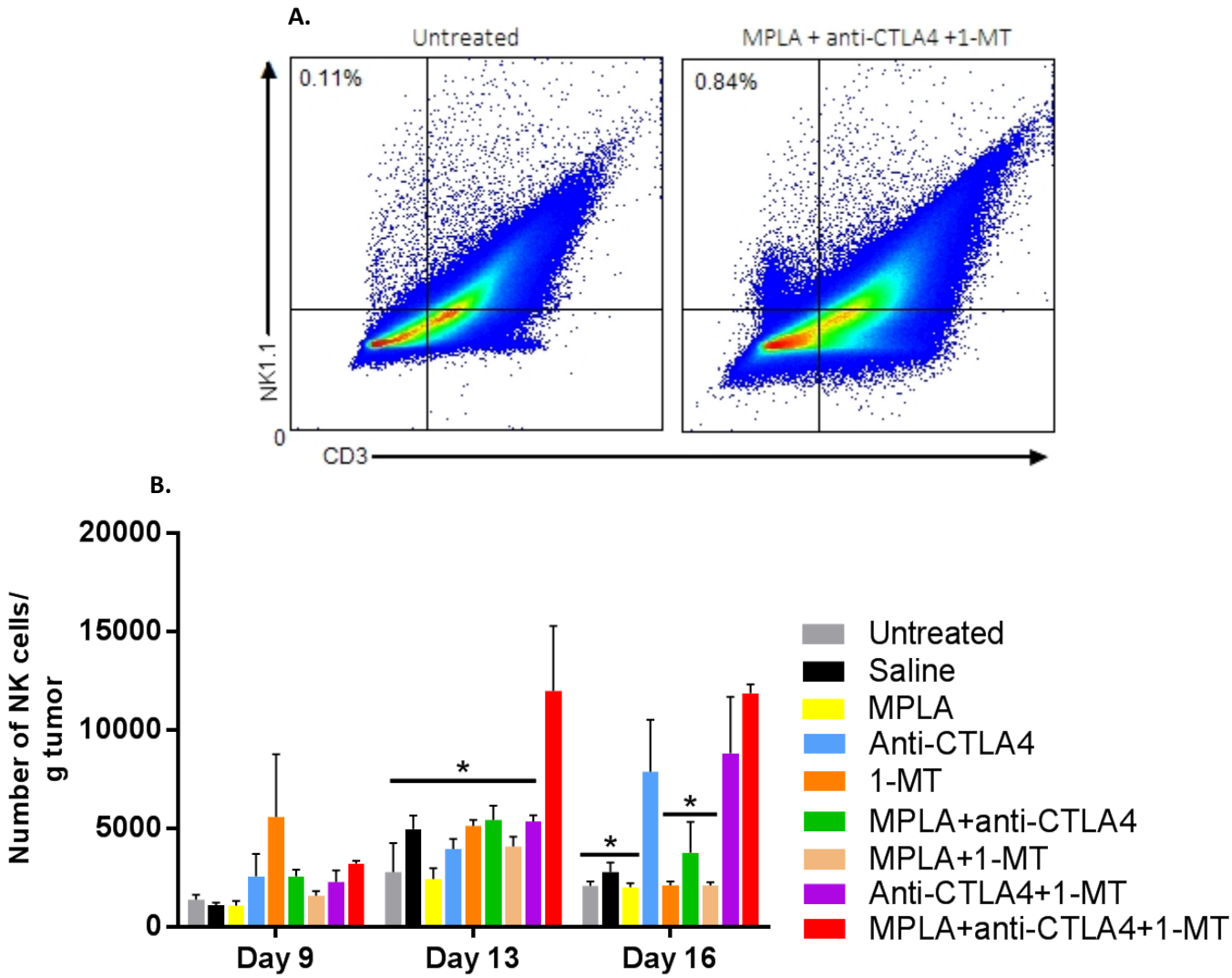

Figure 16: Tumor infiltrating NK cells. (A) Representative dot plots of NK cells for the untreated group and the group treated with the triple immunotherapy; (B) Number of NK cells per gram tumor in the B16F10 melanoma mouse model treated with various immunotherapeutic regimens (n = 3). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group.

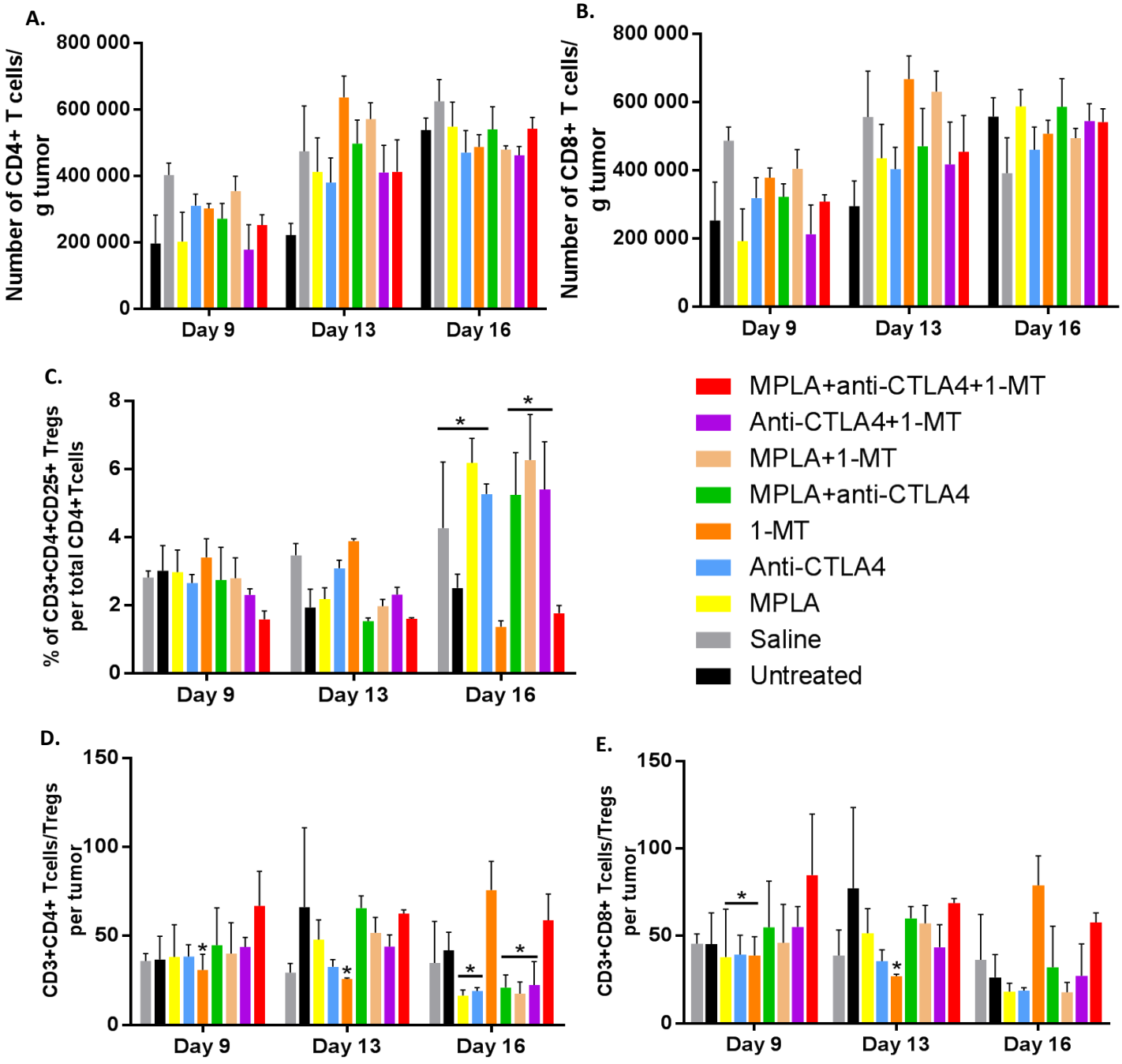

Figure 17: Tumor infiltrating immune cells. (A) and (B) show the numbers of CD3+CD4+ Tcells and CD3+CD8+ Tcells per gram tumor; (C) shows the percentages of Tregs per total CD4+ T cells per tumor; (D) shows the ratio of CD4+ T cells to Tregs per tumor and (E) ratio of CD8+ T cells to Tregs per tumor. All tumors are from B16F10 melanoma mouse model treated with various immunotherapeutic regimens (n = 3). *p < 0.05 compared to the MPLA + CTLA-4ab + 1-MT-treated group.

CHAPTER V

DISCUSSION

A. Absence of adverse events

The specific combination of immunotherapeutic agents examined in this dissertation was selected to provide enough elements for both the innate and adaptive compartments of the immune system to attack cancer cells effectively and for this attack to be sustained for an extended time through the inhibition of immunosuppressive mechanisms. While providing a much needed enhancement of the anti-cancer immunity, the use of immunotherapeutic combinations is often linked to the risk of eliciting immune related adverse events with a broad severity range. Therefore, the first concern for implementing our proposed therapy was the degree of adverse effects it might cause. However, the absence of abnormal clinical signs and the comparable weight gain rates between all treatment groups and the control group indicate that no major adverse events result from any of the single, dual or triple therapies tested. This interpretation is further verified by examination of the histological sections from the heart, lungs, liver and kidneys which showed normal anatomy for all experimental groups. Together, these results imply that all the tested treatment regimens had neither acute nor chronic toxic effect. One of the factors that potentially contributed to the prevention of chief adverse events might have been the intratumoral administration of MPLA. This mode of administration limits the systematic exposure to the drug and allows for the same therapeutic effect while using up to 25 times lower doses than those used in systemic administration such as intraperitoneal injections (Van De Voort et al., 2012).

B. Tumor model:

It is worth noting that the B16F10 cells used to create the melanoma mouse model in this study represent a very severe type of tumor that is notoriously hard to treat compared to other cell lines used in the development of murine melanoma models, therefore making the extrapolation of these outcomes to a wider variety of cancer types potentially more promising (Potez et al., 2018). In addition, the number of cells we initially injected to produce tumor in mice is the highest reported in the literature for this model and it is 15 to 20 times the minimum tumorigenic dose in wild-type C57BL/6 mice (Overwijk & Restifo, 2001). The purpose for using such an inflated number of cells to initiate cancer is to validate the effect of the tested therapeutic agents in treatment rather than prevention of tumors. The resulting model provides a better representation of what happens in the clinic, where patients only seek treatment after the establishment of the disease or even at late stages of its progression. This approach aims at minimizing the gap often seen between the promising results of preclinical studies and the much less favorable outcomes in the subsequent clinical studies.

C. Demonstration of the therapeutic effect

The tumor progression and survival outcomes verified the initial assumption that a triple immunotherapy consisting of CTLA4 blockade, TLR4 activation and IDO-1 inhibition has therapeutic advantage over all the single and also all dual combinations of the tested treatments. The group treated with anti-CTLA-4 showed the best results among the groups taking single therapies which was expected since both MPLA and IDO-inhibitors are used as treatment adjuvants opposed

to anti-CTLA-4 which is approved for treatment of a subset of melanoma patients as a single therapy (Glitza Oliva & Alqusairi, 2018; P. Sharma & Allison, 2015). Dual therapies showed slightly prolonged survival and delayed tumor progression but were outweighed by the results of the triple therapy. The speculated mechanisms underlying this outcome are an adequate activation of the innate immunity by MPLA and a sufficient inhibition of the tryptophan metabolism by 1-MT, which both complemented the anti-CTLA-4 effect of maintaining the effector T cells activity.

Possibly, MPLA, which is the safe form derived from lipopolysaccharide (LPS) with same antigenicity but reduced toxicity, has activated TLR-4 which is a fundamental member in the family to toll-like receptors (TLRs). Two signaling pathways downstream of TLR4 allows it to fulfill its primary function that is to activate the innate immune cells. The MyD88 independent pathway leading to the production of type-I interferons and the MyD88 dependent pathway leading to the secretion of several cytokines among which are IL-12, interleukin-2 (IL-2) and the tumor necrosis factor (TNF) (Lu, Yeh, & Ohashi, 2008). On one hand, Type-I interferons have been recently shown to have an anti-tumor effect and to improve the clinical outcome of cancer patients treated with immunotherapies, namely checkpoint inhibitors (Brockwell & Parker, 2019). On the other hand, some of the produced cytokines are known to enhance the immune responses against tumors. IL-2, for instance, is a T cell growth factor with multiple essential roles in the immune response and actually was itself one of the first immunotherapy drugs to be approved by the FDA for the treatment of metastatic melanoma.(Sun et al., 2019) IL-2 is a potent inducer of NK cells and cytotoxic T cells, both of which are major players against tumor progression. (Hashimoto et al., 2003) The activation of the TLR4 pathways also result in the release of interferon-γ (IFN-γ) which is an anti-angiogenic, anti-tumor factor. IFN-γ with other cytokines released through the activation of TLR4, such as IL-6, are also responsible for suppressing Tregs. (La Cava, 2008) Taken together, this cascade of events may explain the contribution of MPLA to the favorable outcome of the

combination. Nevertheless, the use of either anti-CTLA4, MPLA or both results in the upregulation of the immunosuppressive enzyme IDO-1 (David H. Munn, 2006; Qin et al., 2017). Hence, the addition of 1-MT to the anti-CTLA-4 and MPLA combination has given an enhanced therapeutic effect since it supposedly compensated for this upregulation.

It is worth noting that, in the current study, a single timeline of the different treatments was tested. The sequence of administration of the different drugs is based on previous studies which reported significant control of tumor progression (Holmgaard et al., 2013; Van De Voort et al., 2012). Using distinct sequences of administration may yield varying therapeutic effects.

D. Tumor infiltrating immune cells and serum cytokines

The mechanisms described above are reflected in the tumor-infiltrating cell population numbers which show a significant increase of NK cells in the triple immunotherapy group and a significant decrease of Tregs, thus giving a possible justification for the survival and tumor progression results which are in favor of the triple combination. In fact, the triple immune therapy resulted in a decreased number of Tregs that was sufficient to enhance the CD3+CD4+/Treg and CD3+CD8+/Treg ratio hence highlighting that this type of therapy alters the immune status towards an anti-tumorigenic environment that curbs regulatory mechanism. On day 16, this observation was also made with the 1-MT treatment despite this type of treatment being rather inefficient in our model indicating that the decrease in Treg numbers is not sufficient by itself and that other anti-tumorigenic effects play a more relevant role. Such anti-tumorigenic effects might be the significant increase in NK cell numbers which was mainly seen in the group receiving the triple immunotherapy. It is also worth noting that the data in this study only shows the numbers of cells.

Investigating the state of the tumor infiltrating immune cells, whether functional or exhausted, would give a valuable additional insight into the mechanisms responsible for the therapeutic effect.

As for the cytokines, we first tested the levels of serum IL-12 because it is a pleiotropic cytokine that is often considered an important player in the coordination of anti-cancer defense mechanisms mainly due to its role in bridging the innate and adaptive immunity, its potent stimulation of IFN-γ production and its inhibition of T-regs leading to the maintenance of the adaptive immune responses (Colombo & Trinchieri, 2002). Among other routes, the production of an active IL-12-p70 heterodimer is increased by "danger signaling" transduced through the toll-like receptor (TLR) family. For, instance, in macrophages, IL-12 is induced following the binding of TLR4 or TLR7/8 receptors to their respective ligands. In fact, we chose MPLA, a TLR4 ligand, as one of the immunotherapeutic agents in our combination partly because of its predicted ability to induce IL-12 production (Lasek et al., 2014). However when we tested for serum IL-12 levels in the different groups, to our surprise, the results showed a decrease of IL-12 levels in the groups with the most favorable outcomes. To explain these unexpected results we propose three possible reasons. First, when MPLA binds to its receptor, TLR4, the downstream signal transducer and activator of transcription 3 (STAT3) is activated through both the MYD88 and the TRIF pathways.(Fu et al., 2020) In a study using the same tumor mouse model as ours of B16 melanoma cells in C57BL/6 mice reported that STAT3 signaling can shift inflammation in the tumor microenvironment from an anti-tumor IL-12 by the transcriptional suppression of IL-12-specific p35 gene.(Kortylewski et al., 2009) Second, also following the activation of the TLR4 signaling pathway, the production of a number of downstream cytokines other than IL-12 is stimulated among which are type I IFNs. Type I IFNs are known for their suppression of IL-12 production. Third, apart from CTLA4, other inhibitory receptors normally function to maintain the immune responses under control. These include PD-1, LAG-3, and TIM-3. It was shown that the blockade of one immune inhibitory

pathway, which in our case was through the use of anti-CTLA4, leads to a compensatory upregulation of other checkpoint receptors, particularly, the T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) which was also found to inhibit the production of IL-12 by dendritic cells within a tumor environment (R. Y. Huang, Francois, McGray, Miliotto, & Odunsi, 2017; Lasek et al., 2014). Therefore, taking into consideration that the group receiving all 3 immunotherapeutic agents together is the one to probably elicit most of these IL-12 suppressing pathways concomitantly, we can justify its low levels of IL-12. This finding infers that, with this specific combination of treatments, the therapeutic effect was not essentially fulfilled by an increase in the cancer-suppressive IL-12 implicating that the addition of IL-12 therapy to the combination could possibly produce a synergistic effect against tumor. On another note, possibly IL-12 is an early marker and the fluctuations in its levels were not detected in the span of the time points used in this study. Finally, testing the levels of IFN-γ in future studies may give mechanistic insight into the mechanisms leading to the unexpected low IL-12 levels and shed light onto the discrepancy between these levels and the increase in NK cells.

As for the levels of serum VEGF, several studies have already demonstrated the association of VEGF expression with melanoma progression and that increased serum levels of VEGF are strongly correlated with poor prognosis in melanoma patients. (Dewing, Emmett, & Pritchard Jones, 2012; Rajabi et al., 2012) Moreover, a recent study showed that raised serum VEGF levels are associated with a decrease in overall survival of patients with advanced melanoma who are treated specifically with ipilimumab (anti-CTLA4). This finding suggests that serum VEGF levels are an important factor for predicting the therapeutic outcomes after immune checkpoint blockade (Ott, Hodi, & Buchbinder, 2015). Accordingly, VEGF being below the detection level in the group treated with our triple immunotherapy is a positive indication of the efficacy of this combination. This finding also sheds light into the mechanistic effect of the triple therapy.

Angiogenesis being an essential factor in tumor progression, the low levels of VEGF in the triple therapy group suggests that angiogenic inhibition is fundamental for the observed therapeutic effect.

Finally, we measured the serum levels of S100B which is a member of the S100 family. S100B is one of the first melanoma markers that was accepted as diagnosis tool for melanoma and still is a key marker in the clinic for staging, prognosis, evaluation of treatment result and metastatic growth. (Pitcovski, Shahar, Aizenshtein, & Gorodetsky, 2017) Possibly, S100B assumes this role of tumor progression indicator because one of its functions is to inhibit p53 calcium-dependent phosphorylation by protein kinase C. This will lead to the suppression of the p53 tumor-suppressor mechanism and therefore to an uncontrolled growth of the tumor. Despite the great efforts made to quantify blood compounds associated to melanoma, including cytokines, adhesion molecules, metalloproteinases, and melanin synthesis metabolites, S100B remains, to date, the most commonly applied biomarker in melanoma patients. An increase in circulating S100B levels strongly predicts a poor outcome including shorter disease-free and overall survival. In addition, several malignant melanoma studies reported that serum S100B values are correlated with tumor stage. It has also been demonstrated that the determination of serological S100B levels is effective for treatment monitoring in melanoma patients, whereby a marked elevation in S100B allows an early detection of treatment failure. (Danciu et al., 2015; Harpio & Einarsson, 2004) The data obtained in our study comes in accordance with this, as the groups with the most favorable tumor growth and survival outcomes presented the lowest, and even undetectable, circulating levels of S100B. With the exception of one observation, which the marked drop of serum S100B in the control groups at the thirst testing time point. A plausible explanation of this result is that the steep decrease in S100B is a sign that tumor in these mice have already reached its maximal proliferation stage especially that survival analysis show that by day 16 more than half of the mice in these groups had already died. S100B being a marker for the progression of the disease it could be argued that when the whole

organism is shutting down, even the disease progression would have slowed down. In support of this possibility is the reported drop of the serum S100B levels in 16-21% (depending on the cut-off value used) of melanoma patients when they reached stage 4 of the disease, which is considered a significant rate of negative results in patients with a high tumor load (Gebhardt, Lichtenberger, & Utikal, 2016). However, the overall conclusion based on the findings of the present study is that, as a prognostic marker of melanoma, S100B is probably not as rigorous in C57BL/6 mice as it is in humans since no linear correlation was shown.

E. Comparison to other combinations

Several combinations with anti-CTLA-4 have been reported in the literature. One of these combinations is that of anti-CTLA-4 with IDO-1 inhibitors. Studies have shown that this combination leads to an enhanced survival in comparison with treatment with anti-CTLA-4 alone (Holmgaard et al., 2013). This effect is not clearly highlighted in the present study. Such a discrepancy might be explained by the difference in the severity of the tumor model and the variable treatment doses and modes of administration among the different studies. Some studies have reported a more significant extension of survival in mice treated with this dual combination compared to the extension reported in this study, however, the current results are better assessed in the light of the severity of the model used which seems to have surpassed a certain threshold where treatments that were proven to work in milder models no longer had an effect. This emphasizes the effectiveness of the triple therapy, which despite the severity of the model, had a significant effect in extending survival and thwarting tumor growth in comparison to all treated groups throughout the monitoring period.

Another studied combination is that of anti-CTLA-4 with anti-PD1 which was approved for use in the clinic for the treatment of melanomas that do not express PD-1 (Glitza Oliva & Alqusairi, 2018; Li et al., 2018; Spranger et al., 2014; Wainwright et al., 2014). It has been demonstrated that this kind of combination has an additive therapeutic effect compared to treatments with either anti-CTLA-4 or anti-PD1 as it impedes tumors more efficiently from evading immune responses, however the response is dependent on the level of expression of PD-1 by tumor cells and therefore cannot be considered as standard treatment for all melanoma patients (Rocco, Gravara, & Gridelli, 2019; Tanvetyanon, Gray, & Antonia, 2017; Terheyden, Krackhardt, & Eigentler, 2019). This information can be used to optimize the triple combination used in the current study by adding more immune-checkpoint blockers and therefore providing a tighter siege around cancer which would further thwart its progression. This approach is particularly appealing for further investigation since some research has shown that combination strategies do not seem to amplify adverse events related to excessive inflammation or autoimmunity while enhancing the treatment efficacy, however this finding is still debatable (Mittal et al., 2014; Rocco et al., 2019; Tanvetyanon et al., 2017). Other tested combinations include the use of anti-CTLA-4 along with chemotherapeutic agents such as Imatinib or Dacarbazine, which have shown a superior effect to chemotherapies used alone but still with a limited success (Balachandran et al., 2011; Hervieu et al., 2013). As opposed to these mentioned combinations, the advantage of the currently proposed triple therapy is that it uses agents with mode of actions that are not dependent on the genetic characterization of the melanoma nor on the level of expression of certain markers such as PD-1 by the tumor cells and therefore could be employed to treat a larger proportion of melanoma patients with an otherwise poor prognosis.

F. Limitations

This study used a single tumor mouse model, the B16F10 melanoma cells in C57BL/6 mice. While this model is widely used and its validity as a representative melanoma model is very well illustrated in the literature, the use of multiple cell types and/or animal models is always of an added scientific value and it enhances the plausibility of extrapolating the findings of the study. Also, to assess the anti-tumor efficacy of the different combinations we used one defined dose for each immunotherapeutic agent. While this served the purpose of our study in determining the most effective treatment combination, testing for multiple dosages is essential to demonstrate a dose response relationship inherent to every drug testing plot. Finally, it should be taken into consideration that the modes of administration used in this study, whether intraperitoneal or intratumoral may not be convenient in the clinical setting. Consequently, the demonstrated lack of adverse events needs to be re-assessed should the drugs be administered differently.

G. Future perspectives

The present study provided a proof of concept that the triple immunotherapy, consisting of anti-CTLA4, MPLA and 1-MT, has an added therapeutic value compared to the single and even dual therapies of these agents, with no detectable, acute or chronic, adverse events. Therefore, it is likely to be a promising new approach to target cancer through hindering its multiple ways of immune evasion. Based on the findings of this study, future perspectives include the use of several doses for each treatment in attempt to establish a dose response trend and possibly optimize the anti-cancer therapeutic outcome of the triple combination. If intralesional administration of MPLA can be also applied in the clinic, an increase in the dose is expectedly safe and would possibly

enhance the therapeutic effect of the triple combination. An interesting future perspective would be also to evaluate the anti-tumor effect of PD1 inhibitors instead of anti-CTLA4 in the triple therapy; especially that recent studies have shown PD1 blockade to be more effective with lower toxicity. In addition, more investigations of the mechanisms underlying the survival and tumor progression outcomes should be conducted. These include phenotyping of the different tumor infiltrating immune cells, especially T cells to check for their level of exhaustion, and their localization in the tumor, histopathological testing on tumors to evaluate inflammatory stroma which is indicative of immune cells influx, as well as the assessment of the circulating levels of various cytokines. The data provided by these investigations may further validate the results of this study and, more importantly, may potentially explore new avenues in defining yet better combinations for melanoma treatment through the identification of key players in the anti-tumor immune responses. Finally, a future aim is to test this triple combination in a distinct tumor model to endorse the applicability of this therapy to a wider range of cancer types.

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