

Immunotherapy: A Novel Approach to Harness Immune System against Melanoma

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Abstract:

The incidence of melanoma, the most aggressive and the deadliest form of skin cancer, is increasing. Although Melanoma is curable if detected early with surgical resection or chemotherapy but with progression and metastasis in later stages, the median survival decreases rapidly and it will be deadly. In the last decade, dramatic advances in understanding the behavior of melanoma cells and their interaction with the immune system have led to several new treatment strategies. The immunotherapy has been known as the most efficient therapies that currently have changed the perspective of oncology. Immune checkpoint blockade is a significant branch of immunotherapy, which has revolutionized the treatment of patients with advanced melanoma. Blockade of inhibitory receptors, cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD-1) via monoclonal antibodies promote antitumor immune responses by T-cell which, cause improve survival and durable responses in melanoma patients. In this work, we will review melanoma risk factors, initiation and development, characteristics, and the pros and cons of the most commonly available treatments with special emphasis on immunotherapy and immune checkpoint blockade.

Table of Contents

Abstract	ii
Table of contents	iii
List of Figures	v
Introduction	1
Chapter 1: Melanoma, the Deadliest form of Skin Cancer	3
1.1 History	4
1.2 Epidemiology	5
1.3 Risk Factors.....	7
1.4 Diagnosis and Staging Melanoma.....	9
1.5 Pathology.....	12
1.6 Prognosis and Therapy	18
Chapter 2: Immune Checkpoint Blockade	21
2.1 Background	22
2.2 Immune System and Immune surveillance in Melanoma	23
2.3 Melanoma Cells Evasion.....	30
2.4 Immune Checkpoint Blockade.....	34
2.4.1 Biology of CTLA-4.....	35
2.4.2 CTLA4 Blockade in the Treatment of Melanoma.....	38
2.4.3 Biology of PD-1.....	40
2.4.4 PD-1 Blockade in the Treatment of Melanoma.....	42

Chapter 3: Other immune-modulatory approaches for Melanoma prevention/treatment.....	46
3.1 The other immunotherapy models.....	47
3.1.1 Adoptive T-Cell Therapy	47
3.1.2 Chimeric Antigen Receptor CAR-T.....	48
3.1.3 Targeting Cytokines.....	49
3.1.4 Cancer Vaccines.....	50
3.2 Targeted Therapy Drugs for Melanoma.....	52
3.2.1 BRAF Inhibitors Therapy	52
3.2.2 MEK Inhibitors.....	54
3.2.3C-KIT Inhibitors.....	55
3.2.4 VEGF Inhibitors	55
3.3 Conclusion	57
References:.....	58

List of Figures

Figure 1: Three type of skin cancer.....	2
Figure 2: Melanoma Incidence Rates, by Age and Sex, 2007–2011.....	6
Figure 3: Melanoma in every stage	12
Figure 4: MAPK and PI3K/AKT pathways.....	15
Figure 5: Four types of melanoma tumor shapes.....	17
Figure 6: The innate and adaptive immune responses.....	24
Figure 7: T cell activation.....	27
Figure 8: The death receptor pathway (apoptosis).....	29
Figure 9: Mechanism of CTLA4 and PD1 action in T-cell inactivation.....	35
Figure 10: Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) Function....	37
Figure 11: PD-1-mediated inhibition of T cells activation.....	42
Figure 12: CTLA-4 and PD-1 pathway blockade.....	45
Figure 13: (CAR)-T cell therapy.....	48

Introduction

Skin cancer is the most common type of cancer worldwide. Skin cancers are generally grouped into three large categories according to the type of skin cell they originate from Basal Cell Carcinoma (BCC), Squamous Cell Carcinoma (non-melanoma), and Melanoma.¹

Basal cell carcinoma (BCC), which is the most common type of skin cancer and composed almost more than 90% of skin cancer incidence, is a cancerous lesion that creates in the skin's basal cells (the deepest layer of the epidermis). These curable skin lesions are formed due to long exposure to UV light from the sun and it is common among whites. It usually starts as an open sore, red patch, shiny bump, or scar. It may also bleed if scratched. Although metastasis occurs rarely in BCC, late treatment can be problematic (figure 1).^{2 3}

Squamous cell carcinoma (SCC) is an uncontrolled growth of abnormal squamous cells (compose most of the epidermis). SCC is mostly created on sun-exposed areas, like the head, neck, ears, lips, back of the hands, and forearms. It is often like a scaly red patch, open sore, wart, and sometimes it has a crust or bleeds. It may also grow rapidly and can in case of progress it can lead to death. SCC is three times more common than BCC and usually, both SCC and BCC are curable by surgery (figure 1).^{2 3 4}

The least common and deadliest of all skin cancers is Melanoma and its incidence is rising rapidly in recent years. Melanoma is the most aggressive and dangerous form of skin cancer because it is rooted in the deepest layers of the skin and if not treated early, it can also spread to other parts of the body (also known as Metastasis). In addition to the alarming increased prevalence of Melanoma, the unexpected development of the disease and the inadequacy of current treatments can be the cause of melanoma being the most deadly of skin cancers. Therefore, considering the potentially lethal nature of the Melanoma,

immediate diagnosis and timely excision and treatment are crucial. Despite advances in diagnostic methods and tools, melanomas are sometimes difficult and this delay in diagnosis leads to the rapid progress of the disease to late stages of cancer at which the treatment is not feasible. Once the Melanoma advances to Stage IV, it will start metastasis and disseminate on other organs and it is very difficult to treat. After Stages III and IV (metastasis to other organs), the survival rate of 5 years is less than 10% and surgery is no longer an option. Until recently, the only treatment option available to patients with late-stage immunotherapy was chemotherapy; where several combinations of anti-cancer drugs were injected into patients to target rapidly dividing melanoma cells. In recent years, however, a new mode of treatment with very promising results has been developed called Immunotherapy, where doctors use patients' immune systems to fight off melanoma cells in the body. This novel and recently developed techniques are rapidly developing and being tested for different cancer types. In this dissertation, I will describe current methods, advancement, and mechanisms of action of this novel therapy in melanoma treatment. ⁵



Figure 1. Three types of skin cancer. Basal Cell Carcinoma (BCC) (A) Squamous cell carcinoma (SCC) (B) Melanoma (C). BCC can be shiny and also it may have a sore (ulcerated) area and fluid-filled sacs (cystic). SCC may look pink or red nodules and it may ulcerated. Asymmetry, irregular border, uneven Color, and diameter larger than 6 mm are characteristic of melanoma. Figure adapted from ^{6 7}

Chapter 1: Melanoma, the Deadliest form of Skin Cancer

1.1 History

Dr. John Hunter, a Scottish surgeon in 1787, did the first successful surgical removal of the melanoma. He described it as a “cancerous fungous excrescence.”⁸ Melanoma was first described by a French physician Rene Laennec (1781-1826) in 1804 in a speech and he coined the term “melanoses” to describe these tumors.^{8 9} It was later in 1820, in which an English general practitioner named William Norris presented the first genetic description of Melanoma, and in 1838; Sir Robert Carswell used the medical term “Melanoma” to describe pigmented malignant lesions of the skin. Also, Samuel Cooper was the first who formally proved, in 1844, that the advanced melanoma was untreatable and its early detection is very important. In 1956, Henry Lancaster suggested a connection between ultraviolet radiation (UV) from exposure to sunlight to increased incidence of melanoma, which showed that the characteristics of the skin include skin color, texture, hair color, eye color and reaction to the sun had an impact on melanoma development.⁸

Recent advancements demonstrate a strong link between UV irradiation and mutations in melanocytes, which promote Melanoma initiation and development and it is clear it is caused by ultraviolet radiation and results from the malignancy of melanocytes. Also, it is understood that some individuals are at higher risk of Melanoma due to genetic susceptibility, skin characteristics (skin color, texture, hair color, eye color, and reaction to the sun), and polymorphisms in the melanocortin receptor-1 (MC1R) or Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A) genes.⁸ Approximately 40% of inherited melanoma is related to a mutation in the CDKN2A gene located on chromosome 9p21.3. It can regulate cell cycle entry at the G1 checkpoint and stabilize the expression of p53. MC1R belongs to the melanocortin receptors group, which has a key role in skin protection against damaging ultraviolet radiation by regulating melanin production.¹⁰ MC1R

activation is important in the regulation of melanocyte cell division and MC1R overexpression cause to create melanoma.¹¹ However, the precise mechanism of the progression of a melanocyte into melanoma remains unknown and, as a result, methods of prevention are limited. This area is an appropriate subject for future research.

1.2 Epidemiology

Melanoma is one of the most aggressive and deadliest types of skin cancer, which comprises about one percent of skin cancer cases reported each year.¹¹ Approximately 50,000 deaths from melanoma are reported each year, so that in 2017, approximately 87,110 individuals were diagnosed with melanoma, in the United States alone. Nevertheless, it still represents less than 5% of all skin malignancies.¹² Incidence of Melanoma increased over the past decades. The diagnosis and spread of invasive melanoma were rare between 1950 and 1954. By 2007 and during those six decades, the overall incidence of Melanoma had increased more than 17-fold in men (1.9 to 33.5 per 100,000) and more than nine-fold in women (2.6 to 25.3 per 100,000). So that, the death rate has increased more than tripled in men (1.6 to 4.9 per 100,000) and doubled in women (1.3 to 2.6 per 100,000).^{13 14}

It has been estimated that 96,480 new cases of Melanoma will be diagnosed in the U.S. in 2019. Fortunately, mortality trends of Melanoma have decreased by about 2% per year in adults 50 years of age and older and by about 4% per year in those younger than 50. The risk of melanoma is more common in young adult women compared with men before the age of 50. It may be related to differences of age and sex in occupational and recreational exposure to ultraviolet radiation (including the use of indoor tanning), and perhaps early detection practices and use of health care. After the age of 50 years old, the rate of incidence in men is much higher than in women. (Figure 2)^{15 16}

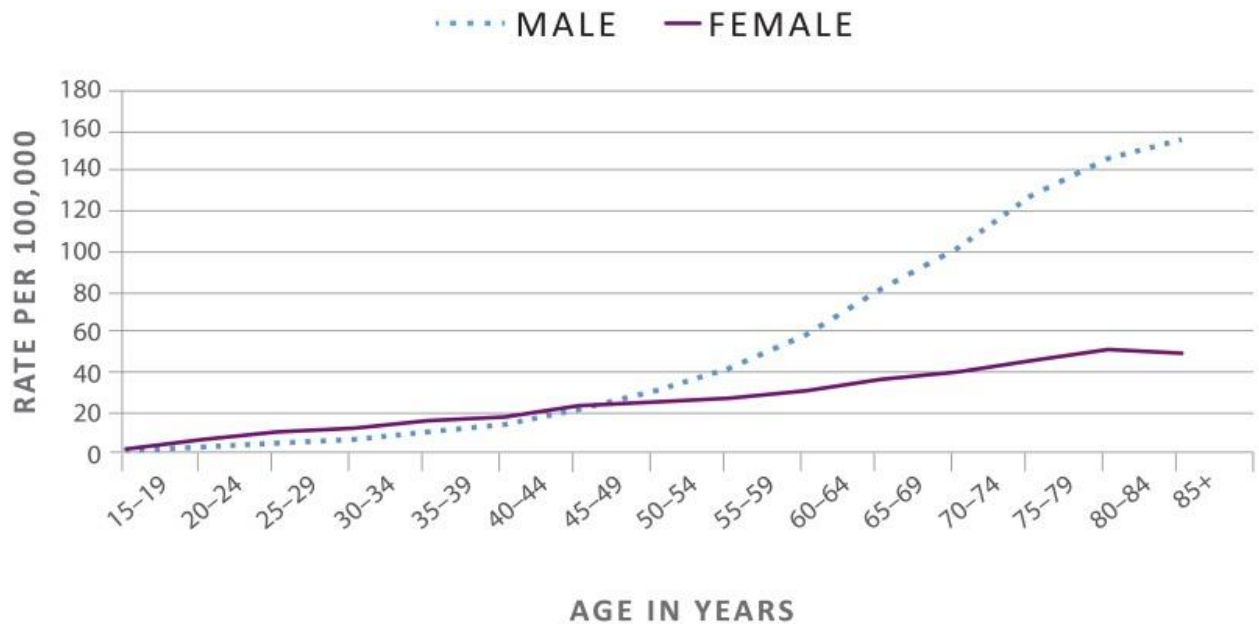


Figure 2. Melanoma Incidence Rates, by Age and Sex, 2007–2011.

After age 50 years old, Melanoma incidence in men is more than in women. Men are less diligent about protecting themselves from the sun and historically, men may have more sun exposure due to outdoor work. Also, men's skin compared with women's skin has more collagen and is thicker, with less fat under it. So, causes more skin damage from UV exposure and increasing the risk of developing Melanoma. Figure adapted from ¹⁶

If detected early, Melanoma is curable with a 5-year relative survival rate of 99% however when it reaches the fourth stage (metastasis) the survival rate drops to only 20%.¹¹ The geographic area and racial skin phenotype and differences in sun exposure are important in the occurrence of melanoma. The prevalence of melanoma is more common in the western world. Australia and New Zealand have the highest melanoma incidence and mortality in the world. Melanoma incidence is more common in the white population (2.4%) because dark skin contains more melanin and better protection against UV-induced damage. However, it may occur in dark skin individuals (0.1%).^{17 18 19}

1.3 Risk Factors

Melanoma like other cancers begins and progresses with gene mutations/abnormalities. These genetic mutations in melanocytes may be hereditary or non-heritable. The most important and harmful environmental risk factor for Melanoma is ultraviolet radiation (UV) from the sun. Besides, artificial UV rays like indoor tanning devices are the other reason for Melanoma development, especially in younger individuals. Recently, scientists have shown that users of indoor tanning devices had a 16–20% higher risk of melanoma development than nonusers. UV rays can both change gene expression and induce mutations. When UV radiation passes through cell skin layers and hits melanocytes' DNA, it causes damage to the DNA, increasing the risk of melanoma. When UV radiation strikes the melanocyte's DNA, chemical changes may occur, which include the opening of bonds and reforming new bonds, creating an error in the DNA.^{14 20}

To evaluate the connection between UV radiation rays and melanoma development, Zaidi et. Al. designed several experiments on genetically engineered mice that their melanocytes express a green fluorescent protein to trace and isolate melanocytes after UV radiation. In sum, their study shows that prolonged exposure to UV light caused sunburn on the skin of these mice. Additionally, they noted an increase in the number of melanocytes in the outer layer of the skin. Cell analysis showed that the melanocytes gene expression profile is different from not irradiate melanocytes. Further analysis showed that melanocytes in UV irradiated mice were more and they are activated by a set of known inflammatory proteins called interferon- γ (IFN- γ) which promote cell proliferation and DNA damage. These changes were not observed when this protein was blocked. This study shows that prolonged exposure to ultraviolet radiation will affect the gene expression profile of melanocytes and promote the expression of chemokines, which attracts macrophages in the irradiated spot, which secrete INF gammas that in

turn activate melanocytes and enhance expression of immune evasion proteins in them. By examining human melanoma tumors, they found that 70% of these melanoma tumors contained macrophages that produce IFN- γ . Thus, macrophages producing IFN- γ play an important role in enhancing the UV-induced melanoma and Interferon- γ links UV to melanocyte activation and promotes melanoma.²¹

Except for tumor microenvironmental alterations, melanoma is associated with genetic alterations of all human tumors. The most frequent mutations that are in sun-exposed skin melanomas, affect genes that control the central cellular process, include proliferation called Rapidly Accelerated Fibrosarcoma (BRAF), Neuroblastoma Rat Sarcoma Virus (NRAS), growth and metabolism [phosphatase and tensin homolog (PTEN) and KIT proto-oncogene receptor tyrosine kinase (RTK)], resistance to apoptosis [tumor protein p53 (TP53)], cell cycle control [cyclin-dependent kinase inhibitor 2A (CDKN2A)] and replicative lifespan [telomerase reverse transcriptase (TERT)]. The BRAF is a human gene on chromosome 7 (7q34) that encodes a protein called B-RAF. B-RAF play role in the mitogen-activated protein kinase (MAPK) signaling pathway that regulates critical cell functions like cellular growth, differentiation, proliferation, senescence, and apoptosis.^{22 23} Activating mutation of the BRAF gene is the most frequent genetic alteration in melanomas that is observed in about 50% of melanoma. The mutation of the BRAF gene increases the proliferation and survival of melanoma cells. The most common BRAF mutation happens at amino acid 600 and in about 90% of cases valine is substituted with glutamate in the 600 codons (V600E) and 10% with lysine (V600K) or arginine (V600R).^{24 25} N-RAS is the second most frequent genetic alteration in melanoma cancer and a subset of the RAS proteins family that encode N-RAS protein. The RAS proteins regulate growth and act as molecular switches that transmit signals from RTKs at the cell surface to transcription factors and cell cycle proteins in the

nucleus. Mutation of N-RAS has observed in 25% of melanomas that can keep RAS protein in the active state and cause to overgrowth of melanoma cells.^{24 26 27}

According to recent studies, host risk factors including the number of congenital and acquired melanocytic nevi (moles), genetic susceptibility, and history of sunburn are the factors in melanoma development. The type and size of nevi are very important so having many moles or unusual moles that are big or asymmetric and with a mixture of colors can increase the risk of melanoma development. The color of hair (blond or red hair) and light-colored eyes are the other risk factors of melanoma.^{14 28}

Since Melanoma development has a multifactorial etiology with risk factors in family history, age, gender, and race, there is no preventive approach that can guarantee melanoma-free life. However, to decrease the chance of melanoma development, individuals, especially children, must be protected against environmental factors such as ultraviolet radiation. For this purpose, it is advisable to use sophisticated hats, sunscreens, and sunglasses in open environments. As mentioned, the use of tanning beds (artificial UV rays) is a risk factor for melanoma incidence because ultraviolet rays can damage the skin in the long run. Avoiding the use of tanning beds and solar bulbs can help prevent melanoma. Permanent skin checking to find the suspicious and abnormal mole and check it by a doctor is one of another way to prevent melanoma.^{29 30}

1.4 Diagnosis and Staging Melanoma

The easiest way to diagnose melanoma is by examining different parts of the body by a dermatologist. These studies evaluate the presence of abnormal moles with itching or bleeding and large lymph nodes. The clinical diagnosis of melanoma is performed in several ways. Since the

lesions resulting from melanoma are specific in terms of color and morphological pattern, it requires skilled physician evaluation. For thin or non-pigmented melanomas, imaging methods (such as CT scans) and dermoscopy (chemiluminescence microscopy) can be very informative. Dermoscopy uses a light source for the Review and diagnosis of skin lesions. Other imaging techniques such as in-vivo reflectance confocal laser microscopy, which is computer-aided and has multispectral digital analysis, and electrical impedance spectroscopy can be among new devices, which aid physicians in the diagnosis of melanoma.³¹

Another powerful and sophisticated approach in melanoma diagnosis is the skin biopsy followed by histological examination. In this method, the dermatopathologist (with a local anesthetic) removes the suspected melanoma and examines it under a microscope by a skin specialist. The sample is intended to be tested in the laboratory. By doing these tests, the pathologist determines the stage of the spread of the tumor by examining the thickness of the tumor and mitotic rate (the portion of cells that are actively dividing). These tests help to choose the best treatment. There are several types of skin biopsies including Shave (tangential) biopsy, Punch biopsy, Incisional and excisional biopsies. Of course, in some cases, the biopsy is not effective in detecting melanoma. The other method is Lymph Node Status that doctors examine the lymph nodes to determine whether any cells have gone beyond the initial site because the melanoma can be very small on the skin while it spreads to the lymph nodes and other organs, such as the lung and the brain. Also, It may there are no symptoms on the skin while it spreads inside the body and is used fine needle aspiration (FNA) Biopsies for large lymph nodes near a melanoma to detect the spread of melanoma.^{32 33}

Genetic testing is most often done for people with advanced melanoma. In this test, the sample is examined to check mutations in a gene like the BRAF V600 gene. The newest test for genetic testing is DecisionDx-Melanoma. In this experiment will show a specific pattern

of genes expressed in melanoma cells that show the early melanoma tends to expand or not.^{34 35}

Biomarkers are tumor- or host-related factors that associate with tumor biological behavior and patient prognosis. A biomarker explains any measurable diagnostic indicator to recognize the risk or presence of disease. Identifying biomarkers is one of the goals of cancer research as early detection is always crucial. In this way, biomarkers are necessary to help in both the diagnosis and prognosis of melanoma and to lay out a proper and personalized treatment plan.³⁶

Once the presence of melanoma is confirmed, the stage of progression for choosing the best treatment should be determined. To determine the stage of melanoma, the lesion and surrounding healthy tissue are examined by microscopic methods and the doctor determines the stage of melanoma based on the thickness of the tumor and other characteristics. In this way, the thickness and extent of the tumor are examined, spread to nearby lymph nodes, and spread (metastasize) to distant parts (other organs). By analyzing these characteristics, determine the stage of melanoma progression.^{37 38} At initial stages When when melanocyte cells start to accumulate mutations and accumulate proliferate, they form "melanocytic neoplasm" under the skin. These neoplasms can be benign or malignant. The benign kind is nevi (Mole) and the malignant form is melanoma. In malignant melanoma create a disorder in the proliferation and growth of melanocyte cells. Eventually, this proliferation lead to penetrating the underlying tissue in the fourth stage such as the lungs, liver, and brain which is not surgical and it occurs metastasis.^{39 40} (Figure 3)

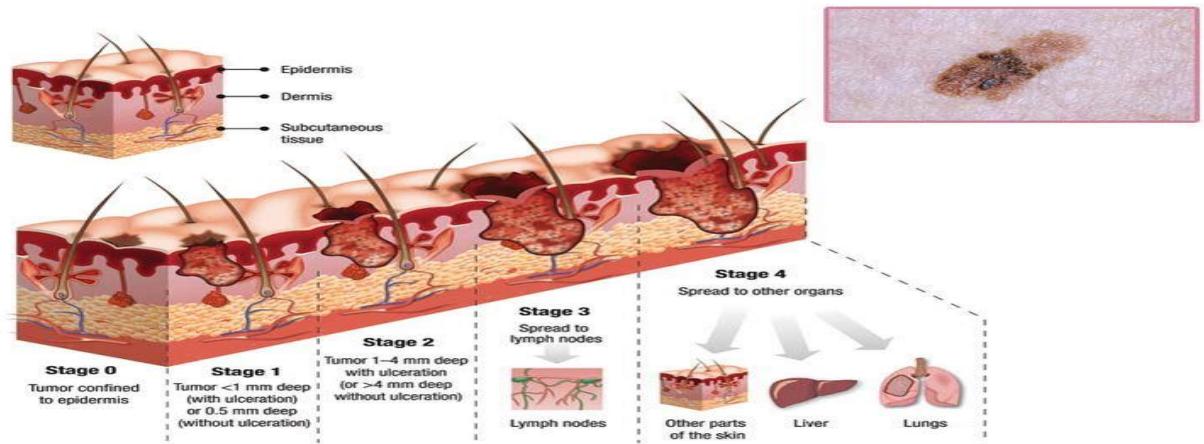


Figure 3. Melanoma in every stage. Stage 0: Melanoma is confined to the epidermis and it has not spread to nearby lymph nodes or distant sites. This stage is also known as melanoma in situ. Stage 1: Thickness is no more than 2mm and without spread to nearby lymph nodes or distant sites. Stage 2: Thickness is at least 2mm and maybe thicker than 4.0 mm. It may or not be ulcerated and without spread to nearby lymph nodes or distant sites. Stage 3: Usually thickness is more than 4.0 mm. It has spread to very small areas of the skin (satellite tumors) or to the lymphatic channels of the skin surrounding the tumor (without reaching the lymph nodes). It may have spread to lymph nodes that are clumped together. It has not spread to distant sites. Stage 4: The melanoma can be any thickness, it might be ulcerated, and It might have spread to nearby lymph nodes. The tumor has spread to distant lymph nodes or organs such as the lungs, liver, or brain. The figure adapted from^{41 42}

1.5 Pathology

Melanoma is an abnormal growth and proliferation of melanocyte cells usually initiated by genetic mutations, which accumulate over time. Melanocytes are neural crest-derived cells, which have the main role in producing pigment. These skin cells produce the protective pigment melanin, which gives a tan or brown color to the skin and helps protect the deeper layers of the skin from the harmful effects of the sun. These cells are found in the epidermis and hair follicles, along with mucosal surfaces choroidal layer of the eye, and in the meninges.²⁸

Studies show the incidence of melanoma increases due to genetic mutations and changes in the tumor microenvironment and dysregulation of the nuclear factor (NF)- κ B pathways, which results in overexpression of proteins promoting tumor invasion and surrounding infiltration. Melanoma is a highly metastatic tumor. Degradation of basement membranes and extracellular matrix is an essential and important step in melanoma cell invasion and metastasis formation. Matrix metalloproteinases (MMPs) and their tissue inhibitors have a key role in this process. Overexpression of two family members of matrix metalloproteinases like MMP-9 and MMP-2, leads to degradation of the components of the extracellular matrix, thus favoring tumor cell infiltration and spreading through the bloodstream. It has been shown DNA methylation, which can also occur by radiation, leads to MMP-9 and MMP-2 overexpression in melanoma. Also, NF- κ B induces the overexpression of MMP-9.⁴³

Physiologically, skin cells, keratinocytes, can also increase melanocyte proliferation by the production of MSH (melanocyte-stimulating hormones) hormone which binds to melanocortin 1 receptor (MC1R) on melanocytes and induces their proliferation. UV irradiation leads to melanocytes malignant transformation by two different mechanisms: 1) the direct transformation of normal melanocytes in neoplastic cells by the occurrence of several mutations affecting the overexpression of proto-oncogene and inhibiting tumor suppressor genes. 2) The transformation of melanocytes into benign "nevi" that in 80% of cases harbor the mutation BRAF V600E. Also, UV rays can determine the onset of additional genetic mutations, like TERT and CDKN2A lead to the malignant transformation of the nevi.^{43 44}

Genomic alterations that were mentioned in 1.3 section, typically lead to the aberrant activation of two main signaling pathways in melanoma: 1) The RAS/RAF/MEK/ERK signaling cascade [the other name is the MAPK pathway]. 2) The phosphoinositol-3-kinase

(PI3K)/AKT pathway. (Figure 4)⁴⁵ The MAPK pathway plays a role in the transduction of extracellular signals, like growth factors and hormones to the nucleus, because of the expression of genes that involve cell proliferation, differentiation, and survival. MAPK signaling helps cell growth and survival and it is active in nevi. RAS family members are activated by receptor tyrosine kinases and signal through effector proteins, including RAF kinases and PI3K. Acquired melanocytic nevi keep oncogenic mutations in BRAF that is the dominant oncogene associated with melanoma. Also, it has been shown that MAPK activation plays an important role in the biology of different types of cancer and is the most common pathway activated in melanoma. The PI3K-AKT pathway is one of the most important mechanistic pathways that have a role in cell proliferation and survival. The evidence showed that activating this pathway has an important role in melanoma especially in regulating activation of RAS-RAF-MEK-ERK signaling at the same time. As a result of this activation is melanoma proliferation and metastasis.^{31 46} Mutation in the KIT gene is found in 2–8% of all malignant melanomas. The receptor tyrosine kinase KIT is involved in melanoma proliferation and survival through the PI3K/AKT and the RAS/RAF/MEK/ERK pathways.

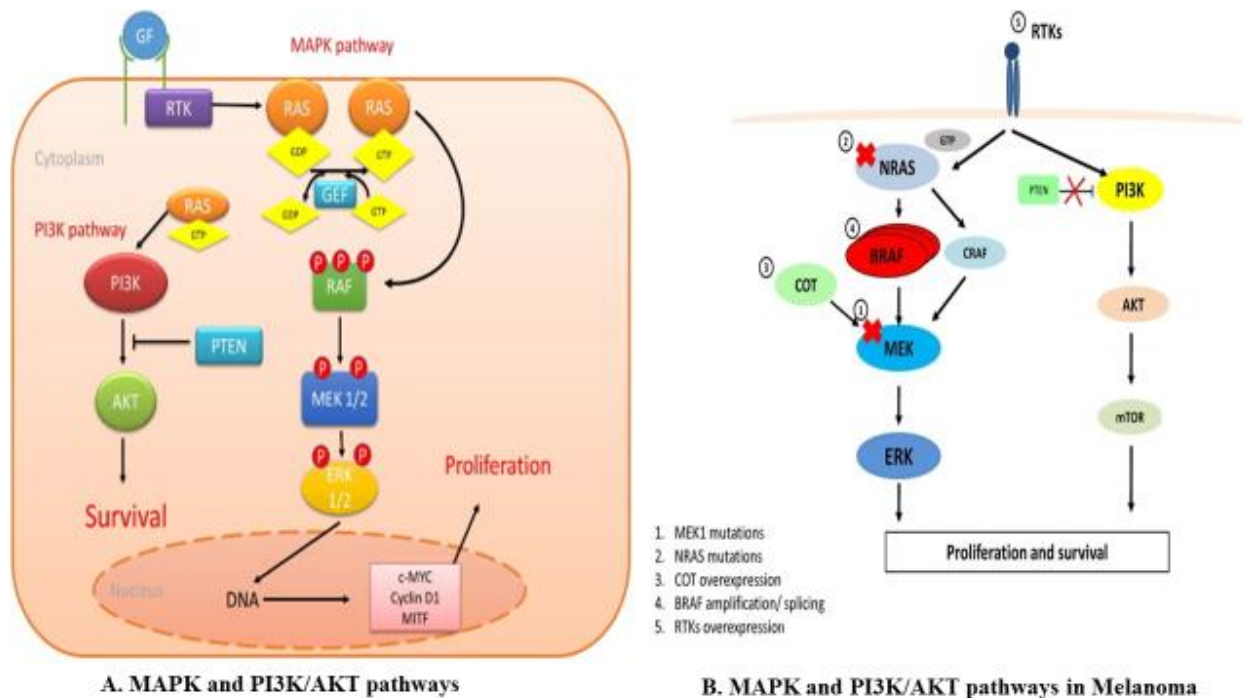


Figure 4. MAPK and PI3K/AKT pathways. **A:** Activation of receptor tyrosine kinases ((RTK) by growth factors (GF) can initiate MAPK signaling. Once RTKs activation, Ras-GDP change to active Ras-GTP. Then activated RAS phosphorylate RAF and activate it. After RAF activation, MEK1 and MEK2 are phosphorylated and activated by RAF and led to ERK1/2 activation. As a result, translocation and regulation of several transcription factors in the nucleus, such as cyclin D1, c-MYC, and MITF. Also, RAS-GDP can promote survival through the PI3K signaling pathway, by activating AKT. **B:** Mutation in genes can change proliferation and survival. The figures adapted from⁴⁵

Usually, this mutation is common in Acral lentiginous melanoma. Point mutations and gene duplications of C-KIT frequently lead to increased expression of the KIT protein, which may alter melanocyte proliferation, cell death and contribute to tumorigenesis.^{40 46} According to studies, there is a BRAF (a serine/threonine-protein kinase encoded on chromosome 7q34) mutation in about 40 to 60 percent of cases of melanoma. It is one of the reasons for the proliferation and growth of the cells. In the development of melanoma, the most common BRAF alteration there is V600E mutation caused by UV radiation, which

changes valine and glutamic acid. This mutation constitutes about 90% of BRAF activating mutations or about 50% of all metastatic melanomas. The second most common mutation is V600K mutation that changes valine to lysine. Studies have shown that BRAF mutation does not have an effect on the onset and early stages of melanoma. In contrast, this mutation is involved in advanced melanoma and metastatic.⁴⁷ NRAS GTPase or neuroblastoma RAS oncogene is the other source for molecular changes associated with malignant melanoma. The activating mutations usually are on the Q60/61 and G12/13 codons. In patients with N-RAS mutations, there are thick vertical growth tumors that its cause is the heightened cell proliferation rates induced by the NRAS mutation. Concurrent mutations in both BRAF and NRAS are rarely found in the same patient. NRAS mutations that lead to melanoma tumors are almost similar to the BRAF mutations.^{46 47} Tumor suppressor gene p53 has been implicated in a variety of human malignancies, such as breast, colorectal, lung, and prostate cancer. The p53 protein is activated by cell stress or DNA damage and induces cell death. The role of p53 protein in melanoma is not clear exactly. Based on immunohistochemistry analysis, p53 mutations are very different so that in some cases mutations in the TP53 gene are found in about 11% of melanomas, and in the others, it maybe 85%.^{40 48}

In general, Melanoma tumors can be categorized into four distinct shapes: 1- Superficial, 2- nodular, 3- Lentigo maligna, and 4- acral lentiginous melanomas. (Figure 5) Superficial spreading melanoma makes almost 70% of all melanoma skin cancers and it is the most common type of melanoma skin cancer. Its growth is radial growth and it means it diffuses on the surface of the skin. Of course, it can grow down into the skin too. It is often flat and thin (less than 1 mm thick) with an uneven border. It is different in color and has different shades of red, blue, brown, black, grey, and white. It usually develops on the central part of the body (trunk), arms, and legs. The second most common type

of melanoma skin cancer is Nodular melanoma (about 15% to 20%). It grows down into the skin. Its color is usually black, but sometimes can be red, pink, or the same skin color and develops on the face, chest, or back. The third type of Melanoma's appearance is referred to as Lentigo maligna. It usually occurs in older people (about 10% to 15%). Its size is large and flat and is reddish-brown with an uneven border. It occurs in areas exposed to sunlight such as the face, arm, and ear. Another less common shape of Melanoma tumors is called Acral lentiginous melanoma that usually presents in people with dark skin (5% of all melanoma). It is small, flat and it is often dark brown or black. It grows on the soles of the feet, on the palms of the hands, or under the nails.³¹

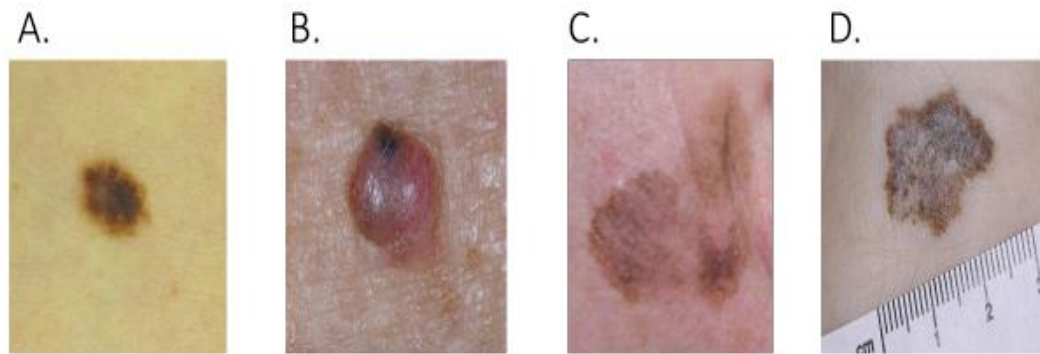


Figure 5. Four types of melanoma tumor shapes. Superficial (A) Nodular(B) Lentigo maligna (C) Acral lentiginous melanomas (D) Superficial spreading: radial growth on the surface of the skin and less than 1 mm thick; Nodular melanoma: Its color is usually black and pink; Lentigo maligna: Its size is large and flat, and is reddish-brown with an uneven border; Acral lentiginous: small, flat and often dark brown or black. Figure adapted from^{49 50}

1.6 Prognosis and Therapy

Cancer prognosis is a general term used to refer to the treatability of cancer. It is very important to know the prognosis of a malignancy before starting any treatment plan. Prognosis and survival depend on many factors. In melanoma cancer, the primary thickness of the tumor is a very important prognostic marker for Melanomas. With the spread of the tumor thicker than 4 mm, the prognosis becomes weaker. Another prognosis factor in Melanoma tumors is the presence or absence of a wound around the tumor. The presence of wounds shows that Melanoma cells are most likely entered the bloodstream and spread to other organs. As a result, this is negatively affecting prognosis in an undesirable direction. Mitotic rate is another factor of melanoma prognosis. It measures the growth and division of mitosis of cancer cells in a specified amount. The prognosis is harder when the mitotic rate increases. Also, the prognosis of older people is weaker. In the higher stages, with the development and spread of melanoma to the lymph vessels and other tissues, the prognosis is usually not positive. Patients with distant metastases (stage IV) usually have the worst prognosis.⁵¹

Depending on the Melanoma location, stage, and genetic profile of the cells the therapeutic options will vary. The first and common treatment option for melanoma in the early stage is surgery.

1.6.1 Surgical Removal

If melanoma is detected in the early stages (stages 0–2 or localized melanoma) and is thin, treatment is achievable with surgery. The surgery procedures differ based on the clinic-pathologic features of the tumor. Excision consists of margins of 0.5 cm for melanoma in its original place, 1 cm for tumors with a thickness of up to 2 mm, and 2 cm for tumors thicker than 2 mm. After determination, the lesion on the skin (as a melanoma part) and a small amount of surrounding normal skin were removed completely by the expert physician. After that, the total

recovery time will vary depending on the thickness of the melanoma and the extent of the surgery required (usually 2 weeks).³¹

1.6.2 Radiotherapy (RT)

After surgery, radiotherapy (RT) is known as the most common and effective non-surgical mode of locoregional therapy of melanoma. RT is a physical agent in which a beam of high-energy particles directed at a tumor to destroy Melanoma cells by damaging their DNA. Generally, radiotherapy is done in several ways based on the treatment intent and the time point which included, upfront radiotherapy as the main treatment modality, replacing surgery, after surgery, radiation for Metastatic Melanoma. Radiotherapy as primary therapy is utilized instead of surgery for all types of primary melanoma lesions (more in lentigo maligna melanoma). Sometimes RT is done in the area where surgery was performed to remove the melanoma. It may reduce the chance that the melanoma will come back in that specific area. In addition, RT is used as adjuvant therapy to help Melanoma patients with metastasis.^{52 53}

1.6.3 Chemotherapy

The earliest treatment option for advanced melanoma is chemotherapy but despite the current improvements, its effectiveness is transient and in many cases, the cancer cells start to develop resistance to anti-cancer drugs and relapse. Nevertheless, this is important in the treatment of melanoma. Dacarbazine and Temozolomide are two examples of chemotherapy drugs for melanoma.⁵⁴ Dacarbazine (DTIC), also known as imidazole carboxamide, is an alkylating agent and it has been approved since 1972 for metastatic melanoma. Since it is a narcotic drug, it is necessary to become 5-(3-methyl-1-triazine) imidazole-4-carboxamide (MTIC) in the liver. Its dose is 850 to 1,000 mg/m² every 3

weeks. The other drug is Temozolomide (TMZ) that administered orally and has been used in advanced melanoma. ³⁵

As it was said, because melanoma is an invasive and highly metastatic cancer, it requires advanced treatments. These methods are usually used after the second stage. Immunotherapy is the advanced method for the treatment of melanoma that will be dealt with in the coming chapters to be detailed. The other current therapies for melanoma is targeted therapy.

Chapter 2: Immune Checkpoint Blockade

2.1 Background

As discussed in the previous chapter, melanoma treatment varies by stage. In the early stages, it can be treated with surgery but at advanced stages of III and IV with metastasis, this cancer becomes unstoppable and it needs further treatments such as chemotherapy. However, this method is not effective because the 5-year survival rate is still very low. Therefore, a more sophisticated treatment option is needed. Recent discoveries about immune checkpoints and mechanisms of immune system interactions with cancer led scientists to a novel way for melanoma treatment called immunotherapy.

Immunotherapy is an approach in oncology that has been used in recent years and it works by promoting or modifying the immune system's response to fight tumors. Immunotherapy has been around for centuries and its origins date back almost 2,000 years, but it is difficult to prove. However, the beginning of scientific reports on immunotherapy dates back to the 18th century. Among the first scientific efforts to modify patients' immune systems to treat cancer were two German physicians, Fehleisen and Busch, who found significant tumor regression after erysipelas infection. Then William Bradley Coley knew as the father of immunotherapy later made significant progress and was able to control the body's immune system to treat bone cancer in 1891. Coley injected streptococcal organisms into a patient with inoperable bone cancer and the infection that he had created caused the side effect of shrinking the malignant tumor. As a result, his work was successful. After years, Ruth and John Grahams conducted extensive studies on immunity and the cancer vaccine in 1945. They tested 114 patients in a cohort of gynecologic cancer patients with adjuvanted tumor lysate. incidence of remission or stable disease was 22% but their work went largely unnoticed because the existence of T cells and their crucial role in immunity was not obvious until 1967. The discovery of interferon was

another important achievement in the field of oncology. Finally, in the 1980s, immunotherapy was again considered and optimism reiterated that immunotherapy might be used to treat many diseases, including cancer.^{55 56}

Using immunotherapy in metastatic melanoma is more because melanoma is one of the most immunogenic tumors. It means it can activate the immune system more. Melanoma studies play an important role in understanding the immune response to cancer. In addition, melanoma is one of the easiest solid cancers that its tissue and cell samples are available to easily research. Therefore, melanoma has been selecting as a model for cancer immunotherapy.⁵⁷

Immunotherapy approaches utilize one's immune system to fight tumors and it can stimulate or boost the natural immune system to work more efficiently to identify and attack cancer cells. Therefore, the study of the connection of the immune system and its components with cancer is very important in immunotherapy. Currently, immune checkpoints blockade has been known as the most important and efficient approach of immunotherapy for melanoma patients that more on that in the following.⁵⁸

2.2 Immune System and Immune surveillance in Melanoma

Immunosurveillance is a theory that the immune system prevents tumor development and does not let cancer cells survive which Paul Ehrlich presented in 1909.⁵⁹ Investigations have indicated that there is a link between the immune system function and the proliferation of melanoma cells. Like other immune responses to external factors, the first line of attack is an innate immune response, which is a rapid response to confine the threat. Macrophages, granulocytes, dendritic cells (DCs), and natural killers (NK) cells are key elements of the innate immune system and respond to the abnormal signals coming from

melanoma cells. In the second phase, adaptive immunity, which is mainly composed of B and T cells, acts specifically to attack and destroy melanoma cells. (figure 6)⁶⁰

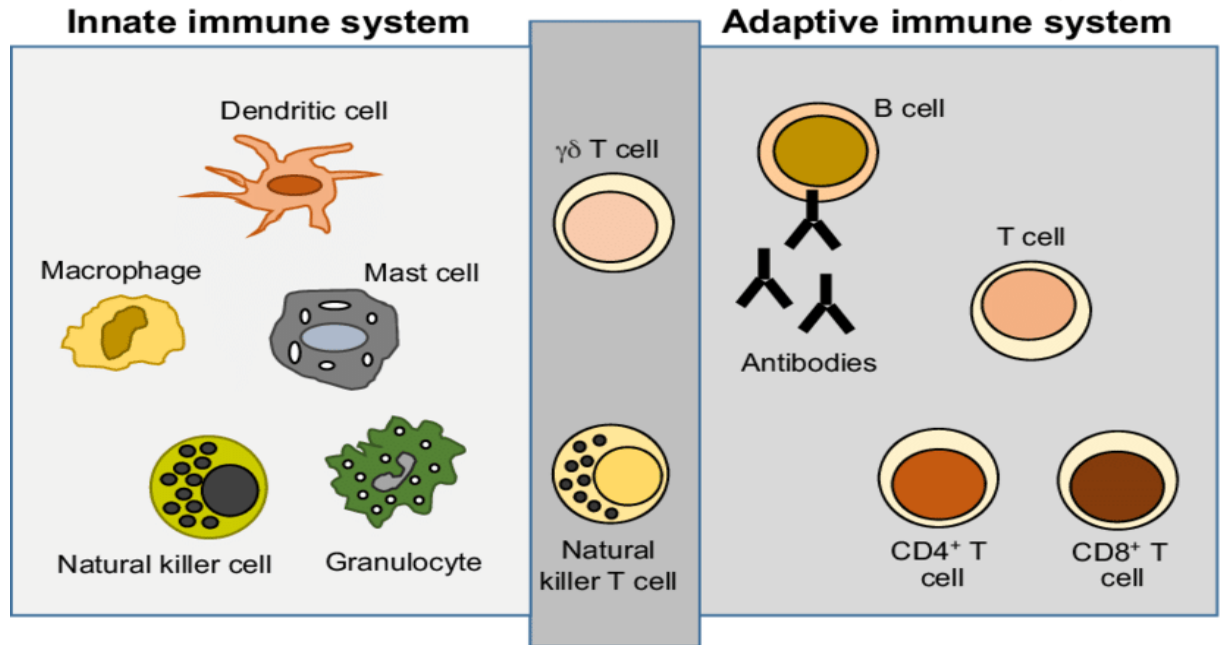


Figure 6. The innate and adaptive immune responses. The innate immune system provides an immediate response to foreign targets, with responses typically within minutes to hours. The second branch of the immune system is the adaptive or acquired immune response, which provides specific, long-lasting immune responses.⁶¹

Innate immunity as the initial immune response is essential to the start and keeping of adaptive immunity. Natural killer cells act as the first line of defense against both external and in this case internal factors such as tumors and infections. NK cells participate in anti-melanoma immunity. As a matter of fact, they can recognize melanoma cells and attack them. All cells in the body present themselves to the immune cells via Major Histocompatibility complex (MHC) I cell surface protein. Immune cells usually recognize abnormal or non-self cells via this

system. If cells expose abnormal signals via MHC class I or lack MHC I cell surface protein that will activate NK cells. Tumor cells usually down-regulate MHC class I and that results in NK cell detection of Melanoma cells and elimination of these cells via NK cell activation. So, the lack of inhibitory signaling causes NK cell activation, leading to the secretion of cytokines and the killing of tumor cells.^{60 62 63 64} Natural killer cells secrete a high level of cytokines. Cytokines are small secreted proteins that can act as opposing factors against the disease and inflammatory processes that exacerbate disease to protect the body. Some important cytokines they secrete include IL-10, tumor necrosis factor- α (TNF- α), and interferon γ (IFN- γ).^{65 66}

Also, natural killer cells secrete lethal protein Perforin and Granzymes onto target cells. Perforin and Granzymes are serine proteases that play an important role in the lysis of tumor cells and programmed cell death (apoptosis). Perforin is a glycoprotein responsible for pore formation in cell membranes of target cells. Granzymes enter the target cell through the Perforin-induced channels and activate intracellular enzymes, called caspases, which play a critical role in the induction of programmed cell death (apoptosis).^{63 67 68 69}

Dendritic cells (DCs) are one of the most important cells of the immune system cell that act as a bridge between innate and acquired immunity. They are in marginal blood and migrate to lymph nodes wherever they face natural and memory T cells. The main role of DCs is the stimulation of inactive T cells to initiate the adaptive immune responses. For activation of T cells, DCs are faced with tumor-specific antigen (TSA) and degrade them to a peptide. Then epitopes bind to major histocompatibility molecules (MHC) to present them to T cells by interaction with the T cell receptor (TCR) and will be ready for activation of T cells. The DCs package antigenic peptides into MHC

molecules and act as a bridge between the innate and adaptive immune systems.^{70 71}

For sustainable immunity, the innate immune response usually is followed by the adaptive immune response. The adaptive immune system appears to be of fundamental importance in the antitumor response. Two types of T cells are involved in adaptive immunity called CD4⁺T cells (including both T helper and regulatory T cell (T-reg) and cytotoxic CD8⁺ T cells (also called cytotoxic T lymphocytes, or CTLs). CD4⁺T cells recognize peptides presented on MHC class II molecules, which are found on antigen-presenting cells (APCs). As a whole, they have a major role in instigating and shaping adaptive immune responses. CD8⁺ T cells are generated in the thymus and express the T-cell receptor and they are critical for immune defense against viruses and bacteria, and tumor surveillance. The CD4⁺ T helper cell type 1 (Th1) cells help to activate CD8⁺ T cells and the CD4⁺ Th2 cells have an important role in stimulation a humoral immune response and suppression of a Th1 response development.^{72 73 74} Regulatory T cells (T-reg) also known as immunosuppressive T cells are T cells, which have a role in regulating or suppressing other cells in the immune system. They have different functions in the control of the magnitude of the immune response but normally they prevent autoimmune diseases by suppression of T-cell activation triggered.⁷² In addition, CD4⁺T cells can represent cytotoxic activity in the tumor microenvironment. CD8⁺ and CD4⁺ T cells can recognize antigens presented as peptides by major histocompatibility complex (MHC) class I or class II molecules.⁷⁵ MHC classes have a pivotal role in the adaptive part of the immune system. MHC class I and class II share the task of presenting peptides on the cell surface for recognition by T cells. The CD8⁺T interacts with melanoma through contact with MHC class I and CD4⁺ T with MHC class II. The MHC complex in humans is called HLA (human leukocyte antigens). MHC class I have a single heavy α -chain (HC) and MHC class II including two

chains (α -chain and β -chain).⁷⁶ MHC molecules play an important role during cell-mediated immunity. Their interaction with TCR is powered by cytokines called IFN- γ and TNF- α .⁷⁷

The first step in the process of T-cell activation initiates with the recruitment of dendritic cells (DC) which are the most specialized APC in the tumor microenvironment. During this process, the tumor antigens become peptides, which are supposed to be loaded onto the MHC. Then the dangerous signals are released from the tumor microenvironment and stimulate Toll-like receptors (TLRs) on DCs and cause DCs maturation and migration to regional lymph nodes to present TSAs on the TCRs. In the next step, the CD28 receptors on the T cell bind to B7 molecules (CD80 or CD86) on the APCs that cause a stimulatory signal, which shows to the T cells that the presented tumor antigen is non-self and they should destroy them. Once activated, T cells access the tumor microenvironment and attack the melanoma cells but before that, they need to identify melanoma cells. (figure 7)^{75 77 78 79}

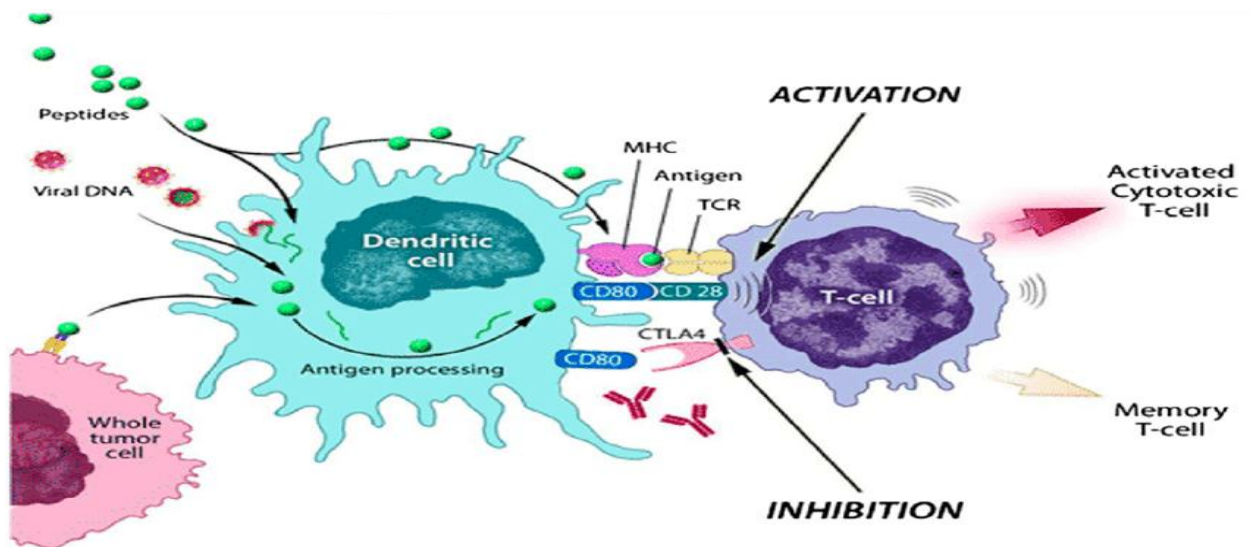


Figure 7. T cell activation. There are two complementary signals: The first signal is the interaction between TCR and peptide-MHC complex. The second co-stimulatory signal is binding CD28 to CD80. Conversely, the interaction between CTLA4 with CD80 provides a control signal that suppresses ongoing T-cell activation. Figure adapted from⁷⁹

After the T cells translocate to the tumor site, they search the region for melanoma cells that express TSA. Transporters associated with antigen processing (TAP) protein, the linker protein tapasin, and β 2-microglobulin are the TSA molecules. They check tumor peptides onto the MHC-I on the melanoma cell surface. After activated T cells connect with the tumor antigen-MHC I complex that is expressed on melanoma cells, they start a cytotoxic signal that causes destruction and lysis of the tumor cells.^{74 77 80}

The death receptor pathway (apoptosis) and the granule exocytosis pathway are two major mechanisms that NK cells and T cells use to kill melanoma cells. Tumor necrosis factor α (TNF- α), Fas ligand (FasL or CD95L), and TRAIL/Apo2L are cytotoxic ligands that belong to the tumor necrosis factor (TNF) family. In the death receptor pathway, the T lymphocytes present the death ligand CD95L on the cell surface that promotes apoptosis via the death receptor CD95, which expresses on the target cells. This leads to intracellular signaling cascades that ultimately activate procaspase-8 to active caspase-8. The resulting protein complex is called the death-inducing signaling complex (DISC). NK cells use another method to kill the target cells. NK cells have a death-ligand called TRAIL (TNF-related apoptosis-inducing ligand), which leads to apoptosis by the death receptors TRAIL-R1 or TRAIL-R2.^{81 82 83} Bid is the only known Bcl-2 family member, which cleaved by active caspase-8 and translocated to the mitochondria, mediates mitochondrial activation. After activation, cytochrome c is an apoptosis-inducing factor and other apoptogenic factors into the cytosol, released by mitochondria. Then the apoptosome that is a complex of Cytochrome c, Apaf-1, adenosine 5'-triphosphate, and procaspase-9, activates caspase-9. Executioner caspases (caspase-3, -6, and -7 that is activated by activated initiator caspases (caspase-8 and -9), break cellular substrates (death substrates), causing to the biochemical and morphological changes characteristic of apoptosis.^{84 85} In the granule exocytosis pathway, Perforin and

Granzymes are involved. They are located within cytotoxic lymphocyte (CTL) granules. Perforin polymerization stimulates changes in the melanoma cell membrane that lead to the transfer of Granzymes from the CTL into the melanoma cells. Granzymes are a family of serine proteases caused to activate caspases in the target cell. Also, the Bcl-2 family member Bid may be cleaved by Granzyme B and led to initiate the mitochondrial death pathway. (Figure 8)^{86 87 88 89}

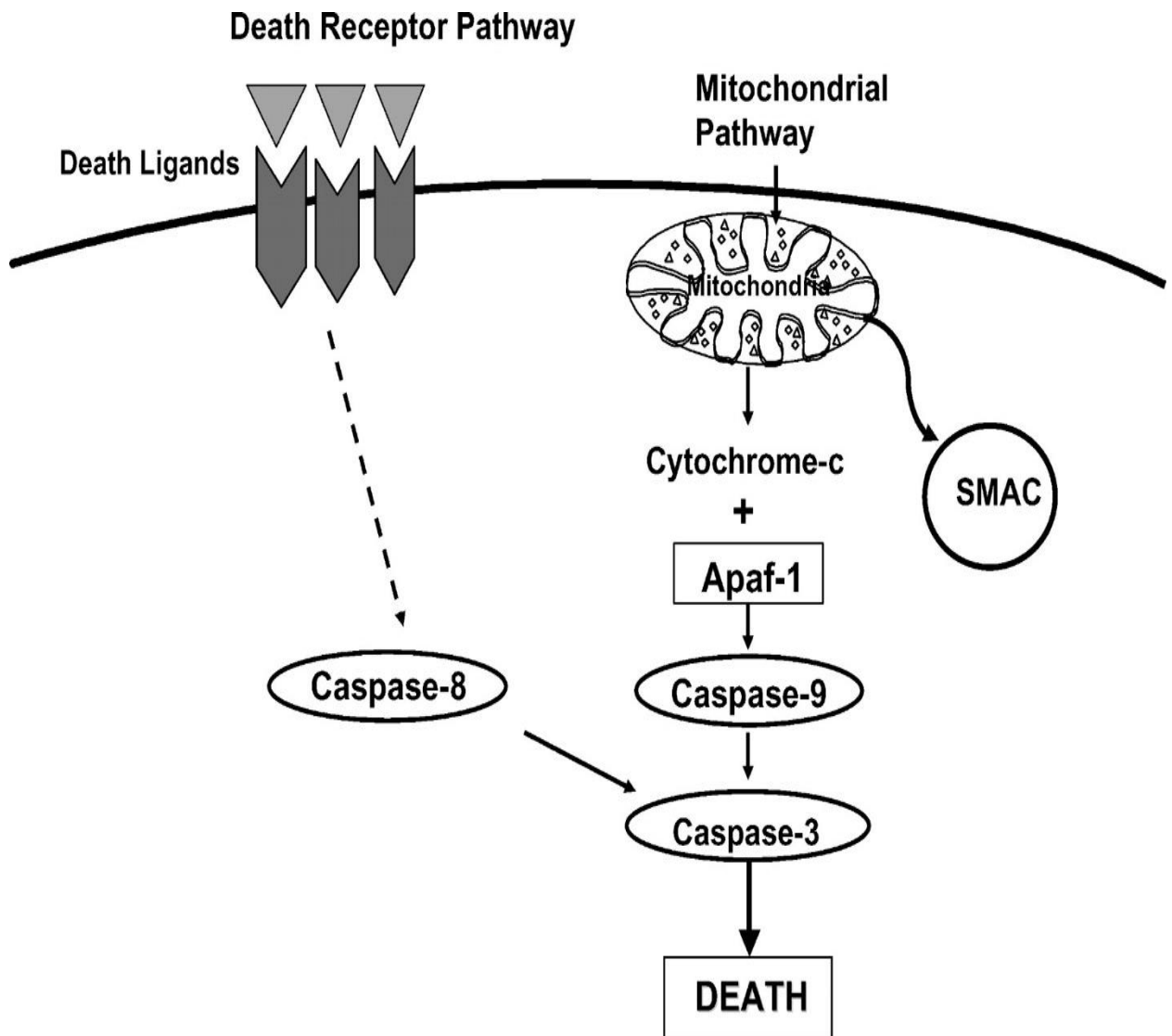


Figure 8. The death receptor pathway (apoptosis). Figure adapted from⁸⁹

Most often tumor cells survive the initial attack by the immune system and they enter a phase called equilibrium, where tumor cells are controlled by the immune system but do not eradicate. Melanoma cells can develop resistance to effector immune cells by the selection of clones with limited immunogenicity and they will be able to survive. In addition, melanoma tumors can create several somatic mutations that help their rapid proliferation and promote their genetic instability, as a result assisting the growth of less immunogenic clones that have a strong propensity to expand within the tumor microenvironment. Tumor cells become weak in the equilibrium phase and stay unedited until they spontaneously modify and escape immune control and develop.^{59 90 91}

2.3 Melanoma Cells Evasion

Immune system evasion is one of the key hallmarks in melanoma progression. Melanoma cell evasion from immune control occurs after the equilibrium phase.⁹¹ Most malignant cells may create new types of mutations that cause an increase in their resistance to immune system control and over-development of them. Melanoma is an example of high mutation-loaded tumors, which because they produce new types of neoantigens cannot more easily be detected by the immune system, and thus can respond better to immunotherapy than other cancers.^{92 93} In the escape phase, the immune system is unable to control the tumor cells because, after mutation, they form new tumor-specific antigens or lose the original antigens previously identified by T cells. For example, T-cells play a critical role in antitumor responses and specifically recognize and react to tumor-expressing antigens. However, in cancers, they are not as effective as expected. Within the tumor microenvironment, T cells (naïve, memory, effector, and T-reg) make a major part of the immune infiltrate. After antigen recognition by TCR, cytotoxic effector cells can clear antigen. Following the clearance of the specific antigen, most

effector T cells die and some memory T cells remain to survive and provide long-term protection against the antigen. Despite this, when tumor cells release countless antigens, antigen-experienced T cells are chronically exposed to the same antigen, leading to major changes in T cell activation and differentiation and as a result, confused immune systems. This state is called "T cell exhaustion" is caused to T cell dysfunction and as a result out of control of tumors.^{94 95} Therefore, gaining new mutations is a way to escape Melanoma cells from immune control. Activation or inhibition of T cells depends on the type and amount of cytokines produced in the tumor environment. Occasionally, cytokines present in the tumor environment are involved in escaping Melanoma cells from immune surveillance. Melanoma cells can prevent maturation and migration of dendritic cells (DCs) through a nuclear factor κ B (NF κ B)-dependent pathway by producing soluble factors such as vascular endothelial growth factor (VEGF), which is a cytokine. Furthermore, DCs maturation inhibits VEGF through Stat-3 (Signal transducer and activator of transcription 3) transcription. VEGF also play role in regulation of TAMs (tumor-associated macrophages), T-regs, and myeloid-derived suppressor cells (MDSCs) recruitment that can suppress immune system activation against melanoma.^{90 96 97 98} The other soluble factors that are expressed and secreted by the by Melanoma cell which can suppress immune response are tumor necrosis factors (TNFs), prostaglandin E2 (PGE2), TGF- β , IL-1, IL-6, IL-8, IL-10, Fas, and Fas-L.^{59 91} The Fas ligand, APO2 ligand/tumor necrosis factor-related apoptosis-associated ligand, and NKG2D ligand/MICA/B are certain ligands which produce by Melanoma as immunosuppression and they can inhibit responses of natural killer cell.⁹⁹

T-regs are deregulated in Melanoma that suppresses and inhibit the activities of CD4⁺ and CD8⁺ lymphocytes, and NK cells by over-production of interleukin-10 (IL-10) and enzyme indoleamine-2, 3-

dioxygenase (IDO). IDO suppresses T cell activation by reducing tryptophan.^{72 100}

Activation of the WNT/ β -catenin pathway in Melanoma cells increases the production of IL-10, which causes reduced maturation of DCs and can suppress IFN- α production by Melanoma-specific CTLs. Failure of DCs to be recruited and their migration to lymph nodes stops T cell activation, and it can help to melanoma immune escape.^{91 101} Chemokines have great importance in the homing of activated T cells into the Melanoma microenvironment. CXCL9, 10, and 11 are important chemokines to help the migration and infiltration of T cells into the tumor microenvironment and IFN- γ increases the production of them.^{59 102} In addition, activation of WNT/ β -catenin pathway in Melanoma cells inhibit homing of activated T cells to the Melanoma microenvironment by inhibiting IFN- γ production through Melanoma-specific CTLs.¹⁰¹

Other mechanisms that help to escape melanoma cells and inhibit T-cell activation are defective expressions of immune checkpoint receptors. CTLA4 and PD-1 are two immune checkpoints which, if bound to the target ligand, can negatively regulate T cell activation which assists hinder autoimmunity.¹⁰³ The CTLA4 expressed on T cell and it competes with CD28 (receptor on the T cell) to connect to B7 molecules on APCs. The CTLA-4 blockade can promote effector T-cell function by the production of IFN- γ and block T-regs activity. On the other hand, melanoma can suppress immune responses through upregulation of the programmed death-ligand 1 (PD-L1) that binds to PD-1 receptors on T cells. Therefore, it needs an Anti-PD-1 agent that blocks the binding of the PD-1 receptors on T cells and the PD-L1 ligand on melanoma cells, like the drugs using an immunotherapy approach. (Table 1) ¹⁰⁴

Table 1. Major mechanisms of Melanoma immune evasion

Mechanism	Population(s)/Pathway(s)	Effect(s)
Releasing lots of antigens by tumor cells	T-cell	T cell exhaustion
Up-regulation and secretion of Chemokines	Migration and infiltration of Regulatory T cells (T-reg) MDSCs (myeloid-derived suppressor cells)	Inhibit the activities of CD4+ and CD8+ lymphocytes
Up-regulation of the immune checkpoints by the production of IFN- γ	CTLA-4 and PD-1 overexpression on T-cells	Inhibition of T cell function
Releasing inflammatory cytokines and soluble factors by Melanoma	Increased production of VEGF, TGF- β , IL-1, IL-6, IL-8, IDO	Suppresses T cell activation Inhibit responses of natural killer cell
Activation of the WNT/ β -catenin pathway by melanoma	Increased expression and secretion of IL-10	Prevent maturation and migration DCs Prevents T cell activation

2.4. Immune Checkpoint Blockade

Both primary and secondary signals are essential for T cell activation. The primary signal is TCR binding to peptide-major histocompatibility complexes (MHCs) on APCs and the second signal is the binding of B7-1 (CD80) or B7-2 (CD86) molecules in APC with CD28 molecules on T cell as a co-stimulatory signal. Interacting CD28 with B7 family ligands (CD80, CD86) helps to promote enhanced proliferation, IL-2 production, and T-cell survival. Immune checkpoints create a critical control mechanism to turn off T-cell responses and prevent destructive inflammation. The most important checkpoints include cytotoxic T-lymphocyte-associated protein-4 (CTLA-4, CD152) and programmed cell death protein-1 (PD-1). Antigens expressed by melanoma cells can stimulate the immune system. In contrast, immune cells make regulatory immune checkpoints against these antigens but melanoma cells exploit these checkpoints to evade immune surveillance. (Figure 9) ^{73 105 106}

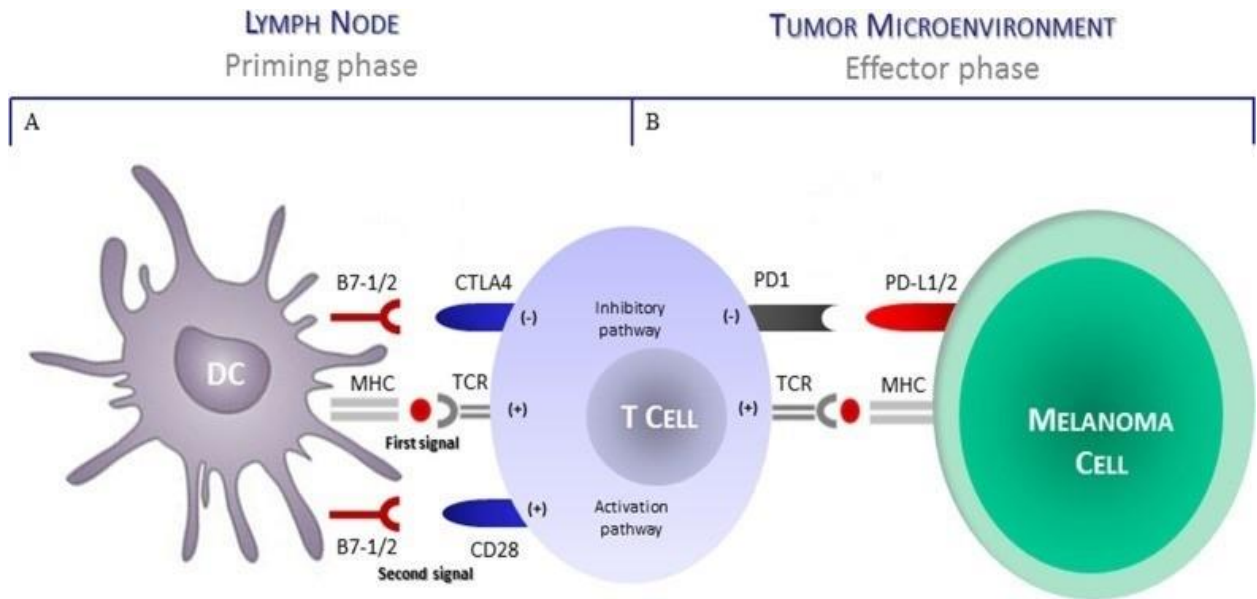


Figure 9. Mechanism of CTLA4 and PD1 action in T-cell inactivation.

Interaction between CD28 and B7 molecules can activate T cells (Stimulating signal). In contrast, the binding of CTLA4 to B7-1/2 (CD80/86) and PD1 to PDL1/2 provides a control signal that suppresses ongoing T-cell activation (Inhibitory signal). Figure adapted from⁷³

2.4.1 Biology of CTLA-4

CTLA-4 belongs to the immunoglobulin (Ig) gene superfamily with homology to CD28 and it is composed of a 223-amino-acid protein. CTLA-4 is expressed on the surface of activated lymphocytes like T-cells and regulatory T (T-reg) cells.¹⁰⁷ CTLA-4 also is known as the godfather of checkpoints that tends to compete with the CD28 molecule on the T-cell to bind to B7-1/2 and act as a co-inhibitory signal.¹⁰³ The binding affinity of CTLA4 is at least 20-fold higher than that of CD28 binding to B7 molecules and it can prevent CD28 from binding CD80 and CD86.¹⁰⁵ The CD80 and CD86 (B7 molecules) are members of the IgSF. Both bear a similar overall structure and they are 60 kDa in size. They were the first ligands to be identified for CD28 and later for CTLA-4.¹⁰⁸ The CTLA-4 expression is precisely regulated and depends

on the T-cell activation. CD28 expressed by all T-cells but expressions of CTLA-4 are not found on the surface of naïve T cells. Upon MHC-peptide/TCR signaling, CTLA-4 is up-regulated and binds B7 molecules, which is controlled via the strength of TCR signals and in result reduced T cell proliferation and lessened cytokine secretion. Therefore, CTLA-4 produces an essential “brake” on T cells to prevent immune responses via producing inhibitory signals like inhibition of IL-2 production and blockade of cell cycle progression in T-cells. Expression of CTLA4 on T cells is up-regulated in melanoma and they promote CTLA4 activity so that the T cells are ready for action but turned off. Tumor cells cause CTLA-4 to produce more inhibitory signals and suppress the T cell activation. Therefore, it can help to the debilitation of the immune response against melanoma cells. In fact, CTLA-4 plays a critical role in maintaining normal immunological homeostasis.^{106 103 107 109 110} A study on mouse models demonstrated the importance of the CTLA-4 in immune cell function. In this experiment, some mice that were deficient in CTLA-4 developed a fatal spontaneous lymphoproliferative disease and they died at 3-4 weeks of age. Therefore, lack of CTLA-4 leads to the activation of peripheral T cells and they can proliferate spontaneously and may damage the tissues.¹¹¹ Based on the studies, T cells-derived from CTLA-4 deficient mice show that expression of Casitas B cell lymphoma-b (Cbl-b) which is a 120-kDa cytoplasmic protein, which acts as both a multivalent adaptor and inhibitor of various protein tyrosine kinases.¹¹² The Cbl-b can be regulated by CTLA-4 and it is important for controlling tolerance for T cell activation and can decrease T cell activation.¹¹³ Various mechanisms, molecular details yet to be discovered, have been described to control T cell response by the CTLA4. Due to the high tendency of CTLA4 to bind to B7 molecules, it can limit the stimulating signal of CD28 and prevent T cell activation. It has been shown that CTLA-4 suppresses activation of transcription factors such as NF- κ B, NF-AT and AP-1 and Ca² mobilization, PLC- γ 1

phosphorylation in activated T cells in vitro.^{114 115} Additionally, the interaction of CTLA-4 with B7 induces expression of indoleamine-2, 3-dioxygenase (IDO), which cause to catabolization of the amino acid tryptophan and suppression of dendritic cell (DC) function, and finally results in inhibition of T cell activation.^{116 117} (Figure 10)

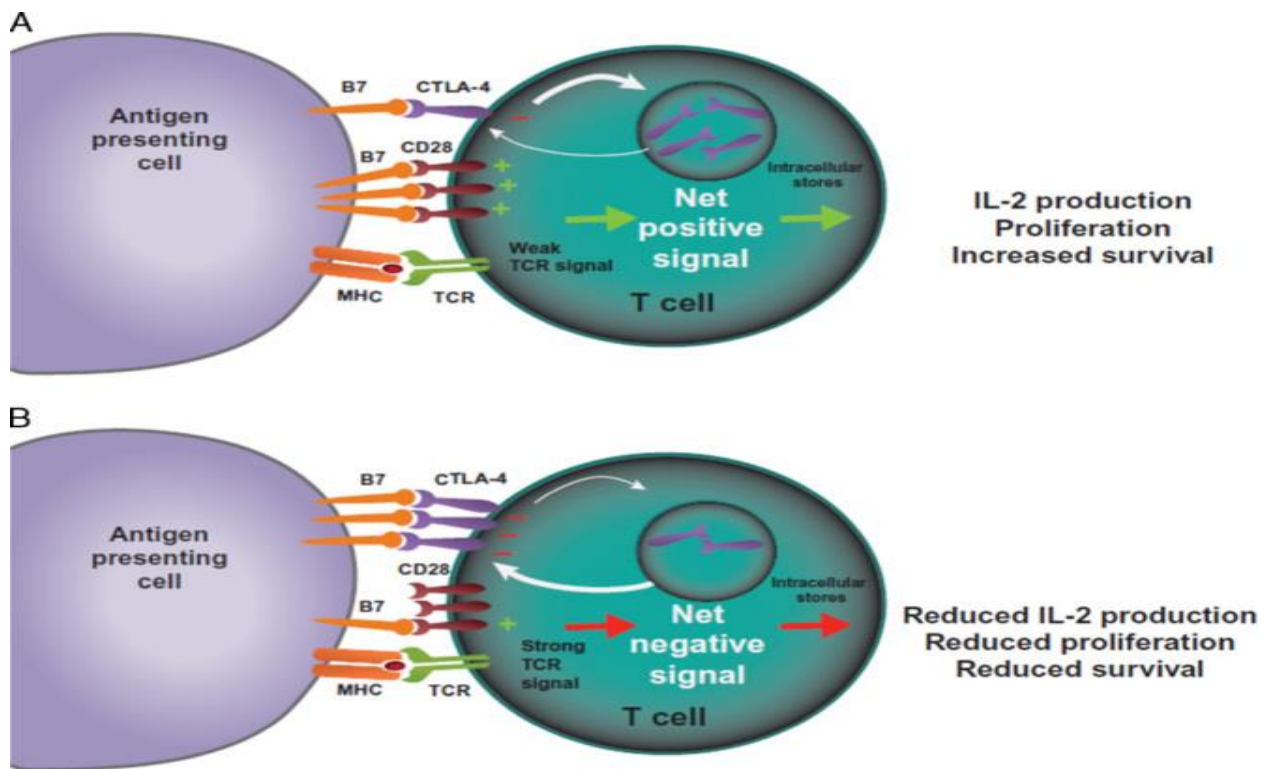


Figure 10. Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4)

Function. Both interaction TCR with MHC and CD28 with B7 led to T-cell activation. CTLA-4 competes with CD28 for the binding of B7 molecules. A) When the TCR signal is weak, CD28: B7 binding predominates. As a result, it creates a net positive activating signal that stimulates IL-2 production, proliferation, and increased survival. B) When a TCR signal is strong, increased transport to the cell surface from intracellular stores upregulates CTLA-4 expression. Increased CTLA-4: B7 binding leads to a net negative signal, which inhibits IL-2 production and proliferation, and limits the survival of the T cell. Figure adapted from¹¹⁶

2.4.2 CTLA4 Blockade in the Treatment of Melanoma

Despite T lymphocytes' efforts to fight and control cancer, cancer cells may eventually escape the tumor T cell response. Recent advances in the understanding of T-cell immunobiology and new therapeutic strategies in the production of monoclonal antibodies have been able to overcome the tumor immune escape. Immune checkpoint blockade is a novel therapeutic approach that utilizes medications known as immune checkpoint inhibitors to fight several types of cancer such as melanoma. Immune checkpoint blockade has been known as one of the most promising therapeutic strategies for patients in the history of cancer treatment especially in patients with advanced malignant melanoma. The first immune checkpoint receptor that was clinically targeted was CTLA4. The blockade of CTLA4 by monoclonal antibodies as a novel therapeutic approach achieved promising successes in preclinical cancer models.¹¹⁸ (Figure 12) Ipilimumab (Yervoy, developed by Medarex and Bristol-Myers Squibb) is a fully-humanized IgG1 monoclonal antibody that received Food and Drug Administration (FDA) approval as anti-CTLA-4 for the treatment of advanced malignant melanoma.^{119 120} Ipilimumab an anti-CTLA-4 antibody, can act as antagonists and suppress the inhibitory effect of CTLA-4 via enhancing pro-inflammatory T-cell cytokine production and increasing clonal T-cell expansion and infiltration in responding tumors.¹²¹ Another role of Ipilimumab in inducing tumor regression by depleting T-reg cells at the tumor microenvironment, which causes an increasing the ratio of intra-tumoral T-effector to T-reg cell and ultimately destroys more tumor cells.¹²² The discovery of Ipilimumab was great progress because it was recognized as the first treatment to increase the median and 5 years survival rate in metastatic melanoma.¹²³

According to clinical studies, Ipilimumab can improve overall survival in patients with advanced melanoma. Ipilimumab was tested at

different doses and the approved dose and for clinical use were based on the phase III study, with 3 mg/kg every three weeks for four doses. In the first reports of Ipilimumab use, 17 patients were studied and two patients showed tumor regression among them. For support, the use of Ipilimumab in the treatment of metastatic melanoma provided two major phases III clinical trials as evidence. In the first trial in 2010, researchers examined the overall survival of patients between 676 HLA-A*0201–positive melanoma patients with stage III or IV melanoma treated with Ipilimumab alone (3mg/kg) with or without a gp100 vaccine. Among them, 403 patients received Ipilimumab plus gp100, 137 patients received Ipilimumab alone (3 mg/kg), and 136 patients received gp100 vaccine as a monotherapy. The result of these investigations was that Ipilimumab in both with or without the gp100 vaccine improved the overall survival of patients with metastatic melanoma. It was 3.7 months higher as compared with the use of gp100 alone. In the second Phase III trial in 2011, 250 patients have randomly received Ipilimumab (10 mg/kg) with Dacarbazine (850 mg per square meter of body-surface area) and 252 were randomly received Dacarbazine plus placebo. The result of the test showed that a combination of Ipilimumab with Dacarbazine responded better (11.2 months) as compared to the use of Dacarbazine alone (9.1 months). These studies demonstrated that Ipilimumab has a long-term anti-tumor effect. Now, the approved dosing schedule of Ipilimumab is 3 mg/kg as a 90-min intravenous infusion every 3 weeks for a total of four doses.^{123 124 125 126}

To date, the toxicity of Ipilimumab has been well investigated in a large clinical trial. Side effects of treatment by Ipilimumab are usually mild to moderate, sometimes deadly, and related to its function (increased immune response). Toxicity increases with an increasing dose of Ipilimumab. Common immune-related adverse events (irAE) at the time of infusions can mention fatigue, fever, headache, nausea, vomiting, diarrhea, dizziness, rash, pruritus, and liver abnormalities (elevated

serum liver tests, hepatitis) that may appear during the infusion, within 24–72 hours or later. It can be cured with diphenhydramine, antipyretics, intravenous, antipruritic, or oral fluids as needed, and the process of using Ipilimumab may be slowed down. The irAEs of Ipilimumab treatment occurs around the third or fourth dose more. Management of irAEs needs to acquire specific skills by the clinicians. To demonstrate the importance of dose rates in the progression of irAEs, a study was done to investigate the association between an increased dose of Ipilimumab and autoimmunity. The results of the studies showed that in higher doses, the tendency toward a greater incidence of grade III/IV autoimmune toxicity is more.^{127 128 129}

Tremelimumab is another anti-human CTLA-4 antagonist immunoglobulin (Ig) G2 antibody. Although the initial results of its activity were promising in phase I and phase II studies, it failed to show its outcome for improving the survival of advanced melanoma patients. Due to its failure to treat malignant melanoma patients, the clinical development for melanoma was stopped, but the evaluation of Tremelimumab in other cancers is continuing.^{58 130}

2.4.3 Biology of PD-1

The other immune checkpoint and immune inhibitory receptor that belongs to the immunoglobulin (Ig) superfamily is PD-1 and it is composed of 288 amino acids. The *Pdcd1* gene encodes the PD-1 receptor protein that is located on chromosome 2 in humans. PD-1 is a member of the CD28 and B7 family and regulates the immune system negatively. PD-1 suppresses T-cell activity as a surface molecule. Activated CD4⁺ and CD8⁺ T cells, B cells, myeloid-derived dendritic cells, and natural killer T cells express this protein. PD-1 limits the proliferation and activity of these cells.^{131 132} Unlike the CTLA-4 that suppress activation of T-cells during the priming phase of immune responses, PD-1 inhibits the activity of T cells in peripheral tissue,

especially in inflammatory response time to infection and autoimmunity.^{106 133} PD-1 maybe play a central role in the prevention of autoimmune diseases. Based on an experiment, disruption of receptor PD-1 in some mice later leads to some kind of autoimmunity including dilated cardiomyopathy with severely impaired contraction and lupus-like arthritis.^{106 133 134} Like CTLA4, PD-1 binding also prevents T-cell proliferation, and decreases secretion of interferon- γ (IFN- γ), tumor necrosis factor- α , and IL-2 and cause to decrease T-cell survival.¹³⁵ High levels of persistent PD-1 expression on T cells cause “T-cell exhaustion” because they have experienced high levels of stimulation that render them dysfunction.¹³⁶ Based on studies in mouse models and human xenografts, PD-1 has an inherent effect in melanoma cells and it can be expressed by some human Melanoma cells. Expression of PD-1 by melanoma cells enhances tumor growth in vitro.¹⁰⁶ Two ligands have been identified for the PD-1 receptor, namely, Programmed death ligand 1 (PD-L1) and Programmed death ligand 2 (PD-L2). PD-L1 that is also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) has 290 amino acid type 1 transmembrane protein, which is encoded by the CD274 gene and located on human chromosome 9.^{105 132} Engagement of the PD-L1 with PD-1 receptor leads to suppress the immune system. PD-L1 is constitutively expressed on hematopoietic and non-hematopoietic cells and most immune cells like B cells, T cells, DCs, macrophages, monocytes, vascular endothelial cells, and many tumor cells.^{137 138} PD-L1 be expressed by cancer cells and immune cells like macrophages. Another method by which Melanoma cells shutdown T cell function is via increasing inflammation. Pro-inflammatory cytokines, which are secreted by melanoma cells such as IFN- γ , can upregulate PD-L1 expression on T-cells, further increasing the opportunity of T cell inhibition. So binding PD-L1 expressed by tumor cells and T cells expressing PD-1 inhibits an effective immune response by inactivating T-cells.^{139 140} PD-L2 also known as B7-DC or CD273 is

the second ligand for PD-1 that is encoded by the *Pdcd1lg2* gene and located near the *CD274* gene. Both PD-L1 and PD-L2 are mainly expressed in antigen-presenting cells and tumor cells, including a few of the melanoma cases.^{105 132 141 142} (Figure 11)

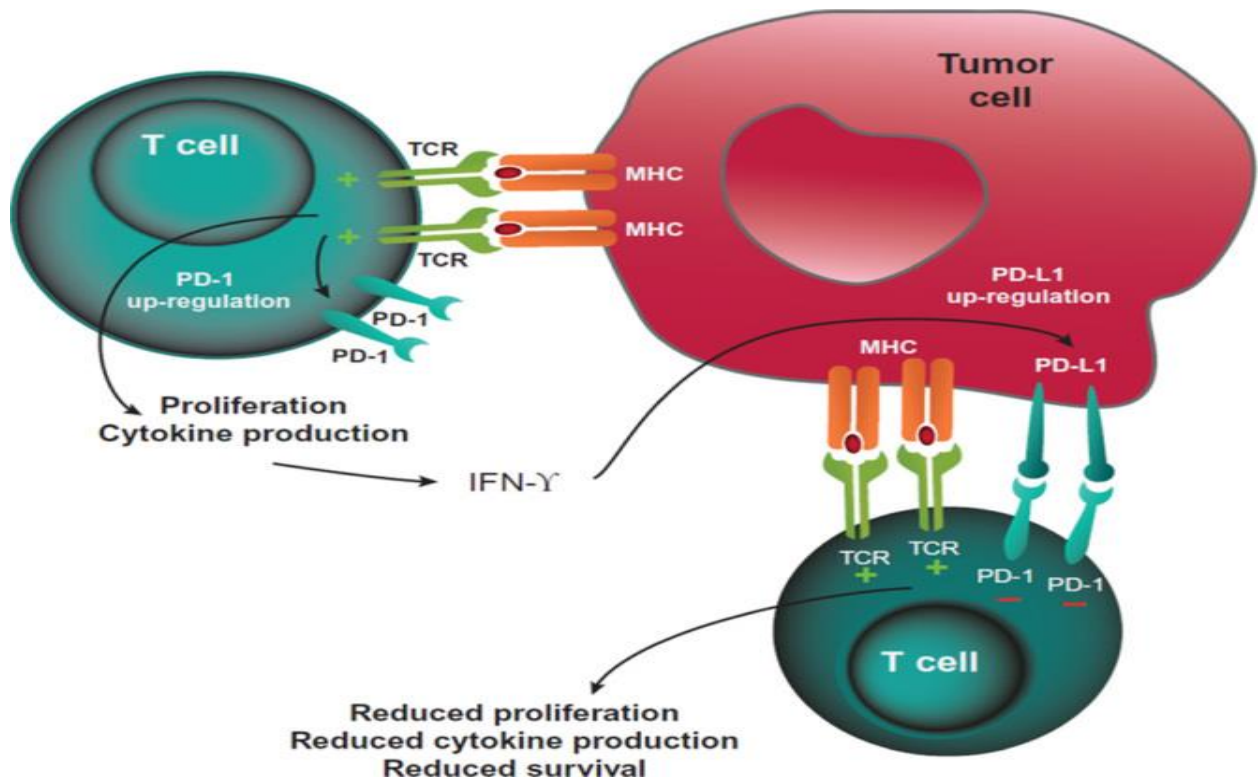


Figure 11. PD-1-mediated inhibition of T cells activation. PD-1 expression is upregulated by prolonged TCR stimulation during an ongoing immune response. PD-L1 is expressed by Melanoma cells as a consequence of inflammatory cytokines (production of IFN- γ) and/or oncogenic signaling pathways. Interaction between PD-1 with PD-L1 limits TCR-mediated positive signaling, causing reduced proliferation, reduced cytokine secretion, and reduced survival. Figure adapted from¹¹⁶

2.4.4 PD-1 Blockade in the Treatment of Melanoma

Once the success of the immune checkpoint blockade in immunotherapy of melanoma, the next checkpoint, PD-1, was investigated. Nivolumab and Pembrolizumab are two anti-PD-1 monoclonal antibodies for melanoma treatment, which have been

approved by the FDA. Nivolumab (BMS-936558, MDX-1106, ONO-4538) is a fully-humanized, monoclonal, immunoglobulin G4 (IgG4) antibody to PD-1 which prevents interaction with PD-L1 and PD-L2 by binding to the PD-1 receptor. Thus, blockade of the PD-1 / PD-L1 signaling pathway could restore the function of exhausted T-cells and cause activation of T cells, and decreasing tumor growth. (Figure 12) In 2010, the first phase 1 trial was performed and after the success of Nivolumab in clinical trials, it was approved in 2014. It also achieved promising results in overall survival and progression-free survival in patients with metastatic melanoma without a BRAF mutation.^{143 144} In the initial phase I clinical trial, Nivolumab was investigated in patients with melanoma, lung cancer, renal cell carcinoma, and a limited number of other malignancies. In this trial, 94 patients with Stage III (in progression) and Stage IV (advanced) melanoma were treated with doses ranging from 0.1 to 10 mg/kg every 2 weeks over an 8 week cycle period. Among them, 26 out of 94 (about 28 %) showed an objective response to treatment and remained free of progression at 2 years. Also, 107 patients were treated with different Nivolumab doses between 0.1 and 10 mg/kg every two weeks for up to 96 weeks which, median overall survival (OS) was 16.8 months and the OS rates in 5 years were 34%.¹⁴⁵ ¹⁴⁶ In phase III clinical trial, 418 patients with metastatic melanoma and wild BRAF without prior treatment were investigated. In this investigation, patients were treated with either Nivolumab 3 mg/kg or Dacarbazine 1,000 mg/m², both every three weeks. Overall survival in a year was 72.9% (Nivolumab) compared to 42.1% (Dacarbazine).^{119 143} ¹⁴⁷ Pembrolizumab (previously known as MK3475 or Lambrolizumab) is the second humanized anti-PD-1 IgG4 isotype antibody with high affinity.¹⁴⁸ In 2014, it was approved by the FDA for the treatment of metastatic melanoma in patients with disease progression following Ipilimumab and, if BRAF V600 mutation-positive. In a phase, I clinical trial, 173 melanoma patients were treated with Pembrolizumab 2 mg/kg

or 10 mg/kg every 2 weeks, and every 3 weeks, and progression-free survival (PFS) was shown. In another phase III study, the efficacy of Ipilimumab versus Pembrolizumab was compared and 834 patients with advanced melanoma were studied in three groups including, 10 mg/kg of pembrolizumab every 2 weeks, 10 mg/kg of pembrolizumab every 3 weeks, or 4 doses of Ipilimumab at 3 mg/kg every 3 weeks. Pembrolizumab showed better results and its immune-related adverse events were less compared with Ipilimumab. Overall, the results of the study showed that anti-PD-1 immunotherapy is promising and it can help improve the treatment of melanoma and increase their overall survival.¹⁴⁶

¹⁴⁹ ¹⁵⁰ Like CTLA4 blockade, PD-1 blockade also has adverse events including low-grade fatigue, diarrhea, nausea, cough, dyspnea, constipation, vomiting, dermatitis, pyrexia, and headache for Nivolumab. Also, Pembrolizumab showed low-grade fatigue, rash, pruritus, arthralgia, amylase elevation, and diarrhea as adverse events. Most of them are treated with a delay in treatment or use of systemic corticosteroids based on safety management guidelines.¹⁴⁵ ¹⁵⁰ ¹⁵¹

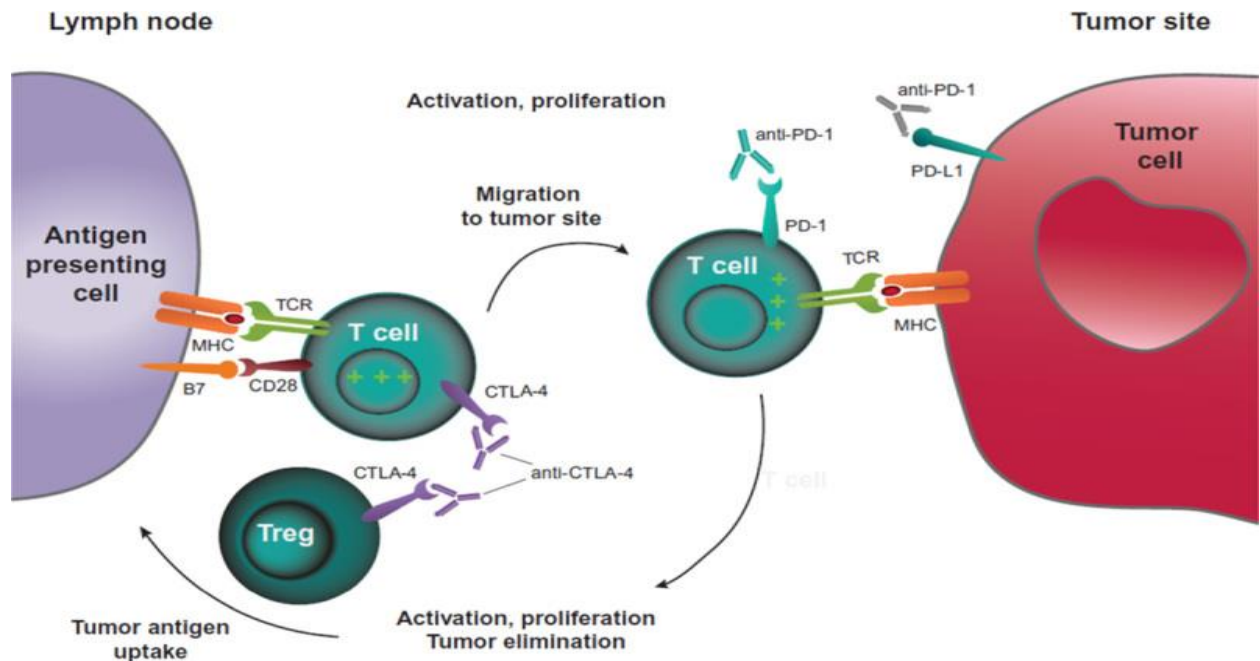


Figure 12. CTLA-4 and PD-1 pathway blockade. The Blockading of CTLA-4 leads to activation and proliferation of more T-cell clones and reduces Treg-mediated immunosuppression. On the other hand, the blocking of the PD-1 pathway restores the activity of antitumor T cells. Figure adapted from¹¹⁶

Chapter 3: Other immune- modulatory approaches for melanoma prevention/treatment

3.1 The other immunotherapy models

The other current approaches to Melanoma immunotherapy including Specific immunotherapy (e.g., Adoptive cell therapy, Chimeric antigen receptor) Non-specific and passive immunotherapy (e.g., targeting cytokines), active and specific immunization (Vaccines).

3.1.1 Adoptive T-Cell Therapy

Adoptive cell therapy (ACT) is known as one of the most effective immunotherapy approaches to treat melanoma patients, which was first described in 1988. It is mostly used for solid cancers like melanoma. In this method, masses of the solid tumor can be resected and fragmented via surgery, then the cells are put in different wells and expanded separately to collect tumor-specific T cells from the tumor-infiltrating lymphocytes (TIL) of melanoma patients. For expansion, TILs are added to a T cell growth factor, such as IL-2 (3000–6000 units/ml). After the initial expansion, T cell populations that have the desired TCR specificity can be selected and expanded. Then 50–100 billion expanded TILs are infused into melanoma patients. Before this adoptive transfer, patients are immunodepleted through either chemotherapy alone or chemotherapy in combination with total-body irradiation. In another way, tumor-specific T cells are extracted from the peripheral blood of melanoma patients via cloning by limiting dilution and expand them with a similar strategy.^{152 153} In metastatic melanoma, ACT seems to have complete and durable responses and is caused to durable complete regression of 24% of the studied patients with metastatic melanoma and a reported median survival of >3 years. Also, studies showed progression-free survival (PFS) of 11 months and 5-year OS of 57% in progressive melanoma, undergoing ACT therapy. The usual AEs of ACT therapy are autoimmune alterations, such as the destruction of normal melanocytes in the eyes and skin and for controlling these AEs is done immunosuppression.^{154 155}

3.1.2 Chimeric Antigen Receptor CAR-T

Chimeric antigen receptor (CAR-) T cell therapy has emerged as a very promising approach to combating solid tumors, including melanomas. The concept of CAR was firstly introduced by Eshhar et al. in 1989.¹⁵⁶ The current CAR structural design is included an extracellular domain that is a monoclonal antibody single-chain variable fragment (scFv), an extracellular hinge and spacer element, a transmembrane domain, and intracellular signaling domains that recognizes a cancer-specific antigen which can stimulate T-cells to destroy the tumor cells.^{157 158} For this therapy, in the first step blood is taken from the patient via apheresis for T-cell enrichment. Then T cells are isolated and genetically transduced with a tumor antigen-specific CAR and are activated to boost the expansion of cells. In the last step, CAR-T cells are infused into patients but before that chemotherapy is received to reducing immunosuppressive cells.¹⁵⁹ (Figure 13)

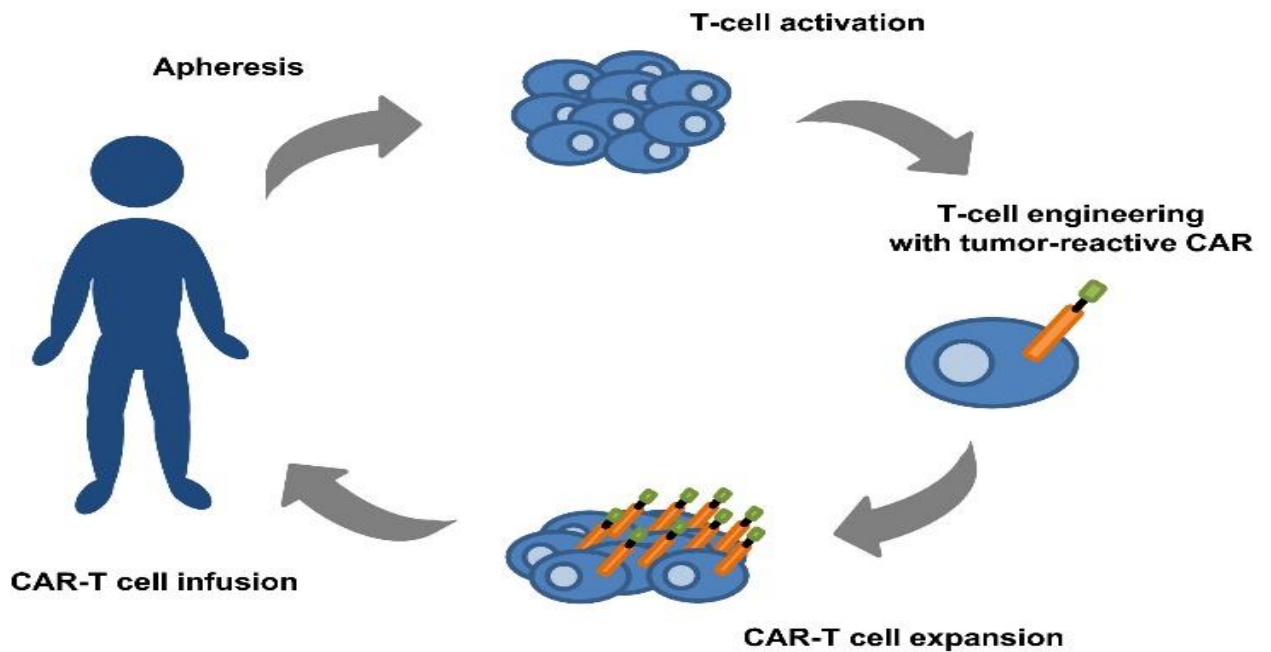


Figure 13. (CAR)-T cell therapy. 1) Blood is taken from the patient; 2) Transformation of T cells with a tumor antigen-specific CAR; 3) CAR-T cells are reinfused into the patient. Figure adapted from¹⁵⁹

3.1.3 Targeting Cytokines:

IL-2 (15.5-kDa four α -helical bundle) is one of the key cytokines that play critical role growth and differentiation of T cells, B cells, natural killer cells, and many other cell types. Considering the important role of this cytokine in immune system function modulation, it has been used as monotherapy as well as in the combination of other cancer therapies such as adoptive T cell transfer therapy, cancer vaccine treatments, immune checkpoint blockade therapies, IL-2 can stimulate T-cell proliferation and function.¹⁶⁰ IL-2 as a cytokine can play a critical role in the activation and enhancement of T lymphocytes and natural killer (NK) cells. In response to IL-2 therapy, T- cells and NK cells can get cytolytic effects, which seems to promote their anti-tumoral properties.¹⁶¹

IL-2 was the first choice for cancer immunotherapy and the FDA for treatment of metastatic melanoma in 1998 approved high-dose IL-2 (HD IL-2).¹⁶⁰ In a clinical trial, 270 patients were treated with HD IL-2. It was administered at a dose of 600,000 to 720,000 IU/kg every 8 hours for up to 14 consecutive doses over 5 days. The objective response rate (ORR) was 16% (complete responses 6% and partial responses 10%). Patients must be evaluated before treatment with IL-2. Major toxicities of HD IL-2 are fever, chills, hypotension, increased capillary permeability, cardiac arrhythmias, oliguria, volume overload, delirium, and rash; these are because of a combination of the capillary leak syndrome and lymphocyte infiltration of tissues. The toxicities of IL-2 could be treated with appropriate monitoring, prophylactic antibiotic coverage, analgesics, and antiemetic drugs.^{162 163}

Interferons (IFNs) are cytokines that leukocytes secrete them in response to viral infections. They have an important role in the immunomodulatory, antiangiogenic, and antitumor activities. The IFNs can stimulate activations of T-cells, B-lymphocytes, natural killer cells,

and dendritic cells and inhibit T-regs and MDSCs activities.¹⁶⁴ Among the IFNs, high-dose IFN α -2b was evaluated clinically and it was approved by the FDA in 1995 as adjuvant therapy for the treatment of melanoma. IFN α -2b molecule is a glycoprotein consisting of 166 amino acids that inhibit the proliferation of tumor cells via cell cycle arrest, apoptosis, or differentiation and it can activate T cells, natural killer cells, and induction of cytokines. Also, It causes an increasing MHC class I expression and enhances antigen recognition.^{165 166} Based on the tests performed, IFN- α can reduce the risk of recurrence and it improves the survival of melanoma patients. However, its antitumor activity is not very efficient ¹⁶⁷

Peginterferon (Peg-IFN) is the combination of IFN α -2b with the molecule polyethylene glycol (Peg). The FDA approved it in 2011 as adjuvant therapy for melanoma patients in stage III. Peg helps the compound to stay longer in the blood and improve the therapeutic effect. The common side effect of IFN α -2b therapy is grade 1 liver toxicities, neutropenia, skin rash, anemia, fever, chills, fatigue, myalgias, sociocognitive impairment, and autoimmune events.^{168 169 170 171}

3.1.4 Cancer Vaccines

With more understanding of the link between the immune system and cancer, melanoma vaccines are being studied. Tumor vaccination is performed to induce immune recognition against tumor antigens. Various vaccination strategies have currently been testing for melanoma treatment. Some of the strategies that are used commonly include, i) vaccination with peptides; ii) dendritic cells (DCs) vaccines; iii) vector-based vaccines. Preclinical models have shown that most peptide-based vaccines do not be able to induce a strong immune response in monotherapy. So, a combination of peptide vaccines with nonspecific immunological adjuvants (e.g. HD IL-2) was used.^{172 173} Peptides are known as the most commonly tumor-associated antigen (TAA) source

for melanoma vaccines. Peptides (protein fragments) present on MHC molecules to be recognized by T-lymphocytes. In this way, cancer-specific peptides were recognized for melanoma over recent years. gp100 peptide vaccine is one of them that is a glycoprotein that is expressed only by melanoma cells and it can be used as adjuvant therapy. In a phase III trial, a combination of HD IL-2 plus vaccination with gp100 peptides vs HD IL-2 alone was tested. This test indicated a significant improvement in the overall clinical response (16% vs. 6%) and the median overall survival was longer in the vaccine–interleukin-2 group than in the interleukin-2–only group (17.8 months vs. 11.1 months).^{174 175} Other strategies like Allovectin-7 are being studied and tested.

Dendritic cells (DCs) have an important role in the induction of T cell activation and antitumor immunity. So, they can use as anti-cancer vaccines in clinical studies in Melanoma. The goal of dendritic cell vaccines is to induce tumor-specific T cell responses in melanoma patients by injecting activated DCs loaded with tumor antigens to facilitate tumor elimination. To prepare a DC vaccine, they should be expanded either in vivo or ex vivo. The first step is infusing immature DC that collects antigens in vivo. Then monocyte-derived DC (MoDC) is taken from patients and is generated at a large scale to gain enough numbers of cells for clinical application. After MoDC maturation, it is re-infused back in melanoma patients with the purpose to induce tumor-specific immune responses. Generally, the response rates using DC vaccines have been usually low ranging from 10% to 20% but it is long in case of response.¹⁷⁶ Despite long-lasting clinical responses that were rarely seen in metastatic melanoma patients after receiving DC vaccines, they are still not completely effective because tumors cells like to be present in immunosuppressive microenvironments that can reduce their effectiveness. In addition, there are more potent treatment options like

blocking immune checkpoints and targeted therapy. However, sometimes they can choose as a treatment option.¹⁷⁷

Viruses can infect cells and they can stimulate an immune response. Vaccine viruses (VVs) have been used as gene therapy vectors. Talimogene laherparepvec (T-VEC) vaccine is identified as VVs, which was approved in 2015 by the FDA as the first oncolytic virus for the treatment of melanoma. Oncolytic viruses (non-pathogenic viruses) are engineered (modified) viruses, which can infect malignant cells. The T-VEC is isolated from a genetically modified herpes simplex virus type 1 (HSV-1). After isolation, the genetic mutation is created in them, which it acquires the ability to cause a productive infection in malignant (but not normal) T-VEC cells. Then the T-VEC is injected into a metastatic melanoma nodule and it can only replicate in the melanoma cells, causing tumor cell lysis and tumor-specific antigens releasing.^{178 179 180} Melanoma patients (stage III or IV) were treated with T-VEC, in result an objective clinical response of 28% was observed in Phase II clinical trial. Side effects of treatment with T-VEC include fatigue, chills, pyrexia, nausea, influenza-like illness, and injection site pain.¹⁸⁰

3.2 Targeted Therapy Drugs for Melanoma

In recent years, advances in understanding the genetic background of melanoma and the identification of activating mutations in the genes have led to new molecular approaches to targeting melanoma inhibitory mutations. Generally, this gene mutation can be identified in approximately 70 % of Melanoma patients. The most common is the BRAF gene mutation, which has been successfully used as a target in melanoma therapy. NRAS is the other gene mutation that has been identified in almost 20 % of melanoma and has been utilized in target

therapy. Mutation in c-KIT can be identified in mucosal and acral lesions and studies on it are underway.¹⁸¹

3.2.1 BRAF Inhibitors Therapy

BRAF is known as a key serine-threonine kinase from the mitogen-activated protein kinase (MAPK) signaling pathway and there is a mutation in BRAF in approximately 50% of skin melanomas unrelated to chronic sun damage.¹⁸² BRAF gene mutations cause activation of the MAPK signaling pathway, which increases proliferation, and survival of melanoma cells. The most common BRAF mutation happens at amino acid 600 and in about 90% of cases, valine is substituted with glutamate in the 600 codons (V600E) and 10% with lysine (V600K) or arginine (V600R).¹⁸³ ¹⁸¹ Vemurafenib and Dabrafenib are two BRAF inhibitors for the treatment of advanced melanoma with BRAF-mutant. Vemurafenib is a potent and selective oral inhibitor of mutated BRAF, which was approved by the FDA in 2011 for the treatment of metastatic melanomas having to activate BRAF V600E mutations.¹⁸⁴ Vemurafenib increased clinical response rates, progression-free survival (PFS), and OS in metastatic melanoma patients. Based on studies tumor regression occurs in 90% of patients who received vemurafenib. The other selective BRAF-mutant inhibitor is Dabrafenib, which was approved by the FDA in 2013 for the treatment of metastatic melanoma with BRAF V600E mutations. Vemurafenib and Dabrafenib can selectively block BRAF signaling in cells with BRAF mutations. In phase 3 randomized clinical trial, 675 patients were treated with previously untreated, metastatic melanoma with the BRAF V600E mutation for comparing Vemurafenib with Dacarbazine. In this study, patients randomly received 960 mg Vemurafenib orally twice daily or 1000 mg Dacarbazine intravenously every 3 weeks. The 6-month overall survival in the vemurafenib group was 84% and 64% in the dacarbazine group. After investigations, it turned out there was a 63 % relative reduction in the risk of death for

patients treated with vemurafenib. So it was approved as a drug for target therapy of melanoma. The toxicity of vemurafenib were skin reactions consisting of photosensitivity, rash, pruritus, hyperkeratosis, keratoacanthomas, and squamous cell carcinomas. Other AEs including, fatigue, arthralgias, alopecia, headache, nausea, vomiting, and diarrhea.^{181 184 185} In another phase III trial, 733 patients were studied and 250 patients were randomly treated with Dabrafenib (150 mg by mouth twice a day) or Dacarbazine (1000 mg per m² by intravenous administration every 3 weeks). Median PFS was 5.1 months for Dabrafenib and 2.7 months for Dacarbazine. The most common AEs of Dabrafenib were skin-related toxic effects, fever, fatigue, arthralgia, and headache. The most common adverse events with Dacarbazine were nausea, vomiting, neutropenia, and fatigue.¹⁸⁶

3.2.2 MEK Inhibitors

MEK is the next downstream target from BRAF in the MAPK pathway and inhibition of MEK can block signaling in the MAPK pathway. MEK inhibitors indicate activity in NRAS-mutant melanomas. Trametinib was approved by FDA in 2013 as the first MEK inhibitor. Trametinib acts as an inhibitory and anti-tumor drug for MEK1/2 inhibition. The blocking of MEK1/2 prevents growth factors-mediated cell signaling and causes a decrease in tumor cell proliferation. MEK inhibition with Trametinib improves the clinical response rate (PFS and OS) compared to chemotherapy.^{24 181 187} In a phase 3 trial, 322 melanoma patients were studied with either Trametinib or Dacarbazine in patients with BRAF mutations. They randomly received 2 mg Trametinib orally once daily or 1000 mg intravenous Dacarbazine or 175 mg per square meter paclitaxel every 3 weeks. The rate of OS at 6 months improved 81% in the Trametinib group vs 67% in the chemotherapy group and increased rates of PFS and OS among metastatic melanoma patients with a BRAF V600E or V600K mutation. The most common toxic effects in the Trametinib group were rash,

diarrhea, and peripheral edema and it was solved with dose interruption and dose reduction.¹⁸⁸ Selumetinib is another MEK1/2 blocker but its activity in melanoma is modest. However, studies and trials are ongoing on the use of Selumetinib in targeted therapy.¹⁸⁹

3.2.3 C-KIT Inhibitors

C-KIT is a receptor tyrosine kinase, which activates the MAPK signaling pathways, and KIT mutations occur in 2–3% of metastatic melanoma. C-KIT mutations in melanoma including acral (36%), mucosal (39%), and sun-damaged (28%).¹⁹⁰ Imatinib has been approved as an oral CKIT inhibitor and it has shown significant clinical responses in melanoma with C-KIT mutation. The phase II trials have been done for using Imatinib in KIT mutant melanoma. Overall, 43 patients received a dose of 400 mg/d. The objective response rate was 23% and the 1-year OS for the patients in this study was 51%. The AEs are myelosuppression, fatigue, and fluid retention.^{187 189}

The other inhibitors of C-KIT including, sunitinib, dasatinib, and nilotinib that may have activity in melanoma patients with KIT mutations. Clinical trials with these drugs are ongoing, in combination with chemotherapy.¹⁹¹

3.2.4 VEGF Inhibitors

Melanoma expresses a high level of VEGF, which causes poor prognosis, immune suppression, and growth of tumor neovasculature and tumor progression. Angiogenesis is critical for tumor progression and metastasis and is promoted by the VEGF. Therefore, the blockading of VEGF can be a useful approach for melanoma therapy.^{192 193} Bevacizumab was investigated as an anti-VEGF humanized monoclonal IgG antibody that can inhibit tumor growth by targeting VEGF and neutralizing its activity.¹⁹⁴ In a single-arm Phase II clinical trial, 62 melanoma patients (No previous treatment) were treated with combined

therapy of oral Temozolomide (a dose of 150 mg/m² on days 1–7) and intravenous bevacizumab (at a dose of 10 mg/kg on day 1 every 2 weeks). Overall, the objective response rate was 16% and the median PFS was 4.2 months. The OS was 9.6 months and reported an improvement in OS in patients with BRAF V600E-mutated melanoma.¹⁹⁵ In another single-arm, phase II clinical trial, a combination of intravenous bevacizumab (15 mg/kg on day 1 of a 2-week) with IFN α -2b at a dose of 5 MU/m² subcutaneously three times a week during cycle 1 and at a dose of 10 MU/m² subcutaneously three times a week during subsequent cycles was tested. The median progression-free rate was 4.8 months and the OS rate was 17 months. Despite the potential of VEGF as a target but failed to validate this therapy for melanomas.¹⁹⁶

3.3 Conclusion:

In recent decades extensive investigation has been conducted in the field of melanoma immunotherapy. Metastatic melanoma has become a challenging clinical problem. Striking gains in understanding the melanoma pathogenesis and interaction between the immunosurveillance and the melanoma tumor microenvironment has been able to help improve the treatment of patients with advanced melanoma. In addition to providing new therapeutic targets, it has brought important insights into the underlying melanoma biology. Among the new goals for effective clinical treatments can be mentioned blocking immune checkpoints which could have a significant impact on anti-tumor immunity. In this way, it has led to the successful development and implementation of CTLA-4 / PD-1 inhibitor antibodies to treat melanoma patients. However, immunotherapy faces many challenges and there is a lot to be done. Cellular characteristics associated with tumor response and resistance in patients should be investigated to overcome resistance mechanisms. Therefore, the use of combination therapies (such as chemotherapy, immunotherapy, and targeted therapy) may be an appropriate approach. In addition, the toxicity and irAEs of the treatment are other important points that should be minimized, which can be prevented by early detection of irAE from the severity of possible accidents. I think further research to discover the oncogene pathways and remove these challenges will be the major subject of discussion in the coming years.

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