Cancer dormancy-related changes in immune checkpoint molecules and the role of autophagy

by

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Koç University

Graduate School of Health Sciences

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Assist. Prof. MNO

Date: _____

"However bad life may seem, there is always something you can do and succeed at. Where there's life, there's hope."

To my family

ABSTRACT

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Recurrence or relapse of cancer is very critical in the progression of the disease and related deaths. It is caused by metastasis, immune response and drug resistance related mechanisms. Cancer cell dormancy emerges as an important determinant of cancer recurrence. Immune surveillance is one of the mechanisms that limits the number of dormant cancer foci. Therefore, understanding the mechanisms of interaction between dormant cells and components of the immune system is of great importance. Moreover, the role autophagy in this context is not clear. In this study, 2D and 3D cancer models of dormancy have been developed and characterized, and co-culture systems with immune cells have been established. Using these models and systems, a set of immune checkpoint molecules that are differentially regulated in dormant cancer cells compared to proliferative counterparts, have been identified. We focused on one of the immune checkpoint genes/proteins that was differentially expressed in dormant cancer cells compared to actively proliferating counterparts. We also analyzed the role of autophagy in dormancy-related immune checkpoint responses. Our study underlines the importance of less studied immune checkpoint molecules and autophagy in dormant cancer-immune cell interactions, affecting anti-cancer immune responses. The study provides a battery of drug targets for the development of novel anti-cancer and anti-dormancy immune checkpoint inhibitors.

ÖZETÇE

Kanserin uyku halinde bağışıklık kontrol noktası moleküllerindeki değişklikler ve otofajinin rolü

Sıla Turgut

Hücresel ve Moleküler Tıp, Yüksek Lisans

Ocak, 2023

Kanserin nüksetmesi, hastalığın ilerlemesinde ve buna bağlı ölümlerde çok kritiktir. Bu durum metastaz, immün yanıt ve ilaç direnci ile ilgili mekanizmalardan kaynaklanır. Kanser hücresi uyku hali, kanser nüksünün önemli bir belirleyicisi olarak ortaya çıkmaktadır. Bağışıklık sürveyansı, uyuyan kanser hücrelerinin sayısını sınırlayan mekanizmalardan biridir. Bu nedenle, uykuda olan hücreler ile bağışıklık sisteminin bileşenleri arasındaki etkileşim mekanizmalarını anlamak büyük önem taşımaktadır. Ayrıca, bu bağlamda otofajinin rolü net değildir. Bu çalışmada, 2D ve 3D kanser dormansi modelleri geliştirilerek karakterize edilmiş ve immün hücreleriyle ortak kültür sistemleri kurulmuştur. Bu modeller ve sistemler kullanılarak, uyuyan kanser hücrelerinde proliferatif hücrelere kıyasla farklı şekilde düzenlenen bir dizi bağışıklık kontrol noktası molekülü tanımlanmıştır. Bu çalışmada aktif olarak çoğalan hücrelre kıyasla uykudaki kanser hücrelerinde farklı şekilde eksprese edilen bağışıklık kontrol noktası genlerinden/proteinlerinden birine odaklandık. Uyku hali ile ilgili bağışıklık kontrol noktası yanıtlarında otofajinin rolünü de analiz ettik. Çalışmamız, daha az çalışılan bağışıklık kontrol noktası moleküllerinin ve uykuda olan kanser-bağışıklık hücresi etkileşimlerinde otofajinin, kanser önleyici bağışıklık tepkilerini etkileyen öneminin altını çizmektedir. Çalışma, yeni anti-kanser ve anti-dormansi bağışıklık kontrol noktası inhibitörlerinin geliştirilmesi için bir dizi ilaç hedefi sağlamaktadır.

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ABBREVIATIONS

Adenocarcinoma	ADC
Antibody-dependent cell mediated cytotoxicity	ADCC
Antigen presenting cells	APC
Bone morphogenetic protein	BMP
Cancer stem cell	CSC
Cancer-associated fibroblasts	CAF
Carcinoembryonmic antigen-related cell adhesion molecule 1	Ceacam-1
Cluster of differentiation	CD
Cytotoxic T lymphocytes	CTL
Dendritic cell	DC
Disseminated tumor cell	DTC
Epidermal growth factor	EGF
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Fibroblast growth factor	FGF
Focal adhesion kinase	FAK
Forkhead box p3	FOXP3
Galectin-3	Gal-3
Galectin-9	Gal-9
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Growth arrest-specific protein 6	Gas6
Immunoreceptor tyrosine-based activation motifs	ITAM
Interferon gamma	IFN-γ
Killer immunoglobulin-like receptors	KIR
Large cell lung carcinoma	LCLC
Linker of activated T	LAT
Lymphocyte tyrosine kinase	Lck
Lymphocyte-activation gene 3	LAG-3
Major Histocompatibility Complex	MHC
Mitogen-activated protein kinase	MAPK
Myeloid derived supressor cell	MDSC

Natural killer	NK
Nectin Cell Adhesion Molecule 2	Nectin-2
Non-small cell carcinoma	NSCLC
Nuclear factor kappa-light-chain-enhancer of activated B	NF-κB
Nuclear receptor subfamily 2 group F member 1	NR2F1
Peripheral blood mononuclear cell	PBMC
Poliovirus receptor	PVR
Programmed cell death 1 ligand 2	PD-L2
Programmed cell death protein 1	PD-1
Programmed cell death-ligand 1	PD-L1
Recombination-activating gene 2	RAG2
Regulatory T	Treg
Retinoic acid receptor-related orphan receptor-γt	RORγt
Reverse transcriptase PCR	RT-PCR
Signal transducer and activator of transcription	STAT
Small-cell carcinoma	SCLC
Squamous cell carcinoma	SqCC
T cell immunoreceptor with Ig and ITIM domains	TIGIT
T cell receptor	TCR
T helper	Th
T-cell immunoglobulin- and mucin-domain-containing molecule	TIM-3
TNF related apoptosis-inducing ligand	TRAIL
Toll-like receptor	TLR
Transforming growth factor	TGF
Tumor associated macrophage	TAM
Tumor infiltrating B	TIB
Tumor microenvironment	TME
Tumor necrosis factor	TNF
Tumor necrosis factor receptor superfamily member 14	TNFRSF14
Urokinase-type plasminogen activator receptor	uPAR
Vascular endothelial growth factor	VEGF
V-set domaining-containing T-cell activation inhibitor 1	VTCN1
V-Set Immunoregulatory Receptor	VSIR

Chapter 1:
INTRODUCTION

1.1 Hallmarks of cancer

Cancer is one of the diseases with the highest morbidity rate in the world. Many different factors can cause the formation of cancer. It includes nutritional disorders, smoking, alcohol consumption, physical inactivity and infections. In addition to these, the development of cancer can be supported genetically. Understanding the pathology of cancer is important for the development of treatment strategies. Despite a range of advanced treatment options and research, a definitive cure for cancer has still not been found (Vaghari-Tabari et al., 2021).

In cancer formation, normal cells acquire a number of distinguishable and unique abilities and characteristics as they transition to a neoplastic growth state. These abilities and functions called as hallmarks of cancer **(Figure 1.1)**.

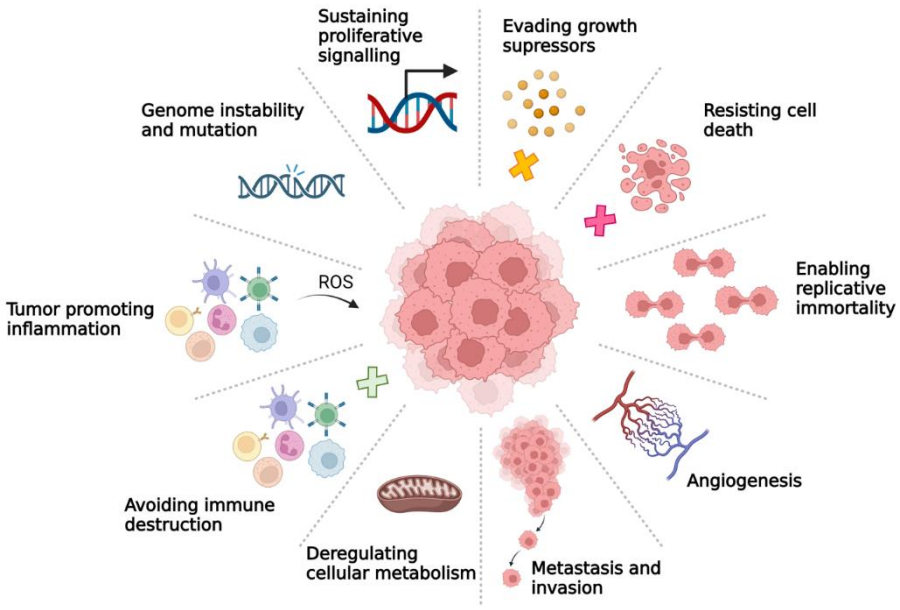


Figure 1.1. Hallmarks of cancer

One of the most important hallmarks is that cancer cells constantly give growth signals. Normal cells maintain cell number homeostasis by controlling growth and division signals. On the contrary, cancer cells support the continuous survival of the cell by ensuring that the cell growth and cycle permanently. Cancer cells also bypass various programs that negatively regulate cell proliferation. Many of these programs depend on the regulation of tumor suppressor genes. While these genes are regulated properly in normal cells, the regulation of these genes in cancer cells show deviations.

Programmed cell death by apoptosis is one of the biggest barriers to cancer development. Cancer cell can resist death by weakening apoptosis in high-grade malignancy and treatment-resistant cases. Cancer cells also have unlimited replication potential. While normal cells keep cell numbers and divisions under control, this cannot be limited to cancer cells.

As in normal tissues, tumors also need to remove their metabolic wastes and need nutrients and oxygen. For this, they created new blood vessels and it is called angiogenesis. While in normal cells this vasculature is largely at quiescent, it remains active and supports the continuation of neoplastic growth in the case of cancer.

Another distinguishing feature of a cancer cell is its ability to invasion and metastasis. Thus, by providing localization to distant tissues, the development of the tumor continues here as well. Acquiring the ability to metastasis and invasion also reveals changes in other cells and extracellular matrix (ECM) connectivity.

The cancer cell can rearrange its energy metabolism to provide unlimited division and growth. Normal cells process glucose under aerobic conditions and promote glycolysis under anaerobic conditions. On the other hand, cancer cells can produce energy in a way called aerobic glycolysis, even in the presence of oxygen, so they can reprogram their energy metabolism.

During tumor formation, cancer cells can escape from the immunological destruction of especially natural killer (NK) cells, macrophages, T and B lymphocytes. This is an ability that supports the formation and growth of tumor. However, it has been shown that when immune cells create inflammatory responses to eliminate infection in the environment, a paradoxical effect is created and it supports the formation and development of the tumor.

Cancer cells become genetically instable over time. This may be caused by mutations in DNA repair genes. This equips the cancer cell with a series of genetic changes that promote tumor progression (Hanahan and Weinberg, 2011).

Consequently, the tumor acquires many distinctive and specific features as it forms, develops and migrates to different tissues. Understanding these is of great importance for the development of new treatment modalities.

1.2 New hallmark of cancer: Cancer dormancy

Cancer recurrence, which occurs weeks, months or even years after many therapeutic approaches, represents an important problem in clinical oncology (Gomatou et al., 2021). The most important reason for cancer recurrence is that the cancer cells can enter the dormancy state and wakes up again after a certain period of time. Cancer dormancy is a state which cells remain hidden and asymptomatic for a long time. While this state is observed in the earliest stages of tumor development, it can also be observed at the stage of micro-metastasis and after removal and treatment of the primary tumor (Almog, 2010; Aguirre-Ghiso, 2007).

In the light of many developments, it shows that in most tumor types, cancer stem cells (CSCs) are responsible for the development of cancer and its evolution to metastasis (Hager et al., 1981). Cancer cells and CSCs can be separated from the primary tumor and spread throughout the body. These cells are called disseminated tumor cell (DTC) and continue to evolve in a new tumor niche (Schardt et al., 2005; Husemann et al., 2008 Magbanua et al., 2013; Pixberg et al., 2017). These cells may remain in dormant state for long periods of time or aggressive proliferation of DTCs can result in metastasis. In dormant state these cells exhibit stable proliferation or no proliferation at all (Holmgren

et al., 1995). Two main mechanisms are observed in cancer dormancy. These are divided into tumor mass dormancy and tumor cell dormancy **(Figure 1.2).**

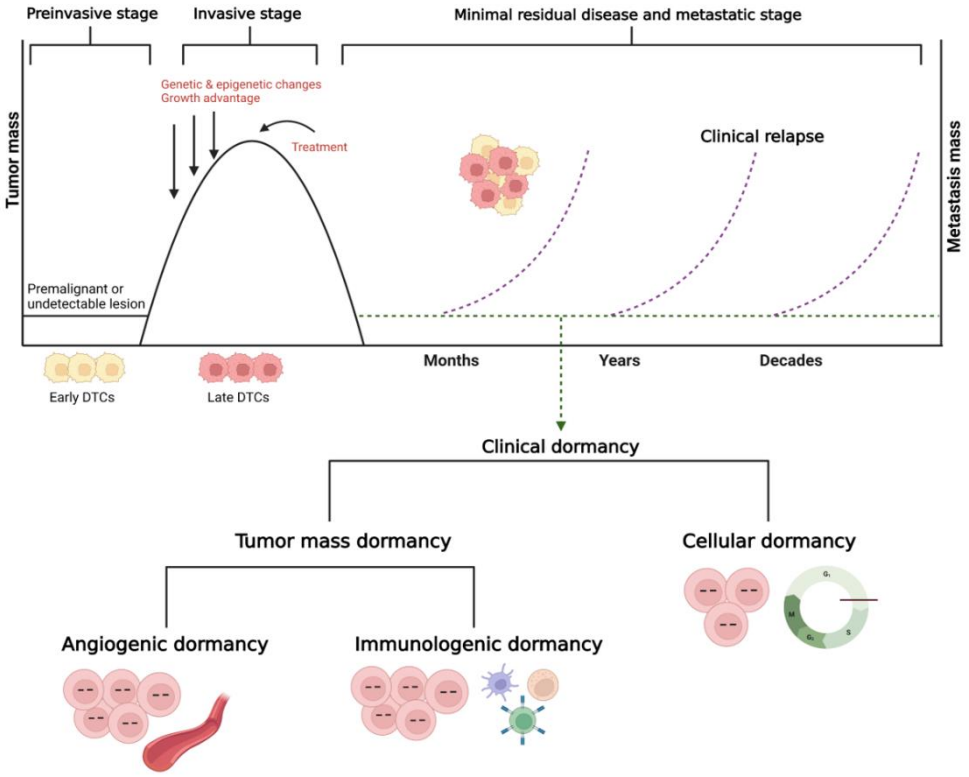


Figure 1.2. Schematic description of cancer dormancy

1.2.1 Tumor Mass Dormancy

Tumor mass dormancy is a model in which the balance between proliferation and cell death is achieved in cancer cells. In this case, no clinical change is observed in the tumor mass. Tumor mass dormancy is described by two different mechanisms. These are angiogenic dormancy and immunogenic dormancy.

Angiogenic dormancy

Cancer cells consume a large amount of energy resources to maintain proliferation. For this reason, tumor growth is accompanied by the formation of a new blood vessel network or angiogenesis (Folkman, 1971). But some tumor cells lack angiogenic capacity and cannot grow above a certain size (Wang and Lin, 2013). This situation causes the cells to remain in balance between proliferation and apoptosis, due to lack of oxygen and

nutrients. This condition is called angiogenic dormancy (Endo et al., 2019). The transition of the tumor to the state of vascularization is called angiogenic switch. This mechanism is regulated by various pro-angiogenic factors and anti-angiogenic factors (Wang and Lin, 2013). Tumor can exit the angiogenic dormancy by the angiogenic switch in the tumor microenvironment, so that tumor growth and disease recurrence can be observed. (Buijs, et al. 2007; Boyerinas et al., 2013; Gao et al., 2012).

Immunogenic dormancy

Immune system plays a critical role in tumor growth and development. Continuous interaction of immune system cells and DTCs with each other can regulate the immunogenicity of tumor cells, causing them to enter an attenuated immunogenic state. This condition is called immunogenic dormancy (Wang and Lin, 2013). Many immune system cells can take part in the formation of immunogenic dormancy. For example, a spontaneous melanoma model was established using metallothionein (MT)-ret/AAD mice and it was shown that CD8+ T cells contribute to tumor dormancy in the metastatic area (Lengagne et al., 2008; Eyles et al., 2010).

In addition, it was stated that the equilibrium state of proliferation and apoptosis of tumor cells in the sarcoma mouse model induced by 3'-methylcholanthrene (MCA) was supported by the secretion of interferon gamma (IFN-γ) and IL-12 by CD4+ and CD8+ T cells (Koebel et al., 2007).

However, these dormant cells can be detected clinically by being reactivated to induce an immunosuppressive tumor microenvironment. For example, DTCs can become resistant to cytotoxic T cell-induced apoptosis by reducing T cell activation over time. They can also reduce the direct T-cell response by blocking the functions of antigen-presenting cells (Hensel et al., 2013; Gelao et al., 2013). In addition, many immune cell types can enable cancer cells to escape from dormancy in the tumor microenvironment. These cells are regulatory T cells (Treg cells), tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC) (Satchi-Fainaro et al., 2012; Quesnel, 2008; Wu et al., 2013). Consequently, the immune system plays a critical role not only in tumor removal, but also in tumor mass dormancy.

1.2.2 Tumor Cell Dormancy

Cellular dormancy is a state of dormancy in which intrinsic and extrinsic factors allow DTCs to become quiescent. This state of sleep can last for a long time. Some cells can awake from this dormant state and become active again thanks to various signaling pathways and mechanisms (Sosa et al., 2014; Phan et al., 2020; Yeh et al., 2015). Dormant cells remain in G0-G1 phase of the cell cycle. These cells are not involved in the normal proliferating cell cycle. Therefore, these cells lack of proliferation markers such as Ki-67, apoptotic markers such as active caspases, and senescence markers such as beta-galactosidase (Spiliotaki et al., 2014; Marlow et al., 2013).

Various cell cycle regulators are known to control the dormant state. Cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ are involved in hematopoietic stem cell dormancy. For example, the APC/CDH1 ubiquitin ligase complex Skp leads to the ubiquitination and degradation of Skp2, which is part of Skp, Cullin, F-box containing complex (SCF). The regulation of the adhesion of lymphoma cells to the bone marrow (BM) stromal cells occurs by downregulation of Skp2 and, accordingly, cell cycle arrest due to the increase in $p27^{Kip1}$ and $p21^{Cip1/WAF}$ (Zou et al., 2011; Lwin et al., 2007). In addition, the DREAM complex formed by the combination of retinoblastoma-like pocket proteins such as p130 and p107, E2F protein, and mutilvulval class B protein (MuvB) plays an important role in cellular dormancy. MuvB is responsible for regulating the cell cycle. If the cell enters quiescent state, MuvB forms the DREAM complex by providing p130 and E2F dimerization and arresting the cell cycle (Sadasivam et al., 2013; Schmit et al., 2007; Sadasivam et al., 2012). Dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) and dual specificity tyrosine phosphorylation-regulated kinase 1B (DYRK1B) phosphorylate subunit of MuvB, namely LIN152, mediating the formation of the DREAM complex and thus thereby transitioning the cell to a non-proliferative stage (Litovchick et al., 2011). DYRK1A and DYRK1B regulate the phosphorylation and stabilization of the $p27^{Kip1}$ and the degradation of cyclin D. Thus, it is ensured that the cell cycle is stopped and the dormant state is activated (Besson et al., 2006; Deng and Friedman, 2009)

Several stress response and survival signaling pathways play an important role in cellular dormancy. For example, the balance of proliferation and dormancy of head and neck cancer cells is specified by the balance between p38 mitogen-activated protein

kinase (p38 MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways. Dormant cells are characterized by inducing p38 MAPK and reducing ERK1/2 pathways, and this is considered an important marker as phenotype (Aguirre-Ghiso et al., 2003; Yu-Lee et al., 2018). The regulation of these kinases is carried out by the urokinase-type plasminogen activator receptor (uPAR). Activation of this receptor increases the expression of focal adhesion kinase (FAK) and Src kinase, thus the ERK1/2 pathway is upregulated. In cases where this is the opposite, high upregulation of the p38 MAPK pathway is ensured. Activation of the p38 MAPK pathway arrests cell proliferation and regulates dormancy by activation of basic helix-loop-helix protein 3 (BHLHB3), nuclear receptor subfamily 2 group F member 1 (NR2F1), and cyclin-dependent kinase inhibitors (Aguirre Ghiso et al., 1999; Bragado et al., 2013; Sosa et al., 2015). Basic helix-loop-helix family, member e41 (BHLHE41) plays an active role in ER positive breast cancer dormancy revealed by an *in vivo* xenograft mouse model. It has also been observed that this transcription factor is induced by the p38 signaling pathway in head and neck squamous cell carcinoma (HNSCC) (Gao et al., 2012; Kim et al., 2012).

In addition to internal signaling, several extrinsic factors in the environment can activate the state of quiescent. For example, growth arrest-specific protein 6 (Gas6), which is formed as a result of AXL receptor tyrosine kinase, MerTK and TYRO3 interactions, supports cell cycle arrest by upregulating the p38 pathway. When Mer expressing leukemia cells and osteoblasts are in contact, Gas6 activation is ocurred and thus the cell enters G0/G1 arrest. (Shiozawa et al., 200). In addition, the p38 MAPK pathway is upregulated by transforming growth factor beta 2/ Transforming growth factor beta receptor III (TGFβ-2/TGFβRIII) activity in HNSCC cells in the bone marrow. However, in prostate cancer, dormancy is supported by activating the p38 pathway thanks to TGF-β2. Dormant cells secrete higher levels of TGF-β2 compared to proliferative cells, thus creating an autocrine cycle (Bragado et al., 2013; Yu-Lee et al., 2018; Yumoto et al., 2016). Several related bone morphogenetic protein (BMP) ligands can contribute to cellular dormancy. For example, BMP7 induces dormancy in prostate cancer cells, while BMP4 expressed in the lungs promotes dormancy in disseminated mammary breast carcinoma cells (Kobayashi et al., 2011; Gao et al., 2012).

Inhibition of signaling pathways that promote proliferation is a phenomenon that induces dormancy. The absence of integrin beta 1 (ITGB1), which promotes adhesion in some of the DTCs, is associated with inhibition of the ERK1/2 signaling pathway that

promotes proliferation. In addition, in the light of pancreatic cancer models, the absence of proliferation-supporting oncogenes such as Kirsten rat sarcoma virus (K-RAS) and c-MYC stimulate dormancy in cancer cells (Barkan et al., 2008; Barkan et al., 2011; Viale et al., 2014; Rajbhandari et al., 2017).

Taking account all these things, several pathways and factors involving the regulation of cellular dormancy. Understanding these mechanisms and further in-vivo studies are of great importance in the development of novel treatment methods for cancer.

1.3 Tumor microenvironment

While many therapeutic treatments are aimed at targeting the tumor itself, cancer progression does not depend on it alone because the tumor mass does not only consist of cancer cells. It is also comprised of the vascular system, non-malignant cells such as immune cells, cancer-associated fibroblasts (CAFs), pericytes, mesenchymal cells, signaling molecules including growth factors, hormones, chemokines, cytokines and extracellular matrix (ECM) (Butturini et al., 2019; Hanahan and Coussens, 2012). All these elements are called tumor stroma. Cancer cells and tumor stroma can form the tumor microenvironment (TME) **(Figure 1.3)**. Alterations in the tumor microenvironment and communication between tumor stroma and cancer cells play an effective role in tumor formation, development, and progression (Spill et al., 2016). Besides, the correct treatment method can be determined as a result of the interactions between the tumor and its environment.

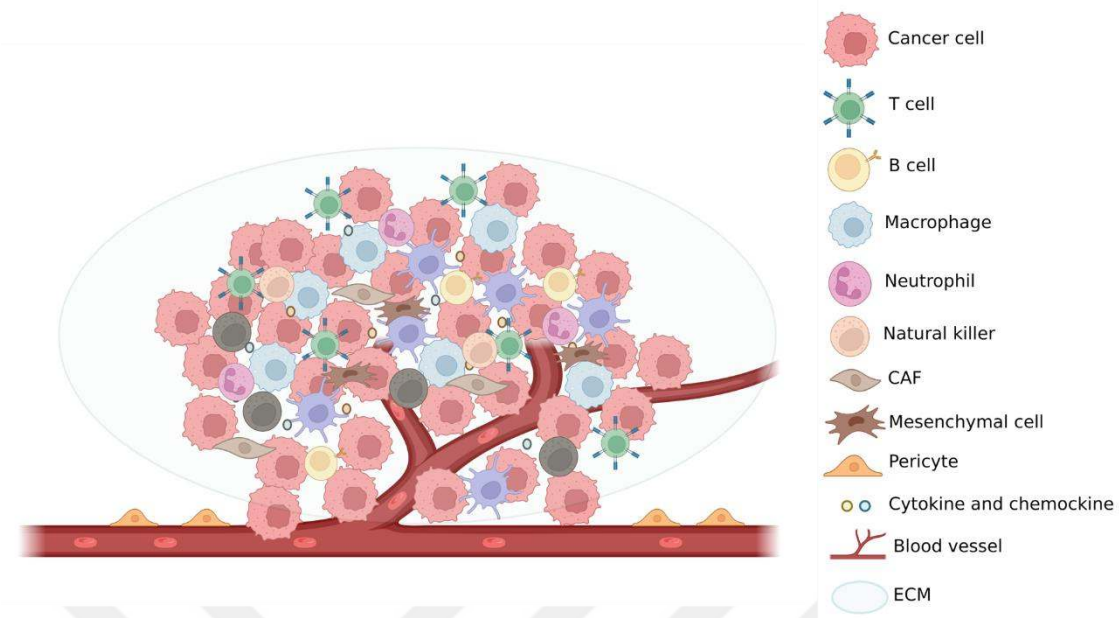


Figure 1.3. Tumor microenvironment

1.4 Cancer Immunoediting:

Immune cells located in the tumor microenvironment are very critical for the process of the cancer progression and development. The idea of tumor immunity was first proposed by Paul Elrich in 1909 (Ehrlich, 1909). In this hypothesis, called immune surveillance, it has been suggested that immune cells are able to identify and eliminate nascent transformed cells before they become clinically evident, thus protecting the host from neoplastic diseases (Lakshmi Narendra et al., 2013; Kim et al., 2007). Due to insufficient empirical evidence, this hypothesis has lost its credibility. In the middle of the 20th century, some experimental studies were conducted by Burnet and Thomas, and it was observed that tumor-associated antigens (TAAs) give an immune response against cancer and that tumor cells can be suppressed by the immune system. Thereby, the basis of the immune surveillance theory was revealed (Burnet, 1957; Thomas, 1959). In studies using animal models, tumor formation in mice is more pronounced when components of innate and adaptive immunity are lacking. For example, it has been observed that recombination-activating gene 2 (RAG2) knockout mice, which lacking the enzyme RAG2 required to entering the somatic recombination, are more susceptible to

spontaneously developing tumor since they cannot produce T, B, and natural killer (NK) cells. It has also been revealed that mice are more prone to develop cancer in the absence of T cell receptor subunits and effector molecules involved in IFN-γ signaling pathways (Shinkai et al., 1992; Billiau et al., 1988). All these observations led to the idea that the immune system has a dynamic effect on cancer, has an inhibitory effect on tumor cells, and also regulates the development of cancer. Unfortunately, despite many evidences and experimental studies supporting the existence of such a process, it is observed that immunocompetent individuals still forming cancer. Thus, in addition to the protective feature of the immune system, it has been identified that it has a stimulating effect on tumors which have attenuated immunogenicity. These binary effects of the immune system on the tumor have caused the "cancer immunosurveillance" hypothesis to evolve into the all-encompassing term "cancer immunoediting" (Lakshmi Narendra et al., 2013; Punt et al., 2018).

1.4.1 The Three phases of cancer immunoediting (Immune elimination, Immune Equilibrium, and Immune Escape)

Cancer immunoediting is a dynamic process that combines all of the processes that take place during an immune response to cancer into the three "Es" of elimination, equilibrium, and escape (Bhatia and Kumar et al., 2015) **(Figure 1.4).**

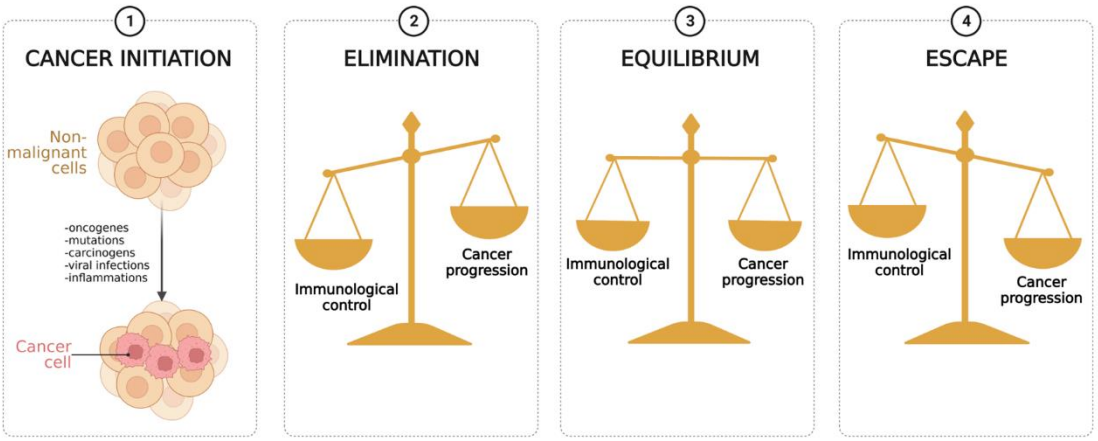


Figure 1.4. The three phases of the cancer immunoediting process

Normal cells can transform to tumor cells due to the activation of various oncogenes, mutations in intrinsic tumor suppressor pathways, carcinogens, viral infections and inflammations (Lakshmi Narendra et al., 2013). After this transformation, the elimination

phase occurs. The elimination step comprises the concept of cancer immune surveillance. In this step, the immune system detects newly formed tumor cells before they become clinically visible and activates various tumor suppressor mechanisms to destroy them. Innate and adaptive immune system work together to abolish tumor cells (Mittal et al., 2014). After this stage, equilibrium phase occurs. At this stage, the growth of the tumor cell is controlled by the immune system and prevents its features such as invasion and metastasis. Nevertheless, immune cells cannot completely destroy the tumor (Dunn et al., 2004). In this way, cells enter the escape stage and form variants that are resistant to immune attacks. These resistant variants increase immunosuppressive signaling pathways to reduce antitumor responses and exhibit low immunogenicity. At this stage, the tumor grows very aggressively and can be detected clinically (Smyth et al., 2006).

1.5 Relationship between the immune system and cancer

Innate and adaptive immune cells, which are involved in the three phases of cancer immunoediting, have different and effective roles at every stage. NK cells, macrophages, dendritic cells, neutrophils form the innate immune system and represent the first line of defense against the cancer cells. T lymphocytes, B lymphocytes and their secreted humoral mediators, cytokines and antibodies, form an adaptive immune system and serve as an antigen-specific secondary barrier.

1.5.1 Role of innate immune cells in cancer

NK cells

NK cells represent a third cytotoxic lymphocyte population as well as T and B cells along with the innate immune response (Imai et al., 2000). NK cells in large granulated lymphocyte morphology are not subjected to rearrangement of receptor genes unlike T and B lymphocytes. NK cells are responsible for defense against viral infections and eliminating malignant cells (Wolf et al., 2022). These immune cells can stimulate by cytokines such as IL-2, IL-12, IL-18, IL-15 and type I interferons (Wiedemann et al., 2021). The most important feature of NK cells is that they cannot attack the target cells which express normal levels of Major Histocompatibility Complex Class I (MHCI) molecules. However, these immune cells destroy the MHCI deficient cancer cells. NK-mediated killing due to the absence of MHCI in a target cell is called a missing-self recognition (Ljunggren and Kärre, 1990; Liao et al., 1991). This condition of NK cells is

provided by the activator and inhibitor receptors found on their surfaces. While inhibitor receptors include killer immunoglobulin-like receptors (KIRs), CD94/NKG2A; activator receptors contain CD16 (FcγRIII), natural cytotoxicity receptors (NKp30, NKp44 and NKp46), DNAM-1 and, NKG2D. If the target cell expresses MHCI, inhibitor receptors connect to this molecule, thus it prevents NK cell activity. On the contrary, if the target cell does not express the MHCI molecule, the activator receptors bind to the activator receptor ligands therefore NK cell activates and kills the target cell (Wolf et al., 2022). The cytotoxic activity of NK cells can be provided by granules that containing perforin and Granzyme B in cytoplasm. While perforin creates pores on the membrane of the host cells, procaspase cleaving Granzyme B trigger the apoptosis (Voskoboinik et al., 2015). In addition, NK cells express the ligands of apoptosis receptors such as TNF related apoptosis-inducing ligand (TRAIL) and Fas ligand (FASL) and therefore induce the apoptosis of target cell (Zamai et al., 1998). Moreover, NK cells contribute to the interaction of dendritic cells and T lymphocytes by providing IFN-γ secretion (Lakshmi Narendra et al., 2013). Last but not least, NK cells can destroy antibody coated cells. The connection of antibody to the CD16 on the NK cell, causing proteolytic enzyme release and activates the antibody-dependent cell mediated cytotoxicity (ADCC) program to kill target cell by apoptosis (Bhatnagar et al., 2014). All of this shows that NK cells have an important role in cancer progression.

Macrophage

Macrophages are one of the elements involved in the innate immune system and also known as antigen presenting cells (APC). These immune cells are highly effective at removing foreign substances, including pathogens, cellular debris, and cancer cells, from the site of infection by phagocytosis (Zhou et al., 2020). Blood monocytes are the source of the population known as macrophages. With the entry of monocytes into the tissue, macrophages are divided into different subclasses according to their functions and structures. Macrophages are mainly classified into two main populations called classically activated macrophages (M1) and alternatively activated macrophages (M2) (Solinas et al., 2009). In addition, when monocytes enter the tumor microenvironment and establish a dynamic relationship with the tumor, they transform into tumor associated macrophages (TAM) (Dehne et al., 2017). M1 macrophages are activated by IFN-γ, bacterial lipopolysaccharides (LPS), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and some toll-like receptor (TLR) ligands (Mantovani et al., 2002). This

macrophage type can be differentiated by cell surface markers such as MHC II, CD68, CD80 and CD86 expressed on the cell surface. M1 macrophages create a pro-inflammatory and cytotoxic suppressor effect on the tumor by secreting various cytokines such as IL-6, IL-12, TNF-α, IL-1β and IL-23 (Chen et al., 2019; Verreck et al., 2004). Besides, M2 macrophages are activated by macrophage colony-stimulating factor (M-CSF), IL-4 and IL-13 (Mantovani et al., 2004). Unlike M1 macrophages, M2 macrophages secrete cytokines such as IL-10 and TGF-β and contribute to tumor growth by showing an immunosuppressor and anti-inflammatory effect (Denning et al., 2007). M2 macrophages express surface markers such as CD163, CD206 and Arginine 1 (Arg1) on their surface (Chen et al., 2019). Two different types of macrophages also functioning by secreting different chemokines. For example, M1 macrophages can support to CD8+ cytotoxic T lymphocytes (CTL) and natural killer cells by expressing proinflammatory chemokines such as CXCL9, CXCL15 and CXCL10 (Mantovani et al., 2004). On the contrary, M2 macrophages contribute to tumor development by supporting Treg cells by secreting anti-inflammatory chemokines such as CCL17, CCL22 and CCL24 (Xu et al., 2022). Tumor associated macrophages are not a typical macrophage type and different from M1 and M2 macrophages. These cells exhibit a dynamic structure in the tumor microenvironment. However, it appears to be more similar to the M2 phenotype and promotes tumor growth and dissemination. TAMs contribute to tumor development by secreting various cytokines and chemokines such as IL-8, IL-10, CCL2 and CCL5 (Solinas et al., 2009; Chen et al., 2019). In addition, these macrophages secrete growth factors including, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-β (Mantovani et al., 2002; Bingle et al., 2006) and also matrix metalloproteases (MMP-2, MMP-7, MMP-9 and MMP-12). TAMs also secrete various ECM proteins to support angiogenesis, tumor growth and metastasis (Pollard, 2004; Denardo et al., 2008; Mantovani et al., 2008). TAMs can activate Treg cells and also inhibit the maturation of dendritic cells. In addition, TAMs can provide an immunosuppressive effect due to the expression of immune checkpoint molecules. Considering all these things, TAMs can contribute to tumor development by supporting genetic instability, feeding cancer stem cells, inducing the metastasis and reducing adaptive immunity (Mantovani et al., 2017).

Dendritic cells

Dendritic cells (DCs) are the APCs that receive, filter and process various types of antigens and presents them to antigen specific naive T cells (Wylie et al., 2019). The antigen-presenting functions of dendritic cells are provided by the MHCI and MHCII complexes on their surface. These innate immune cells act as a bridge between the innate and adaptive immune system and plays a crucial role in anti-tumor T cell immunity. Dendritic cells also affect other immune cell populations by secreting many cytokines, chemokines and growth hormones (Steinman, 2019& Eisenbarth, 2019). Dendritic cells are functionally grouped as immature and mature cells. Immature dendritic cells express fewer MHCI and MHCII complexes and are weaker in T cell stimulation (Dhodapka et al., 2008). Maturation of dendritic cells stimulated by the interaction of pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) (Garg et al., 2013& Gallo and Gallucci, 2013). Mature dendritic cells activate *naïve* T cells in peripheral tissues by presenting antigens and upregulating co-stimulatory molecules such as CD40, CD80 and CD86 (Palucka and Banchereau, 2012). After the maturation of dendritic cells, they can differentiate into several subpopulations based on variety in morphology, function, cell surface markers and cytokine secretion. These subpopulations are named as conventional or classical DCs (cDC), divided to conventional type 1 dendritic cells (cDC1) and conventional type 2 dendritic cells (cDC2), plasmacytoid DCs (pDC) and monocyte-derived DCs (moDC). Although dendritic cells have mostly anti-tumor effect in the tumor microenvironment, they are also known to support tumor growth. The anti-tumor properties of dendritic cells are mostly realized by cDC1 and cDC2 (Salah et al., 2021& Gardner et al., 2020). cDC1s activate CD8+ and CD4+ T cells under favor of cross presentation of tumor antigens via cytokine production and MHC-1 and MHC-II molecules (Wculek et al., 2019& Ferris et al., 2020). On the other hand, cDC2s induce CD4+ T cells through expressing high levels of MHCII molecule (Leal et al., 2017& Eisenbarth, 2019). Monocyte derived dendritic cells (moDC), another subtype of dendritic cells, occur by differentiation of monocytes into dendritic cells under of inflammation and infection. While this cell type may play a complementary role to cDC1 and cDC2, it can also support the formation of an immunosuppressive environment. While they promote the differentiation of CD4+ T cells, these cells also contribute to cross antigen presentation to CD8+ T cells (Verneau et al., 2020& Kuhn et al., 2015). On the contrary, by expressing cytokines such as inducible nitric oxide synthase (iNOS) and IL-10 at high levels, T cell proliferation is

inhibited by moDCs (Laoui et al., 2016). Plasmacytoid DCs (pDCs) are involved in viral infection, autoimmune diseases and cancer. They are well known for being the primary sources of type I interferons, which prevent angiogenesis, tumor growth, and metastasis. In this way, they play an active role in anti-tumor immunity (Lucarini et al., 2012& Zitvogel et al., 2015). Although not as effective as cDC1 and cDC2, pDCs are also known to contribute to low-level antigen presentation by secreting various chemokines and cytokines (Villadangos and Young, 2008). pDCs also promote tumor cell death directly by expressing TRAIL and Granzyme B molecules (Salvi et al., 2017& Wu et al., 2017). Besides all these, pDCs reduce T cell activity and exhibit pro-tumor effect by secrete factors such as IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO) to induce Treg cell formation (Sisirak et al., 2012). All this reveals that dendritic cells have a crucial role in regulating the immune system and determining the course of cancer.

Neutrophils

The most prevalent innate immune cells in bone marrow and peripheral blood have been characterized as neutrophils. Neutrophils are capable of phagocytosis and play a role in inflammation (Borregaard, 2010). When neutrophils are in a dynamic relationship with the tumor, they are referred to as tumor associated neutrophiles (TANs). Tumor associated neutrophils are examined in two groups according to their functions. While these neutrophils exhibit the anti-tumor (N1 neutrophils) phenotype, they also show pro-tumor (N2 neutrophils) activity (Wang et al., 2018). This neutrophil plasticity is mediated by cytokines and chemokines in the tumor microenvironment. While the N2 phenotype is induced in the presence of TGF- β in the tumor microenvironment, stimulation of TGF-β prevents N1 formation (Fridlender et al., 2009). However, in the presence of endogenous IFN- β, N1 neutrophils are occurred, while the formation of N2 neutrophils is blocked. The emergence of the N1 phenotype creates a cytotoxic effect and contributes to the formation of immune memory. N2 neutrophils provide an immunosuppressive environment and support angiogenesis, metastasis, invasion and, tumor growth (Piccard et al., 2012). Eventually, neutrophils are a strong target for therapeutic approaches against cancer.

1.5.2 Role of adaptive immune cells in cancer

B lymphocytes

B cells are one of the main cells of humoral immunity, which originate from hematopoietic stem cells and functioning by secreting immunoglobin (Guo and Cui, 2019). B cells can interact with the tumor and affect either its development or inhibition. When B cells infiltrate with tumor tissue, they are called as tumor infiltrating B (TIB) cells. Numerous variables in the tumor microenvironment have been found to cause TIBs to develop into different subtypes. TIB subtypes serve dual distinct roles in tumor immunity by secreting cytokines, immunoglobins, and presenting antigens (Martin and Chan, 2006). When the *naïve* B cells encounter the antigen, it becomes active and transforms into a memory B cell (MBC) or plasma B cell. Memory B cells can remain in the bloodstream for decades. Their primary task is to induce an accelerated and dynamic secondary immune response upon next encounter with the same B cell activating antigen (Weisel and Shlomchik, 2017). Plasma B cells produce antigen specific antibodies that are found on the surface of target pathogen. This B cell type is the main component of humoral immunity. These antibodies stimulate ADCC, phagocytosis (Gilbert et al., 2011& Kurai et al., 2007), and antigen presentation by dendritic cells to enhance the anti-tumor immunity (Carmi et al., 2015). While B cells can directly present TAAs captured by B-cell receptors to CD4+ and CD8+ T cells (Rivera et al., 2001& Bruno et al., 2017& Rossetti et al., 2018), they can also facilitate the acquisition TAAs by dendritic cells and macrophages. In addition, B cells can create an anti-tumor effect by secreting cytokines such as IL-12 and IFN-γ (Sharonov et al., 2020). B cells also contribute to the direct destruction of tumor cells by inducing the expression of Granzyme B and TRAIL (Kannan et al., 2011). On the contrary, regulatory B (Breg) cells, a subunit of TIBs, create an immunosuppressive environment by secreting cytokines such as IL-10, TGF-β and IL-35 (Sun et al., 2015& Lee et al., 2014& Chen et al., 2020). Breg cells inhibit CD8+ and CD4+ T cell responses. These cells also stimulate the anergy of CD4+ and CD8+ T cells and also promote the transformation of naive CD4+ T cells into Treg cells (Flores-Borja et al., 2013& Parekh et al., 2003& Chen et al., 2020). As a result, understanding B cells and analyzing their relationship with the tumor microenvironment play an important role in the development and eradication of cancer.

Activation of T lymphocytes

Hematopoietic stem cells in the bone marrow are the source of T cell progenitors (Lai and Kondo, 2008). After that, maturation process occurs in the thymus. In thymus there are several stages take place for the formation of T lymphocytes. For instance, according to VDJ recombination, (also known as somatic recombination) T cell receptors (TCR) are produced. In addition, cell surface proteins which are called as cluster of differentiation (CD) molecules characterized in the thymus. Naive CD4+ and CD8+ T cells move through secondary lymphoid organs in the body after these stages, such as the lymph nodes and spleen, until they contact with their cognate antigen (Drijvers et al., 2020). T cells have different functions according to their cell surface proteins. T helper cells are referred to as having CD4+ surface protein, while cytotoxic T lymphocytes are referred to as having CD8+ surface protein. The activation of naive CD4+ and CD8+ T lymphocytes depends on certain signal sequences (**Figure 1.5**).

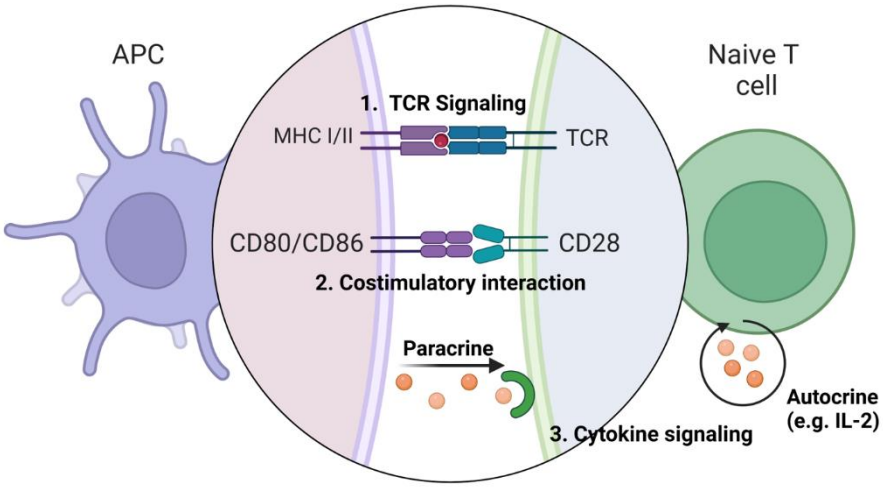


Figure 1.5. Signal cascade for the activation of *naïve* T cell

Firstly, T cells bind to the antigens via cell surface receptors. TCRs can recognize peptide antigens which are presented by MHC molecules. There are two different kinds of MHC molecules. MHCI molecule presents antigen to CD8+ T cells, while the MHCII molecule presents antigen to CD4+ T cells. MHCI molecule is expressed on the surface of nucleated cells, whereas MHCII molecule is expressed on the surface of antigen

presenting cells such as dendritic cells and macrophages (Wieczorek et al., 2017). The main TCR complex includes six CD3 and two TCR chains (Wucherpfennig et al., 2010& Kuhns and Badgandi, 2012). CD3 complex consist of one delta (δ), one gamma (γ), two epsilons (ε), and two zeta (ζ) chain. The antigen binding site of T cell receptor composed of one alpha (TCRα) and one beta (TCRβ) chain. Each of TCR chains contain variable and constant domain. The variable regions of α and β chains bind to the antigen and the remainder of the CD3 complex binds to MHC molecules (Shah et al., 2021). The constant domain has a transmembrane segment and a short cytoplasmic tail (van Boxel et al., 2010). The transmembrane segment contains cysteine residues which allow to adjacent chains to form a disulfide bond connecting the chains to one another. Variable domain of alpha and beta chain forms a single antigen binding site (Clevers et al., 1988& Koning et al., 1988). Interaction between TCR and MHC molecules lead to activation of downstream pathways. These downstream pathways are mediated by critically conserved receptors called immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of T cell receptors. ITAM is a part of the peptide chain that includes two tyrosine amino acids (Underhill and Goodridge, 2007). These amino acids need to be phosphorylated for the T cell activation. Interaction between TCR and MHC molecules lead to activation of lymphocyte tyrosine kinase (Lck) which is a member of Src tyrosine kinase family (Samelson et al., 1986; June et al., 1990; Samelson et al., 1990). After this activation, Lck phosphorylates all of the ITAMs which are located on TCR complex. Once that happens another Syk family tyrosine kinase, ZAP-70 (Zeta chain associated protein kinase 70) binds to phosphorylated ITAMs and become activated (Chan et al., 1992). Activated ZAP-70 then phosphorylates the linker of activated T (LAT), the tyrosine kinase Pyk2 and SH2 domain containing leukocyte protein of 76kDa (SLP-76) (Zhang et al., 1998; Bubeck Wardenburg et al., 1996). After that SLP-76 and LAT work together to trigger chain of events that ultimately activates the major signaling pathways include Ras/RAF (Shan et al., 2001), ERK1/2, calcium flux, nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) (Herndon et al., 2001), phospholipase C (PC) (Akimzhanov et al., 2010), etc. These signal pathways lead to activation of genes that regulate survival, proliferation, differentiation and effector functions of T cells.

The second signal required for T cell activation is called co-stimulation. In this signaling pathway, CD28 mainly needs to interact with one of the B7-1 and B7-2 molecules (CD80 and CD86) which are expressed on antigen presenting cells. If a T cell encounter the

antigen without co-stimulation, it becomes anergic and can not become activated (Chen and Flies, 2013).

The final signal in the activation of T cells is polarized signaling mediated by various cytokines produced between the antigen-presenting cells and the T cell. When cytokines attach to surface cytokine receptors, a series of intracellular signals are triggered that promote cellular proliferation, survival and differentiation (Lee et al., 2020). IL-2 is one of the major cytokines that is essential for T cell proliferation. After the activation of T cells, IL-2 cytokine binds to IL-2 receptor to form an autocrine stimulation. Then the activated T cells start to rapidly undergo cell division a process called clonal expansion. In addition to that type of cytokines, polarizing cytokines produced by APCs, T cells and innate lymphoid cells determine the type of effector cell that T cell develops into (Toribio et al., 1989; Punt et al., 2018).

CD4+ T helper Cells

After the recognition of specific antigens by naïve CD4+ T cells, they polarized into several subsets of T helper (Th) cells that differentially regulate protective immune responses. These polarizations are controlled by many different transcription factors and cytokine signaling pathways. These subsets are classified like Th1, Th2, Th17, Th9, T follicular helper (T_{fh}), and Treg cells **(Figure 1.6)**. Each T helper cell serves a different purpose and regulates the immune system in different ways (Swain et al., 2012& Basu et al., 2021).

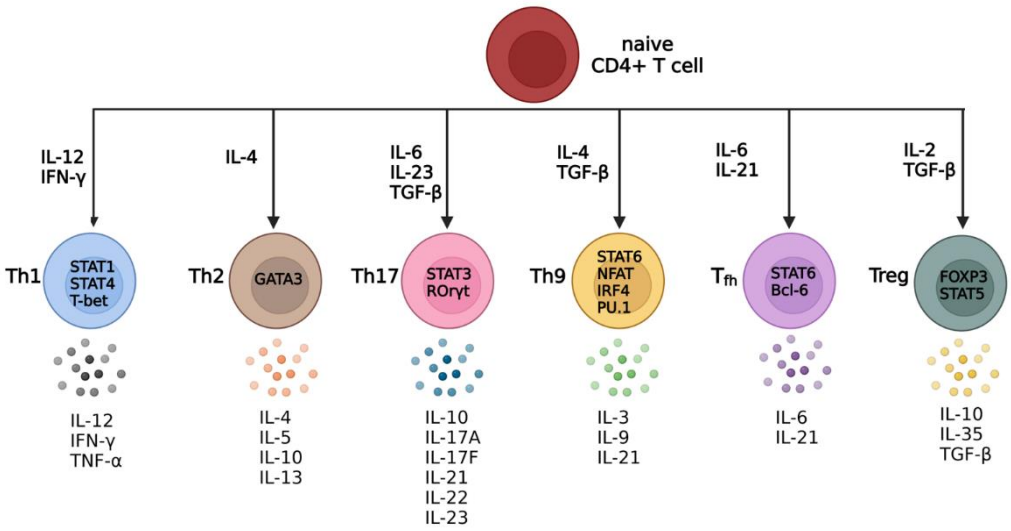


Figure 1.6. Functional subsets of CD4+ T cells

Th1 is one of the most dominant classes of CD4+ Th cells and is characterized by the production of cytokines such as IFN-γ, IL-2 and TNF-α (Saravia et al., 2019). The formation of the Th1 subset is dependent on the secretion of the cytokines such as IL-12, IFN-γ, and type I interferons (Cousens et al., 1999). Th1 cells provide activation of signal transducer and activator of transcription 1 (STAT1) and signal transducer and activator of transcription 4 (STAT4) signaling pathways for the expression of T-bet gene which is a member of the T-box transcription factor family. T-bet plays a dynamic role in the expression of the IL-12 receptor, inhibition of Th2 cell differentiation and induction of IFN-γ secretion (Dobrzanski, 2013; Saravia et al., 2019; Zhu, 2018). IFN-γ and TNF-α secreted from Th1 cells directly support tumor cell apoptosis. In addition, Th1 cells promote the anti-tumor response by up-regulating the activation of NK cells and B cells. Last but not least, Th1 cells secrete IL-12 cytokine, the ligand of CD8+ T cells expressing IL-2Rα, which mediates the proliferation of CD8+ T cells, thereby creating a cytotoxic effect on the tumor (Swain et al., 2012, Basu et al., 2021).

The formation of Th2 cells, an important subset of CD4+ T cells, occurs by the secretion of IL-4 cytokine. After this step, STAT6 signal is stimulated for the expression of GATA 3 transcription factor (GATA binding protein 3) (Saravia et al., 2019; Zhu, 2018). According to that activation Th2 cells are responsible for the high expression of IL-4, IL-5, IL-10 and IL-13 cytokines (Dong, 2006). Thus, it is effective in stimulating B cell mediated humoral immunity, activating mast and eosinophils and eliminating helminth infection. Also, Th2 cells are really crucial for allergic reactions (Kumar et al., 2019). Similar to Th1, differentiation of Th2 cells is caused by IL-4 cytokine with a positive feedback loop. It inhibits the Th1 differentiation and therefore cytokines which are secreted by Th1 cells. In addition to these, IL-4, IL-5, IL-10 and IL-13 also have tumor growth promoting functions. These cytokines induce T cell anergy and inhibit T cell mediated cytotoxicity. Also, these cytokines promote to M2 macrophage polarization, and inhibit the MHCII antigen presentation (Nappo et al., 2017; Li et al., 2008; Bankaitis and Fingleton, 2015; Kidd, 2003).

Naïve CD4+ T cells differentiate into a Th17 cell phenotype by stimulation of IL-6, IL-23 and TGF-β. (Stritesky et al., 2008) Th17 cells promote the expression of transcription factors such as retinoic acid receptor-related orphan receptor-γt (RORγt) and signal transducer and activator of transcription (STAT3) (Saravia et al., 2019; Zhu, 2018). Th17 cells characterized by the secretion of IL-10, 1L17A-F, IL-21, IL-22 and IL-23 cytokines

(Harrington et al., 2005; Liang et al., 2006; Chang and Dong, 2007; Dong, 2008). The Th17 lineage is stabilized by autocrine amplification caused by IL-21 and IL-23 secretion from antigen presentation cells (APC) (Saravia et al., 2019; Zhu, 2018). The most interesting feature of Th17 cells is their plasticity. In other words, while Th17 can differentiate into Th1-like cells that express T-bet and produce IFN-γ and ensure tumor cell elimination (Lee et al., 2009), it can differentiate into Treg cells that express forkhead box p3 (FOXP3) and create an immunosuppressive environment (Ye et al., 2011). Thus, Th17 cells may play a dual role in cancer (Bailey et al., 2014).

Naïve CD4+ T cells polarize to the Th9 cell lineage by the combinations of TGF-β and IL-4 are cytokines (Veldhoe et al., 2008). These cytokines activate the STAT6 signal pathway and expression of transcriptional regulators such as nuclear factor of activated T cells (NFAT), interferon-regulatory factor 4 (IRF4) and PU.1 (Staudt et al., 2010; Jash et al., 2012; Ouyang et al., 2000; Chang et al., 2010). According to these activations, various of cytokines such as IL-3, IL-9 and IL-21 are secreted (Basu et al., 2021). According to IL-9 cytokine secretion, mast cells become more active and cytotoxic (Abdul-Wahid et al., 2016). Furthermore, IL-9 induces the production of CCL20 from epithelial lung cells, which in turn activates CD8+ T cells and DCs in the tumor microenvironment (Lu et al., 2012). NK cytolytic activity is increased and CD8+ T cell proliferation is encouraged by IL-21 secretion (Végran et al., 2014). Furthermore, it has been observed that the cytokine IL-3 increases the production of the anti-apoptotic protein Bcl-xL and induces the activation of the p38, ERK, and STAT5 signaling pathways, both of which stimulate DCs survival (Park et al., 2014). Th9 cells also directly kill cancer cells by supporting the production of Granzyme B (Purwar et al., 2012). In addition to all these, Th9 cells also have pro-tumor properties. IL-9 cytokine contributes to the development of Treg cells. Thus, the activity of immune system cells is inhibited and the development of tumor cells is supported (Feng et al., 2011).

T_{fh} cells are polarized by the presence of IL-6 and IL-21 cytokines and these cells upregulate the expression of the Bcl-6 transcription factor via the STAT3 signaling pathway. T_{fh} cells contribute to production of high-affinity antibody responses via B cell proliferation and immunoglobulin class switching by secreting IL-6 and IL-21 cytokines. Thus, T_{fh} cells represent the anti-tumor effect on cancer cells (Zhu, 2018).

Treg cells, which make up 5-7% of CD4+ T cells, are directly produced in the thymus (tTreg cells), as well as in the peripheral tissues (pTreg cells). High amounts of the cytokines such as TGF-β and IL-2 are necessary for the development of Treg cells. During Treg cell development, it carries out the expression of the FOXP3 gene, a member of the FOX protein family (Raffin et al., 2020). Treg cells control the anti-tumor activities through the secretion of vital immune suppressive cytokines including IL-10, IL-35, and TGF-β (Chaudhry et al., 2011). TGF-β signaling is primarily responsible for the suppression of CD8+ T cell mediated cytotoxic activity (Chen et al., 2005). IL-10 is also prevalent immune suppressor cytokine that can act on CD4+ T cells and other inflammatory mediators. STAT5 signaling pathways in Treg cells reduce the IL-2/IL-2 receptor signaling to establish immunosuppressive activity and limit CD8+ T cell proliferation (Pandiyan et al., 2007; Chinen et al., 2016). However, Treg cells play important roles in preserving self-tolerance and preventing a number of autoimmune disorders (Togashi et al., 2019). In patients with many forms of cancer, high levels of Treg cells are linked to disease progression and poor survival since it is hypothesized that the activation of Treg cells reduce the efficiency of several targeted treatments and immunotherapies (Núñez et al., 2020; Tanaka and Sakaguchi, 2017).

CD8+ cytotoxic T cells

Differentiation to a specific CD8+ cytotoxic T (Tc) cell lineage is determined by the presence of a polarizing cocktail of cytokines during activation of a *naïve* CD8+ T cell. These cytokines stimulate the production of specific transcription factors, which in turn encourage lineage commitment and the adoption of unique effector phenotypes. Each Tc subgroup exhibits a wide range of cytokines and exhibits variations in their capacity for cytotoxicity **(Figure 1.7).**

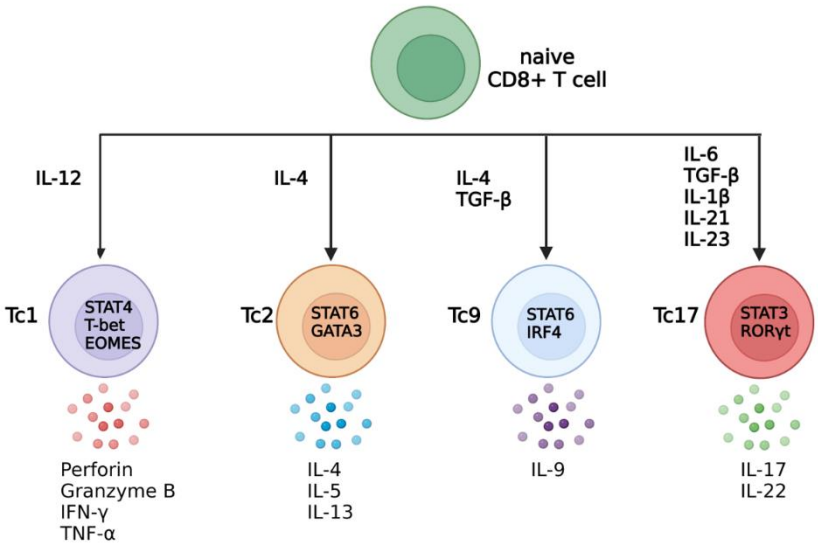


Figure 1.7. Functional diversity of CD8+ T cell subsets

Typical cytotoxic T lymphocytes (CTL), also known as Tc1 cells, are the best-studied effector CD8+ T-cell subset and are essential for the removal of intracellular pathogens and cancer cells (St Paul and Ohashi, 2020). The cytokine IL-12 which is normally produced by APCs, mediates the induction of Tc1 cells (Croft et al., 1994). Tc1 cells can kill the target cancer cells by releasing cytotoxic chemicals such as granzymes and perforins into the immunological synapse. These cells also secrete the cytokines including IFN-γ and TNF-α. Perforin is a pore-forming protein and granzyme is a serin protease that stored within the cytotoxic granules of cytotoxic CD8+ T cells. When cancer cell and CD8+ T cell interact with each other, CD8+ T cells produce perforin to generate pores on the tumor cells plasma membrane. Released granzymes enter the cancer cells and cleave their intracellular substrates (Basu et al., 2016; Mittrücker et al., 2014). When CTLs encounter a target cell with a foreign antigen, they activate the Fas pathway. Thus, it provides the induction of caspases through the release of cytochrome c in the target cell and supports entry into apoptosis (Fu et al., 2016). Cytokines which are released by CD8+ T cells accelerate the innate and adaptive immune response against pathogens and cancer cells (Kaech and Cui, 2012). In addition, activation of innate immune cells such as neutrophils and macrophages, which are involved in body defense, is also provided. STAT4 (Yang et al., 2007), T-bet (Joshi et al., 2007), and eomesodermin (EOMES) (Intlekofer et al., 2008) are important transcription factors that influence the polarization of Tc1 cells.

Tc2, a CD8+ T cell subunit, is characterized by the production of type II cytokines such as IL-4, IL-5 and IL-13. Tc2 cells produce reduced amounts of IFN-γ (Mittrücker et al., 2014; Mingari et al., 1984; Salgame et al., 1991; Seder et al., 1992). IL-4-induced polarization of Tc2 cells causes the expression of transcription factors such as STAT6 and GATA3 (Pai et al., 2004). Tc2 cells also express high levels of Granzyme B, therefore they have robust cytotoxic properties (Kemp and Ronchese, 2001). Tc2 cells play a critical role in allergic reactions, production of IgE and the recruitment of pathogenic cells, including eosinophils (Schaller et al., 2005; Hilvering et al., 2018).

Tc9 cells can generate higher amounts of IL-9 cytokine but they express very reduced levels of Granzyme B, EOMES, T-bet, and IFN-γ (Lu et al., 2014). In contrast to Tc1 and Tc2 cells, Tc9 cells exhibit weak cytotoxic properties. Polarization of Tc9 cells occurred by IL-4 and TGF-β cytokines and interceded by transcription factors including STAT6 and IRF4 (Visekruna et al., 2013).

A specific group of CD8+ T cells called Tc17 cells produce high levels of IL-17, IL-22 and low levels of IFN-γ (Intlekofer et al., 2008; Huber et al., 2009; Hinrichs et al., 2009). Differentiation of Tc17 cells occurred by the interaction between IL-6 and TGF-β, and this process is further aided by the presence of IL-1β, IL-21, and/or IL-23 (Yen et al., 2009). STAT3 and RORγt are activated by IL-6 signaling, which is necessary for polarization of the Tc17 lineage (Huber et al., 2009; El-Behi et al., 2014). Tc17 cells express low levels of granzyme B and have poor cytotoxic functions like Tc9 cells (Huber et al., 2009). While Tc17 cells promote tumor growth in some cancer types, they also show anti-tumor properties in some cases. This suggests that it plays a dual role in the tumor microenvironment.

CTLs have dynamic relations with other cells in tumor microenvironment. These cross-talks between CTLs and cancer cells are really crucial for tumor progress. CTLs show positive cross-talking with immunostimulatory cells including NK cells, M1 cells, CD4+ T cells, and DCs. In contrast they represent negative cross-talking with immune-inhibitory cells like CAFs, cancer cells, Tregs, and M2 cells. NK cells secrete IFN-γ to induce CTL anti-tumor functions (Smyth et al., 2002). IFN-γ is also produced by both CTLs and M1 cells to activate M1 polarity (Albini et al., 2018). Additionally, TNF-α is released into the TME by both M1 cells and CTLs (Bertrand et al., 2017). In addition, CTLs release IFN-γ to trigger the release of chemokines, which aid in recruitment of

CD4+ T cells to the TME (Borst et al., 2018). In contrast, cancer associated fibroblasts (CAFs) play a crucial role in create an immunosuppressive environment. CAFs mediate a residual glycolytic metabolic rate that elicits glucose deficiency in the tumor microenvironment. This deficit encourages malignant cells and effector T cells to compete with each other. When exposed to such circumstances, CTLs have a tendency to become fewer, which CAFs use as a way to control the quantity of these cells (Kato et al., 2018). However, Treg cells play an immunosuppressive role and reduce the translocation of CTL to the tumor nucleus (Maj et al., 2017). By secreting TGF-β, it provides inhibition of gene products of CTLs that perform tumor cytotoxicity such as granzyme, perforin, IFN-γ and FasL. In addition, TGF-β inhibits the proliferation of CD4+ and CD8+ T cells (Thomas and Massagué, 2005).

Considering all these interactions and the dynamic role of CD8+ T cells, it is of great importance in terms of cancer cell development, invasion and target therapeutic approaches.

Jurkat T cells

Jurkat T cells are an immortalized human T lymphocyte cell line. Originally, these cells were isolated from 14-year-old-boy who has a T cell acute lymphoblastic leukemia (TALL). These cells are important in studying T cell signaling. (Abraham & Weiss, 2004). Jurkat T cells are known as cytotoxic CD4+ T cells containing TCR and CD3 complex (Dong et al., 1999). CD4+ cytotoxic cells have the potential to directly kill target cells containing MHCII by secreting granzyme B and perforin. There were some differences between Jurkat T cells and peripheral T cells. ZAP-70, LAT, and SLP-76 phosphorylation are common events for both in TCR-mediated signaling. On the contrary, Pyk2, ERK1/2, PC phosphorylation is seen more in Jurkat T cells and a higher rate of calcium influx is provided. In addition, Jurkat T cells secrete more IL-2 and IL-8 cytokines than peripheral T cells (Bartelt et al., 2009).

1.6 Lung cancer

Despite all the technological developments in the field of health, lung cancer is one of the cancer types with the highest death rate. The most important reason for the formation and development of lung cancer is tobacco smoke. However, according to studies conducted in recent years, an increased incidence of lung cancer is observed in people

who have never smoked and who do not smoke (Aj et al., 2013). Also, the total death is strongly correlated with the age.

Lung cancer is a diverse disease with numerous subgroups that have clinical and pathological significance (Travis et al., 2013; Travis et al., 2013; Fujimoto and Wistuba, 2014). Lung cancer is divided into two groups according to histology and therapeutic approaches: small-cell carcinoma (SCLC) and non-small cell carcinoma (NSCLC). NSCLC represents 85% of all lung cancer patients, while SCLC represents 15% of all lung cancer patients (Rodriguez-Canales et al., 2016). NSCLC is classified into adenocarcinoma (ADC), squamous cell carcinoma (SqCC), and large cell lung carcinoma (LCLC) (Rodriguez-Canales et al., 2016). ADC symbolized the majority of NSCLC and accounts for 38.5% of instances of lung cancer. SqC represents approximately 20% and LCLC symbolized 2.9% of all lung cancers (Dela Cruz et al., 2011).

The most important indicator of the approach to treatment in lung cancer is the stage of the disease. In stage I NSCLC patients, the cancer is in a small portion of the lung and has not yet spread outside. In stage II, the lung is totally infected with cancerous cells. In stage III, it began to spread outside the lung. In stage IV, the cancer cells have expanded to the lung, surrounding tissues, and it has begun to gradually migrate to other organs. Treatment methods of lung cancer also depend on its histological type and stage of the disease. Surgical methods are used in patients with stage I to stage III NSCLC (Beckles et al., 2003). In addition, administration of chemotherapy increases the survival rate in NSLCLC patients. Radiotherapy combined with chemotherapy can give positive results in unresectable species. In addition to these, treatment methods based on the molecular mechanisms underlying NSCLC can also be applied (Jones and Baldwin, 2018).

1.6.1 Lung Cancer Immunology

Alterations in the immune system are involved in the formation and development of lung cancer. Lung cancer cells impair the presentation of tumor-associated antigens to T cells by APCs through the cytokines they secrete. For this, changes can be observed on costimulatory molecules. Under normal conditions, antigen presentation is provided by interacting with B7 in APC and CD28 in lymphocytes. However, due to changes in the tumor microenvironment and immunosuppressive properties, B7 molecule is interacted

with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which inhibits TCR signaling (Egen et al., 2002). CTLA-4 is constitutively expressed on regulatory T cells. Therefore, high percentage of Treg cells show functionality in lung cancer. In studies, a high rate of CD13+/CD4/CD25^{high} Treg cell population is observed in the peripheral blood of lung cancer patients. These cells can express higher amounts of proteins such as CTLA-4 and TGF-β1, which have immunosuppressive functions. (Ju et al., 2009). However, there are Treg cells that express FOXP3 at high rates in tumor-infiltrating lymphocytes in lung cancer patients (Petersen et al., 2006; Shigematsu et al., 2012). In addition, high levels of M2 macrophages have been observed in the lung cancer microenvironment. In this case, it supports angiogenesis, wound healing and IL-10 release in lung cancer cells (Mantovani et al., 2011; Ma et al., 2010).

TGF-β is one of the most well-known cytokines secreted from lung cancer cells. Studies have shown that lung cancer patients have higher levels of TGF-β in their serum compared to healthy individuals (Hasegawa et al., 2001). However, TGF-β expression has been demonstrated in all lung cancer types include SCLC and NCLC (Jeon et al., 2010; Wojciechowska-Lacka et al., 1996). TGF-β expression blocks T cell proliferation, and induce Treg differentiation. All in all, it can stimulate the lung cancer survival (Cottrez and Groux, 2001).

IL-10 is a cytokine that expressed by lung cancer cells. According to research, more IL-10 mRNA was observed in the serum of NSCLC patients compared to healthy individuals, which was associated with a poor prognosis. In addition, increased IL-10 level is also related with lung cancer metastasis (Hatanaka et al., 2000). IL-10 has a high and critical importance on Th2 polarization in lung cancer. It also inhibits Th1 differentiation, provides an immunosuppressive environment by blocking antigen presentation and NK cell activation. IL-10 works with TGF-β to provide an environment with anti-tumor effects for the development of lung cancer cells (De Vita et al., 2000; Hatanaka et al., 2000; Teng et al., 2011).

IL-17A has been associated with poor prognosis in studies on lung cancer (Andreev et al., 2012). For example, high levels of IL-17A and various Th17 transcription factors have been observed in lung cancer patients compared to healthy individuals (Reppert et al., 2011). The pro-tumor feature of IL-17A is due to the fact that the function of STAT3 expression. While STAT3 regulates the expression of IL-17A, IL17A signaling provides

a positive feedback loop by providing the expression of STAT3 with the cytokine IL-6 (Wang et al., 2009). STAT3 is associated with the survival, proliferation, and angiogenesis of lung cancer cells. It also mediates the expression of IL-23, which stimulates lung cancer cell proliferation, while blocking the cytokine IL-12 (Hatton and Weaver, 2009). IL-17A secretion is also associated with Treg differentiation. According to studies, activation of Th17 specific transcription factors in lung cancer patients was correlated with FOXP3 expression. Moreover, IL-17A blockage has been associated with decreased FOXP3+ Treg cells in mouse models (Reppert et al., 2011).

Blocking the factors that provide an immunosuppressive environment in the lung cancer microenvironment has a therapeutic potential on this disease.

1.7 Evasion of immune responses by tumor

The tumor evades from attacks of immune cells by several pathways. These events are sometimes provided by the changes in cancer cell and sometimes by the factors which are secreted from tumor. In addition, inhibition of some immune cells may occur due to the cancer cell before it enters the tumor microenvironment (Kim et al., 2022). For example, cancer cells inhibit DC maturation by secreting tumor derived-factors like IL-10 (Williams et al., 2004), MCSF (Nefedova et al., 2004), TGF-β (Zong et al, 2016), prostaglandin (Sá-Nunes et al., 2007), IDO (Munn and Mellor, 2016), and VEGF (Gabrilovich et al., 1996). Also, tumor blocks the activation of T lymphocytes by downregulating the expression of MHC and co-stimulatory molecules. In addition, T cells express chemokine receptors such as CXCR3 on the cell surface during the activation process (Kuo et al., 2018). Cancer cells reduce the expression of receptor ligands such as CXCL9, CXCL10 and CXCL11 to escape the T cell attack, thus preventing the migration of CD8+ cells into the tumor microenvironment (Karin, 2020). When immune cells enter the tumor microenvironment, the tumor tries to escape from the immune system in different ways. Cancer cells alter, decrease, or delete MHCI molecule from their surface in an effort to avoid being recognized by T cells (Dhatchinamoorthy et al., 2021). Cancer cells either directly or indirectly block peptide-MHC components or control MHCI genes or proteins. Additionally, by mutation, genetic loss, transcriptional inhibition, or epigenetic suppression of gene expression, cancer cells fail the expression of tumor associated antigens to prevent recognition by immune cells (Taylor and Balko, 2022). Last but not least, expression of immune checkpoint molecules on both cancer cells and

immune cells are really fundamental for the escape cancer cells from immune cells (Kim et al., 2022).

1.8 Immune checkpoint molecules

Immune checkpoint molecules, including inhibitory and stimulatory molecules, are ligand-receptor combinations that block or stimulate immune responses **(Figure 1.8).**

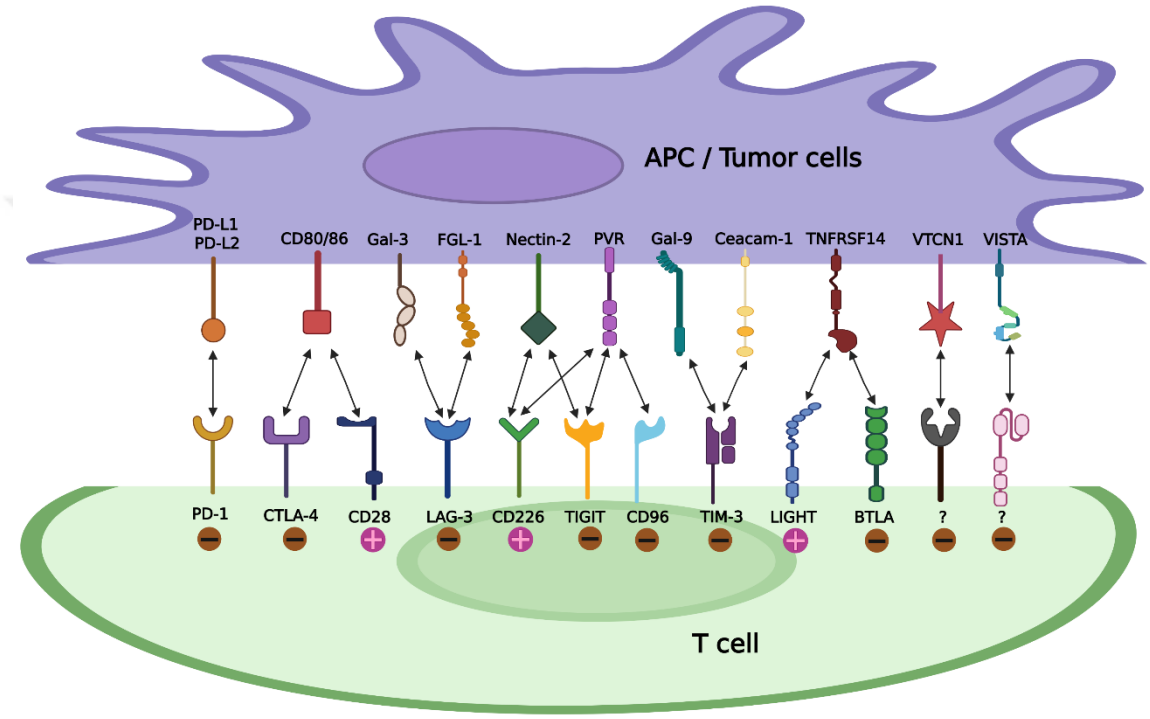


Figure 1.8. Immune checkpoint receptors and their respective ligands

Most of the immune checkpoint molecules are expressed on adaptive immune system cells, especially on T lymphocyte surface, they can also be expressed on innate immune system cells. Respective partners of these immune checkpoint molecules are developed on the surface of antigen presenting cells and also many tumor types (Zhang and Zheng, 2020).

Firstly, antigen-specific T cells identify cognate antigens via the interaction between TCR and MHC molecules (Sharpe, 2009). After that, a signal from CD80/CD86 (stimulaton the APCs to CD28 on T cells is required. The quality and duration of the

effector response are then determined by signals that T cells receive from a variety of ligands on DCs. Effector T cell responses are amplified via receptor-ligand interactions, such as those between CD40-CD40 ligand (CD40L), OX40-OX40L, 4-1BB-4-1BBL (also known as CD137L), inducible T cell co-stimulator (ICOS)-ICOSL, and CD27-CD70. According to these interactions T cell proliferation and activation can be occurred (Wykes and Lewin, 2018). On the contrary, generally the immune checkpoint molecule interactions between immune system and antigen presenting cells or cancer cells involve the inhibition of T cell activation. While this situation limits anti-tumor effect, it enables the metastasis, invasion, drug resistance, anti-apoptosis and gained energy metabolism of tumor (Zhang and Zheng, 2020). Blocking of the immune checkpoint molecules is one of the most novel immunotherapy modalities. This method of treatment does not directly kill cancer cells, but instead utilizes the host's immune system to regain endogenous anti-tumor activity (Marin-Acevedo et al., 2018).

The programmed cell death protein 1 (PD-1) and its ligand pairs programmed cell death-ligand 1 (PD-L1, is encoded by PDCD1LG1 gene), and programmed cell death 1 ligand 2 (PD-L2, is encoded by PDCD1LG1 gene) are one of the most dominant and studied immune checkpoints operating in the tumor microenvironment (TME). As a result of the interaction of these molecules, immune homeostasis is controlled and cancer cells are stimulated to escape from the immune attack (Pardoll, 2012; Pardoll, 2015). Also the activation of these receptor-ligand pairs lead to inhibition of T cell activation, proliferation and cytokine secretion which can be cause to degeneration of anti-tumor immune responses (Ljunggren et al., 2018). In addition, CTLA-4 and its ligand pairs CD80 and CD86 are the most well-known immune checkpoint molecules (June et al., 1994; Linsley et al., 1994). CTLA-4, which has a common ligand combination with CD28, is responsible for inhibiting T cell attack and IL-2 secretion by interacting with ligand pair (Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1996). These immune checkpoint molecules are the most targeted combinations in immunotherapy. However, there are also immune checkpoint molecules that have not been studied much and are unknown.

1.8.1 Galectin-3

Galectins are a family of lectins that have the ability to recognize and bind to β-galactoside glycoconjugates due to their conserved carbohydrate recognition domain

(CRD). Galectins contain 15 members and these members are examined in 3 main groups depending on number and structure of CRDs. These are prototype galectins, tandem repeat galectins and chimera galectins (Fortuna-Costa et al., 2014) **(Figure 1.9).**

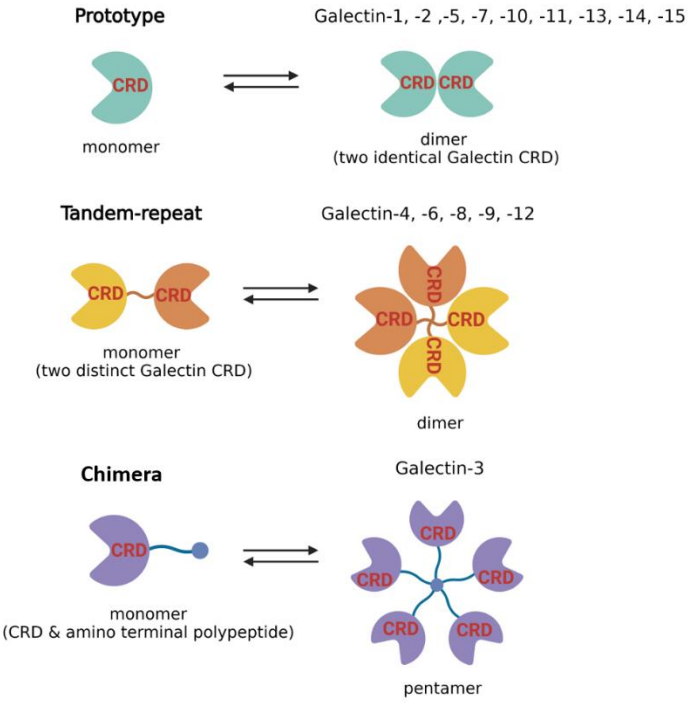


Figure 1.9. Classification of galectins

Prototype galectins are composed of galectin-1, -2, -5, -7, -10, -11, -13, -14,- 15 and contain only one type of CRD that can form homodimers. The tandem-repeat galectins are composed of galectin-4, -6, -8, -9, -12 and contain the linking of two distinct CRDs via a peptide linker. These galectins can form dimers. Finally, chimera galectin consists of Galectin-3 (Gal-3), which is encoded by LGALS3 gene, identified in vertebrates and involves binding to a single CRD and an amino-terminal polypeptide. This galectin type can form pentamer also known as oligomer (Yang et al., 2008; Nabi et al., 2015; Johannes et al., 2018). The oligomeric structure of Gal-3 distinguishes it from all other galectins (Dumic et al., 2006) and contributes to several biological functions (Ahmed and AlSadek, 2015; Ahmad et al., 2004). Extracellularly, Gal-3 can binds to its substrates via CRD on their surface. This binding mediates the cell-cell and cell-ECM interactions and activates many signaling pathways including migration, invasion and cell adhesion. (Obermann et al., 2017) **(Figure 1.10).** In the intracellular space, Gal-3 binds to its substrates through protein-protein interaction (Dumic et al., 2006). Gal-3 can be expressed in many cell

tissues, immune cells, as well as tumors. Galectin-3 regulates many intracellular signaling pathways including proliferation, apoptosis and cell cycle **(Figure 1.11).**

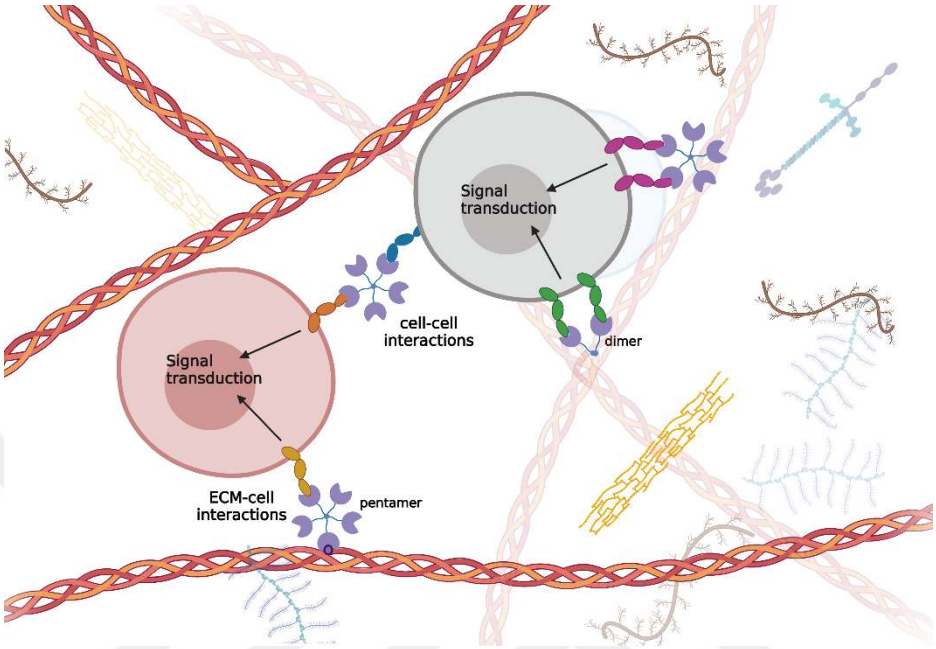


Figure 1.10. Extracellular functions of Galectin-3

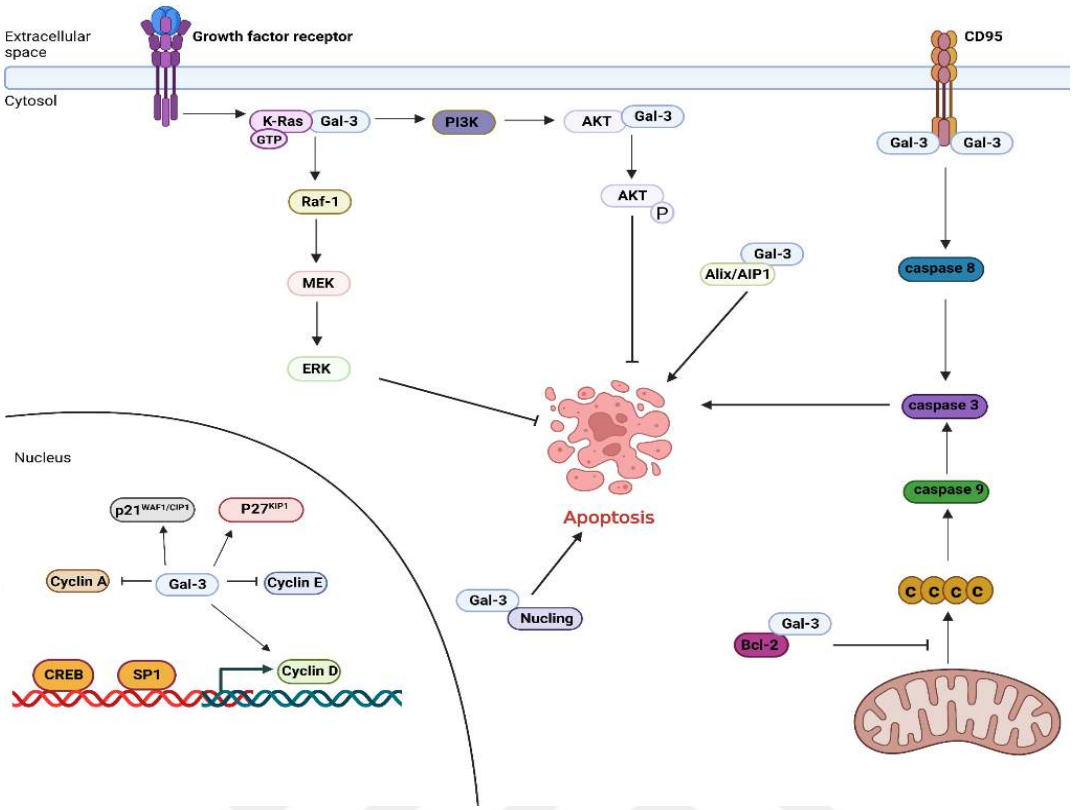


Figure 1.11. Intracellular functions of Galectin-3

The first cytosolic molecule identified as the galectin-3 ligand in *in vivo* studies is B-cell lymphoma 2 (Bcl-2), which is responsible for apoptosis regulation. Gal-3 binds to Bcl-2 via its CRD and as a result of this interaction, an anti-apoptotic effect is created (Yang et al., 1996). In addition, it has been shown that Gal-3 interacts cytosolically with CD95 (APO-1/Fas), one of the apoptosis receptors, by immunoprecipitation and confocal microscopy. As a result of this interaction, it was revealed that the induction of apoptosis through caspase 8 was increased (Fukumori et al., 2004). Nucling (Liu et al., 2004) and Alix/AIP1 (Liu et al., 2002) proteins can interact with Gal-3 and are involved in the regulation of apoptosis. The involvement of cytosolic Gal-3 in cell proliferation, differentiation and survival signaling is also demonstrated by K-Ras and Akt proteins. For example, Gal-3 can bind to GTP-bound K-Ras in breast carcinoma. As a result of this binding, the Raf-MEK-ERK signaling pathway is activated. Thus, a resistance against apoptosis is demonstrated and the proliferation of the breast carcinoma cell is supported. (Shalom-Feuerstein et al., 2005). However, GAL-3 is also effective on TRAIL-induced

apoptosis resistance in bladder carcinoma cells. In case of TRAIL-receptor relationship, the PI3K complex and its sub-signaling pathways are activated. Akt is one of the important downstream PI3K target, reported to be involved in TRAIL-induced apoptosis. After PI3K activation, Akt is translocate to the interior of the plasma membrane. Interaction between Akt and Gal-3 cause the phosphorylation and activation of Akt. As a result, BID cleavage is inhibited, mitochondrial membrane depolarization is impaired, caspase-9 and caspase-3 activation is blocked. All of these events provide the resistance to TRAIL-induced apoptosis. (Oka et al., 2005). Gal-3 is involved in the regulation of the cell cycle. For example, Gal-3 has been shown to stimulate cyclin D expression in human breast epithelial cells (BT549) via cis-elements, including the SP1 and CRE regions. (Lin et al., 2002). In addition, in another study in the same cells, it was observed that overexpression of Gal-3 can induced G1 arrest without cell death and upregulated G1-S cyclin kinases (cyclin E and cyclin A) and inhibitor proteins $p21W^{AF1/CIP1}$ and $p27^{KIP1}$ (Kim et al., 1999).

Gal-3 has a very important role in cell-cell and cell-ECM interactions. The reason for this, Gal-3 can be able to bind to cell surface glycoproteins and glycosylated components of the ECM. Gal-3 binds to laminin (Massa et al., 1993; van den Brûle et al., 1995), fibronectin (Sato and Hughes, 1992), hensin (Hikita et al., 2000), elastin (Ochieng et al., 1999), collagen IV (Ochieng et al., 1998), tenascin C, and tenascin R (Probstmeier et al., 1995). In addition, induced Gal-3 interacts with N-linked oligosaccharides modified with Alpha-1,6-Mannosylglycoprotein 6-Beta-N-Acetylglucosaminyltransferase (Mgat5) in breast carcinoma cells and stimulates tumor cell spreading and motility by activating α5β1-integrin (Lagana et al., 2006). However, it has been shown that exogenous or tumor-derived Gal-3 interacts with cancer-associated transmembrane mucin protein (MUC1) in colon cancer and melanoma cells, increasing the aggregation, adhesion and survival of tumor (Bresalier et al., 1996).

Gal-3 also supports angiogenesis in various cell types. Neuron-glial antigen 2 (NG2) is a proteoglycan expressed by pericytes in newly formed blood vessels. Interaction of NG2 with endothelial cells affect cell motility and network formation *in vitro*. It has been shown that the realization of angiogenesis is occurred by the interaction of NG2–Gal-3–α3β1 integrin. Gal-3-dependent oligomerization has been shown to enhance NG2-mediated activation of α3β1 integrin (Fukushi et al., 2004).

Gal-3 is involved in the regulation of endosomal sorting complexes required for transport (ESRCT) and autophagy systems to create protection system against lysosomal damage (Jia et al., 2018; Radulovic et al., 2018). Gal-3 recognizes and binds to glycans on the lysosome during lysosomal damage. This binding mediates the interaction of the ESCRT component ALIX with Gal-3. After this step, Gal-3 support the connection of ALIX and downstream ESCRT-III effectors charged multivesicular body protein 4a (CHMP4a) and charged multivesicular body protein 4b (CHMP4b) during lysosomal repair (Jia et al., 2020). One of the receptors involved in the regulation of autophagy is the tripartite motif16 (TRIM16) receptor (Kimura et al., 2015). Most TRIMs contain E3 ligase domains and combine with the active forms of Unc-51-like kinase 1 (ULK1) and Beclin-1 (BECN1) (Mandell et al., 2014). During lysosomal and phagosomal damage, TRIM16 interacts with Gal-3 in a ULK1-dependent manner on the damaged lysosome. The interaction of TRIM16, ULK1 and Gal-3 mediate the ubiquitin-based processes and connection of ATG16L1 and BECN1. According to these interactions, autophagic activation of damaged lysosome is stimulated (Chauhan et al., 2016) **(Figure 1.12).** Gal-3 also interacts with the lysosomal surface glycoproteins such as lysosomal-associated membrane protein 1 (LAMP1) and lysosomal-associated membrane protein 2 (LAMP2). Therefore, it can regulate the repair of damaged lysosomes and control the autophagosome and lysosome fusion process during autophagy.

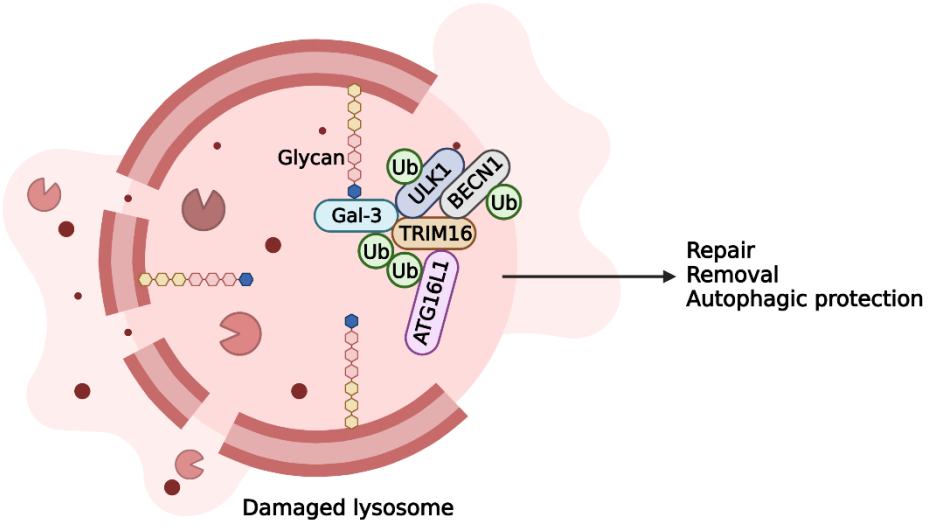


Figure 1.12. Relationship between Gal-3 and autophagy

Gal-3 also has a critical role in the regulation of immune cells. Gal-3 expressed in APCs or tumor. Gal-3 is involved in apoptosis, activation, TCR signaling, etc. of T lymphocytes. For example, CD8+ T cell activation was observed in Gal-3-deficient melanoma cells and increased secretion of IFN-γ cytokine was demonstrated (Melief et al., 2017& Radosavljevic et al., 2011). Also, Gal-3 acts as a ligand and has a high affinity for inhibitory receptor lymphocyte-activation gene 3 (LAG-3). It is a type I transmembrane protein that is mostly find on the surface of activated T cells (including CD4+, CD8+ and Treg) and NK cells. The extracellular domain of the LAG-3 protein is similar to CD4+ T cells, so this protein also interacts with MHCII molecule (Triebel et al., 1990). This interaction modulates the inhibition of Th1 cell proliferation and activation. When Gal-3 and LAG-3 interacts with each other CD8+ T cell mediated cytotoxicity is impaired by reducing the secretion of IFN-γ. In addition, this interaction restricts early TCR activation and provides TCR downregulation (Chen et al., 2009). Gal-3 and LAG-3 also contribute to tumor growth, drug resistance, and the ability to gain metastasis and invasion. Inhibiting this interaction supports a very serious healing effect on cancer (Nakajima et al., 2021). Gal-3 is also effective on macrophages. M1 macrophage activation has a negative effect on Gal-3 expression, while IL-4/IL-13 mediated M2 activation increases Gal-3 secretion (MacKinnon et al., 2008; Novak et al., 2012). Gal-3 then becomes a factor of the feedback loop by binding to the CD98 or CD98-β1 complex leading to M2 stimulation via PI3K activation. Thus, Gal-3 has the capacity to transform M1 macrophages into M2 macrophages in the tumor microenvironment (MacKinnon et al., 2008).

Lung cancer is one of the cancer types that Gal-3 is abundantly expressed. One of the cancer types in which Gal-3 is most expressed is lung cancer. It has been reported that Gal-3 plays a critical role in tumor formation, metastasis and migration in lung cancer (Cardoso et al., 2016). While Gal-3 is less expressed in SCLC type, it is expressed at high levels in NSCLC type (Yoshimura et al., 2003). In studies, CSC contribute to the development of the tumor in a sphere form. CSC plays a critical role in relapse and resistance to chemotherapy. Lung cancer spheres composed of H1299 NSCLC cells showed high expression of Gal-3. Suppression of this high expression is inhibited spheroid forming ability, tumorigenicity and resistance to treatment in mice. It has been shown that the capacity of H1299 cells to develop stem cells is provided by the interaction of Gal-3 and β-catenin, and if this interaction is impaired, the growth of the tumor is

blocked (Chung et al., 2014). In another study, increased Gal-3 expression in mice with Lewis lung carcinoma (LLC) had a positive effect on tumor growth by mediating the migration of MDSCs into the tumor microenvironment (Wang et al., 2014). In all major areas of the healthy lung, Gal-3 is expressed in the endothelium. Thus, the presence of Gal-3 in normal lung tissue, providing adhesion to other cells, is thought to be a critical element for lung metastasis. For example, Gal-3 in the endothelium can bind to poly-N-acetyllactosamine (polyLacNAc) in melanoma cells. This can cause melanoma cells to spread to the lungs. According to studies, polyLacNAc downregulation in melanoma cells inhibited MMP9 secretion, and metastasis to lung (Dange et al., 2014). Conversely, in some cases, the presence of Gal-3 may have a negative effect on tumor growth. For example, Gal-3 has been shown to have a critical role in the regulation of carcinogenesis-related B-cell receptors. In this case, Gal-3 knockout mice had upregulated lung tumor survival compared to Gal-3 positive mice (Abdel-Aziz et al., 2008).

1.8.2 Fibrinogen-like Protein 1

Fibrinogen-like protein 1 (FGL-1) is a physiological secretory protein from hepatocytes in the liver, therefore its expression regulated as a self-protective mechanism to counteract foreign stimulation or injury (Wang et al., 2019; Han et al., 2019; Jin et al., 2019).

FGL-1 is expressed through STAT3 and hepatocyte nuclear factor-1α (HNF-1α) signaling pathways. Inflammatory cytokine IL-6 activates the JAK2-STAT3 pathway in hepatocytes. HNF-1α creates a complex with high mobility group box 1 (HMGB1 and CREB proteins in cytoplasm. This complex moves into the nucleus and interacts with pSTAT3 via HPS promoter, thus increasing the transcription of FGL-1 (Yu et al., 2009; Thilakasiri et al., 2021; Marzec et al., 2008; Wang et al., 2020). FGL-1 is expressed in solid tumors. FGL-1 controls the AKT-mTOR pathway to modulate downstream signaling pathways involved in cancer cell proliferation (Nayeb-Hashemi et al., 2015; Grabinski et al., 2012). Epithelial indicators (E-cadherin and ZO-1) and mesenchymal markers (N-cadherin, vimentin, Snail, Twist, MMP12 and fbronectin) are controlled by FGL-1 to mediate EMT for cancer cells (Bie et al., 2019; Onder et al., 2008; Kim et al., 2019; Mohan et al., 2020; Tsai et al., 2019). By affecting EGFR phosphorylation, FGL-1 also inhibits the PARP1-caspase apoptotic pathways for cancer cells (Sun et al., 2020). FGL-1 has a high affinity to inhibitory receptor LAG-3. When FGL-1 and LAG-3

interacts with each other, it may cause the inhibition of proliferation, activation, homeostasis and effector functions of CD8+ and CD4+ T cells. Also FGL-1-LAG-3 pathway increase pro-tumor effects and reduce the cytotoxic activation of immune cells. Thus tumor cells can evade from the immune cell attacks (Grosso et al., 2009). Taking account all these things, blockade of the FGL-1-LAG-3 pathway is a remarkable immunotherapeutic strategy for cancer patients.

1.8.3 Nectin Cell Adhesion Molecule 2

Nectin cell adhesion molecule 2 (Nectin-2), also known as CD112 or poliovirus receptor-related 2 (PVRL2), has a single transmembrane region, a cytoplasmic tail containing an afadin-binding motif, and an extracellular region containing three Ig-like domains (Samanta et al., 2015). Nectin-2 is a ligand that is highly expressed on APCs and cancer cells. This molecule can either activate the immune system or inhibit the anti-tumor effects according to interaction with its pair receptor.

CD226, also known as DNAX accessory molecule-1 (DNAM-1), is a co-stimulatory receptor expressed on monocytes, NK cells, B cells, and T cells (Stamm et al., 2018). When it interacts with Nectin-2, it creates an activating response on T cells and supports the regression of tumor growth. (Shibuya et al., 2003). In contrast to this receptor, TIGIT (T cell immunoreceptor with Ig and ITIM domains), a co-inhibitory receptor belonging to the immunoglobulin super family, is expressed on memory and effector T cells, Treg cells, T_{fh} CD4+ T cells and NK cells (Stamm et al., 2018). TIGIT and Nectin-2 interaction reduces the immune cell response in an extracellular and cell-specific manner (Yu et al., 2009; Dougall et al., 2017). CD112R, another co-inhibitory receptor with the greatest binding affinity for Nectin-2, is expressed on T cells and NK cells. As a result of interaction with Nectin-2, immune cell attack decreases and thus the anti-tumor effect is eliminated (Zhu et al., 2016). Thus, the inhibition of these immune checkpoint interactions will reveal a curative treatment option on the tumor.

1.8.4 Poliovirus receptor

Poliovirus receptor (PVR), also known as CD155, is a transmembrane protein and contains three Ig-like extracellular domains (Bernhardt et al., 1994). PVR, is a ligand that is expressed on APCs and the tumor. PVR shows different functions as a result of the receptor it binds to (Zeng et al., 2021). Like Nectin-2, PVR also binds to the CD226

receptor. Unlike Nectin-2, it shows greater binding affinity to CD226 (Stamm et al., 2018). When PVR and CD226 interacts with each other, immune cell activation and anti-tumor effect can be observed. In addition, PVR can binds to CD96, also known as T cell activation increased late expression (Tactile) receptor, which is a type 1 transmembrane protein belonging to the immunoglobulin superfamily (Sanchez-Correa et al., 2019). CD96 is expressed on T cells and NK cells. Although the interaction of CD96 and PVR has a negative effect on immune cells (Wang et al., 1992), it is also known to support CD8+ T cell activation and effector response in some cases (Chiang et al., 2020). However, PVR interacts with the TIGIT receptor such as Nectin-2. TIGIT receptor shows greater binding affinity to PVR than Nectin-2 (Kong et al., 2016). TIGIT and Nectin-2 have a far higher affinity than CD226 and Nectin-2, therefore it outcompetes the stimulatory receptor CD226 in terms of CD8+ T cell activation and effector responses. TIGIT-PVR interaction promotes tumor growth on the cancer cell (Stamm et al., 2018).

1.8.5 Galectin-9

Galectin-9, which is encoded by LGALS9, is a member of chimera-type galectin family has conserved two separated carbohydrate-recognition domains (CRDs) that joined by a linker sequence. Gal-9 can crosslink glycoproteins to control a variety of biological activities (Yang et al., 2008). Gal-9 is crucial for angiogenesis (Aanhane et al., 2018), proliferation (John and Mishra, 2016), T cell- homeostasis (Chen et al., 2020), the stability of the central nervous system (Chen et al., 2014), the preservation of liver homeostasis (Golden-Mason and Rosen, 2017), and the control of lysosomes and autophagy (Sudhakar et al., 2020).

Tumor cells or tumor tissue consist high levels of Gal-9 compared to normal para-cancerous tissues in many types of malignancies. Gal-9 which is expressed on the cancer cell acts as a ligand and interacts with various receptors (Lv et al., 2022). T-cell immunoglobulin- and mucin-domain-containing molecule (TIM-3) is a type I transmembrane protein and one of the co-inhibitory receptors for Gal-9 (Zhu et al., 2005). TIM-3 is expressed on CD8+ T cells, Treg cells, macrophages, DCs, and NK cells. The interaction of Gal-9 and TIM-3 causes the exhaustion of T cell which impairs T cell tolerance and inhibits the release of IFN-γ. In addition, this interaction induces the apoptosis of Th1 and Th17 cells (Oomizu et al., 2012). As a result of the interaction of Gal-9 and TIM-3, Treg cells are stimulated in the tumor microenvironment and the anti-

tumor effect on cancer is reduced (Yan et al., 2013). In case of blocking the Gal-9 and TIM-3 pathways, the activation of tumor infiltrating lymphocytes is provided and thus, a curative effect on cancer is demonstrated. However, the immunotherapeutic approach applied to this interaction reveals an anti-tumor effect by increasing the release of molecules such as IFN-γ, IL-2, perforin and granzyme from immune cells (Li et al., 2012; Nagahara et al., 2008; Klibi et al., 2009).

1.8.6 Tumor necrosis factor receptor superfamily member 14

Tumor necrosis factor receptor superfamily member 14 (TNFRSF14), also known as herpesvirus entry mediator (HVEM), is a member of TNFR family that can be expressed in various cell types like hematopoietic and non- hematopoietic cells (Steinberg et al., 2011; Murphy and Murphy, 2010). Defined as a canonical receptor, TNFRSF14 mediates activation of the NF-κB pathway through its association with TNF receptor-related factors (Marsters et al., 1997). While TNFRSF14 acts as a receptor when it binds to TNF superfamily molecules, it acts as a ligand when binds to Ig super family molecules. Thus, both positive and negative immunological reactions can be caused under different contexts. TNFRSF14 can interact with TNF superfamily molecule LIGHT, also known as TNFSF14 (Shui and Kronenberg et al., 2013). LIGHT is expressed on T cells, NK cells, monocytes and granulocytes. When TNFRSF14 and LIGHT interact with each other, LIGHT functions as a ligand. This pathway activates IFN-γ cytokine secretion from T cells and therefore, T cell can be activated. According to that LIGHT and TNFRSF14 interaction seems to be a T cell co-stimulatory effect with the capacity to stimulate proinflammatory responses (Wang et al., 2001). TNFRSF14 also interacts with an Ig superfamily molecule B and T Lymphocyte attenuator (BTLA) (Sedy et al., 2005; Cai et al., 2008). In this case, TNFRSF14 acts as a ligand. BTLA molecule is expressed on T cells, B cells, NK cells and macrophages. The BTLA-TNRFSF14 pathway causes immune cells to be suppressed and unable to produce cytokines. Thus, a negative effect on immune tolerance is created (Shui et al., 2013). BTLA/HVEM pathway is thought to represent a novel method of immune evasion and is an important player in the physiological processes of inflammation and cancer.

1.8.7 V-set domain-containing T-cell activation inhibitor 1

V-set domaining-containing T-cell activation inhibitor 1 (VTCN1), also known as B7-H4, is expressed on tumors and APCs (Podojil et al., 2017). Inflammatory cytokines and other factors such as IL-2, IL-6, TNF-α, IFN-γ and hypoxia in the tumor microenvironment induce the expression of VTCN1 (Xu et al., 2014; Jeon et al., 2015). VTCN1, which binds to a still unexplored receptor on various immune cells, exerts an inhibitory effect. VTCN1 mediates many immunosuppressive mechanisms by regulating T cell functions. It inhibits the production of cytokines such as IFN-γ and TNF-α from effector CD4+ and CD8+ T cells that will cause cytotoxic effects. On contrary, it regulates the secretion of cytokines such as IL-10, which will create an immunosuppressive environment. It also mediates the blocking of T cell proliferation by arresting cell cycle to the G0/G1. VTCN1 contributes to the functional regulation of Treg cells by blocking CD8+ and CD4+ T cell differentiation (Sica et al., 2003).

1.8.8 V-Set Immunoregulatory Receptor

VSIR (V-Set Immunoregulatory Receptor), is a member of B7 family which is expressed on CD4+ and CD8+ T cells, Foxp3+ CD4+ T cells, APCs and tumor (Wang et al., 2011; Chen and Flies, 2013; Flies et al., 2011). VSIR regulates T cell responses through both extracellular and intracellular mechanisms. When VSIR is expressed on APC and/or cancer cells, it binds to an inhibitory receptor present on the T cell, which is still not fully defined and suppresses T cell proliferation and cytokine production (Xu et al., 2018). VSIR is involved in regulating innate and adaptive immunity against cancer. It prevents the production of effector CD4+ and CD8+ T cells and the release of cytokines that act as inhibitors on cancer types, such as IFN-γ. At the same time, the polarization of Th17 CD4+ cells are blocked by VSIR molecule. VSIR can provide an anti-tumor effect by regulating the expression of FOXP3+ Treg cells. It impairs T cell homeostasis by blocking IL-17 cytokine secretion from T cells. VSIR also negatively regulates T cell development by reducing the release of cytokines such as IL-12, IL-6 and TNF-α when interacting with the mediator receptor (Le Mercier et al., 2014). Eventually by altering the inflammatory environment within the TME, blocking VSIR pathways can skew the effector role of tumor-specific T lymphocytes.

1.8.9 Carcinoembryonic antigen-related cell adhesion molecule 1

Carcinoembryonmic antigen-related cell adhesion molecule 1 (CEACAM1), also known as CD66a, is a member of the carcinoembryonic antigen (CEA) gene family that expressed on APCs and cancer cells. It is a type I transmembrane protein with a C2-like immunoglobulin domain and an extracellular N-terminal variable domain (Beauchemin et al., 1999). CEACAM1 is involved in the regulation of many biological events such as angiogenesis, apoptosis (Nittka et al., 2004), metastasis (Beauchemin et al., 2013), cell motility, tumor development (Ebrahimnejad et al., 2004), and regulation of innate and adaptive immunity (Huang et al., 2015). CEACAM1 can interact with TIM-3 molecule. According to this interaction, T cell tolerance is negatively affected and T cell exhaustaion is induced. In the absence of CEACAM1, the HLA-B-associated transcript 3 (BAT3) molecule binds to the cytoplasmic domain of TIM-3 and to the tyrosine kinase LCK, which regulates TCR signaling. When CEACAM1 binds to the inhibitory receptor TIM-3, it causes phosphorylation of the intracellular domain of TIM3 and the removal of BAT3 from the cytoplasmic tail. Thus, TCR signaling pathway is inhibited and T cell activation is prevented (Rangachari et al., 2012). In addition to T cell signaling, this pathway plays a negative role in NK cell regulation. However, the interaction of TIM-3 and CEACAM1 decreases the expression of CD4+ and CD8+ T cells and accordingly contributes to tumor growth, metastasis and invasion (Köhler et al., 2021). As a result, CEACAM1 specific inhibitors may be able to attack cancer cells because CEACAM1 is a tumor-associated molecule in various cancer types. The most appropriate implications for therapeutic drugs targeting CEACAM1 will be revealed by future research (Dankner et al., 2017).

1.9 Autophagy

Autophagy is a process that mediates the recycling of many damaged or long-lived structures. This pathway functions by several evolutionarily conserved autophagy-related proteins (ATGs) (Ravikumar et al., 2010; Mizushima et al., 2011). Autophagy is divided into many stages. These are initiation, nucleation, elongation, fusion, degradation and recycling **(Figure 1.13).**

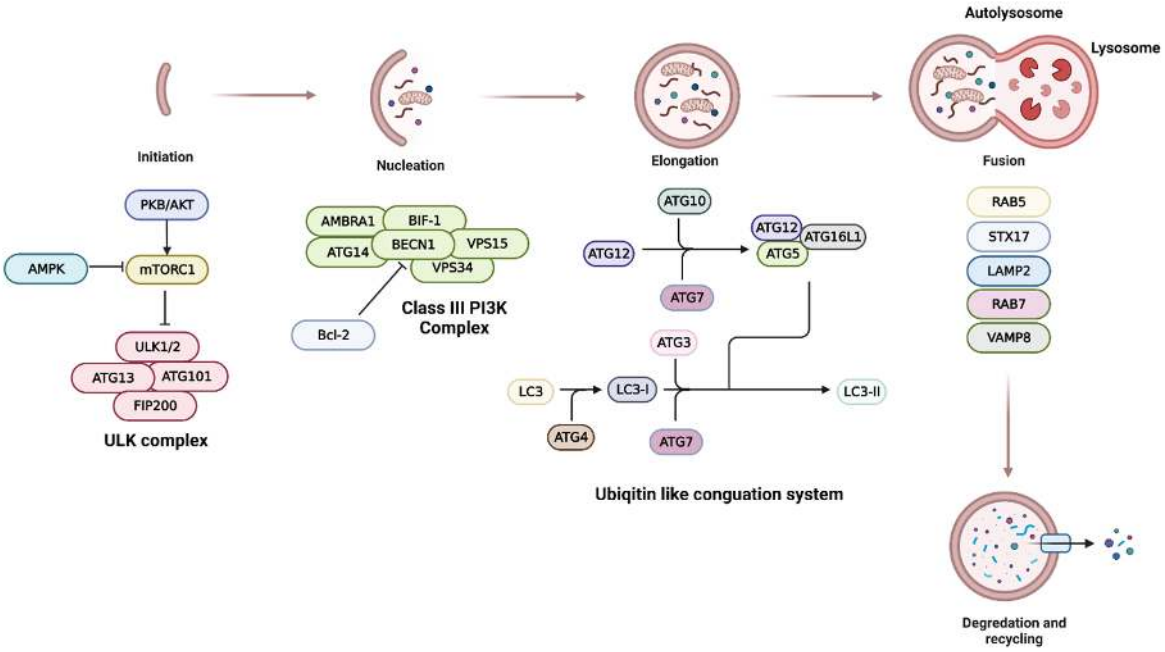


Figure 1.13. Molecular mechanism of autophagy pathway

The main role in the initiation phase belongs to the mammalian target of rapamycin (mTOR) kinase. This kinase creates two autophagy-related protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes regulate cellular energy homeostasis and also autophagy (Bar-Peled and Sabatini, 2014; Oh et al., 2010). While mTORC1 consists of factors such as mTOR, rapamycin-sensitive adapter protein of mTOR (RAPTOR), Tti/Tel2, PRAS40, DEPTOR, and LST8; mTORC2 consists of rapamycin-insensitive companion of mTOR (RICTOR), Tti/Tel2, DEPTOR, Rictor, LST8, and Sin1 factors (Akkoc et al., 2020). mTORC1 is the master regulator in autophagy. Under normal conditions, mTORC1 is activated and autophagy is inhibited. But under several stress conditions such as starvation, ER stress, hypoxia, metabolic

stress, etc. mTORC1 is downregulated and autophagy is induced. Various biological pathways are involved in mTORC1 regulation. The PKB/AKT pathway is responsible for mTORC1 activation and autophagy inhibition (Dan et al., 2014; Zalckvar et al., 2009). On the contrary, AMP-activated protein kinase (AMPK), which is responsible for regulating cellular energy and ATP levels, inhibits mTORC1. In the event of a decrease in ATP, AMPK becomes active by interacting with ADP (Kocaturk et al., 2019). However, AMPK induction regulated by liver kinase B1 (LKB1) and calcium/calmodulin dependent protein kinase kinase-β (CaMKKβ) (Hawley et al., 2005; Shaw et al., 2004). Under basal states, mTORC1 blocks autophagy by inactivating the ULK1/2 complex which is consist of ULK1 kinase or ULK2 kinase, ATG101, ATG13, and FIP200. The lack of nutrients reveals mTORC1 inhibition and autophagy activation by providing ULK1/2 autophosphorylation and ATG13, FIP200 phosphorylation by ULK1/2 complex (Hosokawa et al., 2009).

In the second step, nucleation, class III phosphatidylinositol 3-kinase (PI3K) complex has the main role. The class III PI3K complex consists of VPS34, VPS15, ATG14, BIF-1, AMBRA1, and BECN1. PI3K is responsible for the aggregation of phosphatidylinositol 3-phosphate (PI3P) molecules on membranes. These membranes may include the outer leaflet of the endoplasmic reticulum (ER) (Gozuacik et al., 2017). BCL2 and BECN1 interaction has a negative effect on autophagy (Pattingre et al., 2005). Blocking of this interaction mediates the connection of BECN1 with VPS34, thus promoting nucleation. BECN1 can also interacts with RUBICON, ATG14L (Kohichi et al., 2009), UVRAG (Liang et al., 2008), AMBRA1 (Yazdankhah et al., 2014), and VMP1(Molejon et al., 2013; Molejon et al., 2013). In addition, PI3P molecule contributes to the induction of autophagy by interacting with autophagy proteins such as WIPI1–4 and DFCP1 (Mauthe et al., 2011; Mercer et al., 2008).

During autophagy, cargo molecules are compressed into vesicles called the autophagosomes. Two ubiquitination-like conjugation systems known as, ATG12-ATG5-ATG16 and ATG8/LC3, have a dynamic role in the elongation and completion phase of these membranes (Mizushima et al., 2011). In the first system, ATG12 and ATG5 interact with each other by E1-like enzyme ATG7 and E2-like enzyme ATG10 (Hamasaki et al., 2013). ATG12-ATG5 connects with ATG16L1 as a complex (Kuma et al., 2002). In the second system, the conjugation of the ATG8/LC3 molecule to a lipid molecule such as phosphatidylethanolamine (PE) is provided. In order for this

conjugation to take place, LC3 is converted to the LC3-I form and this is mediated by the ATG4 protein. E1-like enzyme ATG7, E2-like enzyme ATG3 and E3-like enzyme ATG12-ATG5-ATG16 complex are required for conjugation of LC3-I and PE. This exposes the autophagic membrane-bound form of LC3-II, which mediates autophagic membrane elongation and closure of this vesicle (Gozuacik et al., 2017).

In the fusion step, SNARE complexes such as VAMP8 and STX17, lysosomal integral protein LAMP2, and RAB proteins like RAB5 and RAB7 have a critical role in the assembly of autophagosome and lysosome (Jäger et al., 2004). In addition, the interaction of BIF1 and UVRAG proteins with BECN1 is important at this stage (Liang et al., 2006; Takahashi et al., 2007). Eventually, autophagosomes combine with lysosomes to form autolysosomes. Thus, the degradation of transported cargo molecules and autophagic components is ensured. Finally, the degraded structures are recycled back into the cytosol. Thus, it becomes reusable by the cell (Gozuacik et al., 2017).

Although autophagy has been identified as a biological event involved in non-selective cellular homeostasis, a selective aspect of autophagy is also determined by the appearance of various receptors. These receptors include SQSTM1/p62 (Bjørkøy et al., 2005), NBR1 (Lamark et al., 2009), NDP52 (also named as CALCOCO2) (von Muhlinen et al., 2012), OPTN (Korac et al., 2013), and NIX (also named as BNIP3L) (Zhang and Ney, 2019). These receptors provide an interaction between LC3 and cargo molecules. In this way, degradation of autophagy receptors is seen as a marker of autophagic degradation function.

1.9.1 Autophagy in Tumor Immunity

Increasing evidence suggests that autophagy has a dual role in the formation and development of cancer **(Figure 1.14).**

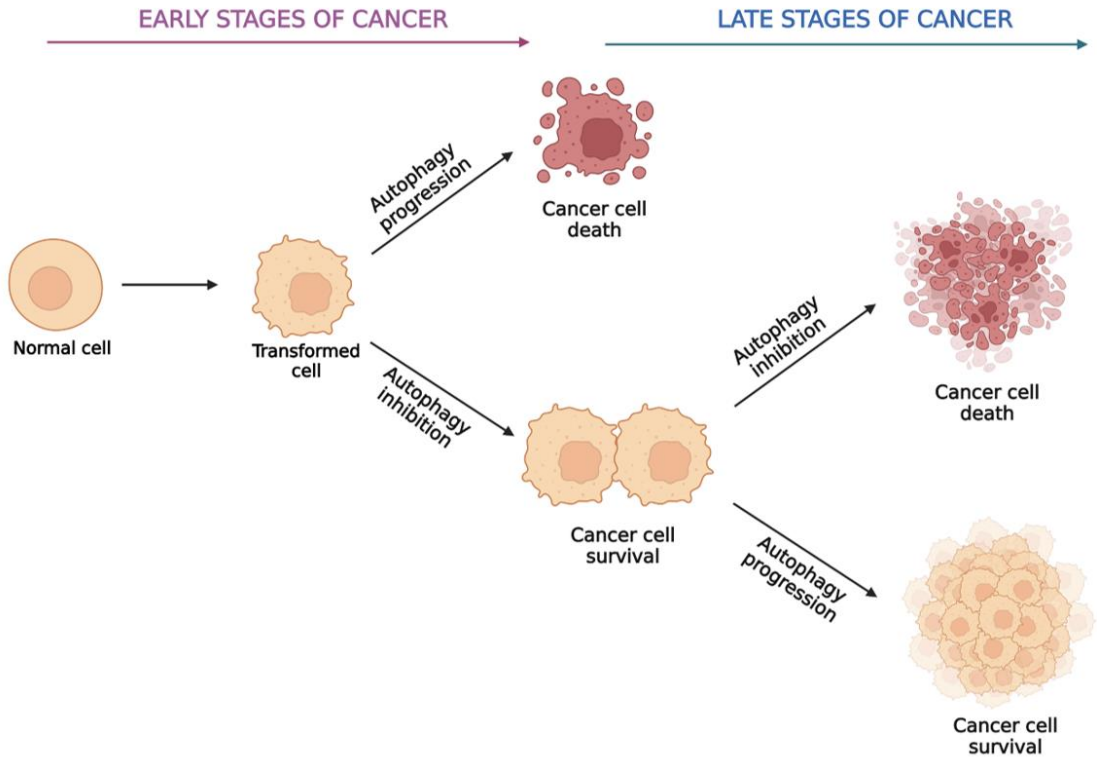


Figure 1.14. Dual role of autophagy in cancer

Autophagy acts as a tumor suppressor in the early stages of cancer formation. This may be due to the elimination of dysfunctional organelles, minimizing ROS production and inhibiting the accumulation of damaged DNA. In addition, it is a mechanism that promotes tumor growth in the later stages of tumor development because autophagy upregulates under stress conditions such as nutrient deprivation and hypoxia (Karakas et al., 2014). Activation of autophagy can be regulated by several innate and adaptive immune system cells. Activation of innate immune system-related autophagy is achieved by the expression of innate immune receptors including TLRs and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Pan et al., 2016). TLRs can induce the autophagy by upregulating some signaling pathways and factors such as of JNK/ERK, p38-MAPK, and myeloid differentiation factor 88 (MyD88) (Fang et al., 2014; Delgado et al., 2008; Xu et al., 2007; Zhan et al., 2014; van der Vaart et al., 2014).

NOD-like receptors stimulate autophagy by interacting directly with ATG16L1, which has a critical role in autophagosome formation (Travassos et al., 2010). Autophagy is involved in several process in adaptive immunity via antigen presenting, preparing inflammatory niche by regulating the secretion of inflammatory cytokines, thymus selection and lymphocyte differentiation and development. In addition to this, ATG8/LC3 mediated autophagy activation may also contribute to antigen presentation (Jiang et al., 2019).

Autophagy can have negative or positive effects on tumor growth and development by interacting with innate and adaptive immune system cells. Induction of autophagy contributes to the blocking of tumor growth by increasing the activation of CD8+ cytotoxic T cells in lymphoid tissues (Sowell et al., 2014). However, inhibition of autophagy induces the differentiation of Treg cells, thus positive effect on tumor development is created (Michalek et al., 2011). The interaction of cytosolic phosphorylated FoxO1 and ATG7 regulates the development of NK cells by stimulating autophagy and has an anti-tumor effect (Wang et al., 2016). Autophagy also plays a role in increasing the production of IgM and IgG on B cells. Lack of autophagy impairs the production of cytokines and antibodies from B (Arnold et al., 2016). Autophagy has an active role in monocyte/macrophage differentiation and migration (Jacquel et al., 2012; Zhang et al., 2012). Autophagy can also inhibit the M1 like TAM activation and contributes M2 like TAM polarization by IL-6 and CCL2. According to that anti-inflammatory cytokines are released and tumor growth is induced (Chen at al., 2014; Liu et al., 2015; Mantovani et al., 2013; Li et al., 2015). Autophagy has a critical and dual role in the cross-presentation of antigens to MHC I and MHCII molecules. For example, activation of autophagy on DCs improves antigen presentation and increases CD8+ T lymphocyte-mediated cytotoxic response. Nano activators which are conjugated to antigens increase cross-presentation to T lymphocytes by stimulating DCs through autophagy-related mechanisms and create an anti-tumor effect (Wang et al., 2019). In addition, using semi-synthetic vitamin E derivative alpha-tocopheryloxyacetic acid (a-TEA) in LLC, DC-mediated cross-antigen presentation is improved and CD8+ T lymphocyte response is enhanced by the regulation of autophagy (Li et al., 2012). In addition, ATG5 deficiency in DCs adversely affects the release of various cytokines and impairs MHCII molecule-mediated antigen presentation (Liu et al. 2015; Lee et al., 2010). In some cases, autophagy has a negative effect on antigen presentation. For example,

inhibition of autophagy in pancreatic ductal adenocarcinoma (PDAC) cells decrease MHCI expression and increased infiltration of CD8+ T lymphocytes. Thus, a negative effect on tumor growth is demonstrated (Yamamoto et al., 2020). In addition, the autophagy inhibitor chloroquine (CQ), and low concentrations of 5-fluorouracil (5-FU), increase the DC maturation and CD8+ T cell response in HCT-116 colorectal cancer cells (Zamame Ramirez et al., 2020). Considering all these, it is revealed that autophagy has a very dynamic effect on the tumor and its microenvironment. Targeted therapeutic approaches that stimulate or inhibit autophagy have also been increasing and gaining importance in recent years.

1.9.2 Autophagy and Cancer Dormancy

Autophagy has a critical role in cancer formation, development, metastasis and resistance to treatments. In the light of these, it is thought that autophagy has an important role in cancer dormancy. Although the relationship between autophagy and cancer dormancy is still not fully understood, studies in different cancer types have shown that autophagy is active in non-proliferative dormant cells. For example, imatinib treatment in gastrointestinal stromal tumor (GIST) cells helped the accumulation of the cell cycle inhibitor p27 and thus induction of dormancy is occurred. It has been observed that autophagy is active in these cells in dormant state (Gupta et al., 2010). The relationship between autophagy and dormancy has also been studied in breast cancer. MCF7 breast cancer cells entered dormancy and showed autophagy induction when cultured with farnesyl transferase inhibitors (FTIs) (Chaterjee et al., 2011). Autophagy stimulation in MDA-MB-231 breast cancer cells resulted in reversible cell cycle arrest (Carcereri de Prati et al., 2017). In further studies, this state of breast cancer was modeled with two cell lines. D2.A1 cells exhibit a metastatic phenotype, while D2.0R cells are more compatible with the dormancy system. It was observed that the factors required for autophagy regulation were more expressed in D2.0R cells compared to D2.A1 cells (La Belle Flynn et al., 2019; Vera-Ramirez et al., 2018). The interaction of dormant state and autophagy of ovarian cancer, another cancer type, are also researched areas. Inducible re-expression of DIRAS3, a tumor suppressor factor frequently downregulated in ovarian cancer, induced autophagy in *in vivo* studies. This demonstrated a dormancy-like state and inhibiting tumor growth (Lu et al., 2006; Washington et al., 2015). High copper levels in PDAC are related with the degree of cancer progression. According to *in vitro* studies,

inhibition of copper levels allowing PDAC cells to enter a dormant phase. In dormant state, autophagy is activated, thus anti-tumor effect in PDAC cells is promoted and tumor growth is impaired in *in vivo* studies (Yu et al., 2019).

Autophagy can induce dormancy in disseminated tumors by providing several amino acids and other nutrients. In addition, autophagy can also support the elimination of mitochondria, thus this situation can stimulate the dormancy. In this case, it is called mitophagy. However, autophagy can induce dormancy by regulating redox balance and stem cell status (Mowers et al., 2018).

Chapter 2:
MATERIALS AND METHODS

2.1 Cell culture and chemicals

A549 ATG5 KO and A549 LGALS3 KO cells were previously established by respectively Dr. Yunus AKKOÇ and Ramiz DEMIR by using the CRISPR/Cas9 method in our laboratory. gRNA sequences to target the ATG5 and LGALS3 gene were designed using the sgRNA Designer: CRISPRko Broad institute online tool. Complementary oligonucleotides including restriction enzyme BsmBI (Thermo, ER0451) cleavage sites were purchased. Oligos were annealed and after that cloned into lentiCRISPRv2 (Addgene #52961). Calcium phosphate salt precipitation procedure was applied for lentivirus production. Human embryonic kidney (HEK 293T) cells were transfected with lentiCRISPRv2 vector containing gRNA-ATG5 and gRNA-LGALS3 with pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) vectors. After 48 hours, the medium was collected, centrifuged and filtered with a 0.45 μm pore syringe. HEK 293T cells were transduced with lentiviruses in the presence of 5 μg/ml polybrene. At the end of 48 hours, selection was made with the selection antibiotic puromycin (1 μg/ml) for a certain period of time. Individual clones were encouraged to grow. A549 ATG5 KO-luc cells (cells that are stably expressing luciferase gene) were previously generated by Dr. Yunus AKKOÇ in our laboratory. The virus required to make A549 LGALS3 KO cells luciferase stable was delivered by Dr. Yunus AKKOÇ. Selection was made in puromycin for 2 weeks. Luciferase stability was determined by luciferase assay. GFP viruses taken from Dr. Yunus AKKOÇ to generate GFP-tagged Jurkat T cells. Jurkat T cells were transduced with GFP viruses in the presence of 5 μg/ml polybrene. At the end of 48 hours, selection was made with the selection antibiotic blasticidin (12,5 mg/ml) for a certain period of time. Individual clones were encouraged to grow.

HEK 293T, A549 WT obtained from ATCC (USA). Jurkat T cells were kind gift from Prof. Dr. Penelope Mavromara. HEK 293T was cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, #BI01-050-1A). A549-WT, A549 WT-luc, A549 ATG5KO-luc, A549 LGALS3KO-luc, Jurkat T and Jurkat-GFP T cells were cultured in RPMI 1640 medium (SIGMA-Aldrich #R8758). All of the culture mediums were supplemented with 10% (v/v) fetal bovine serum (FBS, PAN, #P30- 3302), antibiotics (Penicillin/streptomycin, Biological Industries, #BI03-031-1B) and L-

Glutamine (Biological Industries, #BI03-020-1B) in a 5% CO_2 humidified incubator at 37°C. HEK 293T cells were transiently transfected with calcium phosphate transfection method according to standard protocols. In order to stimulate autophagy by starvation, cells were cultured in Earle's balanced salt solution (EBSS; Biological Industries, #BI02-010-1A) for 4 hours. For chemical induction of autophagy, incubation was carried out for 4 hours in medium containing Torin 1 dissolved in DMSO. Autophagic flux experiment was performed absence of 10mM lysosome inhibitor chloroquine for 3 hours.

2.2 Peripheral Blood Mononuclear Cell (PBMC) isolation

20 ml of blood was taken from individuals in a heparin tube. The collected blood was diluted (1:1) with PBS with 2% FBS. Density gradient medium Lympoprep™ (STEMCELL Technologies, #00321) was put into another tube and the diluted blood was carefully poured onto the Lymphoprep™. Centrifugation was done at 400 g (acc:2 dec:0) for 30 minutes. The blurry phase (PBMC) was transferred to another tube and washed twice with PBS with 2% FBS.

2.3 T cell isolation from PBMC

Invitrogen™ Dynabeads™ Untouched™ Human T Cells Kit (#11344D) was used. Dynabeads contains 4×10^8 beads/mL in phosphate buffered saline (PBS), pH 7.4, with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide as preservatives. Antibody Mixture contains monoclonal mouse anti-human IgG antibodies in PBS with 0.5% BSA and 0.02% sodium azide.

10^6 PBMCs were prepared in PBS with 2% FBS. The desired amount of dynabeads were transferred to a tube and PBS with 2% FBS was added to make up to 1 ml. The tube was placed on magnets and the supernatant was removed. The tube was removed from the magnet and the same amount of PBS with 2% FBS was added. An appropriate amount of antibody mix was put into the prepared PBMC at the beginning and incubation was carried out at 4 ^oC for 20 minutes. Centrifugation was done at 350 g for 8 minutes at 4 ^oC. The pellet was washed with PBS with 2% FBS. Pre-washed dynabeads were added and incubated on the roller at room temperature for 20 minutes. The tube was placed on magnet for 2 minutes and the supernatant containing the untouched human T cell was transferred to another tube.

2.4 Immunofluorescence analysis

The isolated T cell suspension was centrifuged at 1500 g for 5 minutes and washed with PBS with 2% FBS and transferred to a tube. Cells were fixed with 70% ethanol and incubated on ice for 10 minutes. 500 g for 5 minutes centrifugation was done and cells were washed with PBS with 2% FBS. Incubation was performed for 2 hours using primary antibodies including purified mouse anti-human CD3 clone OKT (eBioscience, #14-0037-82, 1:100 dilution) and purified mouse anti-human CD28 (BD Pharmingen, #555726, 1:100 dilution). Anti-mouse Alexa Fluor 48831 (Invitrogen, #A32723, 1:1000 dilution) was used as secondary antibody. Cells were washed with PBS with 2% FBS. Hoescht (BD, #33342) was used for nuclei staining and incubated for 10 minutes. The slides were mounted and inspected under 60X magnification using OLYMPUS microscope.

2.5 Activation of Jurkat T cells

Three different methods were tried for Jurkat T cell activation.

$30x10^4$ Jurkat T cells were seeded in 6-well petri dishes before experiment. After 24 hours Jurkat T cells were collected and centrifuged at 500g for 5 minutes. 160 nM Phorbol-12-myristate-13-acetate (PMA; Merk, #524400) was added to the Jurkat T cell pellet in RPMI and incubated for 8 hours. After incubation, co-culture experiment was carried out.

$50x10^4$ A549 WT-luc cells were dissolved with PBST after centrifugation at 500g for 5 minutes. 10^6 Jurkat T cells were added onto the dissolved cells and incubated on roller for 8 hours. For control experiment 10^6 Jurkat T cells dissolved with only PBST and incubation was done on roller for 8 hours. After incubation, co-culture experiment was carried out.

Final concentrations of 2 µg/ml soluble purified mouse anti-human CD3 clone OKT (eBioscience, #14-0037-82) and purified mouse anti-human CD28 (BD Pharmingen, #555726) and 5 µg/ml anti-mouse Ig (Sigma, #M5905) were added on to desired amounts of Jurkat T cells. Co-culture experiment was carried out without incubation.

2.6 Co-culture

For co-culture analyses to be performed in 2D culture, 12500 luciferase stable A549 cells were seeded into a 12-well petri dish. After 24 hours, the required amount of control Jurkat cells (1250, 12500 and 125000 cells) and activated Jurkat cells (1250, 12500 and 125000 cells) were added directly onto the cells seeded the previous day. After 48 hours, cells were harvested for the luciferase assay.

For co-culture analyses to be performed in 3D culture, 2000 luciferase stable A549 cells were seeded into a 96-well petri dish. On day 12, the required amount of Jurkat and/or Jurkat-GFP cells (200, 2000 and 20000 cells) was added directly onto the seeded cells in low glucose DMEM with 2% FBS and 2% matrigel. On day 14, cells were harvested for the luciferase assay.

2.7 Luciferase assay

Luciferase stable A549 WT, A549 ATG5 KO and A549 LGALS3 KO cells were used for measurement. At the end of the experiment cells lysed with Chris buffer (50 mM Tris pH: 8,0, 200 mM NaCl, 0,1 mM EDTA, 10% glycerol, 0,5% Nonidet P-40, 1x PI, 1x PMSF) for 5 min at 37 °C. Cell lysates centrifuged for 1 min at 13200 x rpm and then vortexed. Supernatant was taken to a new tube and kept on ice. Supernatant was mixed with homemade fresh luciferase reagent (1,07 mM (MgCO3)4xMg(OH)2x5H2O, 200 mM Tricine, 267 mM MgSO4, 250 mM EDTA, 666 µM mM DDT, 10 mM Coenzyme A, 530 mM ATP and 47 mM Luciferin). 150 µL of luciferase reagent was mixed with 50 µL supernatant. Mixture in white 96-well plates (SARSTEDT, #9335911) was incubated for 5 min at room temperature without light. Luciferase activity was detected bt using microplate reader (SYNERGY H1).

2.8 Establishment of the Cancer Dormancy Model

Growth reduced Matrigel (final concentration 4 µg/µl) was used to construct the 3D culture system. The liquid Matrigel was spread at 50 µl/well in a 96-well petri dish. 30 minutes of incubation for polymerization was provided. A549 cells were seeded with a medium containing 0.08mg/ml Matrigel to a final concentration. Cells were prepared with 150 µl of medium per 2000 cells with dormancy trigger medium (2% Matrigel, Low glucose DMEM with 2% FBS, Pen/Strep antibiotics, and L-glutamin) and seeded on

polymerized Matrigel. 3D cultures were observed for 14 days and mediums were changed every 4 days.

2.9 Total RNA Isolation and RT-PCR Analysis

Total RNA isolation was performed using TRIzol reagent (Sigma-Aldrich, #T9424) according to the manufacturer's instructions. Total RNA was treated with DNase I (Thermo, EN0521). cDNA was reverse transcribed from DNase-treated total RNA using RevertAid enzyme (ThermoScientific, EP0441) RiboLock RNase inhibitor (Thermo, EL0012) and random hexamers (Invitrogen, 48190-011).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (q-RT-PCR) for mRNA quantification. For real-time RT-PCR, SYBR Green Quantitative RT-PCR kit (Roche, 04-913- 914-001) and Roche Light Cycler 480 machine were used. To activate the SYBR green, an initial cycle of 95°C, 5 min performed followed by, PCR reactions: 45 cycles of 95°C for 10 sec and 60°C for 1 min. Then a thermal denaturation protocol was used to generate the dissociation curves for the verification of amplification specificity (a single cycle of 95°C for 5 sec, 55°C for 60 sec and 80 cycles of 55°C for 10 sec). Changes in mRNA levels were quantified using the 2-ΔΔCT method using GAPDH (Gyceraldehyde-3- phosphate dehydrogenase) mRNA as control.

Primers used during the study were in Table 2.1

Table 2.1: qPCR primer sequences for gene

Gene	Primer Sequence	
MKI67	Forward (5'-->3')	TTTGTCCTCGGTGGCGTTAT
	Reverse (5'-->3')	GAGGAGAAACGCCAACCAAG
MCM7	Forward (5'-->3')	GCCAAGTCTCAGCTCCTGTC
	Reverse (5'-->3')	CCTCTAAGGTCAGTTCTCCA
CDK1	Forward (5'-->3')	GGAAACCAGGAAGCCTAGCA
	Reverse (5'-->3')	GGATGATTCAGTGCCATTTTT
PCNA	Forward (5'-->3')	CAAGTAATGTCGATAAGAG
	Reverse (5'-->3')	GTGTCACCGTTGAAGAGAGT
TGFβ1	Forward (5'-->3')	ACTGCAAGTGGACATCAACG
	Reverse (5'-->3')	TGCGGAAGTCAATGTACAGC
FN1	Forward (5'-->3')	ACAACACCGAGGTGACTGAG
	Reverse (5'-->3')	GGACACAACGATGCTTCCTG
PVR	Forward (5'-->3')	GCTAGAAGGACTCACTAGACTCAGGAA
	Reverse (5'-->3')	GTCGCCTCATCTGTCGTGGAAC

VSIR	Forward (5'-->3')	GATCCCTGCTCTTCGCTCTC
	Reverse (5'-->3')	TGCGGTACCACGTCTTGTAG
VTCN1	Forward (5'-->3')	AGGGAGTGGAGGAGGATACAG
	Reverse (5'-->3')	GCAGCAGCCAAAGAGACAG
TNFRSF14	Forward (5'-->3')	TTCTCTCAGGGAGCCTCGTCAT
	Reverse (5'-->3')	CTCACCTTCTGCCTCCTGTCTT
NECTIN2α	Forward (5'-->3')	GATCCGAAAGCTCAGGTGTT
	Reverse (5'-->3')	TCCATGTCATCCTCGAGTGC
NECTIN2δ	Forward (5'-->3')	GTCTGCACAGTCACCAATGC
	Reverse (5'-->3')	GCAGATAAGGATGCCCGTGG
FGL1	Forward (5'-->3')	TCCCTTGCGGGGAATTTTCA
	Reverse (5'-->3')	CTCTGTCCCACGTGCTGAAT
LGALS9	Forward (5'-->3')	TAGTGGGTGTGAAAGGCAGC
	Reverse (5'-->3')	GAAGTCCGTCCTGGAGACCT
CEACAM1	Forward (5'-->3')	GGCCCTGGTTGCTCTGATAG
	Reverse (5'-->3')	GGTCATTGGAGTGGTCCTGA
LGALS3	Forward (5'-->3')	CACCTGCACCTGGAGTCTAC
	Reverse (5'-->3')	TGTTATCAGCATGCGAGGCA
PDCD1LG1	Forward (5'-->3')	AAATGGAACCTGGCGAAAGC
	Reverse (5'-->3')	GATGAGCCCCTCAGGCATTT
PDCD1LG2	Forward (5'-->3')	AGTGATTTCCAAAAGCAGAGGTG
	Reverse (5'-->3')	ATTATGATTTGAGTTTGTGCGAAGC
CD80	Forward (5'-->3')	CTCACTTCTGTTCAGGTGTTATCCA
	Reverse (5'-->3')	TCCTTTTGCCAGTAGATGCGA
CD86	Forward (5'-->3')	CCATCAGCTTGTCTGTTTCATTCC
	Reverse (5'-->3')	GCTGTAATCCAAGGAATGTGGTC
TIGIT	Forward (5'-->3')	GTCGCTGACCGTGAACGATA
	Reverse (5'-->3')	CCCAGTGTACTGCCCATCAG
CD226	Forward (5'-->3')	AGCCCCCATGGGTTTCTTGC
	Reverse (5'-->3')	CTGAGCAAACTGCCACCCCA
CD96	Forward (5'-->3')	CTTCTCCAGCCAGCAAGTGTAT
	Reverse (5'-->3')	AAGAAACAGGCATGAATGATGCAA
BTLA	Forward (5'-->3')	ACCCTGGCTCCTGTATCGT
	Reverse (5'-->3')	AATTTTGCCTGGTGCTTGCT
TIM-3	Forward (5'-->3')	GAATACAGAGCCGAGGTCCG
	Reverse (5'-->3')	TCAAACACAGGACAGGCTCC
PD1	Forward (5'-->3')	GGATTTCCAGTGGCGAGAGA
	Reverse (5'-->3')	AATGGTGGCATACTCCGTCTG
CTLA-4	Forward (5'-->3')	TGTACCCACCGCCATACTAC
	Reverse (5'-->3')	AGGAAGTCAGAATCTGGGCAC
CD28	Forward (5'-->3')	GAGAAGAGCAATGGAACCATTATC
	Reverse (5'-->3')	TAGCAAGCCAGGACTCCACCAA
ATG3	Forward (5'-->3')	CGGTCCTCAAGGAATCAAAG

	Reverse (5'-->3')	TTGGACAGTGGTGGACTAGGT
ATG5	Forward (5'-->3')	ATGACAGATGACAAAGATGT
	Reverse (5'-->3')	ATCTGTTGGCTGTGGGATGA
ATG12	Forward (5'-->3')	TTGTGGCCTCAGAACAGTTG
	Reverse (5'-->3')	CCAAAAACACTCATAGAGAGTTCCA
LC3II	Forward (5'-->3')	GAGAAGCAGCYYCYYGTTCTGG
	Reverse (5'-->3')	GTGTCCGTTCACCAACAGGAAG
BECN1	Forward (5'-->3')	ACCGCAAGATAGTGGCAGA
	Reverse (5'-->3')	AGCYGCTGTCGTTTAATTCACT
GAPDH	Forward (5'-->3')	AGCCACATCGCTCAGACAC
	Reverse (5'-->3')	GCCCAATACGACCAAATCC

2.10 Protein extraction, Immunoblot analysis and antibodies

Protein extraction was performed at indicated time points with RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with complete protease inhibitor cocktail (Roche, 04-693-131-001) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, P7626). Protein extracts (50µg) were separated in 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, IPVH00010). Membranes were blocked in 5% non-fat milk in PBST (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween 20, pH 7.4) for 1 hour at RT, membranes were incubated in 3% BSA-PBST solutions containing primary antibodies (ab): anti-LC3B ab (CST, 2775, dilution 1:1000), anti-SQSTM1/p62 ab (Abnova, H00008878, 1:1000) anti-Galectin-3 ab (Abcam, ab53082, dilution 1:1000), anti-Gal3 (Sigma, A9482,1:2000), anti-β-ACTIN (SigmaAldrich, A5441). Then, secondary anti-mouse (Jackson Immunoresearch laboratories, 115035003, 1:10.000 dilution) or anti-rabbit antibodies (Jackson Immunoresearch Laboratories, 111035144, 1:10.000 dilution were applied for 1 hour at RT and protein bands were revealed by chemiluminescence. Band intensities were quantified using ImageJ software.

2.11 RNA-sequencing (RNA-Seq) analyses

Samples for RNA-Seq analysis were prepared by Trizol method and RNA quality was validated. The samples were dissolved in DEPC water. It was determined by FastQC method that the samples were suitable for analysis. Adapter sequences and poor-quality reads detected during the fit phase were filtered out with the Trimmomatic tool. A total

of 4 single-ended mRNAs were sequenced, two biological repeats, for active growth and dormancy models (2 x 2D proliferating state, 2 x 3D dormancy state). Gene expression levels for each sample were assessed with reads aligned to the human GRCh38 reference genome. The gene expression level was normalized by the number of fragments per kilobase per million mapped reads (FPKMs). The DESeq2 program, which generates p-values, was used for comparison analyses.

2.12 Statistical analyses

Statistical analyses were performed using Student's two-tailed t-test. Data represented as means of ±S.D. of≥3 independent experiments. Values of $p<0.05$ considered as significant.

2.13 Ethics Statement

The blood samples used in the study were approved by Koc University Ethics Committee (Ethics committee approval number 2021.130.IRB2.027).

Chapter 3:
RESULTS

3.1 Identification of the in vitro 3D Matrigel NSCLC Dormancy Model

A 3D Matrigel cancer dormancy model was optimized in our laboratory by Dr. Yunus AKKOÇ and Dr. Nesibe PEKER. Spheroid formation of the cells were featured by various assays. A549 cells were examined in 3D Matrigel cancer dormancy model for 14 days. These cells formed as 3D spheroids on Matrigel **(Figure 3.1 A)**. The images of the spheroids were taken with the aid of a microscope and the diameters of the spheroids were measured. As a result of the measurements, slowdowns were observed in the growth of spheroids between 12-14 days **(Figure 3.1 B).**

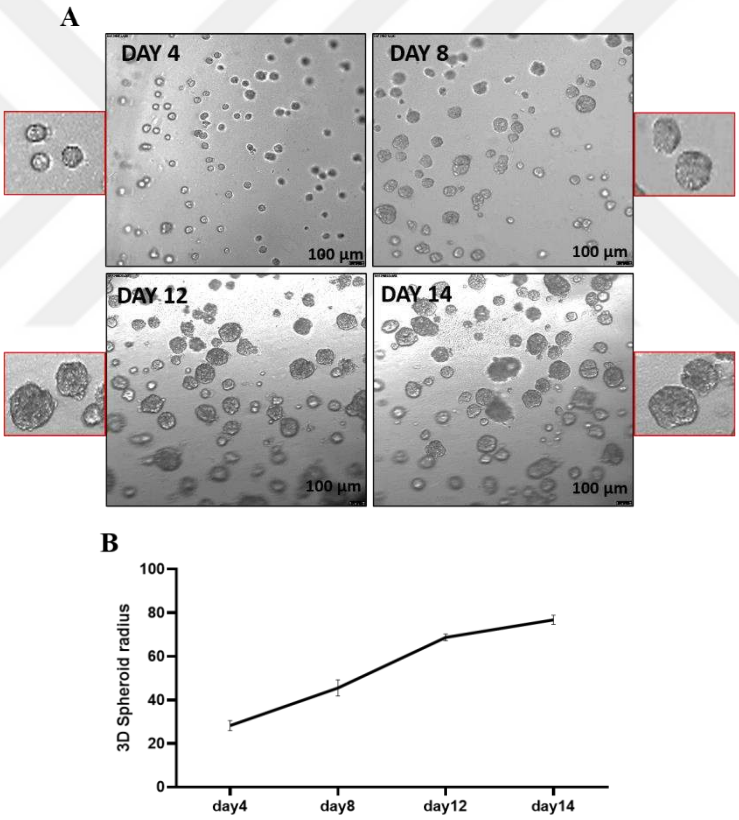


Figure 3.1. 3D spheroid formation of A549 cells in Matrigel dormancy model. **A.** Representative microscopy images of A549 cells in every 4 days. **B.** Diameters of 150 spheroids were measured using the Image J program. (mean±SD of independent experiments, n=3)

According to the literature, some genes are known to be associated with dormancy. These genes include MKI67, MCM7, CDK1, PCNA, TGF-β1 and FN1. According to

RNA-Seq studies, these genes are more expressed in A549 cells in the 3D dormant model than in the 2D proliferative state **(Table 3.1).** qPCR was performed to validate the RNA-Seq results. Accordingly, the expression of all these genes was upregulated in the 3D dormant system compared to the 2D system **(Figure 3.2 A).**

Table 3.1: RNA-Seq results of selected dormancy markers

A

Gene	Gene name	Fold change (3D to 2D)	P-value
MKI67	Marker of proliferation Ki-67	0,029586731	1,94E-73
MCM7	Minichromosome maintenance complex component 7	0,292578922	1,07E-15
CDK1	Cyclin dependent kinase 1	0,053342217	2,63E-52
PCNA	Proliferating cell nuclear antigen	0,388878215	3,30E-10
TGF-β1	Transforming growth factor beta 1	2,697572216	1,42E-09
FN1	Fibronectin 1	4,03347784,3	6,21E-16

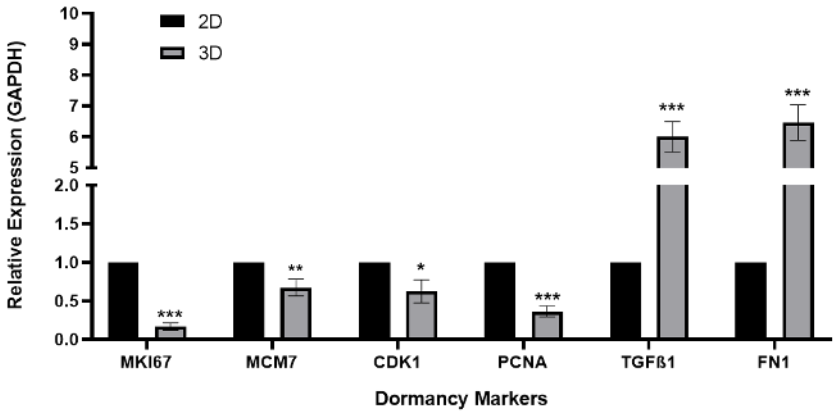


Figure 3.2. Validation of RNA-Seq analysis for dormancy markers in proliferative versus dormant NSCLC cells. Expression of dormancy markers analyzed by qPCR (mean±SD of independent experiments, n=3, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

3.2 Immune checkpoint molecules in non-dormant vs. dormant NSCLC

Expression levels of immune checkpoint molecules show changes in 3D dormant state when compared to non-dormant state. For more detailed research, RNA-Seq analysis is performed **(Table 3.2).** According to RNA-Seq results, all immune checkpoint genes are highly expressed in 3D environment, except PVR, PDCD1LG1 and PDCD1LG2. qPCR analyses were performed to validate the RNA-Seq results **(Figure 3.3).** CD80 and CD86 genes that were not in the RNA-Seq analyses were also included in the qPCR analyses. According to the qPCR results, all immune checkpoint molecules except PDCD1LG, PDCD1LG2 and CD86 were found to be highly expressed in dormant NSCLC cells.

Table 3.2: RNA-Seq results of immune checkpoint molecules

Gene	Gene name	Fold change (3D to 2D)	P-value
PVR	PVR cell adhesion	0,721406922	0,0313994
VSIR	V-set immunoregulatory receptor	2,290347141	8,48E-07
VTCN1	V-set domain containing T cell activation inhibitor 1	18,42186934	1,39E-07
TNFRSF14	TNF receptor superfamily member 14	11,42594976	0,0004539
NECTIN2	Nectin cell adhesion molecule 2	3,16886346	2,15E-13
LGALS9	Galectin-9	14,38989597	1,05E-34
CEACAM1	CEA cell adhesion molecule 1	77,9014781	1,26E-100
LGALS3	Galectin-3	3,77380539	5,85E-17
PDCD1LG1	Programmed cell death1 ligand 1	1,214962309	0,6611738
PDCD1LG2	Programmed cell death 1 ligand 2	0,156656368	0,18809

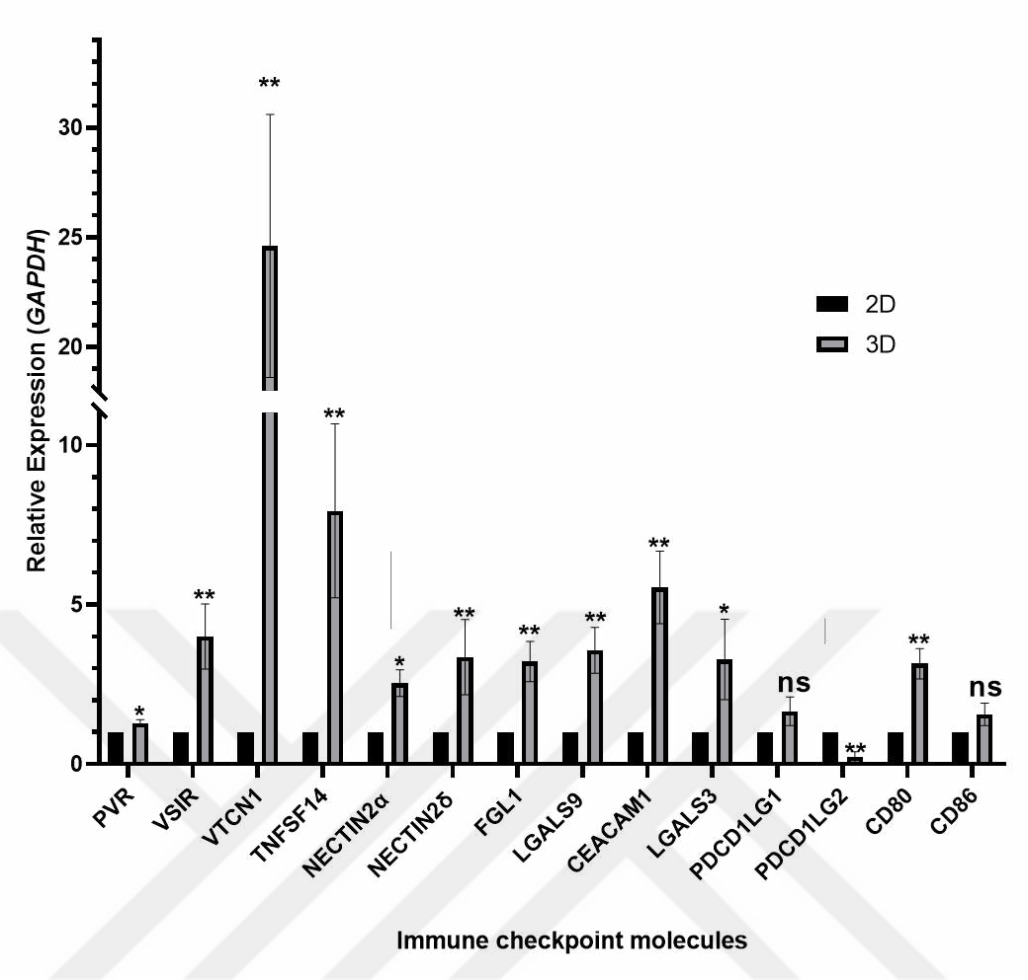


Figure 3.3. Expression analysis of immune checkpoint molecules in proliferative versus dormant NSCLC cells. Expression of immune checkpoint molecules analyzed by qPCR (mean±SD of independent experiments, n=5, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

3.3 Immune checkpoint receptors in Jurkat T cells

Receptor pairs of immune checkpoint receptors are expressed on Jurkat T cells. Therefore, expression of immune checkpoint receptors analyzed by qPCR **(Figure 3.4).** All receptors except BTLA have been demonstrated to be expressed on Jurkat T cells.

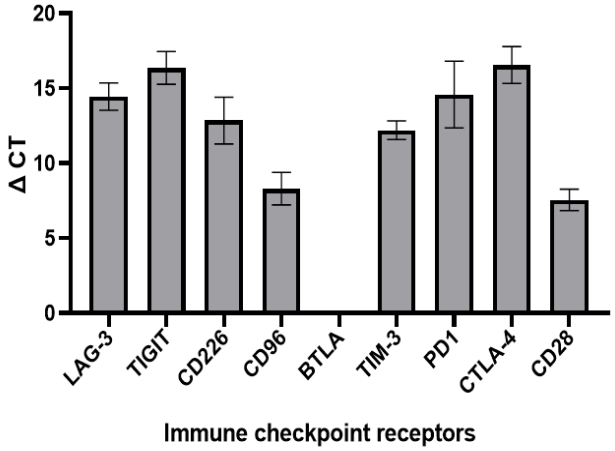


Figure 3.4. Expression analysis of immune checkpoint receptor pairs on Jurkat T cells Expression of immune checkpoint receptor pairs analyzed by qPCR (mean±SD of independent experiments, n=5)

3.4 Co-culture analysis of NSCLC cells in non-dormant state

Firstly, co-culture experiments were started with peripheral blood mononuclear cells (PBMCs). A549 cells expressing luciferase gene in stable manner (A549-luc) were co-cultured with different numbers of PBMCs **(Figure 3.5 A)**. After 48 hours, proliferation of A549 cells was analyzed by the luciferase reporter assay. A decrease in the growth of A549 cells was observed as the number of co-cultured PBMCs increased **(Figure 3.5 B)**. Furthermore, T cells are isolated from PBMC and co-cultured with A549-luc cells **(Figure 3.5 A)**. Co-culture analyses with T cells did not reveal a reduction in proliferation of A549 cells compared to control **(Figure 3.5 C)**. For validation of T cell isolation, CD3 and CD28 markers on T cells were observed under microscope by immunofluorescence method **(Figure 3.5 D)**.

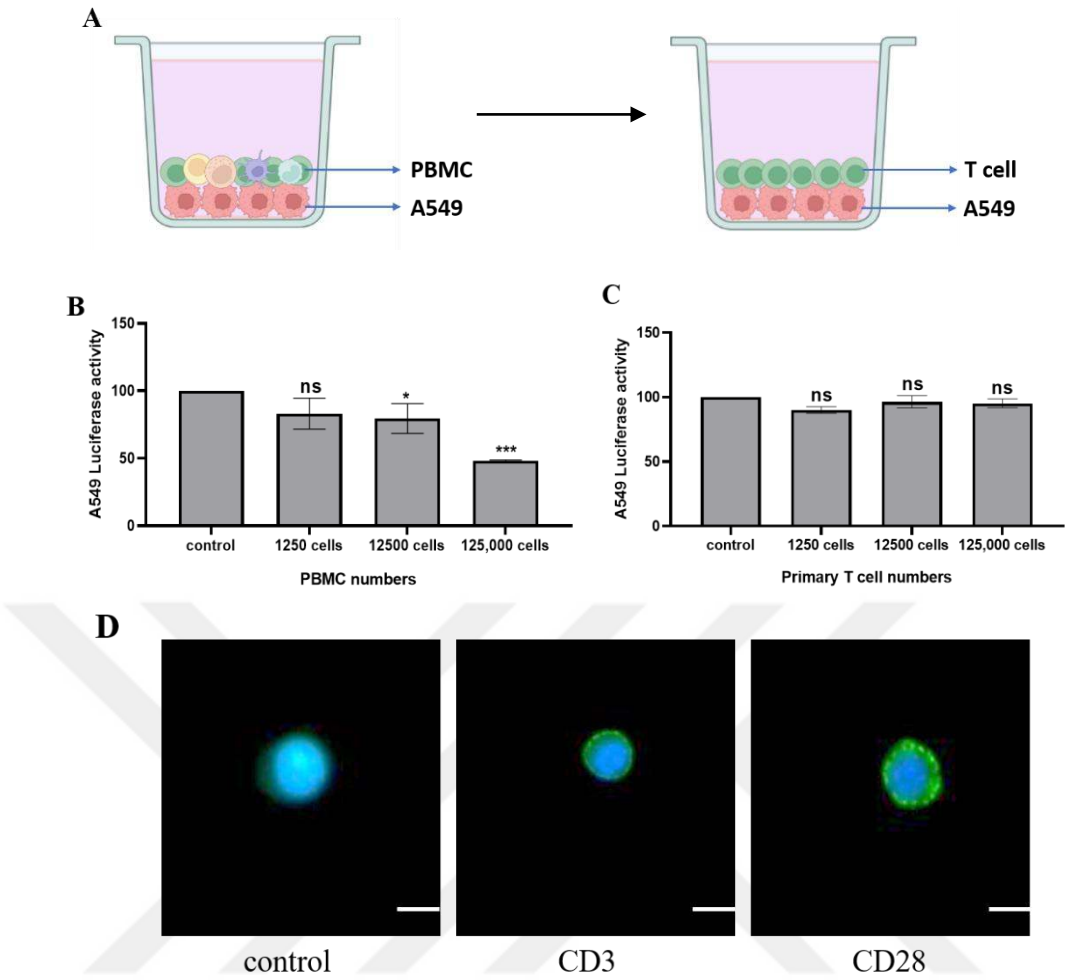


Figure 3.5. 2D co-culture analysis of PBMC or primary T cells with A549 cells expressing luciferase gene in stable manner (A549-luc)**. A.** Schematic view of co-culture conditions **B.** Luciferase activity of A549 cells as a result of PBMC co-culture for 48h **C**. Luciferase activity of A549 cells as a result of T cell co-culture for 48h **D.** Representative microscope image of T cell (mean±SD of independent experiments, n=3, , ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

Co-culture experiments were continued with Jurkat T cells **(Figure 3.6 A)**. Various activation processes were carried out to make Jurkat T cells more effective on the death of A549 cells. Firstly, A549-luc cells were treated with 160 nM PMA for 8 hours. According to the luciferase reporter assay, PMA treatment was not effective on the proliferation of A549 cells compared to the control group **(Figure 3.6 B)**. After that, A549-luc cell pellets treated with Jurkat T cells for 8 hours. According to co-culture analysis, cell pellet and Jurkat T cell treatment did not show any change in the growth of A549 cells **(Figure 3.6 C)**. Finally, A549-luc cells were treated with anti-CD3, andi-CD28 and anti-mouse Ig. Antibody treatment was more effective than all procedures, but did not reveal a great difference compared to control **(Figure 3.6 D)**. Considering all the

results, it was observed that Jurkat T cells were most active on death of A549 cells without any extra activation treatment **(Figure 3.6 E)**.

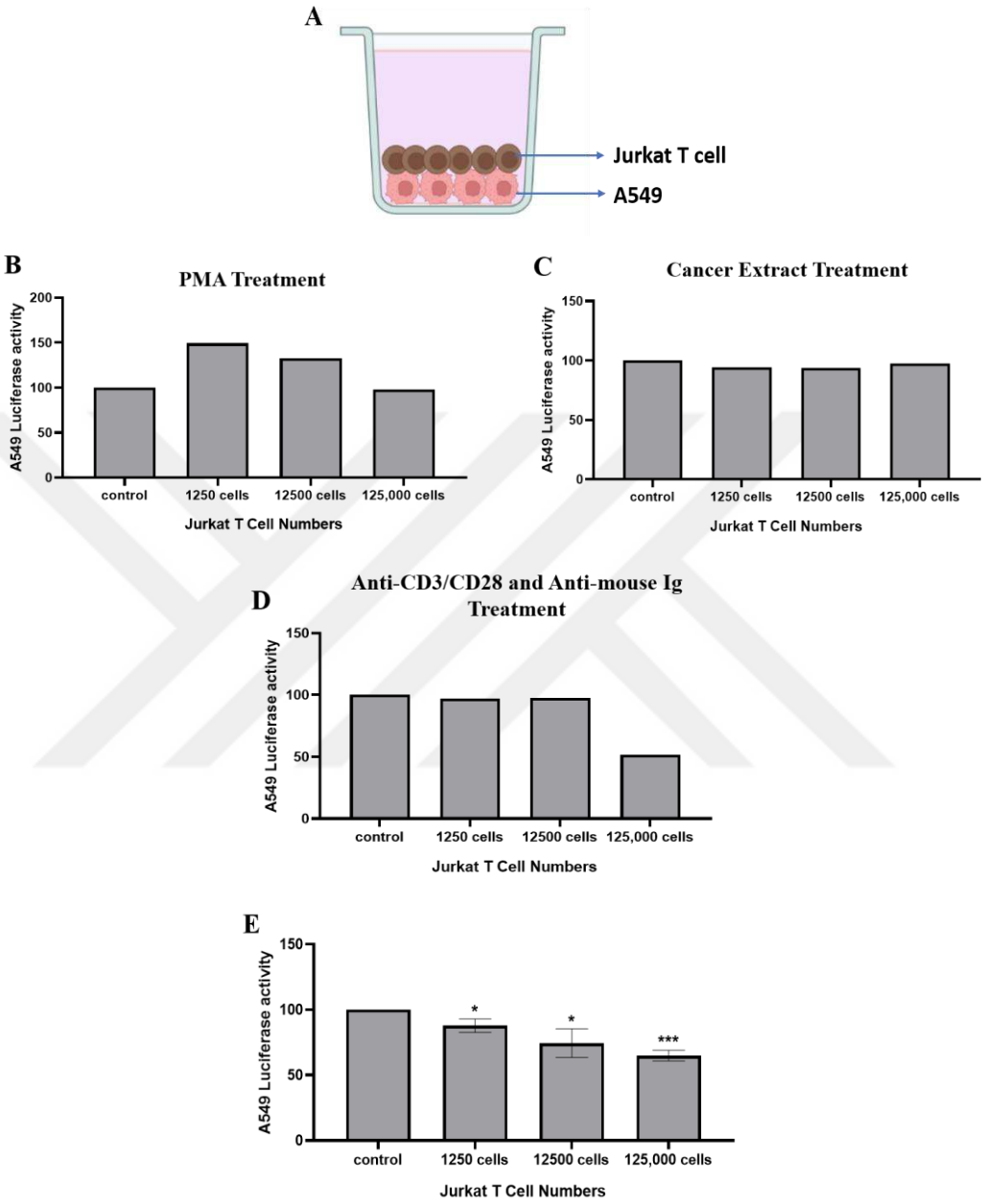


Figure 3.6. 2D co-culture analysis of Jurkat T cells with A549-luc cells. **A.** Schematic view of co-culture conditions **B.** Luciferase activity of A549 cells as a result of co-culture with PMA treated Jurkat T cells for 48h (n=1) **C**. Luciferase activity of A549 cells as a result of co-culture with A549 cell pellet treated Jurkat T cells for 48h (n=1) **D.** Luciferase activity of A549 cells as result of co-culture with the absence of anti-CD3/CD28 and anti-mouse Ig treated Jurkat T cells for 48h (n=1) **E.** Luciferase activity of A549 cells as a result of co-culture with Jurkat T cells for 48h (mean±SD of independent experiments, n=4, , ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

3.5 Co-culture analysis of NSCLC cells in 3D dormant state

The effect of Jurkat T cells on the proliferation of A549 cells was tested in 3D dormant environment **(Figure 3.7 A)**. Jurkat-GFP T cells were used for microscopic examination of co-culture analyses **(Figure 3.7 B).** Compared to proliferative A549 cells, dormant A549 cells are more resistant to Jurkat T cells. However, when the highest number of Jurkat T cells was given, a decrease in the proliferation of dormant A549 cells was also observed **(Figure 3.7 C)**.

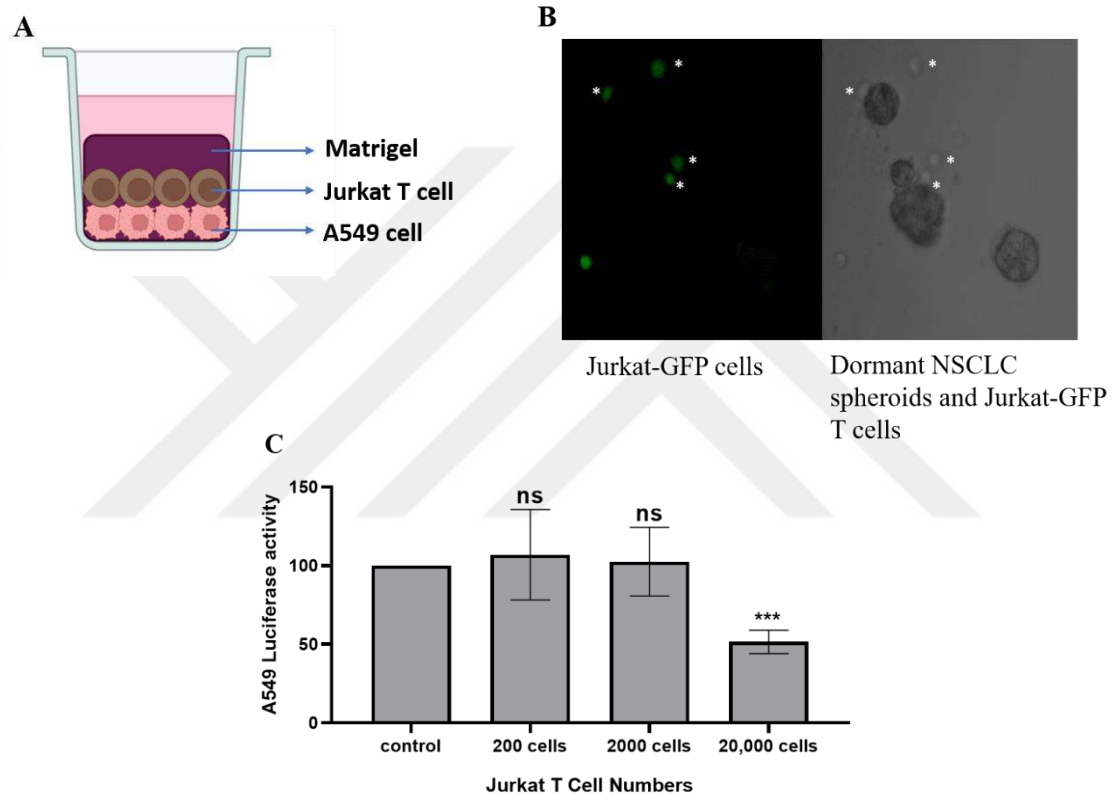


Figure 3.7. 3D co-culture analysis of Jurkat T cells with A549-luc cells. **A.** Schematic view of co-culture conditions **B.** Representative microscopy images of Jurkat-GFP T and A549 cells in day 14 **C.** Luciferase activity of dormant A549 cells as a result of co-culture with Jurkat T cells for 48h (n=4, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) Luciferase activity of dormant A549 cells as a result of co-culture with Jurkat T cells for 48h

3.6 Effect of LGALS3 on the co-culture system in proliferative NSCLC cells

LGALS3, was chosen as the target gene because it showed higher expression in dormant NSCLC cells compared to proliferative cells. LGALS3 gene was knocked out in A549 cells by CRISPR method **(Figure 3.8 A).** Luciferase stable A549 LGALS3 KO (A549 LGALS3 KO-luc) cells were co-cultured with Jurkat T cells. Initially, A549 LGALS3

KO cells showed a more resistant phenotype than A549 WT cells. However, in the most active state of Jurkat cells, proliferation of A549 LGALS3 KO cells is decreased. **(Figure 3.8 B).**

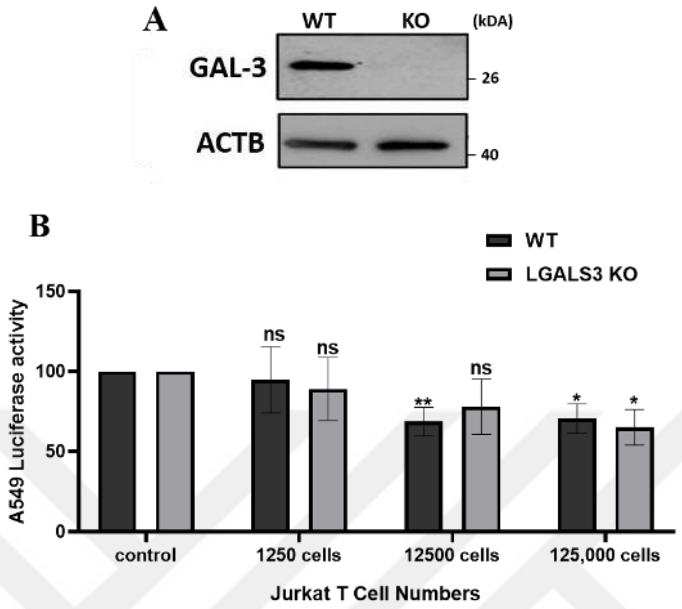


Figure 3.8: 2D co-culture analysis of Jurkat T cells cells with luciferase stable A549 LGALS3 KO (A549 LGALS3 KO-luc) cells. **A.** KO confirmation by western blot **B.** Luciferase activity of A549 W-luc versus A549 LGALS3 KO-luc cells as a result of co-culture with Jurkat T cells for 48h (mean±SD of independent experiments, n=3, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (Each cell type compared with its control group) (WT: Wildtype KO: Knockout clone)

3.7 Effect of LGALS3 on NSCLC 3D dormancy behavior

A549 LGALS3 KO cells were cultured in dormancy environment for 14 days. Compared to A459 WT cells, LGALS3 KO cells did not grow in a spheroid form and spread on Matrigel **(Figure 3.9 A)**. The dormancy state of these cells was tested with various markers and compared with A549 WT cells (**Figure 3.9 B).** Also, it has been shown that these cells do not represent a complete dormant cell character **(Figure 3.9 C)**.

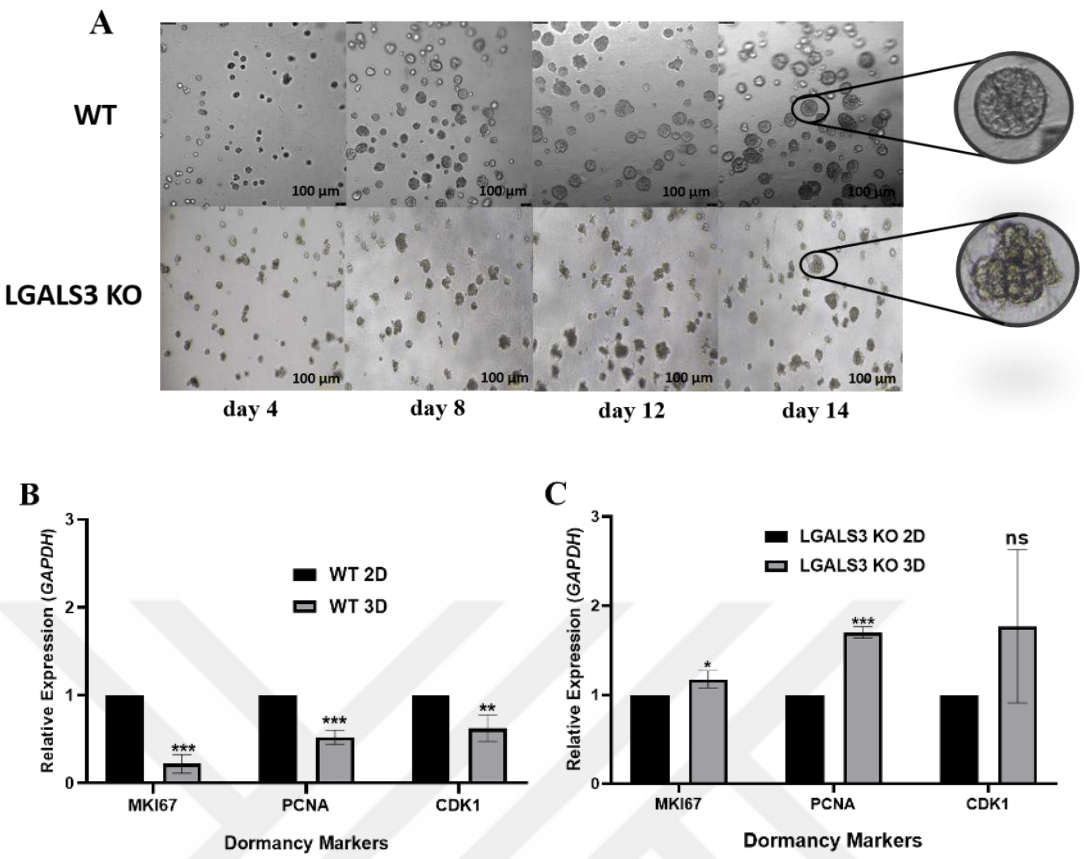


Figure 3.9: Dormancy state analyses of LGALS3 KO cells**. A.** Representative microscopy images of A549 WT and A549 LGALS3 KO cells in every 4 days **B.** Expression of dormancy markers analyzed by qPCR in A549 WT cells **C.** Expression of dormancy markers analyzed by qPCR in A549 LGALS3 KO cells (mean±SD of independent experiments, n=3, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (WT: Wildtype KO: Knockout clone)

3.8 Effect of LGALS3 on the co-culture system in dormant NSCLC cells

A549 LGALS3 KO cells were co-cultured with Jurkat T cells in 3D culture. It has been demonstrated that A549 LGALS3 KO cells resistant to death and continue to grow **(Figure 3.10 A).** To analyze the reason for this, the level of immune checkpoint molecules expressed by A549 LGALS3 KO cells in 3D environment was compared with A549 WT cells. PVR, VSIR, CEACAM1 immune checkpoint molecules are expressed more in A549 LGALS3 KO cells than in A549 WT cells in 3D culture **(Figure 3.10 B).**

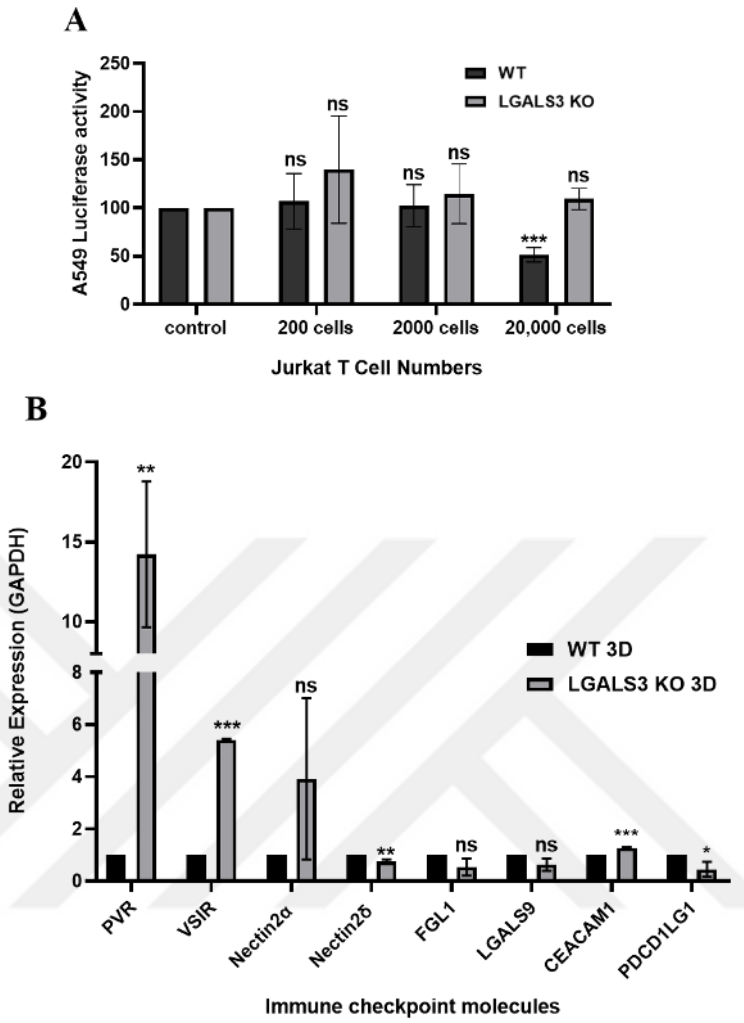
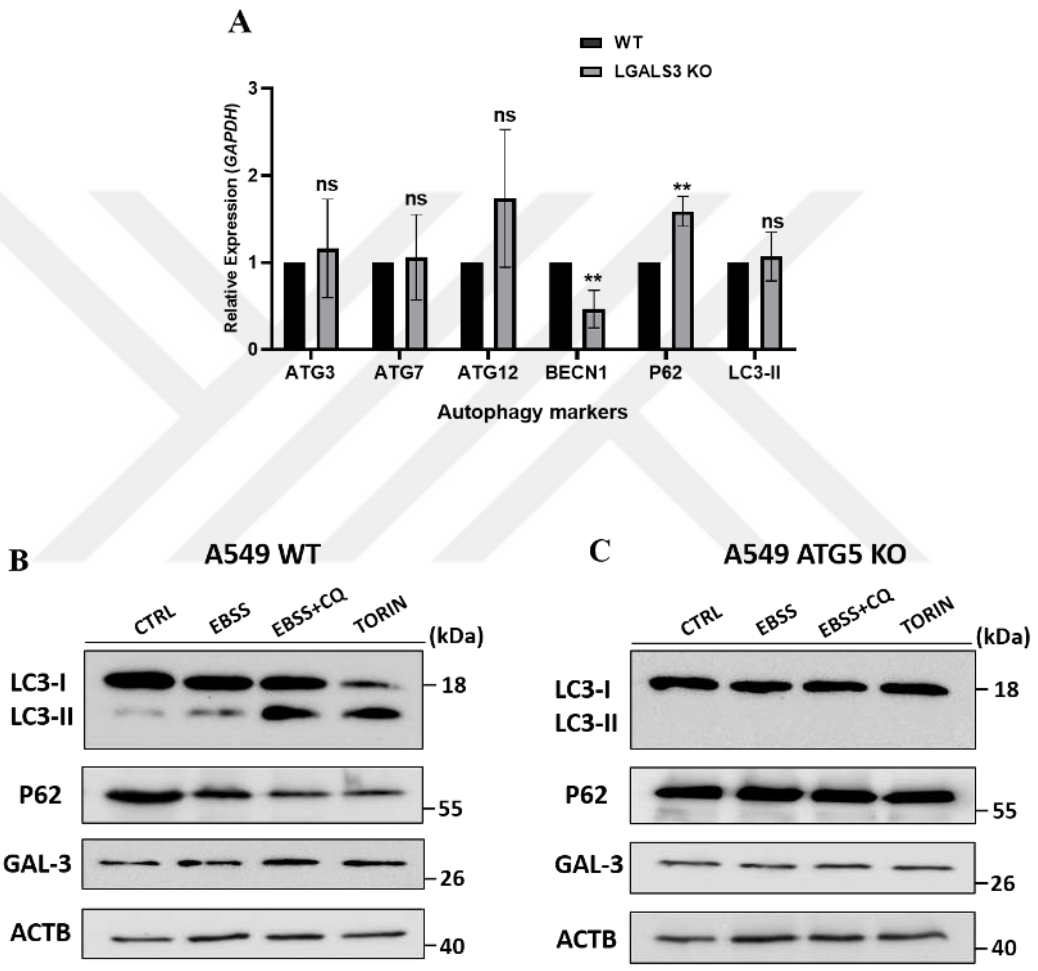


Figure 3.10: 3D co-culture analysis of Jurkat T cells with A549 LGALS3 KO-luc cells. **A.** Luciferase activity of 3D A549 WT-luc versus 3D A549 LGALS3 KO-luc cells as a result of co-culture with Jurkat T cells for 48h (n=3) **B**. Expression of immune checkpoint molecules analyzed by qPCR (n=2) (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (Each cell type compared with its control group) (WT: Wildtype KO: Knockout clone)

3.9 Relationship between LGALS3 and autophagy

The relationship between LGALS3 and autophagy was analyzed. For this purpose, the expressions of various autophagy marker genes in A549 WT and A549 LGALS3 KO cells were compared. According to the qPCR results, while BECN1 expression decreased in LGALS3 KO cells, there was an increase in P62 expression **(Figure 3.11 A),** indicating a defect of autophagy in these knockout cells.

In addition, the expression level of Galectin-3 protein was checked in A549 WT and A549 ATG5 KO cells. Firstly, it was revealed that autophagy is induced under the EBSS, EBSS+CQ and TORIN conditions. Also, it is observed that, although it was reported to bind damaged lysosomes, under normal conditions Galectin-3 was not the direct target of autophagy in A549 WT cells **(Figure 3.1 B, D-F)**. Also, under autophagy inducing stress conditions, expression level change in Galectin-3 in WT cells, was not observed in A549 ATG5 KO cells in 2D cultures **(Figure 3.11 C, G, H).**



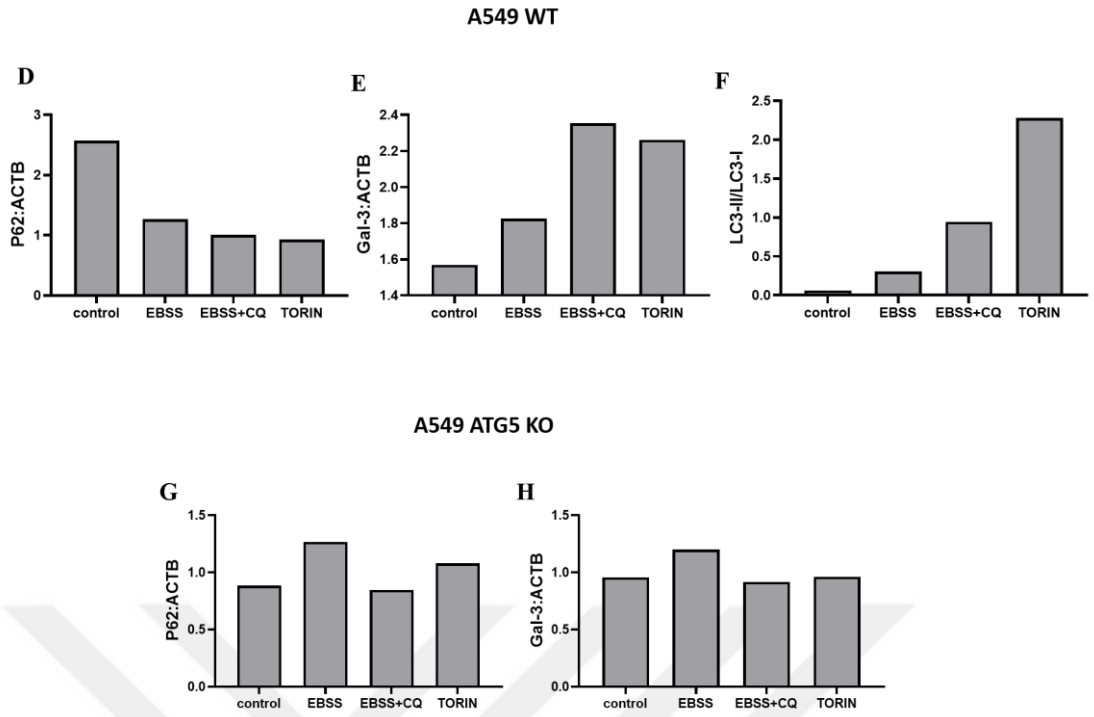


Figure 3.11: Characterization of autophagy and LGALS3 immune checkpoint gene relationship. **A.** Expression of autophagy markers analyzed by qPCR (mean±SD of independent experiments, n=3, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) **B, C.** LC3 shift, P62 cargo degradation, and Gal-3 expression analyzed by western blotting in A549 WT and A549 ATG5 KO cells. EBSS, EBSS+CQ and TORIN used for flux control. Actin used as a loading control. **D, E, F.** Band intensity analysis of p62, Gal-3 and LC3-II/LC3-I are corrected according to control actin in A549 WT cells. **G, H.** Band intensity analysis of p62 and Gal-3 are corrected according to control actin in A549 ATG5 KO cells (n=1) (WT: Wildtype KO: Knockout clone)

3.10 Effect of autophagy on the co-culture system in proliferative NSCLC cells

For analysis of the contribution of autophagy to cell death, A549 ATG5 KO cells and Jurkat T cells were co-cultured. Decreased proliferation of A549 ATG5 KO cells was observed depending on the Jurkat T cell number in non-dormant state **(Figure 3.12)**

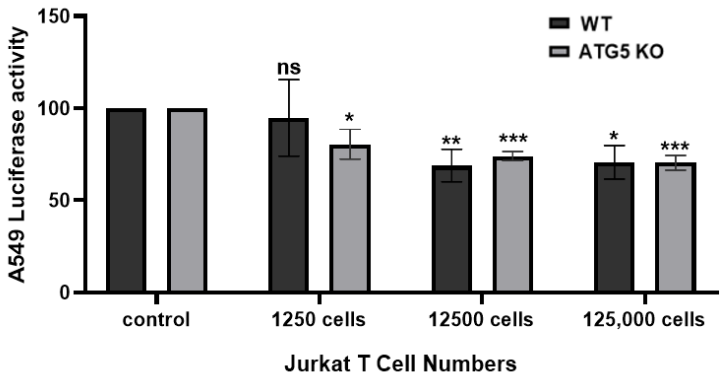
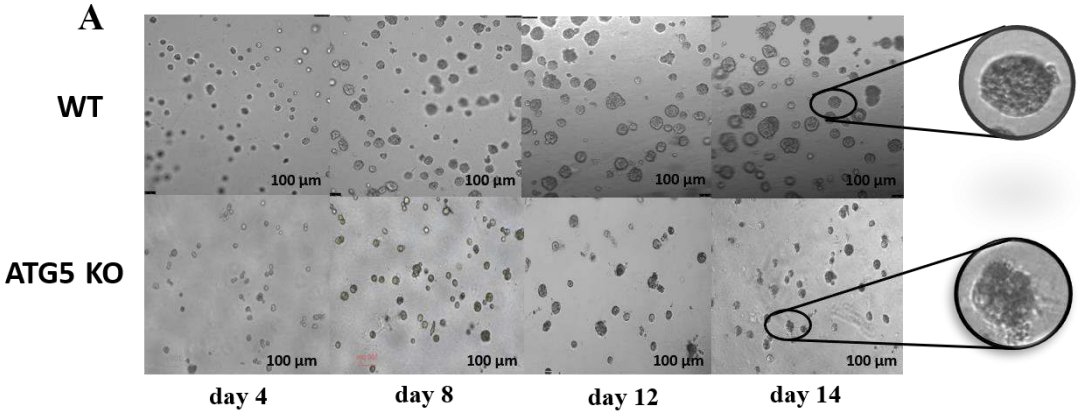


Figure 3.12: 2D co-culture analysis of Jurkat T cells with luciferase stable A549 ATG5 KO (A549 ATG5 KO-luc) cells**.** Luciferase activity of A549 WT-luc versus A549 ATG5 KO-luc cells as a result of co-culture with Jurkat T cells for 48h (mean±SD of independent experiments, n=3, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (Each cell type compared with its control group) (WT: Wildtype KO: Knockout clone)

3.11 Effect of autophagy on NSCLC 3D dormancy behavior

To analyze the contribution of autophagy to dormancy, A549 ATG5 KO cells were grown in 3D culture for 14 days. Compared to A549 WT cells, A549 ATG5 KO cells did not grow in a spheroid and regular structure. In addition, they dispersed into the Matrigel after a certain day **(Figure 3.13 A).** The dormancy validation of A549 ATG5 KO cells was tested by dormancy markers and it was observed that these cells did not enter dormancy **(Figure 3.13 B).**



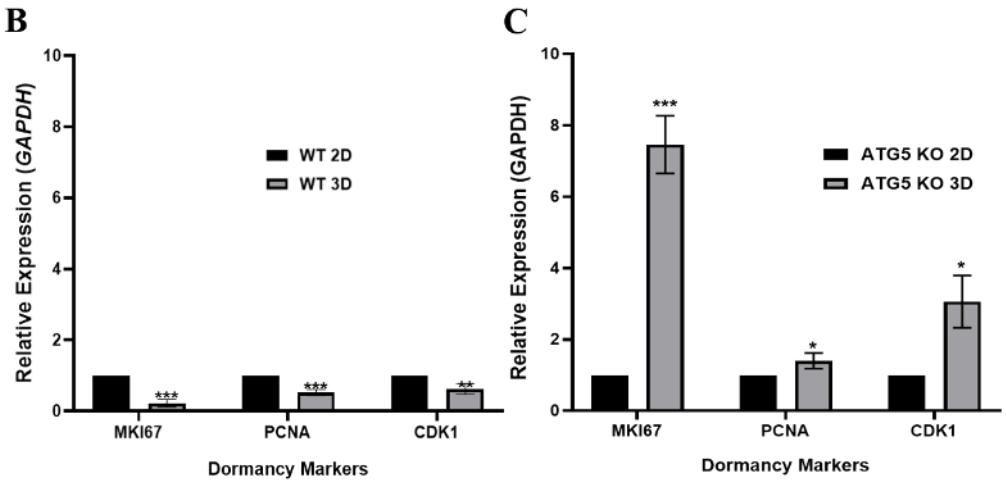
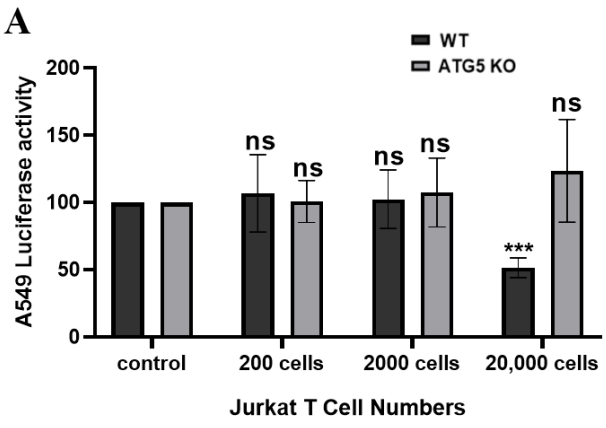


Figure 3.13: Dormancy state analyses of ATG5 KO cells **A.** Representative microscopy images of A549 WT and A549 ATG5 KO cells in every 4 days **B.** Expression of dormancy markers analyzed by qPCR in A549 WT cells **C.** Expression of dormancy markers analyzed by qPCR in A549 ATG5 KO cells (mean±SD of independent experiments, n=3, ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (WT: Wildtype KO: Knockout clone)

3.12 Effect of autophagy on the 3D co-cultures of NSCLC and Jurkat T cells

The effect of autophagy on cell death in 3D culture was analyzed. A549 ATG5 KO cells were grown in 3D culture environment and were co-cultured with Jurkat T cells. A549 ATG5 KO cells continued to grow against Jurkat T cells in 3D culture compared to A549 WT cells **(Figure 3.14 A).** To analyze the reason for this, the level of immune checkpoint molecules expressed by A549 ATG5 KO cells in 3D environment was compared with A549 WT cells. VSIR, TNFRSF14, NECTIN2δ, LGALS3, PDCD1LG1 and CD86 immune checkpoint genes are expressed more in A549 ATG5 KO cells than in A549 WT cells in 3D culture **(Figure 3.14 B).**



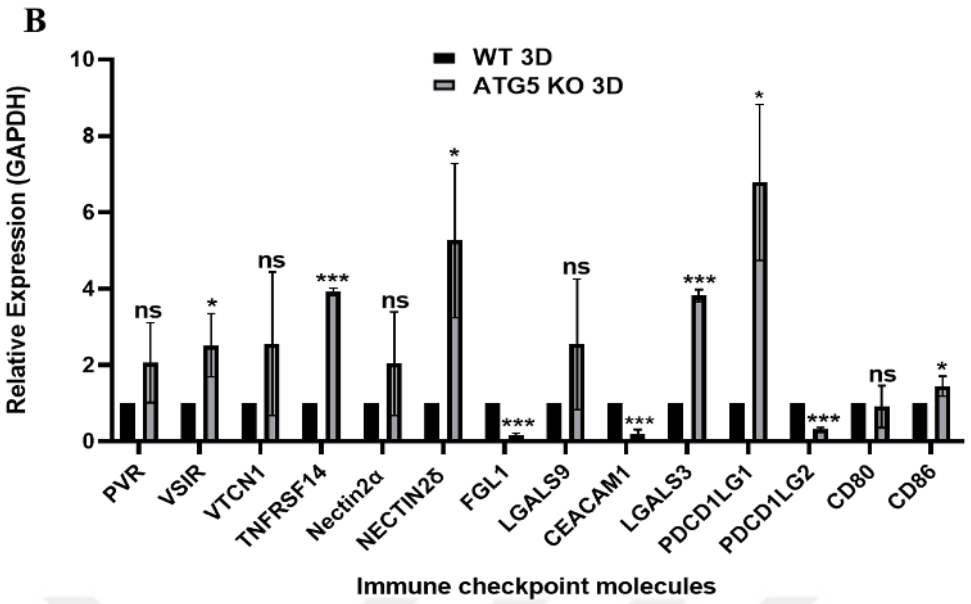


Figure 3.14: 3D co-culture analysis of Jurkat T cells with A549 ATG5 KO-luc cells. **A.** Luciferase activity of 3D A549 WT-luc versus 3D A549 ATG5 KO-luc cells as a result of co-culture with Jurkat T cells for 48h **B.** Expression of immune checkpoint molecules analyzed by qPCR (mean±SD of independent experiments, n=3, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (Each cell type compared with its control group) (WT: Wildtype KO: Knockout clone)

Chapter 4:
CONCLUSION

Cancer recurrence is one of the major causes of mortality in cancer patients. Relapse or recurrence generally results from the reaction of dormant cancer cells that stay in tissues for months or even years. These cells cannot be detected with imaging methods since these methods are not sensitive enough with current tools in hand. Therefore, defining and understanding cancer dormancy at a molecular level is crucial for development of new treatments for cancer and prevention of relapse. However, there are currently not enough studies about cancer dormancy.

Immune surveillance is one of the hallmarks of cancer and mechanisms that has the potential to limit the number of dormant cancer cells. Therefore, involvement of the immune system in disease progression and understanding the mechanism of interaction between dormant cells and components of the anti-cancer immune system is important.

Autophagy is an important molecular mechanism involved in tumor-stroma interaction affecting cancer progression (Akkoc et al., 2022). Moreover, autophagy may also play an important role in the regulation of immune system. Indeed, autophagy was involved in several crucial processes, including antigen presentation, immune cell activation, thymic selection, preparation of an inflammatory niche by regulating the secretion of cytokines and/or lymphokines (Kuballa et al., 2012). Yet, the role of autophagy in tumor-stroma interaction, and especially in tumor immunity is not clear.

In this study, a 2D and 3D *in vitro* cell culture model was established using NSCLC cells and a co-culture system with T cells was characterized. Regulation of immune checkpoint molecules in 2D and 3D cell co-culture models was also investigated. In addition, involvement of autophagy in immune checkpoint controls was characterized.

According to RNA-Seq analyses, a set of immune checkpoint genes were found to be upregulated in dormant NSCLC cancer cells compared to proliferative counterparts. RNA-Seq results were confirmed by qPCR analyses. In the literature, the most targeted immune checkpoint molecule pairs in cancer immunotherapy were PD-1/PD-L1 (PDCD1LG1) and CTLA-4/CD80. According to our RNA-Seq and qPCR results, PDCD1LG1 expression did not change significantly in dormant cancer cells in 3D

culture, while we observed expression changes in less studied immune checkpoint molecules. Therefore, we focused on the effects of these less studied, alternative immune checkpoint genes/proteins on cancer dormancy.

Anti-tumor responses are mainly mediated by T cells and NK cells although contribution of other leukocyte types cannot be excluded. In this study, PBMC were isolated from blood samples and initial tests were perform by using these primary immune cells. PBMC consists of 60% T lymphocytes, 10% B lymphocytes, 15% monocytes and 15% NK cells. Co-culture of PBMC with cancer cells resulted in significant amounts of cell death in cancer cells. Then, T cells were isolated from PBMC samples and co-culture test with cancer cells were repeated. Unfortunately, under our experimental conditions we could not observe the same effect with isolated T cells. One of the most important reasons for this, T lymphocytes contain 70% of T helper cells and these cells include Treg cells that mediate the progression of cancer. 30% of T lymphocytes isolated from PBMC are cytotoxic. In addition, the isolated T cells were not specific to lung cancer cells because they were isolated from the blood of healthy individuals. Hence, T cell training and activation against tumor cells and antigens must have been planned. However, another immune cell group instead of T cell could have a more active role on NSCLC cells such as NK cells or macrophages.

We continued our study with a well-established T cell line, Jurkat T cell line. We performed T cell training and activation assays using this cell line. Interestingly, Jurkat T cells were potent enough to kill NSCLC cells and all our efforts of T cell training and activation (PMA treatment, cancer extract treatment and antibody treatment) did not result in an improvement of their anti-tumor activity. Jurkat T cells reduced the proliferation of NSCLC cells when they were co-cultured with these cells in 2D cell culture. Strikingly, dormant lung cancer cells in 3D cultures did not show resistance to Jurkat T cells. Using this co-culture system, we studied effects of changing immune checkpoint genes/proteins on dormancy and cell death responses of NSCLC cell.

The gene of Galectin-3 protein, LGALS3, is an immune checkpoint gene that is more expressed in 3D culture than 2D culture, according to our RNA-Seq and qPCR results. Therefore, it has been selected as a target for further studies. Galectin-3 was defined as an immune checkpoint molecule. It generally showed an anti-tumor immune suppressive and tumor promoting effect in various cancer studies in the literature. In lung cancer, it

has been revealed that Galectin-3 expression is high in NSCLC patients and cells (Terzioğlu-Uşak et al., 2021; Blair et al., 2021) and high levels of the protein in the blood was associated with advanced metastatic disease (Kim et al., 2022). Moreover, Galectin-3 inhibition potentiated chemotherapy responses or anti-tumor activity of PD-L1 antibodies in lung cancer (Glinsky et al., 2009; Zhang, 2021; Vuong et al., 2019). Yet, there are currently no studies in the literature about Galectin-3 and cancer dormancy.

In the light of these data in the literature and our results in obtained in 3D dormancy model, we tested the effect of LGALS3 deletion on the dormancy behavior. LGALS3 knockout (KO) A549 NSCLC cells were created using CRISPR/Cas9 system and analyzed their responses. KO of the LGALS3 gene inhibited dormant spheroid formation in 3D cultures and resulted irregular cell cluster morphologies infiltrating into the Matrigel. These results are in line with Galectin-3's importance in cell-cell and cell-ECM interactions. Loss of dormant spheroid phenotype may be related to changing cell interactions with its surrounding.

We next analyzed the response of A549 LGALS3 KO cells to T cells in co-culture experiments. We observed that, LGALS3 KO cells in 3D culture showed resistance to Jurkat T cell induced cell death while WT control cells were dying. In other words, deletion of an immune checkpoint molecule from cancer cells conferred resistance to anti-tumor immune attack. These results in contrast with observations in the literature. There might be several explanations for these unexpected results. Firstly, we observed compensatory upregulation of several immune checkpoint molecules, including PVR, VSIR, NECTIN2δ and CEACAM1 on LGALS3 KO cells. These alternative immune checkpoint molecules contributed to immune escape of KO cells. Furthermore, in addition to serving as an immune checkpoint molecule, LGALS3/Galectin-3 has several documented functions in apoptosis, cell proliferation, cell-cell contact and cell cycle. Effect of the KO on these crucial biological events could have contributed to the observed phenotype. Additionally, the protein plays a prominent role in lysosome quality control by autophagy. As discussed below, autophagy incompetent cells showed resistance to T cell mediated destruction.

In order to test contribution of autophagy to immune cell sensitivity of cancer cells, we created autophagy-defective A549 NSCLC cells through CRISPR/Cas9-mediated deletion of the key autophagy protein, ATG5. Interestingly, ATG5 KO cells were equally

sensitive to cell death induced by Jurkat T cells in 2D co-cultures. But under 3D co-culture conditions, in addition to a change in spheroid morphology (larger, more infiltrative and dispersed spheroid formations), ATG5 KO cells showed significant resistance to Jurkat T cell-induced cell death. We observed that, in 3D culture, expression of alternative immune checkpoint molecules, VSIR, TNFRSF14, NECTIN2δ, LGALS3, PDCD1LG1/PD-L1 and CD86 genes were increased in A549 ATG5 KO cells compared to A549 WT cells. Off note in WT cells, we did not observe a significant upregulation of PD-L1 or CD86 in 3D dormant cultures compared to 2D proliferating cells. PD-L1 immune checkpoint molecule, that is the most targeted immunotherapy molecule in the literature. In addition, the blockade of the CTLA-4 and CD86 interaction is very effective in T cell activation and thus inhibition of lung cancer progression (Sabel et al., 2005). Therefore, differences in expression of PD-L1 and CD86 could have further contributed to the observed resistance. Notably, PD-L1 protein degradation by autophagy was documented as well, supporting the idea that autophagy-deficient cells might have higher levels of PD-L1 protein (Li et al., 2021).

In summary, several classical, alternative and clinically relevant immune checkpoint molecules were overexpressed in dormant lung cancer cells in our model. Increase in the expression of immune checkpoint molecules protected lung cancer cells from immune cell-induced elimination. Our study underlined the importance of Galectin-3/LGALS3 as well as autophagy in immune responses against dormant cancer cells.

How and when to eliminate dormant cancer cells is a matter of debate in the cancer community. Currently, specific treatment of metastatic disease is not an accepted clinical practice. Therefore, in the light of our data, inclusion of immunotherapy approaches to classical treatment regiments might provide additional benefits through elimination of undetectable dormant foci and cells, further improving prognosis and by residual disease and preventing relapse.

Chapter 5:

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