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THE REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
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MSc. Behnam AHMADZADEH

Program Of Tumor Biology And Immunology
MASTER OF SCIENCE THESIS

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APPROVAL PAGE

Investigation of Lung Cancer Cell Culture the Supernatants Effect of Activated with TLR Agonists on Macrophage Polarization

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This thesis study has been approved and accepted as a Master dissertation in "Tumor Biology and Immunology Program" by the assesment committee, whose members are listed below, on 12 March 2018.

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This dissertation has been approved by the above committee in conformity tothe relatedissues of Hacettepe University Graduate Education and Examination Regulation.

15 Mart 2018

Institute Manager

Prof. Diclehan ORHAN, MD, PhD

YAYIMLAMA VE FİKRİ MÜLKİYET HAKLARI BEYANI

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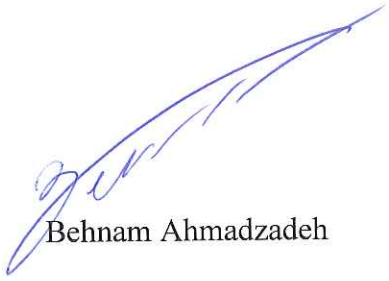
17 / 03 / 2018

Behnam Ahmadzadeh

ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in the data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor (Prof. Dr. Dicle Güç and Dr. Hande Canpınar) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.



Behnam Ahmadzadeh

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“You see things, and you say Why? But I dream things that never were, and I say Why not?”

George Bernard Shaw

(1856–1950)

I would like to thank decedent President John F. Kennedy sincerely for his great words in his speeches that were my companion and gave hopes to my heart to see far horizons in a long way between my home and university. Just as he quoted from President Woodrow Wilson, "Every man sent out from a university should be a man of his Nation, as well as a man of his time.". I will do my best to be.

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ABSTRACT

Ahmadzadeh B, Investigation of Lung Cancer Cell Culture Supernatants Activated with TLR Agonists Effect on Macrophage Polarization, Hacettepe University Institute of Health Sciences Tumor Biology and Immunology Master of Science Thesis, Ankara, 2018. It is well known that inflammation is involved in the development of lung cancer. Macrophages in the tumor microenvironment are the anti-inflammatory M2 type and anti-tumor type M1. Inflammatory cytokines, such as IL-1β, IL-6, and IL-8, released from tumor-associated macrophages (TAM) in the tumor microenvironment, facilitate the development and metastasis of cancer cells. TAMs are found intensely in many tumors, and it is known that the higher the number of TAM is associated with the poor prognosis of the disease. For this reason, the control and regulation of TAMs in the tumor microenvironment is a key factor tumor prognosis. Various Toll-Like Receptor (TLR) agonists are used in clinical practice because they can direct the immune response against the tumor.

Immune response against the tumor is stimulated by TLRs on antigen presenting cells and increasing tumor-specific T cell response. Tumor-releasing soluble factors are known to be efficient in converting macrophages to TAMs, but the effects of soluble factors released from small-cell lung cancer cells on macrophage polarization are unknown. Therefore, we have investigated the effects of the mediators released from small cell lung cancer cell line stimulated by TLR 3, TLR 5 and TLR 8 agonists on the macrophage polarization. Furthermore, we have detected the cytokines released from TLR stimulated lung cancer cell line.In our study, cell culture supernatants obtained from NCI-H82 small cell lung cancer cell lines stimulated by TLR agonist, polarize THP-1 monocytic cells into the M1 type macrophages. These macrophages were expressed CD68, CD11b, and CXCR7. We have detected an increase in the inflammatory cytokines, IL-1β, IL-6, TNFα, IL-12, and IFNγ. Furthermore, phagocytosis capacity of these cells was increased and accumulation of cells in G0/G1 phase in the cell cycle were detected. However, incubation of THP-1 cells with supernatants of NCI-H82 cell lines without TLR agonists end up with the polarization of M2 type macrophages.

In conclusion incubation of TLR3, TLR5 and TLR8 agonists with small cell lung cancer cell line polarized THP-1 monocytic cell line to M1 type macrophages and TLR agonists may improve the effects of immunotherapeutic agents.

Keywords: Lung Cancer, TLR agonist, Tumor-Associated Macrophages (TAM), Phagocytosis, inflammatory cytokine.

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ÖZET

Ahmadzadeh B, TLR Agonistleri ile Aktive Olan Akciğer Kanseri Hücre Kültür Süpernatanlarının Makrofaj Polarizasyonuna Etkisinin Araştırılması, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Tümör Biyolojisi ve İmmünolojisi Yüksek Lisans Tezi, Ankara,2018. Akciğer kanserinin gelişiminde inflamasyonun etkili olduğu çok iyi bilinmektedir. Tümör mikroçevresinde bulunan makrofajlar anti inflamatuvar ve tümöre karşı M1 tipi veya tümör gelişimine yardımcı olan M2 tipindeki makrafajlardır. Tümör mikroçevresinde bulunan M2 tipi makrofajlardan gelişen TAM'lardan salınan IL-1β, IL-6 ve IL-8 gibi inflamatuvar sitokinler kanser hücrelerinin gelişimini ve metastazını kolaylaştırırlar. TAM'lar, bir çok tümörde yoğun olarak bulunur ve tümörü infiltre eden TAM sayısı ne kadar yüksek ise hastalığın kötü prognozu ile ilişkili olduğu bilinmektedir. Bu nedenle TAM'ların kontrolü ve regülasyonu tümörlerin prognozunda anahtar rol oynar. Çeşitli, Toll Like Reseptör (TLR) agonistleri, tümöre karşı immün yanıtı yönlendirebildiği için klinik uygulamalarda kullanılmaktadır.Tümörden salınan soluble faktörlerin, TAM'lara dönüşümde etkili olduğu bilinmektedir. Fakat küçük hücreli akciğer kanseri hücrelerinden salınan soluble faktörlerin makrofaj polarizasyonuna olan etkileri bilinmemektedir. Bu nedenle çalışmamızda, TLR 3, TLR 5 ve TLR 8 agonistleriyle uyarılmış küçük hücreli akciğer kanser hücrelerinden salınan faktörlerin makrofaj hücrelerinin kutuplaşmasına olan etkileri araştırılmıştır. Bunun yanı sıra bu hücrelerden salınan sitokinler ve makrofajların fagositoz fonksiyonları değerlendirilmiştir. Çalışmamızda, NCI-H82 küçük hücreli akciğer kanseri hücrelerinin TLR agonistleriyle uyarılmasından sonra elde edilen hücre kültür süpernatanlarının THP-1 monositik hücrelerini CD68, CD11b ve CXCR7 taşıyan M1 tipi makrofajlara dönüştürdüğü ve inflamatuvar sitokinlerden özellikle IL-1α, IL-1β, IL-6, TNFα, IL-12 ve IFNγ salınımını arttırdığını gösterdik. Bu koşullardaki THP 1 hücrelerinde fagositoz kapasitesinin arttığı ve hücrelerin hücre siklusunun G0/G1 fazında yığılım gösterdiği tespit edildi.

Bu çalışmadan elde edilen sonuçlar, TLR3, TLR5 ve TLR8 agonistleri ile inkübe edilen küçük hücreli akciğer kanseri hücrelerininin THP 1 monositik hücreleri M1 tipte makrofajlara dönüştürdüğünü göstermiştir. Bu da TLR agonistlerinin, immünoterapötik ajanların etkisini arttırabileceğini düşündürmektedir.

Anahtar Kelimeler: Akciğer Kanseri, TLR agonist, Tümör ile ilişkili Makrofajlar (TAM), Fagositoz, inflamatuvar sitokin.

Destekleyen Kurumlar: HÜ BAB, Hızlı Destek projesi. Bu tez, Hacettepe Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından THD-2017-12283 numaralı proje olarak desteklenmiştir.

CONTENTS

LIST OF ABBREVIATIONS

AAM	Alternatively activated macrophage
Ang-2	Angiopoietin-2 protein
AP1	Activator protein 1
APC	Antigen Presenting Cells
APCs	Antigen-Presenting Cells
Arg-1	Arginase-1
ATMs	Adipose-tissue macrophages
BCG	Mycobacterium bovisbacillus Calmette– Guérin
CCL18	C-C motif chemokine ligand 18
c-fms	CSF-1 receptor
CREB	Cyclic AMP-responsive element-binding protein
CSF-1	Colony Stimulation Factor 1
CX3CL1	Fractalkline
CXCL8	C-X-C motif chemokine ligand 8
DC	Dendritic cells
ECM proteins	Extra cellular matrix proteins
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FliC	Recombinant Salmonella typhimuriumflagellin
G-CSF	Granulocytes colony-stimulating factor
GM-CSF	Colony-stimulating factors
G-MDSCs	Granulocytic myeloid-derived suppressor cells
GPCRs	G-protein coupled 7-transmembrane receptors
HIF	Hypoxia-inducible factor
HMGB	High-mobility group box
HMW	High molecular weight
HSC	Hematopoietic stem cell
HSPs	Heat shock proteins
ICX	Immune complex
IFN-c	Interferon-c
IFNs	Type I interferons

IFN-γ	Interferon gamma
IGP	Immunological Genome Project
IL	Interleukin
iNOS	Inducible nitric oxide synthase enzyme
IRAKs	IL-1R-associated kinases
IRF	Interferon regulatory transcription factor
IRFs	Interferon-regulatory factors
ISG	Interferon stimulated gene
JMY	Junction-mediating besides regulatory protein
JNK	JUN N-terminal kinase
LBP	LPS binding protein
LMW	Low molecular weight
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
M2-PK	Type M2 pyruvate kinase isoenzyme
MAPKs	Mitogen-activated protein kinases
M-CSF	Monocyte colony-stimulating factor
MD-2	Myeloid differentiation protein 2
MDSCs	Myeloid-derived suppressor cells MDSCs)
MHC II	MHC class II
MMP	Matrix metalloproteases
MPS	Mononuclear phagocyte system
MYD88	Myeloid differentiation primary-response protein 88
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NKT	Natural killer T cells
NO	Nitric oxide
NSCLC	Non-small-cell lung cancer
ODN	Oligodeoxyribonucleotide
ORNs	Oligoribonucleotides
pDC	Plasmacytoid dendritic cells
poly I	Polyinosinic acid

poly I:C	Polyriboinosinic-polyribocytidylic acid
poly A:U	Polyadenylic-polyuridylic acid
PPAR-γ	Peroxisome proliferator-activated receptor gamma
Prok1	Prokineticin 1
PRRs	Pattern Recognition Receptors family
REPs	Repetitive extragenicpalindromics
RES	Reticuloendothelial system
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SCLC	Small-cell lung cancer
ssRNAs	Single-stranded RNAs
TAM	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TCR	T-cell receptor
TFH	T follicular helper
TGF-β	Transforming growth factor
TGM2	Transglutaminase 2
TH	T helper
Th1	T helper 1
Th2	T helper 2
Tie2	Tunica internal endothelial kinase 2
TIR	Toll/interleukin-1 receptor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
TRAFs	TNF receptor-associated factors
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cells
TRIF	TIR domain-containing adaptor protein inducing IFNβ
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelium-derived growth factor

VEGFR2	VEGF receptor 2
Ym1	Chitinase-3-like protein 3
βFGF	Basic fibroblast growth factor

FIGURES

TABLES

1. INTRODUCTION

Lung cancer is one of the most common types of cancer and is often associated with poor disease progression. The progression of lung cancer is complicated and consists of a known multistep process such as transformation, hypoxia, invasion, migration, and metastasis. The understanding of these mechanisms will lead to overcoming the difficulties in the treatment of malignant lung cancer. Tumor-associated macrophages (TAM) that are macrophages in the tumor microenvironment. Various growth factors, cytokines, chemokines, and inflammatory mediators are responsible for the development of TAM. These factors, which are best known for tumor growth, poor prognosis and effective in metastasis are VEGF, PDGF, and IL-10. In addition, the presence of a high number of TAMs in the tumor microenvironment is a significant marker that facilitates the invasion, angiogenesis, and early metastasis. Inflammatory cytokines, such as IL-1β, IL-6, and IL-8, released from TAM in the tumor microenvironment, facilitate the development and metastasis of cancer cells.

TAMs arising from M2 type macrophages are intensely present in many tumors, and the high number of TAM infiltrating tumors is also associated with poor prognosis of the disease. For this reason, the control and regulation of TAMs is a key factor in improving the prognosis of tumors. Activation TLR on antigen presenting cells increase tumor-specific T cell response. The use of TLR agonists is involved in the new therapeutic approaches in control cancer. Soluble factors released by tumor cells induced by TLR agonists are predicted and envisaged to increase immunoreactivity to the tumor by promoting macrophage polarization in the tumor periphery towards M1.

The polarity of macrophages found in the tumor microenvironment is determined by soluble factors released from tumor cells. In this thesis study, THP-1 cells, a human monocytic cell line was selected in order to search the polarization of macrophages after the stimulation of cancer cells by TLR agonists. The phenotypic characteristics and the functions of monocytes were further investigated.

2. LITERATURE OVERVIEW

2.1. Macrophage and Its Specifications

Monocytes are one type of the white blood cells, that are a little bigger than lymphocytes, and identified by a light blue cytoplasm and an unsymmetrical nucleus. Progenitor cells in bone marrow develop Monocytes (Figure 2.1). Provoked granulocyte–monocyte precursors by common myeloid precursors finally produce monocytes. Monocytes go into the blood from the bone marrow which is their origin and then migrate into various body tissues, where they develop into matured macrophages (1). Macrophages are myeloid immune cells that are characterized by keen phagocytosis. The father of cellular immunology, Ilya Metchnikoff, gives the name of Macrophages which referred “the big eaters” in Greek to these cells after the finding these cells. Anticipating the remarkable macrophage biology and their focal function in disease and health defines, and he won a Nobel prize rightfully. To visualize swarms of macrophage in infected water fleas and pricked starfish larvae, he used intravital microscopy; he mentioned the substantial homologies of vertebrate blood cells and invertebrate phagocytosis (2). Aschoff in 1924 defined these cells to the reticuloendothelial system (RES) (3) but, because the RES consists of cells of non-macrophage lineage, this system doesn’t seem to be favorable; According to the fact that macrophages shared essential functional characteristics in vivo and derived from monocytes but endothelial cells and fibroblasts were not, “Mononuclear phagocyte system” (MPS) introduced in 1969 (4). Mononuclear phagocyte phylogenetically is a type of very primitive cells and found in early life forms with related cells. Exhibiting similar features to the mammalian macrophages come single-cell protozoa discovered. The yolk sac is the first (5) afterward bone marrow in adult man (6) are places which macrophages ontogenetically originate. In tissues, Macrophages are classified in circumscribed sorts which each particular district recruit the special type of cell (7). Macrophages are classified in several ways, the mononuclear phagocytic system (MPS) is the most prosperous definition method to classify this type of cells, which involves macrophages bone marrow progenitors and these professional highly phagocytic cells. In this type of classification, mature tissue macrophages are the end cells of the mononuclear phagocytic lineage which resulting

from circulating monocytes that originate in the bone marrow (8). Macrophages have several origins during the process of ontogeny, and each of these divergent lineages remains in tissue-specific macrophages, and this type of classification seems to be inadequate to define macrophage classification (4).

Another binary functional classification of macrophages introduced by researchers that refer to inflammatory conditions. This classification comprises the activated macrophage and alternatively activated macrophage(AAM) categories, and in the conditions which not caused by pathogens derivative M1 and M2 subclasses for these categories of macrophage (9). These two states respectively are elucidated by reactions to the interferon-c(IFN-c) cytokine and Toll-like receptors(TLRs) activation and to interleukin-13(IL-13) and IL-4. Even though this ordering probably reflects extreme conditions, likewise triggered macrophages throughout immune responses mediated by T helper-1 cells which secrete IFN-c or of AAMs through parasitic infections (5), Such binary categorizations don’t have the ability to exemplify the complicated in-vivo milieu for the most macrophage classes, where various other growth factors and cytokines interact to characterize the latest differentiated form. Undoubtedly, transcriptional profiling of resident macrophages by the Immunological Genome Project(IGP) demonstrate extraordinary transcriptional diversity by a slight overlap between these populations, which suggest the existence of many unique classes of macrophages (10).

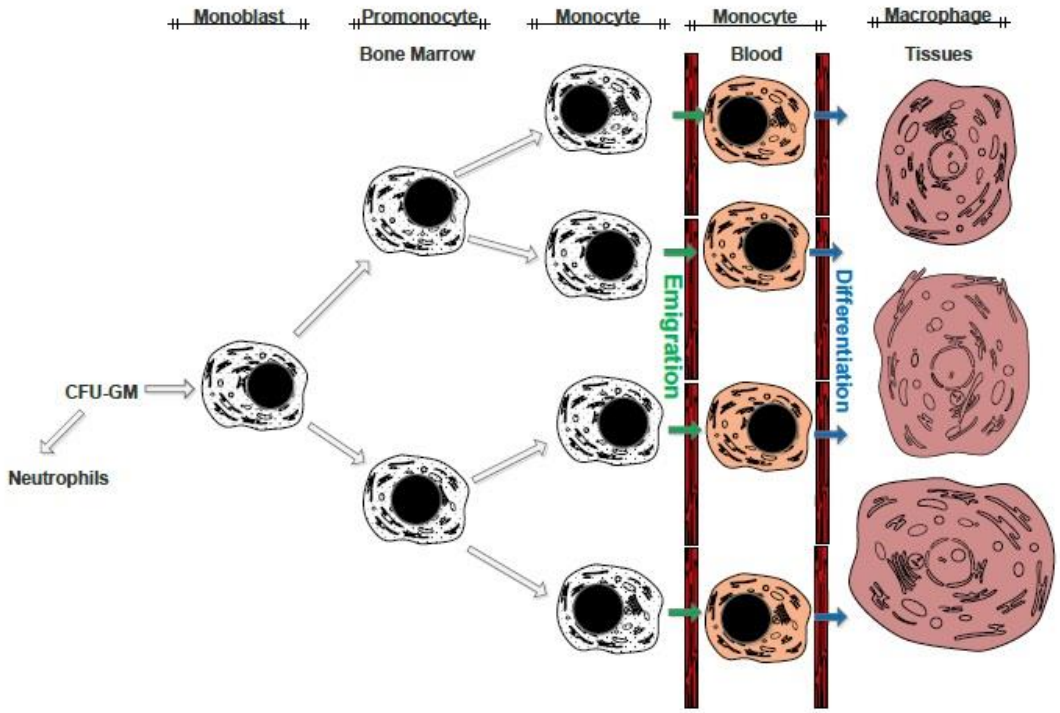

Figure 2.1. Monocyte development from bone marrow. The mononuclear phagocyte system, presenting the macrophages origin in the bone marrow. Adopted and designed based on (1).

Having classical knowledge about the origin and differentiation of macrophages let's have a closer look at additional routes. Varol et al. in 2007 demonstrated some other routes of monocyte excursion in the mice that monocytes which are present in blood can return to their origin that is bone marrow (1) and the reservoir spleen forms, which is the outcome of the monocytes mobilization (11). The extent of monocytes which in homeostatic situations replenish the macrophage populations in the different tissues is not clear yet. Studies in the mouse propose despite the fact that monocytes can replenish macrophages in tissues similar to lung and kidney (12), they do not have any contribution in the brain microglia (13). Moreover, in bone marrow transplantation it has been demonstrating that macrophages in the lung transform into the donor type, this fact indicates that they are originated from immigrating monocytes (14). Moreover, monocytes possess the ability to differentiate and transform into dendritic cells (DC), but there is uncertainty whether monocytes arerisingDCs of bona fide tissue or DCs of the most tissue develop from specific precursor cells of DCs, which are different from the

monocytes. Whatsoever the destiny of monocytes, in homeostatic conditions they circulate in the blood for about 1–3 days (3). Chemokines are released locally in tissue infections and inflammations. Afterward, blood monocytes are activated and attracted to the site. In such conditions, mature macrophages are essential to innate immune defense in the place of infection. Mouse model studies reveal that in the time of nematode infection, lung macrophages in the location of infection were increased but blood monocyte remained fixed (15). But another model of inflammation (peritonitis) shows monocytes immigrate to participate in the local population and even they can induce macrophages which are proliferating. Because of the limitation of proliferation potential of human macrophages (16), additional experiments are necessary to figure out if this is applicable to the man. The blood monocytes probably assist to defense against infections besides to being tissue macrophage progenitors. For instance, monocytes can phagocyte microbes that entered the blood and demolish them. Studying blood monocytes offer a model that supplies a view of the monocyte-macrophage system situation.

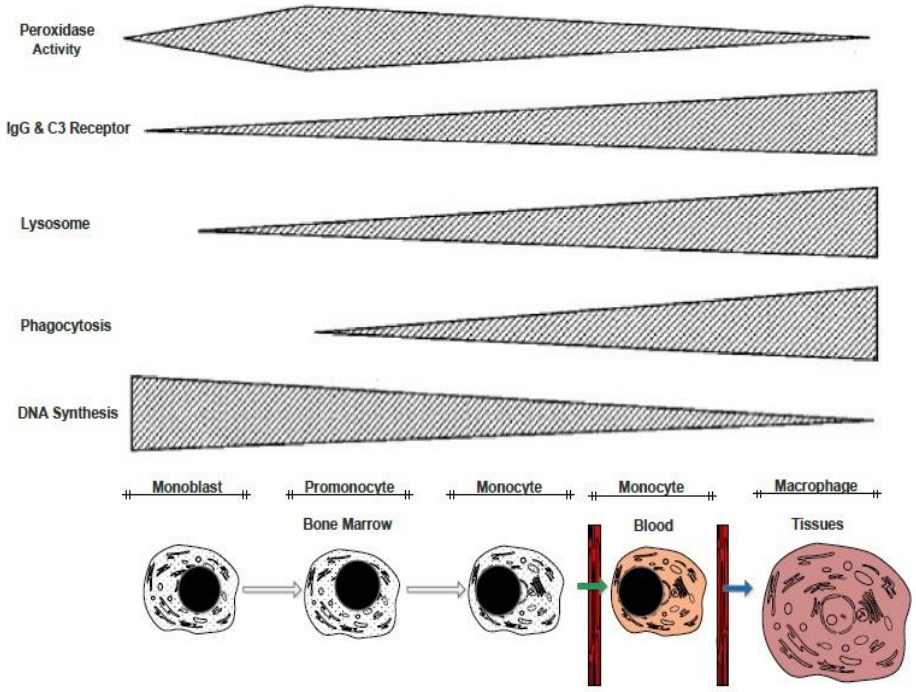

Figure 2.2. Characteristics of mononuclear phagocyte system cells, showing the development of phagocytic activity, lysosomes, IgG and C3 receptors, and peroxidase activity. Adopted and designed based on (1,11-16) .

2.2. Monocyte Subsets

Because of the ease of access to human blood, evidence for Monocytes heterogeneity was first presented in human (17). Monocyte subsets were precisely distinguished in CD16-positive and CD16-negative by flow cytometry technique. Based on their functional aspect, we can further dissect the CD16-positive monocytes into separate subsets(18). All of these findings finally led to human blood monocytes nomination, which defines non-classical (CD14+CD16++), intermediate (CD14++CD16+) and classical (CD14++CD16−) (19). According to some genuine evidence, the classical monocytes generate intermediate monocytes and subsequently to non-classical monocytes in man, as a consequence, this is according to time course studies in severe infection and after chemotherapy and monocytes ablation (20). In man monocyte subsets extensively studied since the 1980s (21). There has been an ample concern regarding the mouse model blood monocyte subset only in the twenty-first century (22). By using markers Ly6C and CD43 comparable subgroups can be defined in the mouse. Based on these markers, the Ly6C++ CD43+ present the classical monocytes subset, the Ly6C+ CD43++ present the non-classical monocytes subset while intermediate levels of both markers indicate the intermediate monocytes subset. The mouse intermediate cells show the morphology of the monocyte and express intermediate levels of CCR2, CX3CR1, andCD62L (23). In the mouse, by using the method of depletion and tracking, the study revealed that classical monocytes are able to give rise to non-classical monocytes. Subsequent to TLR8 or 9 agonists injection, the intermediate monocytes temporary emergence accompanied by non-classical monocytes expansion (24). The non-classical and classical monocytes half-life for the mouse model has been defined and was demonstrated to be about 48 hours for the non-classical and around 24 hours for the classical monocytes. Analogous subclass specific information for a human is impressive (25).

A significant number of similarities and differences in monocyte subsets have been shown in man and mouse. Findings according to mice monocytes, whether it be in disease models or exceptional physiological conditions before testing and confirmation cannot be transferred directly to the man. A contemporary gene expression survey of three appropriate inflammatories “trauma, burn, and LPS

infusion simulating sepsis" conditions verify this result, which confirmed that generated mouse models do not restate any of the conditions in human(26). Monocyte subpopulations exist in man, mouse and various different species similar to primates which are genetically far from human(27), pigs (28), and rats(29). Hence, apparently, man, mouse, and all the other different species monocytes shouldn't be called as a single cell type.

2.3. Using Cell Lines as Study Models

Most widely used cell lines of human monocyte, are Mono Mac 6, U937, and THP-1. The earliest cell line of this family to be explained and characterizes a somewhat immature cell type was U937 without or with a slight expression of CD14 which is a marker of monocytes(30). The second is THP-1 cell line that is rather further mature and can be differentiated slightly more by Vitamin D3 in cell culture(31). Our third cell line is Mono Mac 6 that expresses CD14 and quickly shows the reaction to lipopolysaccharide(LPS)(32). The Mono Mac 6 treatment with VD3 will lead this cell line to more maturation and in series of studies on leukotrienes treatment with TGF-beta plus VD3 has been used (33). Because gene transfer has more efficiency in comparison to primary monocytes in blood, such cell lines are useful models for molecular studies. Due to not expressing of CD16, these cell lines should be avoided to be used as intermediate or non-classical monocytes models.

Various cell lines models representing macrophages and monocytes in the mice are commercially available. The non-phagocytic WEHI-3B Cell line which could represent monocytes from bone marrow (34), but the cell expresses the Ly6C antigen (35). The trustable cell line that could represent the Ly6C++ classical blood monocyte may not exist, but the Pu5 line seems to be the best match cell line to the non-classical monocyte of blood (36). Beyond the level of the monocyte of blood, the cell lines P388D1 (37), J774 (38), and RAW 264.7 (39) are more mature. They represent specifications of macrophages in tissue. These cell lines probably could be used as exudate macrophages which are newly emigrated. Several cell lines existence represent macrophages specifications in the mice but not in human, might imply that

the normal mature tissue macrophages which are not transformed have more capacity of proliferation in mice compared to human.

2.4. Monocyte Function

Properties of purified monocytes from blood which are very sensitive can transform by any operation or handling. The ficoll-hypaque technique isolation which is very typical and is used for mononuclear cells separation in addition to affect the expression of the receptor can lead to activation of monocytes (40). Moreover, using antibodies to target cells surface molecules for positive selection of monocytes can stimulate and activate these cells whereas the binding antibody to cell surface may trigger signal pathways when these cells are cultured. Monocytes are highly sensitive to stimulation by microbial products especially LPS and we have to be aware of this sensitivity. Such microbial products may contaminate blood monocyte during purification, and culture and this will end to stimulation of these cells. As a consequence of monocytes Pre-activation, responses may enhance or decrease in following functional studies. Therefore, minimal handling and treating and no-touch isolation are the most favored procedure for the study of monocytes. Whole blood analysis is the recommended approach (Figure 2.3).

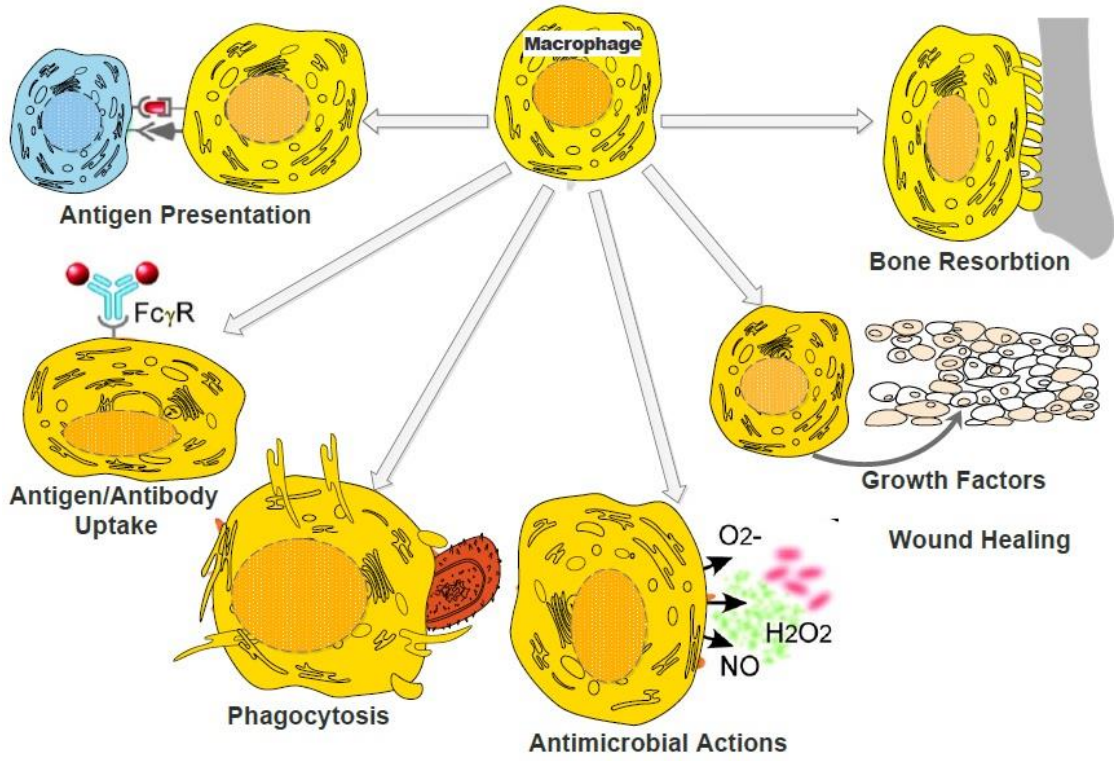

Figure 2.3. Monocyte functions, macrophages have specific functions in immunity and homeostasis, which are dependent on their activation status and resident tissue (Adopted from Chawla A, 2010, Stanford University). Adopted and designed based on (41-59).

2.5. Phagocytosis

By having the ability to engulf large particles, Monocytes belongs to professional phagocytosis. The phagocytosis progress through the ordinary procedure, but precise methods like coiling phagocytosis applied for up taking Legionella and Borrelia(41) or phagocytosis through the looping method exists. In phagocytosis, several receptors like receptors for the Fc-region of antibodies, scavenger receptors, lectins, and complement receptors take part. Another type of phagocytosis is apoptotic cell phagocytosis which is essential to development and repair (42). Phagocytosis of microbes proceeds with antigen presentation, and production of cytokine but apoptotic cells phagocytosis is a silent procedure. These processes are critical duties of tissue-resident macrophages, but monocytes in the blood may take part in immune responses in various infections because the also can perform all of these steps.

The Fc-receptor phagocytosis pattern is obvious in the monocytes subsets withCD16-positive specifications. However, the CD16-positive monocytes showed decreased ability in phagocytosis of antibody-coated erythrocytes (43). Also, it has been noted that reduction of Escherichia coli bacteria uptake was seen(20). The contradictory results in the CD16-positive monocytes showed the higher tendency for phagocytosis of 0.5 μ beads of latex(44)and for E. coli (45). Dissimilar manipulation of monocyte cells in the various studies or diverse properties of the different used beads may lead to conflicting results. For the different monocytes subsets, we need a comprehensive research at the whole spectrum of particles, in addition to various ligands. Also in non-classical, classical, and intermediate monocytes coiling and looping phagocytosis have not been studied yet. Although the classical-monocytes subsets presented poor activity in apoptotic granulocyte uptake, the uppermost activity has been seen in intermediate and non-classical subsets of monocytes (46).

2.6. Cytokine Production

The cytokines production includes the main anti-inflammatory, pro-inflammatory cytokines, IL-10 and TNF, respectively studied in human blood monocytes. The CD16-positive cells secrete more TNF in response to Lipopolysaccharides(LPS) and to TLR7/8 ligands (47).Non-classical monocytes in man stimulated with tumor micro vesicles and produced higher TNF. Scientific findings did not show extra cytokine secretion in the CD16-positive subtype cells; this may be the result of alternatively variant outcomes of cell manipulation earlier to the assay or variability among the blood donors. Results without processing, that gained with whole-blood analyzes are Most convincing (48). Also, in patients diagnosed with sepsis, the CD16-positive subset monocytes produced the highest amount of CCL5 and CXCL10 only when control monocytes from donor stimulated by lipid A in-vitro. It was shown that CD16 molecule triggers the signal to support this higher expression (49). Also, it has been demonstrated that the intermediate-monocytes subset could show excessive TNF secretion among the CD16-positive subset monocytes (46). The highest TNF secretion rates throughout the monocyte subsets were reported in intermediate-monocytes (50). Moreover, the intermediate

monocytes expressed the highest level of IL-10 (46). Analysis of whole blood when monocytes are stimulated with LPS in the non-classical mouse monocytes showed raise in TNF production. Moreover, during infection with L. monocytogenes, non-classical subtype monocytes produce much higher levels of TNF than classical subtype monocytes subsequent to exuding into the peritoneum (51). Monocytes have the ability to produce a wide spectrum of the cytokines and chemokines. Different expression of cells membrane receptors and intracellular signaling pathway molecules illustrate distinctive expression and production of such mediators throughout monocyte cell subsets.

2.7. Antigen Presentation

Monocytes are among of professional Antigen Presenting Cells(APC), besides they present exogenous peptide antigens to T helper(TH) cells. This performance in the adaptive immune response is crucial. The monocytes have taken up the exogenous protein, digested it into peptides, and load those particles onto MHC class II (MHC II) pathway and eventually present them by MHC class II molecules which are located on the cell surface of the antigen-presenting cell. T helper cells by specific T cell receptor molecules recognize Class II peptide complex. The CD4 receptor stabilizes the interaction, and this results in activation of T cell then proliferation, and cytokines production by this cell. High levels of MHC class II fundamentally expressing human monocytes. Therefore, monocyte cells can take part in T cell activation which initially activated by exogenous antigens or superantigens. The rates of monocyte subsets MHC class II cell surface expression has been reported, and the CD16-positive subset monocytes showed a high rate in HLA-DR expression(18,21). In stimulation with mycobacterial antigen, the CD16-positive cells induce excessive rates of IFN-γ production at T cells (18). Newly proliferation of T cells by superantigens was discovered to be the most potent for the intermediate subtype of monocytes (52).

2.8. Migration

Chemokines govern migration of leukocytes, by targeting G-protein coupled 7-transmembrane receptors(GPCRs) which are receptors of chemokine on the cell

membrane. The mechanism of leukocytes migration from the blood into the issue explained by four steps rolling, activation, arrest, and transmigration. Receptors of the CCR type predominantly express by monocytes and react to the respective ligands, the role of CXC receptor-ligand pairs have been discovered. Monocytes strongly express CX3CR1 which is encoded in the major CCR cluster and is located on the chromosome 3 of human and this chemokine receptor is more likely belongs to the receptors of CCR. In man and the mouse monocyte subsets, CCR2 is not expressed by the non-classical subsets of monocytes (53).

The CCL2 chemokine-induced in many infections and inflammations and classical monocytes respond to it exclusively. Also, to leave bone marrow into blood under homeostatic conditions, the classical subsets of monocytes require CCR2.The lower amount of the Ly6C++ monocytes subset in the blood of CCR2−/− mouse is a good illustration in order to prove it. Moreover, The classical subset of monocytes in the CCR2−/− mouse has shown less increase during infection (54). On the non-classical monocytes, the expression levels For CX3CR1 were presented to be higher at both the mRNA levels and the produced levels of protein(55). In triggering by fractalkline(CX3CL1), this differential expression accompanies a privileged arrest and exodus of the CD16-positive subsets of monocytes. The mechanism for leukocyte adhesion is altered in the presence of fractalkine/CX3CL1. By mediating cellular adhesion through the initial tethering and transmigration steps Fractalkline/CX3CL1 may boost extravasation of leukocytes (56). CD16-positive monocytes selectively increase with excessive exercise subsequent to the action of catecholamines (50). Epinephrine Infusion leads to expansion of CD16-positive subsets of monocytes and quick set off is possible while non-classical subtype of monocytes settles in the marginal pool (57). In vivo study in mouse vascular endothelium which demonstrates the steady motion of non-classical subtype of monocytes supports this concept (51). The non-classical subtype of monocytes is in the strategic position, and by receiving signal of inflammation, these subtypes can quickly migrate to neighbor tissue.

2.9. Monocyte Maturation to Macrophages

Maturation to various kinds of macrophages takes place by monocyte migration into tissues. This fact clarifies the various phenotypic and functional attributes which macrophage types show in different tissues. Macrophage characterization also changes by different cytokines in pathophysiological processes like inflammatory processes and malignancies. The converting quantity of exuding monocytes to the macrophages pool depends on tissue type. In infecting mouse with nematodes, local proliferation replenishes lung macrophages more than the influx of monocytes (15). Putting monocyte into culture with/without the addition of different cytokines in many in vitro studies reveal the differentiation process of monocyte to macrophage which ends to differentiation of monocytes to an extensive amount of macrophages in a few days. Monocyte culture in the existence of LPS and IFG-gamma develop classically activated pro-inflammatory macrophages is best known as M1 type whereas the culture of monocytes in the existence of IL-4 or IL-13 develops alternatively activated macrophages which are known as M2 type (5). By using no-touch selection techniques, various monocyte subsets are used to show a contrasting express in molecules in the cell surface which characterize the cells and a higher capacity of phagocytosis by CD16-positive subsets of monocytes which transformed to macrophages in comparison to classical subsets of monocytes that transformed to macrophages (58). The CD16-positive subsets of monocytes co-cultured with T cells generate macrophages which produced exceeding levels of CCL24 and CCL2 than same cultures contain CD16 negative subsets of monocytes (59). The data in this study indicate that different types of macrophages produce the differential chemokine, but no further characterization of the macrophages was done. These data show that the unique types of macrophages in man may derive from classical subsets and the non-classical subsets of monocytes. This concept is not examined yet but holds true concept for intermediate monocytes.

2.10. Macrophage Polarization

The definition M1 and M2 macrophages in mammals are related to the Th1 and Th2 cytokines associated which respectively polarize them. In this mouse strains study macrophages activated with either the lipopolysaccharides (LPS) or interferon

gamma (IFN-γ) cytokine and T helper 1 (Th1) or T helper 2 (Th2) made a difference in their metabolism of arginine(60).Converting l-arginine to nitric oxide (NO) and l-citrulline by inducible nitric oxide synthase enzyme(iNOS) is done in M1-type macrophages, but M2 macrophages convert l-arginine to l-ornithine by using arginase. The l-ornithine is a major component in tissue repair because of being a progenitor of polyamines and proline constituents of collagen. The arginase or iNOS pathways byproducts restrain the enzymes which are reciprocal and respectively stabilize the M1 macrophages or M2 macrophage polarizations (61).

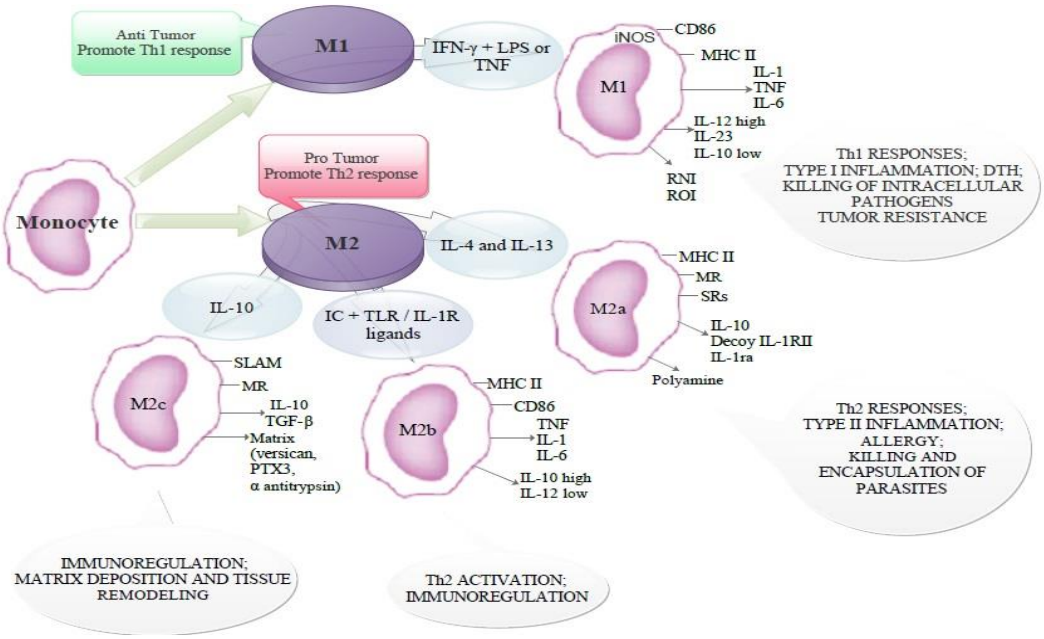

Figure 2.4. Macrophage Subgroups. Properties of M1 and M2 macrophages, Cancer Inhibiting and Cancer Promoting (Adopted from Fernando O. et al, 2014). Adopted and designed based on (60-73).

Classical M1 type activation in mammals is provoked by bacterial cell wall constituents, intracellular pathogens, and Th1 cytokines indicators such as IFN-γ. The pro-inflammatory production of mediators including interleukin 1 beta (IL-1β), interleukin 23 (IL-23), interleukin 18 (IL-18), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNFα); additionally, the nitric oxide synthase-2 dependent reactive nitrogen intermediates and reactive oxygen intermediates (ROI) production; and moreover, an efficient pathogen-killing phenotype which is a consequence of high antigen-presenting activities all together results in this activation.

The M2 type macrophage activity in comparison, described as an alternative pathway in the activation of macrophage characterize through interleukin 4 (IL-4) induction of mannose receptor activity augmentation (62). New studies show that M2-like activation can be amplified by interleukin 10 (IL-10), transforming growth factor (TGF-β), components of the complement system (i.e., proenzymes), immune complexes, apoptotic cells, fungal pathogens, and parasites further than the classical Th2 cell cytokines IL-13 and IL-4. The M2 type macrophages are well known for producing cytokines such as TGF-β and IL-10 (immunosuppressive cytokines) and being highly phagocytic. A positive feedback loop which is established by TGF-β and IL-10 enhance M2 type macrophage polarization and commonly ease the inflammations removal. Nevertheless, M2 type macrophages are also able to facilitate chronic infection with intracellular pathogens, aid in tumors development, and cause allergic inflammation (6).

	M1	M2
		
Polarizing stimulus	IFN-γ, LPS, IFN-γ+LPS	IL-4, IL-13, Ic, IL-10, GC, GC+TGF-β
Phenotype	Proinflammatory	Anti-inflammatory
Effects on Immune System	Immune Stimulation	Immune Regulation
Effects on Tcells	Promotion of Th1 response	Promotion of Th2 response
In vitro morphology	Round/oval	Elongated, fibroblast-like
Products/Markers	TNFα, IL-1J3, IL-6, IL-12, IL-23, CXCL 10, pSTAT1, MMP9	IL-10, TGF-β, CCL 17, CCL22, CD163, CD206, pSTAT3/6
Phagocytic activity	High	Low
Antigen presentation	High	Low
Arginine metabolism	iNOS: Arginine => NO	Arg1: Arginine => Ornithine
Antibacterial capacity	High	Low
Effect on tumors	Tumoricidal	Protumorigenic
Effects on Tissue	Tissue Injury/Destruction	Tissue Remodeling and Repair

Figure 2.5. Macrophage subgroup specifications (Adopted from Immunobiology of Macrophages, David S. Nelson). Adopted and designed based on (60-73).

The M1/M2 macrophage activation still requires further studies and adjustments to consider the extensive functional flexibility and high variety of

macrophage populations to be able to combine to the innovative findings. Similar toTh1/Th2 paradigm (including Treg, Th9, Th17, Th22, and TFH cell types) which represent two opposite ends of a variety and might not entirely consider all the diverse activation schemes we should reconsider the M1/M2 paradigm. Recently, based on both the activation stimuli and the tissue source of macrophages a uniform terminology was recommended (63). For example, depending on the stimuli M2 macrophage responses end to the representation of various M2 subtypes including M2a which is induced by IL-13 and IL-4; M2b that could be activated by immune complexes, Toll-like receptors (TLRs), and apoptotic cells; and M2c which deactivate by IL-10, TGF-β, and glucocorticoids. The M2c cells mainly involve in immune suppression, and tissue remodeling but M2a and M2b macrophages drive Th2 responses (63). In vivo polarization of M1/M2 macrophage is not absolute and presumably, shows a not uncomplicated and complex process.

Subpopulations of macrophages still are growing, and new subsets including CD169+ macrophages, tumor-associated macrophages (TAM), and TCR+ macrophages are actively investigated(64). In define activated macrophage subsets, species-specific macrophage variations are an extra challenge. Albeit of inconstancy in markers applied to define M1type and M2 type polarization among man and mice macrophage cell lines, relatively consistent molecular signatures are found among the species. To highlight the interspecies dissimilarities between man and mice macrophages, the Ym1(chitinase-3-like protein 3) is a good instance. This molecule is the mouse M2 marker and has no homologs in human (65). For more accuracy, it is better to consider the transcriptional regulators, cytokine secretions, surface expressions in macrophages subcategories, but still M1/M2 model provides a proper framework. To understand methods of macrophages to adjust their function in reactions to microbial and physiological signals, studies targeted at illustrating the macrophages functional expansion and the underlying mechanisms of their activation in other spices which are evolutionary far from Human seems to be necessary.

2.11. M1 and M2 Macrophages

By finding the alternative activation of macrophage which is inducible by IL-13 and IL-4, the idea of the plasticity in mononuclear phagocytes turn out to be more

complicated (62). IFN-γ alone or with inflammatory cytokines (GM-CSF and TNF) or microbial stimuli (LPS) was the first Th1 cytokine that finds out to stimulate classical effector functions in macrophages. Subsequently, IL-13 and IL-4 were found to be effective in activation of "alternative" type of macrophage (M2) (5). Upon LPS or IFNγ stimulation, macrophages revealed different nitrogen metabolism pathways (arginine versus Nitric oxide) depending on their derivation from Th2 mice strains (e.g., Balb/c) or Th1 mice strains (e.g., C57/BL6) and respectively describe as M2 or M1 macrophages (60).

Mantovani and colleagues developed this concept to suggest a general pattern for polarization of macrophages. In this pattern, the classically activated macrophages are represented as M1 and the alternatively activated macrophages as M2 (66). Other mediators in addition to IL-13 and IL-4 can also drive polarization of M2, for instance, IL-33 which is a cytokine from the IL-1 family amplifies alveolar macrophages M2 phenotype polarization by induction of IL-13. This phenotype is responsible for lung inflammation and eosinophilia. Similarly, M2 macrophage activation is triggered by IL-21 which is one of Th2-associated cytokine. It has been suggested that macrophages polarization to M2 phenotype is done by IL-34 and CSF-1 (67). In a study CSF-1 and GM-CSF-derived macrophages from bone marrow in the response of the LPS primarily to induce more IL-23 and IL-12 representing an M1 type state and the latter more induction of IL-10 without induction in IL-23/12, representing polarization toward M2 type (68). M1 polarization in the presence of Activin A molecule for GM-CSF- derived macrophages has been reported (69). Another type is an M2-like state which shares some of M2 cells characterizations is between the different pathways activation that describes the intricacy of macrophages. Diverse stimuli, such as IL-10, transforming growth factor-β (TGF-β), immunoglobulin complexes, and glucocorticoids expand M2-like functional cell phenotypes which show characterizations similar to IL-13 or IL-14 activated macrophages (e.g., high expression of angiogenic factors, IL-10, and mannose receptor) (70). Conditions similar to helminth or Listeria infection, cancer, inside the placenta and embryo, and obesity all are in-vivo situations could be marked by the development of M2-like phenotypes (51). In a practical aspect, responses from the polarized Th1 cell, and mediate resistance against tumors and intracellular parasites,

M1 macrophages play an essential role. In fact, M1 cells produce high rates of IL-23 and IL-12 and inflammatory cytokines (IL-6, TNF, IL-1β) with effector molecules (e.g., reactive oxygen(ROI) and nitrogen(RNI) intermediates), but small amounts of the IL-10 immunoregulatory cytokine.

Most types of the M2 cells typically secrete high rates of IL-10, low rates of IL-23 and IL-12, in addition, they exhibit the inconstant capacity in the secretion of the inflammatory cytokines. The M2 cells commonly characterize by mannose, galactose-type, and scavenger receptors expression and Arginase-1(Arg-1), that has effect in polyamines and ornithine production. Also, the M2 type cells express low rates of caspase I and IL-1β, high rates of type II decoy receptors and IL-1ra (71). In responses of polarized Th2 such as clearance of parasite, M2 cells mainly have a function. Besides, M2 cells in accompany with M2-like type macrophages show immunoregulatory characterizations, angiogenesis, enhance tissue remodeling and participate in the progression of tumors. M2- and M1-polarized macrophages differentially express chemokine receptor and product chemokines. CXCL9 and CXCL10 chemokines express by M1 macrophages to attract Th1, while CCL17, CCL22, and CCL24 chemokines express by M2 macrophages (72). Also, chemokines solely can influence macrophage polarization, for instance, CCL2 promote M2-like phenotype in contrast CXCL4 induce an exclusive phenotype of macrophage which express both characteristics of polarized types of M2 and M1. Eventually, cellular regulation in the metabolism of folate, glucose, and iron-controlled with M2and M1 polarized macrophages separately. In man and mice, Transglutaminase 2(TGM2) enzyme expression which is responsible for protein metabolism creates macrophages with M2 characteristics. Lastly, compatible with the concept of polarization, the macrophage cells in-vivo or ex-vivo in pathological situations such as tumors, allergic reactions, and parasite infections express functional phenotypes which reveal several features of M2 and M1 polarized macrophages (6). Nevertheless, macrophages showed overlapping M1–M2 specifications and are able to shifts their polarization in the pathological states this fact suggests the plasticity of these cells (73).

2.12. Macrophage Phenotype in Tumors

Macrophages and monocytes are the key constituents of the host immunological response to tumor cells (74). These cells negatively and positively together with other lymphoid and myeloid cells influence the tumor development. However, the factors that specify the outcome of the host–tumor communication are not well figured out, many tumors recruit premature myelomonocytic cells and by arresting their differentiation and perverting their cytotoxicity and suppressing lymphoid effector cells and lastly induce peripheral tolerance. Tumor cells imitate and utilize macrophage functions to enhance growth, by producing a stroma and promote angiogenesis, local invasion of their microenvironment and metastasis (75). Prostaglandins and TGF-beta can suppress antitumor inflammatory responses and uptake of apoptotic tumor cells.

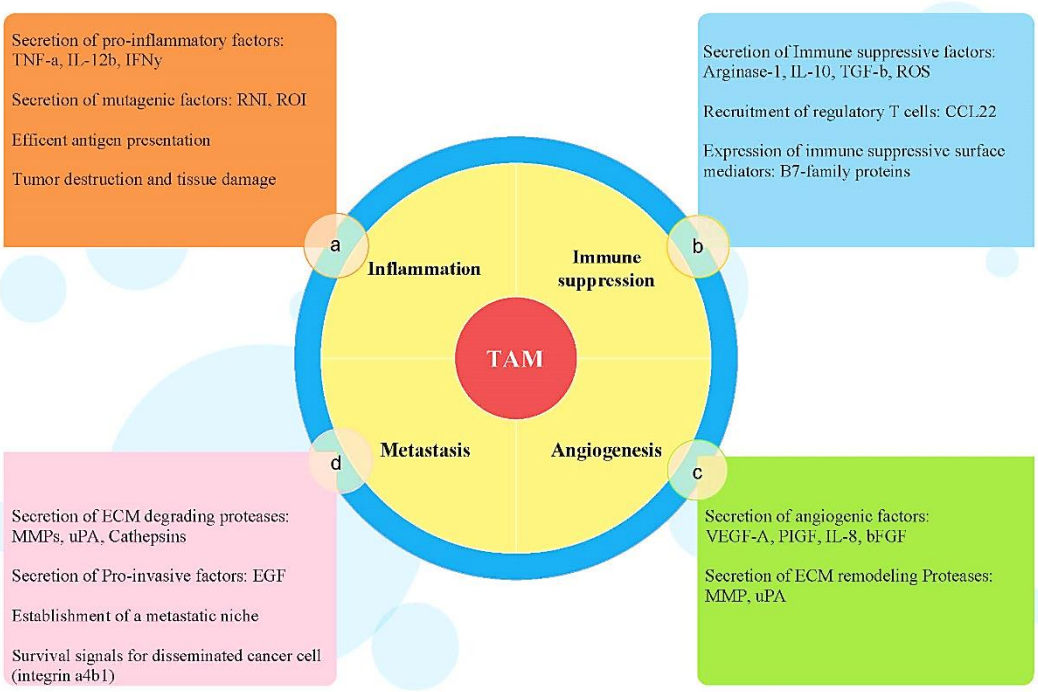

Figure 2.6. Various roles of TAMs in the tumor microenvironment (Adapted from Michael C Schmid et al. 2012). Adopted and designed based on (74,75).

The Colony Stimulation Factor 1(CSF-1) is the factor for macrophage growth and modulates its phenotype besides stimulates macrophage recruitment, limit the activation of cytotoxic function effects; IL-4 and IL-13, act on shared and specific

receptors, induce the trophic and known as alternative M2 activation phenotype, this phenotype is distinct from cytotoxic M1 classically activated macrophages which is Interferon-gamma-dependent (74). The IL-10 cytokine is a powerful deactivator of macrophage inflammatory attributes while TGF-beta another deactivator that boosts fibrosis and vascular remodeling. A broad spectrum of chemokines such as MCP-1 often produce by tumor cells and attract myeloid and mononuclear cells. TNF-alpha also signifies in tumorigenesis. A broad range of plasma membrane receptors expresses on Monocyte-macrophages which control their response to growth factors, cytokines, chemokines, and the other tumor-derived and host-derived ligands (76). Regulating cellular responses to diverse agonists and enhancing or inhibiting macrophage effector mechanisms done by other membrane molecules. Such molecules are useful markers for the characterization, presence, and possible functions of tumor-associated macrophages and are objects for therapeutic interference.

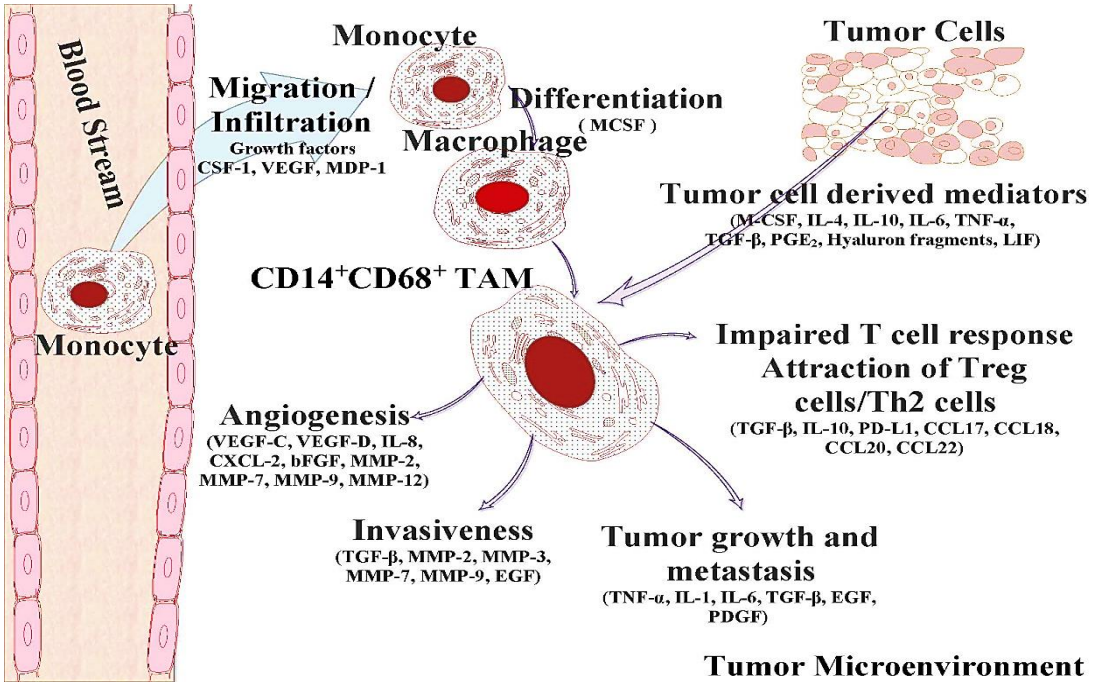

Figure 2.7. Tumor-Associated Macrophage specifications. Induction of TAMs in the tumor microenvironment (Adapted from Michael R Mallmann et al. 2012). Adopted and designed based on (74-76).

2.13. Macrophage Heterogeneity in Tumors

The earliest studies on the existence and probable role of macrophages in tumors done by Mantovani, Pollard, Alexander, Evans and their colleague (75). The topic became the center of researchers attention recently (77). The terms such as myeloid-derived suppressor cells(MDSC) and tumor-associated macrophages (TAMs) which are currently in use and myeloid cell heterogeneity made lots of confusion. The TAM contains the cells with macrophage surface markers such as F4/80 and activation markers of alternative pathway such as Arginase-1(Arg-1) (74), and the MDSC includes phenotype of immature monocytic cells (Gr-1 low) and granulocyte characterizations (Gr-1 high). The diversity of Mononuclear Phagocytes in association with differentiation phase and activation conditions give rise to significant plasticity within and among the cell populations. In some studies, to define precursors of tissue macrophages in various inflammatory and pathologic states and during development, adult life, physiologically, researchers utilized the fractalkine receptors, in combination with other receptors of chemokines. Some studies have been done to illustrate the origins and population kinetics of myeloid dendritic cells in relation to monocyte/macrophages (78). In mouse tumors, they used fluorescence and transgenic methods to trace precursors of myelomonocytic cells.

Tumors not only in their microenvironment (lung, liver, bone and lymph nodes), origin (epithelial, mesenchymal and hemopoietic), vascularization, and in their ability to invade (benign or malignant) are heterogeneous but also within individual tumors besides among different primary or secondary tumor populations we can see the heterogeneity. Induction of matrix synthesis and catabolism by tumors, undergoing hypoxia, necrosis, and apoptosis makes other differences in tumors. The simultaneous presence of CD4+, CD8+ lymphocytes, FoxP3 positive suppressor cells, together with innate lymphoid cells (NKT and NK cells) modulates myeloid cells, mutually. Characteristic properties of leukocytes sometimes expressed by tumor cells which can be related to their migration and invasion. Lymphocyte suppression by cell contact or secretory products can also be characteristics of tolerogenic macrophages. Tumor- or other myeloid-derived products can also corrupt dendritic cell maturation and antigen presentation. Many difficulties prevent experimental research of macrophage phenotype in tumors. Ideally, rather than

transplantable models, it is better to study naturally occurring tumors in situ. If FACS analysis is not associated with immunocytochemistry in situ, isolation of myeloid cells especially macrophages is complicated and prone to the artifact. By using an oncogene transgenic mouse, it becomes possible to coordinate defined stages of experimental tumors (79). Complete replication of human tumors does not occur in mouse and studies are limited to late stages, or after chemotherapy and irradiation.
Lastly, used macrophage markers in the human and mouse may be strictly different between species. The new gene expression profiles in both cells type are the outcome of the interactions between tumor cells, and macrophages and this gene expression only partially reproduced during co-cultivation in vitro. Still, the necessity of microarray and proteomic analyses refinement is required, while these tools are reliable indicators of signatures, e.g. of type 1 interferon activation pathways. Even rapid progressing of development in morphologic and diagnostic pathology methods is not sufficient to interpret functions at the single-cell grade.

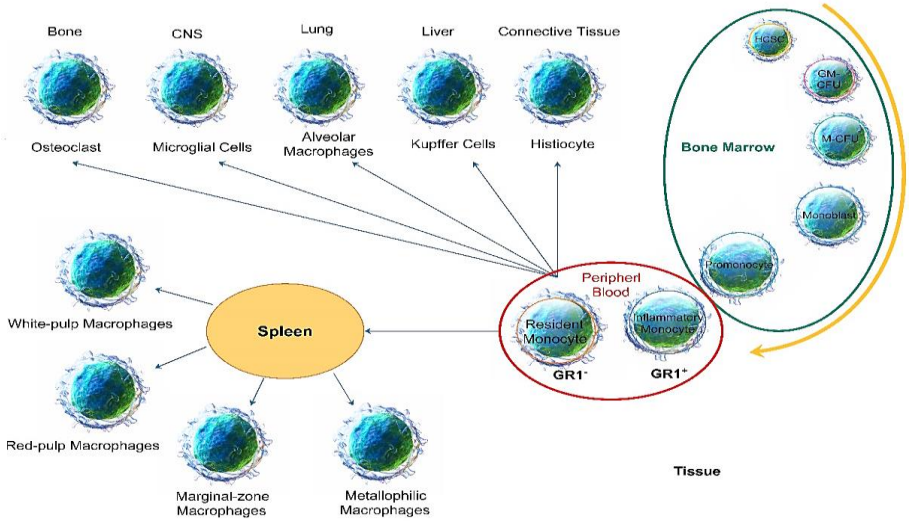

Figure 2.8. Monocyte heterogeneity (Adapted from Nature Reviews, Immunology). Adopted and designed based on (74-79).

2.14. Tumor-Associated Macrophages Origin

Initial steps of tumorigenesis before the lymphocytes, macrophages infiltrate neoplastic lesions. Hematopoietic precursors of monocytes which circulate in the blood are the origin of the TAMs. CD11b+ Ly6C+ CCR2+ monocytes are TAM

progenitors in the mice (80). M-CSF, G-CSF and GM-CSF, IL-3, and Angiotensin II are the hematopoietic growth factors which frequently produce tumors and end to stimulate bone marrow myeloid progenitors and subsequently enhance monocytes/macrophages production (81). In tumor-bearing mice, throughout tumor development myeloid progenitors in the spleen proliferate. This suggests that Tumor-associated macrophages (TAM) can derive directly from spleen-borne myeloid cells. It has been identified that macrophage expansion in peripheral tissues is the determinant of macrophage accumulation and this is evident at sites of M2-polarized inflammation (82). However, accumulation of TAMs mostly relies on blood monocytes because differentiated macrophages have very limited proliferating potential factors of chemotaxis (chemotactic factors), especially CC family chemokines regulate monocyte recruitment in tumors. Another tumor-derived chemotactic factors which induce chemotaxis in blood monocytes to recruit them is chemokine CCL2 (83).

Other chemotactic factors, such as colony-stimulating factors (GM-CSF and M-CSF) and TGF-β and VEGF are secreted from stromal cells and tumor (84). Locally produced M-CSF in tumors results in monocytes differentiation to mature active macrophages. The secretion of monocyte colony-stimulating factor (M-CSF) especially in ovarian, breast, and endometrial cancer in human correlates with poor prognosis (85). The mononuclear phagocytes are multifunctional and capable of stimulating or suppressing immune responses or promoting or restraining inflammation (86). Response to local indications regulates mononuclear phagocyte's functional plasticity. Bacterial infection is a case in point, macrophages during bacterial infections primarily to eliminate invading pathogens set up the acute inflammatory response; after a while, they alter to tissue debris scavengers; then, they provoke healing proliferative phase by releasing a multiple and growth factors and cytokines. This cytokines and growth factors stimulate the fibroblast cells which ends to activation of fibroblasts, recruit them then make new vessels (86). Macrophage polarization to M2 and M1 macrophages which possess distinct characterizations shows this functional heterogeneity (87). Classically activated macrophages(M1) which stimulate by Th1 secreted cytokines (e.g., IFN-γ) and bacterial products are strong effector cells that secrete immunostimulating besides

inflammatory cytokines to provoke response of the adaptive immune, release reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) and possibly against transformed cells even have a cytotoxic activity. Alternatively activated macrophages best known as M2 type cells differentiate in tumor micromilieu that are rich in Th2 secreted cytokines (IL-13, IL-4); with their extraordinary scavenging activity and producing numerous growth factors which trigger tissue repair mechanism, they suppress adaptive immune responses (88). Monocytes differentiate into tumor-educated macrophages in the tumor microenvironment. Factors such as M-CSF, IL-10, TGF-β, IL-6, PGE2, and prostaglandins have the capability to polarize monocytes predominantly into M2-like macrophages that show immunosuppressive and pro-tumoral effectors. TAM in mouse molecular profiling studies, mainly expresses YM1, VEGF, arginase - I, MGL2, FIZZ1, and MMPs which are M2 macrophage hallmarks, also high IL-10 and TGFβ and low MHC II, IL-12 and RNI that are immunosuppressive phenotype specification. Reduction in cytotoxicity and antigen-presenting capacity correlate functionally with these findings. TAMs from ovarian cancer patients shows the similar characterizations (89). RNA profiling of human TAM showed several genes (scavenger, fibronectin, osteopontin, and mannose receptor) in TAM upregulated in in-vivo and in-vitro polarized M2 macrophages. The global profiling of TAM by the Principal Component Analysis showed extreme similarity with M2-polarized macrophages (90). Relying upon the location of the tumor (hypoxic vs. normoxic), the tumor type, stage of cancer (early vs. late), or other microenvironmental clues; TAM heterogeneity has become known(91). To define TAM subsets differential expression of particular markers especially MHC II, CD163, CD206, Ly6C, CCR2 or transcriptional profiling signatures have been used. Common factors which induce by M1 type cells such as IFN-inducible chemokines (e.g., CCL5, CXCL9, CXCL10, CXCL16) express on TAM from murine fibrosarcoma (92).

2.15. TAM's Pro-Tumor Functions

In cancer, TAM take part in cell proliferation, and tumor survival to invasion and eventually metastasis and modulate various features of tumor cell biology besides myeloid cells such as MDSC, and Tie2+ monocytes accompany them in

these steps. Former studies in macrophage-depleted animals have been showed that experimental generated tumors for studies possess gradual progression and dissemination (93). New researches revealed that tumor macrophages maintain the survival and persistence of cancer stem cells or tumor-initiating cells. This epiphany clearly influences on tumor evolution, development and resistance to therapy (94). Elongated protrusions in the cytoplasm of TAM for extended physical interaction with cancer cells demonstrated by in-vivo imaging experiments. Expression of growth and trophic factors (nourish factors) such as members of PDGF, FGF, EGF families have a direct effect on tumor proliferation. Tumor cells exploit, recruit and maintain macrophages sustainability by releasing monocyte colony-stimulating factor (M-CSF) and in interaction response TAM produces EGF in a paracrine. Upon ECM degradation active form of Matrix-bound growth factors are produced by TAM (95).

TAM produce IL-6 and TNF cytokines that display a major role in growth of tumor; IL-6 by activation of the STAT3 pathway induce the essential gene expression for progression of the cell cycle (e.g., PCNA and cyclin D) besides suppressing apoptosis by expressing of Bcl-2, Bcl-XL, and Mcl-1; In tumor cells, the transcription factor NF-κB is induced by TNF and therefore survival program become activated. Macrophages are the primary source of IL-6 in murine models of colon and pancreatic cancers (96). Cytokines, matrix proteins, coagulation factors, and enzymes which released by tumor cells via systemic circulation can achieve to distant sites and contrive the soil of a pre-metastatic niche. In these niches, macrophages and other recruited myeloid cells actively collaborate to the permanence of disseminating tumor cells in the site which is not suitable for them (97).

2.16. Matrix Remodeling, Tumor Invasion, and Metastases

Local stromal cells such as endothelial cells, leukocytes, and fibroblasts in normal tissues do not activate and the matrix roles to mix complex signaling among these constituents are quiescent. It retains several cellular processes such as gene expression, growth, differentiation, death, migration, and adhesion in accurate control; in the case of injury is pertinence to maintain homeostasis and manage

repairing of tissue (98). Especially in not well-differentiated carcinoma the stroma of tumor is characterized by a important demolish of the tissue structure and includes leukocytes, activated fibroblasts and abnormal vessels. Moreover, ultrastructural and immunohistochemical analyses showed not only an altered structure of some extra cellular matrix proteins (ECM proteins) (biglycan, alpha-smooth muscle actin, tenascin, decorin, fibronectin, and fibulin-1) but also the truncated protein isoforms emersion that generally do not express in normal cells. Multiple various ECM proteins generate and secrete by TAM. In the human tumors, up-regulation of several genes responsible for coding different proteins in matrix revealed by isolated TAMgene expression profile. Osteopontin, fibronectin, osteonactivin, several collagens, and a fibronectin truncated isoform is known as stimulation factor of migration are among these upregulated genes (90). Dependent on patient clinical outcome shifted ECM- related genes expression by stromal myeloid cells have been investigated(99). In hepatocellular carcinoma, the MMP7 and VEGF expression prognosticate the poor prognosis risk. The upregulation of metalloproteases (MMP1); TGF- related genes such as thrombospondin 1; stromal activation markers, for instance, fibroblast activation protein alpha (Fap-α); junction-mediating besides regulatory protein (JMY) characterize the conversion from pre-invasive lesions in stomach cancer to invasive form(100).

In the non-malignant fraction of diffuse large-B-cell lymphoma, expressing of ECM components in stroma enhanced and included: collagen, SPARC, fibronectin, and laminin isoforms. Macrophages secrete several proteolytic enzymes in addition to producing multiple ECM proteins. TAM considered as the main cell type which expresses protease enzymes activity in tumor tissues even if fibroblasts or neoplastic cells are potent producers of protease enzymes (101). Specific proteases degrade ECM proteins which can be clustered into significant categories and encompass not only hyaluronidases, cathepsins, ADAM proteases, and matrix metalloproteases (MMPs)but also urokinase-type plasminogen activator (uPA), elastase, plasmin, heparinase, and others (102).

In human ovarian carcinoma, TAM Gene profiling revealed that several proteolytic enzymes are among the high upregulated genes: lysosomal enzymes, ADAM proteases, MMPs (1, 9, 12 and 14), uPA, and cathepsins(B, C, L, and Z

types) (90). By altering stroma rigidity and removing the physical barriers among cells, proteolytic degradation facilitates neoplastic and endothelial migrating cells invasion. Invasion of cancer cell into the adjacent places critically needs interruption of focal adhesion kinase (FAK) and integrin-mediated anchorage. IL-4 stimulated TAM's cathepsin protease activities, promotes tumor dissemination (103). IL-4 is produced by CD4 T-cells which infiltrate the tumor, and rising evidence demonstrate its pertinence in the polarization of macrophage with pro-tumor functions. Matrix molecules degradation reveal available binding molecules which were concealed previously to cell surface receptors: such as, cleavage of collagen by MMP-2 reveals sites of integrin-binding that protect melanoma cells from apoptosis (104). Angiogenesis and tumor growth trigger by mysterious epitopes of fibronectin (104). The ability of ECM fragments in regulating innate immune cells multiple functions through the Toll-like receptors activation recognized over the last decade.Activation of TLR2 and TLR6 on TAM and expression of TNF and-6that are two cancer-related inflammation prototypic cytokines all trigger by versican (105). Inflammatory genes which are expressed in immune cells induced by hyaluronan fragments through the TLR2 and TLR4 also the CD44 receptor activation (106). The matrix also serves as a growth factor reservoir and FGF, EGF, and the members of TGFβ family, along with VEGF and PDGF bind to various ECM constituents and store in a passive form. Releasing active growth factors due to increased proteolytic activities, stimulate tumors as well as stromal cells (104). Angiogenic factor FGF-beta is released by Plasmin, MMPs, and heparinase besides MMP-3 breaks decorin and release active TGF-beta and MMP13 delivers VEGF (107). This process can also produce angiostatic active ECM fragments; the equilibrium between pro-and anti-angiogenic factors determines neo-angiogenesis. Tumor metastasis augmentation with TAMs and related expressed factors (TNF and IL-1) revealed since a long time (108).

The TAMs function in cancer cell invasion determined by multiphoton microscopy of experimental in vivo tumors. Wyckoff and colleagues by using fluorescently labeled cells demonstrate that tumor cell intravasation takes place alongside perivascular macrophages (95). After inducing of macrophage recruitment by M-CSF, SNAIL-expressing keratinocytes become locally invasive, and this

supports the idea of a mutual interaction between tumor cells and TAM (109). For their promoting effect on metastases in tumor-associated myeloid cells, TGFβ signaling is also essential. Less metastasis observed in a murine model which has a deletion in Tgfbr2 specifically in myeloid cells. Boosting the phenotype of immune suppressive and inhibiting adaptive immunity are consequences of TGFβ signaling. By paracrine production of TGF-β, TAM plays an appropriate role in tumor progression which is promoted by epithelial-mesenchymal transition(EMT). A positive correlation among intratumoral macrophages, levels of intraepithelial TGF-β, EMT markers and grade of tumor identified in the Immunohistochemical examination of cancer patients' NSCLC tumor samples (110).

2.17. Hypoxia and Angiogenesis

The consequence of an oxygen supply and consumption balance results in developing of tumor hypoxia in proliferating tumors. Hypoxia arises from falling pO2 levels. Thus the angiogenesis process elevates, and the new blood vessels generation support metabolic requirements of the tumor. The uncontrolled tumor growth which increases the risk of metastasis is a consequence of intratumor hypoxia and characterizations of malignant tumors (111). Regardless of oxygen availability and unlike normal cells which can adjust their metabolism to their environmental pO2, tumor cells constantly privilege glycolysis, this phenomenon is well-known as Warburg effect (112). The "aerobic glycolysis" is controlled by the type M2 pyruvate kinase isoenzyme (M2-PK) gene, which is an HIF-dependent gene. Moreover, oncogene-mediated and hypoxia-independent HIF-1 stabilization can explain the upregulation of M2-PK (113). The mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes transcription upregulate to react to the hypoxia levels in macrophages which preferentially accumulate in the tumor regions which poorly vascularized. For the recruitment and activation of TAMs into solid tumors, the pathway of hypoxia-inducible factor (HIF) seems to be essential and develop their pro-tumor functions. The positioning and function of TAMs, stromal cells, and tumor cells are regulated by the hypoxic induction of HIF-1α effect in TAMs which particularly up-regulates the chemokine receptor CXCR4 expression. The activation

of HIF-1 induces expression of the CXCL12/CXCR4 chemokine ligand; this chemokine plays a role in angiogenesis and as well in cancer metastasis (114).

When expression of HIF-inducible pro-angiogenic genes increased, TAMs adapt to hypoxia. These genes include basic fibroblast growth factor (βFGF), VEGF, and C-X-C motif chemokine ligand 8 (CXCL8), MMP-12, MMP-9, MMP-7, glycolytic enzymes, PDGF, TNF-α, and COX-2 (91). In tumor microenvironment, hypoxia directly affects the functions and accumulations of TAMs and myeloid cells population; myeloid cell includes the angiogenic monocytes which express the tunica internal endothelial kinase 2 (Tie2) and the population of heterogeneous MDSCs. Hypoxic parts of solid tumors are the main reservoir of Tie2+ monocytes; the hypoxia-inducible chemotactic factors such as the CXCL12/CXCR4 chemokine ligand and Angiopoietin-2protein (Ang-2) recruit Tie2+ type monocytes near nascent tumor vessels. Peptibodies (peptide-Fc fusions), monoclonal antibodies, or CovX Bodies which inhibit ANG2, produce strong antiangiogenic and antitumor responses in a diverse murine tumor models (115). In murine tumors, Myeloid-derived suppressor cells (MDSCs)produce high rates of matrix metalloproteases, especially MMP9. VEGF has sequestered in the ECM, and MMP9 increases its bioavailability. The MDSCs in the conditions such as the tumor microenvironments and proangiogenic cultures express the endothelial markers such as VEGF receptor 2 (VEGFR2) andCD31 and get the capability to join directly into endothelium of tumors. CD11b+GR1+ myeloid cells mediate resistance of the tumor to anti-VEGF therapy and can provide resistance to VEGF therapy of tumor by secretingBv8 proteins, also known as prokineticin 1 (Prok1); exposure to granulocytes colony-stimulating factor (G-CSF) -derived from a tumor cell, stimulates secretion of this proangiogenic cytokine (116). In TAM, hypoxia enhances the differentiation of MDSC mediated by HIF-1α and associate to their intratumor accumulation. Also, as observed in broncho alveolar carcinoma, hypoxia-induced expression of CXCL8 promotes neutrophils accumulation in tumor tissues, and in low oxygen conditions survival increased (117). In this regard, neutrophils accumulations in tumors is gaining relevance, as Fridlender and colleagues recently suggested that resident tumor-associated neutrophils (TANs) get phenotype of pro-tumor angiogenic, and TGFβ cytokine drives them to develop “N2 type polarized neutrophils” (118).

2.18. Immunosuppression

The main component of inflammatory reactions consists of myeloid cells, and recent studies put these cells at a notable point in the regulation of immune suppression which associate by the tumor. Myeloid progenitors in healthy evaluated individuals differentiate into granulocytes, mature macrophages, or dendritic cells, but in pathological conditions, such as cancers, they differentiate into MDSCs. The MDSCs detected in chronic infectious diseases, autoimmunity, and cancer. MDSCs accumulation was seen in primary and metastatic murine tumors, in the spleen, peripheral blood, and bone marrow. Also, MDSCs have been distinguished in the blood samples of a cancer patient. Myeloid-derived suppressor cells (MDSCs) known as a heterogeneous population, contain the granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). MDSCs common attributes are their immature state and the T-cell responses suppression capacity either in vitro and in vivo (119). MDSCs use various techniques for immune suppression including the production of ROS and TGFβ, depletion of cysteine and arginine which is mediated by Arg1 and iNOS, induction of Tregs and post-translational alterations of the T-cell receptor (TCR) conducted by peroxynitrite generation.Human MDSCs express the ordinary myeloid marker CD33, do not express the mature myeloid and the lymphoid cells common markers and able to suppress the activation of T-cell (120). The cell to cell contact is not required in M-MDSC-mediated immune suppression, However, upregulation of iNOS and Arg1, and production of immunosuppressive cytokines utilize in such immune suppression (119). The granulocytic myeloid-derived suppressor cells (G-MDSCs) can suppress antigen-specific responses utilizing mechanisms, include releasing ROS, which requires extended T cell and MDSC cell-cell contact (121).

A significant relation between TAMs and MDSCs exists. MDSCs can drive TAM polarization into tumor-promoting type-2 phenotype. The cell-cell contact is a requirement for cross-talk between macrophages and MDSCs, MDSCs by releasing IL-10 and affecting macrophages reduce secretion of IL-12. The MDSCs in tumor microenvironment which enriched by IL-1β secrete additional IL-10 amounts and are more efficient down-regulators of IL-12 cytokine which release by macrophages. Despite the fact of exhibitions both M1 and M2 macrophages characteristics,

MDSCs contribute with type-2 immune responses because they promote tumor activities (122). The partly M2-like phenotype of TAMs, distinguishable by an IL-12low/IL-10high phenotype observed in established tumors. Some studies reveal defective productions of IL-12 and autocrine IL-10 secretion. The multiple factors including IL-4, IL-6, IL-10, TGF-β1, PGE2 and CSF-1 produced by TAMs and this factors are responsible for suppressing the proliferations and the cytotoxicity of NK cells and T-cells (66). Moreover, production of VEGF and IL-10 by TAMs prevents the progress of dendritic cells maturation process and impair effective presentation of tumor antigens in these cells (66).

TAMs produce CCL2, CCL22, CCL18 chemokines and in the ascites fluid from human ovarian, the C-C motif chemokine ligand 18 (CCL18) recognized as the most abundant chemokine. The M2 cells might induce anergy in a peripheral microenvironment by promoting accumulation of naïve T-cells which attract to CCL18. In TAM and MDSC expression and production of the suppressive enzymes, arginase and iNOS enhance in hypoxia (123). Immune suppression may also affect TANs (118). Neutrophil depletion is associated with more intratumoral activated CD8+ T-cells and reduced growth of the tumor (118). Degranulation of neutrophils and arginase-1 release which is pre-stored in their granules may happen to apply their immunosuppressive properties (124).

2.19. Targeting Macrophages and Re-switching them

Specific clinical targeted therapies of macrophages are developing (6). Also, therapeutic approaches do not target the macrophage activations and polarizations directly. The main determining factor of sustaining macrophage counts at inflammation and immunity sites is recruitment. In particular members of the chemokines superfamily, CCL2/MCP-1, and growth factors interact with tyrosine kinase receptors, VEGF and CSF-1 and attract monocytes (80). In melanoma and acute myeloid leukemia models, inhibitors of CSF-1 receptor (c-fms) kinase show anti-angiogenic and anti-metastatic activity (125). Chemokines and CSF-1 are more powerful attractants than other attractants for monocytes; they also enhance macrophage functions toward M2-like. In breast and prostate cancer, the activity of antibodies against CCL2/CCR2 proved (126). In preclinical cancer models and

vascular pathology, bindarit (CCL2 inhibitor) inhibits monocyte recruitment. Antisense oligonucleotides and Anti-CSF-1 antibodies result in suppressing macrophage infiltration and growth in mice xenografts mammary tumors. The anti-angiogenic activity of VEGF may be responsible for diminishing macrophage recruitment (127).

In responses to neoadjuvant chemotherapy in breast cancer patients, the shifted combination in the immune microenvironment with the boosted percentage of infiltrating myeloid cells was found. Some studies suggest that during inflammation in the lung and peritoneum, in situ proliferation is the key determining factor of macrophage accumulation (128). IL-4 is responsible for preserving and supporting macrophage proliferation. Considering proliferation as a common dominant mechanism which preserves and promote macrophage accumulation, proposes unique methodologies to decrease macrophage numbers in situ and a diverse outlook for anti-CSF-1/CSF-1R targeted therapy strategies. Nevertheless, figuring the absolute concern of macrophage proliferation particularly in TH2-mediated inflammation in humans is a significant obstacle in this viewpoint. Remodeling and reshaping disarranged polarization of macrophage is a goal that is sought after for its great significance in macrophage therapeutic targeting therapies. Macrophages polarized phenotypes are reorientable in vivo and in vitro (129). To confirm this theory and in extensive clinical studies in patients diagnosed by ovarian cancer, IFNγ found to trigger the tumoricidal activity of TAMs which induces a phenotype switching with unambiguous confirmation of clinical responses (122). In pancreatic ductal adenocarcinoma model which is the most common form of pancreatic cancer, CD40 agonist antibodies enhanced an important antitumor effect and promoted high expression level of M1 type markers (MHC class II and CD86) in macrophages (130). For some various therapeutic agents, manipulation of macrophage functions is an inaccurate effect. Thiazolidinediones (PPAR-γ agonists) prescribed for a long time in the clinical treatment of diabetes. The evidence link peroxisome proliferator-activated receptor gamma (PPAR-γ) to M2 type polarization and therefore to the homeostatic roles of adipose-tissue macrophages (ATMs) illuminated their action mode. Based on clinical evidence PPAR-γ promotes M2-like phenotype polarization and function of metabolic homeostasis in ATMs, and this conversion is a crucial

pathogenic feature in diabetes (131). TLR ligands (imiquimod and CpG), trabectedin (132), zoledronic acid (also known as Zometa or zoledronate a therapeutic agent used for preventing recurrence of breast cancer bone metastasis) and statins (133) have been reported to affect macrophage polarization in other therapeutic studies. Therapeutic macrophage targeting is on the verge of clinical use; increasing confirmations demonstrate the recognition of molecules and mechanisms associated with polarized activation and macrophage plasticity. These confirmations provide a cornerstone for strategies that focus on macrophage diagnostic and therapeutic potentials.

2.20. Toll-Like Receptors

A member of Pattern Recognition Receptors family (PRRs) are Toll-like receptors (TLRs) which play a crucial function in immediate defense mechanism against invading pathogens and are responsible in reactions to endogenous warning signals. So far ten type of TLRs in human (TLR1-TLR10) and 12 type in the mouse (TLR1-9, TLR11-13) have been classified. The ligand specificity, expression patterns, cellular localization and signal transduction, are characteristics of TLRs. TLRs are a member of the type I transmembrane receptors family and compose section of the Toll/interleukin-1 receptor homology (TIR) domain superfamily which contains the IL-1 receptors (IL-1Rs) since their cytoplasmic regions share homology (134). TLRs ectodomains (extracellular regions) in contrast are extremely different. TLR ectodomains possess leucine-rich repeats (LRRs) which are leucine-rich regions tandem repeats, whereas IL-1Rs possess three immunoglobulin-like domains. To enable binding each TLR to a specific ligand, the composition of LRR side chains gives an exclusive combinatory code special for each TLR. Also, detection of ligand depends on the TLR cellular localization (135). The different cellular site of various TLRs and the exclusive combinatory code of their LRRs grant them the capability to cope with exogenous and endogenous ligands which are structurally different.

2.21. Localization of TLRs

TLRs localize on the plasma membrane which is cell surface or in the intracellular compartments. TLRs location is related to the origin of the ligands

which they recognize. TLR1, TLR2, TLR4, TLR5, and TLR6 mainly involved in the recognition of bacterial products in just extracellular space, and their expressions are on the plasma membrane. In comparison, the location of TLR3, TLR7, TLR8, and TLR9 are inside endocytic compartments of cells which nucleic acids of viral origin are presented to these TLRs (136). This sort of localization is furthermore necessary for the “self” and “non-self” discrimination. For instance, in comparison to most TLR ligands, the nucleic acids origin can be of self(endogenous) and foreign(exogenous). Relevant studies have shown that a chimeric TLR9 that consist a cytoplasmic and transmembrane domain of other TLRs localize in the cell plasma membrane (137). At this place TLR9can identify and reply to mammalian DNA but so far is unresponsive to nucleic acids from viral origins; this illustrates the significance of TLR unique locations. The endogenous TLR9 can only activate by viral DNA which is processed and acidified within cellular endosomes because it is not encountered with mammalian DNA. The activation of Intracellular TLRs in in-vitro trials needs the exert of cell-permeable ligands or ligands mixed through cationic lipids in the direction of aid and boost their uptaking process.

2.22. Signal transduction of TLRs

In cell-based assays, for the relevant reporter or readouts genes selection the science of the various signaling pathways triggered and generated by TLRs is imperative. Signaling of TLRs is started by the coupling of an exogenous or endogenous respective ligand to TLR. Pathogen-associated molecular patterns known as PAMPs recognize raiding microbes specifically. Danger-associated molecular patterns known as DAMPs are molecules which are consequent of damaged cells imputed to as endogenous molecules and also are detected by TLRs. As soon as PAMPs or DAMPs distinguished by TLRs, signaling pathways triggering occur and this will induces the chemokines, cytokines and costimulatory molecules upregulations.Subsequent to the coupling of a ligand, dimerization of two chains of TLR receptor starts the signal transduction. In TLR4, when the Myeloid differentiation protein 2(MD-2 is TLR4 coreceptor) attached to the lipid A segment of gram-negative bacteria Lipopolysaccharide (LPS),a homodimer is induced (138).

For structural studies, the crystal configurations of some TLR dimers as well asTLR3 (139–141), TLR2/1 (142) and TLR4 (143), TLR5 (144), TLR8 (145), and TLR10 (146)dimers have been explained. Combination of the two cytoplasmic receptor TIR domains leads by conformational transformations in the receptor after dimerization (147). By ligand binding, the general structure of the TLR cytoplasmic regions, transmembrane, and ectodomain, in turn, constitutes a molecular switch which is known as “turned-on” state. The combination of cytoplasmic TIR domains inTLR generates two uniform relevant coupling segments for the particular adaptor molecules which comprise TIR domains assignment (148). The complex of post-receptor signaling which associates pertinent adapter molecules to the active domains ofTIR in TLR dimers is the consequence of this process. Afterward, by recruitment of such adaptor signaling cascades are activated. These consist of TRIF/TICAM-1, MyD88, TRAM/TICAM-2, Mal/TIRAP, and SARM (135).

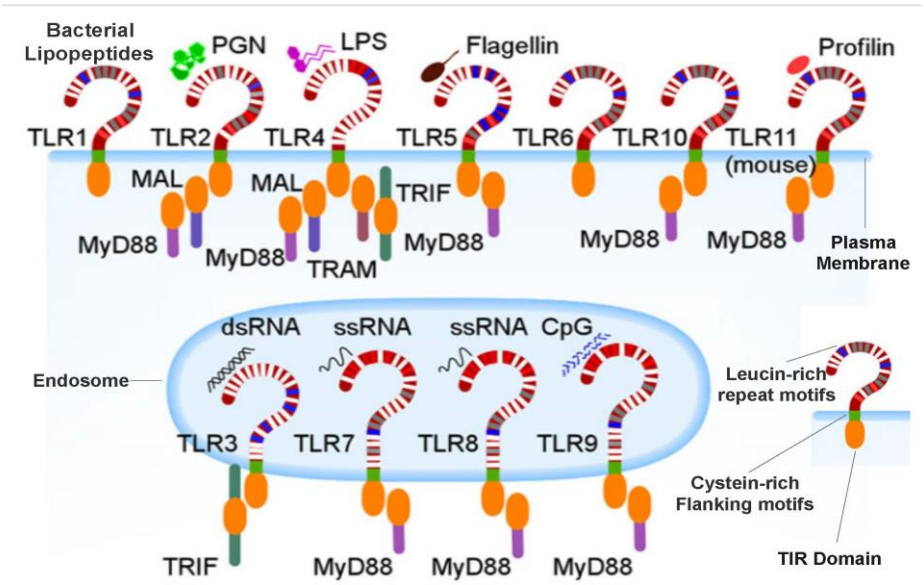

Figure 2.9. Structure, location, and specificities of mammalian TLRs (Adapted from cellular and molecular Immunology, 2012). Adopted and designed based on (135-138).

The activation of MyD88-dependent and MyD88-independent pathways which are two major signaling cascades is the consequence of the proximal events of binding ligands and recruitment of the adaptor molecule to the active TIRdomains of

TLRs. The MyD88-independent pathway induces the Type I interferons(IFNs) and interferon-inducible genes (is an Interferon-stimulated gene (ISG)) via interferon regulatory transcription factor (IRF), while the MyD88-dependent pathway outcomes to Nuclear Factor kappa B (NF-κB) translocation in addition to induction of proinflammatory cytokines(e.g., IRF3/7). Except for TLR3, all TLRs use MyD88 and trigger the MyD88-dependent pathway activation which exploits NF-κB and mitogen-activated protein kinases (MAPKs)(136). For activation of MyD88-dependent pathway, TLR4 and TLR2 require Mal/TIRAP along with MyD88 molecule(149). TLR3stimulate the endocytic compartments of IRFs and activate it through TRIF which ends to the expression of IFNs (150). To activate IRF3 molecule, TLR4 signaling activates both theMyD88-independent pathway by TRAM and TRIF. In the same state to activate NF-κB molecule, TLR4 signaling activates the MyD88-dependent pathway by MyD88 and Mal/TIRAP. Stimulation of pathways which directed by TRAM and Mal concurrently or inevitable when assignation of each adapter is reciprocally specializing TLR4 dimer signaling remains unclear (148). The statement that TLR4 signaling through TRAM and TRIF as well directs the final phase of NF-κB activation is particularly interesting (151). In TLR2 endosomal signaling, TRAM pays a part in the induction of Type I IFNs. TLR7, TLR8, and TLR9 signaling via MyD88 induce proinflammatory cytokine secretion as well as the IFNs. When high doses of CpG is present, the reaction of TRIF is taking part in TLR9-induced IFNs (152). To activate IFNs, the signaling proteins utilized by TLR7-8 not discovered except for MyD88.

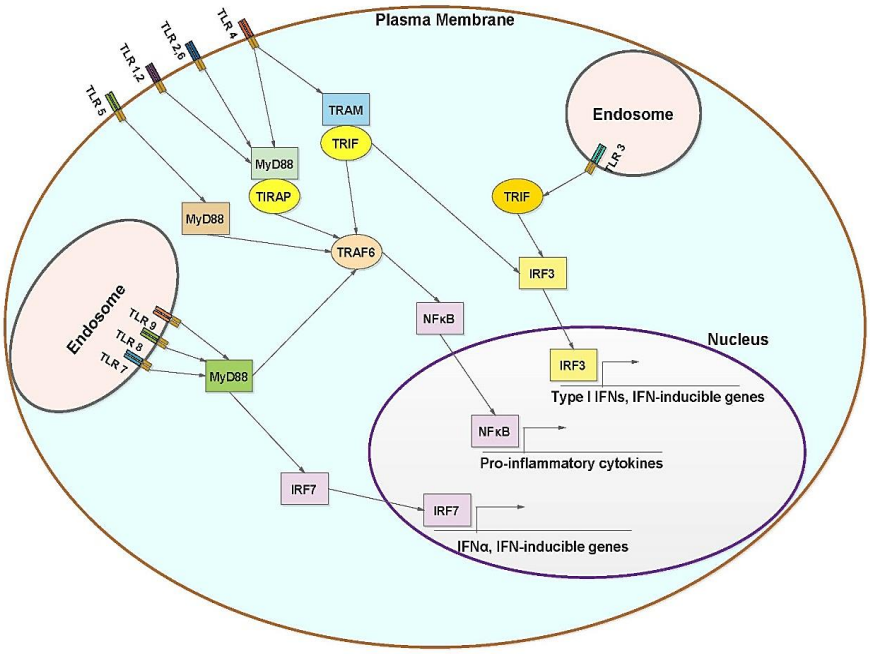

Figure 2.10. Mammalian TLR signaling pathways(Adapted from Nature Review, Immunology). Adopted and designed based on (135-176).

2.23. TLRs ligands

Understanding the ways of pathogenic ligands recognition by TLRs also their activation for designing therapeutic compounds which target this receptors family for inflammatory or chronic diseases seems to be necessary and essential. Microorganisms such as viruses, bacteria, fungi and various endogenous ligands can activate TLRs. Remarkably TLRs ligands are natural complexes and undefined, but research demand specific defined ligands. Also, novel synthetic TLR ligands and TLR inhibitors mostly for TLR7,8 and 9 are drawn up by several pharmaceutical companies. TLR ligands potential for induction of homodimerization or heterodimerization and receptor chains configuration changes determine the potency of TLR ligands (153). In several trails, direct coupling of various TLRs to specifically identified ligands has been proved for TLR1/2 (142), and TLR3 (139–141), TLR4 (143), TLR5 (144), TLR8 (145), TLR9 (154), and TLR13 (155).

2.24. TLR1

Functional heterodimers for TLR1 has been made by using TLR2. Heterodimers of TLR1/2 are triacyl lipopeptides receptors which present in mycobacteria and bacteria (156). In a simulated model, it has been showed that triggered signals via homodimers of TLR1 are weak which could be characterized by the TNF promoter activation (157). The Pam3CSK4 is a synthetic ligand for TLR1/2, and it is activation is at 10 ng/ml.

2.25. TLR2

TLR2 always tends to forms TLR2/6, TLR2/1heterodimers, and probably TLR2/10 to identify various microorganisms and It is hard to demonstrate specific ligand for it (158). In the absence of TLR1 or TLR6, gram-negative bacteria lipoproteins or mycoplasma lipoproteins (156), lipoteichoic acid, yeast cell wall Zymosan (159) and lipoarabinomannan from mycobacteria and bacteria (160) all can activate TLR2. Pam3CSK4, Heat-killed Listeria monocytogenes, high-mobility group box (HMGB) 1 (161), and peptidoglycan of Staphylococcus aureus when TLR1 and TLR6are not exist, can activate TLR2 in vitro (156). It has been shown that the cytosolic receptor NOD1 recognize peptidoglycan (162).

2.26. TLR3

TLR3 is localized in endosomes and is one of intracellular TLRs; it is specified to binds to viral dsRNA. Also, polyriboinosinic-polyribocytidylic acid (poly I:C) and polyadenylic-polyuridylic acid (polyA:U) which are two synthetic TLR3 ligands mimic dsRNA (163). TLR3 could be activated by both high molecular weight (HMW; 1–1.5 kb) and low molecular weight (LMW; 0.2–1 kb) fragments of Poly I:C. The large fragments of Poly I:C are more influential than the low molecular weight fragments. In mouse bone marrow-derived dendritic cells, macrophages, and B cells it has been revealed that Polyinosinic acid (poly I) can activate TLR3.It should be noted that TLR3 ligands at 10–50 μg/ml concentration in the medium are active. TLR3 ligands admixture with a transfection reagent which is lipid-based ends to a concentration with lower effectiveness, but this delivery system independently of

TLR3 can trigger cytoplasmic receptors activation such as cytoplasmic RNA sensors MDA-5. Activation of TLR3 occurs if both poly I:C HMW and LMW directly add to the cultures of human neuroblastoma cell line (CHP212) and primary macrophages(164). However, to induce activation of TLR3 in other cell-based assays the transfection of poly I:C was necessary. Methods of delivery, size of the ligands and cell types all are various factors which influence the activation efficiency of TLR3by poly I:C.

2.27. TLR4

TLR4 is one of the most studied TLRs which initially found in mammals. TLR4 firstly considered as lipopolysaccharide(LPS) receptor, but future studies revealed that TLR4 requires co-receptors such as MD2, CD14, and LPS binding protein (LBP) to function. Polysaccharidemoiety and the active component lipid A are parts of LPS. Lipid A consists of a glucosamine disaccharide (the backbone of lipid A) bound to residues of fatty acids. The number of fatty acids determines the potency to activate TLR4. Lipid A in less pathogenic strains of bacteria such as Rhodobacter sphaeroides, mutated strains of E. coli, and P. gingivalis contains 4 or 5 residues of fatty acids whereas, in pathogenic strains of bacteria such as E. Coli, Salmonella species lipid A contains six fatty acids residues. Because of the potential for inhibiting the TLR4 activation induced by Hexa acylatedLPS the first type LPS are considered as antagonists (165). By application of a 100X more antagonistic LPS derived from Rhodobacter sphaeroides, the full competitive inhibitory effect on LPS activity become possible (available from InvivoGen).

Based on the morphology of bacteria colonies: smooth or rough, agonistic LPS are divided into two categories S-LPS (denoted for smooth) or R-LPS (denoted for rough), respectively. O-polysaccharide residues in S-LPS are not present in R-LPS. S-LPs are synthesized by wild-type Gram-negative bacteria and need CD14 to signal through TLR4. S-LPS signaling via TLR4/CD14 triggers signal transduction from MyD88-dependent and MyD88-independent pathways simultaneously. In the absence of CD14, the activation of the MyD88 independent pathway does not happen, but R-LPS continue signaling. Such TLR4 activation is incomplete, but this attribute might be beneficial in the absence of TLR4 coreceptors for in vitro

experiments. Monophospholipid A which is a vaccine adjuvant is the best choice in TLR4 signaling to stimulate just TRIF/TRAM arm of it(166). This ligand is an encouraging tool for MyD88-independent response study which TLR4 activation induced it. Activation of TLR2 and TLR4 have been seen since it's purification protocols is simple that lead to lipoproteins which contain LPS. To maintain only a TLR4 activity we require LPS Phenol re-extraction. Several strains of bacteria produce LPS and their purified LPS showed activity at 10 ng/ml concentration. Notably, mouse TLR4 (but not human) discerns the paclitaxel which is an antitumor chemotherapy medication for treating a number of cancer types (167). TLR4 is also activated by viral origin proteins, for instance, mouse mammary tumor virus and the respiratory syncytial virus are among such proteins (158). In an MD2-independent and CD14-dependent manner, it has been shown that heme could activates TLR4. Endogenous ligands are also recognized by TLR4; these comprise fibrinogen, hyaluronate, and HMGB1. After tissue injury, these substances are released and through TLR4. Possessing the potency for triggering a danger signal in such substances, the TLR4 activation is plausible. Activation of TLR4 as a result of heat shock proteins (HSPs) stimulation is argumentative and the probability of contamination with endotoxin which ends to such activation has been shown by some authors (168).

2.28. TLR5

Monomeric flagellin that is a constituent protein of bacterial flagella is recognized by TLR5 (169). Bacillussubtilisis or Salmonella typhimurium purified flagellins are commercially available for trial studies (InvivoGen, Bio-Techne). Recombinant Salmonella typhimurium flagellin (FliC) produced in mammalian cells that unlike bacterial origin unable to trigger TLR2/4 activity (InvivoGen). The flagellins showed activity at 0.1–1 μg/ml concentrations.

2.29. TLR6

A member of lipopeptide family known as diacyl lipopeptides such as FSL-1 and MALP-2 are recognized by TLR6 in association with TLR2. A synthetic ligand for TLR2/6 namely Pam2CSK4 at 1–10 ng/ml concentrations could trigger TLR6-

expressing cells activation. By the addition of CD14 and CD36 to medium, the level of activation elevates. For TLR2/6 activation, besides commercially available standard bacterial cell wall components, fully prepared heat-killed Gram-negative and Gram-positive bacteria are available (InvivoGen). TLR ligands in the absence of TLR2seem unable to activate TLR6. However, theoretically signaling via homodimers of TLR6 is conceivable by artificial stimulation of a TLR6-specific signaling cascade (158).

2.30. TLR7

TLR7 is viral ssRNA receptor which expresses in endosomes, particularly U or GU-rich oligoribonucleotides (ORNs) (e.g., RNA40 [ORN] derived from the HIV-1 U5 region). Using complexed single-stranded RNAs(ssRNAs) murine TLR7 can be activated while human TLR7 didn't show any activity. TLR7 could recognize Imidazoquinolines (a tricyclic organic molecule), which are artificial compounds and synthetic by their antiviral specifications, encompassing resiquimod (R848) and its relevantCL097 which is a water-soluble descendant (InvivoGen), gardiquimod also imiquimod (when used at less than 1 μg/ ml are active) (170). Furthermore, TLR7 is triggered explicitly by analogs of nucleoside (e.g., ≈1 mM loxoribine which is cognate and analog of guanosine), and the analogs of adenine (CL264 and CL307 from InvivoGen). Also, siRNA is able to activate TLR7 precisely. A motif with the length of nine-base inside a 19-mer sequence is responsible for this activity. Self-RNA and RNA-specific antibodies form a complex and such immune complexes (ICX) can activate TLR7; eventually, this takes part in the appearance and development of autoimmune diseases. Thus, ICX purified small nuclear ribonucleoproteins could be used as TLR7 ligands (171).

2.31. TLR8

Due to the high homology with TLR7 sequences, most of the TLR7 ligands could be recognized by TLR8, and human TLR8 could be activated by ssRNAs. Using different nucleotide analogs concentrations makes it conceivable to distinguish between TLR7 and TLR8 activity. A fantastic instance of this is 3M-002 (a thiazoloquinolone derivative) produce by 3M Pharmaceuticals which vended as

CL075 by InvivoGen and when used at 0.1 μg/ml equals to 0.4 μM can activate human TLR8specifically whereas to activate TLR7higher concentrations of this ligand should be used. At first, TLR8 activation was thought to be just in human, but a mixture of ~10 μM 3M-002 and 3M-003 in combination with ~1–3 μM poly T oligodeoxyribonucleotide (ODN) showed the ability to activate mouse TLR8 (172).

2.32. TLR9

TLR9 is an intracellular TLR that recognize viral and bacterial DNA besides self-DNA in the immune complex (ICX)(137). The unmethylated CpG motifs existence make nonself-DNA detectable. To identify CpG DNA motifs which have immunostimulatory effects; colossal research has been done and lastly ended to describe of two major classes have. Described classes named alphabetically to A and B. The Class-A motifs that optimally stimulate plasmacytoid dendritic cells (pDC). Such molecules on a mixed phosphorothioate/phosphodiester backbone involve a palindromic DNA sequence and polyG DNA motifs and can configure large arrangements by multimerization. The second class is Class-B motifs can activate B cells and involve one or more CpG DNA, but they lack polyG DNA motifs on a phosphorothioate backbone. Another class that participates class-A and class-B properties and characteristics are known as class C motifs. TLR9+ cells activation is usually done by adding CpG ODNs (CpG oligodeoxynucleotides) to the culture medium despite the fact of TLR9 endosomal location, and that is why understanding the activation mechanism by CpG is challenging. Interaction of Class A CpG with HMGB1 make a complex and Internalization of this complex is effectively done by RAGE mechanism that includes the distinctive receptors for following glycation end products (173). It is believed that the RAGE-dependent mechanism eases class-A CpG delivery to TLR9. For in vitro immunostimulatory the optimal necessary concentration ofCpG ODN is ~1 μM and the concentrations above this usually show less efficiency. Species specificities in some CpG ODNs have been reported (in human and mouse). Activationspecificity of CpG ODN could be definite by using control CpG ODN including the identical sequences as immunostimulatory CpG ODN in whatever place CpG dinucleotides is substituted by GpC dinucleotides. For instance, at 50 μg/ml E. coli DNA which is purified and is endotoxin-free able to

trigger TLR9 signaling and could be used as a ligand. Isolated repetitive extragenic palindromics (REPs) that are natural CpG sequences from Gram-negative bacteria which exhibit the ability to activate innate immune responses through TLR9 signaling. Through a mechanism that involves the cell surface receptor FcγR (CD32), immune complexes derived from SLE patients serum could trigger TLR9 activation. TLR9-expressing cells could be activated in vitro by patients serum.Also, immunostimulatory ODNs acting via TLR9, also ODNs with inhibitory effects have been recognized. These inhibitory types bind to TLR9 but are not capable of inducing the switch to an active form of TLR9. The inhibitory ODNs arrest signaling and compete with immunostimulatory ODNs for the TLR9binding site possession. Heretofore two varieties of inhibitory ODNs is recognized: the first is recurring TTAGGG motifs that could be found in telomeres and the second is ODN which contains either methylated CG or unmethylated GC. Hemozoin which is a TLR9 ligand from Plasmodium falciparum help to bring parasite DNA near TLR9 to produce a reaction (174).

2.33. TLR10

Expressing on B cells, TLR10 is closely linked to TLR1 and TLR6 respectively with the identity of 48%, and 46 % amino acid sequence besides it probably could interact with TLR2 because of its characteristics. Up to date, there is no known ligand for TLR10. The similarity of the binding positioning of a human TLR2-TLR10 heterodimer with TLR2/1 and TLR2/6 have been shown in recent studies by using of homology modeling.Furthermore, the study revealed that Pam3CSK4 as a ligand might activate human TLR10/2 complex and human TLR10 homodimer and TLR10/1 heterodimer may be activated by PamCysPamSK4 (175). By using chimeric CD4TLR10, promoters could be stimulated and initiated through signaling via TLR10 include NF-κB, IL-4, CXCL5 and on a smaller scale TNF, AP-1 and IL-2(157). TLR10 with its suppressive effect acts as an inhibitory receptor. Using specific blocker antibodies to TLR10 blockade, upregulation of cytokine production mediated by TLR2 significantly has been shown (176).

Table 2.1. TLRs expression in different immune cells.

TLR	Immune Cell Expression	PAMPs	DAMPs	Signal Adaptor	Production
TLR1+ TLR2	Cell surface Mo, MΦ, DC, B	Triacylated lipoproteins (Pam3CSK4), Peptidoglycans, Lipopolysaccharides	(TLR2 DAMPs listed below)	TIRAP, MyD88, Mal	IC
TLR2+ TLR6	Cell surface Mo, MΦ, MC, B	Diacylated lipoproteins (FSL-1)	Heat Shock Proteins (HSP 60, 70, Gp96), High mobility group proteins (HMGB1), Proteoglycans (Versican, Hyaluronic Acid fragments)	TIRAP, MyD88, Mal	IC
TLR3	Endosomes B, T, NK, DC	dsRNA (poly (I:C)), tRNA, siRNA	mRNA, tRNA	TRIF	IC, type1 IFN
TLR4	Cell surface/ endosomes Mo, MΦ, DC, MC, IE	Lipopolysaccharides (LPS), Paclitaxel	Heat Shock Proteins (HSP 60, 70, 72, Gp96), High mobility group proteins (HMGB1), Proteoglycans (Versican, Hyaluronic Acid fragments), Fibronectin, Tenascin-C	TRAM, TRIF, TIRAP, MyD88, Mal	IC, type1 IFN
TLR5	Cell surface Mo, MΦ, DC, IE	Flagellin		MyD88	IC
TLR7	Endosomes Mo, MΦ, DC. B	ssRNA, Imidazoquinolines (R848), Guanosine analogs (Loxoribine)	ssRNA	MyD88	IC, type1 IFN
TLR8	Endosomes Mo, MΦ, DC, MC	ssRNA, Imidazoquinolines (R848)	ssRNA	MyD88	IC, type1 IFN
TLR9	Endosomes Mo, MΦ, DC, B,T	CpG DNA, CpG ODNs	Chromatin IgG complex	MyD88	IC, type1 IFN
TLR10	Endosomes Mo, MΦ, DC	profilin-like proteins		MyD88	IC

Table 2.2. TLRs expression in different cancer cells(Adapted from Yusuke Sato et al. 2009).

TLRs	Natural Ligand-origin	Expressing Cancer Cells and Tissues
TLR1	Bacterial Triacyl lipoproteins and Triacyl Lipopeptides	Colorectal cancer,
TLR2	Peptidoglycan, Lipoprotein, Lipoteichoic acid, Zymosan	Gastric cancer, Colorectal cancer, Ovarian cancer, Lung cancer, Melanoma, Brain cancer, Breast cancer, Hepatocellular carcinoma, Laryngeal cancer,
TLR3	Viral dsRNA	Colorectal cancer, Ovarian cancer, Cervical cancer, Lung cancer, Melanoma, Breast cancer,Hepatocellular carcinoma, Laryngeal cancer,
TLR4	Heat shock proteins 60/70, Lipopolysaccharides, RSV fusion protein, MMTV envelope proteins, Paclitaxel	Gastric cancer, Colorectal cancer, Ovarian cancer, Cervical cancer, Lung cancer, Prostate cancer, Melanoma, Brain cancer, Breast cancer, Hepatocellular carcinoma, Laryngeal cancer,
TLR5	Flagellin	Gastric cancer, Colorectal cancer, Ovarian cancer, Cervical cancer,
TLR6	Lipoteichoic acid, Triacyl lipoproteins, Zymosan	Hepatocellular carcinoma,
TLR7	viral ssRNA	Chronic lymphocytic leukemia
TLR8	viral ssRNA	
TLR9	CpG-containing Bacterial and viral DNA	Gastric cancer, Colorectal cancer, Cervical cancer, Lung cancer, Prostate cancer, Breast cancer, Hepatocellular carcinoma,
TLR10	Unkown	

2.34. Toll-Like Receptors and Cancer

By ability of PAMPs and DAMPs recognization, Toll-Like Receptors are the cornerstone of innate immunity. The TLRs are not restricted just to immune cells, expression of these receptors occurs almost all over, from epithelial to immune

competent cells. New studies showed the expression of functional TLRs on cancer cells which implicate roles of TLRs in tumor destiny. Coalescence of TLRs leads to TLR signaling, several adapters proteins and downstream kinases initiate signaling which causes anti-inflammatory and pro-inflammatory mediators and several cytokines production. TLRs keeps host homeostasis by triggering innate and adaptive immune responses against the pathogens and tumor cells (177). By their ability to enhance antigen presentation and induction of the immune response against the tumor, TLRs are utilized as potential targets against infectious disease and in vaccine therapy and treatment of cancer. However, some studies indicate adverse effects of TLRs on tumor cells. In tumor cells, uncontrolled signaling of the TLRs supplies a microenvironment which is a prerequisite to proliferate and escape from the host immune system. In neoplastic diseases, based on evidence TLRs are act similar to a double-edged sword. Under diverse conditions, they could have pro-apoptotic and pro-survival effects (177).

2.35. TLRs as Negative Regulators of Cancer: (Anti-tumor Effects)

The first person who observed the positive effect of microbes on the tumor regression was the Deidier at the beginning of the 18th century. Afterward William Coley at the end of the 19th century realized that repeated injections of a bacterial toxins mixture from the Streptococcus pneumoniae (Gram-positive bacteria) and Serratia marcescens (Gram-negative bacteria) have an anti-tumor effect which, later known as Coley's toxin, to successfully treat patients with cancer (178). These results showed the anti-tumor effect of microbial products. Shear and Turnerc in 1943 found that the Coley's toxin antitumor effect was due to lipopolysaccharides (LPS) and we currently know that the LPS is a constant component of the gram-negative bacterias' outer membrane and stimulate TLR4. Other microbe-derived therapeutic agents are able to activate TLRs and can have an anti-tumor effect. OK-432 which is a lyophilized preparation of group A streptococcus utilized in the treatment of oral, gastric and cervical squamous cell carcinoma recently was shown to stimulate TLR4 (179). One of the potent activators of TLR2- and TLR4-dependent signaling is Mycobacterium bovisbacillus Calmette–Guérin (BCG) that has been used as an effective treatment of bladder cancer by the intravesicular injection of

these mycobacteria. Either in mice and humans, application of TLRs ligands against established tumors in the both local (at the site of the tumor) and systemic delivery has been shown to have potent anti-cancer effects.

In phase II clinical trials, systemic administration of LPS for colorectal and lung cancer treatment when injected directly into adoptively transferred tumors cause to tumor regression. Flagellin application is another similar example. Local applications of imiquimod (ligands for TLR7/8) used for skin cancer and may also be beneficial for systematic utilization in chronic lymphocytic leukemia. CpG is the TLR9 activator ligand which is for the treatment of skin, brain, lymphoma, renal and lung cancer and is the most studied and promising TLR ligand. TLR agonists may conduct their anti-tumor activity by a different mechanism. For example, poly (IC) which is the TLR3 agonist via TRIF adaptor protein can cause apoptosis tumor cells and their microenvironment cells like vascular endothelium by increasing vascular permeability that mediated by TNF (179). Activation of TLRs may also mediate recruiting leukocytes like macrophages by tumors which leading to tumor cells lysis by cytotoxic T-cells and NK cells. Activated TLR enhances the tumor regression via MyD88 and TRAIL(pro-apoptosis) (TLR4, 2) pathways, granzyme B/perforin and TNF.

Accumulating evidence suggests, TLRs in cancer therapy can be caused to adaptive immune system stimulation, and the immune system became sensitive against tumor self-antigens, and co-stimulatory signals are up-regulated to the adaptive immune response, a property is known as an adjuvant. Nevertheless, often under the physiological circumstances, TLR activation has a pro-tumorigenic effect. Although there is some evidence which emerged in mice with TLR4-MyD88 deficiency, the chemotherapeutic agent's ability in killing cancer are decreased. Died tumor cells with chemotherapeutic agents release HMGB1 that can bind to TLR4 and this will induce T-cell immunity against the tumor (179).

2.36. Lung Cancer

Lung cancer is one of the main causes of death worldwide. This type of cancer is non-immunogenic and immune surveillance-resistant. Lung cancer

representing 28% of all cancer deaths and is the leading cause of death even more than pancreatic, breast, prostate, and colorectal combine. Annually in the United States alone, more than 170,000 new cases of lung cancer are diagnosed, and among them, more than 90% of the cases will pass away. Lung cancer similar to many other cancers initiates by oncogenes activation or tumor suppressor genes inactivation (180). In 10–30% of lung adenocarcinomas K-ras proto-oncogene mutations occur and in small-cell lung carcinoma in 60-75% of cases amplification and mutations of EGFR, and p53 tumor suppressor gene mutation commonly occur in lung carcinomas. PIK3CA, c-MET, NKX2-1, BRAF, and LKB1 are other genes that often mutated or amplified.

Besides the advances and improvements in the clinical treatment of lung cancer, the prognosis did not progress as well as treatment. Many pieces of evidence proved that smoking has the direct relation with lung cancer (181). The primary reason for about 85% of the lung cancer incidence is tobacco Smoking. There are other carcinogen factors which are responsible for the remaining 15% of lung cancer, such as radon or asbestos and genetic factors that yet are unknown. Risk of lung cancer remarkably reduces in people who quit smoking before the age 30 rather than people at age 50 or 60 who quit smoking. Recent studies on smokers show off multiple genetic lesions in clonal patches of epithelium cells (182). Attempts for classification of Lung cancer leads to distinct it into two major types according to histological type: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). The NSCLC encompasses about 80% of lung cancers reported cases and can be subdivided into three subtypes: Squamous cell, Adenocarcinomas, Large-cell carcinomas. The most critical subgroups are Squamous cell carcinomas and adenocarcinomas (183). SCLC that are the remaining 20% of lung cancers diagnosed cases amazingly show properties of neuroendocrine cells.

Both large-cell neuroendocrine carcinomas and SCLC have the very high potential for proliferative and metastatic characteristics. NSCLC and SCLC have different characteristics which refer to their distinct genetic pattern. Also, have different responses to treatment with chemotherapy and radiation. For lung cancer, the current overall 5-yr survival rate is ~ 9%. The survival rates in developed countries are higher than developing countries (15% vs. 9%) (183,184).

2.37. Lung Cancer and TLRs

TLRs are expressed on infiltrating cells and resident lung cells which are originated from myeloid and lymphoid. According to some studies, cancer cells which could evade the immune system can be stimulated by activated TLRs which are expressed on tumor cells, and immune system activation could mediate tumor regression. However, other studies revealed that TLRs act like two edge sword. It means that although TLRs activation can have an anti-tumor effect, other studies demonstrate that TLRs possess a pro-tumor effect on cancer cells and mention as an essential regulator of tumor biology (177,184). In the lung cancer TLR2, TLR4, and TLR9 activation have pro-tumor activity. TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 highly express by pulmonary epithelial cells. In lung carcinoma TLR2, TLR4, TLR7/8, and TLR9 are overexpressed compared with the normal lung (184). Toll-like receptors are widely expressed on immune cells as mentioned before and have an essential role in determining the microenvironment of the tumor and its progression or regression (185). During lung cancer, the interaction between the stromal and the hematopoietic cell lineage is critical because TLRs activation can recruit both tolerogenic and immuno-stimulant cells to the lung. Activation of the TLRs on lung epithelial cells increase the chemokines production, like CXCL-8 (IL-8), (neutrophil chemoattractant), and the growth factors release, such as vascular endothelium-derived growth factor (VEGF) which cause to angiogenesis and finally damaged lung epithelium (184). Likewise, activation of the TLRs on innate immune system cells, such as Antigen-Presenting Cells (APCs) can stimulate the activation of adaptive immune system, by antigen processing and presentation enhancement, increasing co-stimulatory molecules such as CD80/86 expression which are required for activation of CD4+ T-cell, and regulation of T regulatory cells (Treg) activity by secreting IL-6. Pro-Inflammatory cytokines produced by TLR could lead to polarization of the adaptive immunity. For example, TLR produces IL-12p19 or IL-12p35 and IL-27, which respectively boost Th1 or Th17 immunity (184). In lung carcinoma, activation of Th1, cause an antitumor immune response. However, TLR-mediated Th17 immunity depends on the tumor model (in mice), and the tissue or organ can have both anti-tumor or pro-tumor effects, in lung carcinoma, Th17 immunity has anti-tumor activity.

In the lung, TLRs activation on the stromal and immune cells cause to inflammatory mediators secretion, which as a result these mediators determine the tumor-microenvironment that could be led to tumorigenesis (177). In contrast, the activity of the Th2 downregulates the host response and cause to chronic inflammation establishment which leads to progression of a tumor in the lung. Also, T regulatory (Treg) cells and other immunosuppressive cells can cause tumor immune escape in the lung. Increasing evidence in several studies in tumor immunology has indicated that TLRs activation could be a crucial decider for the destiny of cancerous cells in the lung to the involvement of TLRs. In cancer the exact role of TLRs is not clear yet, they have two contrary effects (Figure 2.11.). They can either stimulate the immune system against cancerous cells or by augmenting chronic inflammatory responses evoke carcinogenesis. Some effects of activated TLRs on the immune cells and stromal cells are indicated here: in lung cancer, TLR2 and 4 activations on lung-derived DCs cause to Th1 or Th2 immune responses which is co-related with a dose of PAMPs inhaled. TLR3 activation by Poly I:C on mDCs facilitated NK cell-mediated cytotoxicity, it is also implicated that TLR3 activation via TRIF pathway can lead to the apoptosis (184). In lung cancer, activation of TLR7 and 9 on B cells give B cells both pro-tumor and anti-tumor effect: B cells countered T-cell, transform to regulatory B cell which mediates tumor cell growth. CpG which is the TLR9 ligand leads B cells toward a B1 phenotype and cause to tumor regression (186).

Activation of TLR2, TLR4, and TLR9 on stromal cells determine the fate of immune cell phenotype. Depends on the anti-inflammatory or pro-inflammatory cytokines they produce, can ease lung tumor progression by inducing the release of both extracellular matrix proteins and immune suppressive cytokines, such as IL-10 and TGFβ and growth factors (e.g., EGF, VEGF, and FGF2) (187).

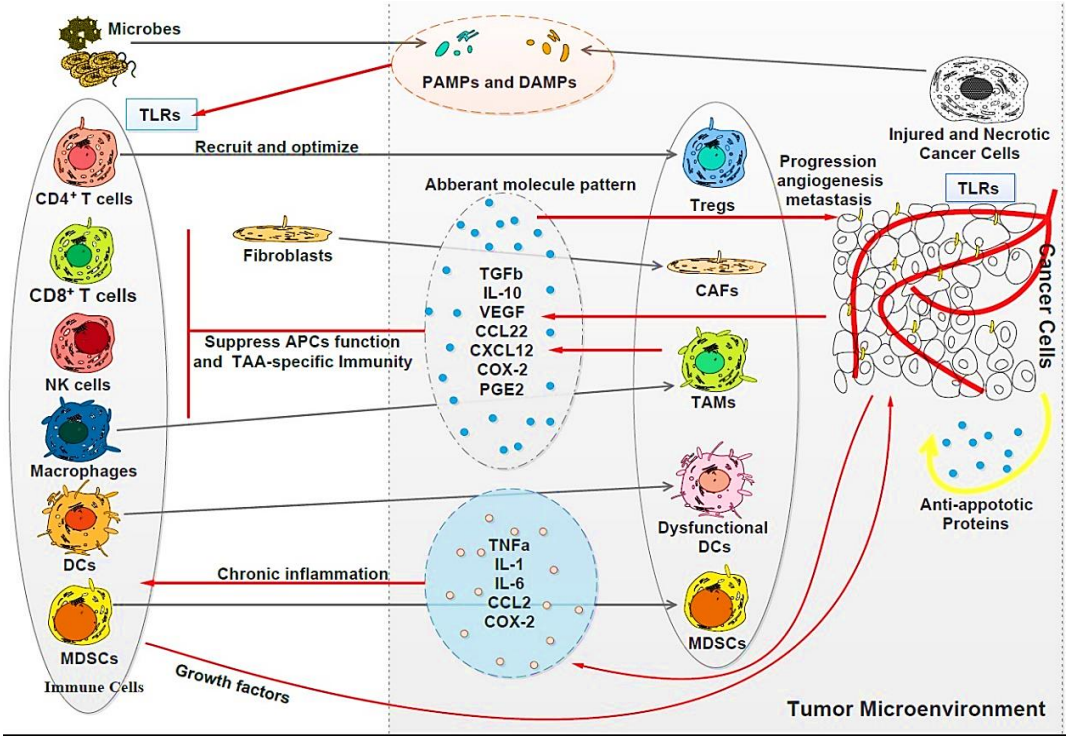

Figure 2.11. In the tumor microenvironment, TLR signals contribute to tumor progression. Adopted and designed based on (177-187).

In lung carcinoma, the role of TLR3 and TLR7 is not clear yet. Mast cells are highly represented in this type of cancer, and when activated by TLR2 and IL-6 antitumor activities promotes CD8+ Cytotoxic T-cell and NK cell recruitment into the tumor. Although in mast cell, IL-6 can enhance the STAT-3 pro-tumor activity and TLR2 is also crucial for Treg expansion. In lung cancer, activation of TLR2 on myeloid-derived suppressor cells (MDSCs) leads to tumor immune evasion and administration of CpG-ODN (TLR9 ligand) recruits MDSC with Treg to the lung tumor which leads to tumor progression (184,186,187).

3. MATERIALS AND METHODS

This study has been done in Hacettepe University Cancer Institute, Department of Basic Oncology Laboratories through November 2016- June 2017.

3.1. Materials

3.1.1. Cell culture materials used in this study

Cell Lines
NCI-H82 (Small Cell Lung Cancer Cells, ATCC; USA)
THP-1(Acute Monoblastic Leukemia Cells, ATCC; USA)

3.1.2. Cell Culture Medium

Fetal Bovine Serum (FBS, Biochrom, Deutschland)
L-Glutamine (Biochrom, Deutschland)
Penicillin-Streptomycin (Biochrom, Deutschland)
RPMI-1640 (Sigma, R-0883)
Phorbol Myristate Acetate (PMA P 8139 Sigma-Aldrich, USA)

3.1.3. Flow Cytometry materials used in this study

Monoclonal Antibodies

CD68 PE (Catalog no:333806, Clone Y1/82A BioLegend)
CD11bPE (Catalog no:101208, Clone M1/70 BioLegend)
CD206 FITC (Catalog no:321106, Clone 15-2 BioLegend)
HLA-DR PE (Catalog no:307606, Clone L243 BioLegend)
CXCR2 PE (Catalog no:320706, Clone 5P8/CXCR2 BioLegend)
CXCR7 FITC (Catalog no:331104, Clone 8F1-M1b BioLegend)

3.1.3.2. Isotype Controls

MsIgG1 FITC (BioLegend)
MsIgG1PE(BioLegend)

3.1.4. Phagocytosis Kit

Cayman Chemical Phagocytosis Assay Kit (IgGFITC) (Catalog no:500290, USA)

3.1.5. Cell Cycle Analysis Reagent

Propidium Iodide (Sigma, P-4170)
RNase (Sigma, R-4875)
Phosphate Buffer Solution (Sigma)

3.1.6. TLR Ligands

TLR3 agonist- (poly(I:CL), InvivoGen,USA),
TLR5 agonist- (Purified Flagellin B subtilis, InvivoGen,USA)
TLR8 agonist- (ssRNA40, InvivoGen,USA)

TLR induced Cytokines ELISA kit
Multi-Analyte ELISArray ELISA Kit (Qiagen,www.Qiagen.com)

3.2. Methods

3.2.1. Cell Culture

In this study, we used THP-1 human monocytic cell lines and the NCI-H82 Small Cell Lung Cancer (SCLC) cell line. These cell lines has obtained from the cell lines collection of Basic Oncology Department. Characteristics of NCI-H82 cells are cells that are growing in aggregate forms in suspension, THP-1 cells are an adherent cell line. Cells were resuspended in RPMI 1640 medium containing 10% FBS, 1%

Penicillin-Streptomycin and 1% L-Glutamine as a culture medium and are incubated in an incubator with 5% CO 2 and 37 ° C. Since THP-1 cells are adherent cells, the experiments carried out by removing cells from culture flasks with trypsin EDTA and transferring into the test tubes.

3.2.2. Activation of Cells with TLR Agonists

NCI-H82 cells were incubated with TLR3 agonist poly-ICL, TLR5 agonist flagellin, and TLR8 agonist ssRNA40 / LyoVec for 48 hours. Also, the cells were incubated without the TLR agonist as a control. THP-1 cells were stimulated with 200 nM Phorbol myristate acetate (PMA) for evaluation and controlling monocyte differentiation. Both TLR agonists and supernatants of PMA-stimulated cells were collected and supernatants were added to THP-1 cells which previously cultured on 6-well plates and 72 hours' incubation was done. Cell experiments were performed at the end of the incubation. Cell culture supernatants were also collected and stored at -80 °C for cytokine release measurements.

3.2.3. THP-1 Cells Morphological Investigation

The effect of TLR agonists stimulated NCI-H82 cell supernatants on the THP-1 cells morphology were observed in the dark field microscope after 72 hours' incubation.

3.2.4. Determination of THP-1 Cells Macrophage Polarization by Flow Cytometry

After incubation of THP-1 monocytic cells with supernatants harvested from TLR stimulated lung cancer cells macrophage polarization was analyzed by flow cytometry CD11b, CD68, CXCR7, HLA-DR, CD206, and CXCR2 specific monoclonal antibodies were used in order to analyze M1 and M2 phenotype respectively. 10,000 cells were analyzed for each experiment.

3.2.5. Analysis of Cytokine Release

Following the incubation of THP-1 macrophages with supernatants of small cell lung cancer cells stimulated TLR agonists for 72h, supernatants were collected

for the evaluation of cytokines TNFα, IL1b, IL6, IL12, IL17A, IL8, MCP-1, RANTES, MIP-1a, MIP-1b, MDC and Eotaxin secretion were analyzed by Multi-Analyte ELISArray.

3.2.6. Evaluation of Phagocytosis in Macrophages

Following the incubation of THP-1 macrophages with supernatants of small cell lung cancer cells which were activated with TLR agonists. Macrophages phagocytosis capacity were analyzed using Fagotest kit. First, $2x10^5$ cells/ml were plated. The complex of latex beads rabbit IgG-FITC at a final dilution of 1:100 in media was added to the cells. Then the cells were incubated at 37 ° C for 24 hours. After incubation, the cells were centrifuged, and the supernatant was discarded, and 500 µl assay buffer was added to the cells and analyzed on a flow cytometry device.

3.2.7. Analysis of Cell Proliferation and Cell Cycle

The effect of presence and absence of TLR agonists on macrophages cell cycle were evaluated by flow cytometry. After incubation macrophage cells with supernatants of NCI-H82 cells in the present and absence of TLR agonists they were removed from 6 well plates, then centrifuged and fixed in 95% ethyl alcohol and were incubated at +4° C overnight. Cells were washed with PBS then 70 µl of RNase was added to the cells. This followed by the addition of 70 µl of propidium iodide dye and incubation for further 30 minutes in the dark place. After incubation, 10,000 cells were evaluated in a flow cytometry device. The results were evaluated by histograms of cell numbers and fluorescence intensities of cells in two variable histograms, the Mcycle software program used to measure the cell cycle phases and rates.

3.2.8. Statistical Analysis

The data obtained from experiments was assessed by Student's t-test with an instant software program (GraphPad Instat Dr. Granger, LSU Medical center, 1993) by comparing two parameters.

4. RESULTS

In this study, NCI-H82 small cell lung cancer cell line and THP 1 monocytic cell line were used. NCI-H82 small cell lung cancer cells were treated separately with TLR3, TLR5, TLR8 agonists, and also in combined form of TLR3, TLR5 and TLR8 agonists for 48 hours. After stimulation, cell culture supernatants were collected and added to THP 1 cells and incubated for 72 hours. The THP 1 cells were also stimulated with PMA only.

4.1. Evaluation of the Small Cell Lung Cancer Cell Culture Supernatants Effects on the Morphology of THP1 Monocytic Cells

After 48 hours' stimulation of NCI-H82 small cell lung cancer cells separately with TLR3, TLR5, and TLR8 agonists, and in the combined form of TLR3, TLR5 and TLR8, cell culture supernatants were collected and added to THP 1 cells and incubated for 72 hours. THP1 cells were also stimulated just with PMA. THP1 cells were typically found to be round morphology (Figure 4.1.), In the presence of TLR agonists or PMA THP 1 cells were expressed elongated and fusiform morphology. Figure 4.2. shows the morphological image of THP1 cells after incubation with NCI-H82 cell culture supernatant alone. The morphologic image of THP-1 cells after incubation with culture supernatant collected from of NCI-H82 cells stimulated with TLR3, TLR5, and TLR8 agonists and shown in figure 4.3 C, D, E respectively. Figure 4.3F shows the morphology of THP1 cells after combined activation of TLR3 + 5 + 8 agonists, In Figure 4.4. shows the morphology of THP1 cells after stimulation with PMA.

After treatment withNCI-H82 cell culture supernatants and TLR agonists, THP1 monocyte showed prolonged fusiform morphology and cells adhesion and aggregation enhancement were observed.

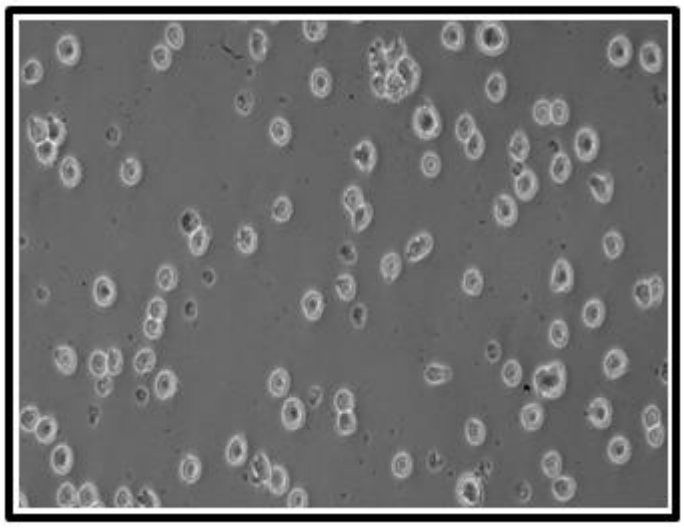

Figure 4.1. Morphological image of THP1 cells in cell culture medium (Control Cells).

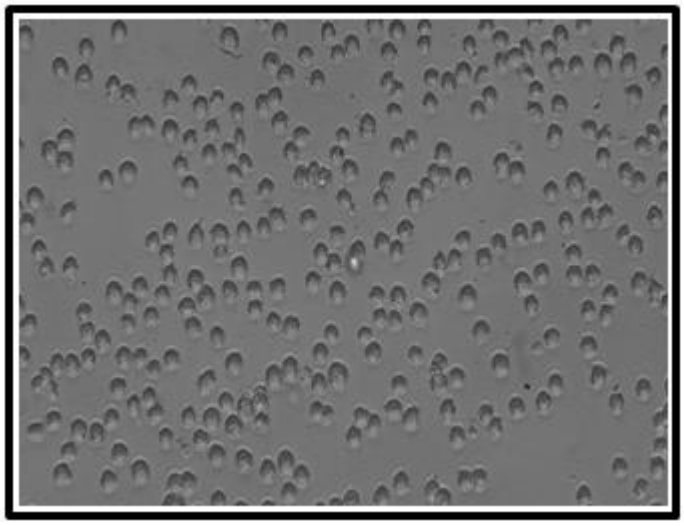

Figure 4.2. The morphological image of THP 1 cells after incubation with NCI-H82 cell culture supernatant.

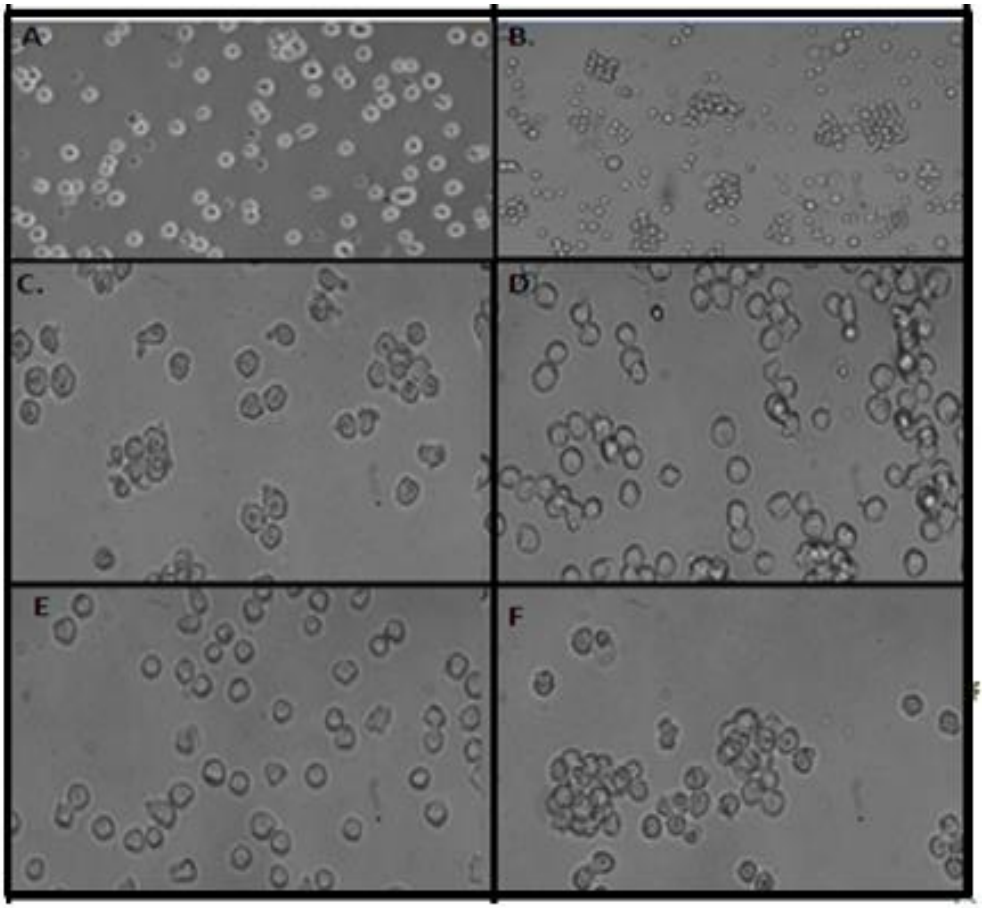

Figure 4.3. The image of THP1 cells in cell media (A), the morphological image of THP1 cells in the presence of supernatant of NCI-H82 cells (B), in the presence of supernatant of NCI-H82 cells activated with: TLR3 agonist (C), TLR5 agonist (D), TLR8 agonist (E) and combined form of the TLR3 + 5 + 8 agonists (F).

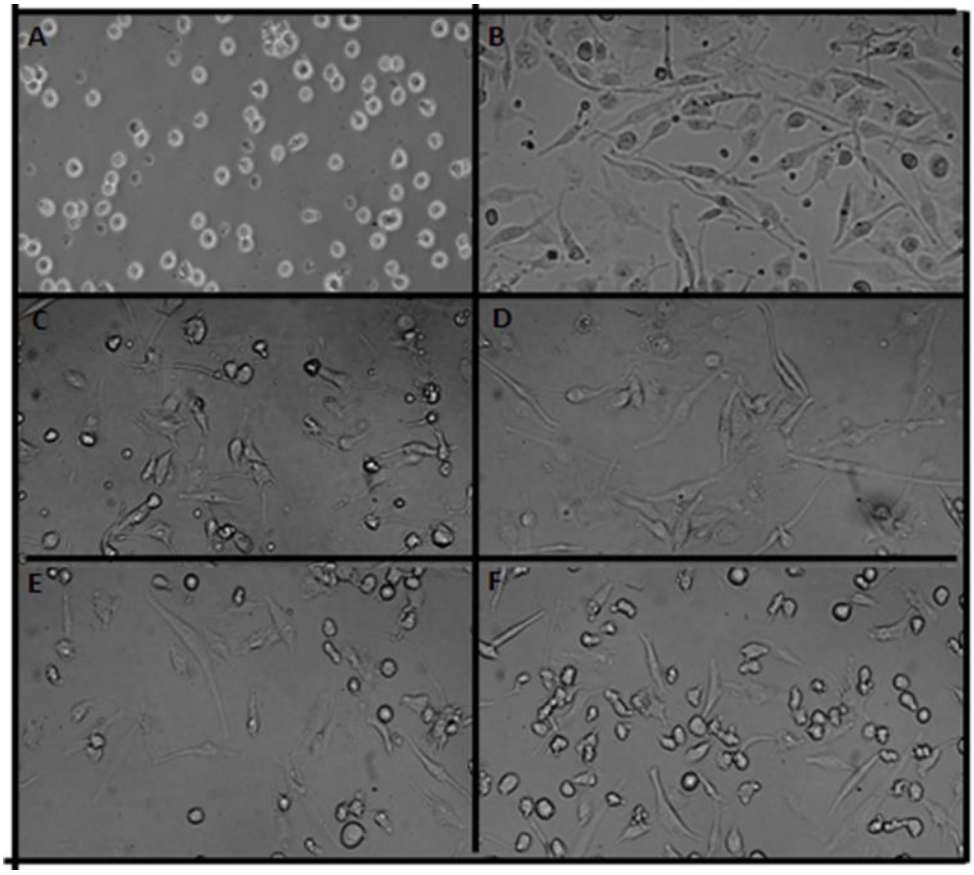

Figure 4.4. Shows the image of THP1 cells. THP1cells in culture media (A), The morphological image of THP1 cells after stimulation with NCI-H82 supernatant and PMA (B), In the presence of PMA and supernatants of NCI-H82 cells activated with: TLR3 agonist (C), TLR5 agonist (D), TLR8 agonist (E) and combined form of TLR3+5+8 agonists (F).

4.2. The effect of Small Cell Lung Cancer Cell Culture Supernatants Effect on Surface Markers Expressions of THP-1 Cells

After 48 hours' stimulation of NCI-H82 small cell lung cancer cells separately with TLR3, TL5 and TLR8 agonists, and combined TLR3, TL5 and TLR8 agonists, cell culture supernatants were collected and added to THP 1 cells and incubated for 72 hours. The THP 1 cells were also stimulated with PMA.

THP 1 cells surface markers were evaluated by flow cytometry after THP 1 cells stimulated with PMA in combination with supernatants of NCI-H82 cells activated with TLR3, TLR5, TLR8 agonists separately and combined TLR3, TLR5, TLR8 agonists.

CD68, CD11b, CD206, HLA-DR and chemokine receptors CXCR2 and CXCR7 surface markers were screened with specific monoclonal antibodies as surface determinants. The table 4.1. shows the expression of cell surface markers in all experimental conditions of THP-1 cells.

The expression CD68 and CD11b were detected high in all experimental conditions.

Cells bearing the CD206 M2 type macrophage marker show an increase in the presence of NCI-H82 supernatant (SN) and Phorbol myristate acetate (PMA) stimulation. THP1 cells showed decreased CD206 surface expression in the presence of TLR3, TLR5, TLR8 separately and TLR3 + 5 + 8, combined agonists.

The CXCR7 chemokine receptor is expressed in the M1 type macrophage polarization. THP1 cells incubated with supernatants of NCI-H82 cells activated with: TLR3, TLR5, TLR8 separately and combined form of TLR3 + 5 + 8 ligands. The obtained results showed increased expression of CXCR7 and decreased CXCR2 chemokine receptor.

There was no significant change in the distribution of HLA-DR expressing cells. As a result, while THP-1 cells incubated just with tumor cell culture supernatants differentiation to M2 type macrophage was obvious, the cell culture supernatants obtained in the presence of TLR ligands have been found to differentiate macrophages toward M1 type.

Table.4.1. The surface marker expression of THP 1 cells treated with NCI-H82 cells culture supernatant (SN) and/or PMA

	M1/M2	M1/M2	M2	M1	M2	M1
	CD68 %	CD11b %	CD206 %	HLA-DR %	CXCR2 %	CXCR7 %
THP1	88	99	11	15	8	33
THP1+SN	45	91	25	53	88	21
THP1+SN+TLR3L	28	98	14	46	43	67
THP1+SN+TLR5L	41	95	11	26	40	63
THP1+SN+TLR8L	52	88	16	33	56	48
THP1+SN+TLRLK	60	96	29	72	35	72
THP1+PMA	37	80	34	16	16	39
THP1+PMA+SN	52	90	26	18	20	27
THP1+PMA+TLRL3	42	82	27	7	35	33
THP1+PMA+TLRL5	28	79	20	16	33	38
THP1+PMA+TLRL8	33	73	27	12	30	41
THP1+PMA+TLR3+5+8	46	85	23	10	49	49

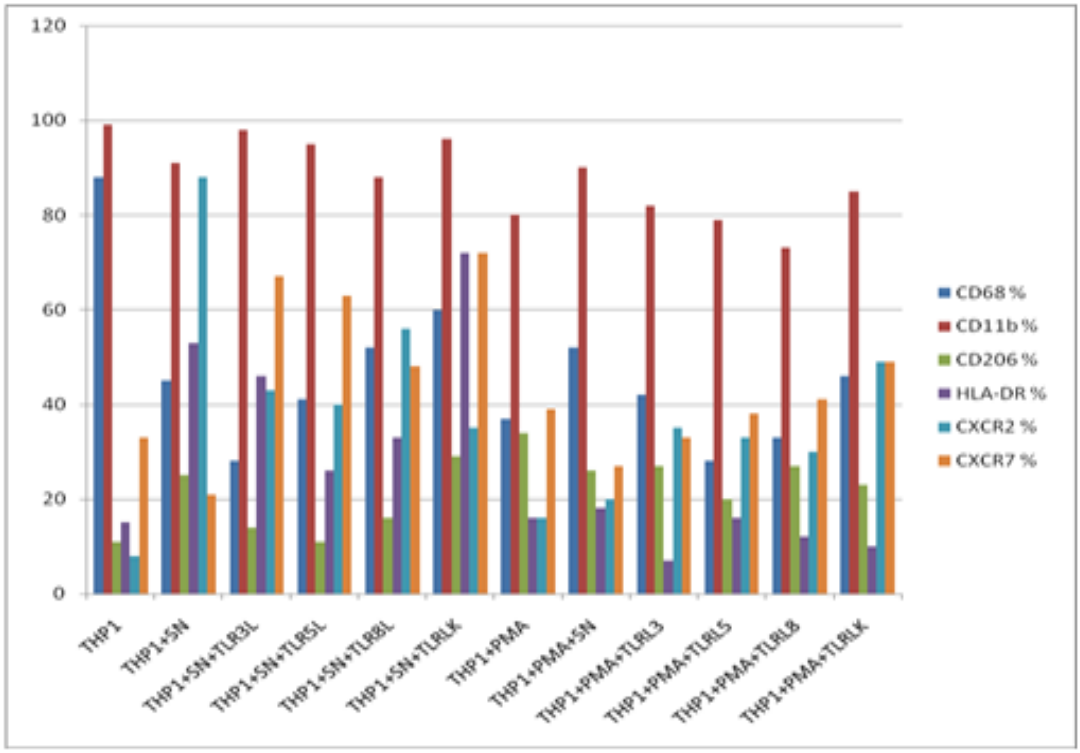

Figure 4.5. Shows the flow cytometry histograms of the macrophage surface markers distribution.

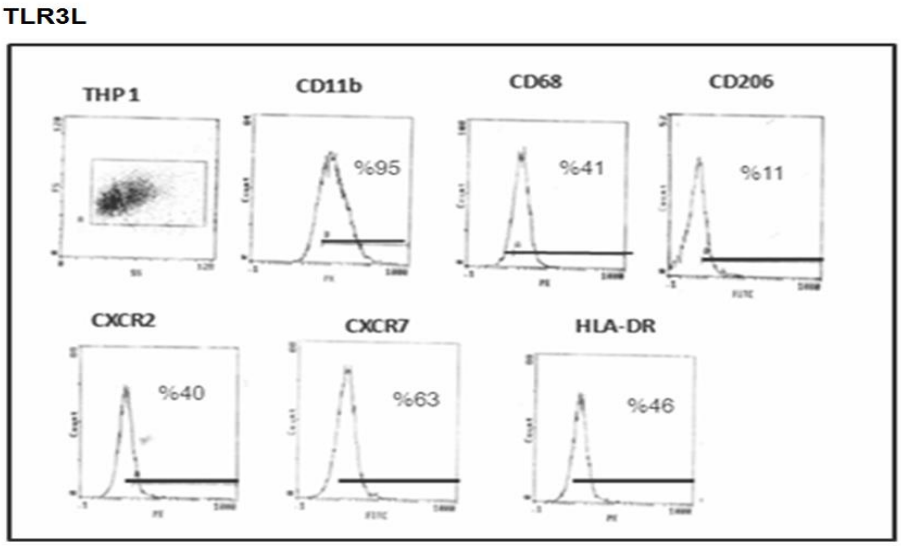

Figure 4.6. Surface expressions of CD68, CD206, HLA-DR, CXCR2, and CXCR7 in THP-1 cells in the presence of TLR3 ligands of NCI H82 cells.

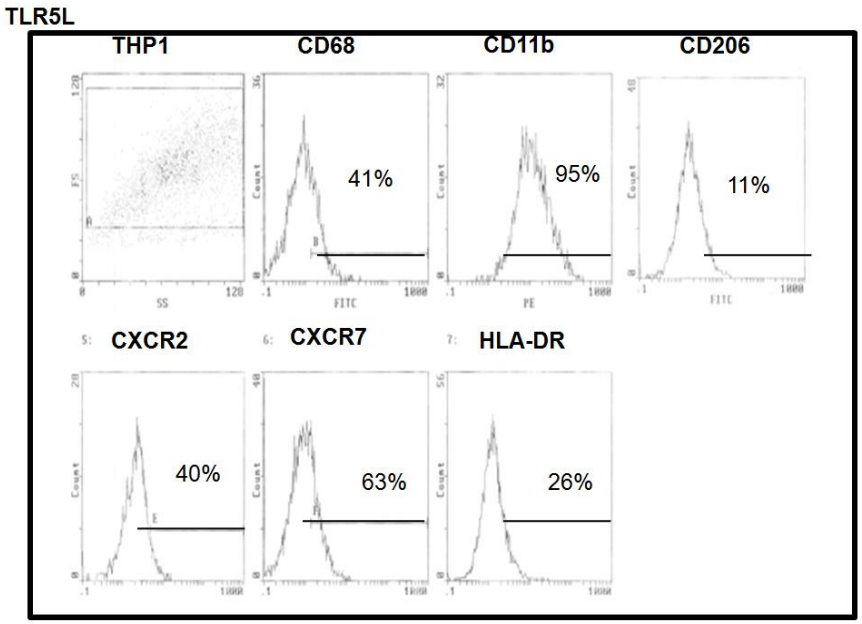

Figure 4.7. Surface expressions of CD68, CD206, HLA-DR, CXCR2, and CXCR7 in THP-1 cells in the presence of TLR5 ligands of NCIH82 cells.

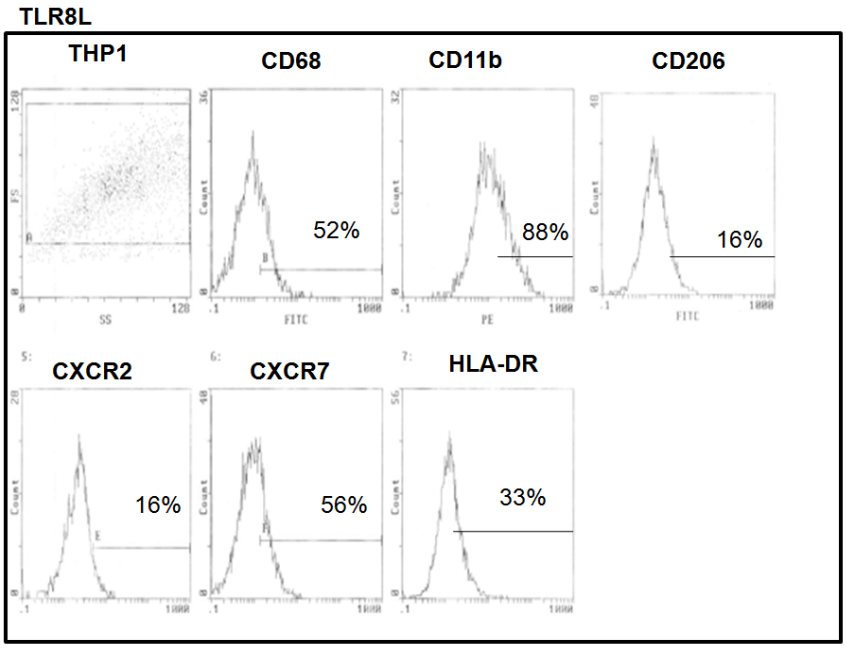

Figure 4.8. Surface expressions of CD68, CD206, HLA-DR, CXCR2, and CXCR7 in THP-1 cells in the presence of TLR8 ligands of NCIH82 cells.

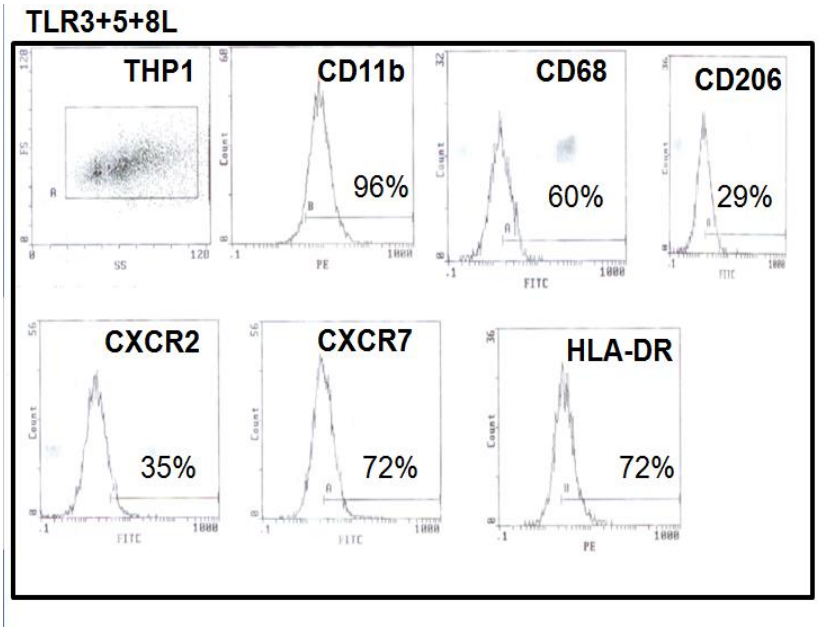

Figure 4.9. Surface expressions of CD68, CD206, HLA-DR, CXCR2, and CXCR7 in THP-1 cells in the presence of TLR3 + 5 + 8 ligands of NCIH82 cells.

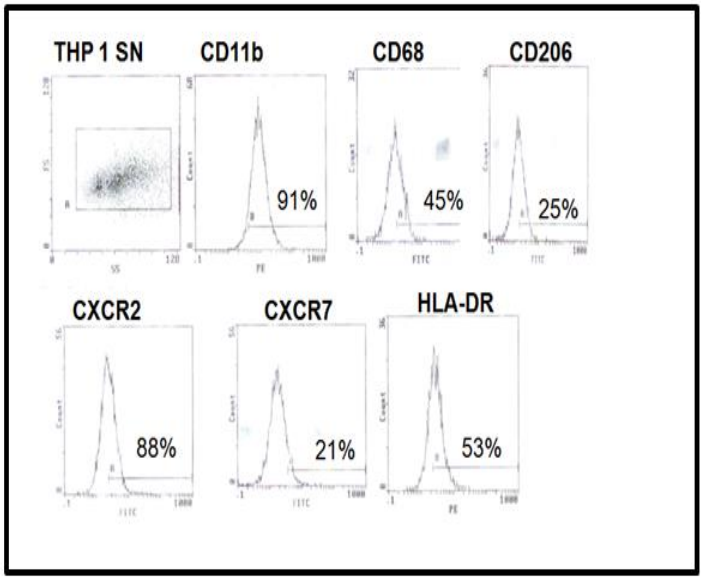

Figure 4.10. Surface expressions of CD68, CD206, HLA-DR, CXCR2, and CXCR7 in THP-1 cells incubated with supernatants of NCI-H82 cells activated with the combined form of TLR3 + 5 + 8 ligands.

4.3. Analysis of small cell lung cancer cell culture supernatants effects on cytokine release from THP1 monocytic cells

Cell culture supernatants were collected after 48 hours' incubation of NCI-H82 small cell lung cancer cells afterward THP-1 cells incubated for 72 hours with supernatants of NCI-H82 cells activated with: TLR3, TLR5, TLR8 separately and combined form of TLR3 + 5 + 8 ligands. After incubation, the supernatants were collected and stored at -80° C until the time of the study. Multiarray cytokine assay was performed by ELISA.

Determination of the inflammatory cytokines; IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFNγ, TNFα and GM CSF has been performed. The presence of inflammatory cytokines was detected at low levels as a result of incubation of THP-1 cells with supernatants of small cell lung cancer cells. In contrast, the presence of inflammatory cytokines was evident in the culture of THP-1 cells incubated with supernatants of small cell lung cancer cells with TLR3, TLR5, TLR8 agonists separately and TLR3 + 5 + 8 agonists in combined form.

Notably, the supernatants from combined use of TLR3 + 5 + 8 agonists significantly increased cytokine release from macrophages. This increase specifically was significant in IL-1α, TNFα, IFN-ϒ, and GM-CSF production (Figure 4.11).

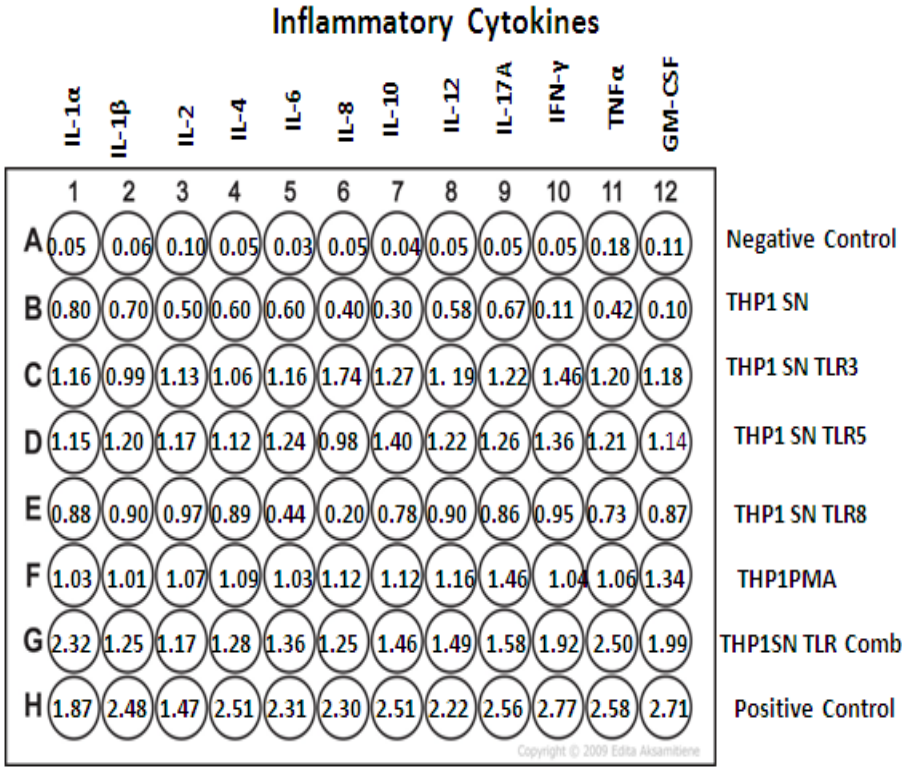

Figure 4.11. Multi-Analyte ELISArray Analysis used to determine cytokine presence in THP-1 cells culture supernatants which were previously incubated by activated supernatants collected from NCI-H82 cell line stimulated with TLR3, TLR5, and TLR8 agonists separately and combined form of TLR3+5+8 agonists.

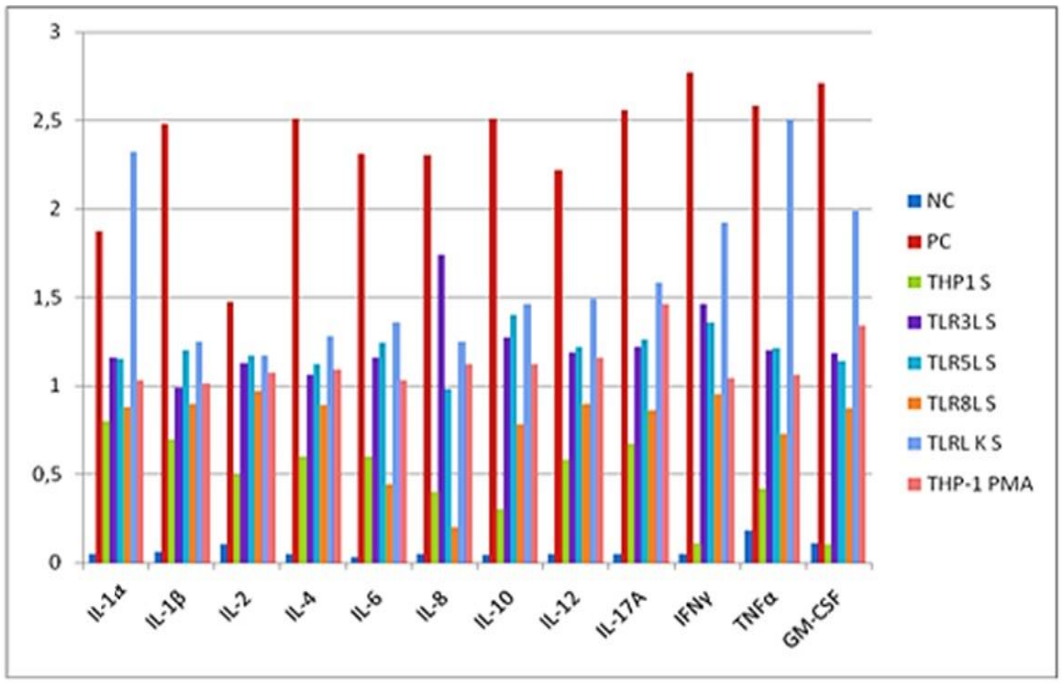

Figure 4.12. Multi-Analyte ELISArray analysis of inflammatory cytokines secreted by THP-1 cells which incubated with NCI-H82 cell supernatants activated by TLR agonists.

4.4. Analysis the effect of small cell lung cancer cell culture supernatants on the THP-1 cell phagocytosis

After stimulation of NCI-H82 small cell lung cancer cells with TLR3, TLR5 and TLR8 agonists separately, and TLR3 + 5 + 8 agonists in combined form for 48 hours, cell culture supernatants were collected and added on to THP-1 cells, then incubated for 72 hours. Likewise, THP-1 cells were incubated with PMA and same collected supernatants under the same conditions. The THP-1 cells phagocytosis activity was then assessed by flow cytometry. There was a significant increase in phagocytic activity of THP-1 cells incubated with supernatants of tumor cell stimulated TLR3, TLR5, and TLR8 when compared to THP-1 control cells. Also, there was a significant increase in phagocytic activity of THP-1 cells stimulated with PMA in all conditions compared to THP-1 control cells.

Table 4.2. Phagocytosis activity of THP-1 cells in different culture conditions.

	Fagositoz %
THP-1	5
THP-1 + PMA	61
THP-1 + PMA + SN	56
THP-1 + PMA + TLR 3	62
THP-1 + PMA + TLR 5	63
THP-1 + PMA + TLR 8	58
THP-1 + PMA + TLR 3,5,8	85
THP-1+SN	28
THP-1SNTLR3	55
THP-1SNTLR5	52
THP-1SNTLR8	54
THP-1SNTLR3+5+8	57

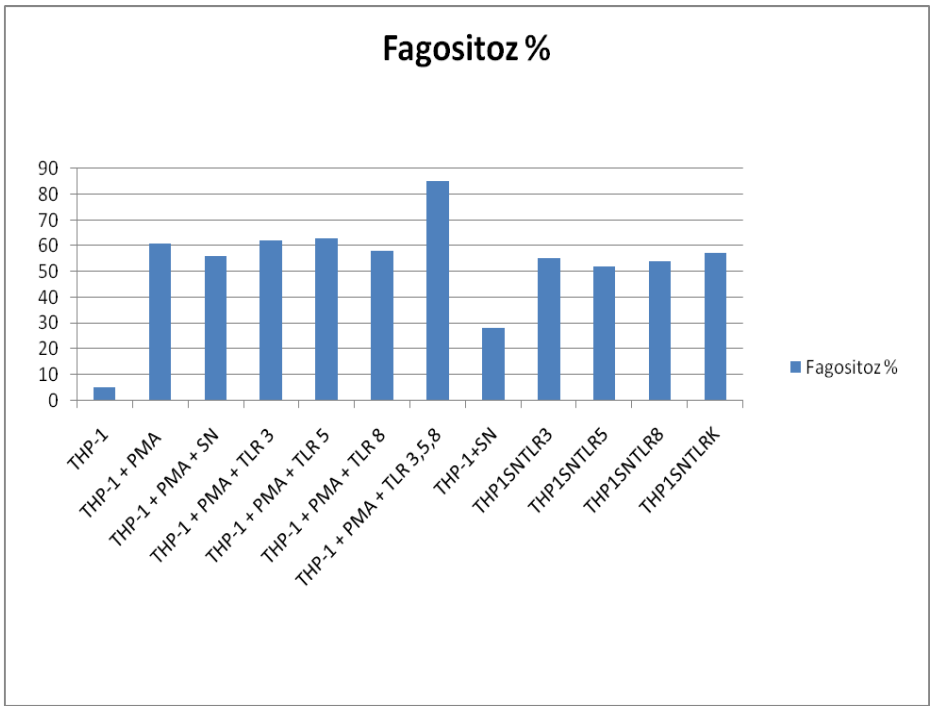

Figure 4.13. Phagocytosis activity of THP 1 cells in different culture conditions.

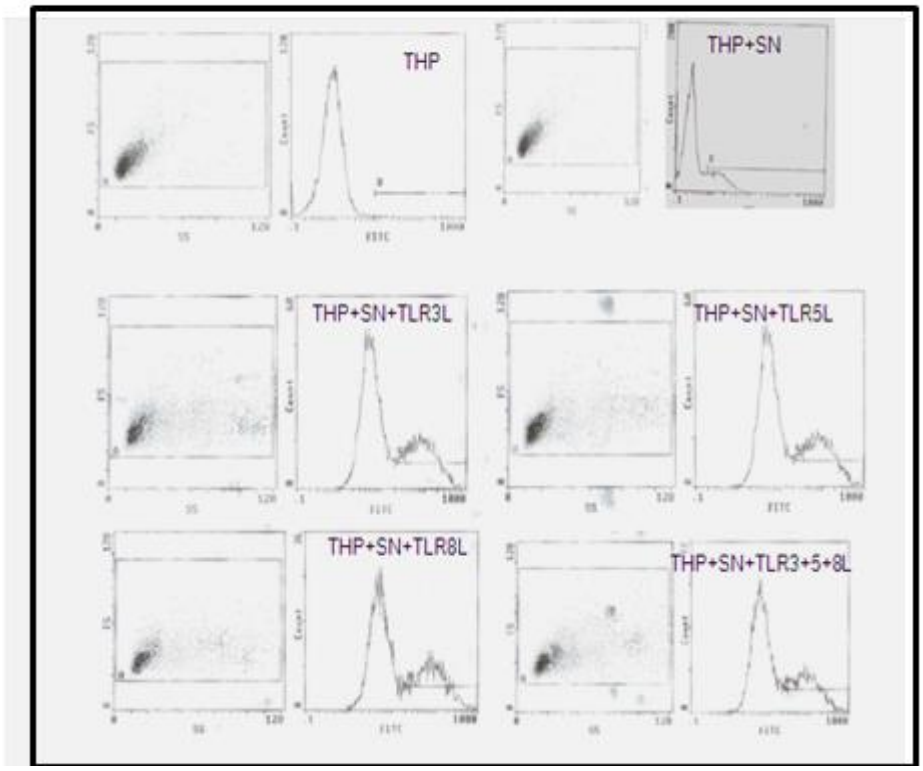

Figure 4.14. Flow cytometry histograms for phagocytosis activity in THP-1 cells incubated with supernatants of NCI-H82 activated with TLR3L, TLR5L, TLR8L, and TLR3+5+8L.

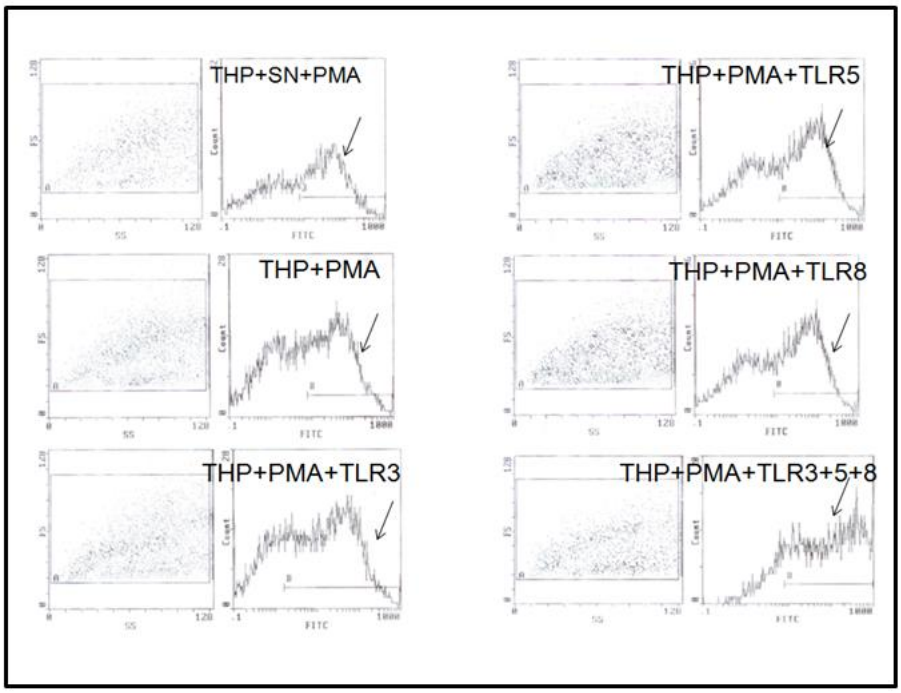

Figure 4.15. Flow cytometry histograms for phagocytosis activity in THP-1 cells incubated with PMA and supernatants of NCI-H82 activated with TLR3L, TLR5L, TLR8L, and TLR3+5+8L.

4.5. Analysis of Small Cell Lung Cancer Cell Culture Supernatants Effect on Cell Cycle of THP-1 Cells

After 48 hours' incubation of NCI-H82 small cell lung cancer cells with TLR3, TL5, TLR8 agonists separately, and TLR3 + 5 + 8 agonists combined cell culture supernatants were collected and added to THP-1 cells and incubation was allowed for 72 hours. Likewise, THP-1 cells were incubated with PMA and TLR agonists under the same conditions. Then, the cell cycle and DNA analysis were evaluated by flow cytometry in THP-1 cells.

When cell cycle phases of untreated THP-1 cells compared with THP-1 cells incubated with supernatants of NCI-H82 activated with TLR3L, TLR5L, TLR8L and TLR3+5+8L, accumulation was detected in G0/G1 phase of cell cycle. This increase was statistically significant ($p<0.001$). Table 4.3. shows cell cycle results in THP-1 cells in different conditions. The figure 4.15,16,17,18. shows flow cytometry histograms which present cell cycle distribution in THP-1 cells.

Table 4.3. Results of flow cytometric cell cycle analysis in THP-1 cells

	G0/G1 %	S%	G2/M %
THP-1	38	45	17
THP-1 PMA	44,8	36,2	18,9
THP1SN PMA	52,7	21,7	25,7
THP1 SN	46,8	40,8	12,4
TLR3LPMA	50,9	24,1	24,8
TLR5PMA	49,6	24,8	25,4
TLR8PMA	50,7	23,4	25,7
TLR3+5+8L PMA	48,8	26,6	25,5
TLR3L SN	59,8	28,4	11,7
TLR5L SN	67,2	24,5	8,1
TLR8L SN	72,4	17,7	9,8
TLR3+5+8L	95,7	4,3	0

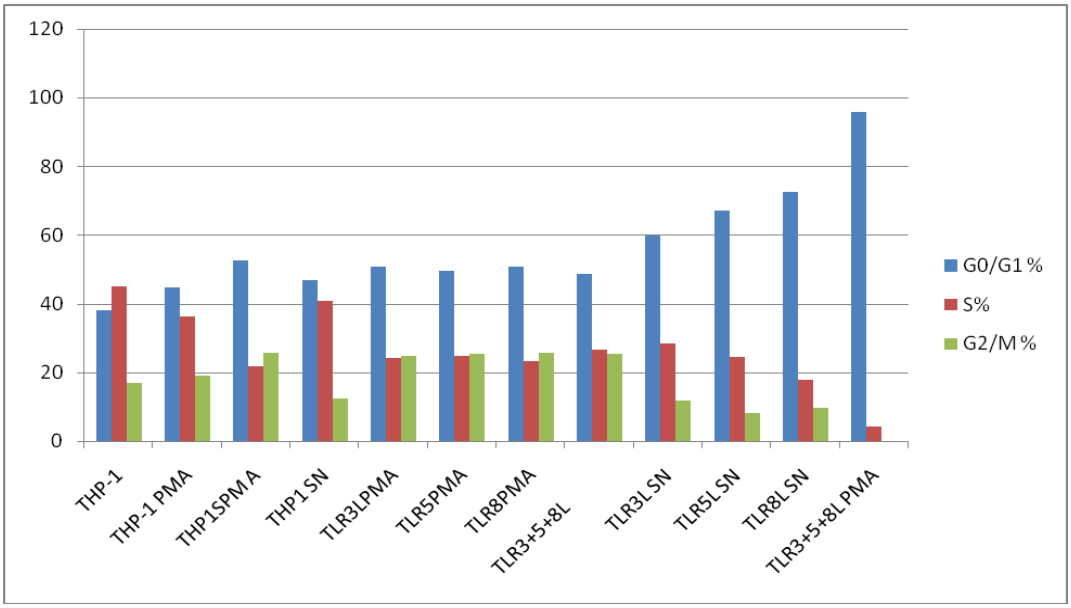

Figure 4.16. Results of cell cycle analysis in THP-1 cells

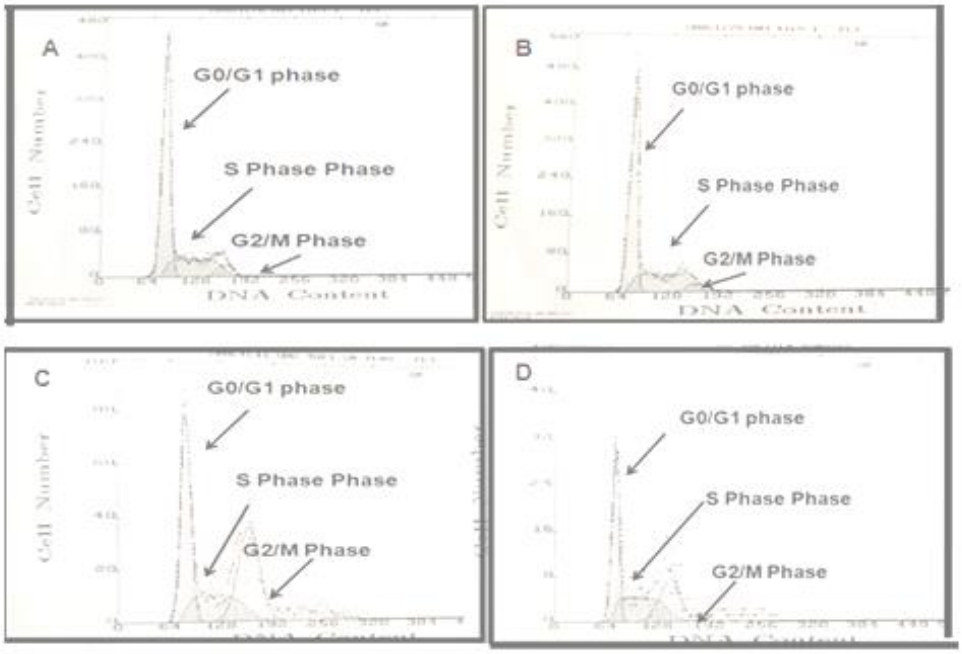

Figure 4.17. Flow cytometry histograms which present cell cycle distribution in THP-1 cells treatment with TLR ligands. A. TLR3 ligand B TLR5 ligand C. TLR8 ligand and D TLR3+5+8 combine ligands.

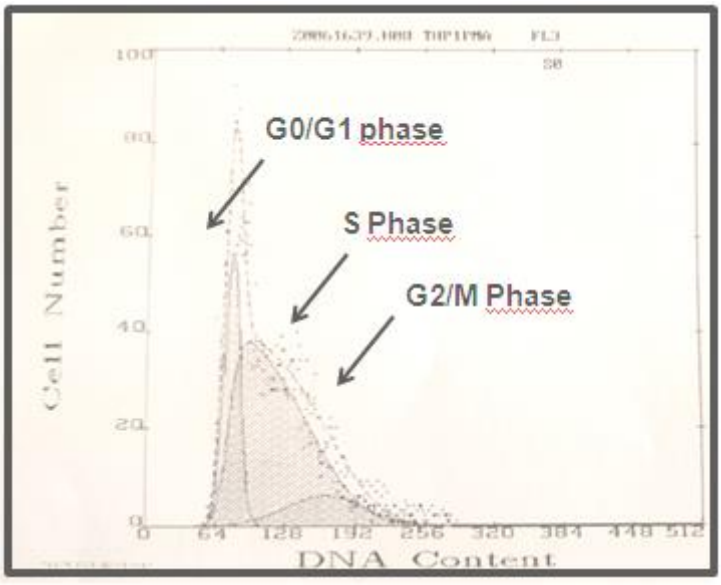

Figure 4.18. Flow cytometry histograms which present cell cycle distribution in THP-1 cells treatment with PMA.

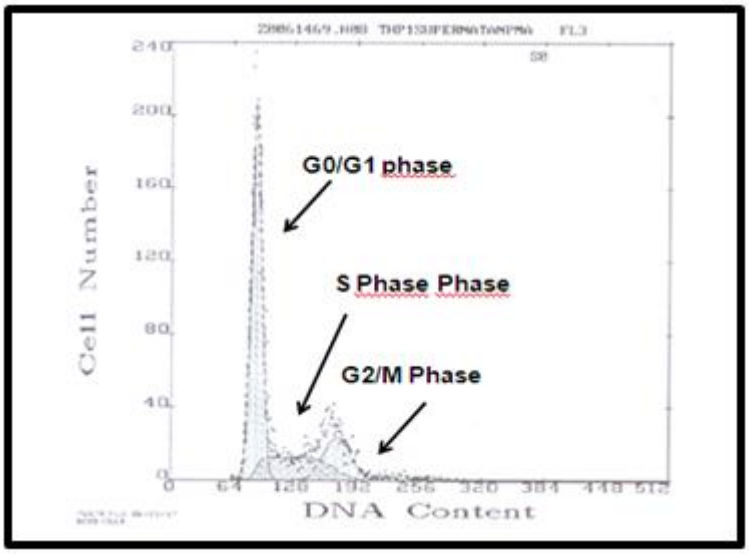

Figure 4.19. Flow cytometry histograms which present cell cycle distribution in THP-1 cells treatment with PMA and SCLC cell supernatant.

5. DISCUSSION

Macrophages show phenotypic heterogeneity and plasticity in response to environmental stimuli. Macrophages present in the tumor microenvironment through the effects of cytokines, chemokines and growth factors released from tumors, change their functional diversity and phenotypic differentiation. (188–190). In this study, small cell lung cancer cells (SCLC) treated with TLR agonists and properties of secreted supernatants containing the factors that have effects on the polarization of macrophage cells was evaluated. In our study, the morphology of THP-1 monocytic cells was evaluated as a result of incubation with SCLC culture supernatants incubated with TLR3, TLR5, TLR8 agonists separately and combined form of TLR3 + 5 + 8 agonists. The THP-1 monocytic cells in the presence of SCLC culture supernatants and TLR agonists changed their round morphology to elongated fusiform and showed adhesion and increase the aggregate formation, and they showed a change in morphological appearance. Similarly, morphology changes of macrophages by tumor cell supernatants has been observed in studies investigated on colorectal, breast, and cervical cancers (191). Increased adhesion by supernatant from tumor cell culture and increased aggregate formation support cell survival. M1-type differentiating macrophages constitute an essential cellular component of the immune system. Macrophages differentiate to M1 (classical activation) and M2 (alternative activation) (188–190). M1 type macrophages play a protective role against intracellular pathogens and cancer. M1 type macrophages stimulate the release of IFNγ and TNFα cytokines as a result of activation by Toll-like receptors. Stimulation of inflammatory response results in the secretion of IL-1, IL-6, IL-12, IL-23, and TNFα. These cytokines show a cytotoxic effect against microorganisms and cancer cells and increase Th1 response. M1 macrophages also form immunoreactive responses against tumor as antigen presenting cells.

M2 type macrophages facilitate tumor growth and metastasis. CD68 is the surface antigen that determines macrophages. CD163 and CD206 are used as surface antigen determinants that determine M2 macrophages. The results of numerous studies have shown that TAM is associated with poor clinical prognosis according to tumor type and localization. Human THP1 cells are a monocytic cell line used as an alternative to peripheral blood monocytes. The ability of these cells to proliferate

rapidly in the culture medium and to show no change up to 20 passages and their surface determinants, cytokine release and phagocytosis capacities resemble macrophages biological behaviors in-vivo. Due to these specifications THP 1 cell line frequently used for research purposes (192).

We have also used THP-1 cells in our studies to demonstrate macrophage polarization by the factors secreted by small cell lung cancer cells. Previously, numerous studies in the literature have shown that THP-1 cells differ to M2 type macrophage especially when incubated with colorectal and gynecologic tumor cell culture supernatants. We also carried out our studies with NCI-H82 cells (small cell lung cancer cell line) that have not been studied before. After incubation of THP 1 monocytic cells with culture supernatants of small cell lung cancer cells, CD206 and CXCR2 chemokine cell surface expressions were increased. It was also found that the phagocytosis capacity of these cells was decreased and the inflammatory cytokine release decreased markedly in this macrophages. These results show that soluble factors released from small cell lung cancer cells have an essential role in M2 type macrophage polarization.

M2 type macrophages highly express CD206 (Mannose receptor), and CD68 (Scavenger receptor). M2 type macrophages also secrete inflammatory cytokines as IL-10high, IL-12low, IL-23low. TAMs arising from M2 type macrophages are effective in cellular functions like proliferation, apoptosis, and angiogenesis of tumor cells (193). Proinflammatory cytokines play an essential role in the process in the biological behavior of tumors. IL-1β, IL-6, and IL-8 participate in physiological and pathological responses such as inflammation, immunological response and tumor growth(194). The chemokine (C-C motif) ligand CCL2, CCL5, CCL7 and chemokine (C-X3-C motif) ligand (various chemokines such as CX3CL91), furthermore macrophage colony-stimulating factor(M-CSF), granulocyte-macrophage-colony-stimulating factor(GM-CSF), vascular endothelial growth factor(VEGF), and cytokines produced by tumor cells enhance infiltration of monocytes and macrophages in tumor (195).

Inflammatory stimulation has been shown to be involved in the development and progression of various cancers, but the mechanisms which underly proinflammatory cytokine production has not been fully understood. TAMs in tumor

stroma constitute the main cell source. TAMs generally have M2 macrophage phenotype. M2 macrophages are activated by IFN- γ and this activation increases the response of Thelper2 type cell. When M2 macrophages are stimulated by different cytokines, they are divided into three different subgroups. M2a (alternative) type macrophages are developed by IL-4 and IL-13 exposure, M2b macrophages are also induced by toll-like receptor ligands (LPS) and immunocomplexes.

Both M2a and M2b macrophages activate the Th2 type response and accumulate T helper 2 type cytokines and T helper 2 cells. Both M2a and M2b macrophages exhibit protumorigenic character by developing a response to inflammation. The M2c type macrophage subgroup is also activated by IL-10 and glucocorticoid hormones, and the M2c macrophage response is responsible for tissue repair and has immune regulatory characteristics (194).

Elevated levels of IL-1β in the serum of non-small cell lung cancer (NSCLC) patients have been a detected at the early-stage tumors (195). IL-6 has been shown to be an antiapoptotic and pleiotropic inflammatory cytokine that facilitates tumor growth (196). In our study, we investigated inflammatory cytokines using the Multiarray ELISA to demonstrate the effect of tumor cell supernatants on macrophage differentiation. In particular, we have shown that IL-1α, IL-1β, IL-6, IL-8, IL-12, and TNFα cytokines are secreted. These cytokines are functionally involved in the polarization into the M2 macrophage. After activation of THP-1 macrophages with PMA, similar secretion of inflammatory cytokines has also been detected. We have found that phagocytosis capacity of THP-1 macrophages treated with small cell lung cancer cell culture supernatants is reduced when compared to untreated control group THP-1 macrophages. The decrease in phagocytosis capacity of M2 macrophages favors the escape of tumor cell from the immune system.

In the innate immune response, Toll-like receptors (TLRs) recognize microorganisms pathogen-associated molecular patterns (PAMPs) and play an essential role in host defense. Up to now, 11 TLRs have been identified in human. These receptors are divided into two main classes based on lipid and nucleic acids content. TLR1, 2, 4, 6 are lipid base and TLR3, TLR5, TLR7, TLR8, 9, 10 and 11 are nucleic acid based. In the TLRs Tri-acyl lipopeptide is ligand of TLR1; LPS, glycolipid, and HSP70, are ligands of TLR2; double helix RNA is the ligand of

TLR3; LPS, HSP60, and HSP70, are the ligand of TLR4; flagellin is the ligand of TLR5; di-acyl lipopeptide is the ligand of TLR6; imidazoquinoline and single-stranded RNA are Ligands of TLR7 and 8; and the CpG ODN is the ligand of TLR9. The ligand of TLR10 is not yet known. The ligand of TLR11 is a proline-like protein. TLR 4 as the binding ligand at the cell surface recognize lipopolysaccharides which are glycoproteins of outer membranes of Gram-negative bacteria. Heat shock proteins (HSP), HSP60, HSP70, and Gp96 also use TLR 4 and 2 as ligands (197). Activation of TLRs by microorganisms stimulates many defense mechanisms. Thus, phagocytosis, increased production of reactive oxygen and nitrogen, increased inflammatory cytokines are seen. TLRs act as key regulators in the development of metastasis and chemoresistance in tumor cells. TLRs are found as a "sensor" on the cell surface, and by binding to TLR ligands, signal transduction pathways become active in the cell, leading to tumor cell proliferation, inhibition of apoptosis and resistance to chemotherapeutic drugs.

Recent studies have shown the importance of TLRs in the process of invasion, and metastasis of breast cancer, glioma, gastrointestinal system, and laryngeal cancer cells by escaping the immunosuppression of the tumor, (197). In a mouse model of metastatic breast cancer, tumor cells which induced by lipopolysaccharide (LPS) cause to enhance tumor cell invasion by an increase in angiogenesis and the vascular permeability. In the progression of tumor cells, beta-1 integrin has been shown to be directly associated with adhesion to extracellular matrix proteins. Blockage of TLR4 has also been shown to regress in tumor growth. Although even if TLRs have benefit to tumor cell growth in tumor cells, the use of appropriate adjuvants increases immunological response to tumor antigens and results in increased antibody production and NK cell function. Double-stranded RNA (dsRNA) activates TLR3 on dendritic cells (DC), results in secreting Type-I IFN by DCs and increase NK cell cytotoxicity which ends to tumor cells become apoptotic. Likewise, B cell lymphoma cells carry TLR9. TLR9 recognizes CpG ODN and shows their effects as a potent adjuvant therapy in lymphoma treatment. “Imiquimod” is a TLR7 agonist and approved for the treatment of basal cell carcinoma. The use of TLR7 agonists in phase I studies in chronic lymphocytic leukemia (CLL) also has yielded good results.

The induction of TLR7 and 9 by their agonists increases NK and cytotoxic T cell activity, and eventually, CLL cells are gradually removed by apoptosis. TLR agonists also inhibit angiogenesis by altering the tumor microenvironment. Activation of TLR7, 8 and 9 enhances antigen presentation by Type-I IFN synthesis increase. As a result, the activation of cytotoxic T cells leads to an effective immune response in the direction of Th1 response (194,198).

In the presence of this information, where is the Toll-like receptors location in the macrophage differentiation process? As an answer to this question, we also investigated the effect of macrophage polarization in small cell lung cancer cells after stimulation with TLR agonists. For this purpose, when we investigated the effect of collected cell culture supernatants on the polarization of THP-1 monocytic cells after small cell lung cancer cells were incubated with TLR3, TLR5, TLR8 agonists separately (standalone) or with TLR3 + 5 + 8 agonists in combined form. We showed that THP-1 cells expressed CD11b, CD68, and CXCR7 chemokine receptors on their cell surface, indicating that TLR agonists are efficient on macrophage differentiation toward M1 type macrophage. M1 type macrophages produce a pro-inflammatory response in macrophage differentiation, while supernatants collected from small cell lung cancer cells without TLR agonist cause differentiation to M2 type macrophages. Cell culture supernatants collected by stimulation of small cell lung cancer cells with TLR agonists also affect inflammatory cytokine release in cells showing M1 differentiation. In these conditions, IL-1α, IL-1β, IFNγ, IL-12, TNFα cytokines were found in M1-type differentiated macrophages. These cytokines are proinflammatory cytokines and act as antitumor agents.

Increased phagocytosis capacity in M1-differentiating cells suggests that debris in the tumor microenvironment is cleared and defense mechanisms against tumor and chronic inflammation functionally. M1 type macrophages in the tumor microenvironment, stimulated by TLRs exerting phagocytosis activity. In the literature up to date, expression of TLR2/6 and TLR5 in small cell lung cancer cells has been shown in particular. TLRs cause differentiation of TAMs toward M1 type macrophages which with their secreted cytokines create the antitumor response. In our study, we have shown that TLR agonists increase the proinflammatory response

by macrophage-induced cytokines in lung cancer and increase cytokine release from immune cells in the inflammatory microenvironment of the tumor microenvironment. These findings can be explained as a novel mechanism regulated via TLR agonists. The TLR3 agonist we use in our study could be linked to the endosomal TLR3 and is a Poly I:C (ds RNA analog), consisting of Poly (I) and poly (C). Poly-ICL will induce Th1 type cytokine secretion, IL-12, TNFα, IFNγ, and IL-6, increase the release of various chemokines, helping to collect other immune system cells mainly macrophages, in the tumor microenvironment in vivo (188).

Administration of Poly I:C in glioma patients has been shown to increase the efficacy of chemotherapy and radiotherapy, while at the same time reducing the toxic and side effects of the treatment. After administration of the TLR3 agonist in tumor models generated by subcutaneous transplantation of Lewis lung cancer cell lines and melanoma B16H10 cell lines into mice, the reduction in M2 type macrophages bearing CD206 and increase in M1-type macrophage markers and IL-1β, TNFα and iNOS expressions were found (199). TLR3 agonists activate the inflammatory response by providing activation of the TLR3 signaling pathway and in M1 type macrophage differentiation. In this study, in addition to the TLR3 agonist, other TLRs agonists which enhance inflammatory cytokine release is used. Flagellin which is a TLR5 agonist and the ssRNA which is TLR8 agonist they both induce IFNα and TNFα release. Moreover, these agonists increase the expression of chemokines such CXCL9, CXCL10 and allow the accumulation of immune cells in the tumor microenvironment (197). M2 and TAMs infiltrate various tumors and are associated with poor prognosis of tumors. Therefore, the control and regulation of M2 and TAM is a key factor for the prognosis of tumors. There are very few studies on TLR3 agonist poly-ICL, TLR5 agonist flagellin, and TLR8 agonist sRNA40/LyoVec. In our study, the effects of small cell lung carcinoma culture supernatants and TLR agonists on cell cycle and cell proliferation were also evaluated in M1 and M2 type differentiating macrophages. TLR agonists cause macrophages accumulation in the G0 / G1 phase of the cell cycle, and cells proliferation was arrested.

Lung cancer is the leading cause of death in the world, and its development consists of a complicated multistep process (200). In this process transformation, hypoxia, invasion, migration, and metastasis are the most common well-known

specifications. Lung cancer cells are cells that escape from immunosurveillance. For this reason, recent studies have begun to investigate new targets for the recognition of cancer cells by the immune system. Tumor-associated macrophages in the tumor microenvironment and their presence and activation by TLRs are among these targets. The cytotoxic immune response enhanced by the TLR activation signal and arresting the growth and proliferation of tumor cells cause the death of lung cancer cells (188,197).

Our study results showed that TLR agonists on small cell lung cancer cells provide M1 type macrophage differentiation and antitumor effect by increasing proinflammatory cytokine release from macrophages. Therefore, it is promising that TLR3, TLR5, and TLR8 agonists differentiate TAMs toward M1 type macrophage in the treatment of small-cell lung cancer and should be included in alternative treatment strategies as well as conventional therapies in for development of new promising treatment.

5.1. Conclusion

Our results show that activation of small cell lung cancer cells by TLR3, TLR5 and TLR8 agonists play an essential role in the polarization of macrophages in the tumor microenvironment and this process is also responsible for inflammatory cytokine release from macrophages. Activation of TLRs, which express on small cell lung cancer cells with TLR agonists, suggests that TLR signal transduction pathways are a novel regulator axis moreover, support the tumor formation of lung cancer by inflammation. This results may help to modulation of immunotherapeutic approaches by TLR agonist in lung cancer.

6. RESULTS and RECOMMENDATIONS

1. THP1 cells which treated with NCI-H82 cell culture supernatants and TLR agonists, as well as PMA, changed their morphology increased aggregation and adhesion.
2. Cell culture supernatants obtained in the presence of TLR ligands have been found to induce M1 type macrophage polarization. While tumor cell culture supernatants induce M2 type macrophage differentiation.
3. Inflammatory cytokines secretion was low after the incubation of THP1 cells with supernatants of small cell lung cancer cells. In contrast, inflammatory cytokines secretion was higher in the presence of THP1 cells with TLR3, TLR5, TLR8 agonists separately and TLR3 + 5 + 8 agonists in the combined form of small cell lung cancer cells. Notably, the combined use of TLR3+5+8 agonists significantly increased cytokine release from macrophages Especially was increased IL-1α, TNFα, and GM CSF levels.
4. Phagocytosis capacity was significantly increased in THP 1 cells stimulated with NCI-H82 culture supernatants and TLR3, TLR5, TLR8 agonists separately and TLR3 + 5 + 8 agonists in combined form.
5. An accumulation of G0/G1 phase was detected in untreated THP-1 cells in comparison to THP-1 cells stimulated with TLR3+5+8 agonists in combined form, and THP 1 cells stimulated with TLR3, TLR5, and TLR8 agonists separately.
6. Similar experiments might be studied with samples obtained from small cell lung carcinoma patients.
7. The high rate of proinflammatory cytokines assessed by Multiarray ELISA could be quantitated by cytokine-specific ELISA so that the amount of cytokine that affects macrophage polarization can be assessed.
8. Investigation of TLR signal transduction pathways in small cell lung cancer cells induced with TLR3, TLR5, TLR8 agonists separately and with TLR3 + 5 + 8 agonists in combined form may be suggested in future studies.

7. REFERENCES

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8. APPENDICES

APPENDIX 1: Ethics Committee Approval

T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557 –400
Konu :

14.03.2017

Öğr. Gör. Dr. Hande CANPINAR
Kanser Enstitüsü
Temel Onkoloji Anabilim Dalı
Öğretim Elemanı

Sayın Öğr. Gör. Dr. CANPINAR,

Kurulumuza değerlendirilmek üzere sunduğunuz GO 17/241 kayıt numaralı ve ***"TLR Agonistleri ile Aktive Olan Akciğer Kanseri Hücre Kültür Süpernatanlarının Makrofaj Polarizasyonuna Etkisinin Araştırılması*** başlıklı proje Kurulumuzun 14.03.2017 tarihli toplantısında değerlendirilmiş olup, çalışma materyalinin ticari olarak satın alınmış hücrelerde yapılacağı insandan elde edilen primer kültürlerin kullanılmayacağı görülmüştür Klinik Araştırmalar Yönetmeliği gereği gönüllü insanlar üzerinde gerçekleştirilecek nitelikte olmayan bu tip çalışmalar Etik Kurulların kapsamı dışında kalmaktadır.

Bu yazı Etik Kurul kararı yerine geçmek üzere hazırlanmıştır.

Prof. Dr. Nurten AKARSU
Başkan

EK :
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9. CURRICULUM VITAE

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