T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

THE EFFECT OF FASL ON M1, M2A, AND M2C POLARIZATION OF PRIMARY HUMAN MACROPHAGES

RESUL ÖZBİLGİÇ

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCES THESIS

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Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı, Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Resul Özbilgiç **'THE EFFECT OF FASL ON M1, M2A AND M2C POLARIZATION OF PRIMARY HUMAN MACROPHAGES'** konulu Yüksek Lisans tezini 24.02.2021 tarihinde başarılı olarak tamamlamıştır.

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LIST OF ABBREVIATIONS

ADAM: A Disintegrin And Metalloproteinase

AF647: Alexa Flour 647

AICD: Activation-Induced Cell Death

APC-Cy7: Allophycocyanin-Cyanine7

BMDMs: Bone Marrow-Derived Macrophages

BSA: Bovine Serum Albumine

BV421: Brilliant Violet 421AF700: Alexa Flour 700

BV605: Brilliant Violet 605

BV785: Brilliant Violet 785

cDNA: Complementary DNA

CIAP2: The Cellular Inhibitor of Apoptosis 2

CXCL-10: C-X-C Motif Chemokine Ligand 10

DAXX: Death Domain-Associated Protein

DCs: Dendritic Cells

DD: Death Domain

DED: Death Effector Domain

DISC: Death-Inducing Signaling Complex

DNA: Deoksiribonucleic Acid

DRs: Death Receptors

ef450: Eflour 450

ef660: Eflour 660

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

FACS: Fluorescence-Activated Cell Sorting

FADD: Fas-Associated Protein with Death Domain

FBS: Fetal Bovine Serum

FITC: Fluorescein Isothiocyanate

FLIP: Fas-Associated Death Domain-Like Interleukin 1β-Converting Enzyme-Inhibitory Protein

FSC-H: Forward Scatter- Height

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HMDM: Human Monocyte Derived Macrophages

HMGB: High Mobility Group Box 1

IAPs: Inhibitors of Apoptosis Proteins

IDO-1: Indoleamine 2,3-Dioxygenase

IFNg: Interferon Gamma

IL-10: Interleukin 10

IL-1b: Interleukin 1 Beta

ILCs: Innate Lymphoid Cells

IRAK4: Interleukin-1 Receptor-Associated Kinase 4

JNKs: C-Jun N-Terminal Kinases

LPS: Lipopolysaccharide

MAPK: A Mitogen-Activated Protein Kinase

MCSF: Macrophage Colony-Stimulating Factor

MEKK1: MAP/ERK Kinase Kinase 1

mFasL: Membrane-Bound FasL

MFI: Mean Fluorescence İntensity

MISC: Motility-Inducing Signaling Complex

MMP: Matrix Metalloproteinase

MPs: Macrophages

MRC1: Mannose Receptor C-Type 1

MyD88: Myeloid Differentiation Primary Response 88

NFKB: Nuclear Factor-Kappa B

NK Cells: Natural Killer

NLRs: NOD-Like Receptors

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PD-1: Programmed Cell Death Protein 1

PE: Phycoerythrin

PE-Cy7: Phycoerythrin Cyanine 7

PerCp-Cy5.5: Peridinin Chlorophyll Protein Cyanine 5.5

PI3K: Phosphoinositide 3-Kinase

PRRs: Pathogen Recognition Receptors

qPCR: Quantitative Polymerase Chain Reaction

RBCs: Red Blood Cells

RIPK: Receptor-Interacting Serine/Threonine-Protein Kinase 1

RNA: Ribonucleic Acid

sFasL: Soluble FasL

TAMs: Tumor-Associated Macrophages

TCR: T Cell Receptor

TGM2: Transglutaminase 2

Th1 Cells: Type 1 Helper Cells

Th2 Cells: Type 2 Helper Cells

TLRs: Toll-Like Receptors

TNF: Tumor Necrosis Factor

TNFRSF: Tumor Necrosis Factor Receptor Superfamily

TRADD: TNF Receptor-Associated Protein with Death Domain

TRAF2: TNF Receptor-Associated Factor 2

TRAIL: TNF-Related Apoptosis-Inducing Ligand

Tregs: T Regulatory Cells

XIAP: X-Linked Inhibitor of Apoptosis

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THE EFFECT OF FASL ON M1, M2A, AND M2C POLARIZATION OF PRIMARY HUMAN MACROPHAGES

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ABSTRACT

Macrophages, the body's first line of defense, play a pivotal role in inflammation. With the plasticity ability, macrophages can be polarized into classically-activated (M1) or alternatively-activated (M2) phenotypes with appropriate signals. While M1 macrophages are responsible for generating a pro-inflammatory response against pathogens or tumors, M2 macrophages induce the anti-inflammatory response such as tissue regeneration and angiogenesis. Death receptor Fas is a member of the TNF receptor superfamily. Binding of Fas Ligand (FasL) to its receptor Fas can initiate apoptosis or non-apoptotic mechanisms. Previous studies have shown that Fas/FasL interaction enhances the expression of pro-inflammatory factors by inducing non-apoptotic mechanisms rather than causing cell death in macrophages. However, there is no comprehensive study in the literature investigating the effect of Fas activation on human primary macrophage polarization. To investigate this effect primary human macrophages were polarized into M1, M2a, and M2c phenotypes in the presence or absence of soluble FasL. The effect of FasL stimulation on macrophage phenotype was analyzed by qPCR and flow cytometry. In conclusion, our results demonstrated that FasL stimulation did not alter M0 macrophage response. In M1 macrophages, while FasL stimulation increased the expression of proinflammatory markers at mRNA level, but not at protein level. FasL stimulation significantly reduced the expression of the M2a surface marker CD206 at protein level in M2a polarized macrophages. Finally, M2c polarization was not affected by FasL stimulation. Our findings suggest that FasL, whose expression increases in inflammatory diseases such as cancer, has no functional effect on primary human macrophage polarization.

Keywords: Fas (CD95), FasL (CD178), human monocyte-derived macrophages, macrophage polarization

FASL UYARIMININ PRİMER İNSAN MAKROFAJLARININ M1, M2A VE M2C POLARİZASYONUNA ETKİSİ

Resul Özbilgiç, Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü,

ÖZET

Vücudun ilk savunma hattı olan makrofajlar enflamasyonda merkezi bir rol oynar. Makrofajlar plastisite yeteneği sayesinde uygun sinyallerle klasik olarak aktive edilmiş (M1) veya alternatif olarak aktive edilmiş (M2) fenotiplerine polarize olabilirler. M1 makrofajlar tümörler ve patojenlere karşı pro-enflamatuar yanıt oluşturulmasından sorumluyken, M2 makrofajlar doku yenilenmesi ve anjiyogenez gibi anti-enflamatuar yanıt oluştururlar. Bir ölüm reseptörü olan Fas, TNF reseptörleri ailesinin bir üyesidir. Fas ligandı, Fas reseptörüne bağlandığında hem apoptozu hem de sağkalım mekanizmalarını başlatabilir. Önceki çalışmalar, Fas/FasL etkileşiminin makrofajarda hücre ölümüne sebep olmaktansa sağkalım mekanizmalarını tetikleyerek pro-proenflamatuar faktörlerin ekspresyonunu arttırdığını göstermektedir. Ancak literatürde Fas aktivasyonunun insan primer makrofajlarının farklı polarizasyon durumlara etkisini araştıran kapsamlı bir çalışma bulunmamaktadır. Fas ligandının primer insan makrofaj polarizasyonuna etkisini araştırmak için primer insan makrofajları çözünebilir Fas ligand varlığı veya yokluğunda M1, M2a ve M2c fenotiplerine polarize edildi. FasL uyarımının makrofaj fenotipine etkisi qPCR ve akış sitometrisi ile analiz edildi. Sonuç olarak, FasL uyarımının polarize olmamış makrofajlarda bir yanıta sebep olmadığını gösterdi. M1 makrofajlarında Fas ligand uyarımı proenflamatuar belirteç ekspresyonunu mRNA düzeyinde arttırdı ancak protein veya sitokin üretiminde anlamlı bir değişime yol açmadı. İlaveten, Fas ligand uyarımı M2a fenotipine polarize olan makrofajlarda, M2a yüzey belirteci olan CD206 ekspresyonunu protein düzeyinde anlamlı bir biçimde azalttı. Son olarak M2c polarizasyonu FasL uyarımından etkilenmedi. Bulgularımız, kanser gibi enflamatuar hastalıklarda ifadesi artan FasL'ının primer insan makrofaj polarizasyonuna fonksiyonel bir etkisinin olmadığı yönündedir.

Anahtar Sözcükler: Fas (CD95), FasL (CD178), insan monositten türetilmiş makrofajları, makrofaj polarizasyonu

1. INTRODUCTION AND GOALS

1.1. Definition and Importance of the Problem

Macrophages are localized throughout almost all tissues and play a key role in inflammation and tissue homeostasis. While M1 macrophages, which have proinflammatory features and fight with pathogens, M2 macrophages have anti-inflammatory properties and are involved in tissue regeneration and angiogenesis. Many diseases, especially inflammatory diseases and cancer, are associated with a disruption of macrophage polarization and response. The possibility to re-polarize macrophages towards the desired direction would be promising immunotherapy for such diseases. Therefore, it is important to identify novel modulators that play a role in macrophage polarization.

Fas, a member of the TNF receptors superfamily, is one of the death receptors. When Fas interacts with its relevant ligand FasL, two different mechanisms can be activated. The first is the apoptotic pathway, which has been studied and many mechanisms of which have been elucidated before, and the latter is the non-apoptotic pathway, which has not been clarified yet, although it has been discovered for a long time. Studies on the role of Fas/FasL interaction on immune cells have generally focused on adaptive immune cells, especially T cells. Importantly, FasL level is increased in the tumor microenvironment and patient sera in many cancer types. However, the effect of this increase on the polarization of macrophages which orchestrate the immune response is not clear. A limited number of studies on the effect of Fas activation on macrophage response and polarization have reported that Fas activation increases the production of pro-inflammatory cytokines in mouse bone marrow-derived macrophages and some cell lines. However, there is no comprehensive study investigating the effect of FasL stimulation on primary human macrophage polarization.

1.2. Research Objective

The objective of this study was to investigate the impact of natural sFasL on primary human macrophage polarization. To this end, primary human macrophages were polarized into M1, M2a, or M2c macrophages with appropriate stimulants in the presence or absence of sFasL. The effect of sFasL stimulation on macrophage phenotype was analyzed by qPCR and flow cytometry.

1.3. Hypothesis

We hypothesize that sFasL stimulation drives primary human macrophages to an M1 phenotype.

2. GENERAL INFORMATION

2.1. Macrophages

2.1.1. Origin and Development of Macrophages

The immune system is a host defense system consisting of numerous biological molecules, cells, and tissues which protect organisms against pathogens and diseases. In mammals, the immune system consists of innate immunity and adaptive immunity (Parkin & Cohen, 2001).

Innate immunity, previously evolved, responds immediately to pathogens through the innate cells with a limited diversity of receptors. The innate immune system comprises physical barriers, immune cells, and humoral components. Physical barriers, providing the first line of defense against foreign substances, are the skin, mucosal tissues, pH, lysozymes, and other secretions. Monocytes, macrophages, dendritic cells (DCs), mast cells, eosinophils, neutrophils, basophils, natural killer (NK) cells, innate lymphoid cells (ILCs), and innate-like T cells such as natural killer T cells are involved in the innate immune response (Turvey & Broide, 2010). Innate immune cells have an invariant and limited variety of receptors called pathogen recognition receptors (PRRs) which recognize damaged host cells and some conserved microbial constitutes (Amarante-Mendes *et al.*, 2018). Humoral components of the innate immune system include elements of the complement system, pentraxins, antimicrobial peptides such as defensins. These soluble proteins are responsible for both recognizing pathogens and triggering effector mechanisms to eradicate the infection. Another important role of innate immunity is the activation of adaptive immunity during inflammation (Shishido *et al.*, 2012).

Unlike innate immunity, more specialized and slower responses to the infections occur through adaptive immunity. The adaptive immune system comprises B and T lymphocytes and their products such as antibodies (Yatim & Lakkis, 2015).

Some lymphocytes, such as natural killer T cells, mucosal-associated invariant T cells, and gamma-delta T cells, which have the characteristic feature of adaptive immune cells, respond quickly like innate immune cells and called innate-like lymphocytes (Verykokakis *et al.*, 2014).

Macrophages were discovered by Ilya Metchnikoff in the late 19th century and were classified into the mononuclear phagocytic system along with monocytes and dendritic cells. Macrophages are myeloid-derived innate immune cells that are localized throughout the tissues to recognize and eliminate pathogens or cells that are damaged or infected. In addition, macrophages have a regulatory role on other immune cells at the inflammatory site (Varol *et al.*, 2015). Macrophages are derived from three different sources which involve the yolk sac, fetal liver-derived monocytes, and bone marrow-derived monocytes. In general, yolk sac and fetal liver-derived macrophages are positioned in tissues and named tissue-resident macrophages. However, monocyte-derived macrophages can be recruited into tissues and participated in tissue homeostasis as well. One of the most important differences between embryonic-derived macrophages and monocyte-derived macrophages is the self-renewal capability (Epelman *et al.*, 2014).

Monocytes, the precursors of monocyte-derived macrophages, have a critical role in inflammatory processes such as cancer, cardiovascular diseases, and infection. There are three major monocyte populations defined in humans; CD14+CD16− classical, CD14lowCD16+ non-classical, and CD14+CD16+ intermediate monocytes. The equivalent of these subtypes in mice are Ly6C+ (including two subtypes based on Ly6C expression level on the cell surface) classical, and Ly6C- (also called Ly6Clow) non-classical monocytes (Coillard & Segura, 2019). In the case of inflammation, in mice, Ly6C+ and Ly6C- monocytes are recruited into relevant tissues and more likely differentiate into inflammatory M1 macrophages (Audoy-Remus *et al.*, 2008) and anti-inflammatory M2 macrophages, respectively, (Auffray *et al.*, 2007). A similar situation has been observed in humans during inflammation such as cancer (Sade-Feldman *et al.*, 2018; Müller *et al.*, 2017; Liao *et al.*, 2017).

At steady state, the presence of monocyte-derived macrophages was demonstrated in the small intestine (Bujko *et al.*, 2018), skin (McGovern *et al.*, 2014), heart (Bajpai *et al.*, 2018), lung (Eguíluz-Gracia *et al.*, 2016), and liver (Patel *et al.*, 2017) in humans.

In the literature, studies have mainly focused on the development of mouse tissue-resident macrophages. Therefore, there is insufficient information available about the origin of human tissue-resident macrophages. Recent studies have demonstrated that the development of human yolk sac-derived tissue-resident macrophages resemble that of the mice (Stremmel *et al.*, 2018). Typically, microglia originate only from yolk sac macrophages, Langerhans cells, Kupffer cells, and intestinal gut macrophages originate from both yolk sac macrophages and fetal liver-derived monocytes in mice (Epelman *et al.*, 2014).

2.1.2. Macrophage Polarization

The term "polarization" has come to be used to refer to the process by which macrophages acquire a certain phenotype in response to extrinsic factors. These extrinsic factors involved in the macrophage polarization are cytokines produced during inflammation, some bacterial products such as lipopolysaccharide (LPS), and some survival factors such as CSF-1 (Murray, 2017). There are two types of macrophages: classically activated (M1) and alternatively activated (M2) macrophages. Macrophages have a pivotal role in inflammation. Simply, in the initial phase of inflammation, macrophages polarize towards M1 to induce the pro-inflammatory response to get rid of the pathogens. Then, M1 macrophages switch to M2 for the resolution of inflammation and prevention of tissue damages (Martinez, 2014). Type 1 helper cells (Th1) cells and Type 2 helper cells (Th2) are subsets of CD4 + cells. Basically, while Th1 cells triggers pro-inflammatory response producing IL-2, IL-12, IFNg and TNFa, Th2 cells triggers anti-inflammatory response producing interleukins 4, 5, 10, 13, and 25 (Romagnani *et al.*, 2000). Proinflammatory Th1 cytokines (such as TNFa and IFNg), some microbial products, or granulocyte-macrophage colony-stimulating factor (GM-CSF) alone or in combination can induce the polarization of macrophages towards the M1 phenotype (Murray *et al.*, 2014; Murray, 2017; Sica *et al.*, 2015).

M1 macrophages secrete proinflammatory cytokines and chemokines such as TNFa, IL-1b, IL-6, IL-12, and CXCL10. Therefore, M1 macrophages enhance the Th1 response and the recruitment of Th1 lymphocytes (Mantovani *et al.*, 2004; Fujiwara *et al.*, 2005).

M1 macrophages have a role in the clearance of intracellular bacteria, suppression of tumors, induction of Th1 responses, and inhibition of tissue repair (Sica *et al.*, 2015; Murray *et al.*, 2014; Martinez *et al.*, 2014).

On the contrary, Th2 cytokines (IL-4, IL-10, or IL-13), macrophage colony-stimulating factor (MCSF), some parasites, and the components of the complement system can induce M2 macrophage polarization (Gordon *et al.*, 2003; Murray *et al.*; 2014). M2 macrophages produce IL-10, TGFb, CCL17, CCL22, and CCL24 (Shapouri-Moghaddam *et al.*, 2018; Rözser *et al.*, 2015). Hereby, M2 macrophages increase the Th2 response and the recruitment of Th2 lymphocytes and suppressive T regulatory cells (Tregs). Moreover, M2 macrophages can recruit eosinophils and basophils via secreting chemokines to remove the fungal and helminth infections (Mantovani *et al.*, 2014). The antigen-presenting capability of M2 macrophages is low (Murray *et al.*, 2014). Generally, M2 macrophages participate in tissue repair, angiogenesis, fibrosis, and suppression of anti-tumorigenic response (Novak *et al.*, 2013; Ostuni *et al.*, 2015). Moreover, M2 macrophages are powerful phagocytes and attend the clarification of some parasites (Braga *et al.*, 2015; Sica *et al.*, 2015).

M2 macrophages are divided into four subsets such as M2a, M2b, M2c, and M2d macrophages (Chistiakov *et al.*, 2015; Martinez *et al.*, 2009). Table 1 demonstrates the stimulators to drive the macrophage subsets, the cytokines/chemokines produced by, and the surface molecules expressed on the macrophage subsets.

Disruption of the balance between M1 and M2 macrophages influences the progress of diseases as was stated in the "Macrophage Function" section. For example, in most cancers, macrophages tend to become M2 phenotype and support the tumor due to the increase of anti-inflammatory cytokines in the tumor microenvironment (Noy *et al.*, 2014; Mantovani *et al.*, 2010).

Besides, it has been reported that some bacteria including Mycobacterium tuberculosis, Helicobacter pylori, etc. can predispose macrophages to the M2 phenotype, thereby supporting their survival (Rajaram *et al.*, 2010; Parveen *et al.*, 2013; El Kasmi *et al.*, 2008).

Importantly, it should be emphasized that macrophages are highly plastic cells and can switch between M1 and M2 polarization states with appropriate signals. (Mantovani *et al.*, 2015). Since macrophages are the maestro in inflammation, the switch between M1 and M2 macrophages is promising to cure many diseases, especially cancer and autoimmune diseases.

Subset	Stimulator	Cytokines/Chemokines	Surface Molecules
M1	IFNg, TNFa, LPS, GM-CSF, CXCL-9, CXCL-10	TNF-α, IL-1β, IL-6, IL-12, IL-23	CD86, MHC-II, IL-1R, TLR2, TLR4
M2a	IL-4, IL-13	IL-10, TGFb, IL-1R antagonists, CCL17, CCL22	CD206, IL-1R II, Decoy Receptor, CD200R
M2b	Immune Complex, TLR agonists, IL-1R ligands	IL-10, IL-1b, IL-6, TNF	CD86, MHC-II
M2c	Glucocorticoids, IL-10	IL-10, TGFb	CD163, TLR1/8
M2d	Adenosine	IL-10, TGFb, TNFa, IL-12	VEGF

Table 1. The phenotype of M1 and M2 subsets (Sica et al., 2015; Shapouri-Moghaddam et al., 2017; Murray, 2017)

2.1.3. Macrophage Function

Macrophages have a role in both homeostasis and inflammation processes. Macrophages generally function through three mechanisms; (1) Phagocytosis of pathogens, as well as infected or damaged cells, (2) the induction of adaptive immunity via antigen presentation, and (3) the regulation of immune response by producing cytokines and chemokines (Sica *et al.*, 2015; Wynn *et al.*, 2013).

Macrophages are equipped with receptors called pattern recognition receptors (PRRs) to perform these functions. PRRs consist of Toll-like receptors (TLRs), RIG-I-like receptors, C-type lectin receptors, and NOD-like receptors (NLRs). In addition to PRRs, macrophages have some cytokine, hormone, and scavenger receptors (Gordon & Plüddemann, 2017). The repertoire of mouse macrophage receptors is shown in Figure 1.

In the development process, embryonic-derived macrophages participate in the central nervous system vascularization (Nucera *et al.*, 2011; Fantin *et al.*, 2010), retina development (Lobov *et al.*, 2005), neuronal survival, and cell death (Wake, 2013; Nayak, 2014), clarification of senescent cells (Munoz-Espin *et al.*, 2013; Storer *et al.*, 2013), and the formation of bone and tooth (Ortega *et al.*, 2010; Kodama *et al.*, 1991).

In the case of infection, the balance between M1 and M2 macrophages is critical for both eradication of the pathogen and repairing the damaged tissue. M1 macrophages generally contribute to wiping out bacterial, viral, parasitic, and fungal infections while M2 macrophages are responsible for repairing the damaged tissue (Shapouri-Moghaddam *et al.*, 2018). In some parasite and fungal infections, however, M2 macrophages help the clearance of pathogens (Wager *et al.*, 2014; Biswas *et al.*, 2012).

The tumor microenvironment has not harbored only cancer cells, but also immune cells and stromal cells. The macrophages, called Tumor-Associated Macrophages (TAMs), are found intensely in the tumor microenvironment of most cancer types (Ramanathan *et al.*, 2014).

In the early stages of tumorigenesis, macrophages are polarized into the M1 phenotype and together with other immune cells generate an anti-tumorigenic response (Najafi *et al.*, 2018). In the advanced stages, tumor or stromal cells-derived factors switch macrophages into M2-like tumor-associated macrophages which have generally the tumor-promoting phenotype and induce the pro-tumorigenic immune response, angiogenesis, and metastasis (Belgiovine *et al.*, 2016; Ramanathan *et al.*, 2014). A growing body of literature has demonstrated that there is a correlation between the number of infiltrated macrophages in the tumor microenvironment and poor clinical outcomes (Yang *et al.*, 2018).

Moreover, TAMs limit the efficiency of chemotherapy, radiotherapy, and newly developed immunotherapies such as PD-1 inhibitors (Mantovani *et al.*, 2017; De Palma *et al.*, 2013). Therefore, the recruitment and polarization of macrophages make a very attractive target in cancer therapy and promising initial results have been reported (Anfray *et al.*, 2020; Ramesh *et al.*, 2019; Kulkarni *et al.*, 2018).

As macrophages can orchestrate the immune response, they play a central role in inflammatory diseases. For example, in allergic and bronchial asthma, M2 macrophages and Th2-associated cytokines augment the pathogenesis (Murray *et al.*, 2014; Wyn *et al.*, 2013). A similar clinical picture is painted in fibrosis (Wick *et al.*, 2010; Xu *et al.*, 2012). Conversely, the infiltration and M1 polarization of macrophages negatively influence obesity and type 2 diabetes in human patients (Castoldi *et al.*, 2015; Meshkani *et al.*, 2016). Lastly, macrophages are also included in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, inflammatory bowel disease. Mostly, M1 polarization and Th1-related cytokines such as TNFa exacerbate the disease progression. Nevertheless, it has been reported that in some autoimmune diseases, both types of macrophages and relevant cytokines can be observed (Ma *et al.*, 2019).

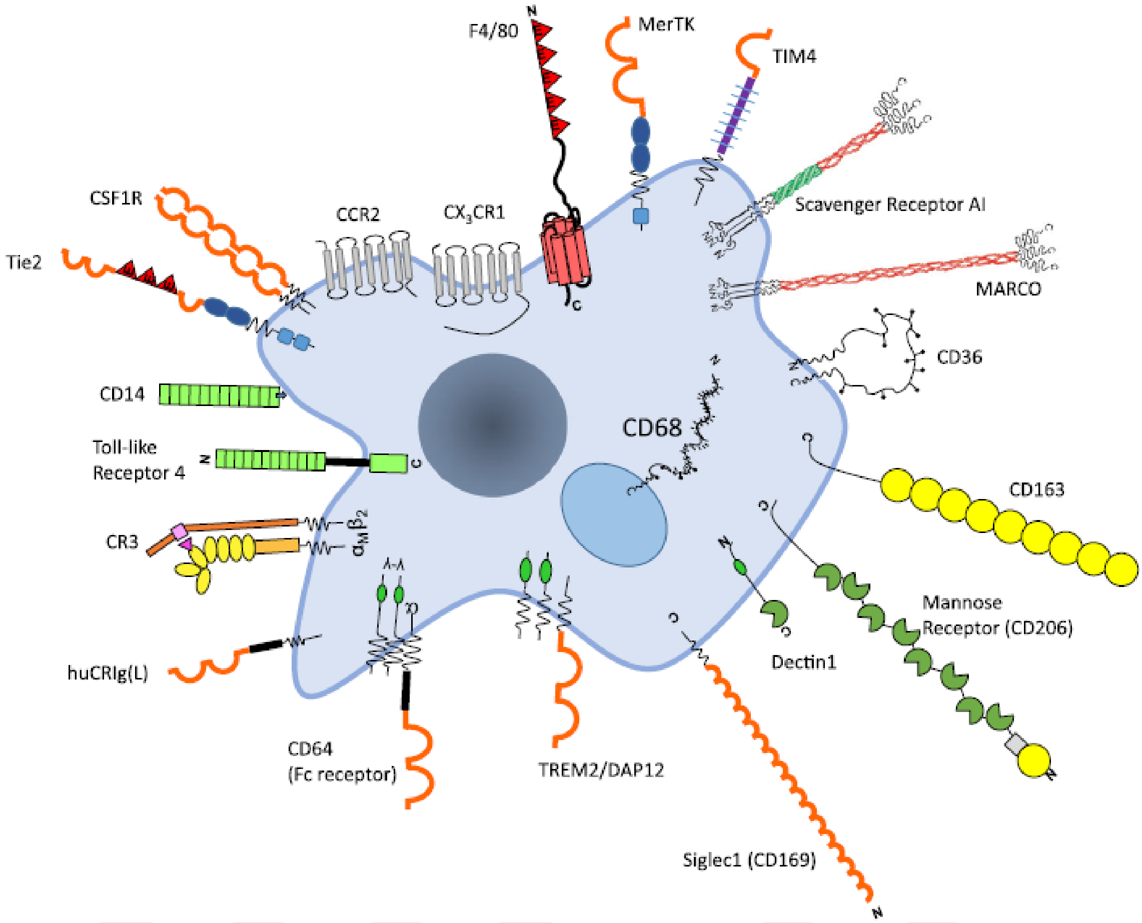


Figure 1. The repertoire of mouse macrophage receptors (Gordon & Plüddemann, 2017)

2.2. Fas/FasL Pathway

2.2.1. Cell Death

Cell death is an essential process for the formation of organs, removal of cells which are infected or irreversibly damaged, depletion of immune cells at the end of inflammation, and cancer prevention (Wang *et al.*, 2010; Buss *et al.*, 2006; Holler *et al.*, 2000; Sever *et al.*, 2014). Fundamentally, there are 3 types of cell death: apoptosis, necrosis, and autophagy. This classification is based on the appearance of dying cells (Galluzzi *et al.*, 2018).

Apoptosis is generally defined by the apoptotic body formation as a result of membrane blebbing, DNA fragmentation, and chromatin condensation. Two different pathways can initiate apoptosis: the intrinsic pathway and the extrinsic pathway (Galluzzi *et al.*, 2007). First, the intrinsic pathway takes place due to stress conditions such as ER stress, reactive oxygen species, or lack of growth factors (Buss *et al.*, 2006). The family of Bcl2 proteins, including pro-apoptotic and anti-apoptotic proteins, is crucial for the regulation of intrinsic apoptosis. Once mitochondrial membrane integrity is impaired, pro-apoptotic factors accumulate in the cytoplasm and initiate apoptosis (Green *et al.*, 2015). Secondly, the extrinsic pathway is activated by death receptors (DRs). More details on DR-mediated apoptosis will be given in section 2.2.3.

Necrosis is the energy-independent and unprogrammed form of cell death. Exogenous stressors such as inflammation or chemicals trigger necrosis. Necrosis is characterized by discomposed cell membranes and the dissemination of cytoplasm (D'Arcy *et al.*, 2019). Necroptosis is a type of cell death that is classified under the title of necrosis but occurs programmatically. Necroptosis is tightly regulated by receptor-interacting protein kinase 3 (RIP3) (Degretev *et al.*, 2005). TLR, TCR, and DR stimulations can initiate the necroptosis via RIP3 activation (He *et al.*, 2011; Oliveira *et al.*, 2018; Edward *et al.*, 2014). It is well illuminated that the Fas/FasL pathway, which has an important place in this thesis, can also initiate necroptosis (Holler *et al.*, 2000).

Finally, autophagy is a mechanism that is triggered by stressors such as nutritional inadequacy and rarely results in death. The protective mechanism of autophagy is better known than the death mechanism. Generally, protein aggregates, damaged organelles, or infected cells are delivered to the lysosome by vesicles and are destroyed (Galuzzi *et al.*, 2017; Kroemer *et al.*, 2010). Autophagic cell death has been observed in the developmental process and the removal of senescent cells (Mizushima *et al.*, 2008).

2.2.2. Death Receptors (DRs)

The term "death receptors" refers to the members of the tumor necrosis factor receptor superfamily (TNFRSF) that induce apoptosis. DRs, like other members of TNFSFR superfamily, are type 1 transmembrane proteins and consist of a cytoplasmic tail, a transmembrane region, and an extracellular ligand recognizing part. The distinguishing feature of death receptors is the death domain, which gives these receptors the ability to initiate cell death (Guicciardi *et al.*, 2009). DRs consist of TNF receptor 1 (TNFR1), Fas (CD95 or Apo-1), death receptor 3 (DR3), TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAILR1 or DR4), and TRAILR2 (DR5) (Wajant*,* 2003; Chinnaiyan *et al.*, 1995). These receptors exist in a ligand-independent oligomerized form (Guicciardi *et al.*, 2009). In general, their stimulation and downstream processes are comparable. Simply, when ligands are bound to the relevant receptors, the adapter proteins (FADD: Fas-associated protein with death domain or TRADD: TNF receptor-associated protein with death domain) interacts with the death domain and recruit caspase 8 and caspase 10, and thus, resulting in apoptosis (Siegmund *et al.*,2016). However, stimulation of DRs (especially Fas and DR4/5) with relevant ligands induces not only apoptosis but also pro-inflammatory cytokine production (Cullen *et al.*, 2015). The non-apoptotic mechanism of DRs will be described in details in section 2.2.4.

2.2.3. Structure of Fas and its ligand FasL

Fas is a type I transmembrane glycoprotein consisting of 319 aa and a 16 aa long signal peptide sequence. Its structure consists of 3 main parts: the ligand-binding extracellular N-terminal part (157 aa), the transmembrane part, and the intracellular C-terminal part (145 aa) which activates downstream pathways (Cheng *et al.*, 1995; Lavrik *et al.*, 2012). This receptor has 3 cysteine-rich domains that provide FasL recognition (Siegmund *et al.*, 2016). Moreover, Fas can also be found in a soluble form (sFas) as a result of metalloprotease activity or transcriptional splicing. The major differences between these two forms are that sFas does not have a transmembrane domain and inhibits apoptosis (Marsik *et al.*, 2003).

Noteworthily, there is a positive correlation between tumor progression and increased serum sFas levels in patients with many cancers (Jodo *et al.*, 1998; Konno *et al.*, 2000; Kondera-Anasz *et al.*, 2005).

FasL is a type 2 transmembrane protein and can be found in 2 different forms. Initially, membrane-bound FasL (mFasL) is produced and then its soluble form (sFasL) is formed via proteolytic cleavage of the stalk region or C terminal TNF domain by various metalloproteases (MMP3, MMP7, MMP9, and ADAM) or by plasmin, respectively (Guegan *et al.*, 2018; Bajou *et al.*, 2008). These two forms have different functions. mFasL is mostly found on the cells located in immune-privileged areas (neurons and epithelial cells), and activated lymphocytes (NK cells and T cells) at the inflammatory site (Takahashi *et al.*, 1994; Singer *et al.*, 1994). mFasL is the essential molecule in the elimination of cancer cells and infected cells by immune cell-mediated apoptosis (Gallo *et al.*, 2017). Moreover, mFasL has a central role in the apoptosis of autoreactive or overactivated T cells in the periphery in order to maintain the peripheral immune tolerance, called activation-induced cell death (AICD) (Arakaki *et al.*, 2014). While mFasL is able to induce apoptosis, sFasL mainly activates pro-survival or pro-inflammatory pathways instead of apoptosis (O'Reilly *et al.*, 2009; Siegmund *et al.*, 2016). Although the cause of this effect cannot be adequately clarified, it has been thought that these ligands trigger different pathways due to the trimerization capability of Fas. Fas is located as a monomer on the cell membrane and can be trimerized when stimulated with mFasL, but this cannot occur when stimulated with sFasL (Matsumoto *et al.*, 2015).

Importantly, sFasL reduces the apoptosis activity of mFasL via blocking the relevant receptors (Suda *et al.*, 1997; Hohlbaum *et al.*, 2000). This situation may explain the increase in sFasL in the serum of patients with poor cancer prognosis (Peter *et al.*, 2015). It should be noted that, in addition to these FasL forms, another ligand form was detected on vesicles, for example on tumor-derived exosomes. It has been demonstrated that cancer-derived exosomes suppress the immune response by FasL-mediated apoptosis of cytotoxic T cells (Abusamraa *et al.*, 2005; Kim *et al.*, 2005)

2.2.4. Apoptotic Mechanisms of Fas/FasL Pathway

Binding of FasL to its receptor Fas activates the downstream molecules to start apoptosis. Fas molecules are present on the cell membrane as monomers or sometimes as oligomers (dimers or trimers) in an unstimulated cell. The PLAD domain of the Fas receptor facilitates the assembly as dimers or trimers in a steady-state (Siegmund *et al.*, 2016). Once stimulated, the ligand with heterotrimer structure first assembles 3 Fas receptor molecules, then this ligation forms a cluster with the other Fas/FasL complex. Fas-associated death domain (FADD), an adapter protein, binds to the cytoplasmic tail of the clustered ligand-receptor complex via the death domain (DD) (Wajant *et al.*, 2014; Schleich *et al.*, 2012). FADD interacts with procaspase 8 via the death effector domain (DED) and forms the death-inducing signaling complex (DISC). This receptor-adapter protein-procaspase 8 structure causes the recruitment of more procaspase 8 (Dickens *et al.*, 2012). This aggregation both stabilizes the FADD-Fas interaction and triggers the enzymatic dimerization of procaspase 8. The resulting heterodimers autocatalytically constitute the mature heterotetrameric form which provides the maturation of other executioner caspases, such as caspase 3 and 9. As a consequence, the cell undergoes apoptosis (Gueagan *et al.*, 2017; Siegmund *et al.*, 2016; Yamada *et al.*, 2017).

It is crucial to state that apoptosis is a controlled process that is achieved by the balance between pro-apoptotic and anti-apoptotic molecules. The balance in Fas/FasL signaling is provided through reciprocal negative regulation of the caspase activity and NFκB-induced anti-apoptotic molecules (Hayden *et al.*, 2012; Wajant *et al.*, 2003).

As Fas signaling can also activate the NFκB pathway like TRAIL and TNF receptors, NFKB-induced anti-apoptotic molecules such as FLIP and cIAP2 inhibit apoptosis (Wajant *et al.*, 2003). Similarly, apoptosis-related caspases suppress anti-apoptotic molecules at the translational level (Clemens *et al.*, 2000). In Fas-mediated apoptosis, according to the mitochondrial dependence, cells are classified as type-1 and type-2 cells. The main difference between these types is that mitochondrial involvement in apoptosis of type 2 cells (Ozoren & El-Deiry, 2002).

While rapid formation of DISC and accumulation of procaspases are observed following FasL stimulation in type 1 cells, this mechanism is postponed by various anti-apoptotic molecules in type 2 cells (Scaffidi *et al.*, 1998; 1999). In type 2 cells, inhibitors of apoptosis proteins (IAPs), such as the x-linked inhibitor of apoptosis (XIAP), bind to the executioner caspases and inhibit apoptosis (Deveraux *et al.*, 1997; 1998). Therefore, although FasL stimulation activates both extrinsic and intrinsic apoptosis pathways, in type 2 cells, apoptosis usually occurs through the intrinsic pathway (Jost *et al.*, 2009). In addition to IAPs, the PI3K signal pathway increases survival by preventing Fas/FasL clustering (Beneteau *et al.*, 2008; Pizon *et al.*, 2011).

FasL stimulation activates not only the apoptosis pathway but also the necroptosis pathway. In this process, cFLIPs are very determining. cFLIPs generally have 3 isoforms with similar structures: small, Rajii, and long isoform. The main function of these isoforms is inhibition of apoptosis. Noteworthily, the short isoform of cFLIP enhances the FasL-mediated necroptosis, whilst the long isoform does the opposite (Tsuchiya *et al.*, 2015).

2.2.5. Non-apoptotic Mechanisms of Fas/FasL Pathway

Fas stimulation not only provokes apoptosis but also activates some kinases and pathways involved in important biological activities. These biological activities are pro-inflammatory responses, migration, metastasis, proliferation, and differentiation (Hayden *et al.*, 2012; Tauzin *et al.*, 2011; Lavrik *et al.*, 2007; Park *et al.*, 2005). FasL binding can induce important pathways such as NFKB, MAPK, RIPK, and PI3K (Siegmund *et al.*, 2017).

First, Cullen and colleagues have demonstrated that FasL stimulation both induces apoptosis and activates the NFKB pathway, providing a small amount of secretion of some proinflammatory molecules in sensitive cells. In this study, it has been observed that apoptotic and non-apoptotic pathways can occur simultaneously (Cullen *et al.*, 2013). However, it should be noted that, as mentioned previously, there is an inhibitory communication between the apoptotic pathway and the NFKB pathway (Wajant *et al.*, 2003).

The most important reasons for the superiority of the non-apoptotic pathway are the soluble form of FasL and the level of expressed anti-apoptotic molecules (cFLIPs and IAPs) (Gallo *et al.*, 2017). FasL-mediated NFKB activation occurs in different ways. One of them is an apoptosis-dependent way, which starts with the maturation of caspase 3 by caspase 8. Mature caspase 3 undergoes proteolytic cleavage of MAP/ERK kinase kinase 1 (MEKK1) and forms a 91 kDa activated kinase (Deak *et al.*, 1998). MEKK1 triggers the NFKB pathway through c-Jun N-terminal kinases (JNKs) activation and provides the production of pro-inflammatory factors (Faris *et al.*, 1998). Another way is independent of apoptosis. Following FasL stimulation, FADD and procaspase 8 are recruited to DD of Fas. Then NFKB activation takes place within the recruitment of RIP and TRAF2 without procaspase 8 processing. It has been thought that caspase 8 constitutes a skeletal structure without any enzymatic activity in this mechanism (Cullen *et al.*, 2013; Kreuz *et al.*, 2004). Noteworthily, although it has been shown that NFKB activation is abolished in the lack of FADD or caspase 8, pro-inflammatory molecules can be produced via receptor-interacting protein 3 (RIP3)-mediated NFKB activation (Kreuz *et al.*, 2004; Moriwaki *et al.*, 2014, Siegmund *et al.*, 2017). However, more research is required to explain the mechanism of this type of pro-inflammatory cytokine production.

Another important pathway initiated by Fas/FasL stimulation is the MAPK pathway. MAPK operates many biological processes: cell motility, cell death/survival, and inflammation (Kyriakis *et al.*, 2012). Death receptors can activate MAPK in different ways due to the recruitment of different molecules to their death domain. One of these ways is the apoptosis-associated pathway which is similar to MEKK1-mediated JNK activation, Fas stimulation causes caspase 3-mediated cleavage of another MAPK signaling cascade protein, serine/threonine kinase 4 (STK4, MST1). STK4 initiates the ERK, JNK, and p38 cascades, resulting in pro-inflammatory molecule production (Graves *et al.*, 1998). However, FasL-mediated MAPK activation is also possible without apoptosis by two different mechanisms: dependent or independent of caspase 8 activity.

In the caspase activity-dependent mechanism, mature caspase 8 or procaspase 8-FLIPL heterodimer induces JNK signaling and causes the production of pro-inflammatory factors via the proteolytic cleavage of Rho-kinase or the recruitment of RIP and TRAF2, respectively (Ehrenschwender *et al.*, 2010; Tummers & Green, 2017). Caspase-independent MAPK activation happens in several different ways. One of these is occurred by death domain-associated protein (DAXX), which is closely associated with Fas in the database named STRING and is recognized by DD of Fas. DAXX can induce JNK activation in the absence of caspase 8 and FADD by interacting with apoptosis signaling kinase-1 (ASK1) (Yang *et al.*, 1997; Chang *et al.*, 1998).

Finally, the PI3K/Akt pathway is induced by Fas stimulation. Activation of the FasL-mediated PI3K pathway generally results in the accumulation of Ca^{+2} in the cytosol. This accumulation has been associated with both apoptosis and survival in the literature (Boehning *et al.*, 2005; Wozniak *et al.*, 2006; Khadra *et al.*, 2011). In peripheral blood lymphocytes, FasL stimulation-mediated Ca^{+2} accumulation is achieved by a protein called phospholipase C γ1 (PLCγ1) and reduced FADD and procaspase 8 recruitment (Khadra *et al.*, 2011). The increase in Ca^{+2} in the cytosol leads to the interaction of calmodulin, a calcium sensor protein, and Src kinase family proteins with Fas and the induction of the Akt pathway (Yuan *et al.*, 2011). In this process, the Src substrate TRIP6 binds directly to PLCγ1, which is interacted with Fas and decreases the recruitment of FADD (Poissonnier *et al.*, 2016). Thus, instead of DISC formation, which is fundamental for cell death, it creates a complex called motility-inducing signaling complex (MISC) that provides cell migration (Lai *et al.*, 2010). As stated in Section 2.2.4, these mechanisms are usually carried out through sFasL, which is cleaved by metalloproteases (Poissonnier *et al.*, 2016). It has been reported that tyrosine kinase Yes protein, a component of MISC, can activate the PI3K/Akt pathway by an indirect association of EGF receptor tyrosine kinase with Fas receptor (Malleter *et al.*,2013). Basic schematization of apoptotic and non-apoptotic mechanisms of Fas/FasL pathway is given in Figure 2.

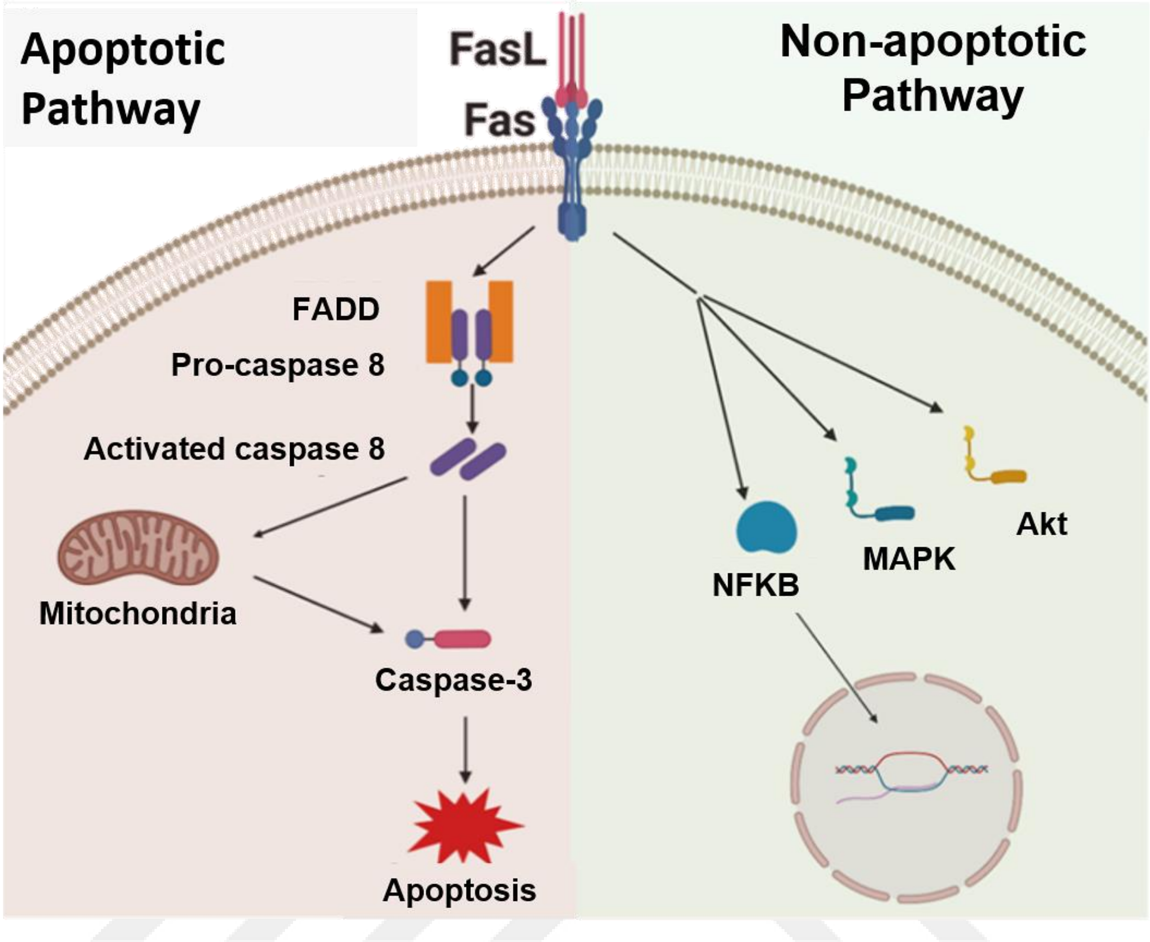


Figure 2. Apoptotic and non-apoptotic mechanisms of Fas/FasL pathway

2.3. The Role of Fas Stimulation in Macrophages

Macrophages are generally resistant to FasL-induced apoptosis due to the Fas-associated death domain-like interleukin 1β-converting enzyme-inhibitory protein (FLIP) upregulation during differentiation from monocytes (Perlman *et al.*, 1999). Therefore, FasL can cause apoptosis of monocytes but not macrophages (Kiener *et al.*, 1997; Park *et al.*, 2003; Brown *et al.*, 2004; Altemeier *et al.*, 2007; Isayama *et al.*, 2016; Wang *et al.*, 2010). In murine bone marrow-derived macrophages (BMDMs), sFasL stimulation fails to recruit FADD and therefore cannot initiate apoptosis. Besides, FasL stimulation increases the production of pro-inflammatory cytokines and chemokines in both monocytes and macrophages (Park *et al.*, 2003).

Pro-inflammatory cytokine production causes migration of neutrophils, thereby not only creating a response against the tumor but also impairing post-injury repair. (Shimizu, 2005; Lettelier *et al.*, 2010).

FasL-induced pro-inflammatory cytokine production in macrophages is low. For example, a study by Cullen *et al.* observed that Fas stimulation antibody-treated murine BMDMs secreted IL-6, TNFa, and MIP2 in a dose-dependent manner but in a relatively small amount (Cullen *et al.*, 2013). Importantly, FasL significantly increases the production of proinflammatory cytokines (eg TNFa, IL-6, and NO) by synergistic effect with various M1 polarization factors such as LPS and IFNg (Brennan *et al.*, 2018; Chakour *et al.*, 2009). Consistently, alveolar macrophages isolated in the chest trauma rat model produced more FasL-induced IL-6 and less IL-10 compared to those isolated from healthy rats. (Seitz *et al.*, 2011). Therefore, the Fas / FasL pathway is thought to reduce the function of M2 macrophages. Lastly, while FasL ligation significantly increases M1-like cytokine and chemokine production in M1 polarized macrophages but significantly decreases M2-like cytokine and chemokines in murine BMDMs (Isayama *et al.*, 2016).

The molecular mechanism of Fas/FasL signaling in macrophages has not yet been adequately uncovered. The most interacting molecules with death receptors are FADD, caspases, and some kinases. First, unlike monocytes, FasL-mediated proinflammatory response occurs caspase-independently in macrophages (Park *et al.*, 2003; Altemeier *et al.*, 2007).

Lettelier and colleagues (Lettelier *et al.*, 2010) revealed that FasL stimulation increased the phosphorylation of Src family kinases, Syk kinase, and Akt in murine BMDMs. It was also reported in this study that FasL-induced Syk activation increased macrophage migration and adhesion. In another study, Fas activation increased both extracellular secretion and intracellular accumulation of high mobility group box 1 (HMGB1) protein. The neutralization of HMGB1 decreased sharply pro-inflammatory factor production. Moreover, this pro-inflammatory response was abrogated in the absence of TLR4 or IRAK4 (Wang *et al.*, 2010). Noteworthily, the production of different proinflammatory cytokines may be dependent on different proteins.

For example, TNFa production occurs in MyD88-independent but IL-6 production in a MyD88-dependent manner (Chakour *et al.*, 2009). A recent study reported that, in macrophages, while Fas activation induced the NFKB pathway, it sharply reduced IFNb production through the FADD-DD domain, the negative form of FADD (Brennan *et al.*, 2018).

3. MATERIALS AND METHODS

3.1. Type of Research

This study is an *in vitro* experimental study.

3.2. Date and Location of Research

All experiments were performed at the Izmir Biomedicine and Genome Center, between 2017 and 2020.

3.3. Universe and Sample of Research

Human primary peripheral blood monocyte-derived macrophages were used in this study.

3.4. Variables of Research

Independent Variables: FasL concentration, Polarization Factors

Dependent Variables: M1, M2a, and M2c macrophage polarization markers

3.5. Materials

3.5.1. General laboratory equipment

Cell Analyzer	BD LSRFortessa
Centrifuge	Eppendorf, 5702 and 5415R
CO_2 Incubator	Memmert, INCO153
Electrical Pipette Pump	ISOLAB
Freezer (-20^oC)	Bosch
Freezer (-80^oC)	Thermo Scientific, Forma 700 series
Ice Maker	Hoshizaki FM300
Inverted Microscope	Carl Zeiss, Axio Vert.A1
Laminar Flow Cabinet	Thermo Scientific, Thermo Safe 2020

Microcentrifuge	Thermo Fisher Scientific, Thermo Micro CL 17R
Microplate Reader	Thermo, Varioscan Flash
Mini Spin Centrifuge	Thermo Scientific, MYSPIN 6
Multichannel Pipette	200 µl Rainin
Nanodrop 2000	Thermo Scientific™
Pipette	Eppendorf :0,2-2µl, 0,5-10µl, 2-20µl, 20-100µl, 30-200µl, 200-1000µl
Real-Time PCR	Roche, LightCycler 480 II
Refrigerator	Bosch
Vortex	Thermo Scientific, LP
Water bath	Nüve, NB 9

3.5.2. Chemicals, reagents, and commercial kits

10X Phosphate Buffered Saline, w/o Ca^{+2}, w/o Mg^{+2}	Gibco, ThermoFisher Scientific
1X Phosphate Buffered Saline, w/o Ca^{+2}, w/o Mg^{+2}	Gibco, ThermoFisher Scientific
cDNA Conversion Kit	EvoScript Universal cDNA Master kit
Cytofix/Cytoperm	BD Biosciences
Cytofix™ Fixation Buffer	BD Biosciences
Dimethyl Sulfoxide (DMSO)	Applichem

Dithiothreitol (DTT)	Bioshop Canada
FastStart Essential DNA Probes Master	Roche
Fetal Bovine Serum	Biochrome
Ficoll Paque PLUS	Sigma-Aldrich
Penicillin (10.000U/ml) /Streptomycin (10.000mg/ml)	ThermoFisher Scientific
Percoll	Sigma-Aldrich
Perm/Wash Buffer	BD Biosciences
Recombinant human FasL	R&D Systems
Recombinant Human IFN-γ	R&D Systems
Recombinant human IL-10	R&D Systems
Recombinant Human IL-4	R&D Systems
Recombinant Human M-CSF	Peprotech
RPMI 1640, w/o Phenol red, with 2.0 g/l $NaHCO_3$, w/o L-Glutamine	Gibco, ThermoFisher Scientific
RPMI 1640, with 2.0 g/l $NaHCO_3$, w/o L-Glutamine	Gibco, ThermoFisher Scientific
StemPro Accutase Cell Dissociation Reagent	Gibco, ThermoFisher Scientific
Total RNA Isolation Kit	GeneMark
Trypan Blue (0.4%)	Biological Industries

Ultrapure LPS	Sigma
Zombie UV Live/Dead Staining Kit	Biolegend

3.5.3. Consumables

0,2 ml PCR Tube	Axygen
0,5 ml PCR Tube	Sarstedt
1,5 ml Tube	Greiner
250 ml Bottle Filtration System 0.22um	Millipore
5 ml FACS Tubes	Corning
Cryo Vials	Greiner
Disposable serological pipette: 5,10 and 25 ml	Greiner
Filter Pipette Tips: 10, 200, and 1000 µl	Neptune
Hemocytometer	Sigma-Aldrich
LightCycler 480 Plates	Roche
LightCycler® 480 Sealing Foil	Roche
Nunc-Immuno™ MicroWell™ 96 well solid ELISA plates	Thermo Scientific
Pipette Tips: 10, 200, and 1000 µl	Vertex
Polypropylene Tubes: 15 and 50 ml	Greiner

The LiteTouch System (LTS) Pipette Tips	Greiner
Tissue Culture Plates: 6, 12, 24, 48, and 96 well	Greiner
Ultra-Low Attachment 6 well plates	Corning

3.5.4. qPCR primers

Macrophage Phenotype	Marker Gene	Catalog Number
M1	TNFα	100134777
	IDO-1	100134768
	CXCL-10	100134759
	IL-1b	100126016
M2a	MRC-1	100134731
	TGM-2	100134722
M2c	IL-10	100133437
	CD-163	100134801
Housekeeping	β-actin	100074091

Table 2. List of qPCR probes used in this study

3.5.5. Antibodies

	Macrophage Phenotype	Specificity	Conjugate	Clone	Origin	Isotype	Company
Surface Markers	M1	CD86	BV605	IT2.2	Mouse	IgG2b, κ	BioLegend
		HLA-DRα	APC-Cy7	L243	Mouse	IgG2a, κ	BioLegend
			eF450	L243	Mouse	IgG2a, κ	e-Bioscience
			PE	L243	Mouse	IgG2a, κ	e-Bioscience
		CD64	PerCP-Cy5.5	10.1	Mouse	IgG1, κ	BioLegend
	M2a	CD200R	BV421	OX-104	Mouse	IgG1, κ	BioLegend
		CD206	AF700	15-2	Mouse	IgG1, κ	BioLegend
	M2c	CD163	PE-Cy7	GHI/61	Mouse	IgG1, κ	BioLegend
Intracellular Markers	M1	TNFa	AF700	MAb11	Mouse	IgG1, κ	BioLegend
			PerCP-Cy5.5	MAb11	Mouse	IgG1, κ	e-Bioscience
			PE	MAb11	Mouse	IgG1, κ	e-Bioscience
		CXCL10	AF647	33036R	Mouse	IgG1	Biolegend
		IFNγ	BV785	4S.B3	Mouse	IgG1, κ	BioLegend

			PE	4S. B3	Mouse	IgG1, κ	e-Bioscience
		IL-6	PE	MQ213A5	Rat	IgG1, κ	e-Bioscience
	M2a	IL-4	PE-Cy7	8D4-8	Mouse	IgG1, κ	ThermoFisher
	M2c	IL-10	eF660	JES3-9D7	Rat	IgG1, κ	ThermoFisher
		TGF-β1	PE	TW4-2F8	Mouse	IgG1, κ	BioLegend
	Common	CD68	FITC	Y1/82A	Mouse	IgG2b, κ	BioLegend

Table 3. List of the antibodies used in the study

3.5.6. Solutions

Complete RPMI	RPMI 1640 with 1% Penicillin-Streptomycin and 5% FBS
Percoll Solution	46.25% Percoll, 3.75% 10XPBS, 50.00% Complete RPMI
FACS Buffer	1X PBS with 1% BSA and 0,1 % sodium aside
Freezing Medium	70% Complete RPMI, 20% FBS and 10% DMSO
RNA Lysis Solution	RNA Lysis Buffer with 15mM DTT
Antibody Cocktail for Cell Surface Staining	Antibodies diluted in FACS buffer: CD86 (1:400), HLA-DRα (1:400), HLA-DRα (1:400), CD200R (1:400), CD206 (1:400), CD163 (1:200)
Antibody Cocktail for Intracellular Staining	Antibodies diluted in Perm/Wash Buffer: CXCL-10(1:100), TNFa (1:50), CXCL10 (1:100), IL-6 (1:100)
Protein Transport Inhibitor Cocktail	BD GolgiPlug™(1 mg/ml in DMSO) / BD GolgiStop™ (2 mg/ml in ethanol)
Ficoll Paque PLUS	Ficoll PM400 and sodium diatrizoate with calcium disodium ethylenediamine-tetra acetic acid

Table 4. Contents of the solutions used

3.6. Data Collection Methods

3.6.1. Generation and stimulation of monocyte-derived macrophages

Buffy coat samples which include leukocytes and platelets from the whole blood sample were supplied by the Blood Center of Dokuz Eylul University. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat by density gradient. Since the density of PBMCs (1,077-1,04 g/ml) does not allow these cells to pass through into Ficoll-Paque PLUS, these cells collect as a concentrated phase at the interface of the gradient. Then, monocytes were separated from lymphocytes on a high-density hyper-osmotic Percoll density gradient and cultured with complete RPMI containing human macrophage colony-stimulating factor (M-CSF) for 1 week to generate HMDMs. PBMCs and monocytes were isolated by density gradient centrifugation (Figure 3)

3.6.1.1. Isolation of human peripheral blood mononuclear cells from the buffy coat

Approximately 20 ml of the buffy coat suspension was transferred into a 50 ml tube and centrifuged at 400g for 7 minutes at room temperature (RT). The upper plasma layer was discarded and the pellet was suspended in 25 ml 1X PBS. Then, 15 ml of Ficoll-Paque® Plus was added to 50 ml tubes first and a 25 ml diluted buffy coat was carefully laid on top. Tubes were centrifuged at 400g for 30 minutes at RT without any break. The middle layer containing peripheral blood mononuclear cells (PBMC) was collected using Pasteur pipette and it was taken care not to take any Ficoll-Paque® Plus since it inhibits the pellet formation. The collected cells were washed twice with 1X PBS at 400g for 7 minutes at RT. The pellet was collected and then suspended in 30 ml of complete RPMI (w/o Phenol Red).

3.6.1.2. Isolation of monocytes from peripheral blood mononuclear cells

Two tubes were prepared for each PBMC sample. The first 25 ml of the Percoll solution was added to the tubes and 15 ml of PBMC samples suspended in complete RPMI (w/o Phenol Red), were carefully laid on top. Tubes were centrifuged at 550g for 30 minutes at RT without any break. The middle layer, containing monocytes, was collected and washed twice with 1X PBS at 400g for 7 minutes at RT. The pellet was suspended in 20 ml of complete RPMI.

Acquired monocytes were counted with a hemocytometer. 20 µl of the cell suspension mixed with an equal volume of Trypan Blue. The mixture was filled into both sides of the chamber and counted using an inverted phase-contrast microscope.

3.6.1.3. Generation of monocyte-derived macrophages

<u>Macrophage generation:</u> Monocytes were diluted to a concentration of $1x10^6$ cells/ml in complete RPMI in the presence of 10 ng/ml recombinant human M-CSF and seeded in ultra-low attachment 6-well plates ($5x10^6$ cells/well/5 ml). Cells were cultured at 37^oC and 5% CO_2 and 95% humidity for 7 days. Afterward, the supernatant was removed and adherent cells were collected with pipetting. Collected cells were washed twice with 1X PBS at 400g for 7 minutes at RT. The pellet was resuspended in complete RPMI and counted.

<u>Cryopreservation of macrophages:</u> To freeze, the cell suspension was centrifuged and suspended to obtain a solution containing $5x10^6$ cells/ml in a freezing medium. Each milliliter of the solution was added to a cryovial tube and cryovial tubes were placed inside Mr. Frosty Freezing Container (a passive freezer). The freezer was filled with isopropyl alcohol and placed at -80^oC for 24 hours. Frozen tubes were transferred to a storage box and stored at -80^oC for up to 1 month. Frozen macrophages were thawed in a 37°C water bath, quickly transferred to a 50 ml tube containing 10 ml of 37°C complete RPMI. The cells were washed twice with complete RPMI at 400g for 7 minutes at RT and suspended in complete RPMI.

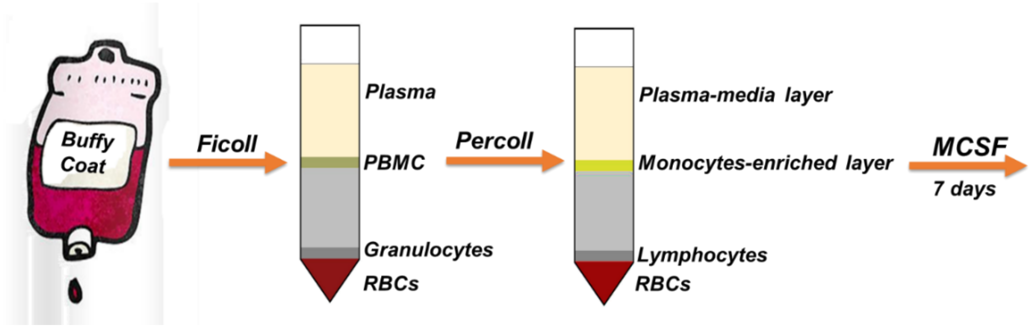


Figure 3. Macrophage generation procedure.

3.6.1.4. Stimulation and polarization of monocyte-derived macrophages

HMDMs were seeded in a 24-well cell culture plate at a concentration of $7x10^5$ cells in 1 ml of complete RPMI and incubated overnight at 37ºC and 5% CO_2 and 95% humidity. The supernatant was removed and HMDMs were stimulated with soluble FasL at a concentration of 50, 100, and 200 ng/ml in 500 µl of complete RPMI for 2 hours and polarized simultaneously with appropriate stimulants: LPS (100 ng/ml) plus IFNγ (20 ng/ml) for M1 macrophages, IL4 (20 ng/ml) for M2a macrophages and IL-10 (10 ng/ml) for M2c macrophages in accordance with the literature (Vogel, 2014; Raggi, 2014; Genin, 2015; Iqbal, 2015). Stimulation time was determined as 6 hours for gene expression and intracellular cytokine staining studies and 12 hours for surface staining.

3.6.2. Gene expression analysis of M1 and M2 markers by quantitative PCR

Macrophage subsets have different transcriptomic levels and certain genes are used as markers in the classification of macrophage phenotypes (Gerrick *et al.*, 2018). To understand the effect of the FasL on macrophage polarization at the transcriptomic level, qPCR was performed with the markers given in Table 2.

3.6.2.1. RNA isolation and cDNA conversion

The concentration and purity of RNA samples were measured using the NanoDrop Spectrophotometer (Thermo Scientific). The measurement was performed using 1 µl of RNA samples. It was expected that the concentration of RNA samples to be 50-100 ng/µl because of the cell number. A260/280 ratio of 2.0 was accepted as pure for RNA. Since a low A260/280 ratio indicates potential DNA or protein contamination. A260/230 ratio was used as another measurement of RNA purity. A260/A230 ratio of >2.0 was accepted as pure for RNA. While the A260/230 ratio could be lower than 2.0 due to failure to completely clean the spin columns, this impurity didn't influence the next steps.

<u>cDNA conversion:</u> EvoScript Universal cDNA Master kit was used to convert the RNA samples to complementary DNA. cDNA synthesis was performed equalizing the RNA concentration to 1 µg/20 µl. 14 µl of RNA template and 4 µl of 5X Reaction Buffer containing the mixture of dNTP, random primers, reaction buffer, and RNAse inhibitors were added in the 200 µl tubes. Tubes were mixed and centrifuged shortly and incubated on the ice for at least 5 minutes to let primers anneal to RNA. Finally, 2 µl of 10X Enzyme Mix was added and mixed. Thermal Cycler (BioRad) was used to carry out cDNA synthesis. The reaction mixture was heated to 42°C for 15 minutes, 85°C for 5 minutes, and +65°C for 15 minutes and cooled to +4°C with unlimited holding time. The obtained cDNA was diluted to 10ng/µl and was stored at -20°C.

3.6.2.2. qPCR analysis of M1 and M2 marker genes

qPCR was performed on the LightCycler480 II in a 10µl final volume containing 0,5 µl of 20X primer-probe mix, 5 µl of 2X TaqMan Probe Master, 2µl of nuclease-free water, and 2,5 µl of diluted cDNA in accordance with manufacturer's instructions. The PCR cycling conditions were an initial denaturing step of 15 minutes at 95° C followed by 45 cycles of 95° C for 10 seconds, 60° C for 30 seconds, and 40° C for 30 seconds, and then a cooling at 40 °C for 30 seconds. Three replicates per sample were carried out and differences were analyzed by the two-tailed Student's t-test.

The housekeeping gene β-actin and the cycle threshold method were used for the normalization of the results. Relative quantification of gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

3.6.3. Analysis of M1 and M2 cell surface markers by flow cytometry

Cell surface markers are displayed on the cell surface and give information about the type and function of the cell. Macrophage polarization can be identified based on the cell surface markers. To understand the effect of the FasL on macrophage phenotype and polarization, cell surface staining was performed with the antibodies given in Table 3.

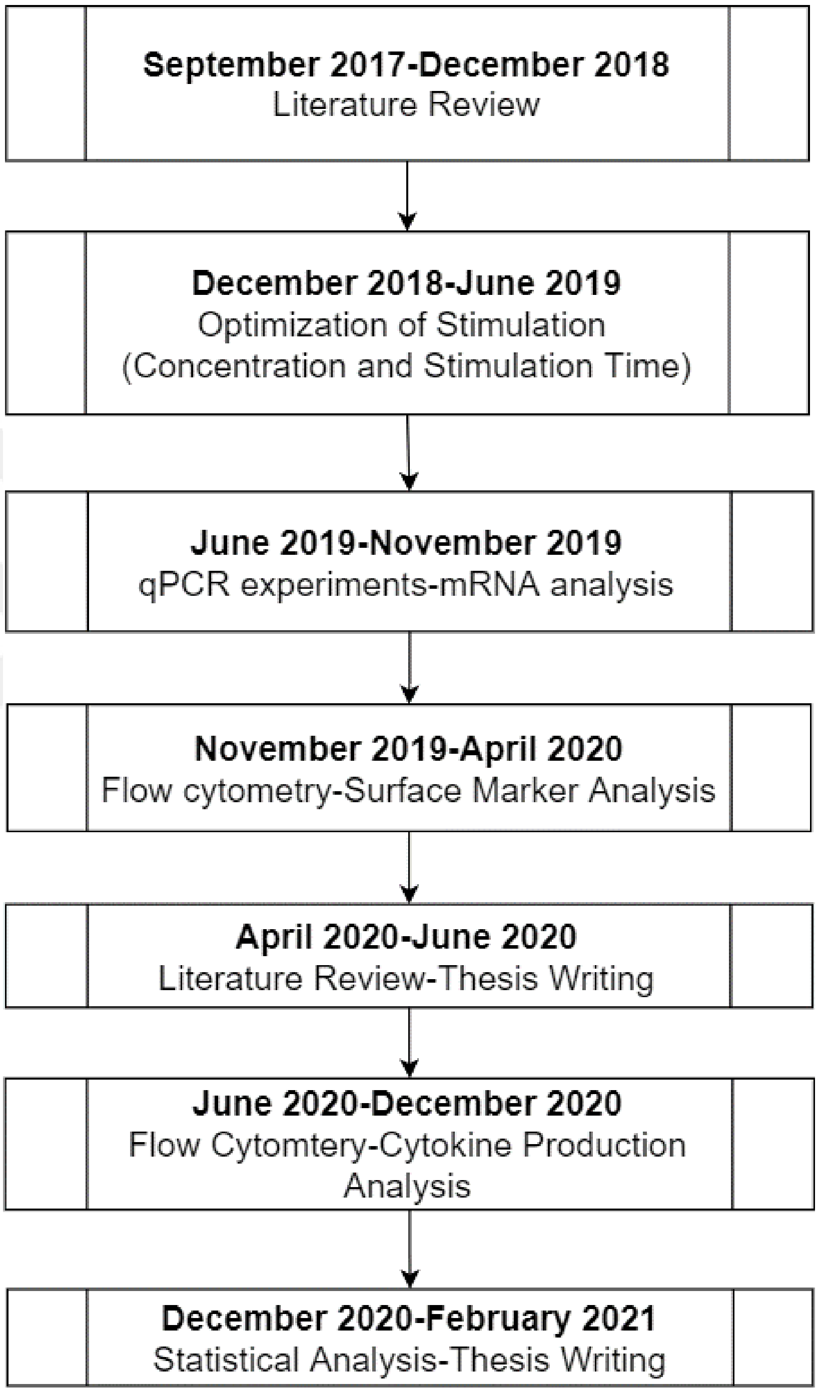
The supernatant was discarded and 150 µl of StemPro Accutase® compatible with flow cytometry was added to each well and incubated for 1 hour. Following incubation, cells were harvested and transferred to a V-bottom 96-well plate with 0,6-0.8x10^6 cells/well. The cells were washed twice with 100 µl of 1X PBS by centrifugation at 400xg for 5 minutes at 4°C. Zombie UV dye used for live-dead staining was diluted with 1x PBS. 50 µl of diluted Zombie UV dye was added to the live-death compensation control and samples. 50 µl of 1X PBS was added to other compensation controls and the plate was incubated for 15 minutes on ice in the dark. The cells were washed twice with 100 µL of 1X PBS by centrifugation at 400xg for 5 minutes at 4°C. The pellet was suspended with 25 µl of FACS buffer containing 1:100 diluted αCD16/32 (Fc receptor) antibody and incubated for 15 minutes on ice. Then, the cells were resuspended with 25 µl of antibody cocktail mix and incubated for 40 minutes on ice in the dark. At this stage, 25 µl of single dyes were added to relevant compensation controls. After incubation, the cells were washed twice with 100 µl of FACS buffer by centrifugation at 400xg for 5 minutes at 4°C. The pellet was suspended with 100 µl of BD Cytofix™ buffer and incubated for 15 minutes on ice in the dark. The stained and fixed cells were washed twice with 100 µl of FACS buffer by centrifugation at 450xg for 5 minutes at 4°C and suspended with 100 µl of FACS buffer. The expression of cell surface markers was analyzed on BD LSRFortessa™ flow cytometer and the data analyses were processed using FlowJo (TreeStar) software.

3.6.4. Analysis of M1 and M2 intracellular markers by flow cytometry

Macrophage polarization can be defined according to their intracellular molecules, such as some transcription factors or cytokines. To determine the effect of the FasL on macrophage polarization intracellular staining was performed with the antibodies given in Table 3.

The supernatant was discarded and 150 µl of StemPro Accutase® compatible with flow cytometry was added to each well and incubated for 1 hour. Following incubation, cells were harvested and transferred to a V-bottom 96-well plate with 0,6-0.8x10^6 cells/well. The cells were washed twice with 100 µl of 1X PBS by centrifugation at 400xg for 5 minutes at 4°C. Zombie UV dye used for live-dead staining was diluted with 1x PBS. 50 µl of diluted Zombie UV dye was added to the live-death compensation control and samples. 50 µl of 1X PBS was added to other compensation controls and the plate was incubated for 15 minutes on ice in the dark. The cells were washed twice with 100 µL of 1X PBS by centrifugation at 400xg for 5 minutes at 4°C. The pellet was suspended with 50 µl of FACS buffer containing 1:100 diluted αCD16/32 (Fc receptor) antibody and incubated for 15 minutes on ice. The cells were washed twice with 100 µl of 1X PBS by centrifugation at 400xg for 5 minutes at 4°C. Then, the cells were suspended with 100 µl of BD CytoFix/CytoPerm Buffer and incubated for 15 minutes on ice. Afterward, the cells were washed twice with BD Perm/Wash Buffer at 400xg for 5 minutes at 4°C. The pellet was suspended with 50 µl of antibody cocktail mix and incubated for 30 minutes at RT in the dark. At this stage, 50 µl of single dyes were added to relevant compensation controls. After incubation, the cells were washed twice with 100 µl of BD Perm/Wash buffer by centrifugation at 400xg for 5 minutes at 4°C. The pellet was washed once with 100 µl of FACS buffer by centrifugation at 400xg for 5 minutes at 4°C and suspended with 100 µl of FACS buffer. The expression of cell surface markers was analyzed on BD LSRFortessa™ flow cytometer and the data analyses were processed using FlowJo (TreeStar) software.

3.7. Research Plan



3.8. Evaluation of Data

All data are reported as the standard error of the mean (SEM). Graphs were generated with GraphPad Prism 8 (GraphPad Software, San Diego, CA). Statistical analysis was performed in GraphPad Prism using a 2-tailed Student's t-test. For all analyses, $P < 0.05$ was considered significant, and $p<0.05$, denoted by *, $p<0.01$ was denoted by **, and $p < 0.001$ was denoted by ***. Flow cytometry data were analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

3.9. Limitation of Research

There were no limitations in this study.

3.10. Ethics Committee Approval

This thesis was approved by the Non-Invasive Research Ethical Committee of Dokuz Eylul Universiy with the protocol number 2021/03-38 on 01.02-2021. The thesis approval is presented in Section 8.1.

4. RESULTS

4.1. Macrophage Differentiation

Human peripheral blood monocytes were isolated from healthy donors' buffy coat using Density Gradient Centrifugation. The obtained monocyte-enriched layer was cultured with MCSF for 7 days to generate monocyte-derived macrophages. To test the purity of differentiation, intracellular staining of CD68 marker expression was performed to identify macrophages. With this protocol, approximately 90% HMDMs differentiation efficiency was achieved (Figure 4).

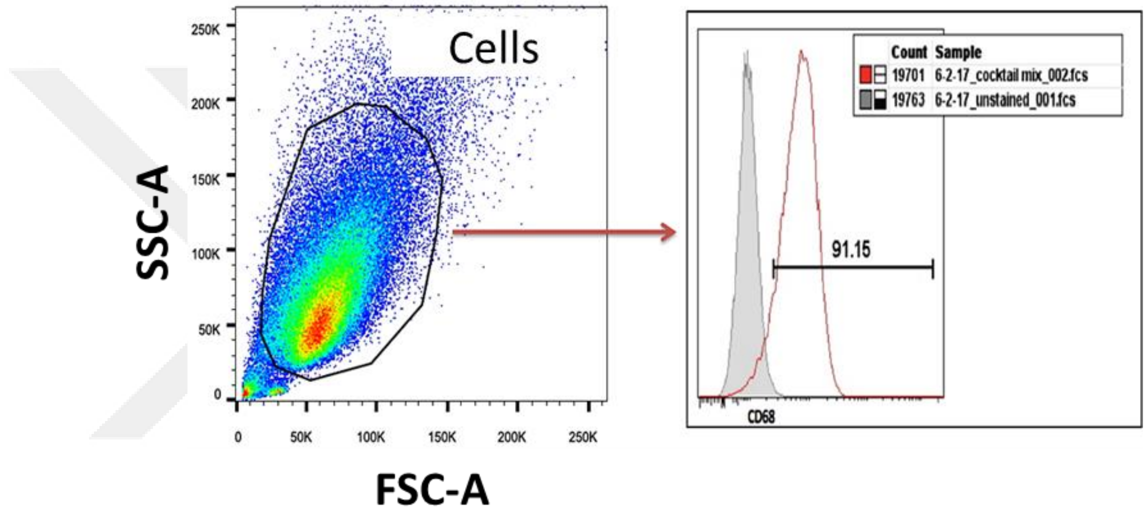
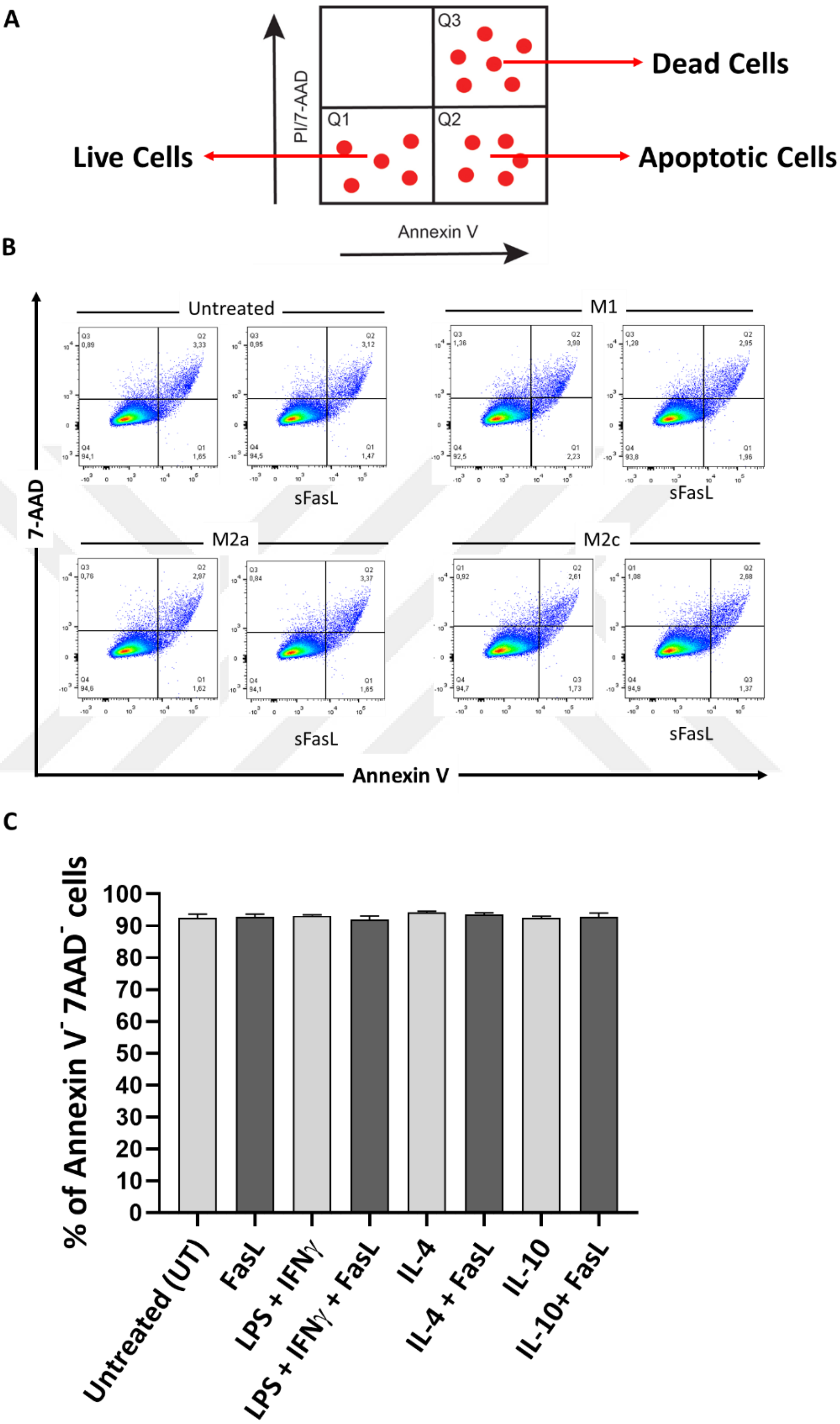


Figure 4. Macrophage Differentiation. CD68: Human Macrophage Marker

4.2. Apoptosis Assay

It was assessed whether the soluble FasL (sFasL) can cause cell death in HMDMs or not. Apoptosis of HMDMs was determined with Annexin V/7-AAD staining and analyzed by flow cytometry. To this end, macrophages were polarized into M1, M2a or M2c phenotypes with LPS/IFNg, IL-4, or IL-10, respectively in the presence or absence of sFasL for 12 hours. With this staining, 3 different populations were observed; double-positive cells were considered as dead, double-negative cells as alive, and Annexin V^+ 7-AAD^- cells as apoptotic (Figure 5A). As a result, it was revealed that sFasL did not cause cell death in HMDMs regardless of the polarization state (Figure 5B and 5C).

A



Q3
Q1
Q2
PI/7-AAD
Dead Cells
Live Cells
Apoptotic Cells
Annexin V
B
Untreated
M1
M2a
M2c
sFasL
sFasL
sFasL
sFasL
7-AAD
Annexin V
C
% of Annexin V⁻ 7AAD⁻ cells
100
90
80
70
60
50
40
30
20
10
0
Untreated (UT)
FasL
LPS + IFNγ
LPS + IFNγ + FasL
IL-4
IL-4 + FasL
IL-10
IL-10+ FasL

Figure 5. sFasL causes cell death in neither non-polarized nor polarized HMDMs. HMDMs were treated with 200 ng/ml sFasL and polarized into M1 with 100 ng/ml LPS and 20 ng/ml IFNg, M2a with 20 ng/ml IL-4 or M2c with 10 ng/ml IL-10 for 12 hours. sFasL-induced apoptosis was detected by labeling Annexin V and 7-AAD. A) Simplified dot plot diagram of Annexin V/7-AAD staining. Q1: Annexin V-7-AAD- healthy cells, Q2: Annexin V+ 7-AAD- healthy cells, Q3: Annexin V+7-AAD+ dead cells. B) Pseudo-color plots of sFasL-induced apoptosis staining of macrophages polarized into different phenotypes. C) Bar graphs show the representative percentages of Annexin V-7-AAD- live cells. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments.

4.3. The Impact of sFasL Stimulation on Non-polarized Macrophages

Fas activation induces the pro-inflammatory response through non-apoptotic mechanisms in different cell types, including macrophages (Park *et al.*, 2003; Cullen et al., 2013). Therefore, it was first investigated whether sFasL affects the primary human macrophage response alone, and if it does, whether this effect is in a dose-dependent manner or not. To this end, HMDMs were stimulated with sFasL for 6 hours. The expression of M1 markers TNFa, CXCL10, IDO-1, and IL-1b; M2a markers MRC1 and TGM2; and M2c markers IL-10 and CD163 at mRNA level were assessed by qPCR. Interestingly, in contrast to the literature (Park *et al.*, 2003; Altemeier et al., 2007), sFasL stimulation had no significant effect on pro-inflammatory M1 gene expression (Figure 6A). Similarly, expressions of M2a (Figure 6B) and M2c (Figure 6C) marker genes were not affected by sFasL. Additionally, there was no significant dose-dependent alteration in the sFasL-induced macrophage response.

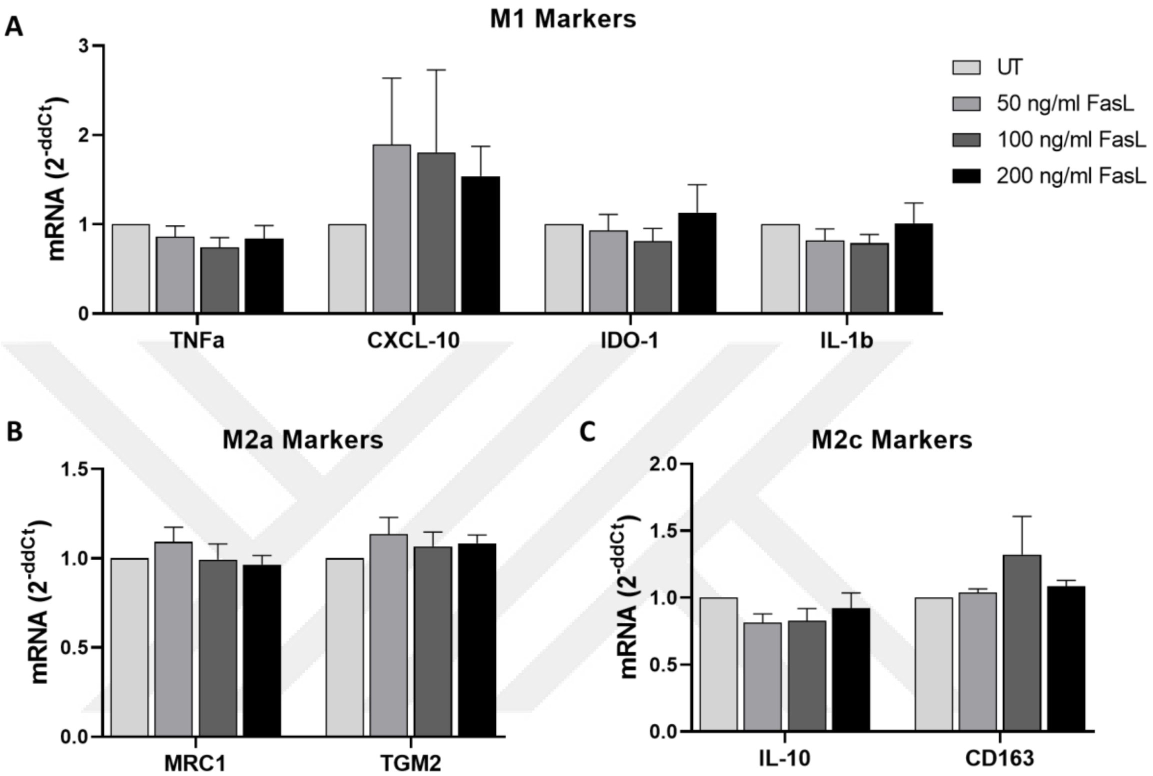


Figure 6. sFasL does not alter the macrophage response. HMDMs were stimulated with sFasL alone for 6 hours. The relative mRNA expressions of A) M1 (TNFa, CXCL-10, IDO-1, and IL-1b), B) M2a (MRC1 and TGM2), and C) M2c (IL-10 and CD163) marker genes were detected by qPCR. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments.

4.4. The Impact of sFasL stimulation on M1 Polarization

Brennan et al. showed that the agonistic Fas antibody CH-11 increased LPS and IFNg-induced M1 polarization in the THP-1 cell line. (Brennan *et al.*, 2018) Therefore, we investigated whether Fas activation leads to such synergy in human primary macrophages. HMDMs were treated with sFasL and M1 polarization factors LPS/ IFNg for 6 hours simultaneously. Gene expression of M1 markers was measured by qPCR. The expression of TNFa, CXCL-10, and IL-1b increased in the presence of 50 and 200 ng/ml sFasL, while IDO-1 expression increased in the presence of 100 and 200 ng/ml sFasL, compared to LPS/IFNg alone. As the next step, the expression of M1 markers were analyzed at protein level. HMDMs were stimulated with M1 polarization factors and sFasL for 6 hours simultaneously. Since the most promising response was obtained with the use of 200 ng/ml sFasL, the next experiments were performed with this concentration. Golgi Plug-Golgi stop cocktail was added to accumulate cytokines in the cell at the second hour of stimulation. Intracellular staining was performed and the expression levels of TNFa, CXCL-10, and IL-6 cytokines were measured by flow cytometry. Unlike the gene expression results, sFasL stimulation did not affect M1-related cytokine production in macrophages polarized to M1 (Figure 8). Finally, M1 surface markers were examined to understand the effect of sFasL on the M1 polarization of HMDMs. For this purpose, HMDMs were treated with LPS and IFNg for 12 hours in the presence or absence of sFasL. Surface markers specific to the M1 phenotype (HLA-DRa, CD86, and CD64) were assessed by flow cytometry. Similar to the cytokine production, sFasL did not cause any change in M1 surface marker levels. Next, to determine the impact of sFasL stimulation on M2 markers, we analyzed M2a and M2c markers in sFasl treated M1 macrophages. It was observed that sFasL slightly increased CD163, an M2c marker, in macrophages polarized into M1 (Figure 10A). As macrophages need to be polarized for at least 12 hours to detect surface markers, this observation may suggest that prolonged sFasL stimulation may have a negative effect on M1 cytokine production, although it did not cause any change in M1 surface markers. Therefore, next, HMDMs were stimulated with LPS and IFNg in the presence or absence of sFasL for 12 hours.

The Golgi Plug-Golgi Stop cocktail was added 2 or 6 hours after the start of stimulation. The purpose of this was to eliminate the early produced CXCL-10 to investigate whether or not the cytokine response of M1 polarized macrophages was impaired due to prolonged sFasL stimulation. However, prolonged sFasL stimulation did not affect CXCL-10 production (Figure 10B).

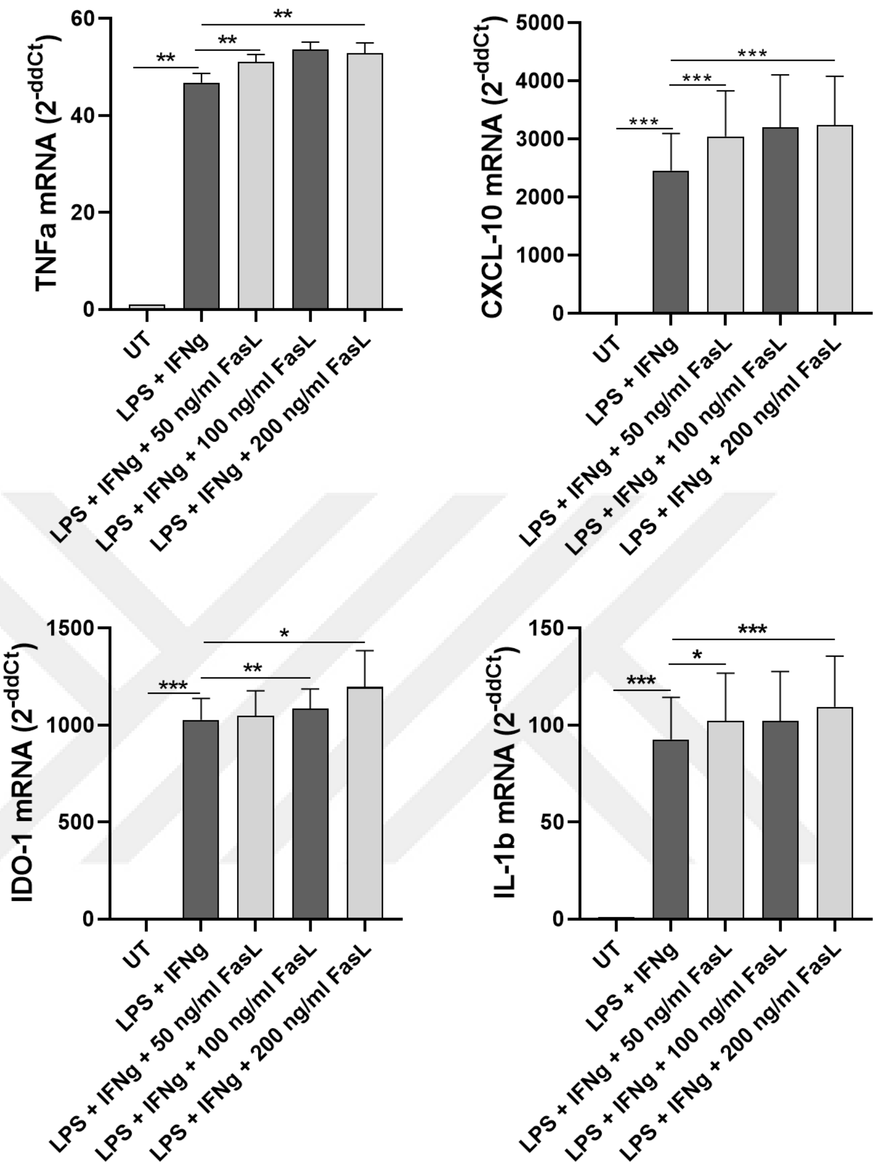


Figure 7. In M1 macrophages, sFasL increases the expression of some M1 markers at mRNA level. HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 6 hours. The relative mRNA expression of M1 markers TNFa, CXCL10, IDO-1, IL-1b was detected by qPCR. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 by Student t test.

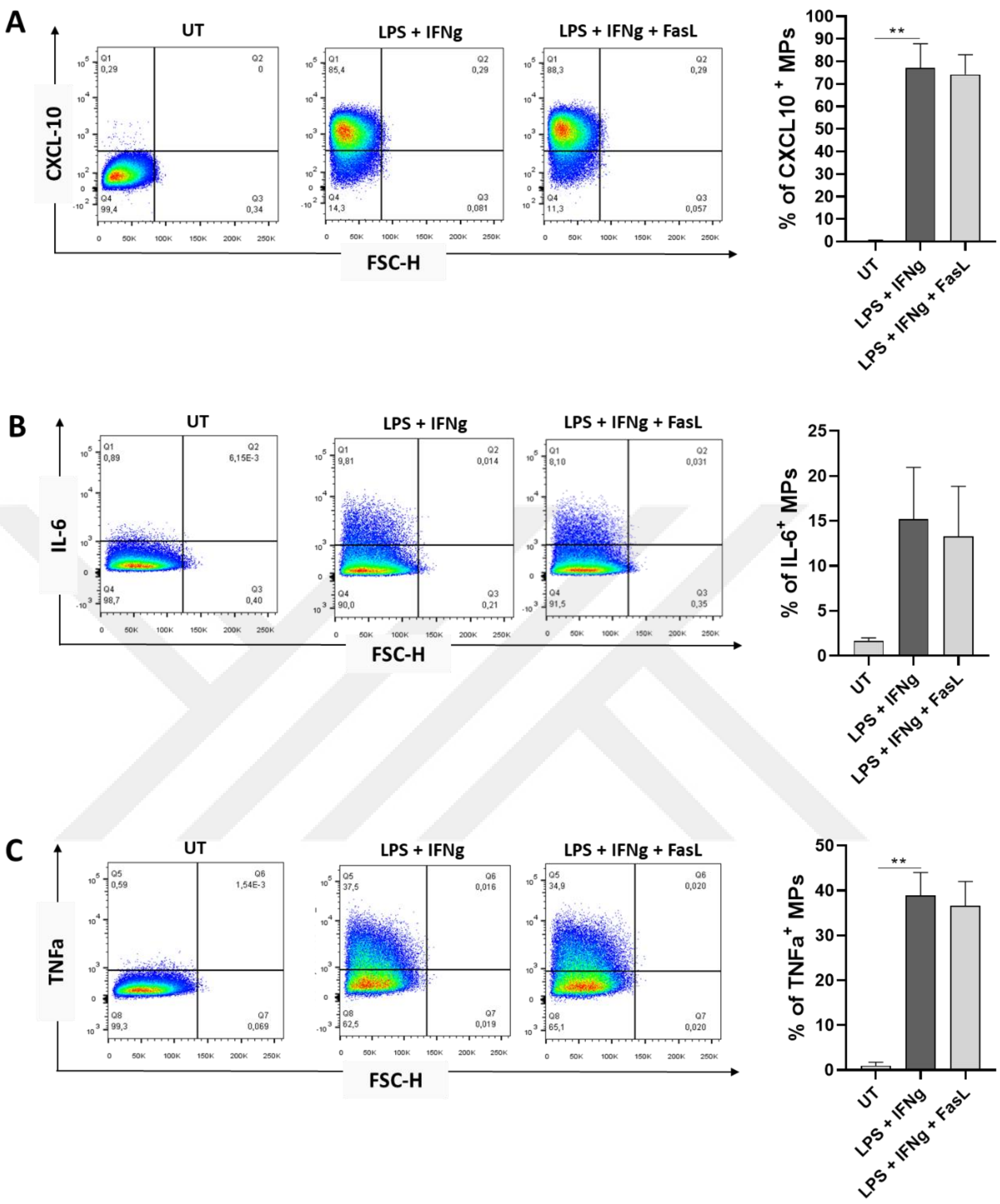


Figure 8. sFasL does not affect M1 cytokine production. HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 6 hours. The cytokine production of the M1 phenotype was measured by flow cytometry. Bar graphs show the percentage of cytokine expression. MPs: macrophages. Representative pseudo-color plots and bar graphs of sFasL-induced CXCL-10 (A), IL-6 (B), and TNFa (C) production in M1-polarized macrophages. ***p* ≤ 0.01 by Student t test.

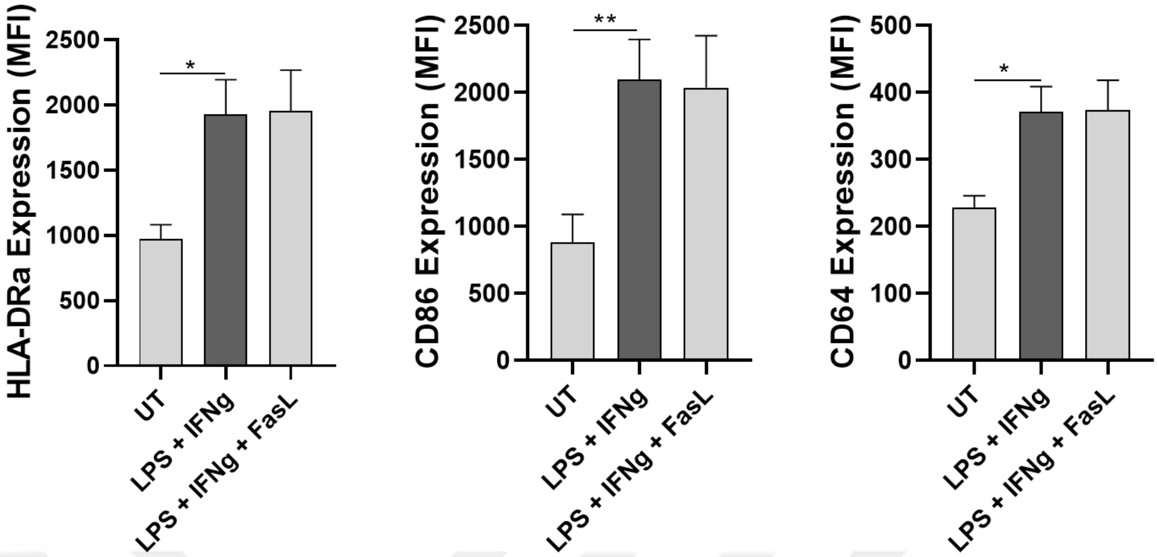


Figure 9. sFasL does not change M1 surface marker expression in M1 polarized macrophages. HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 12 hours. The surface marker expression levels of the M1 phenotype were determined by flow cytometry. Bar graphs show the mean fluorescence intensity of the surface markers. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments. *$p < 0.05$, **$p \leq 0.01$ by Student t test.

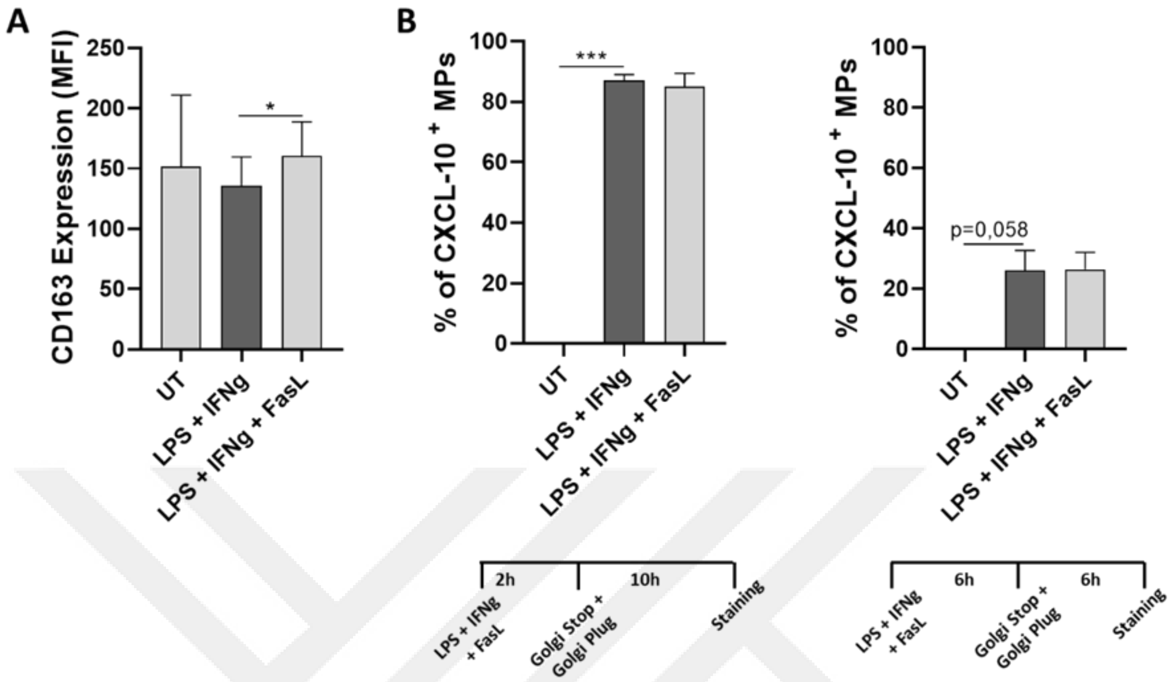


Figure 10. Prolonged stimulation of sFasL does not affect CXCL-10 production in M1 polarized macrophages. A) CD163 expression of M1 polarized macrophages. B) HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 12 hours. Golgi Plug/Golgi stop cocktail was added 2 or 6 hours after the stimulation started. CXCL-10 expression levels of macrophages were compared with flow cytometry. Bar graphs show the percentage of the CXCL-10 production. Data are shown as mean values ± SEM (n = 3). $*p < 0.05$, $***p \leq 0.001$ by Student t test.

4.5. The Impact of sFasL stimulation on M2a Polarization

In the literature, studies have focused on Fas-mediated pro-inflammatory response in macrophages. However, the effect of Fas activation on M2 response is not clear. Isayama et al. (2016) showed that Fas-agonist Jo-2 stimulation dramatically reduced IL-4 induced M2 response in mouse BMDMs and tissue-resident Kupffer cells. Yet, the effect of Fas activation on M2a polarization of human primary macrophages has not been investigated. Therefore, HMDMs were stimulated with IL-4 for 6 hours in the presence or absence of sFasL. M2a marker gene expressions (MRC1 and TGM2) were detected by qPCR. Our results showed that sFasL stimulation did not affect MRC1 and TGM2 gene expressions (Figure 11A). For the analysis of M2a surface markers, HMDMs were then stimulated with IL-4 for 12 hours in the presence or absence of sFasL. M2a surface markers were measured by flow cytometry. It was observed that the expression of CD206 surface marker decreased significantly. However, no significant change was observed in CD200R surface marker expression.

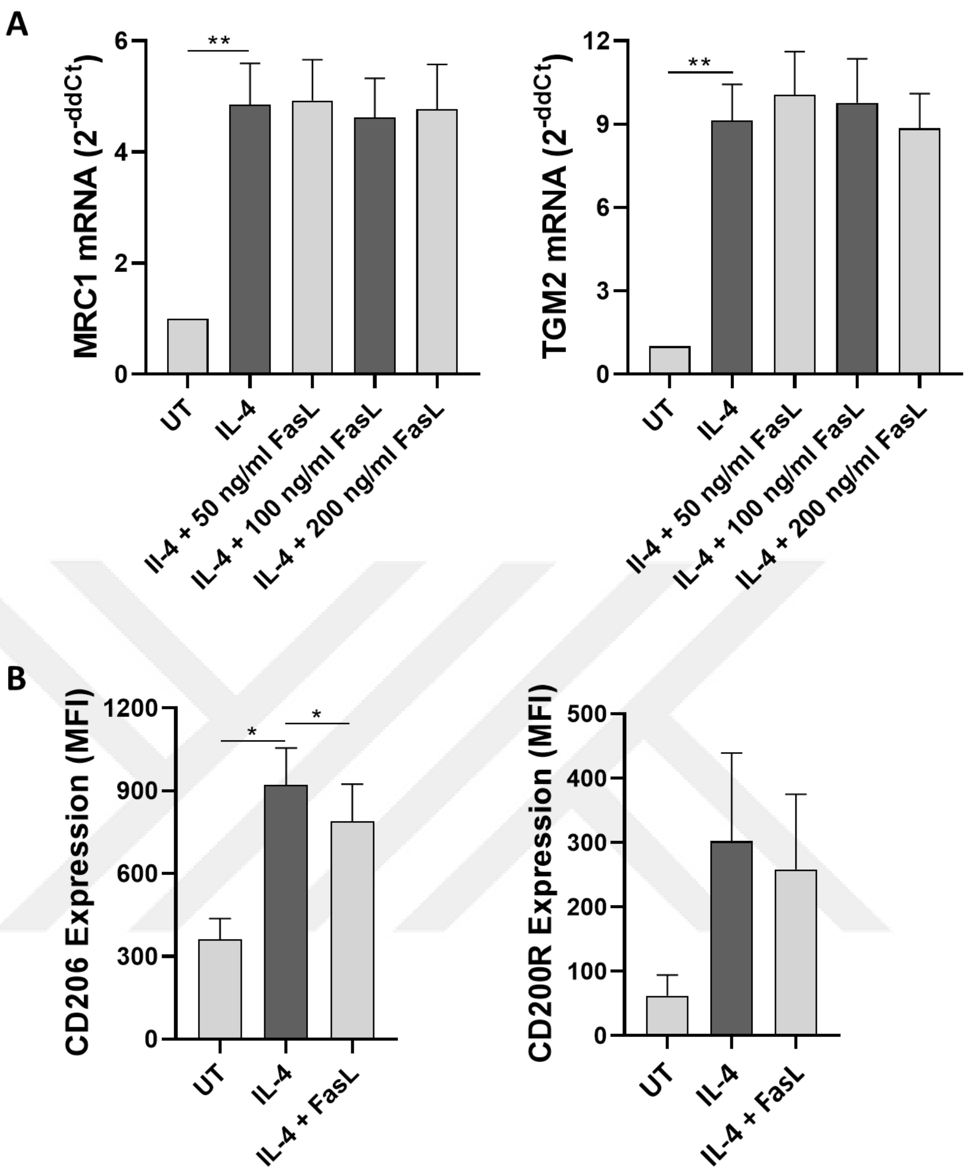


Figure 11. sFasL stimulation decreases CD206 surface marker expression but not CD200R in M2a polarized macrophages. HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 6 or 12 hours to analyze marker gene expression or surface markers, respectively. A) The relative mRNA expression of M2a markers. B) The mean fluorescence intensity of M2a surface markers. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments. **p* < 0.05, by Student t test.

4.6. The Impact of sFasL stimulation on M2c Polarization

In order to determine the impact of sFasL on IL-10-induced M2c polarization, HMDMs were polarized with IL-10 in the presence or absence of sFasL for 6 or 12 hours to analyze marker expression at mRNA or protein level, respectively. To this end, mRNA expression of IL-10 and CD163 marker genes were assessed by qPCR and CD163 surface protein was analyzed by flow cytometry. At mRNA level, there was a significant increase in CD163 expression in IL-10-induced macrophages while no change in IL-10 expression was observed. sFasL stimulation increased CD163 mRNA expression in 200 ng/ml sFasL stimulated group slightly (Fig 12 A, B). However, analysis at protein level revealed that CD163 surface marker expression did not change with the stimulation of sFasL (Fig 12 C). Therefore, we conclude that, sFasL stimulation did not impair or promote IL-10-induced M2c macrophage polarization.

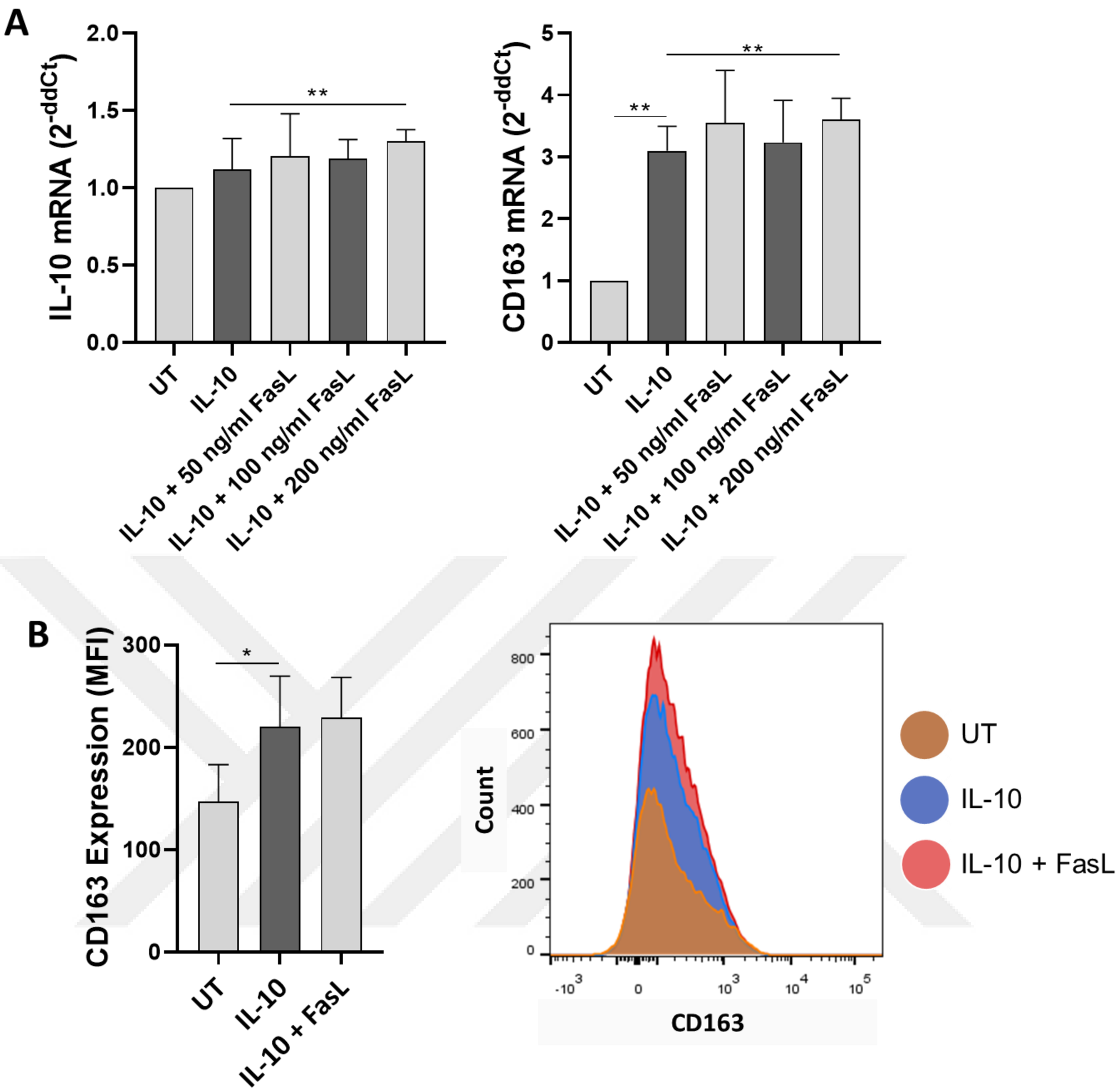


Figure 12. sFasL does not alter the M2c macrophage polarization. HMDMs were polarized into M1 phenotype with LPS and IFNg in the presence or absence of sFasL for 6 or 12 hours to analyze marker gene expression or surface markers, respectively. A) A) The relative mRNA expression of M2c markers; IL-10 and CD163 B) The mean fluorescence intensity of M2c surface marker CD163. Data are shown as mean values ± SEM (n = 3). Results are representative of two independent experiments. $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ by Student t test.

5. DISCUSSION

Macrophages are the innate immune cells localized almost throughout the body. Macrophages can polarize into pro-inflammatory or anti-inflammatory phenotype with appropriate signals. Due to the ability to orchestrate the initiation and resolution of inflammation, macrophages have a crucial role in development, wound healing, tissue homeostasis, and inflammatory diseases. Therefore, uncovering the mechanisms of macrophage polarization will enable us to understand the processes of inflammation and to develop promising immunotherapies.

Although the apoptotic mechanisms, which are the main functions of Fas/FasL interaction, have been clarified, our current knowledge about the non-apoptotic mechanisms of Fas activation is insufficient. Therefore, the non-apoptotic pathways of the Fas activation need to be elucidated. Studies investigating the effect of FasL on immune cells are very few in the literature, and most of the existing studies are related to T cells. However, there is no comprehensive study investigating the interaction between the Fas/FasL pathway and human primary macrophage polarization. Given the increased expression of FasL in many types of cancer and the correlation between increased FasL in the serum of cancer patients and poor prognosis, it was worthwhile to decipher this interaction (Kozlowski *et al.*, 2007; Tsutsami *et al.*, 2000; Rouge *et al.*, 2019).

In the literature, it has been reported that macrophages are resistant to Fas-mediated apoptosis, which is consistent with our results (Park *et al.*, 2003). Park et al previously reported that Fas activation increased the pro-inflammatory response in macrophages (Park *et al.*, 2003). However, contrary to this finding, our results showed that sFasL did not alter gene expression of pro-inflammatory and anti-inflammatory cytokines in non-polarized HMDMs. Several recent studies have reported that Fas activation increased the LPS/IFNg-induced pro-inflammatory immune response synergistically in THP1 cell lines and mouse BMDMs (Isayama *et al.*, 2016; Brennan *et al.*, 2018). Interestingly, our data demonstrated that although sFasL partially increased the M1 marker gene expression level, it did not cause any significant change in the production of M1 cytokines and expression levels of M1 surface markers (Fig 7, 8, and 9).

As for the qPCR data of M1 marker gene expression levels, it can be explained that the statistical significance appears to be significant since it measures whether the result was obtained by chance rather than biological significance. Noteworthily, previous studies showed that the impact of Fas activation on both non-polarized and M1 macrophages was dose-dependent, but our study revealed that the effect of sFasL on human macrophage response was not in a dose-dependent manner. These are probably because the use of agonistic Fas antibody Jo2 (Isayama *et al.*, 2016; Altemeier *et al.*, 2003) or the use of agonistic Fas antibody CH-11 (Brennan *et al.*, 2018; Park *et al.*, 2003) to stimulate macrophages generates a strong response compared to the sFasL used in this study. Also, the different observations may be due to the type of macrophages used in the previous studies and our study. While THP1 cells are frequently preferred due to the challenges in generating and long-term storage of primary macrophages, this cell line cannot fully replace primary macrophages in the cell plasticity studies (Tedesco *et al.*, 2018). Furthermore, although mice are highly valid in vivo model for macrophage biology studies, there are some significant metabolic differences between human and mouse macrophages. For example, M1 polarization factors lead to glycolytic reprogramming and nitric oxide production in mice macrophages, but not in human macrophages (Vijayan *et al.*, 2019; Mestas & Hughes, 2004).

The fact that FasL slightly decreased CD206 surface marker expression in M2a polarization might be an indicator for impairment of the M2a response. Furthermore, although a trend towards a decrease in the CD200R marker was observed, it was not statistically significant due to the variations between samples. Although there are a limited number of similar studies on the effect of Fas activation on M1 and M2a macrophage response in the literature, the effect of FasL on IL-10-induced M2c response was investigated for the first time. However, since measuring M2 cytokines in HMDMs by flow cytometry or ELISA is challenging, the effect of sFasL on the M2a and M2c cytokine response could not be assessed within the scope of this study.

6. CONCLUSION

In this study, we investigated the impact of sFasL on the polarization of primary human macrophages. This could help us to understand whether the increased expression of FasL in inflammatory diseases has a direct effect on the response of macrophages. Based on previous studies, we hypothesized that Fas activation would increase the pro-inflammatory response in human primary macrophages. In conclusion, our results revealed that sFasL had no functional impact on pro-inflammatory cytokine production of non-polarized or polarized HMDMs. There was a significant decrease in M2a surface marker CD206, while no change in M2c markers was observed. However, cytokine production assessment could not be performed due to the difficulties of M2 cytokine measurement. With our data, it can be claimed that sFasL, which is abundant in the tumor microenvironment, has no direct effect on human macrophage polarization. To understand whether sFasL has an indirect effect on macrophage response, coculture experiments with different cells or animal models can be used.

7. REFERENCES

Abusamra AJ, Zhong Z, Zheng X, et al. Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells, Mol Dis*. 2005;35(2):169-173. doi:10.1016/j.bcmd.2005.07.001

Altemeier WA, Zhu X, Berrington WR, Harlan JM, Liles WC. Fas (CD95) induces macrophage proinflammatory chemokine production via a MyD88-dependent, caspase-independent pathway. *J Leukoc Biol*. 2007;82(3):721-728. doi:10.1189/jlb.1006652

Amarante-Mendes GP, Adjemian S, Branco LM, Zanetti LC, Weinlich R, Bortoluci KR. Pattern recognition receptors and the host cell death molecular machinery. *Front Immunol*. 2018;9(OCT):2379. doi:10.3389/fimmu.2018.02379

Anfray C, Ummarino A, Andón FT, Allavena P. Current Strategies to Target Tumor-Associated-Macrophages to Improve Anti-Tumor Immune Responses. *Cells*. 2019;9(1):46. doi:10.3390/cells9010046

Arakaki R, Yamada A, Kudo Y, Hayashi Y, Ishimaru N. Mechanism of activation-induced cell death of T cells and regulation of FasL expression. *Crit Rev Immunol*. 2014;34(4):301-314. doi:10.1615/CritRevImmunol.2014009988

Audoy-Rémus J, Richard JF, Soulet D, Zhou H, Kubes P, Vallières L. Rod-shaped monocytes patrol the brain vasculature and give rise to perivascular macrophages under the influence of proinflammatory cytokines and angiopoietin-2. *J Neurosci*. 2008;28(41):10187-10199. doi:10.1523/JNEUROSCI.3510-08.2008

Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science (80-)*. 2007;317(5838):666-670. doi:10.1126/science.1142883

Bajou K, Peng H, Laug WE, et al. Plasminogen Activator Inhibitor-1 Protects Endothelial Cells from FasL-Mediated Apoptosis. *Cancer Cell*. 2008;14(4):324-334. doi:10.1016/j.ccr.2008.08.012

Bajpai G, Schneider C, Wong N, et al. The human heart contains distinct macrophage subsets with divergent origins and functions. *Nat Med*. 2018;24(8):1234-1245. doi:10.1038/s41591-018-0059-x

Belgiovine C, D'Incalci M, Allavena P, Frapolli R. Tumor-associated macrophages and anti-tumor therapies: complex links. *Cell Mol Life Sci*. 2016;73(13):2411-2424. doi:10.1007/s00018-016-2166-5

Bénéteau M, Pizon M, Chaigne-Delalande B, et al. Localization of Fas/CD95 into the lipid rafts on down-modulation of the phosphatidylinositol 3-kinase signaling pathway. *Mol Cancer Res*. 2008;6(4):604-613. doi:10.1158/1541-7786.MCR-07-0331

Biswas SK, Chittezhath M, Shalova IN, Lim JY. Macrophage polarization and plasticity in health and disease. *Immunol Res*. 2012;53(1-3):11-24. doi:10.1007/s12026-012-8291-9

Boehning D, Van Rossum DB, Patterson RL, Snyder SH. A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A*. 2005;102(5):1466-1471. doi:10.1073/pnas.0409650102

Braga TT, Agudelo JSH, Camara NOS. Macrophages during the fibrotic process: M2 as friend and foe. *Front Immunol*. 2015;6(NOV):602. doi:10.3389/fimmu.2015.00602

Brown NJ, Hutcheson J, Bickel E, et al. Fas Death Receptor Signaling Represses Monocyte Numbers and Macrophage Activation In Vivo. *J Immunol*. 2004;173(12):7584-7593. doi:10.4049/jimmunol.173.12.7584

Bujko A, Atlasy N, Landsverk OJB, et al. Transcriptional and functional profiling defines human small intestinal macrophage subsets. *J Exp Med*. 2018;215(2):441-458. doi:10.1084/jem.20170057

Buss RR, Sun W, Oppenheim RW. ADAPTIVE ROLES OF PROGRAMMED CELL DEATH DURING NERVOUS SYSTEM DEVELOPMENT. *Annu Rev Neurosci*. 2006;29(1):1-35. doi:10.1146/annurev.neuro.29.051605.112800

Castoldi A, De Souza CN, Saraiva Câmara NO, Moraes-Vieira PM. The macrophage switch in obesity development. *Front Immunol*. 2016;6(JAN):1. doi:10.3389/fimmu.2015.00637

Chakour R, Allenbach C, Desgranges F, et al. A new function of the Fas-FasL pathway in macrophage activation. *J Leukoc Biol*. 2009;86(1):81-90. doi:10.1189/jlb.1008590

Chang HY, Nishitoh H, Yang X, Ichijo H, Baltimore D. Activation of Apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science (80-)*. 1998;281(5384):1860-1863. doi:10.1126/science.281.5384.1860

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis. *Cell*. 1995;81(4):505-512. doi:10.1016/0092-8674(95)90071-3

Clemens MJ, Bushell M, Jeffrey IW, Pain VM, Morley SJ. Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. *Cell Death Differ*. 2000;7(7):603-615. doi:10.1038/sj.cdd.4400695

Coillard A, Segura E. In vivo differentiation of human monocytes. *Front Immunol*. 2019;10(AUG). doi:10.3389/fimmu.2019.01907

Cullen SP, Henry CM, Kearney CJ, et al. Fas/CD95-Induced Chemokines Can Serve as " Find-Me" Signals for Apoptotic Cells. *Mol Cell*. 2013;49(6):1034-1048. doi:10.1016/j.molcel.2013.01.025

Cullen SP, Martin SJ. Fas and TRAIL "death receptors" as initiators of inflammation: Implications for cancer. *Semin Cell Dev Biol*. 2015;39:26-34. doi:10.1016/j.semcdb.2015.01.012

D'Arcy MS. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int*. 2019;43(6):582-592. doi:10.1002/cbin.11137

De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell*. 2013;23(3):277-286. doi:10.1016/j.ccr.2013.02.013

Deak JC, Cross J V., Lewis M, et al. Fas-induced proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. *Proc Natl Acad Sci U S A*. 1998;95(10):5595-5600. doi:10.1073/pnas.95.10.5595

Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol*. 2005;1(2):112-119. doi:10.1038/chembio711

Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J*. 1998;17(8):2215-2223. doi:10.1093/emboj/17.8.2215

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature*. 1997;388(6639):300-304. doi:10.1038/40901

Dickens LS, Boyd RS, Jukes-Jones R, et al. A Death Effector Domain Chain DISC Model Reveals a Crucial Role for Caspase-8 Chain Assembly in Mediating Apoptotic Cell Death. *Mol Cell*. 2012;47(2):291-305. doi:10.1016/j.molcel.2012.05.004

Eguíluz-Gracia I, Lawaetz Schultz HH, Sikkeland LIB, et al. Long-term persistence of human donor alveolar macrophages in lung transplant recipients. *thorax.bmj.com*. Published online 2016. doi:10.1136/thoraxjnl-2016-208292

Ehrenschwender M, Siegmund D, Wicovsky A, et al. Mutant PIK3CA licenses TRAIL and CD95L to induce non-apoptotic caspase-8-mediated ROCK activation. *Cell Death Differ*. 2010;17(9):1435-1447. doi:10.1038/cdd.2010.36

El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol*. 2008;9(12):1399-1406. doi:10.1038/ni.1671

Epelman S, Lavine KJ, Randolph GJ. Origin and Functions of Tissue Macrophages. *Immunity*. 2014;41(1):21-35. doi:10.1016/j.immuni.2014.06.013

Fantin A, Vieira JM, Gestri G, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*. 2010;116(5):829-840. doi:10.1182/blood-2009-12-257832

Fujiwara N, Kobayashi K. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy*. 2005;4(3):281-286. doi:10.2174/1568010054022024

Galluzzi L, Maiuri MC, Vitale I, et al. Cell death modalities: Classification and pathophysiological implications. *Cell Death Differ*. 2007;14(7):1237-1243. doi:10.1038/sj.cdd.4402148

Gerrick KY, Gerrick ER, Gupta A, Wheelan SJ, Yegnasubramanian S, Jaffee EM. Transcriptional profiling identifies novel regulators of macrophage polarization. Karpurapu M, ed. *PLoS One*. 2018;13(12):e0208602. doi:10.1371/journal.pone.0208602

Gordon S, Plüddemann A. Tissue macrophages: Heterogeneity and functions. *BMC Biol*. 2017;15(1):1-18. doi:10.1186/s12915-017-0392-4

Graves JD, Gotoh Y, Draves KE, et al. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *EMBO J*. 1998;17(8):2224-2234. doi:10.1093/emboj/17.8.2224

Green DR, Llambi F. Cell death signaling. *Cold Spring Harb Perspect Biol*. 2015;7(12). doi:10.1101/cshperspect.a006080

Guégan JP, Ginestier C, Charafe-Jauffret E, et al. CD95/Fas and metastatic disease: What does not kill you makes you stronger. *Semin Cancer Biol*. 2020;60:121-131. doi:10.1016/j.semcancer.2019.06.004

Guégan JP, Legembre P. Nonapoptotic functions of Fas/CD95 in the immune response. *FEBS J*. 2018;285(5):809-827. doi:10.1111/febs.14292

Guicciardi ME, Gores GJ. Life and death by death receptors. *FASEB J*. 2009;23(6):1625-1637. doi:10.1096/fj.08-111005

Hayden MS, Ghosh S. NF-κB, the first quarter-century: Remarkable progress and outstanding questions. *Genes Dev*. 2012;26(3):203-234. doi:10.1101/gad.183434.111

He C, Levine B. The Beclin 1 interactome. *Curr Opin Cell Biol*. 2010;22(2):140-149. doi:10.1016/j.ceb.2010.01.001

Hohlbaum AM, Moe S, Marshak-Rothstein A. Opposing effects of transmemrane and soluble Fas ligand expression on inflammation and tumor cell survival. *J Exp Med*. 2000;191(7):1209-1219. doi:10.1084/jem.191.7.1209

Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000;1(6):489-495. doi:10.1038/82732

Isayama F, Moore S, Hines IN, Wheeler MD. Fas Regulates Macrophage Polarization and Fibrogenic Phenotype in a Model of Chronic Ethanol-Induced Hepatocellular Injury. *Am J Pathol*. 2016;186(6):1524-1536. doi:10.1016/j.ajpath.2016.02.006

Jodo S, Kobayashi S, Nakajima Y, et al. Elevated serum levels of soluble Fas/APO-1 (CD95) in patients with hepatocellular carcinoma. *Clin Exp Immunol*. 1998;112(2):166-171. doi:10.1046/j.1365-2249.1998.00569.x

Jost PJ, Grabow S, Gray D, et al. XIAP discriminates between type i and type II FAS-induced apoptosis. *Nature*. 2009;460(7258):1035-1039. doi:10.1038/nature08229

Khadra N, Bresson-Bepoldin L, Penna A, et al. CD95 triggers Orai1-mediated localized Ca 2+ entry, regulates recruitment of protein kinase C (PKC) β2, and prevents death-inducing signaling complex formation. *Proc Natl Acad Sci U S A*. 2011;108(47):19072-19077. doi:10.1073/pnas.1116946108

Kiener PA, Davis PM, Starling GC, et al. Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. *J Exp Med*. 1997;185(8):1511-1516. doi:10.1084/jem.185.8.1511

Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas Ligand–Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T Lymphocytes. *Clin Cancer Res*. 2005;11(3).

Kodama H, Nose M, Niida S, Yamasaki A. Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. *J Exp Med*. 1991;173(5):1291-1294. doi:10.1084/jem.173.5.1291

Kondera-Anasz Z, Mielczarek-Palacz A, Sikora J. Soluble Fas receptor and soluble Fas ligand in the serum of women with uterine tumors. *Apoptosis*. 2005;10(5):1143-1149. doi:10.1007/s10495-005-1018-9

Kovats M, Burian DM, Dozmorov M, et al. Macrophage and Dendritic Cell Subsets Characterization of Human Airway Transcriptional Classification and Functional. *Am Assoc Immnol*. Published online 2021. doi:10.4049/jimmunol.1600777

Kozlowski M, Kowalczuk O, Sulewska A, et al. *Serum Soluble Fas Ligand (SFasL) in Patients with Primary Squamous Cell Carcinoma of the Esophagus.* Vol 45.; 2007.

Kreuz S, Siegmund D, Rumpf JJ, et al. NFκB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J Cell Biol*. 2004;166(3):369-380. doi:10.1083/jcb.200401036

Kroemer G, Mariño G, Levine B. Autophagy and the Integrated Stress Response. *Mol Cell*. 2010;40(2):280-293. doi:10.1016/j.molcel.2010.09.023

Kulkarni A, Chandrasekar V, Natarajan SK, et al. A designer self-assembled supramolecule amplifies macrophage immune responses against aggressive cancer. *Nat Biomed Eng*. 2018;2(8):589-599. doi:10.1038/s41551-018-0254-6

Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: A 10-year update. *Physiol Rev*. 2012;92(2):689-737. doi:10.1152/physrev.00028.2011

Lai Y-J, Lin VTG, Zheng Y, Benveniste EN, Lin F-T. The Adaptor Protein TRIP6 Antagonizes Fas-Induced Apoptosis but Promotes Its Effect on Cell Migration. *Mol Cell Biol*. 2010;30(23):5582-5596. doi:10.1128/mcb.00134-10

Lavrik IN, Krammer PH. Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ*. 2012;19(1):36-41. doi:10.1038/cdd.2011.155

Lavrik IN, Golks A, Riess D, Bentele M, Eils R, Krammer PH. Analysis of CD95 threshold signaling: Triggering of CD95 (FAS/APO-1) at low concentrations primarily results in survival signaling. *J Biol Chem*. 2007;282(18):13664-13671. doi:10.1074/jbc.M700434200

Lee PL, Gelbart T, West C, Halloran C, Beutler E. The human Nramp2 gene: Characterization of the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells, Mol Dis*. 1998;24(2):199-215. doi:10.1006/bcmd.1998.0186

Letellier E, Kumar S, Sancho-Martinez I, et al. CD95-Ligand on Peripheral Myeloid Cells Activates Syk Kinase to Trigger Their Recruitment to the Inflammatory Site. *Immunity*. 2010;32(2):240-252. doi:10.1016/j.immuni.2010.01.011

Liao C Te, Andrews R, Wallace LE, et al. Peritoneal macrophage heterogeneity is associated with different peritoneal dialysis outcomes. *Kidney Int*. 2017;91(5):1088-1103. doi:10.1016/j.kint.2016.10.030

Lobov IB, Rao S, Carroll TJ, et al. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature*. 2005;437(7057):417-421. doi:10.1038/nature03928

Ma WT, Gao F, Gu K, Chen DK. The role of monocytes and macrophages in autoimmune diseases: A comprehensive review. *Front Immunol*. 2019;10(MAY):1140. doi:10.3389/fimmu.2019.01140

Malleter M, Tauzin S, Bessede A, et al. CD95L cell surface cleavage triggers a prometastatic signaling pathway in triple-negative breast cancer. *Cancer Res*. 2013;73(22):6711-6721. doi:10.1158/0008-5472.CAN-13-1794

Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol*. 2017;14(7):399-416. doi:10.1038/nrclinonc.2016.217

Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. 2010;22(2):231-237. doi:10.1016/j.coi.2010.01.009

Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. 2010;22(2):231-237. doi:10.1016/j.coi.2010.01.009

Marsik C, Halama T, Cardona F, et al. Regulation of Fas (APO-1, CD95) and Fas ligand expression in leukocytes during systemic inflammation in humans. *Shock*. 2003;20(6):493-496. doi:10.1097/01.shk.0000097248.97298.ea

Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep*. 2014;6. doi:10.12703/P6-13Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep*. 2014;6. doi:10.12703/P6-13

Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: An immunologic functional perspective. *Annu Rev Immunol*. 2009;27:451-483. doi:10.1146/annurev.immunol.021908.132532

Matsumoto H, Murakami Y, Kataoka K, et al. Membrane-bound and soluble Fas ligands have opposite functions in photoreceptor cell death following separation from the retinal pigment epithelium. *Cell Death Dis*. 2015;6(11). doi:10.1038/cddis.2015.334

McGovern N, Schlitzer A, Gunawan M, et al. Human dermal CD14+ cells are a transient population of monocyte-derived macrophages. *Immunity*. 2014;41(3):465-477. doi:10.1016/j.immuni.2014.08.006

Meshkani R, Vakili S. Tissue resident macrophages: Key players in the pathogenesis of type 2 diabetes and its complications. *Clin Chim Acta*. 2016;462:77-89. doi:10.1016/j.cca.2016.08.015

Mestas J, Hughes CCW. Of Mice and Not Men: Differences between Mouse and Human Immunology. *J Immunol*. 2004;172(5):2731-2738. doi:10.4049/jimmunol.172.5.2731

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008;451(7182):1069-1075. doi:10.1038/nature06639

Mocarski ES, Kaiser WJ, Livingston-Rosanoff D, Upton JW, Daley-Bauer LP. True Grit: Programmed Necrosis in Antiviral Host Defense, Inflammation, and Immunogenicity. *J Immunol*. 2014;192(5):2019-2026. doi:10.4049/jimmunol.1302426

Moriwaki K, Balaji S, McQuade T, Malhotra N, Kang J, Chan FKM. The Necroptosis Adaptor RIPK3 Promotes Injury-Induced Cytokine Expression and Tissue Repair. *Immunity*. 2014;41(4):567-578. doi:10.1016/j.immuni.2014.09.016

Muñoz-Espín D, Cañamero M, Maraver A, et al. XProgrammed cell senescence during mammalian embryonic development. *Cell*. 2013;155(5):1104. doi:10.1016/j.cell.2013.10.019

Murray PJ. Macrophage Polarization. *Annu Rev Physiol*. 2017;79:541-566. doi:10.1146/annurev-physiol-022516-034339

Murray PJ, Allen JE, Biswas SK, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*. 2014;41(1):14-20. doi:10.1016/j.immuni.2014.06.008

Müller S, Kohanbash G, Liu SJ, et al. Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol*. 2017;18(1). doi:10.1186/s13059-017-1362-4

Najafi M, Hashemi Goradel N, Farhood B, et al. Macrophage polarity in cancer: A review. *J Cell Biochem*. 2019;120(3):2756-2765. doi:10.1002/jcb.27646

Nayak D, Roth TL, McGavern DB. Microglia development and function. *Annu Rev Immunol*. 2014;32:367-402. doi:10.1146/annurev-immunol-032713-120240

Novak ML, Koh TJ. Macrophage phenotypes during tissue repair. *Wiley Online Libr*. 2012;93(6):875-881. doi:10.1189/jlb.1012512

Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity*. 2014;41(1):49-61. doi:10.1016/j.immuni.2014.06.010

Nucera S, Biziato D, De Palma M. The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. *Int J Dev Biol*. 2011;55(4-5):495-503. doi:10.1387/ijdb.103227sn

O Reilly LA, Tai L, Lee L, et al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature*. 2009;461(7264):659-663. doi:10.1038/nature08402

Oliveira SR, Amaral JD, Rodrigues CMP. Mechanism and disease implications of necroptosis and neuronal inflammation. *Cell Death Dis*. 2018;9(9):1-3. doi:10.1038/s41419-018-0872-7

Ortega N, Wang K, Ferrara N, Werb Z, Vu TH. Complementary interplay between matrix metalloproteinase-9, vascular endothelial growth factor and osteoclast function drives endochondral bone formation. *DMM Dis Model Mech*. 2010;3(3-4):224-235. doi:10.1242/dmm.004226

Özören N, El-Deiry WS. Defining characteristics of types I and II apoptotic cells in response to TRAIL. *Neoplasia*. 2002;4(6):551-557. doi:10.1038/sj.neo.7900270

Park H, Jung Y-K, Park O-J, Lee YJ, Choi J-Y, Choi Y. Interaction of Fas Ligand and Fas Expressed on Osteoclast Precursors Increases Osteoclastogenesis. *J Immunol*. 2005;175(11):7193-7201. doi:10.4049/jimmunol.175.11.7193

Parkin J, Cohen B. An overview of the immune system. *Lancet*. 2001;357(9270):1777-1789. doi:10.1016/S0140-6736(00)04904-7

Parveen N, Varman R, Nair S, Das G, Ghosh S, Mukhopadhyay S. Endocytosis of mycobacterium tuberculosis heat shock protein 60 is required to induce interleukin-10 production in macrophages. *J Biol Chem*. 2013;288(34):24956-24971. doi:10.1074/jbc.M113.461004

Perlman H, Pagliari LJ, Georganas C, Mano T, Walsh K, Pope RM. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to Fas-mediated apoptosis. *J Exp Med*. 1999;190(11):1679-1688. doi:10.1084/jem.190.11.1679

Peter ME, Hadji A, Murmann AE, et al. The role of CD95 and CD95 ligand in cancer. *Cell Death Differ*. 2015;22(4):549-559. doi:10.1038/cdd.2015.3

Pizon M, Rampanarivo H, Tauzin S, et al. Actin-independent exclusion of CD95 by PI3K/AKT signalling: Implications for apoptosis. *Eur J Immunol*. 2011;41(8):2368-2378. doi:10.1002/eji.201041078

Poissonnier A, Sanséau D, Le Gallo M, et al. CD95-Mediated Calcium Signaling Promotes T Helper 17 Trafficking to Inflamed Organs in Lupus-Prone Mice. *Immunity*. 2016;45(1):209-223. doi:10.1016/j.immuni.2016.06.028

Rajaram MVS, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. Mycobacterium tuberculosis Activates Human Macrophage Peroxisome Proliferator-Activated Receptor γ Linking Mannose Receptor Recognition to Regulation of Immune Responses . *J Immunol*. 2010;185(2):929-942. doi:10.4049/jimmunol.1000866

Ramanathan S, Jagannathan N. Tumor associated macrophage: A review on the phenotypes, traits and functions. *Iran J Cancer Prev*. 2014;7(1):1-8. Accessed February 17, 2021. /pmc/articles/PMC4142950/

Ramesh A, Kumar S, Nandi D, Kulkarni A. CSF1R- and SHP2-Inhibitor-Loaded Nanoparticles Enhance Cytotoxic Activity and Phagocytosis in Tumor-Associated Macrophages. *Adv Mater*. 2019;31(51):1904364. doi:10.1002/adma.201904364

Riisbro R, Stephens RW, Brünner N, et al. Soluble urokinase plasminogen activator receptor in preoperatively obtained plasma from patients with gynecological cancer or benign gynecological diseases. *Gynecol Oncol*. 2001;82(3):523-531. doi:10.1006/gyno.2001.6324

Romagnani S. T-cell subsets (Th1 versus Th2). *Ann Allergy, Asthma Immunol*. 2000;85(1):9-18. doi:10.1016/S1081-1206(10)62426-X

Rossin A, Miloro G, Hueber AO. TRAIL and FasL functions in cancer and autoimmune diseases: Towards an increasing complexity. *Cancers (Basel)*. 2019;11(5). doi:10.3390/cancers11050639

Sade-Feldman M, Yizhak K, Bjorgaard SL, et al. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell*. 2018;175(4):998-1013.e20. doi:10.1016/j.cell.2018.10.038

Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 1998;17(6):1675-1687. doi:10.1093/emboj/17.6.1675

Scaffidi G, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem*. 1999;274(32):22532-22538. doi:10.1074/jbc.274.32.22532

Schleich K, Warnken U, Fricker N, et al. Stoichiometry of the CD95 Death-Inducing Signaling Complex: Experimental and Modeling Evidence for a Death Effector Domain Chain Model. *Mol Cell*. 2012;47(2):306-319. doi:10.1016/j.molcel.2012.05.006

Seitz DH, Palmer A, Niesler U, et al. Altered expression of fas receptor on alveolar macrophages and inflammatory effects of soluble fas ligand following blunt chest trauma. *Shock*. 2011;35(6):610-617. doi:10.1097/SHK.0b013e318213665d

Sever R, Brugge JS. Signal transduction in cancer. *Cold Spring Harb Perspect Med*. 2015;5(4). doi:10.1101/cshperspect.a006098

Shapouri-Moghaddam A, Mohammadian S, Vazini H, et al. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol*. 2018;233(9):6425-6440. doi:10.1002/jcp.26429

Shimizu T. Role of macrophage migration inhibitory factor (MIF) in the skin. *J Dermatol Sci*. 2005;37(2):65-73. doi:10.1016/j.jdermsci.2004.08.007

Shishido SN, Varahan S, Yuan K, Li X, Fleming SD. Humoral innate immune response and disease. *Clin Immunol*. 2012;144(2):142-158. doi:10.1016/j.clim.2012.06.002

Sica A, Erreni M. Macrophage polarization in pathology. *Springer*. 2015;72(21):4111-4126. doi:10.1007/s00018-015-1995-y

Siegmund D, Lang I, Wajant H. Cell death-independent activities of the death receptors CD95, TRAILR1, and TRAILR2. *FEBS J*. 2017;284(8):1131-1159. doi:10.1111/febs.13968

Singer GG, Abbas AK. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity*. 1994;1(5):365-371. doi:10.1016/1074-7613(94)90067-1

Storer M, Mas A, Robert-Moreno A, et al. Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell*. 2013;155(5):1119. doi:10.1016/j.cell.2013.10.041

Stremmel C, Schuchert R, Wagner F, et al. Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Commun*. 2018;9(1). doi:10.1038/s41467-017-02492-2

Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble fas ligand blocks the killing. *J Exp Med*. 1997;186(12):2045-2050. doi:10.1084/jem.186.12.2045

Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the fas ligand. *Cell*. 1994;76(6):969-976. doi:10.1016/0092-8674(94)90375-1

Tauzin S, Chaigne-Delalande B, Selva E, et al. The naturally processed CD95L Elicits a c-yes/Calcium/PI3K-driven cell migration pathway. *PLoS Biol*. 2011;9(6). doi:10.1371/journal.pbio.1001090

Tedesco S, De Majo F, Kim J, et al. Convenience versus biological significance: Are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in vitro polarization? *Front Pharmacol*. 2018;9(FEB). doi:10.3389/fphar.2018.00071

Tsuchiya Y, Nakabayashi O, Nakano H. FLIP the switch: Regulation of apoptosis and necroptosis by cFLIP. *Int J Mol Sci*. 2015;16(12):30321-30341. doi:10.3390/ijms161226232

Tsutsumi S, Kuwano H, Shimura T, Morinaga N, Mochiki E, Asao T. Circulating soluble Fas ligand in patients with gastric carcinoma. *Cancer*. 2000;89(12):2560-2564. doi:10.1002/1097-0142(20001215)89:12<2560::AID-CNCR7>3.0.CO;2-Q

Tummers B, Green DR. Caspase-8: regulating life and death. *Immunol Rev*. 2017;277(1):76-89. doi:10.1111/imr.12541

Turvey SE, Broide DH. Innate immunity. *J Allergy Clin Immunol*. 2010;125(2 SUPPL. 2):S24-S32. doi:10.1016/j.jaci.2009.07.016

Varol C, Mildner A, Jung S. Macrophages: Development and Tissue Specialization. *Annu Rev Immunol*. 2015;33(1):643-675. doi:10.1146/annurev-immunol-032414-112220

Verykokakis M, Zook EC, Kee BL. ID'ing innate and innate-like lymphoid cells. *Immunol Rev*. 2014;261(1):177-197. doi:10.1111/imr.12203

Vijayan V, Pradhan P, Braud L, et al. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biol*. 2019;22:101147. doi:10.1016/j.redox.2019.101147

Wager CML, Wormley FL. Classical versus alternative macrophage activation: The Ying and the Yang in host defense against pulmonary fungal infections. *Mucosal Immunol*. 2014;7(5):1023-1035. doi:10.1038/mi.2014.65

Wajant H. Principles and mechanisms of CD95 activation. In: *Biological Chemistry*. Vol 395. Walter de Gruyter GmbH; 2014:1401-1416. doi:10.1515/hsz-2014-0212

Wajant H. Death receptors. *Essays Biochem*. 2003;39:53-71. doi:10.1042/bse0390053

Wake H, Moorhouse AJ, Miyamoto A, Nabekura J. Microglia: Actively surveying and shaping neuronal circuit structure and function. *Trends Neurosci*. 2013;36(4):209-217. doi:10.1016/j.tins.2012.11.007

Wang F, Lu Z, Hawkes M, Yang H, Kain KC, Liles WC. Fas (CD95) induces rapid, TLR4/IRAK4-dependent release of pro-inflammatory HMGB1 from macrophages. *J Inflamm*. 2010;7(1):30. doi:10.1186/1476-9255-7-30

Wang Y, Qin ZH. Molecular and cellular mechanisms of excitotoxic neuronal death. *Apoptosis*. 2010;15(11):1382-1402. doi:10.1007/s10495-010-0481-0

Wick G, Backovic A, Rabensteiner E, Plank N, Schwentner C, Sgonc R. The immunology of fibrosis: innate and adaptive responses. *Trends Immunol*. 2010;31(3):110-119. doi:10.1016/j.it.2009.12.001

Wozniak AL, Wang X, Stieren ES, Scarbrough SG, Elferink CJ, Boehning D. Requirement of biphasic calcium release from the endoplasmic reticulum for Fas-mediated apoptosis. *J Cell Biol*. 2006;175(5):709-714. doi:10.1083/jcb.200608035

Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;496(7446):445-455. doi:10.1038/nature12034

Xu R, Zhang Z, Wang FS. Liver fibrosis: Mechanisms of immune-mediated liver injury. *Cell Mol Immunol*. 2012;9(4):296-301. doi:10.1038/cmi.2011.53

Yamada A, Arakaki R, Saito M, Kudo Y, Ishimaru N. Dual role of Fas/FasL-mediated signal in peripheral immune tolerance. *Front Immunol*. 2017;8(APR). doi:10.3389/fimmu.2017.00403

Yang M, McKay D, Pollard JW, Lewis CE. Diverse functions of macrophages in different tumor microenvironments. *Cancer Res*. 2018;78(19):5492-5503. doi:10.1158/0008-5472.CAN-18-1367

Yang X, Khosravi-Far R, Chang HY, Baltimore D. Daxx, a novel fas-binding protein that activates JNK and apoptosis. *Cell*. 1997;89(7):1067-1076. doi:10.1016/S0092-8674(00)80294-9

Yatim KM, Lakkis FG. A brief journey through the immune system. *Clin J Am Soc Nephrol*. 2015;10(7):1274-1281. doi:10.2215/CJN.10031014

Yuan K, Jing G, Chen J, et al. Calmodulin mediates Fas-induced FADD-independent survival signaling in pancreatic cancer cells via activation of Src-Extracellular Signal-regulated Kinase (ERK). *J Biol Chem*. 2011;286(28):24776-24784. doi:10.1074/jbc.M110.2028

8. APPENDIX

8.1. Ethics Committee Approval

KARAR BİLGİLERİ	**Karar No:2021/03-38**		**Tarih:01.02.2021**				
	Doç.Dr. Duygu Sağ'ın sorumlusu olduğu **"Fas/Fas Ligand Yolağının Primer İnsan Monosit Diferansiyasyonu ve Makrofaj Fenotipine Etkisinin Araştırılması"** isimli klinik araştırmaya ilişkin sorumlu araştırmacı tarafından gönderilen dilekçeye ilişkin; -Çalışma adının "FasL Uyarımının İnsan Primer M1,M2a ve M2c Makrofaj Polirizasyonuna Etkisinin Araştırılması" olarak değiştirilmesi, -Yöntemde, deney gruplarında ve araştırma bütçesinde yapılan değişiklikler ile ilgili bilgi edinilmiş ve uygun bulunmuştur.						
ETİK KURUL BİLGİLERİ							
ÇALIŞMA ESASI	Dokuz Eylül Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu İşleyiş Yönergesi İyi Klinik Uygulamaları Kılavuzu						
ETİK KURUL ÜYELERİ							
Unvanı/Adı/Soyadı	**Uzmanlık Alanı**	**Kurumu**	**Cinsiyet**	**Araştırma ile İlişkili mi?**		**İmza**	
Prof.Dr.Sadık Kıvanç METİN (Başkan)	Kalp ve Damar Cerrahisi	DEU Tıp Fakültesi Kalp Damar Cerrahisi Anabilim Dalı	Erkek	E ☐	H ☒		
Prof.Dr. Sermin ÖZKAL (Başkan Yardımcısı)	Tıbbi Patoloji	DEÜ Tıp Fakültesi Tıbbi Patoloji A.D	Kadın	E ☐	H ☒		
Prof.Dr.Serkan YENER	Endokrinoloji	DEU Tıp Fakültesi İç Hastalıkları Anabilim Dalı	Erkek	E ☐	H ☒		
Prof.Dr.Pınar TUNCEL	Tıbbi Biyokimya	DEU Tıp Fakültesi Tıbbi Biyokimya Anabilim Dalı	Kadın	E ☐	H ☒		
Prof.Dr.Murat BEKTAŞ	Çocuk Sağlığı ve Hastalıkları Hemşireliği	DEU Hemşirelik Fakültesi Çocuk Sağlığı ve Hastalıkları Hemşireliği	Erkek	E ☐	H ☒		
Doç.Dr.Nil Hocaoğlu AKSAY	Tıbbi Farmakoloji	DEU Tıp Fakültesi Tıbbi Farmakoloji Anabilim Dalı	Kadın	E ☐	H ☒		
Doç.Dr.Seher Özyürek	Muskuloskeletal Fizyoterapi - Fizik Tedavi ve Rehabilitasyon	DEU Fizik Tedavi ve Rehabilitasyon Yüksek Okulu	Kadın	E ☐	H ☒		
Doç.Dr.Tufan ÇANKAYA	Tıbbi Genetik	Tıbbi Genetik Anabilim Dalı	Erkek	E ☐	H ☒		
Doç.Dr.Ayfer DAYI	Davranış Fizyolojisi	DEU Tıp Fakültesi Fizyoloji Anabilim Dalı	Kadın	E ☐	H ☒		
Doç.Dr.Korcan DEMİR	Pediatrik Endokrinoloji	DEU Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	Erkek	E ☐	H ☒		
Doç.Dr.Mahmut Cem ERGON	Tıbbi Mikrobiyoloji	DEU Tıp Fakültesi Tıbbi Mikrobiyoloji Anabilim Dalı	Erkek	E ☐	H ☒		
Doç.Dr. Aylin Özgen Alpaydın	Göğüs Hastalıkları	DEU Tıp Fakültesi Göğüs Hastalıkları Anabilim Dalı	Kadın	E ☐	H ☒		
Öğr.Gör.Dr.Kıvanç YÜKSEL	Biyoistatistik ve Tıbbi Bilişim	Ege Üniversitesi Tıp Fakültesi Biyoistatistik ve Bilişim A.D	Erkek	E ☐	H ☒		
Av.Esra FIRTINA	Avukat	DEU Rektörlüğü Hukuk Müşavirliği	Kadın	E ☐	H ☒		
Mehmet Erhan ÖZKUL	Sağlık mensubu olmayan üye	D.E.U Tıp Fakültesi İdari Mali İşler	Erkek	E ☐	H ☒		

Dokuz Eylül Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu Karar Formu

8.2. Curriculum Vitae

Resul Özbilgiç

ID Number:

Date of Birth:

Contact Address:

Phone:

email:

EDUCATIONAL BACKGROUND

15/09/2017 – CURRENT – Dokuz Eylul University Health Campus Mithatpaşa Ave. 58/5 Balcova, Izmir, Turkey

MASTER OF SCIENCE – Dokuz Eylul University, Izmir International Biomedicine and Genome Institute

15/09/2012 – 15/06/2016 – Istanbul University, Faculty of Science, 34134 Vezneciler, Fatih/İstanbul, Istanbul, Turkey

BACHELOR OF SCIENCE – Istanbul University, Department of Molecular Biology and Genetics

ACADEMIC / PROFESSIONAL EXPERIENCE

15/09/2017 – CURRENT – Izmir, Turkey

Researcher– IZMIR BIOMEDICINE AND GENOME CENTER - Sag Cancer Immunology Lab

01/06/2016 – 01/02/2017 – Malatya, Turkey

Voluntary Internship – INONU UNIVERSITY - Molecular Microbiology Lab.

01/09/2014 – 01/06/2016 – Istanbul, Turkey

Undergraduate Research Project – ISTANBUL UNIVERSITY- Genetically Modified Organisms Lab.

07/2013 – 10/2013 – Malatya, Turkey

Internship – INONU UNIVERSITY, Molecular Microbiology Lab.

AWARDS

2020- Winner of SensorPlug Competition – PreSens Precision Sensing GmbH

A microfluidic platform for real-time non-invasive monitoring of microglia polarization

Polatli, E., Yilmaz A., Ozbilgic, R., Erbay, I.H., Sag, D., Guven, S.

2015-Undergraduate Project Funding – The Scientific and Technological Research Council of Turkey (TUBITAK)

"Comparison of the persistence of plant chromosomal and chloroplast DNA in soil"

TUBITAK Project No:1919B0115500645

2014-Undergraduate Project Funding – The Scientific and Technological Research Council of Turkey (TUBITAK)

"Investigation of horizontal gene transfer from genetically modified plants to bacteria"

TUBITAK Project No:1919B011400922

ABSTRACTS PUBLISHED IN CONFERENCE PROCEEDINGS

INTERNATIONAL CONFERENCES

2021-Real-Time Monitoring of Macrophage Activation via Sensor-Integrated Microfluidic Platform

Polatli E, Yilmaz A, **Ozbilgic R**, Sag D, Guven S. 6th World Congress of the Tissue Engineering and Regenerative Medicine International Society (TERMIS2021) (Submitted)

2020-Microfluidic Model for Studying Macrophage Polarization in 3D culture

Yilmaz A, **Ozbilgic, R**, Akbulud O, Kocak G, Sag D, Guven S. 2nd Annual Immune Modulation & Engineering Symposium

2019-Molecular Analysis of Horizontal Transfer of Regulatory Sequences from GM Soybean to A. baylyi

Elmali G, Cavdar C, **Ozbilgic R**, Ari S. II. International Green Biotechnology Congress.

2018-Molecular mechanism for M1 bias of ABCG1-deficient macrophages

Altunay M, Gunalp S, Unuvar-Purcu D, **Ozbilgic R**, Ayyildiz Z, Hapil F, Tavsan Z, Wingender G, Sag D. 5th European Congress of Immunology

2016-Investigation of horizontal gene transfer (HGT) from genetically modified plants to bacteria

Ozbilgic R, Meric S, Sahin O, Tombul N, & Ari S. FEBS JOURNAL (Vol. 283, pp. 333-334). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: WILEY-BLACKWELL.

NATIONAL CONFERENCES

2020-Investigation of the effect of immune checkpoint receptor VSIG4 expression on the inflammatory response of large peritoneal macrophages

Ozbilgic R, Erdal Y, Eskiocak C, Sag D. XXV National Immunology Congress

2020-ABCG1-mediated Macrophage Polarization in Type 2 Diabetes Patients

Korkmaz A, Unuvar-Purcu D, Gunalp S, **Ozbilgic R**, Helvaci DG, Sag D. XXV National Immunology Congress

2020-Effect of stimulation time on the expression of polarization markers in macrophage polarization.

Unuvar-Purcu D, Gunalp S, Korkmaz A, Helvaci DG, Erdal Y, **Ozbilgic R**, Suner A, Sag D. XXV National Immunology Congress

2020-TRAIL: A novel modulator of human macrophage polarization

Gunalp S, Helvaci DG, Unuvar Purcu D, Korkmaz A, **Ozbilgic R**, Karakulah G, Sag D. XXV National Immunology Congress

2019-Immunomodulation in 3D Dynamic Systems
Akbulud OB, Kahraman E, **Ozbilgic R**, Altunay M, Sag D, Guven S. 24th Biomedical Science and Technology Symposium

CONFERENCES AND SEMINARS

2020-Drexel University-2nd Annual Immune Modulation & Engineering Symposium.

2020- 25th National Immunology Congress.

2019- International Molecular Immunology & Immunogenetics Congress IV

2018- Molecules in Immunology, Turkish Society of Immunology

2017- New Horizons in Molecular Immunology

2017-Monoclonal Antibody Engineering

Practised Laboratory Method During M.Sc Education: Primary Cell Culture Techniques, FACS sorting, Flow Cytometry, ELISA, qPCR, animal handling, static and microfluidic 3D cell culture.

Certifications: Certificate of Experimental Animal Usage for Researchers, Dokuz Eylul University, 2017

Digital Skills: Microsoft Office, Graph Pad Prism, FlowJo, VideoScribe.