

CHARACTERIZATION OF ALTERED INNATE AND ADAPTIVE IMMUNE RESPONSES OF PRIMARY IMMUNE DEFICIENT PATIENTS

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We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and in quality, as a thesis for the degree of Master of Science.

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To my highly optimistic beloved parents...

ABSTRACT

CHARACTERIZATION OF ALTERED INNATE AND ADAPTIVE IMMUNE RESPONSES OF PRIMARY IMMUNE DEFICIENT PATIENTS

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The immune system dysregulations led to primary immune deficiencies (PIDs). Immune deficiencies can be divided into two groups, primary and secondary immune deficiencies. The reasons for primary immune deficiencies could be inherited immune dysfunctions originated from autosomal recessive or autosomal dominant mutations. Combinations of ongoing infections, lymphoproliferation, atopies, malignancies, autoimmunity and granulomatous processes are seen in primary immune deficiency disorders. To establish distinctive therapies, characterization of immune deficiencies along with the understanding the course of impaired mechanisms are very critical to offer robust means of cure. In this study, the effects of different mutations (RLTPR, RLTPR-TLR1, and CTLA-4) on innate and adaptive immune systems were investigated.

RLTPR (CARMIL2) is a cytosolic scaffold protein which facilitates CD28 co-stimulation for T-cell activation. RLTPR facilitates recruitment of CARMA1, a cytosolic adaptor, along with the BCL10 and MALT1 which form CBM complex to CD28 site to activate NF- κ B signaling pathway. Besides facilitating CD28 co-stimulation, RLTPR takes place in cell shape control, phagocytosis and endocytosis movements by promoting actin polymerization. Reduced CD4⁺ T-cells, memory B-cells and antibody response along with the impaired B-cell receptor mediated NF- κ B activation were mainly observed in RLTPR deficient patients. Moreover, polarity and migration of the T-cells were affected by RLTPR deficiency in patients. Herein,

immunological phenotypes of RLTPR deficient patient with and without CMV infection were described. As expected, Treg counts of the patient were reduced as compared to healthy controls both with and without infection. Although Treg cell counts of patient were decreased, IL-10 secretion upon cytosolic nucleic acid ligands was increased in infection due to ongoing defense and homeostasis. Under CMV infection, patient showed elevated IL-1 β and TNF- α responses upon activation of TLRs and cytosolic nucleic acid sensors. However, without any viral infections, TLR7 and TLR8 mediated IL-1 β and TNF- α responses were impaired in patient. Moreover, under CMV infection, TLR and cytosolic nucleic acid sensors mediated antiviral IFN- α , IFN- γ and IL-12 responses were substantially increased compared to healthy subjects. Unexpectedly, when patient PBMCs were assessed following infection, we detect that IFN- α and IFN- γ levels of the patient in response to endosomal TLRs and cytosolic nucleic acids stimulations were reduced. Throughout the course of viral infection due to ongoing defense by innate immune cells, one would predict to detect higher type I and II IFN responses. When infection was cleared with medical treatment, it is observed that not only endosomal TLRs but also the cytosolic nucleic acid sensors were impaired in RLTPR patient which makes the patient vulnerable to viral infections.

Thereafter, altered immune responses of RLTPR-TLR1 deficient patient was investigated. Decreased number of Treg cells from whole blood of the patient was confirmed. IL-1 β response from PBMCs of patient was elevated by stimulation with the TLR and cytosolic nucleic acid ligands. Especially, TLR2-6 response was elevated which could be the result of TLR1 deficiency because immune response could be compensated when TLR1 is deficient but TLR6 is not. Also, remaining improved proinflammatory cytokine response to different PRR ligands could be reasoned by ongoing infection. IFN- α secretion was increased by endosomal TLR and cytosolic nucleic acid ligands while IL-12 secretion of patient showed ligand specific modulated responses which suggest an ongoing infection. As expected, there was no detectable TLR1 mediated IL-12 response due to TLR1 deficiency. However, TLR2-6 mediated IL-12 secretion was elevated in patient compared to healthy subjects which could be regarded as a compensatory response against TLR1 deficiency. RLTPR-TLR1 deficient patient PBMCs elicited elevated IL-10 response to TLR and cytosolic nucleic acid ligand triggering even though Treg cells were reduced., implying that other suppressor cells could be involved in this response.

CTLA-4, a negative regulator of T-cells, is expressed on activated T-cells and Treg cells. CTLA-4 binds to CD80/86 molecules on APCs which prevents CD28 co-stimulation. Without CD28 co-stimulation, the T-cell cannot be activated. Patients with insufficient CTLA-4 receptors could have an increased number of Treg cells with reduced function. CTLA-4 haploinsufficiency leads to hyperactive T-cells, enhanced autoreactive B-cells and reduced numbers of circulating B-cells. In this work, immune responses of four CTLA-4 patients were also studied. Patient #1 showed higher CD4⁺/CD8⁺ ratio, elevated LDG frequency, as well as reduced TCR expression on CD3⁺ cells and elevated pAKT protein levels. Patient #2 had increased LDG count, reduced pDC and Treg population in whole blood. Similar blood cell profile to Patient #2, Patient #3 additionally had increased monocyte percentage and lower CD4⁺/CD8⁺ ratio which could indicate an ongoing infection. Patient #4 showed decreased pDC, Treg counts, increased levels of PD-L1 on CD8⁺ cells, Treg cells and B-cells, reduced TCR expression on CD3⁺ T-cells, increased pAKT and p4EBP1 levels which all may contribute to compensate the autoimmune status of the CTLA-4 mutation. B-cell percentages and CTLA-4 expression levels of all patients were not altered while mTOR, pmTOR, STAT3, pSTAT3, AKT, p4EBP1 and Hif-1 α expression levels were impaired in all patients.

Collectively, our findings imply that the complexity of the dysregulation of these deficiencies, and point-out to an unappreciated immune functional status of these patients. We propose that investigation of the innate immune arm of these individuals which were perceived as solely related to and impacting only adaptive immune system is necessary to offer more robust therapies to these patients.

Keywords: Primary Immune Deficiency, RLTPR, TLR1, CTLA-4, innate immune system, adaptive immune system.

ÖZET

BİRİNCİL BAĞIŞIKLIK YETERSİZLİĞİ HASTALARININ DOĞAL VE EDİNSEL BAĞIŞIKLIK YANITLARIN KARAKTERİZASYONU

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Bağışıklık sistemi düzensizlikleri birincil bağışıklık yetersizliklerine (PID'ler) yol açar. Bağışıklık yetersizlikleri doğal ve edinSEL bağışıklık yetersizlikleri olarak iki gruba ayrılabilir. Doğal bağışıklık yetersizliklerinin nedenleri, otozomal resesif veya otozomal dominant mutasyonlardan kaynaklanan kalıtsal immün fonksiyon bozuklukları olabilir. Devam eden enfeksiyonlar, lifoproliferasyonlar, atopiler, maligniteler, otoimmünlikler ve granüloamatöz süreçlerin kombinasyonları birincil immün yetersizlik bozukluklarında görülür. Tedaviler oluşturmak için, bağışıklık yetersizliklerinin karakterizasyonu ve bozulmuş mekanizmaların anlaşılması çok önemlidir. Bu çalışmada, farklı mutasyonların (RLTPR, RLTPR-TLR1, CTLA-4) doğal ve edinSEL bağışıklık sistemleri üzerindeki etkileri araştırılmıştır.

RLTPR (CARMIL2), T-hücresi aktivasyonu için CD28 ko-stimülasyonunu sağlayan bir sitozolik iskelet proteindir. CD28 ko-stimülasyonunu sağlamasının yanı sıra, RLTPR, aktin polimerizasyonunu teşvik ederek hücre şekli kontrolü, fagositoz ve endositoz hareketlerini gerçekleştirir. Farklı çalışmalarda azalmış CD4 + T-hücreleri, bellek B-hücreleri ve B-hücresi reseptörü aracılı NFκB aktivasyonu, RLTPR eksikliği olan hastalarda gözlenmiştir. Ayrıca, T-hücrelerinin polaritesi ve göçü, RLTPR eksikliğinden etkilenmiştir. Burada, CMV enfeksiyonu olan ve olmayan RLTPR eksikliği olan hastanın immünolojik yanıtları incelenmiştir. Beklendiği gibi, hem enfeksiyonlu hem de enfeksiyonsuz hastanın Treg sayıları azalmıştır. Hastanın Treg hücre sayıları azalmış olmasına rağmen, devam eden savunma ve homeostaz

nedeniyle enfeksiyonlu örnekte sitozolik nükleik asit ligandlarına bağılı IL-10 salımı artmıştır. CMV enfeksiyonu altında hasta, TLR'lerin ve sitozolik nükleik asit sensörlerinin aktivasyonu üzerine artmış IL-1 β ve TNF- α salımı göstermiştir. Bununla birlikte, herhangi bir viral enfeksiyon olmaksızın, hastada TLR7 ve TLR8 aracılı IL-1 β ve TNF- α tepkileri bozulmuştur. Ayrıca, CMV enfeksiyonu altında, hastada TLR ve sitozolik nükleik asit sensörü aracılı antiviral IFN- α , IFN- γ ve IL-12 tepkileri artmıştır. Ancak enfeksiyondan sonra, hastanın endosomal TLR'lerden ve sitozolik nükleik asit'lerden IFN- α ve IFN- γ yanıtlarını azaldığı görülmüştür. Viral enfeksiyon altında, doğal bağışıklık hücreleri ve bunların efektör sitokinleri tarafından devam eden savunmaya bağılı olarak daha yüksek yanıt görmesi bekleniyordu. Tıbbi tedavi ile enfeksiyon temizlendiğinde, RLTPR hastasında sadece endosomal TLR'lerin değil, sitozolik nükleik asit sensörlerinin de bozulduğu ve bu da hastayı viral enfeksiyonlara karşı savunmasız hale getirmiş olabileceği gözlenmiştir.

Ardından, RLTPR ve TLR1 eksikliği olan hastanın bağışıklık tepkileri araştırıldı. Hastanın tam kanından azalan Treg hücre sayısı doğrulandı. Hastanın PBMC'leri TLR ve sitozolik nükleik asit ligandları ile uyarıldığında yüksek IL-1 β salımı gözlemlendi. Özellikle TLR2-6 yanıtındaki yüksekliğin TLR1 eksikliğinin bir sonucu olabileceği düşünülmüştür çünkü TLR1 eksikliğinde, TLR6'nın eksik olan immün yanıtı kompanse edilebildiği bilinmektedir. Ayrıca, farklı PRR ligandlarına karşı verilen proinflatuar sitokin tepkisi, devam eden enfeksiyonla gerekebilir. Endosomal TLR ve sitozolik nükleik asit ligandları üzerinden IFN- α salımının arttığı gözlenmiştir. Hastanın IL-12 seviyeleri, devam eden bir enfeksiyonu düşündüren çeşitli yanıtlar göstermiştir. TLR1 eksikliğinden kaynaklanan TLR1 aracılı IL-12 yanıtı ise gözlenmemiştir. Ayrıca, hastada TLR2-6 aracılı IL-12 yanıtı daha yükselmiş ve bu da TLR1 eksikliğinin telafi edilmesine katkıda bulunduğunu düşündürmüştür. Hasta, Treg hücreleri azalmış olsa bile TLR ve sitozolik nükleik asit ligandlarına yüksek IL-10 yanıtı vermiştir.

T-hücrelerinin negatif düzenleyicisi olan CTLA-4, aktive edilmiş T-hücreleri ve Treg hücreleri üzerinde bulunur. CTLA-4, APC'ler üzerindeki CD80/86 ligandlarına bağlanarak CD28 ko-stimülasyonunu engeller. CD28 ko-stimülasyonu olmadan, T-hücresi aktive edilemez. Yetersiz CTLA-4 reseptörü olan hastaların, azalmış fonksiyona sahip artan sayıda Treg hücresine sahip olduğu bilinmektedir. Ayrıca farklı çalışmalarda hastaların azalmış hiperaktif T-hücreleri, artmış otoreaktif B-

hücreleri ve dolaşımında azalmış B-hücreleri olduğu gösterilmiştir. Bu noktada, dört CTLA-4 hastasının bağışıklık tepkileri incelenmiştir. Hasta #1'de, yüksek CD4+/CD8+ oranı, yüksek LDG sayısı, ayrıca CD3+ hücrelerinde azalmış TCR ekspresyonu ve yükselmiş pAKT protein seviyeleri gözlenmiştir. Hasta #2, tam kanda artmış LDG sayımına, azalmış pDC ve Treg popülasyonuna sahipti. Hasta #2'ye benzer kan hücresi profilin ek olarak Hasta #3 artmış monosit yüzdesine ve daha düşük CD4+/CD8+ oranına sahipti, bu da devam eden bir enfeksiyonu gösterebilir. Hasta #4, azalmış pDC, Treg sayıları, CD8+ hücrelerinde, Treg hücrelerinde ve B-hücrelerinde artan PD-L1 seviyeleri, CD3+ T-hücrelerinde azalmış TCR ekspresyonu, artmış pAKT ve p4EBP1 protein seviyeleri göstermiştir ve bunların tümü CTLA-4 mutasyonunun otoimmün durumunu telafi etmeye katkıda bulunabilir. Tüm hastaların B-hücre yüzdeleri ve CTLA-4 ekspresyon seviyeleri değişmezken, mTOR, pmTOR, STAT3, pSTAT3, AKT, p4EBP1 ve Hif-1 α protein seviyeleri bozulmuştu. Özet olarak, bulgularımız bu eksikliklerin düzensizliğinin karmaşıklığına işaret etmekte ve bu hastaların gözardı edilen bir immün fonksiyonel durumuna işaret etmektedir. Hastalıkları sadece edinsel bağışıklık sistemi ile ilişkili olarak algılanan bu bireylerin doğuştan gelen bağışıklık kolunun araştırılmasının, bu hastalara daha sağlam tedaviler sunmak için gerekli olduğunu öneriyoruz.

Anahtar Kelimeler: Birincil Bağışıklık Yetersizliği, RLTPR, TLR1, CTLA-4, doğal bağışıklık sistemi, edinsel bağışıklık sistemi.

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ABBREVIATIONS

AKT	Serine/threonine-specific protein kinase (protein kinase B)
ALRs	AIM2-like receptor
APC	Antigen Presenting Cells
AMPK	5' AMP-activated protein kinase
BCRs	B-cell Receptors
BSA	Bovine Serum Albumin
CMB	CARMA1–BCL10–MALT1
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMPs	Danger Associated Molecular Patterns
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EBV	Epstein-Barr Virus
ERK	ETS domain-containing protein
FBS	Fetal Bovine Serum
HIF-1 α	Heterodimeric transcription factor hypoxia-inducible factor 1
HIV	Human Immunodeficiency Virus
IFN	Interferon
IKK	I κ B Kinase
IRAK	IL-1 Receptor Associated Kinase
IRFs	Interferon Regulatory Factors
LDGs	Low Density Granulocytes
LPS	Lipopolysaccharide
LRRs	Leucine Rich Repeats
MAVS	Mitochondrial Antiviral Signaling Protein
MD-2	Myeloid Differentiation protein-2
MDA5	Melanoma Differentiation Associated 5
MFI	Mean Fluorescent Intensity
mTOR	Mechanistic target of rapamycin

MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear Factor Kappa B
NLRs	NOD Like Receptor
NLRC4	NLR family CARD domain containing 4 protein
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NOD	Nucleotide Oligomerization Domain
ODNs	Oligodeoxynucleotides
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
pDCs	Plasmacytoid Dendritic Cells
poly (dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
poly (I:C)	Polyinosinic-Polycytidylic acid
PIDs	Primary Immune Deficiencies
PRRs	Pathogen Recognition Receptors
RIG-I	Retinoic Acid Inducible gene I
RLRs	RIG Like Receptors
RLTPR	RDG, LRR, Tropomodulin and Proline-Rich containing protein
RNA	Ribonucleic Acid
SCID	Severe Combined Immunodeficiency
ssRNA	Single-stranded RNA
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of IFN Genes
TANK	TRAF family member associated NFκB activator
TBK1	Tank Binding Kinase protein 1
TCRs	T-cell receptors
TLRs	Toll Like Receptors
TNF	Tumor Necrosis Factor
TIR	Toll/IL-1 Receptor
TRAF	TNF Receptor Associated Factor
Tregs	Regulatory T-cells
TRIF	TIR domain containing adaptor inducible IFNβ

CHAPTER 1

INTRODUCTION

1.1 Immune System

Pathogens are as ancient as life. All species had a chance to encounter many pathogens which helped them to develop protection mechanisms for survival during evolution. Identification of the markers that do not resemble itself or lack of resembling markers are the most widely used strategies. Thanks to pathogens, all organisms have some sort of immune systems from primitive such as plants and invertebrates to complex. Complexity of the immune system is correlated with the evolutionary order, so the mammalian immune system is the most sophisticated one compared to others [1].

The Mammalian immune system is composed of two interconnecting parts which are innate and adaptive immune systems. At different levels of the protective barriers, special types of cells, molecules and receptors of the innate or adaptive systems play a role to prevent pathogen invasion into the organism. The first obstacle that pathogens will encounter is the skin or mucosal layers which create physical barriers. If this layer is breached, antimicrobial peptides and complement system are the new protective barriers for already invaded pathogens. There are some cases that complement system become ineffective over pathogens, and innate immune cells arrive on the scene. With the help of germline encoded receptors, innate immune cells give a fast and immediate response to detect pathogen markers that do not correspond itself [2]. In case of undefeated pathogens, antigen presenting cells (APCs) interconnect the innate immune system to the adaptive immune system. In adaptive immune system, unlike innate immunity, germline gene segments are somatically recombined to increase the receptor pool for more extensive recognition which causes slower response against pathogens. Adaptive immunity is composed of cell mediated and humoral immunities. Cell mediated immunity refers to T-cells derived response while humoral immunity is provided by antibodies that are produced by B-cells. Firstly, T-cells that contain T-cell receptors (TCRs) are developed at thymus, and B-cells that carry B-cell receptors

(BCRs) containing immunoglobulins are produced at bone marrow. Cytotoxic T-cells or CD8⁺ T-cells can target and directly kill cells carrying pathogenic peptides. Helper T-cells or CD4⁺ T-cells which gave rise to different subtypes which lead to produce more organized immune response by helping other immune cells. After maturation, both T- and B-cells move to lymph nodes and spleen to be activated in case of any undefeated pathogen infection which escaped from innate immunity. APCs presents pathogen peptides to the T-cells and B-cells, Helper T-cells or APCs activate B-cells to induce immunoglobulin production such as IgG, IgA and IgE to constitute memory as memory T-cells [3].

1.1.1 Innate Immune System

Different types of pathogens also commensal organisms can cause infections when the physical barrier of the organism is breached. Skin and mucosal surfaces introduced by urinary, gastrointestinal and respiratory epithelial tissues are the physical barriers that pathogens encounter first. If pathogens invade through these surfaces, another aspect of this pioneer safety fence takes part by producing antimicrobial proteins and peptides (APPs). Epithelial cells of skin, urinary, gastrointestinal and respiratory tract secrete APPs such as cathelicidins, defensins, calprotectin and bactericidal permeability increasing proteins into the body fluids like saliva, airway surface liquid and gastric juice [4]. Some APPs target negatively charged bacterial surfaces via electrostatic interactions while most of the APPs are produced to permeabilize cytoplasmic membranes of pathogens [5]. However, as the immune system had evolved against pathogens, they were also evolved to pass the immune system defenses. When the physical and APP barriers are overcome by pathogens, the complement system is there to prevent spreading. Complement system is composed of many plasma and cell surface proteins which compose the membrane attack complex on the pathogen membrane. When a pathogen is recognized by the complement system, one of the three mechanisms, which are classical, lectin or alternative pathway, is activated. Cell surface and plasma proteins are recruited on the pathogen surface to opsonize and lyse pathogen via membrane penetration. Also, complement system leads to proinflammatory molecule release for inflammatory response besides targeted pathogen eradication [6]. In some cases, the complement

system may not be enough to clear out invaded pathogens, and innate immune cells which have Pathogen Recognition Receptors (PRRs) take the scene. Neutrophils, monocytes, macrophages and dendritic cells as the members of innate immunity contain PRRs, and identify self and nonself particles to stop invasion of pathogens containing nonself motifs. PRRs are composed of Toll-like Receptors (TLRs), Nucleotide Oligomerization Domain-like Receptors (NLRs), Retinoic Acid Inducible Gene-I-like Receptors (RLRs), C-type Lectin Receptors (CLRs) and Absent In Melanoma 2-like Receptors (ALRs) which can distinguish Pathogen Associated Molecular Patterns (PAMPs) and Danger Associated Molecular Patterns (DAMPs). Pathogens release some components like LPS, unmethylated CpG or flagellin to the extracellular matrix, lymphatic and blood which are recognized as PAMPs while host DNA, histones or ATP released from stressed host cells are identified as DAMPs. Both PAMPs and DAMPs lead to cytokine and/or chemokine release with following innate immune cell recruitment to the site, and inflammatory response [7].

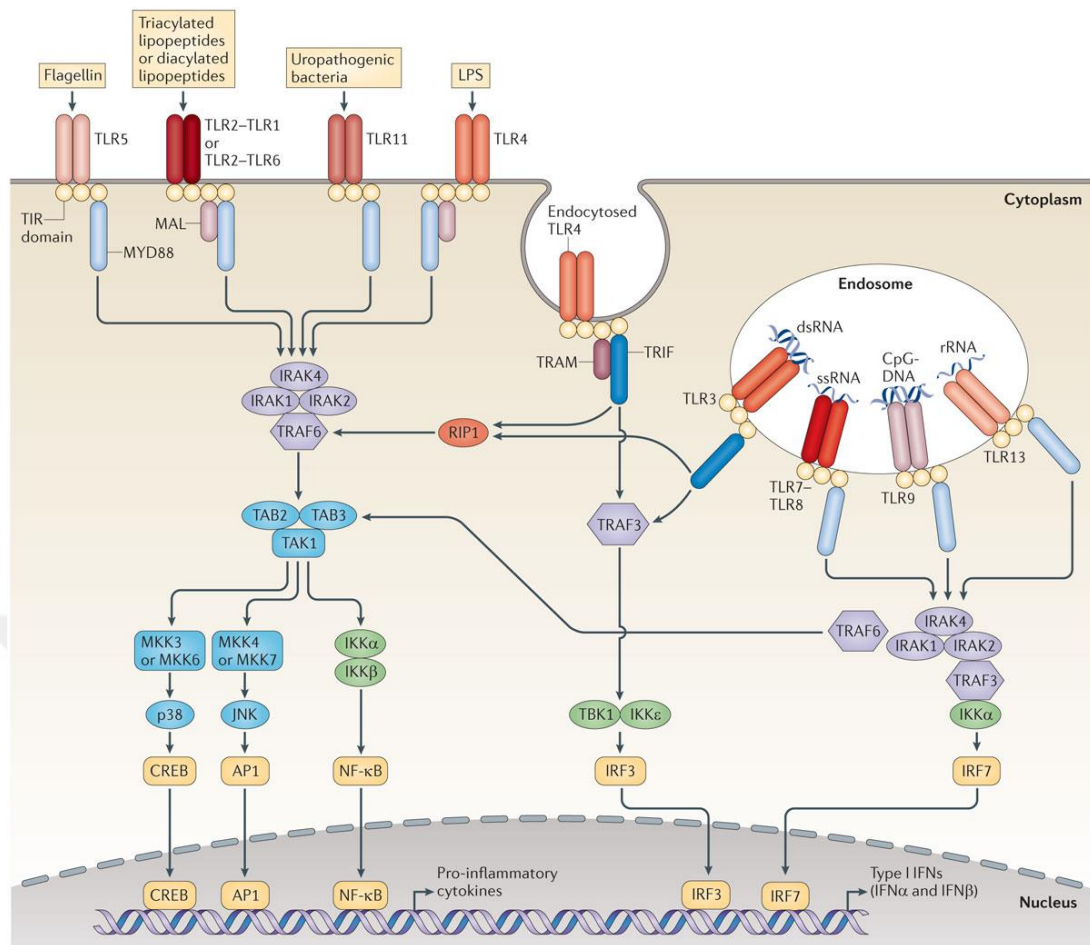
1.1.1.1 Pathogen Recognition Receptors (PRRs)

Host PRRs play a crucial role when pathogens such as protozoa, fungi, bacteria and viruses are entered the organism through breaching physical barriers and antimicrobial proteins and peptides. There are 5 divisions of the PRRs which are most widely studied TLRs, NLRs and RLRs as cytosolic nucleic acids, lastly CLRs and ALRs. If there was any PAMPs or DAMPs to induce different types of PRRs, expression and synthesis of the proinflammatory chemokines, cytokines, immunoreceptors and cell adhesion molecules increases to give a quick early response to infection, also interconnecting to adaptive immunity for intensive late response [8].

1.1.1.1.1 Toll-Like Receptors (TLRs)

Toll-like Receptors, as the name refers, share structural and genomic similarities with Toll receptors of *Drosophila* which functions against Gram-positive bacterial and fungal infections by producing antimicrobial peptides and proteins, also functions for development of embryo [9]. When phylogenetic analyses are examined, variances of Toll family receptors occurred independently in mammals and insects which explains Toll like receptors of mammals only function as host protection instead of additional

embryonic development [10]. Besides insects, also many metazoans contain TLRs and downstream signal transductions according to transcriptome and genome sequencing [11]. Toll-like Receptors are composed of three main structural parts. The first part, an ectodomain which recognizes DAMPs and PAMPs are hydrophobic Leucine Rich Region (LRR) with 45 Leucine rich parts. The second part is a transmembrane domain that emphasizes TLRs are membrane spanning proteins. And the last part is an intracellular domain called the Toll Interleukin-1 Receptor (TIR) which activates downstream transduction [12]. Mouse has 12 functional Toll-like Receptors (TLR1-11, 13) while Human has 10 functional TLRs (TLR1-10). Even though humans have TLR11, it is not functional due to a stop codon in the genome [13]. Toll-like receptors are differentially localized in the cell which means some of them are located at the cell surface while others are present at endosomal structures. This variability enables host cells to identify different pathogens that use diverse invasion mechanisms. For instance, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are placed on the cell surface to identify extracellular PAMPs and DAMPs while TLR3, TLR4, TLR7, TLR8, TLR9 and TLR13 are present inside endosomes to detect cytoplasmic PAMPs or DAMPs. Distinctly, TLR4 is the only Toll-like Receptor that can be localized both on the surface and in the endosomes [14].



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Figure 1.1 Mammalian TLR signaling pathways and their ligands. Adopted from [15].

1.1.1.1.1 Signaling Pathways of Toll-like Receptors

Signaling cascade of Toll-like Receptors start with PAMPs or DAMPs interaction to TLRs, and dimerization of the receptors occur. After receptor dimerization, intracellular TIR domain which is conserved through all TLRs recruits MyD88, TIRAF or TRIF adaptors to the vicinity. When the TIR domain and MyD88 adaptor protein interacts, activated MyD88 associates with the N-terminal death domain of the IL-1 Receptor Associated Kinase (IRAK) to be phosphorylated for activation. Then, IRAK activates Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6) which is a E3 ubiquitin ligase via binding to TRAF domain. Binding of IRAK and TRAF6 to each other causes release of the complex from the TIR domain to interact with TGF-

β Activated Kinase 1 (TAK1) and TAK1 Binding proteins called TAB1 and TAB2. At this point, TRAF6-TAK1-TAB1-TAB2 complex detached from IRAK1 which stands inside the membrane and be degraded later [16]. Activated TAK1 branches out two different downstream signaling which are NF- κ B and MAPK pathways. At NF- κ B pathway, activated TAK1 associates with the I- κ B Kinase (IKK) complex and activates it via phosphorylation. The I- κ B is phosphorylated by the IKK complex which results in I- κ B degradation, and relocation of NF- κ B into the nucleus to enable the production of proinflammatory cytokines such as IL-1 β , IL-6 and TNF α . However, at MAPK pathways, ERK1/2, p38 and JNK are activated by TAK1 which causes the activation of transcription factor AP1 for inflammation response. All Toll-like Receptors except TLR3 recruit MyD88 adaptor to initiate proinflammatory response activation [17]. TLR7-8 and TLR9 initiates not only MyD88 mediated inflammation but also activates Interferon Regulatory Factor 7 (IRF7) by IRAK. Besides the proinflammatory cytokines, Type 1 Interferon mostly IFN α is produced by IRF7 translocation into the nucleus for gene transcription [18].

While Toll-like Receptor other than TLR3 uses MyD88 adaptor to stimulate proinflammatory cytokine and/or Type 1 Interferons production, TLR3 also TLR4 use TRIF adaptor. TRIF recruits either TRAF6 or TRAF3 to the TIR domain that leads to different pathway activations. TRAF6 binds to RIP1 Kinase for TAK1 interaction and activation. When TAK1 is activated, proinflammatory cytokines are produced via NF- κ B and MAPK pathways. However, TRAF3 associates TBK1 and IKK ϵ to IRF3 activation by phosphorylation. Activated IRF3 dimerize and travel inside the nucleus for Type 1 Interferon gene mostly IFN β transcription [19].

1.1.1.1.2 Cell Surface Toll-like Receptors

TLR1, TLR2, TLR4, TLR5 and TLR6 are cell surface Toll-like receptors.

TLR1, TLR2 and TLR6 are found in epithelial cells, endothelial cells, monocytes, macrophages, myeloid dendritic cells, T-cells and B-cells. TLR2 is activated by lipoproteins of Gram-negative bacteria, lipoteichoic acid and peptidoglycan of Gram-positive bacteria, and zymosan of fungi via heterodimerization with either TLR1 or TLR6 to enable proinflammatory cytokine production. TLR1 and TLR6 share very similar genomic structure which was probably caused by evolutionary duplication.

This similarity may cause a functional compensation to recognize triacyl lipopeptides when TLR1 is defected, but TLR6 is not [20]. Triacyl lipopeptides bind to TLR1 and TLR2 hydrophobically that causes TLR1&TLR2 heterodimerization. Triacyl lipopeptides are recognized by TLR1&TLR2 heterodimer, while diacyl lipoproteins are sensed by TLR2&TLR6 heterodimer [21].

TLR4 is found in epithelial cells, monocytes, macrophages, myeloid dendritic cells, T-cells and B-cells. TLR4 can sense lipopolysaccharides (LPS) which is found at the outer membrane of Gram-negative bacteria. On the cell surface, TLR4 is physically attached to Myeloid Differentiation protein 2 (MD2) to form a complex which is required for LPS binding. The hydrophobic motifs of LPS cause aggregation which causes requirement of additional help for LPS transfer to the receptor. LPS Binding Protein (LBP) and CD14 proteins can bind and transfer LPS to the TLR4-MD2 complex, or can cause LPS induced receptor endocytosis [22]. With the LPS binding, conformation of the TLR4 TIR domain changes, and allows homodimerization. Because TLR4 can both be found at the cell surface and endosome, different adaptor proteins are used at each place. MyD88 is used by cell surface bound TLR4 to induce proinflammatory cytokine release, while TRAM&TRIF are used by endocytosed TLR4 to promote both proinflammatory cytokines and IRF3 induced Type 1 Interferons mostly IFN β [23].

TLR5 is found in epithelial cells, monocytes, macrophages, myeloid dendritic cells and T-cells. TLR5 is activated by flagellin, and homodimerize to recruit MyD88 for proinflammatory cytokine release under infections. TLR5 is very important for intestinal infections because when microbiota breaches the physical barrier and starts to invade, bacterial flagellin is sensed by TLR5 [24].

1.1.1.1.3 Endosomal Toll-Like Receptors

TLR3, TLR7, TLR8 and TLR9 located in the endosomes which sense intracellular nucleic acids replicated in the host, and endocytosed nucleic acids [25].

TLR3 is expressed in murine macrophages, and both murine and human myeloid dendritic cells. During viral replication inside the host, double stranded RNA (dsRNA) is produced by viruses, and it is sensed by TLR3. When dsRNAs such as polyinosinic:polycytidylic acid (poly I:C) bind to TLR3 leading homodimerization,

MyD88 is recruited and result in both NF- κ B and MAPK induced proinflammatory cytokine production, and dendritic cell maturation [26]. Besides MyD88, TRIF is also recruited to TLR3 TIR domain with the presence of dsRNA, and promotes IRF3 mediated Type 1 Interferon production for antiviral response [27].

TLR7 and TLR8 senses single stranded RNAs (ssRNA) of viruses, and analogs of ssRNA imidazoquinoline such as R848 and Resiquimod. TLR7 and TLR8 are very similar in structure due to evolutionary duplication, and both receptors are functional in humans while only TLR7 is active in mice [28]. TLR7 is found in plasmacytoid dendritic cells and B cells, and TLR8 is present in neutrophils, macrophages and dendritic cells. With the receptor mediated endocytosis, in the phagolysosome, viral ssRNA is released due to viral coat protein hydrolysis [29]. TLR7 and TLR8 recognize ssRNA which leads to receptor homodimerization and stimulation of proinflammatory cytokine synthesis besides IRF7 mediated Type 1 Interferon production for antiviral response [30].

TLR9 recognizes double stranded DNA from the unmethylated CpG site which is found in viruses and bacteria while mammalian cells contain methylated CpG motifs in DNA which is not sensed. To be detected by the TLR9, viral coat protein is degraded inside the endolysosome, and free the dsDNA from the virion [8]. Other than remaining Toll-like receptors, TLR9 is very specific in response due to presence in only B-cells and plasmacytoid dendritic cells (pDCs) [31]. When dsDNA is sensed by the receptor, homodimerization occurs, and MyD88 is recruited to the site which results in proinflammatory cytokine production to activate macrophages, proliferation and differentiation of B-cells into plasma cells which secrete antibodies along with the IRF7 mediated Type 1 Interferon secretion from activated and proliferated pDCs [32]. TLR9 is not only activated by bacterial and viral dsDNA, but also induced by synthetically produced oligonucleotides (ODNs). Single stranded ODNs which are required for B-cell activation are formed as unmethylated CpG with the two 5' purine and two 3' pyrimidine flanks [33]. There are four different CpG ODN types found so far as K-type (B-type), D-type (A-type), C-type and P-type. K-type ODN is specific to contain multiple CpG on phosphorothioate backbone which is more resistant to nuclease digestion and promotes half-life compared to classical phosphodiester backbone. TNF α production, pDC activation and IgM producing plasma cell differentiation from B-cells are typical causes of sensed K-type ODNs. Also, K-type

ODN is directly transported into the late endosome from early endosome of pDCs. Besides K-type ODN, D-type ODNs contain single CpG with palindromic flanks on a mixed phosphodiester/phosphorothioate backbone with 3' to 5' poly G tail which promotes concatemer formation. Because D-type ODN stays more in the early endosome, IRF7 mediated IFN α secretion and pDC differentiation are induced while no B-cell stimulation occurs. C-type ODN is a mixture of both K- and D-type ODNs. It contains phosphorothioate backbone as K-type ODN, and a palindromic CpG part like D-type ODN. Moreover, C-type ODN gives a combined response by triggering both B-cells and IFN α producing pDCs due to localization at both early and late endosome. In addition to C-type ODN, P-type ODNs are composed of two palindromes which cause higher order formation in structure. B-cells and pDCs are activated by P-type ODN while IFN α secretion is higher than C-type ODN induce pDCs [34].

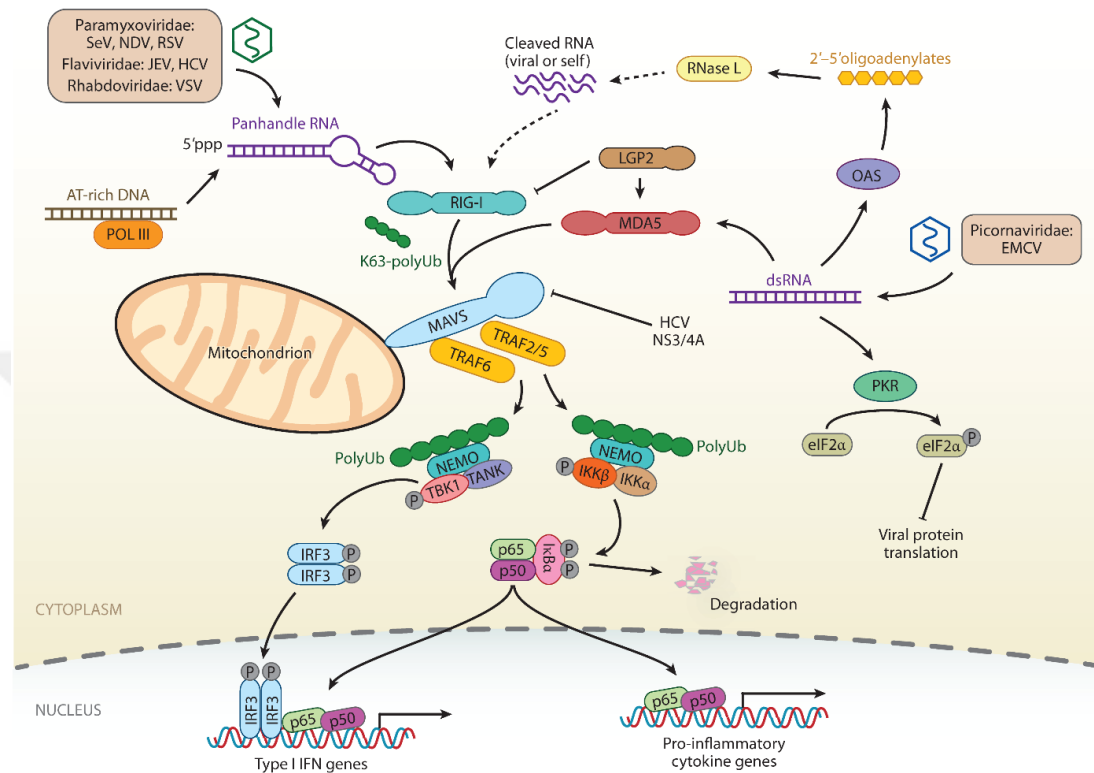
1.1.1.1.2 Cytosolic Nucleic Acid Sensors

Besides endosomal nucleic acid detection receptors, cytosolic nucleic acid sensors are also important during infections to sense any pathogen inside the cell. There is one RNA detecting receptor named as RIG-I Like Receptors (RLRs) while two DNA sensing receptors that are AIM2-Like Receptors (ALRs) and STING-Dependent Activators [35].

1.1.1.1.2.1 RIG-I Like Receptors (RLRs)

Retinoic Acid-Inducible Gene-I (RIG-I) and Melanoma Differentiation Gene 5 (MDA5) are the two RLRs that recognize RNA of pathogens in the host cytosol [34]. Pathogenic RNAs either contain 5' end modifications such as 5' triphosphate, or can be in the form of long dsRNAs which are not found in host RNA. These differences help RLRs to recognize pathogenic RNAs while they are not activated by endogenous RNAs. 5' end modifications are detected by RIG-I while long dsRNAs are sensed by MDA5 [36-37]. When receptors associate with a pathogenic RNA or transfected synthetic polyI:C, CARD domain of the receptors become activated and cause Mitochondrial Antiviral Signaling Protein (MAVS) recruitment along with the self-

polymerization of MAVS which is required for signaling. Then, TRAF associates with the MAVS polymer to promote NF- κ B induced proinflammatory cytokine production and IRF3 mediated Type 1 Interferon release [38].



Wu J, Chen ZJ. 2014. Annu. Rev. Immunol. 32:461–88

Figure 1.2 Cytosolic nucleic acid sensors, RIG-I like receptors (RLRs). Adopted from [39].

1.1.1.1.2.2 AIM-2 Like Receptors

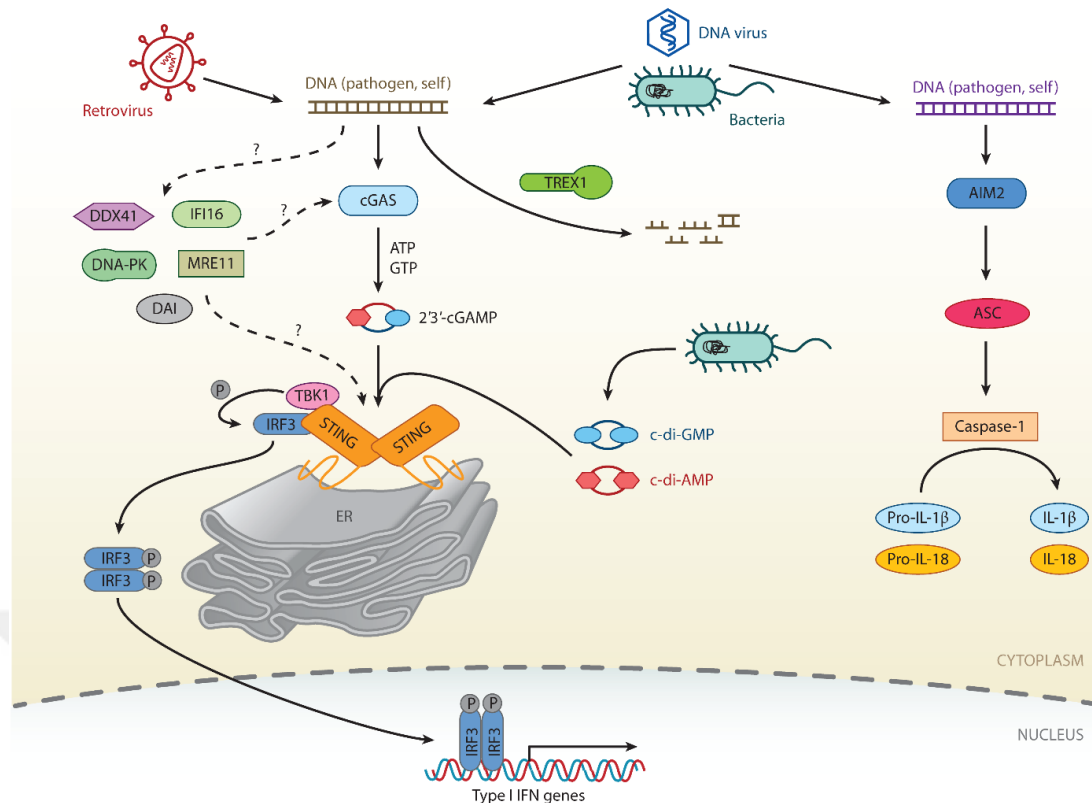
Originally DNA was only found in nucleus. When any DNA inside the cytosol is detected, it works as PAMPs from a pathogen or DAMPs from the infected and stressed host cell. Cytosolic nucleic acid is sensed by AIM-2 which recruits apoptosis-associated speck-like protein containing a CARD (ASC), and ASC pyroptosome is formed to induce NLRP3-independent inflammasome. Then, the inflammasome

complex activates Caspase-1 to promote proinflammatory cytokine activation such as IL1 β and IL18, and rapid cell death which enhances inflammation [40].

1.1.1.1.2.3 STING-Dependent Sensors

There are also more cytosolic nucleic acid sensors except than AIM-2. IFI16, DAI, DDX41, LSm14A and LRRFIP1 recruit Stimulator of IFN Genes (STING) adaptor when dsDNA is sensed to produce IRF3 mediated Type 1 Interferon response. However, Sox2 activates TAK1 instead of STING for NF- κ B promoted proinflammatory cytokine production. DHX9/36 uses MyD88 for proinflammatory response, and IRF7 for antiviral response. Also, RNA Polymerase III/Ku70 induces antiviral immunity by IRF1 and IRF7 [41].

STING is not only an adaptor, but also a receptor which binds cyclic dinucleotides. When the cyclic DNA which is a common pathogenic product is present in the cytosol, cGAMP Synthase (cGAS) converts it into cGAMP by using ATP and GTP. Produced cGAMP is detected by STING which promotes Type 1 Interferon production for antiviral response [42].



Wu J, Chen ZJ. 2014.
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Figure 1.3 Cytosolic nucleic acid sensors, STING dependent pathways. Adopted from [39].

1.1.2 Immune Deficiencies

In a healthy individual, if pathogens breach physical protective barrier, firstly antipathogenic proteins and peptides, then innate immune cells fight against pathogen spread through organism. Also, in case of unstopped infection, antigen presenting cells carry pathogenic antigens to the lymph nodes to activate T- and B-cells which gives more antigen specific but slower response, along with the memory. Then, an immune regulatory response is activated to prevent tissue damage after pathogen clearance. However, there could be cases that any part or a particular mechanism do not work in the organism due to several causes. It results in Immunodeficiencies which do not allow a proper immune response, as a result higher chance of infection occurs. Immunodeficiencies divided into two which are Primary Immunodeficiency Disorders

(PIDs) and Secondary/Acquired Immunodeficiency Disorders. PIDs are reasoned by mutations in genes which could be hereditary, also seen mostly at infancy and childhood. Secondary Immunodeficiency Disorders are caused by multiple reasons such as certain drug exposures like immunosuppressants, chemotherapy drugs, corticosteroids; other disorders like prolonged diabetes, cancers that affect bone marrow or HIV infection; and radiation therapies. Also, undernutrition of all or a particular one is a very potent cause for secondary immune deficiencies because losing 20-30% of the body weight cause severe immune impairments [43].

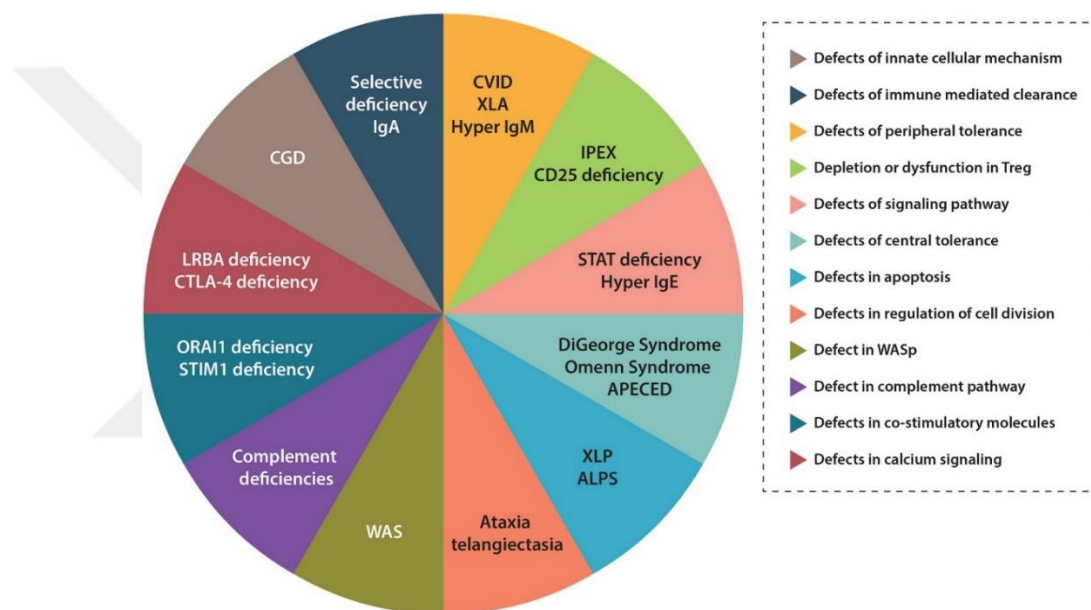


Figure 1.4 Primary Immune Deficiencies. Adapted from [44].

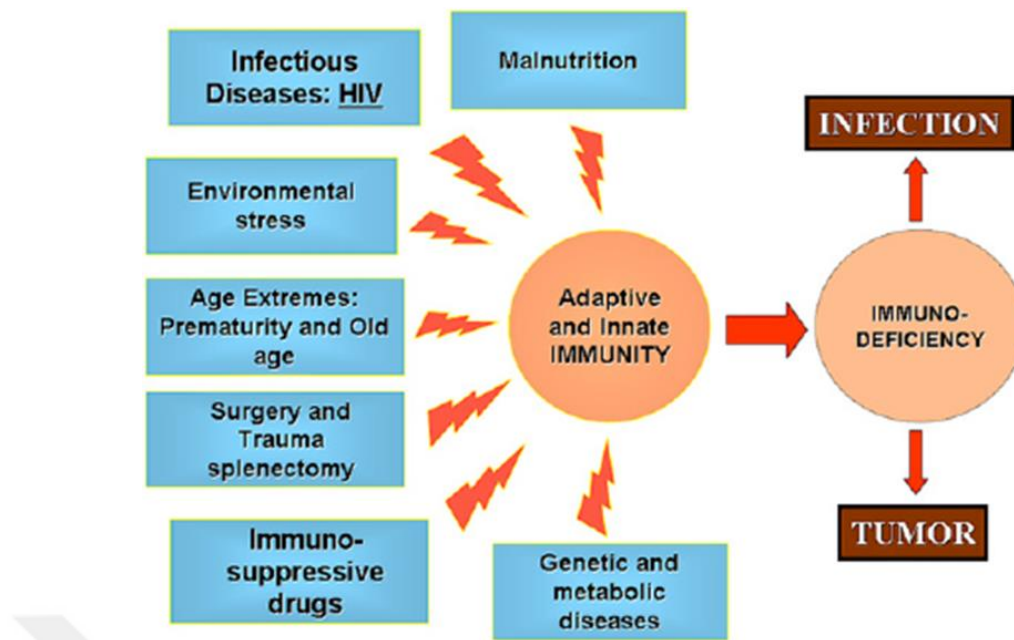


Figure 1.5 Secondary Immune Deficiency causing factors. Adapted from [45].

1.1.2.1 Primary Immune Deficiency Disorders

Some heterogeneous genetic disorders that are PIDs are reasoned by immune system deficiencies which leads immune dysregulation like autoimmunity, malignancies and repetitive infections. Due to autosomal recessive gene inheritance, consanguineous marriages which are between relatives like cousins increase the risks of PIDs in offspring. Besides marriage between relatives, rapid population growth and bigger family size which all three are very common at Middle East and North Africa are the contributors of the PIDs. Morocco, Tunisia, Israel, Egypt, Iran, Kuwait, Saudi Arabia are the countries that have higher combined immunodeficiency cases while antibody deficiencies and autoinflammatory disorders are found dominantly in Turkey [46].

Unlike to secondary immunodeficiencies that occur via malnutrition, infections or drugs, PIDs are inherited immune system disorders which affect development or function. PIDs can be classified into two broad groups as innate immunity disorders and adaptive immunity disorders. Innate immunity is important to prevent spread of infections in the first place and play roles to interconnect adaptive immunity. Complement proteins, dendritic cells and phagocytes such as neutrophils, monocytes, macrophages are the members of large innate immune family. Phagocyte defects

which cannot engulf pathogen and complement defects that cannot opsonize or lyse pathogens are classified in innate immune disorders. Severe pyogenic infections, chronic granulomatous disease and hyper-IgE syndrome are examples of phagocyte defects while autoimmunity can be caused by complement system defects [47].

Adaptive immune defects are also divided into three group as T-cell disorder which is cell mediated, B-cell disorders which is antibody mediated and combined immunodeficiency which are caused by any defect that occur at development, differentiation or maturation stages of adaptive immune cells. Lymphopenia which is having severely low levels of lymphocytes and neutropenia which is having very low levels of neutrophils can be results of T-cell immunodeficiencies. B-cell defects which are the most common type of PIDs can lead to low or lack of immunoglobulin levels in serum, or can be seen as normal or high levels of serum immunoglobulin with abnormal function. Common variable immunodeficiency (CVID), X-linked agammaglobulinemia (XLA) and selective IgA deficiency are the well-known examples of humoral immunodeficiencies. To present a healthy antibody production by B-cell mediated immunity, functionality of T-cells is crucial. With any T-cell defect, antibody production is also compromised, and lead to rare severe combined immunodeficiency disorders (SCIDs). So, either unfunctional T-cells combined with functional B-cells, or both unfunctional T- and B-cells introduce SCIDs which can be cured via bone marrow transplantation; otherwise, if it is not cured, SCIDs can be lethal. Besides abnormal adaptive immune cell functions, immune dysregulation and autoimmunity also relate to combined immune deficiencies. X-linked lymphoproliferative disease, DiGeorge syndrome, Wiskott-Aldrich syndrome and ataxia-telangiectasia are the highly studied examples of SCID [48].

1.1.2.1.1 RLTPR Deficiency

RLTPR (CARMIL2) is a cytosolic scaffold protein which facilitates CD28 co-stimulation for T-cell activation. Two separate signals are required for T-cell activation. TCR and antigen carrying MHC molecule binds for first signal while CD28 of T-cell and CD80/CD86 of APC binds to provide second co-stimulatory signal. When two signals were present, RLTPR help TCR and CD28 to recruit CARMA1, a cytosolic adaptor, along with the BCL10 and MALT1 which form CBM complex to

activate NF- κ B signaling pathway [49]. Besides facilitating CD28 co-stimulation, RLTPR takes place cell shape control, phagocytosis and endocytosis movements by promoting actin polymerization [50].

RLTPR deficiency causes impaired CD28 co-stimulation, and defective T-cell activation mostly along with the defective B-cell response which make patients susceptible to Epstein Barr Virus (EBV). Also, because cytoskeleton of the cells is affected, immune cell migration and T-cell polarity could be impaired [51-52]. The functional RLTPR enable CD4⁺ T-cells differentiate T-helper 1 (Th1) and T-helper 17 (Th17) cells, so RLTPR deficiency interrupts Th1 and Th17 differentiation. Moreover, due to impaired CD28 co-stimulation, especially Treg cell development and homeostasis became defected [53].

1.1.2.1.2 CTLA-4 Mutation

T-cell regulation depends on two main co-stimulatory proteins, CD28 and CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) which have homolog structures, and both bind to CD80 and CD86 ligands on APCs. CD28 is required for T-cell activation while CTLA-4 facilitates suppression of T-cell activity [54]. Both CD28 and CTLA-4 receptors are expressed on CD4⁺ and CD8⁺ T-cells; however, their affinities for CD80/86 ligands differ. CTLA-4 receptor has greater affinity for both CD80 and CD86 ligands than CD28 receptor which enables T-cell regulation. CTLA-4 is not only found on plasma membrane, but also found in intracellular vesicles of activated T-cells and Treg cells [55]. Only the 10% of the CTLA-4 is expressed on plasma membrane, because others internalized to sustain healthy trafficking via recycling and degradation [56]. Working mechanism of CTLA-4 is trans endocytosis to suppress T-cell function. When CTLA-4 binds to CD80 or CD86 on APCs with higher affinity, CTLA-4 bound CD80 or CD86, receptor-ligand complex, is removed from plasma membrane of the APC, and trans endocytosed into CTLA-4 expressing cell such as Treg cells. So, CD28 co-stimulatory receptor cannot receive CD80/86 signal for T-cell survival [57].

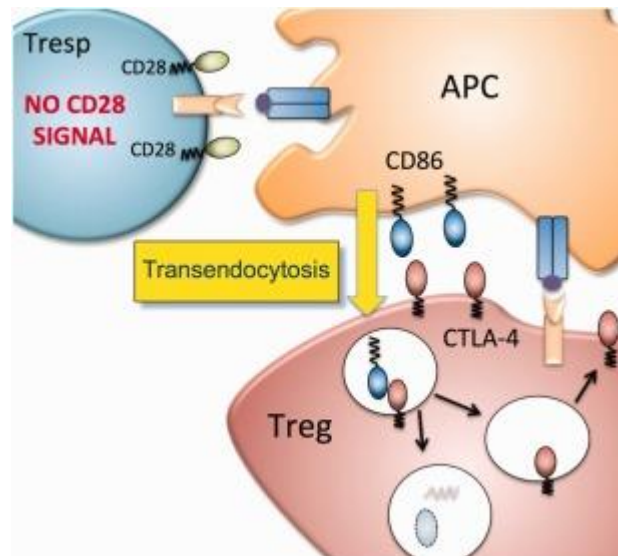


Figure 1.6 Working mechanism of CTLA-4 receptor. Adapted from [56].

In case of unfunctional CTLA-4 receptor, T-cell mediated autoimmune disease are seen. During T-cell development in thymus, most self-reactive T-cells are deleted from repertoire while some of them are not eliminated. When deletion is not complete, autoreactive T-cells can circulate in the organism, and require additional control mechanism like CTLA-4 to prevent autoimmunity by T-cell suppression [58]. With an unfunctional CTLA-4 receptor, co-stimulatory CD28 can bind to CD80/86 ligands and activate autoreactive T-cells against self-antigens. Besides promoted T-cell activation, T-cell infiltration is enhanced while Treg cell activity is impaired in CTLA-4 deficient patients [56].

1.2 Aim of the Study

PIDs, heterogeneous genetic disorders, are reasoned by gene mutations which leads immune dysregulation like autoimmunity, malignancies and repetitive infections. Autosomal recessive gene inheritance is common in consanguineous marriages which increase the risks of PIDs in offspring. Consanguineous marriages, rapid population growth and bigger family size are very common concepts in Middle East and North

Africa, and they contribute the possibility of the PIDs. Turkey, Morocco, Tunisia, Israel, Egypt, Iran, Kuwait and Saudi Arabia are the countries that PIDs are recorded mostly. Investigation of the immune systems of the PID patients is necessary to offer more robust therapies to these patients, and necessary to reveal how aspects of immune system work and interplay under real life conditions. Therefore, molecular mechanisms and immune phenotypes of PID patients (RLTPR, RLTPR-TLR1, CTLA-4) were investigated in this thesis.

For RLTPR and RLTPR-TLR1 deficient patients, Treg cell percentages will be analyzed due to reported low Treg counts in other studies. Also, because RLTPR protein is important for T-cell activity, adaptive immune responses of patients were investigated mostly by others. So, innate immune responses of patients will be characterized by comparing healthy controls' and patients' PBMC cytokine secretion profiles upon different PRR stimulations via ELISA.

For CTLA-4 mutated patients, the effect of mutation on immune cell counts such as monocytes, pDCs, B-cells and Treg cells will be analyzed from whole blood by flow cytometer. Also, downstream proteins of activated CTLA-4 pathway such as mTOR, pmTOR, AKT1, pAKT, 4EBP1, p4EBP1, STAT3, ERK and other proteins that affect CTLA-4 protein expression as HIF1 α and pAMPK will be analyzed by immunoblotting to examine the protein level effect of the mutation. Moreover, PD-L1 expression in CD8⁺ T-cells, B-cells and Tregs will be investigated because PD-L1 is another negative regulator of T-cell activation. Besides PD-L1, TCR expressions levels of CD3⁺ cells will be monitored to understand if altered TCR expression would have an impact on severity of CTLA-4 mutation phenotype. To see the altered adaptive immune response, pSTAT3 levels of patients after PMA/io treatment will be analyzed by flow cytometer.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Culture and Buffers

Heat inactivated Fetal Bovine Serum (FBS), RPMI 1640 with L-Glutamine, Sodium Pyruvate, Penicillin-Streptomycin, HEPES Buffer and Dulbecco's Phosphate Saline Buffer (DPBS) were purchased from Biological Industries, Israel. Ultrapure Cell Culture Water was from Biosera, USA. Lymphocytes Separation Medium and Bovine Serum Albumin (BSA) were supplied from Capricorn Scientific, Germany. Non-Essential Amino Acids (NEAA) was purchased from HyClone, USA. Lipofectamine 2000® Reagent was obtained from ThermoFischer Scientific, USA.

2.1.1.1 Contents of Cell Culture Media

RPMI Medium containing 2% FBS:

- 500 ml RPMI-1640 Media with L-Glutamine (2mM final concentration)
- 10.64 ml heat inactivated FBS
- 5.32 ml Penicillin/Streptomycin (50 µg/ml final concentration)
- 5.32 ml Sodium Pyruvate (1100 µg/ml final concentration)
- 5.32 ml Non-essential Amino Acid (1X final conc. from 100X Stock Solution)
- 5.32 ml HEPES Buffer (10mM final concentration)

RPMI Medium containing 5% FBS:

- 500 ml RPMI-1640 Media with L-Glutamine (2mM final concentration)
- 27.47 ml heat inactivated FBS
- 5.5 ml Penicillin/Streptomycin (50 µg/ml final concentration)
- 5.5 ml Sodium Pyruvate (1100 µg/ml final concentration)

- 5.5 ml Non-essential Amino Acid (1X final conc. from 100X Stock Solution)
- 5.5 ml HEPES Buffer (10mM final concentration)

RPMI Medium containing 10% FBS:

- 500 ml RPMI-1640 Media with L-Glutamine (2mM final concentration)
- 58.14 ml heat inactivated FBS
- 5.8 ml Penicillin/Streptomycin (50 µg/ml final concentration)
- 5.8 ml Sodium Pyruvate (1100 µg/ml final concentration)
- 5.8 ml Non-essential Amino Acid (1X final conc. from 100X Stock Solution)
- 5.8 ml HEPES Buffer (10mM final concentration)

2.1.1.2 Content of Buffers

10X Phosphate Buffered Saline (10X PBS):

- 80 g NaCl
- g KCl
- 15.2 g Na₂HPO₄·2H₂O
- g KH₂PO₄
- Completed up to 1 L with distilled water
- 6.8 pH was adjusted and autoclaved
- Stored at room temperature

1X Phosphate Buffered Saline (1X PBS):

- 10X PBS was diluted with distilled water
- 7.2-7.4 pH was adjusted and autoclaved
- Stored at room temperature

Blocking Buffer for ELISA:

- 25 g BSA (5% final concentration)
- Dissolved in 300 ml 1X PBS
- 250 µl Tween20 (0.025% final concentration)
- Completed up to 500 ml with 1X PBS
- Aliquoted and stored at -20° C

Wash Buffer for ELISA (Freshly prepared):

- 4.5 L distilled water
- 0.5 L 10X PBS
- 2.5 ml Tween20
- Stored at room temperature

Fluorescent Activated Cell Sorting (FACS) Buffer for Flow Cytometry:

- 5 g BSA
- 250 mg Sodium Azide (NaN₃)
- Completed up to 500 ml with 1X PBS
- Stored at +4° C

RIPA Lysis Buffer:

- 72 µl 2M NaCl
- 50 µl 1M Tris (pH=8)
- 10 µl NP-40
- 10 µl 10% Sodium dodecyl sulfate (SDS)
- 815 µl distilled water
- 40 µl protease inhibitor (25X)
- 40 µl phosphatase inhibitor (25X)
- Stored at -20° C

4X SDS Sample Loading Buffer:

- ml 1 M Tris-HCl (pH 6.5)
- ml of 1 M Dithiothreitol (DTT)
- 0.8 g of SDS
- 40 mg of bromophenol blue
- 3.2 mL of glycerol
- Complete up to 10 ml with distilled water

5% Stacking gel:

- 600 µl 1M Tris (pH=6.8)
- 100 µl 5% SDS
- 800 µl 30% Acrylamide mix

- 50 µl 10% APS
- 5 µl TEMED
- 3.4 ml distilled water

7% Resolving gel:

- 3.75 ml 1,5M Tris (pH=8.8)
- 300 µl 5% SDS
- 3.45 ml 30% Acrylamide mix
- 125 µl 10% APS
- 10 µl TEMED
- 7.65 ml distilled water

10X Running Buffer for SDS-Page:

- 144 g Glycine
- 30 g Tris-Base
- 10 g SDS
- Completed up to 1 L with distilled water
- Diluted to 1X with distilled water for usage
- Stored at room temperature

10X Transfer Buffer for Western Blot:

- 144 g Glycine
- 30 g Tris-Base
- Completed up to 1 L with distilled water
- Stored at +4° C

1X Transfer Buffer for Western Blot (Freshly prepared):

- 700 ml distilled water
- 200 ml Methanol
- 100 ml 10X Transfer Buffer
- Stored at -20° C

Blocking Buffer for Western Blot:

- 2.5 g Milk powder or BSA
- 50 µl Tween20
- Completed up to 50 ml with 1X PBS

PBS-T Wash Buffer for Western Blot:

- 999 ml 1X PBS
- 1 ml Tween20

Antibody Dilution Buffer for Western Blot:

- 2.5 g Milk powder or BSA
- 25 µl Tween20
- Completed up to 50 ml with 1X PBS

2.1.2 Ligands of PRR and Cytokine Receptors for in vitro stimulation experiments

Name of the ligands, their target receptor or signaling pathways with the working concentrations, purchased brand and catalog numbers are given in the **Table 2.1**.

Table 2.1 Ligands that are used in this study

Name of the Ligand	Target Pathway	Working Concentration	Brand & Country	Catalogue #
Pam3CSK4	TLR1/2	1 µg/ml	Invivogen-USA	tlrl-pms
Peptidoglycan	TLR2	5 µg/ml	Invivogen-USA	TLRL-PGNB3
Zymosan A (Isolated from <i>S. Cerevisiae</i>)	TLR2	5 µg/ml	Sigma Aldrich-Germany	Z4250-250MG
p(I:C)	TLR3	30 µg/ml	Invivogen-USA	tlrl-picw

LPS (Isolated from <i>E. coli</i>)	TLR4	5 µg/ml	Sigma Aldrich-Germany	L-2880-100mg
Flagellin (Isolated from <i>S. typhimurium</i>)	TLR5	100 ng/ml	Invivogen-USA	tlr-pstfla-5
R848	TLR7	5 µg/ml	Enzo Life Sciences- USA	ALX-420-038-M025
Resiquimod	TLR7/8	5 µg/ml	Sigma Aldrich-Germany	SML0196
D35-3CG*	TLR9	3 µM	Alpha DNA-Canada	590691
K3*	TLR9	1 µM	Alpha DNA-Canada	539665
2'3'-cGAMP**	STING	30 µg/ml	Invivogen-USA	tlrl-cga-23
HSV60**	cGAS-STING	5 µg/ml	Invivogen-USA	tlrl-hsv60n
p(dA:dT)**	AIM2 Inflammasome DAI-STING & RIG-I/MAVS	5 µg/ml	Invivogen-USA	tlrl-patn
LPS (Isolated from <i>E. coli</i>)**	Non-Canonical Inflammasome	5 µg/ml	Sigma Aldrich-Germany	L-2880-100mg
Flagellin (Isolated from <i>S. typhimurium</i>)*	NLRC4 Inflammasome	100 ng/ml	Invivogen-USA	tlr-pstfla-5
p(I:C)**	RIG-I/MAVS	30 µg/ml	Invivogen-USA	tlrl-picw
Alum**	NLRP3 Inflammasome	20 ng/ml	Invivogen-USA	tlrl-alk
Ionomycin	ER Ca ⁺² Channels	1 µg/ml	Calbiochem-Germany	407950

Phorbol-12-myristate-13-acetate (PMA)	Protein Kinase C	50 ng/ml	Sigma Aldrich-Germany	P1585
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*Sequence of D35-3CG CpG ODN: 5' GGtgcatcgatgcaggggGG 3' which has both phosphodiester (upper case) and phosphorothioate (lower case) backbones.

Sequence of K3 CpG ODN: 5' TCGACTCTCGAGCGTTCTC 3' which has only phosphonothioate backbone.

**These ligands were transfected by 0.3 µl Lipofectamine 2000® Reagent for each stimulation.

2.1.3 Antibodies, recombinants and reagent used in ELISA

The names, working concentrations, brand, catalogue numbers and clone information of the recombinant proteins, capture and detection antibodies are summarized in **Table 2.2.**

Table 2.2 Recombinant proteins and antibodies used for ELISA

Name of the Antibody & Recombinant	Working Concentration	Brand & Country	Catalogue #	Clone
Anti-human IFN- α	4 µg/ml	Mabtech-Sweden	3425-1A-20	MT1/3/5
Biotin anti-human IFN- α	1 µg/ml	Mabtech-Sweden	3425-1A-20	MT2/4/6
Human recombinant IFN- α	40 ng/ml	Mabtech-Sweden	3425-1A-20	-
Anti-human IFN- γ	4 µg/ml	Mabtech-Sweden	3420-1A-20	1-D1K
Biotin anti-human IFN- γ	1 µg/ml	Mabtech-Sweden	3420-1A-	20 7-B6-1

Human recombinant IFN- γ	12.5 ng/ml	Mabtech-Sweden	3420-1A-20	-
Anti-human IL-1 β	2 μ g/ml	Mabtech-Sweden	3416-1A-20	MT175
Biotin anti-human IL-1 β	1 μ g/ml	Mabtech-Sweden	3416-1A-20	7P10
Human recombinant IL-1 β	25 ng/ml	Mabtech-Sweden	3416-1A-20	-
Anti-human IL-6	0.5 μ g/ml	Mabtech-Sweden	3460-1A-20	13A5
Biotin anti-human IL-6	1 μ g/ml	Mabtech-Sweden	3460-1A-20	39C3
Human recombinant IL-6	12.5 ng/ml	Mabtech-Sweden	3460-1A-20	-
Anti-human IL-10	2 μ g/ml	Mabtech-Sweden	3430-1A-20	9D7
Biotin anti-human IL-10	1 μ g/ml	Mabtech-Sweden	3430-1A-20	12G8
Human recombinant IL-10	8 ng/ml	Mabtech-Sweden	3430-1A-20	-
Anti-human IL-12/-23 (p40 specific)	2 μ g/ml	Mabtech-Sweden	3450-1A-20	MT86/221
Biotin anti-human IL-12/-23 (p40 specific)	1 μ g/ml	Mabtech-Sweden	3450-1A-20	MT618
Human recombinant IL-12/-23	8 ng/ml	Mabtech-Sweden	3450-1A-20	-

Anti-human TNF α	2 μ g/ml	Mabtech-Sweden	3512-1A-20	MT25C5
Biotin anti-human TNF α	1 μ g/ml	Mabtech-Sweden	3512-1A-20	MT20D9
Human recombinant TNF α	12.5 ng/ml	Mabtech-Sweden	3512-1A-20	-
Streptavidin-Alkaline Phosphatase	1:1000 diluted	Mabtech-Sweden	3310-8-1000	-

2.1.4 Conjugated Antibodies used in Flow Cytometry

The name of the antibodies along with the conjugated fluorochromes, brand, catalogue number and clone information were summarized in **Table 2.3**. FACS Buffer was used as antibody diluent.

Table 2.3 Fluorochrome conjugated antibodies used for Flow cytometry

Name of the Antibody	Brand & Country	Catalogue #	Clone
Anti-human CD4-APC	Immunostep-Spain	4A-100T	HP2/6
Anti-human CD4-FITC	Biolegend- USA	300538	RPA-T4
Anti-human CD8-Pacific Blue	Biolegend- USA	344718	SK1
Anti-human CD14-FITC	Biolegend- USA	982502	M5E2
Anti-human CD14-PE	Biolegend- USA	325606	HCD14

Anti-human CD16-Pasific Blue	Biolegend- USA	302032	3G8
Anti-human CD19-FITC	Biolegend- USA	302206	HIB19
Anti-human CD25-APC	Biolegend- USA	302610	BC96
Anti-human CD25-APC/Cy7	Biolegend- USA	302614	BC96
Anti-human CD38-PE	Biolegend- USA	556604	HIT2
Anti-human CD123-APC	Biolegend- USA	306012	6H6
Anti-human CD123-FITC	Biolegend- USA	306014	6H6
Anti-human CD127-PE	Biolegend- USA	351340	A019D5
Anti-human CD303-APC	Biolegend- USA	354206	201A
Anti-human CD303-FITC	Biolegend- USA	354208	201A
Anti-human CTLA4- PerCP/Cy5.5	Biolegend- USA	369608	BNI3
Anti-human FOXP3	Biolegend- USA	320008	150D
Anti-human PD-L1-PE	Biolegend- USA	329706	29E.2A3
Anti-human PD-L1-PE/Cy7	Biolegend- USA	329718	29E.2A3
Anti-human pmTOR-PE/Cy7	eBioscience- USA	25-9718-42	MRRBY

Anti-human pSTAT3- Alexa Fluor 645	BD Biosciences- USA	557815	4/P-STAT3
Anti-human TCR α /β-APC	Biolegend- USA	306718	IP26
Anti-human TNF α -PE	Biolegend- USA	502909	MAB11

2.1.5 Antibodies used in Western Blot

Tanks, cassettes and combs were purchased from Bio-Rad, USA. Prestained protein ladder was supplied from ThermoFischer Scientific, USA. The name of the antibodies along with the dilution factors, brand, catalogue number and clone information were given in **Table 2.4**.

Table 2.4 Antibodies used in Western Blot

Name of the Antibody	Dilution Factor	Brand & Country	Catalogue #	Clone
Akt	1:1000	Cell Signaling Technology- Germany	4691S	C67E7
MAPK (Erk1/2)	1:1000	Cell Signaling Technology- Germany	4695S	137F5
HIF1 α	1:1000	Cell Signaling Technology- Germany	14179S	D2U3T
mTOR	1:1000	Cell Signaling Technology- Germany	2983S	7C10

pAkt	1:1000	Cell Signaling Technology- Germany	9271S	Ser473
pAMPK α	1:2000	Cell Signaling Technology- Germany	4188S	D79.5E
pmTOR	1:1000	Cell Signaling Technology- Germany	5536S	D9C2
p-p70 S6 Kinase	1:1000	Cell Signaling Technology- Germany	9234S	108D2
p4EBP1	1:1000	Cell Signaling Technology- Germany	2855S	236B4
STAT3	1:2000	Cell Signaling Technology- Germany	9145S	D3A7
4EBP1	1:1000	Cell Signaling Technology- Germany	9644S	53H11
β -actin	1:5000	Cell Signaling Technology- Germany	3700S	8H10D10
HRP- conjugated anti-mouse IgG	1:10000	Cell Signaling Technology- Germany	7076S	-
HRP- conjugated anti-rabbit IgG	1:10000	Cell Signaling Technology- Germany	7074P2	-

2.2 Methods

2.2.1 Healthy Controls and Patients

2.2.1.1 RLTPR Deficient Patient and Healthy Controls

Peripheral blood of female RLTPR deficient patient during and after Cytomegalovirus (CMV) infection were obtained from Hacettepe University, Department of Immunology, Ankara, Turkey. Two healthy controls used in study were age matched with patient to lower variations.

2.2.1.2 RLTPR-TLR1 Deficient Patient and Healthy Controls

Peripheral blood of RLTPR-TLR1 deficient patient was supplied from Selcuk University, Department of Pediatric Allergy and Immunology, Ankara, Turkey. Two healthy controls were used in study.

2.2.1.3 CTLA-4 Mutated Patients and Healthy Controls

Peripheral bloods of three female and one male CTLA-4 mutated patients were obtained from Ege University, Department of Immunology and Allergy, Izmir, Turkey. Two healthy controls were used in study.

2.2.1.4 Probably mTOR/NFκB Mutated Patient and Healthy Controls

Peripheral blood of probably mTOR/NFκB mutated patient was recieved from Ege University, Department of Immunology and Allergy, Izmir, Turkey. Two healthy controls used in study were age matched with patient to lower variations.

2.2.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Whole Blood

Patients' and age matched healthy donors' approximately 10-20 ml peripheral bloods were obtained in EDTA blood collection tubes (BD Biosciences, USA). The collected blood samples were diluted at 1:1 (v/v) with 1X DPBS which is room temperature. Room temperature lymphocyte separation medium was poured into falcons and diluted bloods were layered on the media at 1.5:1 (v/v) ratio. The samples were centrifuged at 540xg for 30 minutes at 21° C. To prevent formed cell layers to be disrupted, deceleration rate of the centrifuge was set to 0 while acceleration rate was kept 9. After this centrifugation step, 4 distinct layers can be seen. From the top to the bottom layer yellow clear plasma, cloudy lymphocyte separation media containing PBMCs, granulocytes and red blood cells can be observed. With the help of the Pasteur pipette, PBMCs collected and transferred to the falcon containing fresh 2% RPMI-1640 medium to wash cells at 540xg for 10 minutes. After the washing step, PBMC pellet was resuspended in 1 ml 5% RPMI-1640 medium to count the cells by Flow cytometer which is mentioned in Section 2.2.5.1 for further experiments.

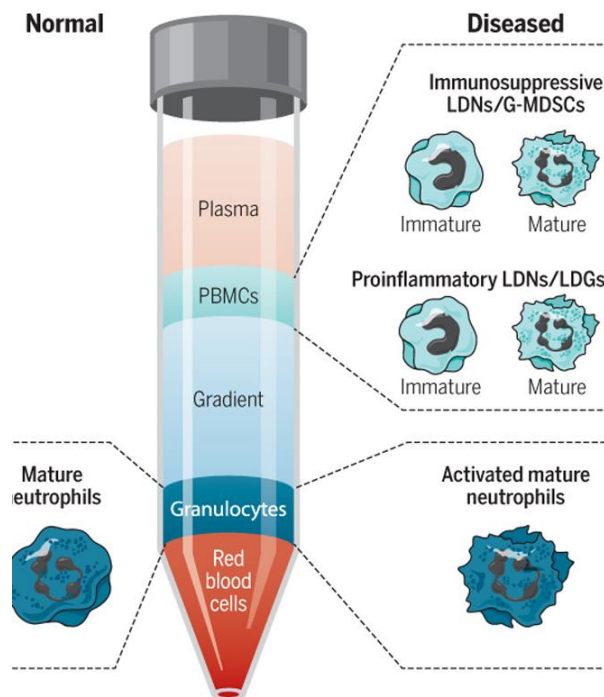


Figure 2.1 Isolated cell layers from whole blood. Adopted from [59].

2.2.3 In vitro Stimulation of PBMC

Isolated patient and healthy control PBMCs were seeded into the flat bottom 96-well plates in 100 μ l with varying concentration according to experimental setup. Stimulants were prepared in 5% RPMI-1640 medium and introduced 50 μ l into each well. Transfected stimulants were prepared in FBS-free medium along with the Lipofectamine 2000® Reagent. Total volume for each well was arranged to 250 μ l by adding the required volume of 5% RPMI-1640 medium. Then, PBMCs were incubated for 24 hours at 37°C, and cells were precipitated at 400xg for 10 minutes to obtain clear supernatant for Cytokine ELISA. Supernatant which contains cytokines can be stored at -20°C.

2.2.4 Cytokine Enzyme Linked Immunosorbent Assay (Cytokine ELISA)

Coating antibodies were prepared in 1X PBS and wells of the 2HB immunoplates (SPL Life Sciences, South Korea) were coated with 50 μ l coating antibodies, and incubated for 12-16 hours at +4°C or for 4 hours at room temperature. After blocking, excess coating antibody solution was removed and wells were blocked with 200 μ l/well blocking buffer for 2 hours at room temperature. When the 2 hours incubation was over, the plate was washed with wash buffer for 5 times and with distilled water for 2 times. Standards and non-diluted or 2X diluted supernatants came from PBMC stimulation were incubated for 12-16 hours at +4°C. Next day, excess standards and supernatants were removed and the plate was washed with wash buffer for 5 times and with distilled water for 2 times. Biotinylated antibodies were diluted in a blocking buffer and 50 μ l antibody solution per well was incubated for 2 hours at room temperature. After 2 hours incubation, the plate was emptied and washed again to proceed 1 hour 50 μ l/well SA-ALP (1:1000) incubation at room temperature. Plate was washed again and 50 μ l/well PNPP solution was introduced to observe yellow color formation and optical density measurement of the plate at 405 nm by ELISA Plate Reader (Molecular Devices, USA). 4-parametric standard curve was used to calculate the concentration of released cytokines from PBMCs.

2.2.5 Flow Cytometry Analysis

2.2.5.1 Cell Counting

20 μ l was taken from 1 ml resuspended PBMCs and diluted in 5 ml isotonic solution (Beckmann Coulter, USA). 100 μ l sample was acquired from 5 ml diluted PBMC suspension by Novocyte 3000 Flow Cytometer to be analyzed. FSC and SSC positioning was used to gate the PMBCs leaving apoptotic cells and debris out of the gate. To obtain the total PBMC count in 1 ml suspension, dilution factor was multiplied with the number of counted events in the gate. Dilution Factor for this experiment was 2500.

2.2.5.2 Cell Surface Marker Staining from Whole Blood

For whole blood cell staining, 5 μ l fluorochrome labeled antibodies were mixed with 100 μ l of the whole blood and vortexed. Then, the mixture was incubated for 30 minutes at room temperature and dark. After 30 minutes incubation, 2 ml 1X RBC lysis buffer (Biolegend, USA) was added and vortexed to eliminate RBCs via lysis, and cells were incubated in this mixture 20 more minutes at room temperature and dark. Later, cells were precipitated at 500xg for 10 minutes to remove RBC lysis buffer. Stained cell pellet was resuspended in 5% RPMI-1640 medium to be analyzed with Novocyte 3000 flow cytometer.

2.2.5.3 Cell Surface Marker Staining from PBMCs

For isolated and stimulated or unstimulated PBMC staining, 1 μ g/ml fluorochrome labeled antibodies diluted in 5% RPMI-1640 medium were mixed with required amounts of PBMCs and vortexed. Then, mixture was incubated for 30 minutes at +4° C and dark. After 30 minutes incubation, cells were precipitated at 500xg for 10 minutes to remove excess antibody. Stained cell pellet was resuspended in 5% RPMI-1640 medium to be analyzed by Novocyte 3000 flow cytometer with at least 30000 events/sample.

2.2.5.4 Intracellular Cytokine Staining (ICS) of CD4⁺ T-Cells

U bottom 96-well plate was used to seed PBMCs 4×10^5 /well in 5% RPMI-1640 medium, and they were treated with PMA (50 ng/ml) and Ionomycin (1 μ g/ml) or kept untreated for 2 hours at 37°C. During 2 hours, cytokines were synthesized, and 5 μ g/ml Brefeldin A was used to prevent their secretion from PBMCs. After 4 hours Brefeldin A treatment at 37°C, cells were precipitated at 400xg for 10 minutes. Supernatants were removed, and 150 μ l 1X Cytofix Buffer (BD Biosciences, USA) per well was applied to fix cells for 20 minutes at dark and room temperature. After incubation, cells were washed twice with 250 μ l FACS buffer at 400xg for 10 minutes centrifugation. To permeabilize cells, 150 μ l cold 1X Cytoperm Buffer (BD Biosciences, USA) was applied into each well for 15 minutes at dark and room temperature. Centrifugation at 400xg for 10 minutes was applied to remove 1X Cytoperm Buffer. Then, cytokine antibodies and/or cell surface markers were prepared in the FACS buffer to stain cells at 1 μ g/ml concentration for 30 minutes at dark and room temperature. After 30 minutes incubation, cells were washed twice as mentioned, and resuspended in 200 μ l FACS buffer to be analyzed by Novocyte 3000 flow cytometer with at least 30000 events/sample gated from CD4⁺ T-cells.

2.2.6 SDS-Page and Western Blot

2.2.6.1 Isolation of Proteins from PBMCs

500 μ l RIPA buffer was used to resuspend PBMC pellets made of at least 5×10^6 cells, and incubated for 20 minutes on ice. To lyse cells better, at every 5 minutes, suspension was vortexed for 15 seconds. Then, 12000xg centrifugation was performed for 20 minutes at +4°C to collect supernatant. It can be stored at -80°C for long term storage.

2.2.6.2 Protein Concentration Quantification by BCA Assay

PierceTM BCA Protein assay kit (ThermoFischer Scientific, USA) was used to determine the protein concentration isolated from PBMCs. 25 µl of 2X serially diluted standards (2000 µg/ml to 31.25 µg/ml) and 25 µl of sample were loaded into the flat bottom 96-well plate. By mixing 1:50 Solution A and Solution B (v/v), working reagent was prepared and 200 µl of the reagent was transferred into each well for 30 minutes incubation at 37°C and dark. Microplate reader (Synergy HT) was used to measure optical densities of the samples at 562 nm. Then, a linear standard curve formed by standards was used to measure protein concentrations.

2.2.6.3 Protein Denaturation and SDS-Page Running

4X Loading dye was mixed with 10 µg samples, and total volume was completed up to 45 µl with ultrapure cell culture water to make loading dye's final concentration 1X. To denature proteins more, +95°C was applied for 5 minutes, and samples were kept on ice until further steps. 5% concentrated stacking gel and 7% concentrated separating gel was used. 1X Running buffer was used to fill the tank and samples were loaded on each well. During stacking gel, 60V power was applied; 100V power was used for further.

2.2.6.4 SDS-Page to PDVF Membrane Transfer

During wet transfer method, the alignments of materials were as such wet sponge, 2 wet Whatman papers (GE Healthcare, USA), 0.45 µm PDVF membrane (ThermoFischer Scientific, USA), running gel, 2 wet Whatman papers and wet sponge which are stacked in the transfer cassette from anode to cathode face. To remove air bubbles which cause inefficient transfer from layers, a roller was used, and cassette was placed properly into the tank filled with cold 1X transfer buffer. 100V power was applied for 2 hours on ice.

2.2.6.5 Blotting and Imaging

Blocking buffer was used to block membrane for 2 hours at room temperature under rotation. Primary antibodies were diluted with antibody dilution buffer, and incubated for 12-16 hours at +4° C. Then, the membrane was washed 3 times with PBS-T wash buffer for 10 minutes. HRP-conjugated secondary antibodies were diluted, and incubated for 2 hours at room temperature. To remove excess antibody solution, the membrane

was washed 3 times with PBS-T wash buffer for 10 minutes. Amersham ECL Prime Western Blotting Reagent (GE Healthcare, USA) was applied on the membrane, and Amersham Imager 600 (GE Healthcare, USA) was used to visualize the proteins.

2.2.7 Statistical Analyses

All statistical analyses were carried out with GraphPad Prism 6 Software (USA).

CHAPTER 3

RESULTS

3.1 Characterization of Immune Responses in RLTPR Deficiency

3.1.1 Decreased Number of Treg Cells From Whole Blood of The RLTPR Deficient Patient Was Confirmed With And Without Viral Load

RLTPR deficiency do not allow proper CD28 costimulation which impair successful T-cell activation and maturation. It is known that the patients with RLTPR deficiency showed significantly decreased Treg cell population [60, 61]. In order to confirm RLTPR deficiency interference, Treg cell percentage of the healthy controls and RLTPR patient with and without viral load was assessed (Figure 3.1 and 3.2, respectively). The whole blood of controls and patient were stained with anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25, anti-human CD127 antibodies and anti-human FOXP3 antibodies for flow cytometry analysis of Treg count. According to flow cytometry, healthy controls of each experiment have 2.82%, 6.18% and 4.08%, 2.83% Treg population in CD4⁺ cells which could satisfy 2-10% range [62]. Moreover, RLTPR patient has 1.25% Treg under viral load and 0.46% Treg after viral load which are reduced compared to healthy controls.

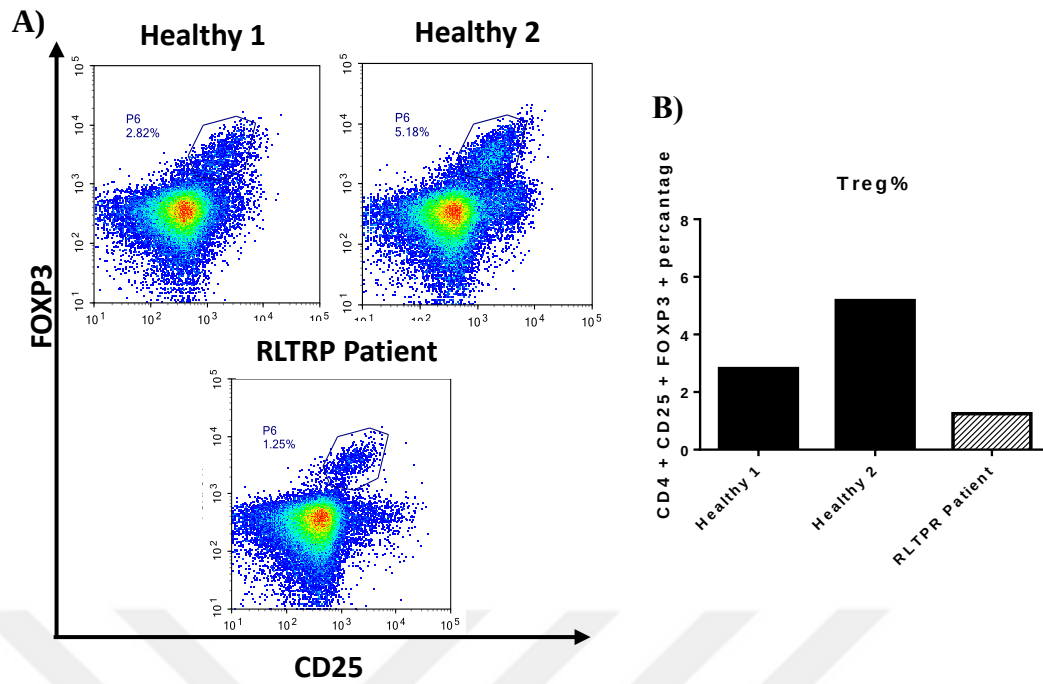


Figure 3.1 CD25 and FOXP3 surface expression of CD45+ CD3+ CD4+ cells. The 100 μ l whole blood of controls and RLTRP patient with viral load were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human FOXP3 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD25+ FOXP3+ cells. **B)** Representative bar graphs of CD4+ CD25+ FOXP3+ cell percentage of healthy controls and patient.

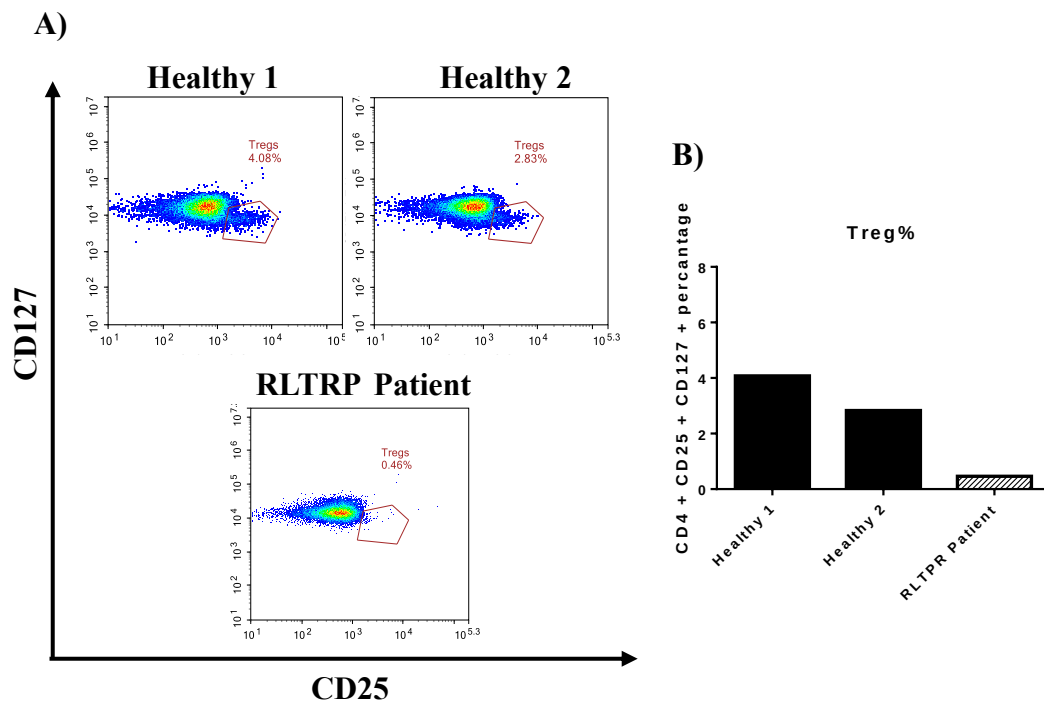


Figure 3.2 CD25 and CD127 surface expression of CD45+ CD3+ CD4+ cells. The 100 μ l whole blood of controls and RLTPR patient without viral load were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD25+ CD127dim cells. **B)** Representative bar graphs of CD4+ CD25+ CD127dim cell percentage of healthy controls and patient.

3.1.2 Characterization of Innate Immune Responses in RLTPR Deficiency Under Viral Load

The effects of RLTPR deficiency to the adaptive immune cells were studied frequently due to impaired T-cell activation and T-cell mediated B-cell response [60, 63]. However, the impact of the deficiency over innate immune system was not fully studied. Therefore, PRR ligand stimulation experiments were done to see the effects of RLTPR mutation on the innate immune system.

3.1.2.1 IL-1 β and TNF- α responses from PBMCs of RLTPR deficient patient were elevated by stimulation with the TLR and cytosolic nucleic acid ligands

Proinflammatory cytokine responses of the healthy controls and patient under viral load were examined by PRR ligand stimulation. Patient under viral infection showed higher TLR4, TLR7, TLR8, TLR9, cGAS-STING, NLRP3 inflammasome activity according to IL-1 β response. Also, showed similar elevated TNF- α response through TLR4, TLR9, STING, NLRP3 inflammasome and non-canonical inflammasome pathway activations compared to controls (Figure 3.3). This improved response could be reasoned by ongoing viral infection.

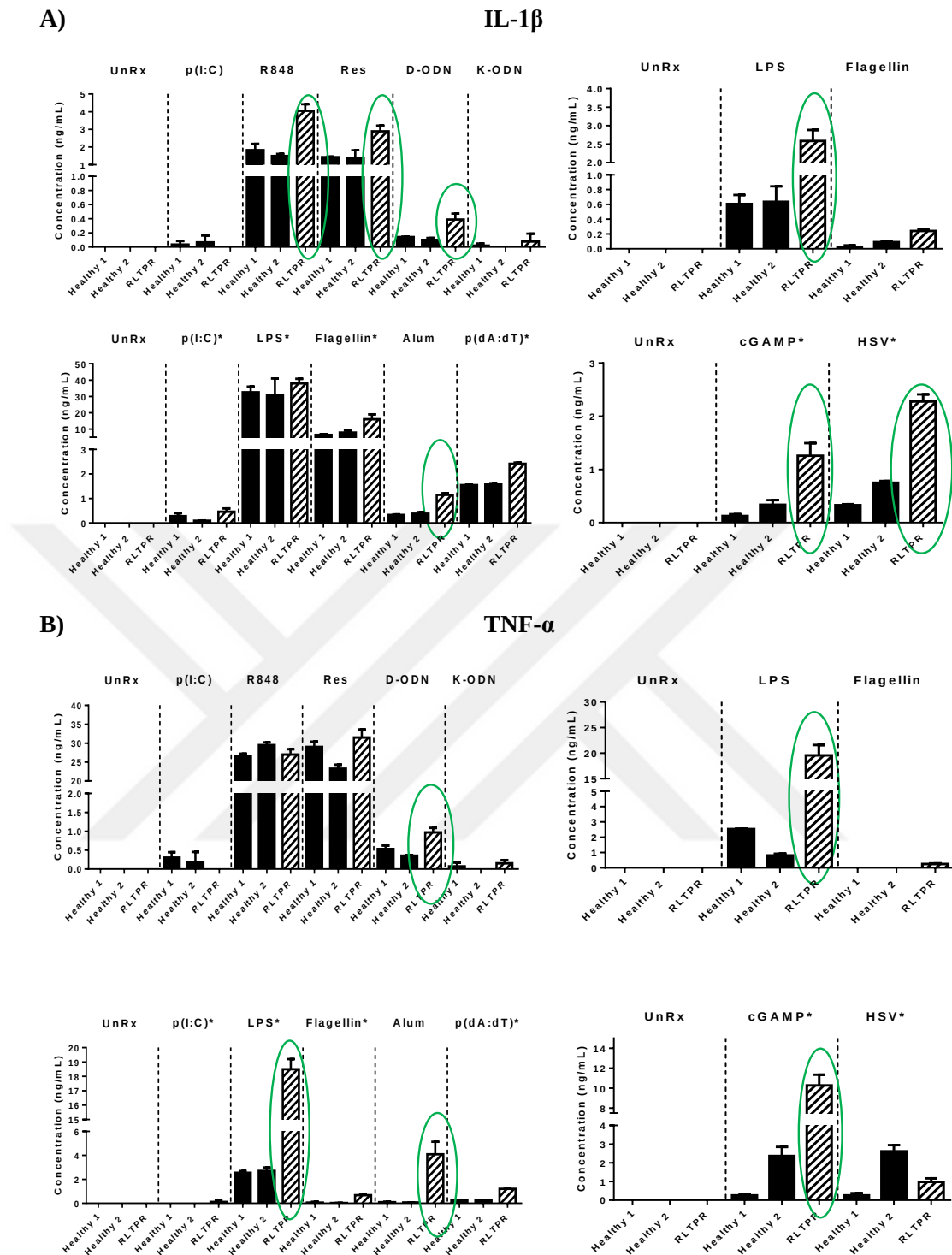
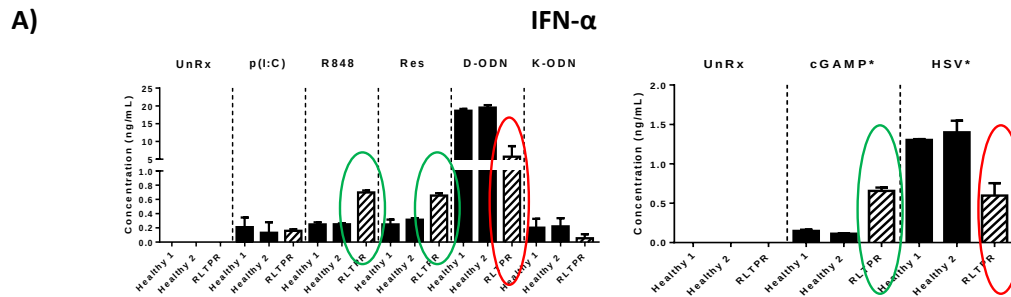


Figure 3.3 IL-1 β and TNF- α cytokine secretion profiles of healthy and RLTPR patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with p(I:C) (TLR3, 30 μ g/ml), LPS (TLR4, 5 μ g/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 μ g/ml), Resiquimod (TLR7&8, 5 μ g/ml), D-ODN (TLR9, 3 μ M), K-ODN (TLR9, 1 μ M), transfected p(I:C) (RIG-I/MAVS, 30 μ g/ml), transfected LPS (Non-canonical Inflammasome, 5 μ g/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome,

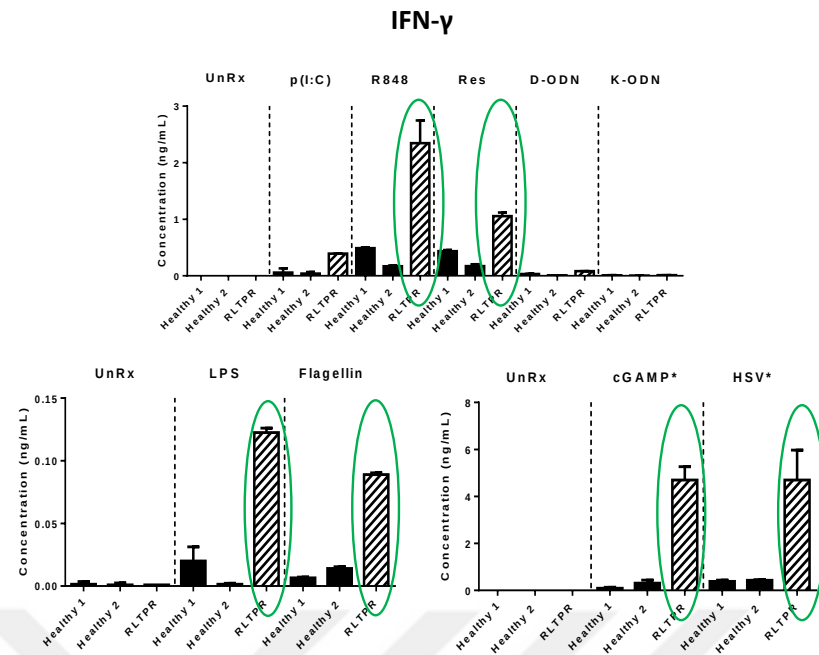
20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 µg/ml), transfected cGAMP (STING, 30 µg/ml) and transfected HSV (cGAS-STING, 5 µg/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A) IL-1β, B) TNFα** secretion levels from healthy controls and patient PBMCs.

3.1.2.2 IFN-γ and IL-12 secretion from PBMCs of RLTPR deficient patient were increased while IFN-α secretion shows various responses through TLR and cytosolic nucleic acid ligand stimulations

RLTPR deficient patients are susceptible to the Epstein Barr Virus which is defeated by a properly functioning immune system after infancy [60]. Because patients are prone to viral infections, Type I and Type II interferon responses were investigated along with the IL-12 response which induces IFN-γ upregulation by PRR ligand stimulation [64]. Type I IFN response was elevated in patient through TLR7, TLR8 and STING pathway activations while Type II IFN and IL-12 responses are increased via TLR4, TLR5, TLR7, TLR8, STING and cGAS-STING signaling compared to healthy controls which could be result of viral infection (Figure 3.4).



B)



C)

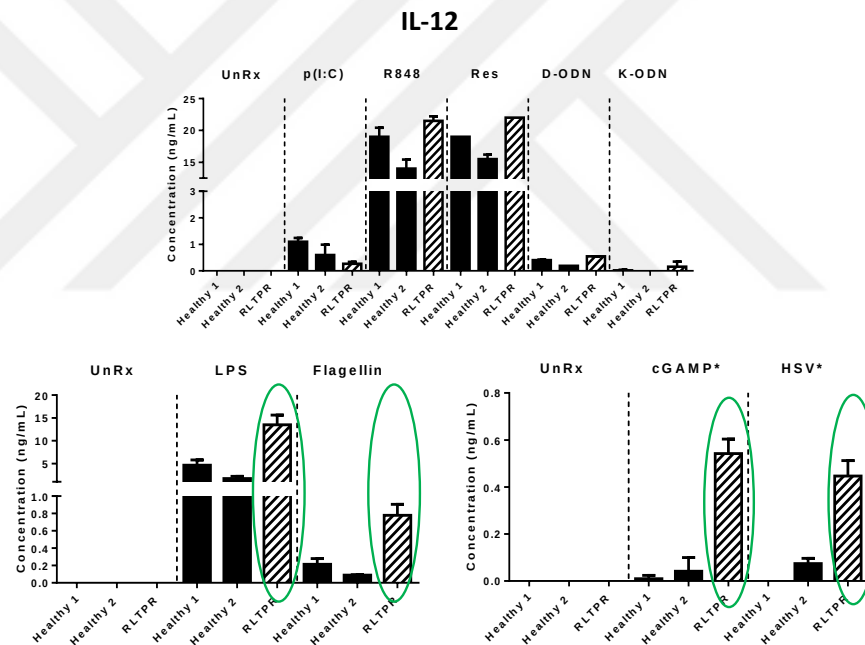


Figure 3.4 IFN- α , IFN- γ and IL-12 cytokine secretion profiles of healthy and RLTPR patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with p(I:C) (TLR3, 30 μ g/ml), LPS (TLR4, 5 μ g/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 μ g/ml), Resiquimod (TLR7&8, 5 μ g/ml), D-ODN (TLR9, 3 μ M), K-ODN (TLR9, 1 μ M), transfected cGAMP (STING, 30 μ g/ml) and transfected HSV (cGAS-STING, 5 μ g/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A)** IFN α , **B)** IFN γ , **C)** IL-12 secretion levels from healthy controls and patient PBMCs.

3.1.2.3 RLTPR deficient patient gives elevated IL-10 response to cytosolic nucleic acid ligands

Because Treg percentage in the RLTPR patient is decreased, IL-10 was investigated to see the effect of reduced Treg count on cytokine response by cytosolic nucleic acid ligand stimulations. Again, due to ongoing viral infection, non-canonical inflammasome mediated IL-10 response is higher in patient compared to healthy controls (Figure 3.5)

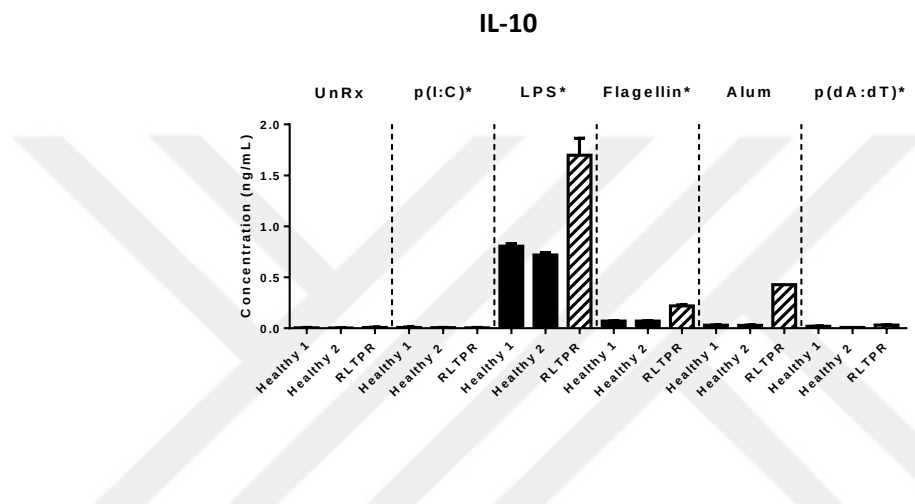


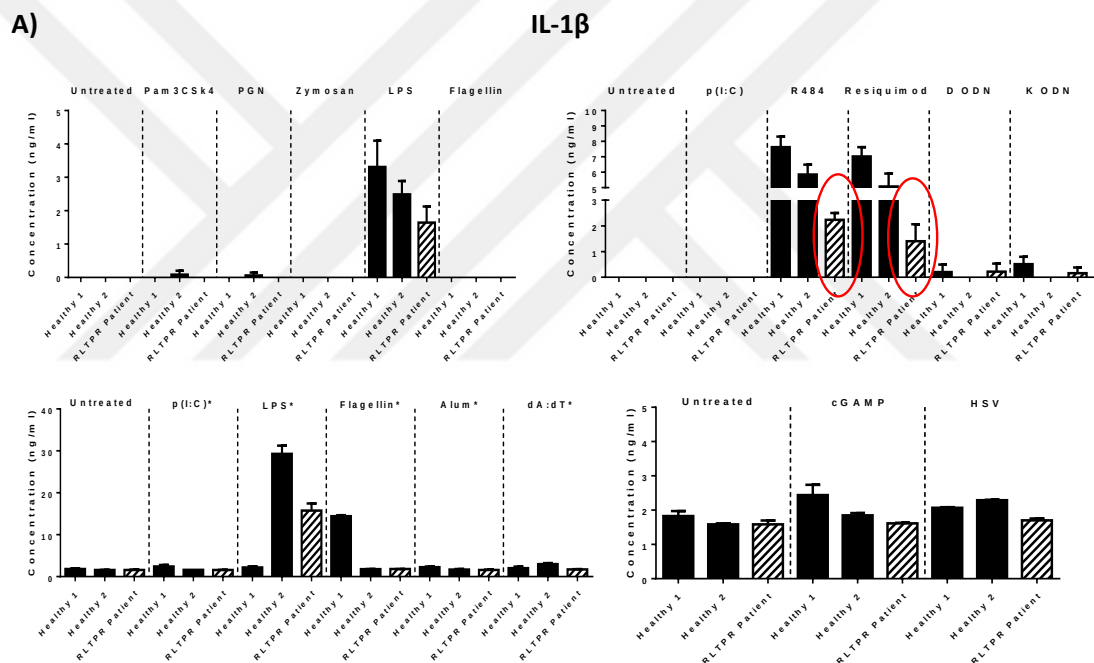
Figure 3.5 IL-10 cytokine secretion profiles of healthy and RLTPR patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with transfected p(I:C) (RIG-I/MAVS, 30 μ g/ml), transfected LPS (Non-canonical Inflammasome, 5 μ g/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml) and transfected p(dA:dT) (AIM2 Inflammasome, 5 μ g/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA/

3.1.3 Characterization of Innate Immune Responses in RLTPR Deficiency Without Viral Load

After viral infection was cleared by drug therapies, the innate immune responses of the patient is not influenced by this invasion, and impact of RLTPR deficiency on innate immune cells was assessed once more.

3.1.3.1 IL-1 β and TNF α responses from PBMCs of RLTPR deficient patient were decreased by stimulation with the endosomal TLR ligands while responses were normal with surface TLR and cytosolic nucleic acid ligands

Proinflammatory cytokine responses of the healthy controls and patient were examined by PRR ligand stimulation. Patient had impaired TLR7 and TLR8 mediated IL-1 β and TNF α response while surface TLR and cytosolic nucleic acid pathways function normally. Reduced TLR7 and TLR8 activity which senses viral single stranded RNA could be reason of the chronic viral infections (Figure 3.6).



B)

TNF- α

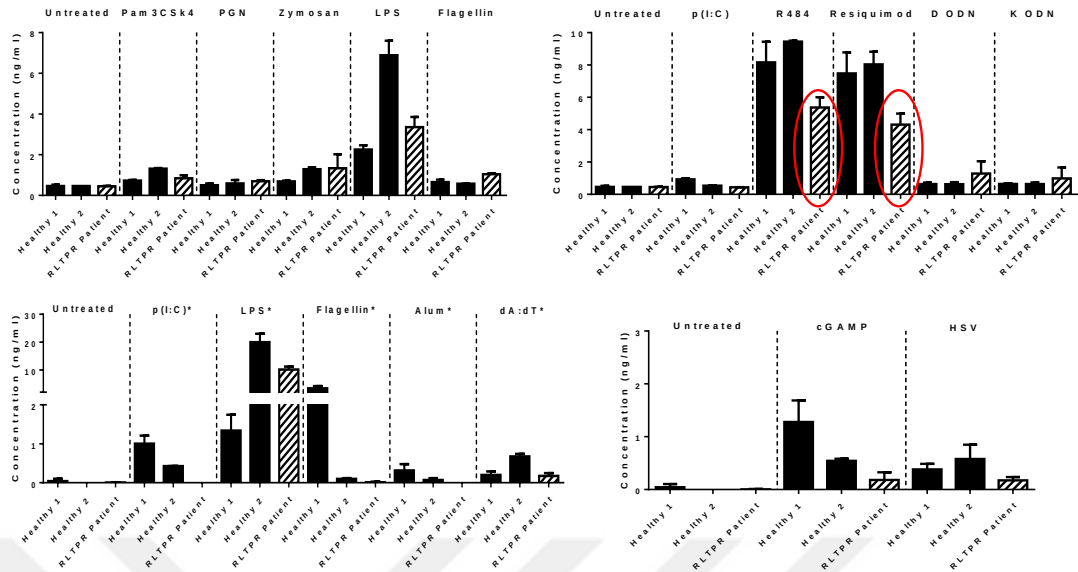


Figure 3.6 IL-1 β and TNF- α cytokine secretion profiles of healthy and RLTPR patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 μ g/ml), Peptidoglycan (TLR2, 5 μ g/ml), Zymosan (TLR2, 5 μ g/ml) p(I:C) (TLR3, 30 μ g/ml), LPS (TLR4, 5 μ g/ml), Flagellin (TLR5, 100 ng/ml), R484 (TLR7, 5 μ g/ml), Resiquimod (TLR7&8, 5 μ g/ml), D-ODN (TLR9, 3 μ M), K-ODN (TLR9, 1 μ M), transfected p(I:C) (RIG-I/MAVS, 30 μ g/ml), transfected LPS (Non-canonical Inflammasome, 5 μ g/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 μ g/ml), transfected cGAMP (STING, 30 μ g/ml) and transfected HSV (cGAS-STING, 5 μ g/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A)** IL-1 β , **B)** TNF α secretion levels from healthy controls and patient PBMCs.

3.1.3.2 IFN- α secretion from PBMCs of RLTPR deficient patient was impaired by both surface and endosomal TLR ligand stimulations while IFN- γ secretion was decreased only at stimulation with endosomal TLR ligands

In order to understand the problem of viral response of innate immune system which lead chronic infections in patient, Type I and Type II IFN responses were examined by PRR ligand stimulation. Endosomal TLR, STING and RIG-I/MAVS mediated IFN- α response was highly reduced in patient. Moreover TLR3, TLR7, TLR8,

STING, RIG-I/MAVS and non-canonical inflammasome mediated IFN- γ response highly impaired in patient compared to controls (Figure 3.7). Because endosomal TLRs play crucial role in viral response, it is reasonable to see repeating viral infections in the patient.

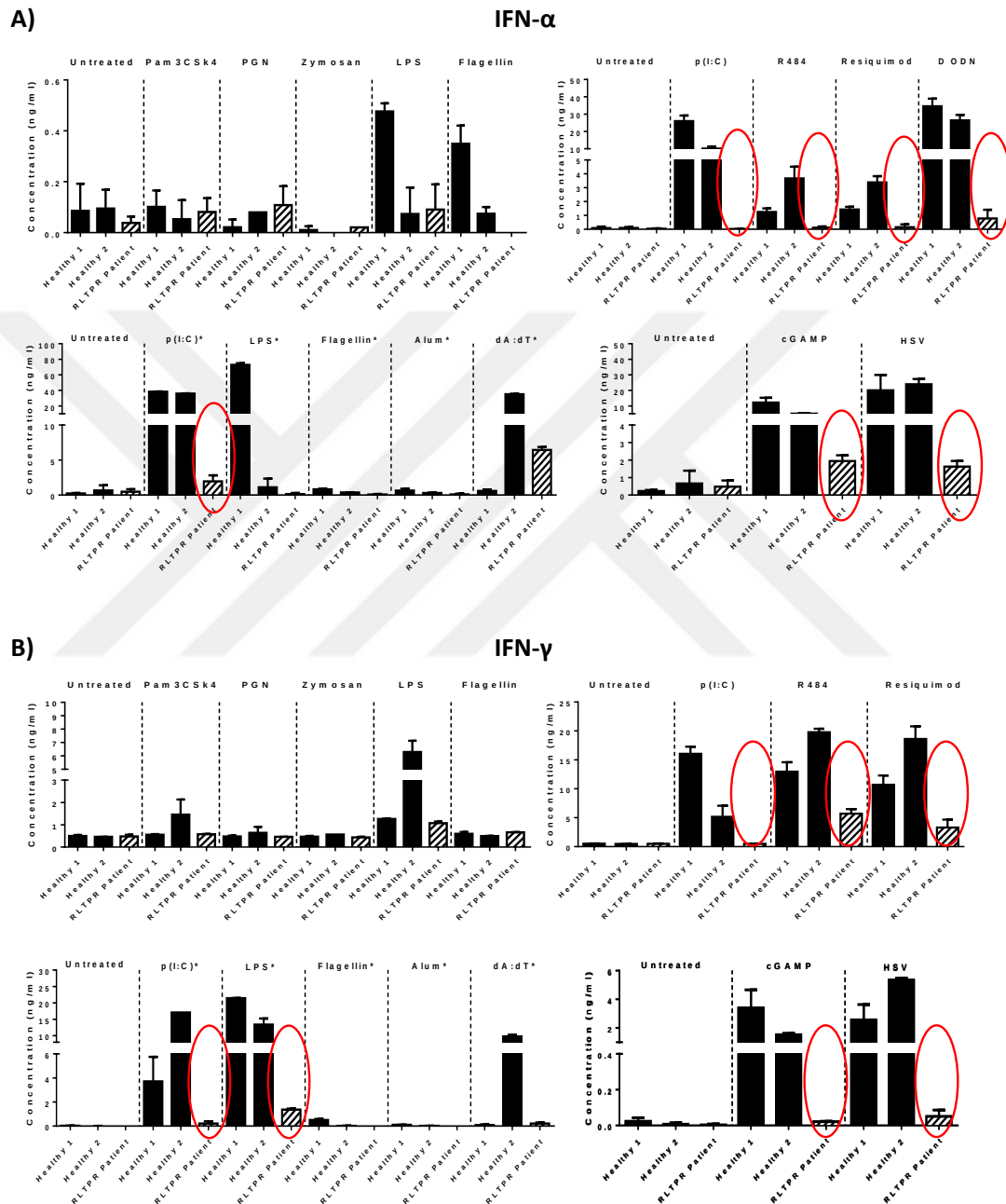


Figure 3.7 IFN- α and IFN- γ cytokine secretion profiles of healthy and RLTPR patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 μ g/ml), Peptidoglycan (TLR2, 5 μ g/ml), Zymosan (TLR2, 5 μ g/ml) p(I:C) (TLR3, 30 μ g/ml),

LPS (TLR4, 5 µg/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 µg/ml), Resiquimod (TLR7&8, 5 µg/ml), D-ODN (TLR9, 3 µM), K-ODN (TLR9, 1 µM), transfected p(I:C) (RIG-I/MAVS, 30 µg/ml), transfected LPS (Non-canonical Inflammasome, 5 µg/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 µg/ml), transfected cGAMP (STING, 30 µg/ml) and transfected HSV (cGAS-STING, 5 µg/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A)** IFN α , **B)** IFN γ secretion levels from healthy controls and patient PBMCs.

3.2 Characterization of Immune Responses in RLTPR-TLR1 Deficiency

3.2.1 Decreased Number of Treg Cells From Whole Blood of The RLTPR-TLR1 Deficient Patient Was Confirmed

To validate RLTPR deficiency interference, Treg cell percentage of the healthy controls and RLTPR-TLR1 patient was investigated. The whole blood of controls and patient were stained with anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies for flow cytometry analysis of Treg count. According to flow cytometry, healthy controls have 13.16%, 5.68% Treg population in CD4⁺ cells while RLTPR-TLR1 patient has 2.79% Treg which is reduced according to controls as expected (Figure 3.8).

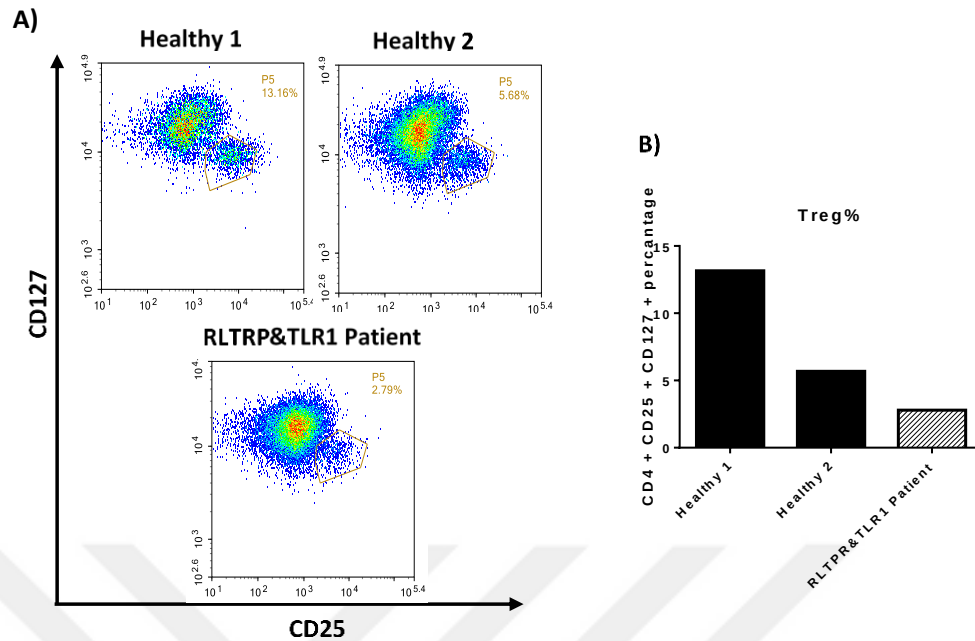


Figure 3.8 CD25 and CD127 surface expression of CD45+ CD3+ CD4+ cells. The 100 μ l whole blood of controls and RLTPR-TLR1 patient were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD25+ CD127dim cells. **B)** Representative bar graphs of CD4+ CD25+ CD127dim cell percentage of healthy controls and patient.

3.2.2 Characterization of Innate Immune Responses in RLTPR Def. & TLR1 Mut.

RLTPR-TLR1 deficiency is a very novel combination to study. Because adaptive immune arm response is well studied for RLTPR deficiency, and because TLR1 is deficient, effect on innate immune response of the RLTPR-TLR1 deficiency combination is studied.

3.2.2.1 IL-1 β response from PBMCs of patient was elevated by stimulation with the TLR and cytosolic nucleic acid ligands

Proinflammatory cytokine responses of the healthy controls and patient were examined by PRR ligand stimulation because TLR1 deficiency lead uncontrolled bacterial infections. Patient showed higher TLR2-6, TLR4, TLR7, TLR8 and non-canonical inflammasome mediated IL-1 β response (Figure 3.9). Enhanced TLR2-6 response could be the result of TLR1 deficiency because it is known that TLR1 and TLR6 are similar in structure, and immune response could be compensated when TLR1 is deficient but TLR6 is not [19]. Also, remaining improved proinflammatory cytokine response to different PRR ligands could be reasoned by ongoing infection which is not stated.

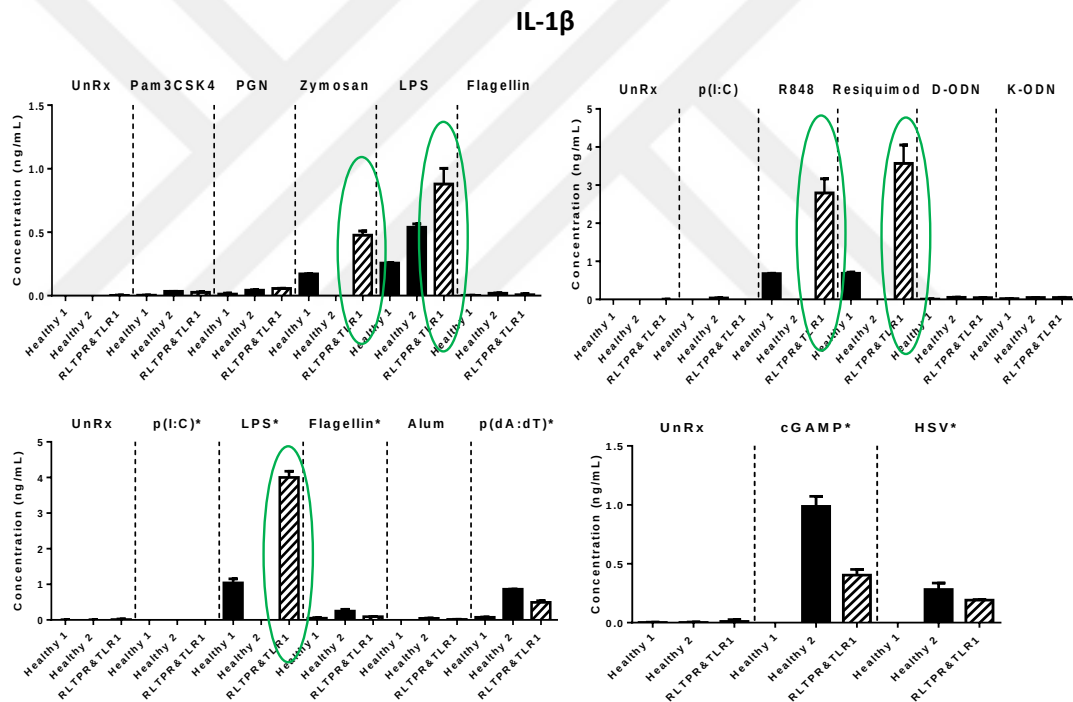


Figure 3.9 IL-1 β cytokine secretion profiles of healthy and RLTPR-TLR1 patient PBMCs through 24 hours PRR ligand stimulation.. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 μ g/ml), Peptidoglycan (TLR2, 5 μ g/ml), Zymosan (TLR2, 5 μ g/ml) p(I:C) (TLR3, 30 μ g/ml), LPS (TLR4, 5 μ g/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 μ g/ml), Resiquimod (TLR7&8, 5 μ g/ml), D-ODN (TLR9, 3 μ M), K-ODN (TLR9, 1 μ M), transfected p(I:C) (RIG-I/MAVS, 30 μ g/ml), transfected LPS (Non-canonical Inflammasome, 5 μ g/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 μ g/ml), transfected cGAMP

(STING, 30 $\mu\text{g/ml}$) and transfected HSV (cGAS-STING, 5 $\mu\text{g/ml}$) for 24 hours, and supernatants were collected to investigate with cytokine ELISA

3.2.2.2 IFN- α secretion was increased by endosomal TLR and cytosolic nucleic acid ligands while IL-12 secretion from PBMCs of patient shows various responses

RLTPR deficient patients are susceptible to the viral infections, so Type I and Type II interferon inducing IL-12 responses were investigated by PRR ligand stimulation. Patient Type I IFN response was elevated in patient through AIM2 inflammasome, RIG-I/MAVS and cGAS-STING pathway activations which suggest an ongoing infection. TLR1 mediated IL-12 response cannot be observed which is caused by TLR1 deficiency. Moreover TLR2-6 mediated IL-12 response is higher in patient which contribute to compensate the TLR1 deficiency (Figure 3.10).

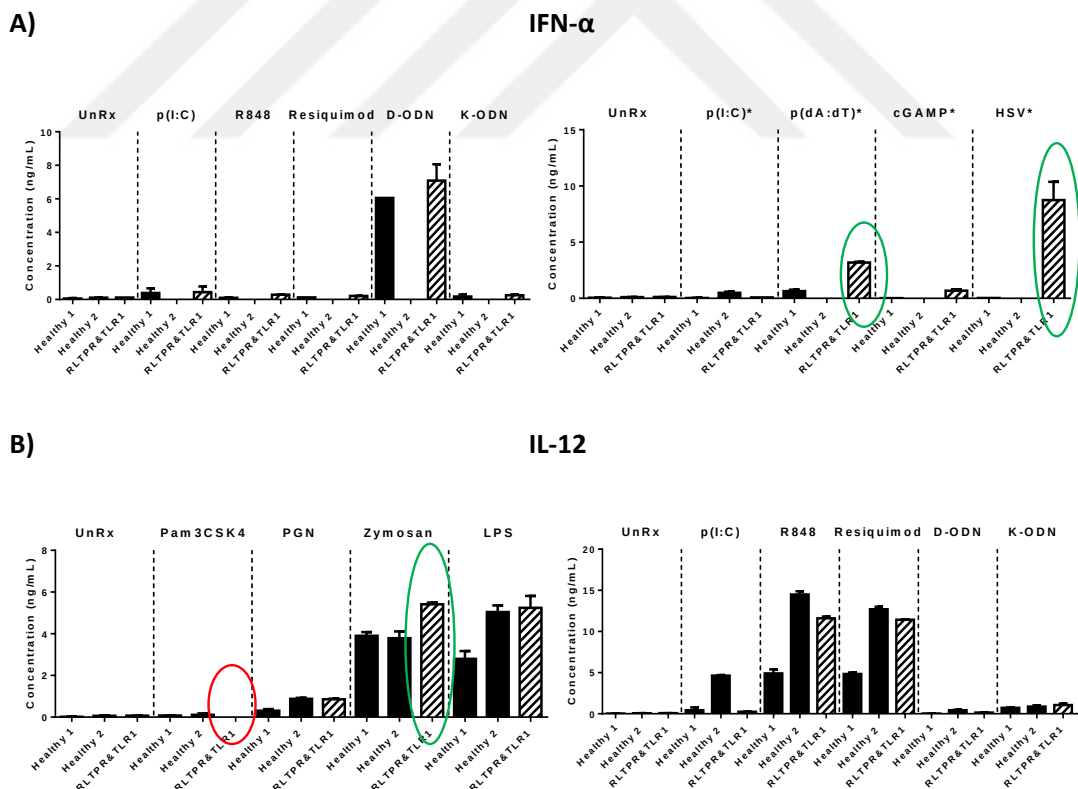


Figure 3.10 IFN- α and IL-12 cytokine secretion profiles of healthy and RLTPR-TLR1 patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 $\mu\text{g/ml}$),

Peptidoglycan (TLR2, 5 µg/ml), Zymosan (TLR2, 5 µg/ml) p(I:C) (TLR3, 30 µg/ml), LPS (TLR4, 5 µg/ml), R848 (TLR7, 5 µg/ml), Resiquimod (TLR7&8, 5 µg/ml), D-ODN (TLR9, 3 µM), K-ODN (TLR9, 1 µM), transfected p(I:C) (RIG-I/MAVS, 30 µg/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 µg/ml), transfected cGAMP (STING, 30 µg/ml) and transfected HSV (cGAS-STING, 5 µg/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A) IFN α** , **B) IL-12** secretion levels from healthy controls and patient PBMCs.

3.2.2.3 Patient gives elevated IL-10 response to TLR and cytosolic nucleic acid ligands

Treg percentage in the RLTPR patient is reduced, so IL-10 was investigated to see the effect of reduced Treg count on cytokine response by PRR ligand stimulations. Patient showed increased IL-10 secretion through TLR2-6, TLR4, TLR7, non-canonical inflammasome and cGAS-STING activations which suggest present infection, and compensation of TLR1 deficiency (Figure 3.11).

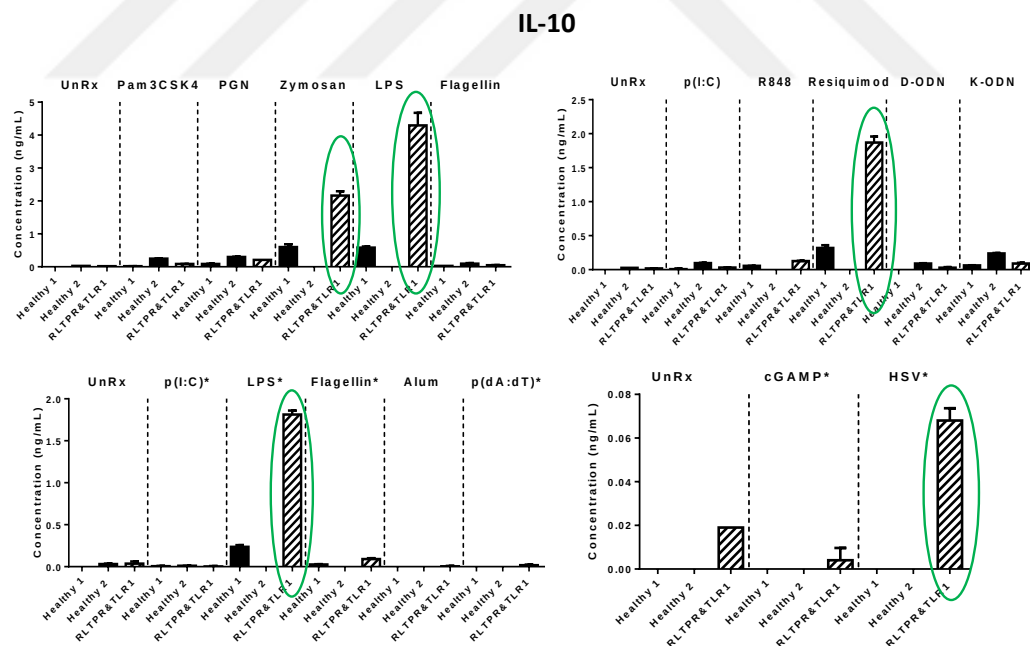


Figure 3.11 IL-10 cytokine secretion profiles of healthy and RLTPR-TLR1 patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 µg/ml), Peptidoglycan (TLR2, 5 µg/ml), Zymosan (TLR2, 5 µg/ml) p(I:C) (TLR3, 30 µg/ml),

LPS (TLR4, 5 µg/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 µg/ml), Resiquimod (TLR7&8, 5 µg/ml), D-ODN (TLR9, 3 µM), K-ODN (TLR9, 1 µM), transfected p(I:C) (RIG-I/MAVS, 30 µg/ml), transfected LPS (Non-canonical Inflammasome, 5 µg/ml), transfected flagellin (NLRC4, 100 ng/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 µg/ml), transfected cGAMP (STING, 30 µg/ml) and transfected HSV (cGAS-STING, 5 µg/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA.

3.3 Investigation of Effects of CTLA-4 Mutation on Immune Cells

3.3.1 Determination of Immune Cell Counts From Whole Blood

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is an immune check point to downregulate T-cell activity, and expressed highly on Treg cells. CTLA-4 mutations cause autoimmunity because T-cell activity and T-cell dependent B-cell activity cannot be controlled [56]. For this reason, adaptive immune response of CTLA-4 mutation is studied well while effect on innate immune system is not fully studied. In this part of the thesis, affects of CTLA-4 mutation on both innate and adaptive immune cells were investigated.

3.3.1.1 CD4/CD8 ratio was reversed only in one patient

Healthy immune system has a balance between CD4⁺ and CD8⁺ T-cells. If CD4⁺/CD8⁺ ratio is lower than 1 it implies viral infections such as HIV while increased ratio could be reasoned by autoimmunity. According to literature, ratio between 1.5-2.5 is normal [65]. Because CTLA-4 mutation cause autoimmunity in patients, CD4⁺/CD8⁺ ratios of 4 patients were assessed. The whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4 and anti-human CD8 antibodies, and analyzed by flow cytometer (Figure. 3.12). Ratios of the patients are 3, 1.8, 0.7 and 1.7 while one healthy donor has 1.6, and the other has 0.7. Therefore, one of the

healthy control probably have an infection as well as Patient 3, and Patient 1 experience autoimmunity.

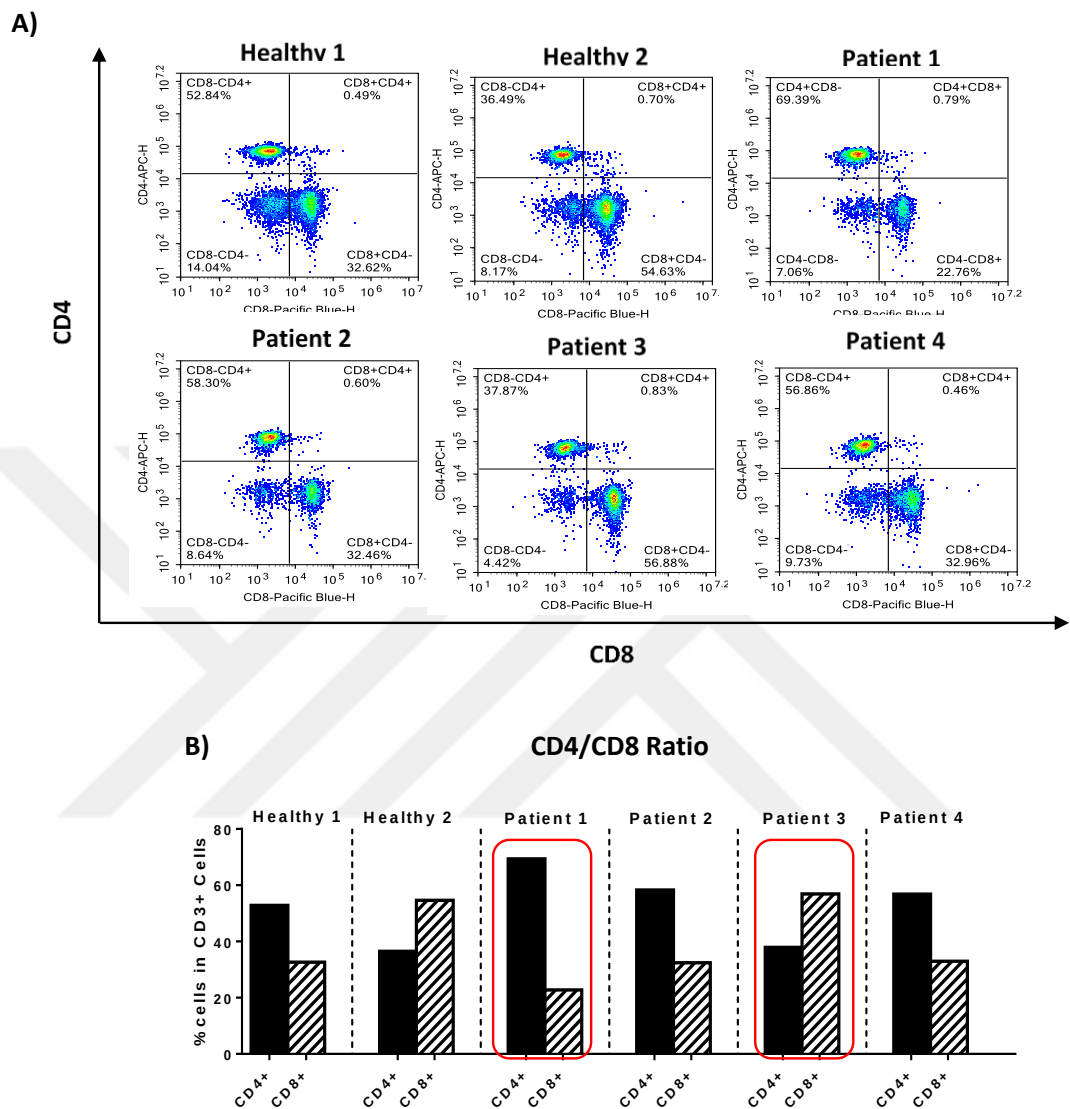


Figure 3.12 CD4 and CD8 surface expression of CD45+ CD3+ cells. The 100 μ l whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4 and anti-human CD8 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD8+ cells. **B)** Representative bar graphs of CD4+ CD8+ cell percentages in CD3+ cells of healthy controls and patients.

3.3.1.2 Low density granulocytes were observed in two patients

Proinflammatory low density granulocytes (LDGs) play a role in systemic autoimmunity [66]. Therefore, LDG counts of PBMCs of healthy controls and patients were determined by flow cytometer which localize between lymphocytes and granulocytes (Figure 3.13). Average LDGs from a healthy person is 1.28% from PBMC count [67]. Patient 1,2 and 3 showed increased percentage of LDGs as 2.41%, 5.41%, 3.18% compared to healthy controls, 0.38% and 1.77%, which could be result of CTLA-4 mutation which make patients prone to autoimmunity.

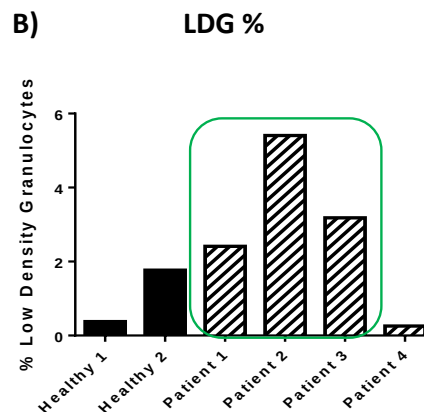
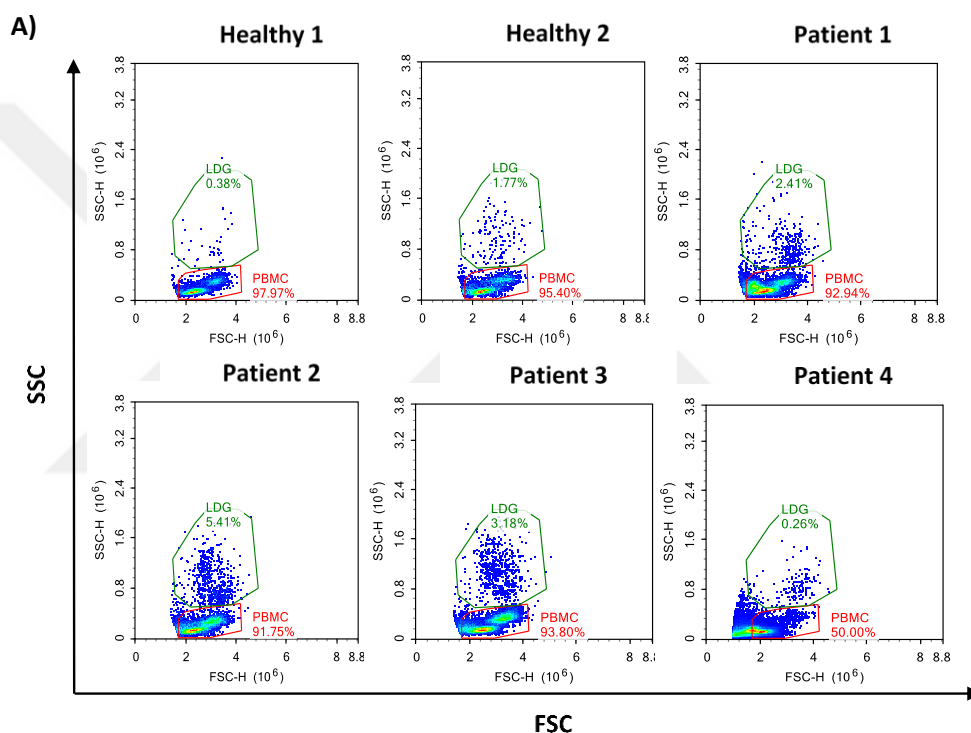
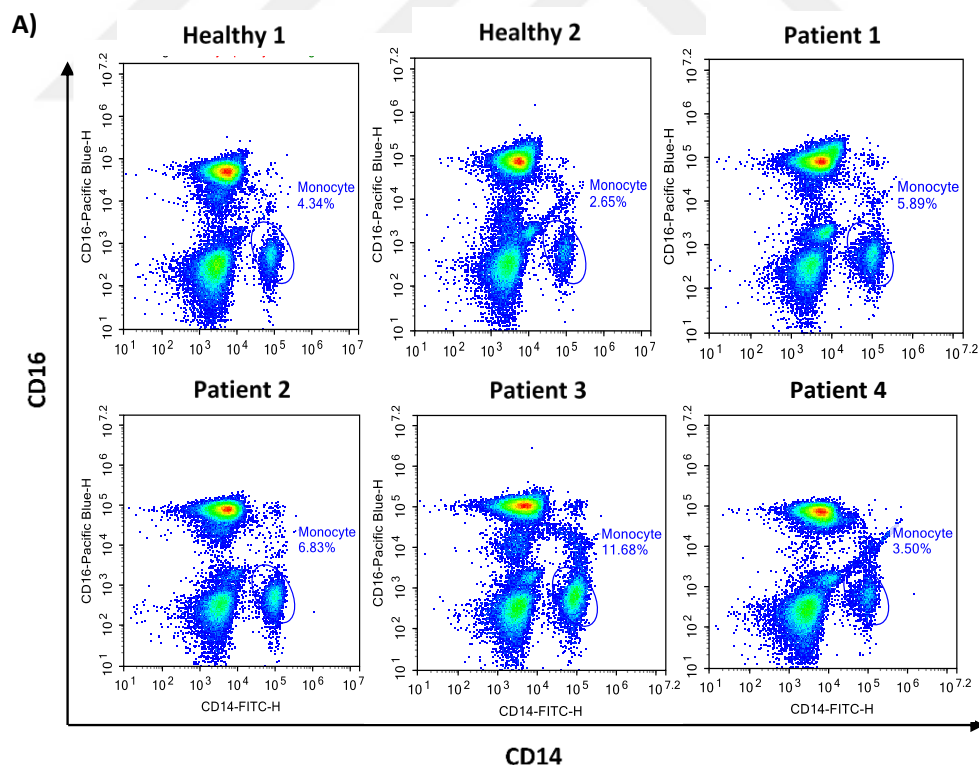


Figure 3.13 Low density granulocyte (LDG) percentages in PBMCs. Gating strategy is performed as forward and side scattering of lymphocytes. **A)** Density plot of LDGs. **B)** Representative bar graphs LDG percentages in PBMCs of healthy controls and patients.

3.3.1.3 Monocyte percentage is higher in all patients

CTLA-4 is expressed by 3% monocytes on their surfaces [68]. The whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD14 and anti-human CD16 antibodies to count monocyte percentage in PBMCs, and analyzed by the flow cytometer (Figure 3.14). Monocyte counts of healthy controls were 4.34% and 2.65% of PBMCs while patients had 5.89%, 6.83%, 11.68% and 3.50%, respectively. Patient 3 had elevated monocyte population in the PBMC.



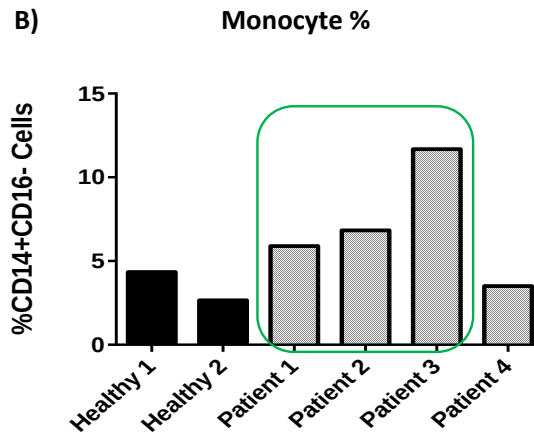
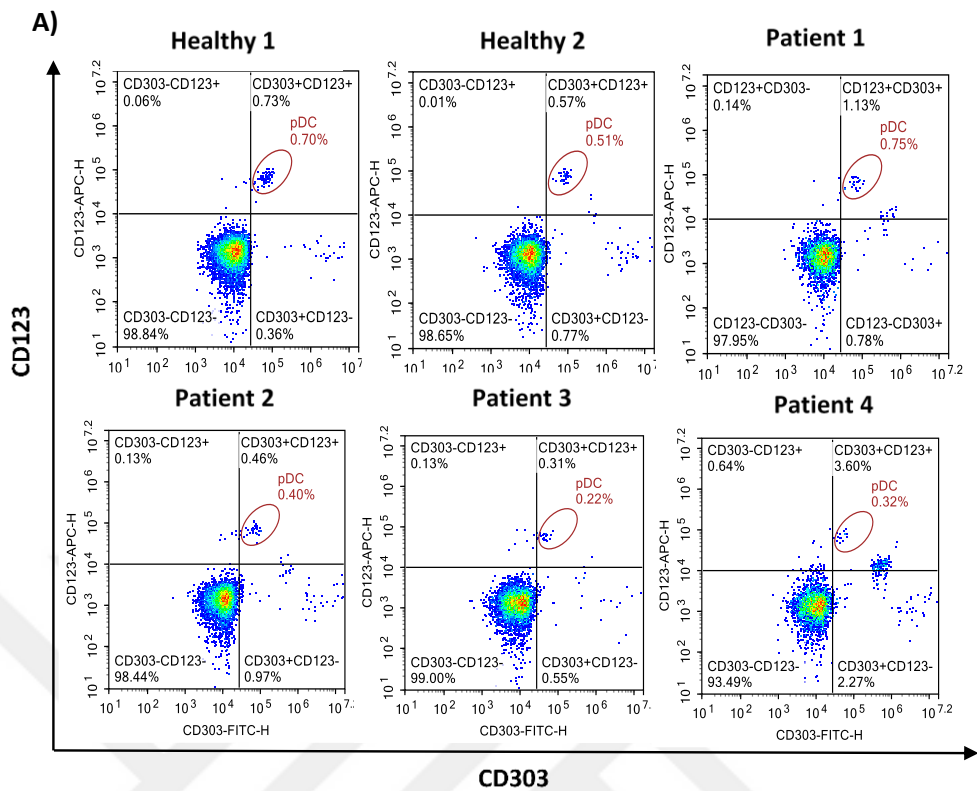


Figure 3.14 CD14 and CD16 surface expression of lymphocytes. The 100 μ l whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD14 and anti-human CD16 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD14⁺ CD16⁻ cells. **B)** Representative bar graphs of CD14⁺ CD16⁻ cell percentages in PBMCs of healthy controls and patients.

3.3.1.4 Three patients have low pDC count

Plasmacytoid dendritic cells (pDC) express CD86 which is a CTLA-4 ligand. By the CD86 and CTLA-4 ligation, Treg cells are stimulated, and T-cell priming is inhibited [69]. The whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD123 and anti-human CD303 antibodies to examine pDC count from PBMCs by flow cytometric analysis (Figure 3.15). Patient 2, 3 and 4 had reduced count of pDCs while Patient 1 had normal pDC count compared to healthy controls.



B) pDC %

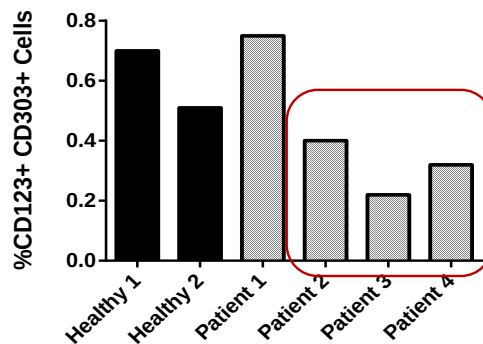


Figure 3.15 CD123 and CD303 surface expression of lymphocytes. The 100 μ l whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD123 and anti-human CD303 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD123+ CD303+ cells. **B)** Representative bar graphs of CD123+ CD303+ cell percentages in PBMCs of healthy controls and patients.

3.3.1.5 B-cell percentage is normal in patients

CTLA-4 prevent T-cell mediated autoimmunity by downregulating T-cell response, and T-cell dependent B-cell response is also controlled by CTLA-4 indirectly. The whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3 and anti-human CD19 antibodies to determine B-cell percentage in PBMCs, and analyzed by flow cytometer (Figure 3.16). All patients had normal B-cell percentage as compared to healthy controls.

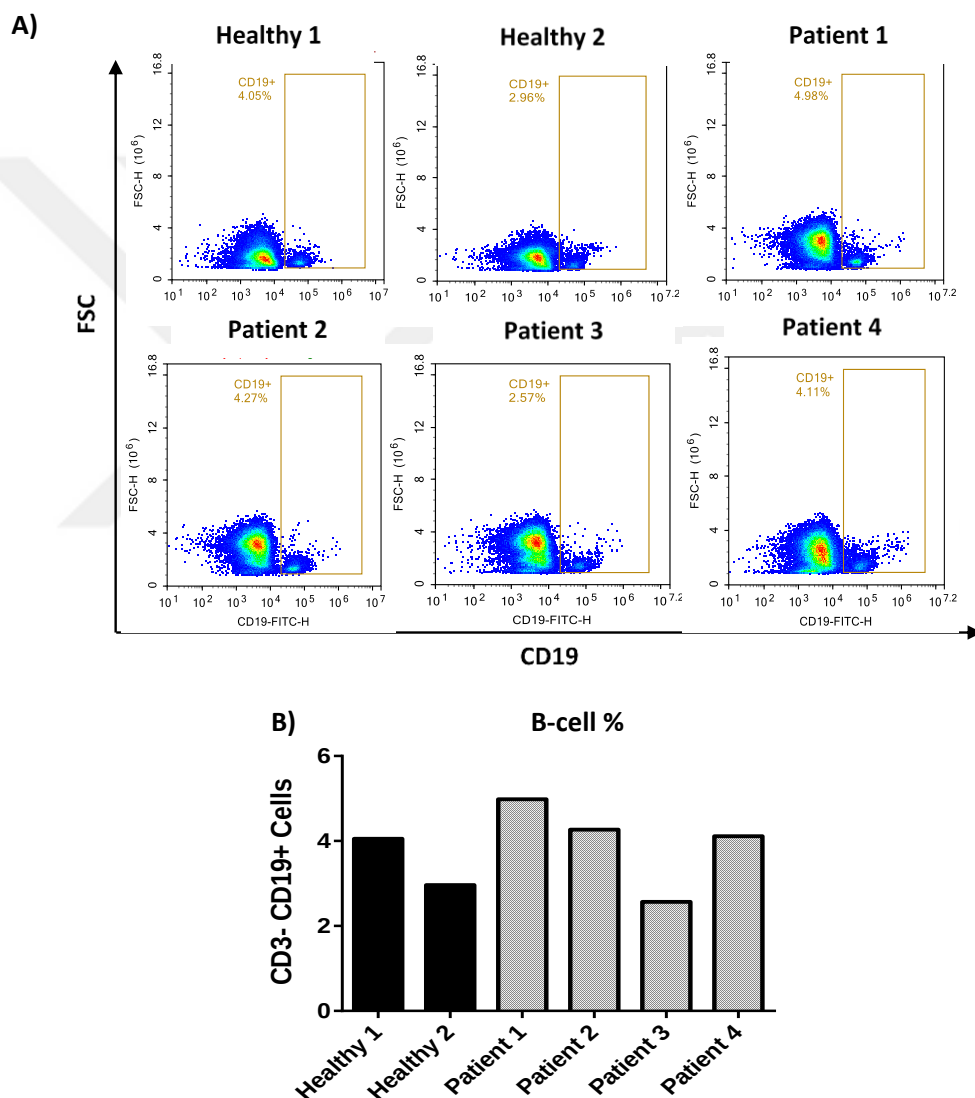
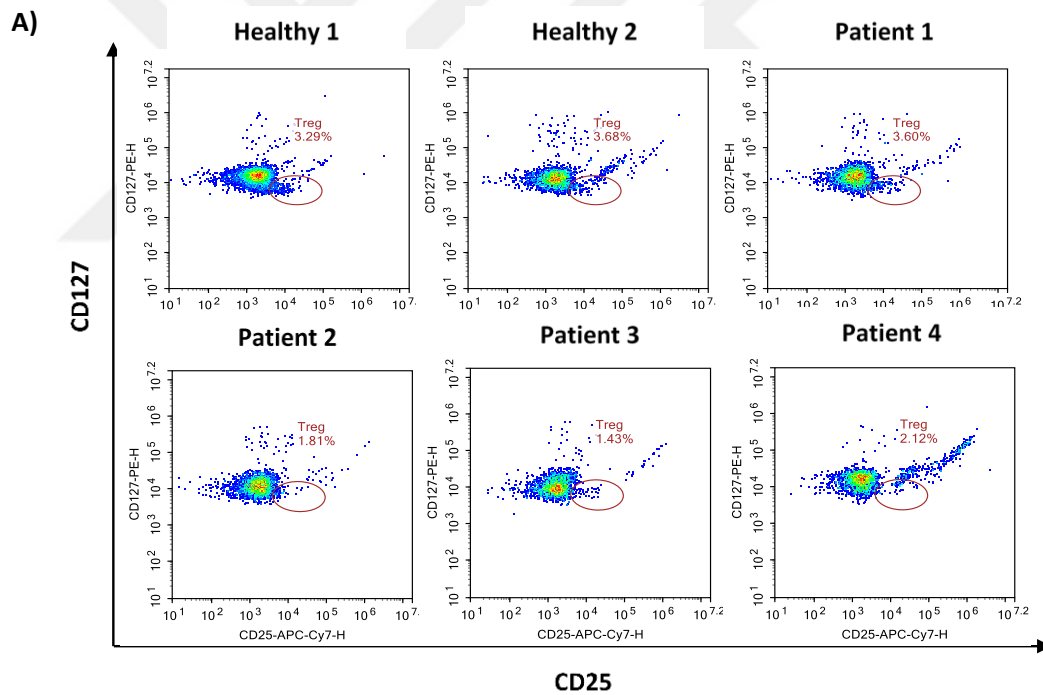


Figure 3.16 CD19 surface expression of CD45+ CD3- cells. The 100 μ l whole blood of healthy controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3 and anti-human CD19 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)**

Density plot of CD3- CD19+ cells. **B)** Representative bar graphs of CD19+ cell percentages in CD45+ CD3- cells of healthy controls and patients.

3.3.1.6 Treg count is decreased in three patients

Treg cells contain CTLA-4 protein to regulate T-cell activity by downregulation. The whole blood of controls and patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies to stain Treg cell population inside the PBMCs (Figure 3.17). Patient 2,3 and 4 had lower Treg count as 1.81%, 1.43% and 2.12%, respectively, while Patient 1 and controls had 2 fold more Treg percentage as 3.29% and 3.68%, respectively.



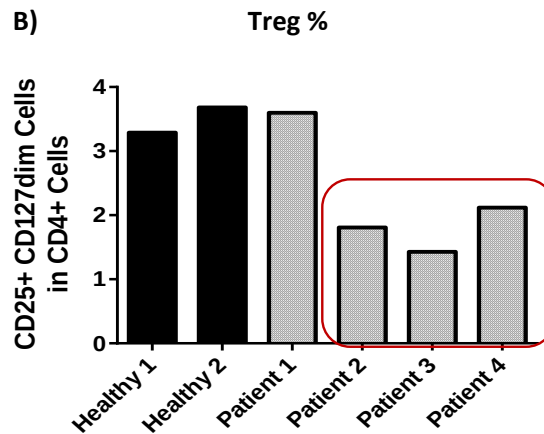


Figure 3.17 CD25 and CD127 surface expression of CD45+ CD3+ CD4+ cells. The 100 μ l whole blood of controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD25+ CD127dim cells. **B)** Representative bar graphs of CD4+ CD25+ CD127dim cell percentage of healthy controls and patients.

3.3.2 Determination of Protein Expressions From PBMCs

3.3.2.1 Increased PD-L1 expression in CD8+ T-cells, B-cells and Tregs is observed in only one patient

PD-L1 is another negative regulatory protein like CTLA-4 to inhibit T-cell activation and to reduce T-cell survival [70]. Therefore, PD-L1 expression levels of different cell types were examined to see whether CTLA-4 mutation could be compensated or not by PD-L1 protein in patients. The whole blood of controls and patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD8, anti-human CD4, anti-human CD25, anti-human CD127, anti-human CD19 and anti-human PDL-1 antibodies to detect surface PD-L1 expression on CD8+ cells, CD4+ cells, Treg cells and B-cells, then analyzed by flow cytometer (Figure 3.18). Only Patient 4 had elevated levels of PD-L1 on CD8+ cells, Treg cells and B-cells as compared to healthy controls. High PD-L1 expression could reduce the CTLA-4 mutation impacts on Patient 4.

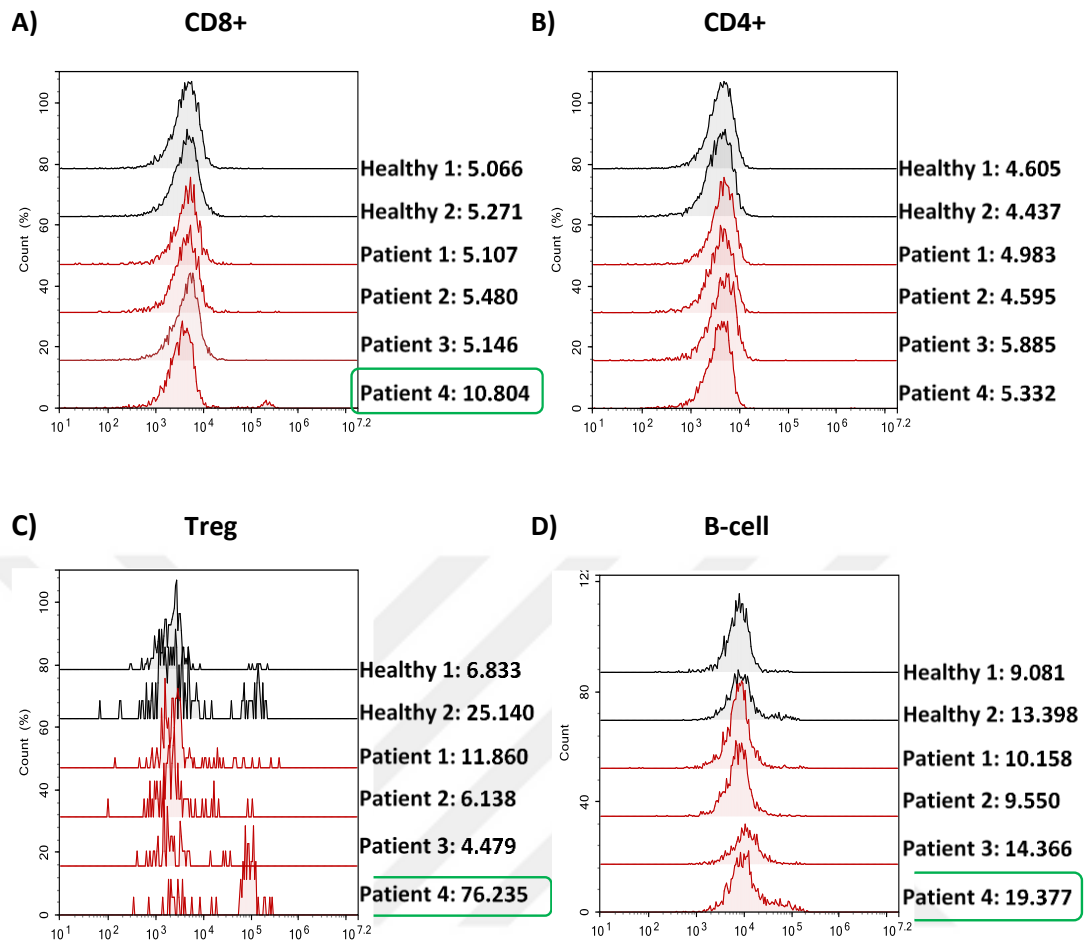


Figure 3.18 PDL-1 surface expression of CD8+ cells, CD4+ cells, Tregs and B-cells. The 100 μ l whole blood of controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD45, anti-human CD8, anti-human CD4, anti-human CD25, anti-human CD127, anti-human CD19 and anti-human PDL-1 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. Mean fluorescence intensity (MFI) of PDL-1 of **A)** CD8+ T-cells, **B)** CD4+ T-cells, **C)** CD25+ CD127dim Tregs, **D)** CD19+ B-cells.

3.3.2.2 Low TCR expression was detected in two patients

Antigen presentation to the T-cell receptor (TCR) along with the costimulatory signal cause T-cell proliferation and survival. Higher TCR expression may worsen the effects of CTLA-4 mutation while lower TCR expression might reduce the impact of CTLA-4 mutation in patients. To see TCR and CTLA-4 levels of the CD3+ cells, the whole blood of controls and patients were stained with fluorochrome conjugated anti-

human CD3+, anti-human TCR and anti-human CTLA-4 antibodies, and flow cytometer analysis was performed (Figure 3.19). Even though, patients had mutation, CTLA-4 expression levels on CD3+ cells were normal as healthy controls. However, Patient 1 and 4 had reduced levels of TCR protein on CD3+ cells as compared to healthy group which might reduce the damaging effect of CTLA-4 mutation.

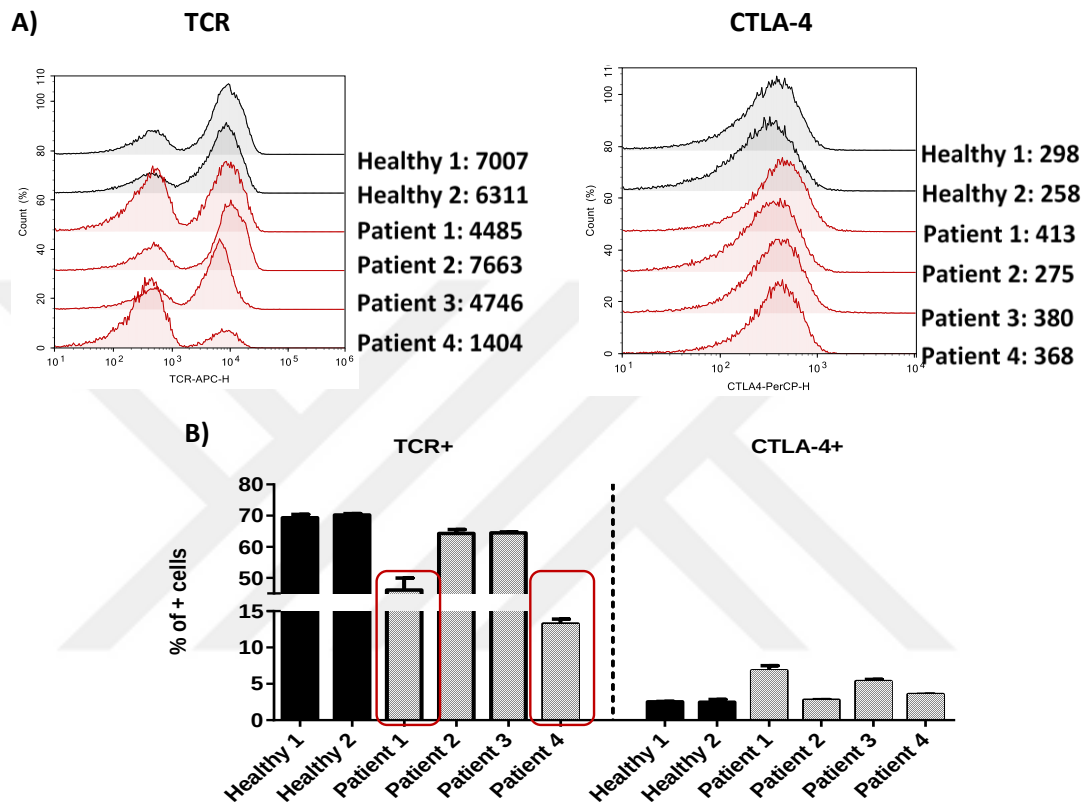


Figure 3.19 TCR and CTLA-4 surface expression of CD3+ cells. The 100 μ l whole blood of controls and CTLA-4 patients were stained with fluorochrome conjugated anti-human CD3+, anti-human TCR and anti-human CTLA-4 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Mean fluorescence intensity (MFI) of TCR and CTLA-4 of CD3+ T-cells. **B)** Representative bar graphs of TCR and CTLA-4 percentage of CD3+ cells in healthy controls and patients.

3.3.2.3 Patients had no detectable mTOR and pmTOR proteins, besides STAT3, AKT and 4EBP1 levels were decreased in patients

Functional CTLA-4 receptor, induce several downstream protein activations for healthy response after activation. CTLA-4 receptor and ligand binding activates ERK and AKT via phosphorylation, and pAKT phosphorylate mTOR for activation. Phosphorylated mTOR further activates 4EBP1 by phosphorylation [71, 72]. Also, healthy CTLA-4 response cause STAT3 phosphorylation to downregulate T helper 1 cells [73]. Transcription factor HIF-1 α in CD8⁺ T-cells increase the CTLA-4 expression on the T-cells, so functional HIF-1 α is required for healthy CTLA-4 response [74]. Moreover, phosphorylated AMPKs cause CTLA-4 and PD-L1 degradation [75]. To see the impact of CTLA-4 mutation over pERK, pAKT, pmTOR, p4EBP1 and STAT3 proteins, also to examine the presence HIF-1 α and AMPK, the 15 μ g protein of controls and patients PBMCs were loaded for immunoblotting (Figure 3.20). mTOR and pmTOR could not be detected in all patients, and ERK expression of the patients was normal according to healthy controls. Even though AKT expression is impaired in patients, pAKT levels were higher in patient 1 and 4. Similarly, 4EBP1 expression was reduced in all patients, but p4EBP1 level of the Patient 4 was elevated. Moreover, as expected STAT3 levels were decreased in all patients especially Patient 2 and 3. Patient 1 and 4 showed increased AMPK phosphorylation which could degrade present CTLA-4. Furthermore, Hif-1 α expression was impaired in patients which could affect CTLA-4 expression in patients.

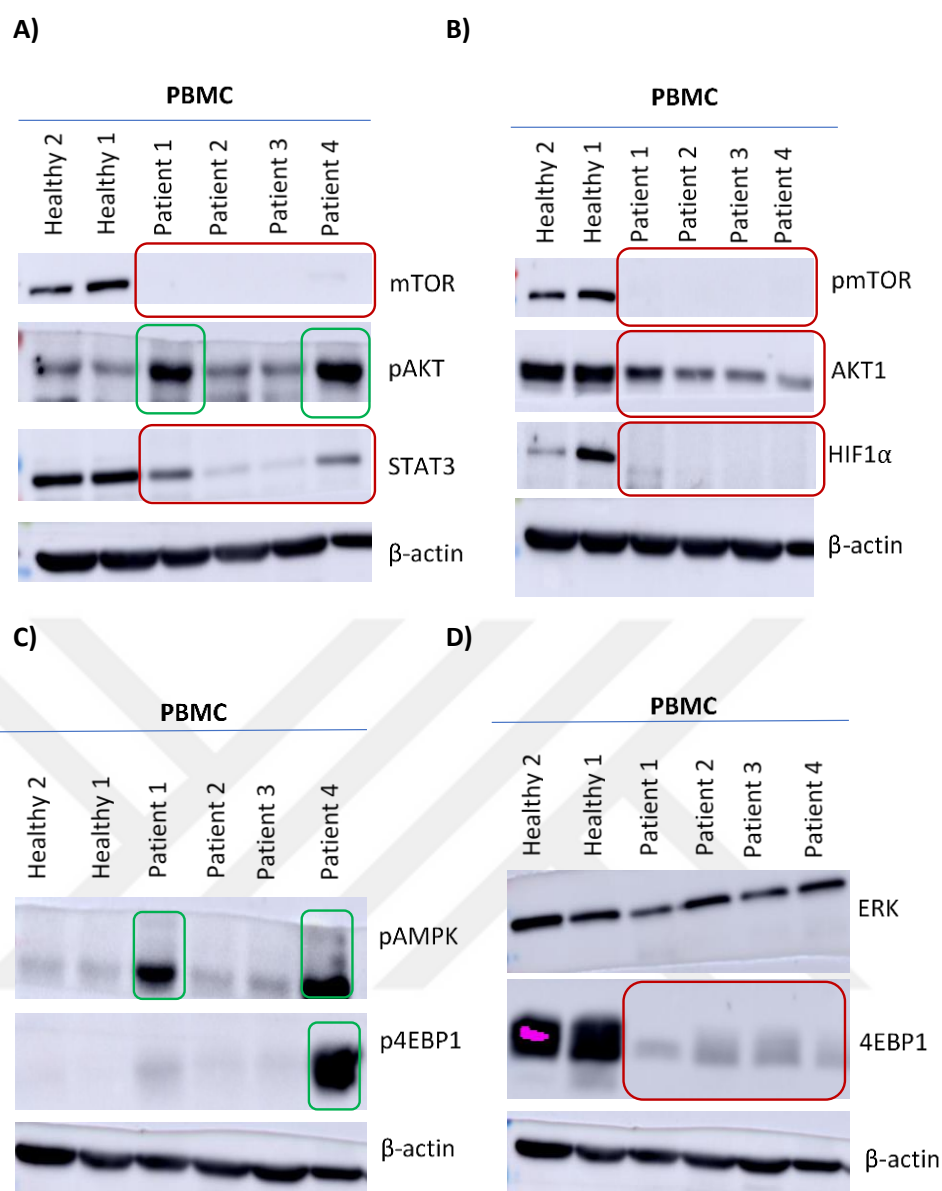


Figure 3.20 Expression of CTLA-4 activated downstream signaling proteins of PBMCs. The 15 µg protein of controls and CTLA-4 patients PBMCs were loaded for immunoblotting. **A)** mTOR, pAKT, STAT3, **B)** pmTOR, AKT1, HIF1α, **C)** pAMPK, p4EBP1, **D)** ERK, 4EBP1 protein expressions of healthy controls and patients.

3.3.2.4 All patients had low pSTAT3 percentage in CD4+ T-cells upon PMA treatment

Functional CTLA-4 response cause STAT3 phosphorylation. STAT3 phosphorylation levels in patients were assessed to impower immunoblotting results. PBMCs of

controls and CTLA-4 patients were stimulated with PMA and Ionomycin or left untreated for 2 hours, then treated with Brefeldin A for 4 hours to stop cytokine secretion. After fixation and permeabilization, cells were stained with fluorochrome conjugated anti-human CD4+ and anti-human pSTAT3 antibodies, then analyzed by flow cytometer (Figure 3.21). After PMA stimulation which causes T-cell activation, all patients showed impaired pSTAT3 levels compared to healthy controls as expected because patients do not have functional CTLA-4 protein to induce STAT3 phosphorylation.

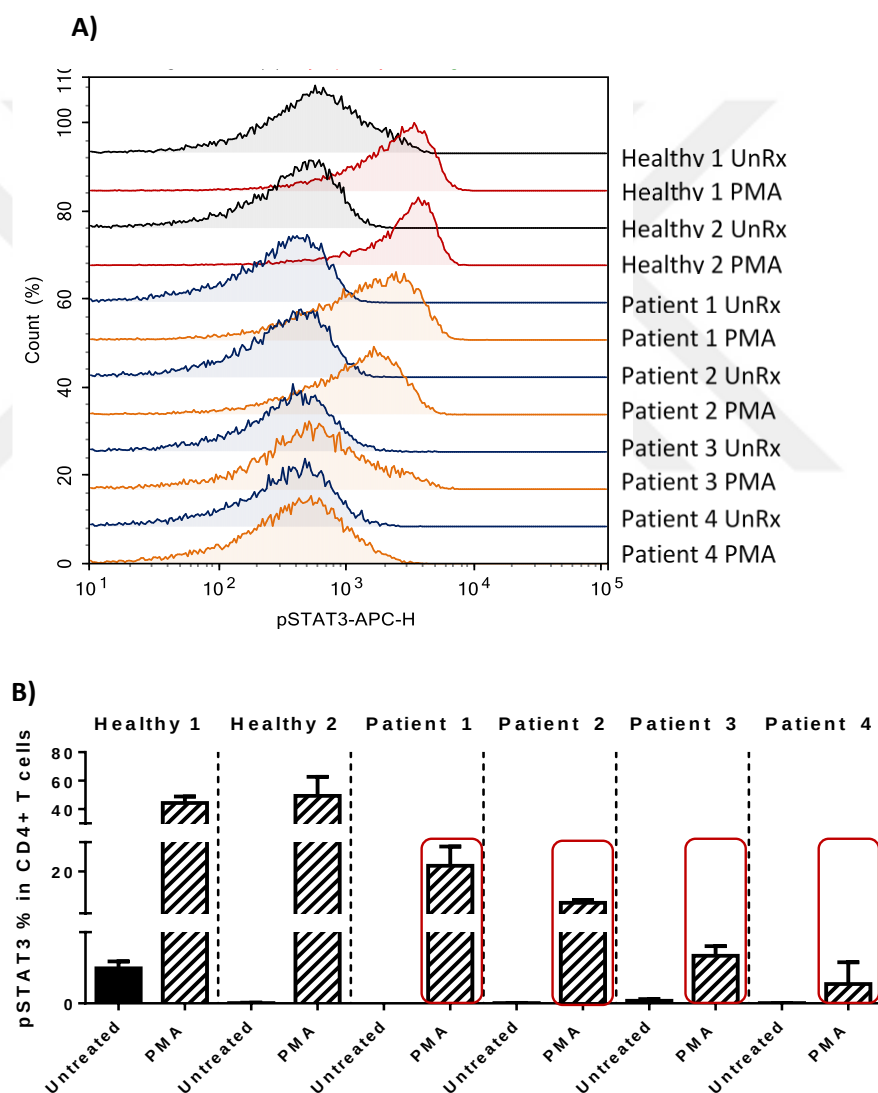


Figure 3.21 pSTAT3 expression of CD4+ cells. 4×10^5 /ml PBMCs of controls and CTLA-4 patients were stimulated with 50 ng/ml PMA and 1 μ g/ml Ionomycin or left untreated for 2 hours, then treated with 5 μ g/ml Brefeldin A for 4 hours, fixed,

permeabilized and stained with fluorochrome conjugated anti-human CD4⁺ and anti-human pSTAT3 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Mean fluorescence intensity (MFI) of pSTAT3 of CD4⁺ cells. **B)** Representative bar graphs of pSTAT3 percentage of CD4⁺ T-cells in healthy controls and patients.

3.4 Investigation of Immune System Alteration in Probably mTOR/NFκB Mutated Patient

3.4.1 Determination of Immune Cell Counts From Whole Blood

3.4.1.1 CD4/CD8 ratio was not reversed

The balance between CD4⁺ and CD8⁺ T-cells is important for healthy functional immune system. The CD4⁺/CD8⁺ ratio lower than 1 indicates viral infections, and increased ratio could imply autoimmunity. To see if patient had any trace of infection or autoimmunity, CD4⁺/CD8⁺ ratios were assessed. The whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4 and anti-human CD8 antibodies, and analyzed by flow cytometer (Figure. 3.22). Healthy controls had normal ratio as 2 and 2.8 while patients had slightly lower ratio as 1.2, but still above the 1 ratio which implies severe viral infections such as HIV.

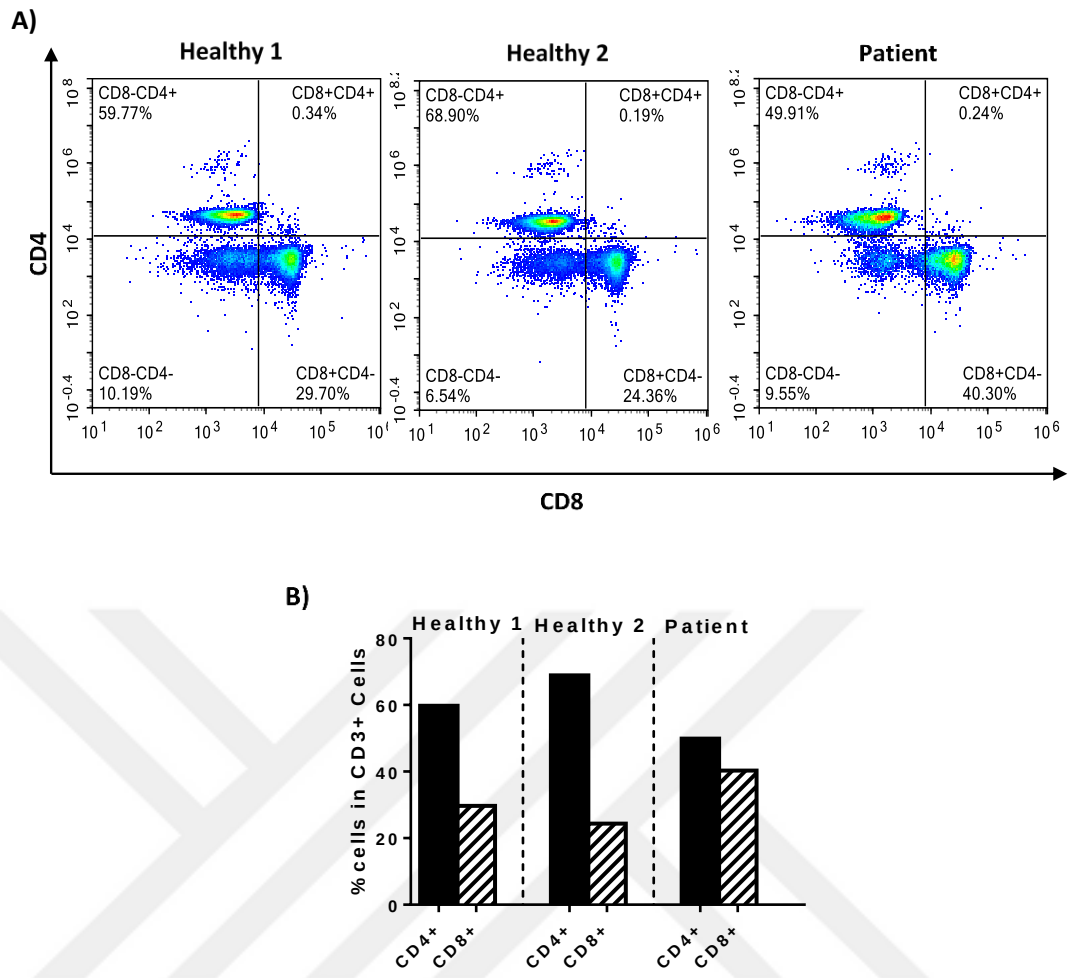


Figure 3.22 CD4 and CD8 surface expression of CD3+ cells. The 100 μ l whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD3, anti-human CD4 and anti-human CD8 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD3+ CD4+ CD8+ cells. **B)** Representative bar graphs of CD4+ CD8+ cell percentages in CD3+ cells of healthy controls and patient.

3.4.1.2 Low density granulocytes were observed in patient

The low density granulocytes (LDGs) have a proinflammatory role in systemic autoimmunity [67]. Therefore, LDG counts which localize between lymphocytes and granulocytes from PBMCs of healthy controls and patient were determined by flow cytometer (Figure 3.23). Healthy controls had 0.03% and 0.05% LDG from PBMCs. However, patient had 12.53% LDGs in the PMBCs which is higher than average

healthy value, 1.28% [68]. High LDG count could provide undesired proinflammatory response.

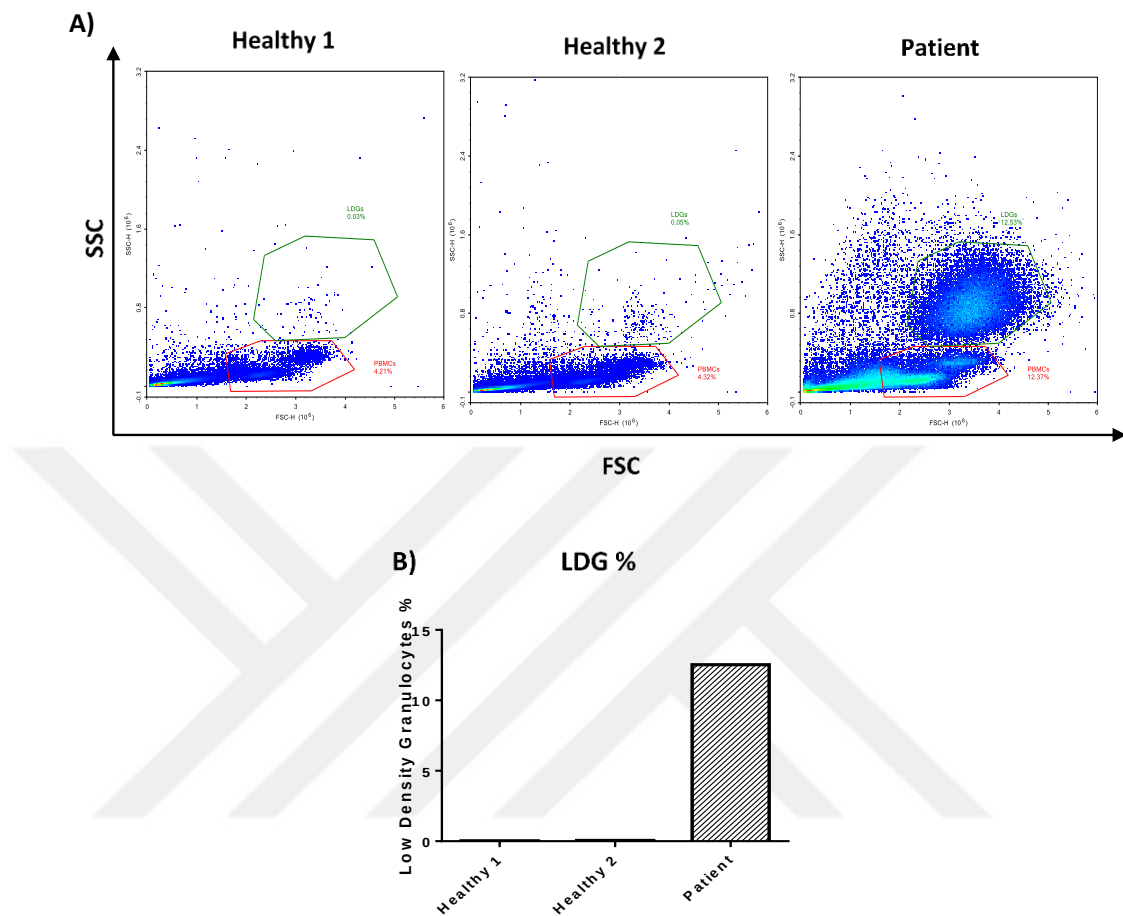


Figure 3.23 Low density granulocyte (LDG) percentages in PBMCs. Gating strategy is performed as forward and side scattering of lymphocytes. **A)** Density plot of LDGs. **B)** Representative bar graphs LDG percentages in PBMCs of healthy controls and patient.

3.4.1.3 Patient has elevated monocyte percentage

Monocytes are important member of the innate immune system which found in blood, and give rise to phagocytic macrophages in tissue. Increased monocyte count would imply an ongoing infection or inflammation. Therefore, the whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD14 and anti-human CD16 antibodies to count monocyte percentage in PBMCs, then analyzed by the flow cytometer (Figure 3.24). Monocyte counts of healthy controls were

12.98% and 9.6 % of PBMCs while patient had 2-fold elevated monocytes as 23.49% of PBMCs which leads undesired inflammation.

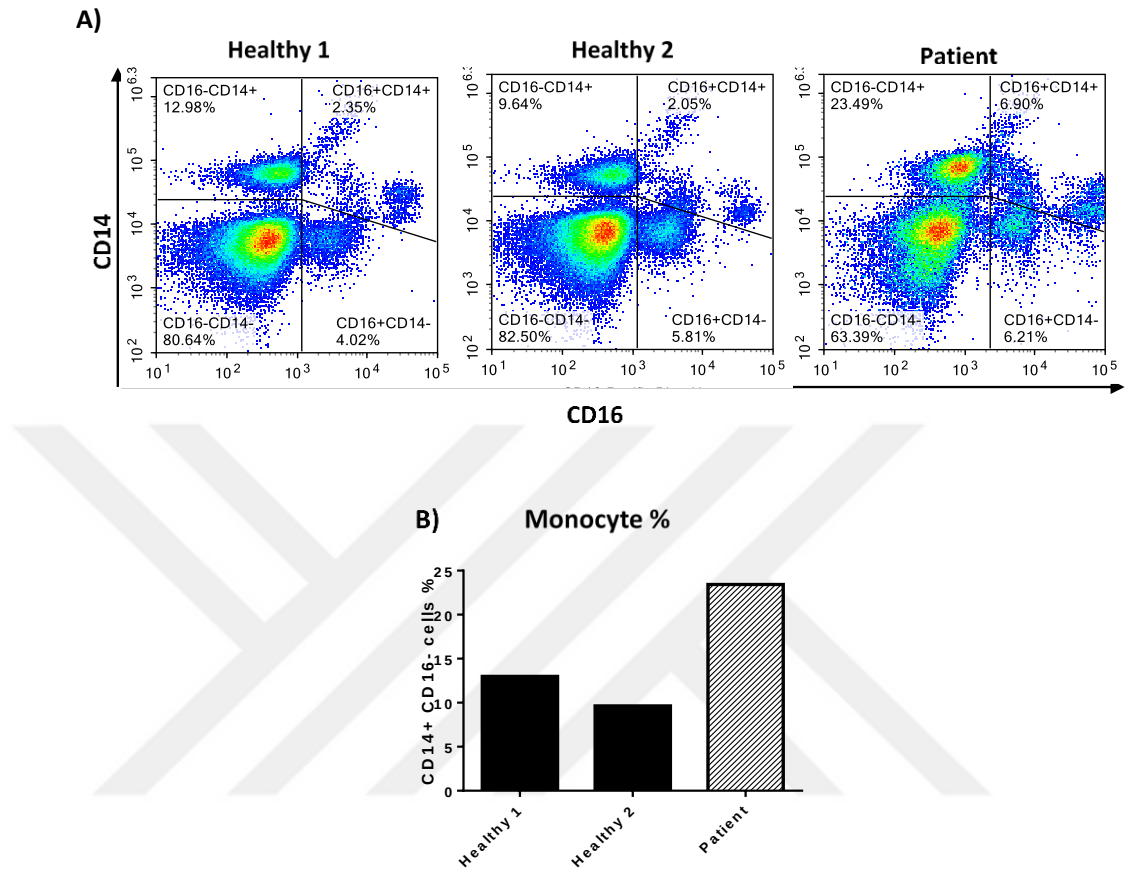


Figure 3.24 CD14 and CD16 surface expression of lymphocytes. The 100 μ l whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD14 and anti-human CD16 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD14+ CD16- cells. **B)** Representative bar graphs of CD14+ CD16- cell percentages in PBMCs of healthy controls and patient.

3.4.1.4 pDC count was normal in patient

Plasmacytoid dendritic cells are critical during viral infections due to Type I IFN release. Increased numbers of pDC from PBMC could suggest presence of viral infections. The whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD123 and anti-human CD303 antibodies, and

examined for pDC count from PBMCs by flow cytometric analysis (Figure 3.25). Patient had similar pDC percent in PBMC population to healthy controls.

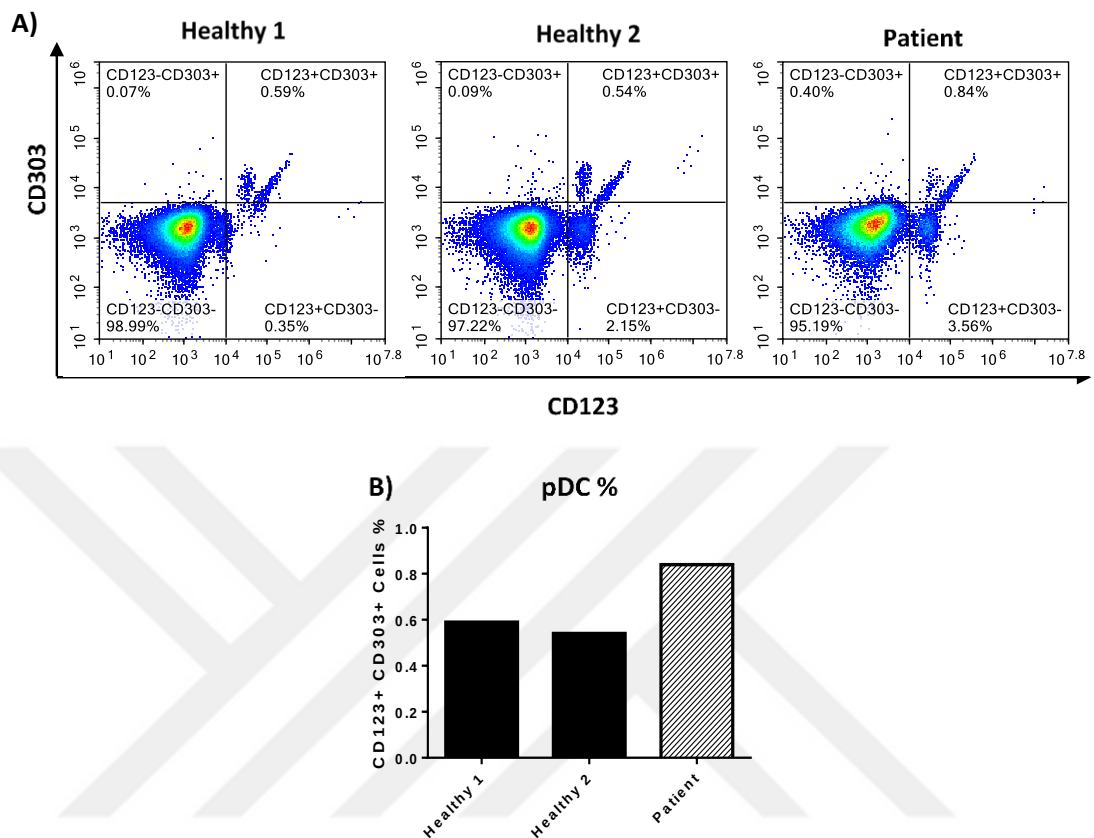


Figure 3.25 CD123 and CD303 surface expression of lymphocytes. The 100 μ l whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD123 and anti-human CD303 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD123+ CD303+ cells. **B)** Representative bar graphs of CD123+ CD303+ cell percentages in PBMCs of healthy controls and patient.

3.4.1.5 Treg percentages were elevated in patients

The functional Treg cells downregulate cytotoxic T-cell activity to prevent excess cellular damage during infections, and required for self-tolerance. Increased Treg activity would support cancer cell development while decreased Treg activity would lead T-cell mediated cell damage and autoimmunity [76]. The whole blood of controls and patient were stained with fluorochrome conjugated anti-human CD45, anti-human

CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies for Treg cell population staining inside the PBMCs (Figure 3.26). Patient had 6-fold elevated Treg count with 29.90% as compared to healthy controls which had 5.79% and 4.34%. Treg count might be increased to balance ongoing autoimmunity. Also, increased Treg cells would suppress T-cell activity which would cause primer immune deficiency.

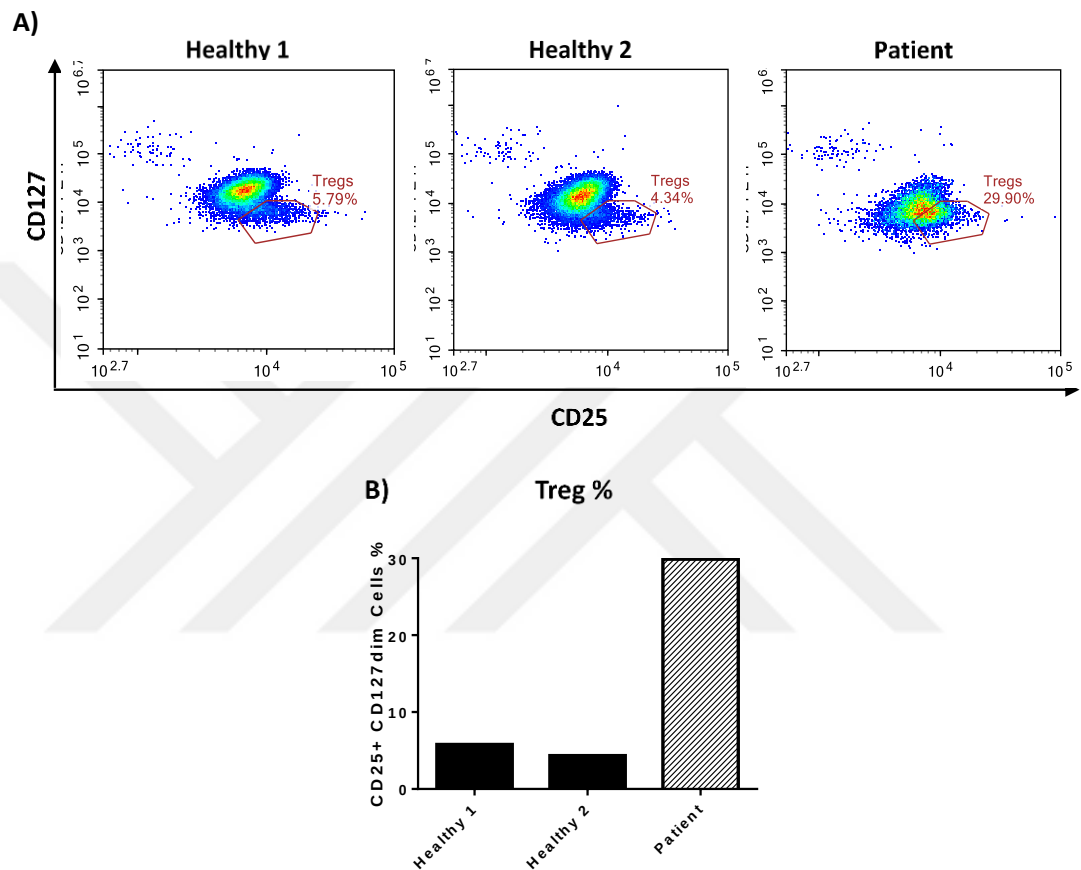


Figure 3.26 CD25 and CD127 surface expression of CD45+ CD3+ CD4+ cells. The 100 μ l whole blood of controls and patient were stained with fluorochrome conjugated anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD45+ CD3+ CD4+ CD25+ CD127dim cells. **B)** Representative bar graphs of CD4+ CD25+ CD127dim cell percentage of healthy controls and patient.

3.4.1.6 Decreased number of B-cells are detected in patient

B-cells are important members of adaptive immunity. Through the activation B-cells give rise to plasma cells which secrete immunoglobulins for humoral immunity during infections. Unfunctional B-cells are one of the reasons for primer immune deficiencies [77]. The whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD45, anti-human CD3 and anti-human CD19 antibodies, B-cell percentages in PBMCs were determined by flow cytometry analysis (Figure 3.27). Healthy controls had 12.90% and 16.38% B-cells in the PBMCs while patient showed highly impaired numbers of B-cells as 1.65% of the PMBCs which would indicate PID or even severe combined immune deficiency (SCID) along with the increased Treg count.

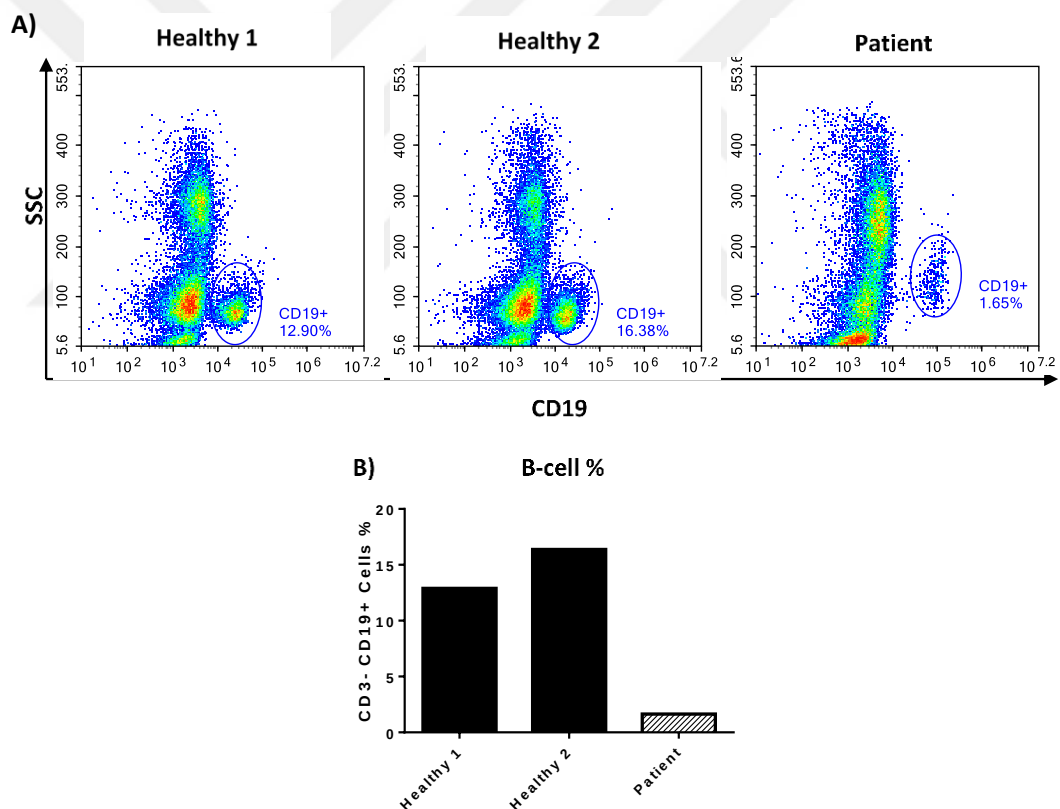
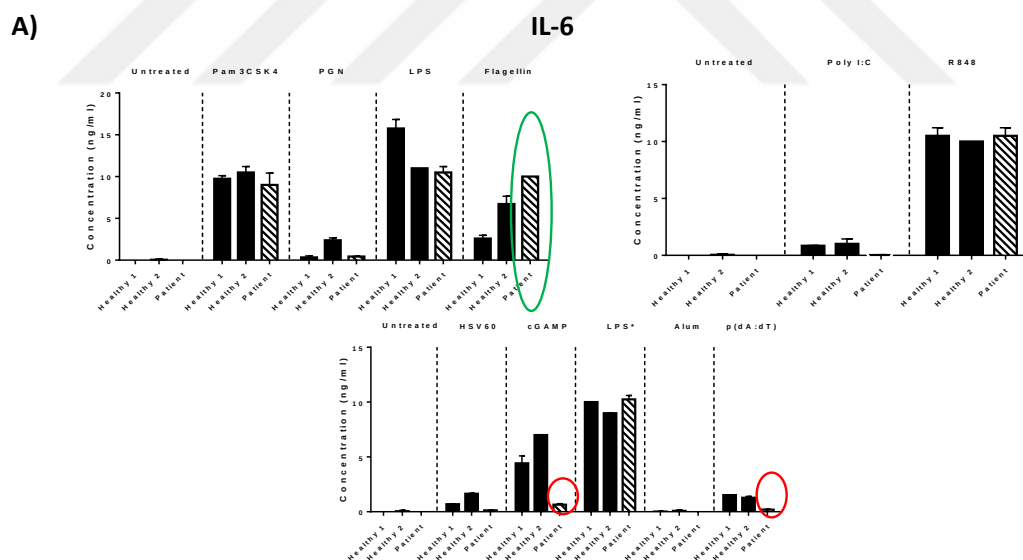


Figure 3.27 CD19 surface expression of CD45+ CD3- cells. The 100 μ l whole blood of healthy controls and patient were stained with fluorochrome conjugated anti-human CD45, anti-human CD3 and anti-human CD19 antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. **A)** Density plot of CD3- CD19+ cells. **B)** Representative bar graphs of CD19+ cell percentages in CD45+ CD3- cells of healthy controls and patient.

3.4.2 Characterization of Innate Immune Responses. IL-6, TNF α and IFN γ responses from PBMCs of patient was elevated by stimulation with the surface TLR ligands while opposite effect is detected through cytosolic nucleic acid ligand stimulations

Proinflammatory cytokine and Type II IFN responses of the healthy controls and patient were examined by PRR ligand stimulation (Figure 3.28). Patient showed elevated TLR5 mediated IL-6 response and TLR4 derived TNF α response as compared to healthy controls. However, STING, AIM2 inflammasome and RIG-I/MAVS mediated proinflammatory responses were impaired in patient. Moreover, TLR4 mediated IFN γ response was higher while AIM2 inflammasome and RIG-I/MAVS derived IFN γ response was reduced in patient.



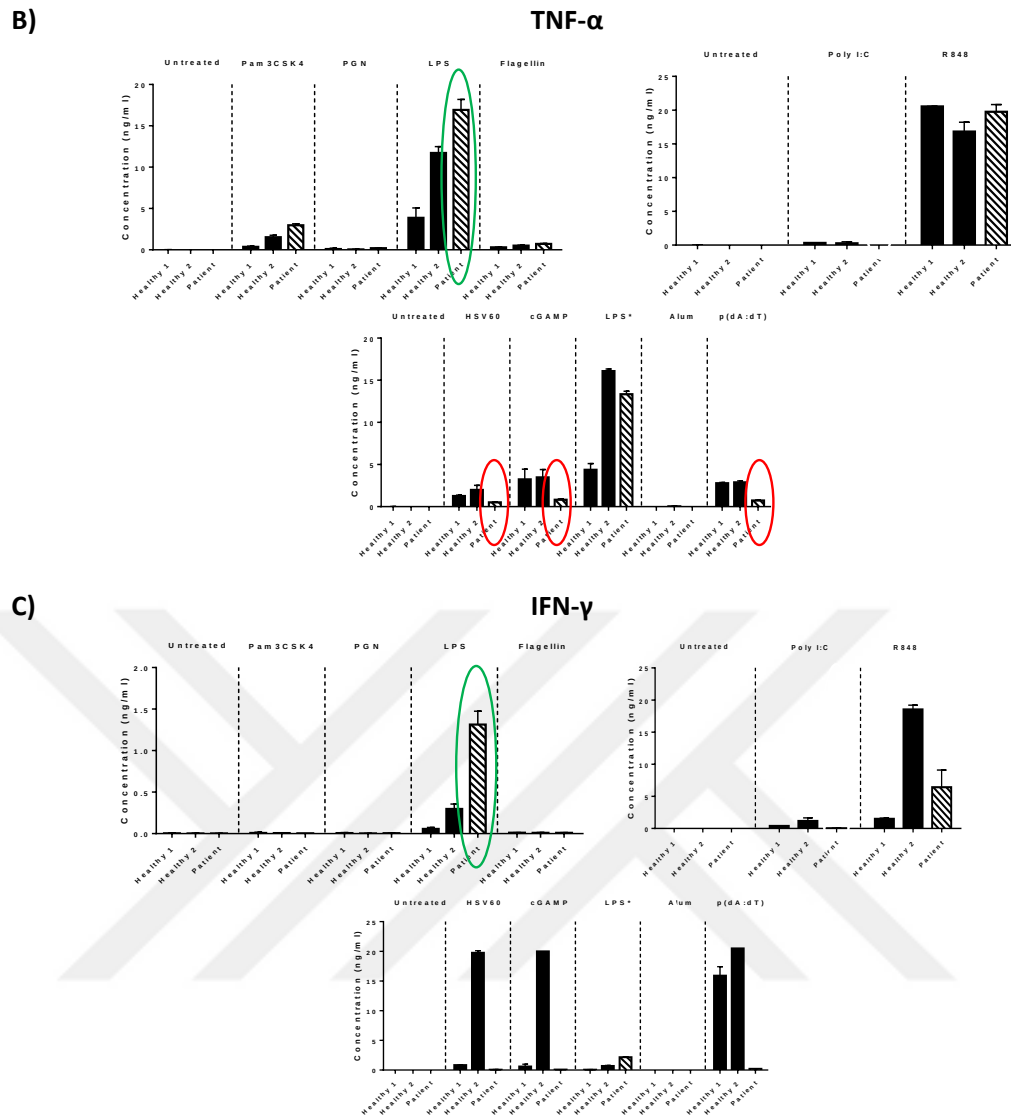


Figure 3.28 IL-6, TNF- α and IFN- γ cytokine secretion profiles of healthy and patient PBMCs through 24 hours PRR ligand stimulation. Control and patient PBMCs (4×10^5 /ml) were stimulated with Pam3CSK4 (TLR1/2, 1 μ g/ml), Peptidoglycan (TLR2, 5 μ g/ml), p(I:C) (TLR3, 30 μ g/ml), LPS (TLR4, 5 μ g/ml), Flagellin (TLR5, 100 ng/ml), R848 (TLR7, 5 μ g/ml), transfected LPS (Non-canonical Inflammasome, 5 μ g/ml), transfected Alum (NLRP3 Inflammasome, 20 ng/ml), transfected p(dA:dT) (AIM2 Inflammasome, 5 μ g/ml), transfected cGAMP (STING, 30 μ g/ml) and transfected HSV (cGAS-STING, 5 μ g/ml) for 24 hours, and supernatants were collected to investigate with cytokine ELISA. **A)** IL-6, **B)** TNF α , **C)** IFN γ secretion levels from healthy controls and patient PBMCs.

3.4.3 Characterization of Adaptive Immune Responses in Probably mTOR/NFκB Mutated Patient

3.4.3.1 Patient had higher pmTOR levels in CD4+ and CD8+ T-cells upon LPS stimulation

In patient, it is suspected that NFκB-mTOR-PI3K pathways or one of the pathway were probably mutated. Therefore, pmTOR levels in CD4+ cells, CD8+ cells and B-cells were investigated through LPS stimulation. The PBMCs of healthy controls and patient were stimulated with for 1 hours, then treated with Brefeldin A for 4 hours. After fixation, permeabilization and staining with fluorochrome conjugated anti-human pmTOR, anti-human CD4, anti-human CD8 and anti-human CD19 antibodies, flow cytometry analysis was performed (Figure 3.29). Patient showed 3-fold elevated pmTOR levels in CD4+ cells and CD8+ cells upon LPS treatment as compared to healthy controls.

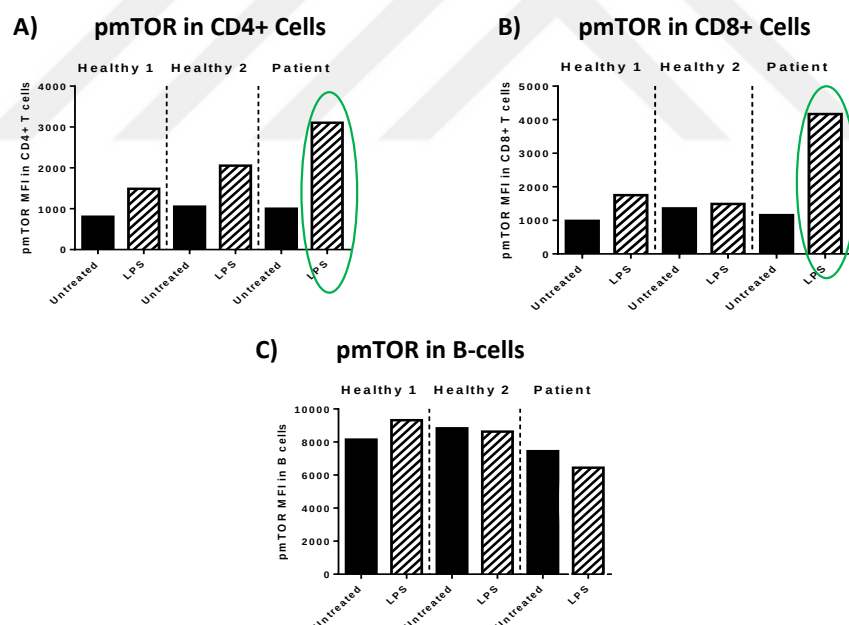


Figure 3.29 MFI values of pmTOR+ cells in healthy and control CD4+, CD8+ and B-cells through LPS treatment. The 3×10^5 /ml PBMCs of healthy controls and patient were stimulated with 100 ng/ml LPS for 1 hours, then treated with with 5 μ g/ml Brefeldin A for 4 hours, fixed, permeabilized and stained with fluorochrome conjugated antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. Representative bar graphs of pmTOR+ cells in **A)** CD4+, **B)** CD8+, **C)** B-cells of healthy controls and patient.

3.4.3.2 Patient had elevated basal TNF- α expression from B-cells, also higher TNF α expression from CD4+ T-cells was observed upon LPS treatment in patient

TNF α expression is under control of NF κ B pathway, so intracellular TNF α levels in CD4+ cells, B-cells and monocytes were analyzed. The PBMCs of healthy controls and patient were stimulated with LPS for 4 hours, then treated with Brefeldin A for 4 hours, fixed, permeabilized and stained with fluorochrome conjugated anti-human TNF α , anti-human CD4, anti-human CD19, anti-human CD14 and anti-human CD16 antibodies for flow cytometry analysis (Figure 3.30). Patient had increased levels of TNF α expression in CD4+ cells and B-cells while monocyte derived TNF α expression was normal through LPS treatment in patient according to control group.

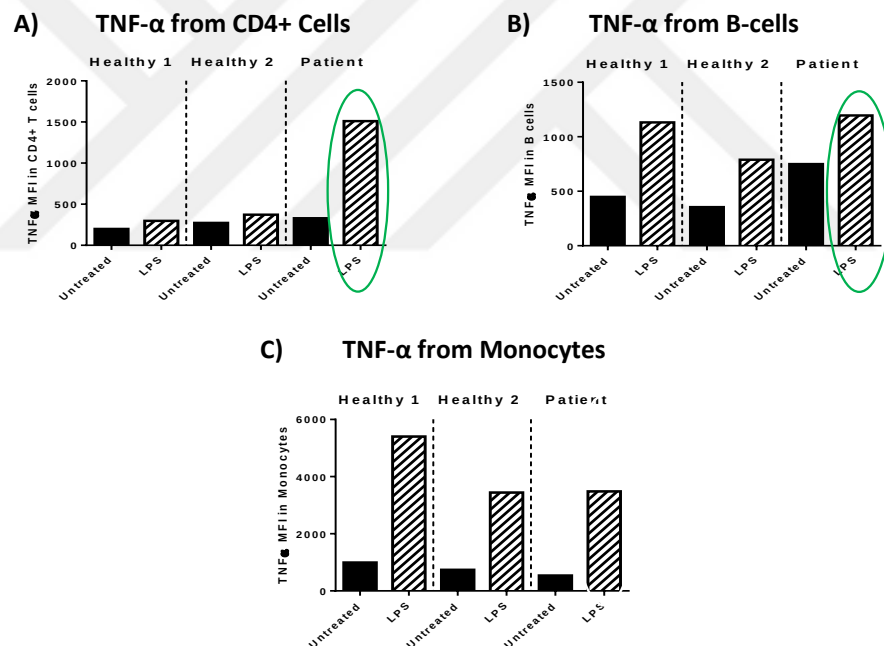


Figure 3.30 MFI values of TNF α + cells in healthy and control CD4+ cells, B-cells and monocytes through LPS treatment. The 3×10^5 /ml PBMCs of healthy controls and patient were stimulated with 1 μ g/ml LPS for 4 hours, then treated with with 5 μ g/ml Brefeldin A for 4 hours then fixed, permeabilized and stained with fluorochrome conjugated antibodies. Gating strategy is performed as forward and side scattering of lymphocytes, further as singlets. Representative bar graphs of TNF α + cells in **A)** CD4+ cells, **B)** B-cells, **C)** monocytes of healthy controls and patient.

CHAPTER 4

DISCUSSION

Immune system is a defense mechanism against invaders. Through evolution, different mutations occurred in the systems, and some of them caused detrimental effects while others provided increased survival to the organisms. Like all other organisms, pathogens also evolved with time which caused the evolution of the defense mechanism in host organisms to protect itself against invaders. Among all species, mammalian immune system is the most complex one [1]. However, this complexity could have some drawbacks such as one nonfunctional protein or the deficiency of one key protein in a complex mechanism may lead undesired system failure due to domino effect which impairs defense mechanisms. Not only mutations, but also secondary factors such as malnutrition, chemical abuse, environmental stress or some other disorders could cause defects in immune system [45]. Immune deficiencies can be divided into two group, primary and secondary immune deficiencies. The reasons of primary immune deficiencies could be inherited immune dysfunctions originated from autosomal recessive or autosomal dominant mutations. Combinations of ongoing infections, lymphoproliferation, atopies, malignancies, autoimmunity and granulomatous processes are seen in primary immune deficiency disorders. B-cell defects make patients more prone to bacterial infections and self-reactive immunoglobulin mediated autoimmunity while T-cell defects could favor asthma and food allergies. T-cell and B-cell combined immune deficiencies cause vulnerability to different pathogens like viruses, bacteria and fungi. Also, malignancies could be seen as leukemias and lymphomas [78]. For different types of PIDs, several therapeutic approaches can be applied. Prophylactic antibiotics and immunoglobulin replacement therapies are widely used for treatment of antibody mediated deficiencies while immunosuppressant biologic drugs are good candidates in common variable immune deficiency treatments [79, 80]. Moreover, gene therapies, hematopoietic stem cell transplantations and enzyme replacements are commonly used against severe combined deficiencies [81]. To establish distinctive

therapies, characterization of immune deficiencies along with the understanding of the impaired mechanisms are very critical.

RLTPR scaffolding protein is crucial for CD28 co-stimulation to activate T-cells via facilitating recruitment of CBM complex which activates NF- κ B pathway [50]. Reduced CD4⁺ T-cells, memory B-cells and antibody response along with the impaired B-cell receptor mediated NF- κ B activation were observed in RLTPR deficient patients [84]. Moreover, polarity and migration of the T-cells were affected by RLTPR deficiency in patients [48]. The adaptive immune responses of RLTPR loss-of-function (LOF) mutations were well studied, the innate immune response of RLTPR deficient patient was investigated. Two different blood samples were collected from patients, one was CMV infected and the other was uninfected. Firstly, Treg cells were examined because RLTPR LOF patients showed decreased numbers of Treg cells due to importance of RLTPR at T-cell survival [47, 48, 63]. As expected, Treg counts of the patient were reduced as compared to healthy controls both with and without infection (Figure 3.1 and Figure 3.2). Although Treg cell counts of patient were decreased, IL-10 secretion upon cytosolic nucleic acid ligand was increased in infection due to ongoing defense and homeostasis (Figure 3.5). Under CMV infection, patient showed elevated proinflammatory IL-1 β response upon activation of TLR4, TLR7, TLR8, TLR9, cGAS-STING, NLRP3 inflammasome pathways, also elevated proinflammatory TNF- α response through activation of TLR4, TLR9, STING, NLRP3 inflammasome, non-canonical inflammasome pathways (Figure 3.3). However, without any viral infections, TLR7 and TLR8 mediated IL-1 β and TNF- α responses were impaired in patient (Figure 3.6). Moreover, under CMV infection, TLR7, TLR8, STING pathway mediated antiviral IFN- α response and TLR4, TLR5, TLR7, TLR8, STING, cGAS-STING mediated antiviral IFN- γ and IL-12 responses were increased in patient (Figure 3.4). But after infection, IFN- α response of the patient from all of the endosomal TLRs, STING and RIG-I/MAVS were reduced along with the impaired IFN- γ response upon TLR3, TLR7, TLR8, STING, RIG-I/MAVS and non-canonical inflammasome pathways (Figure 3.7). Under viral infection, it was expected to see higher response due to ongoing defense by innate immune cells and their effector cytokines. When infection was cleared with medical treatment, it is observed that not only endosomal TLRs but also the cytosolic nucleic

acid sensors are impaired in RLTPR patient which makes the patient vulnerable to viral infections.

In RLTPR-TLR1 deficient patient, decreased number of Treg cells from whole blood was confirmed as expected due to interference of RLTPR mutation in Treg development (Figure 3.8). Because TLR1 deficiency accompanies RLTPR deficiency in patient, innate immune responses were investigated. TLR2-6, TLR4, TLR7, TLR8 and non-canonical inflammasome mediated IL-1 β response of patient was elevated (Figure 3.9). TLR1 deficiency might have an impact on increased TLR2-6 response due to structural TLR1-TLR6 resemblance and functional compensation [19]. However, other increased pathways would indicate an ongoing infection which is very common in PID patients. Besides IL-1 β , increased IFN- α secretion upon AIM2 inflammasome, RIG-I/MAVS, cGAS-STING ligand stimulations, and TLR2-6 mediated IL-12 response support the idea of infection (Figure 3.10). Also, one important confirmation for TLR1 deficiency was provided by lack of IL-12 response through Pam3CSK stimulation in the patient. In addition to all these, elevated IL-10 response through cytosolic nucleic acid ligands resembles a very similar phenotype to RLTPR deficient patient under CMV infection (Figure 3.11). Therefore, it can be concluded that innate immune responses of RLTPR-TLR1 deficient patient were enhanced due to an ongoing infection, still the absence of the TLR1 response can be observed.

CTLA-4, a negative regulator of T-cells, is expressed on activated T-cells and Treg cells. Through binding to CD80/86 ligands on APCs, CTLA-4/CD80 or CD86 complex is trans endocytosed into CTLA-4 carrying cells which removes CD80/86 ligands of CD28 on APCs and prevents T-cell co-stimulation. Without CD28 co-stimulation, T-cell cannot be activated [57]. In case of nonfunctional CTLA-4, enhanced T-cell activation and survival could lead to autoimmunity, repeated infections, lymphocytic infiltration and hypogammaglobulinemia. Patients with insufficient CTLA-4 receptors could have an increased number of Treg cells with reduced function, CTLA-4 expression and CD80/86 trans endocytosis capacity [57, 86, 87]. CTLA-4 is also important for T-cell and B-cell homeostasis. CTLA-4 haploinsufficient patients had hyperactive T-cells along with the enhanced autoreactive B-cells which accumulated non lymphoid organs, although circulating B-cells were decreased [86, 87]. The adaptive immune responses of CTLA-4 mutation

were studied often, the both innate and adaptive immune arms of four CTLA-4 mutated patients were investigated. CD4⁺/CD8⁺ ratio is an indicator of balanced T-cell response. Ratio lower than 1 indicates infections while greater than 2 refers to autoimmunity. Because CTLA-4 mutation can lead to autoimmunity in patients, CD4⁺/CD8⁺ ratios of 4 patients were assessed. Only Patient 1 showed a ratio greater than 2 which implies autoimmunity while Patient 3 had a ratio lower than 1 which indicates an ongoing infection [66]. Patient 2 and Patient 4 showed a normal CD4⁺/CD8⁺ ratio as Healthy 1 (Figure 3.12). Another autoimmunity marker is the presence of proinflammatory low density granulocytes (LDGs) in whole blood. Patient 4 again showed no trace of autoimmunity according to LDGs count which was similar to healthy controls. However, not only Patient 1, but also Patient 2 and Patient 3 had elevated numbers of LDGs as compared to healthy controls (Figure 3.13). Monocytes are the members of innate immune system and 3% of monocytes express CTLA-4 on their surfaces [69]. So, monocyte count of patient and healthy controls were analyzed. Only Patient 3 had three-fold increased monocyte number in whole blood. This finding supports lower CD4⁺/CD8⁺ ratio which indicates an ongoing infection. Other patients showed similar monocyte count to control group (Figure 3.14). The interconnection between innate and adaptive immune system is provided by dendritic cells; therefore, pDC population which express CD86, a CTLA-4 ligand, in whole blood was analyzed by flow cytometer. Patient 1 showed normal pDC percentage in whole blood according to healthy controls while Patient 2, 3 and 4 had lower pDC population (Figure 3.15). Although CTLA-4 is mutated and CD28 can bind un-trans endocytosed CD86 more than normal, reduced pDC count might compensate for enhanced CD28 co-stimulation in Patient 2,3 and 4 if CD86 expression is not elevated. CTLA-4 mutation has an indirect effect on B-cells as increased autoreactive B-cells due to enhanced T-cell activity. Therefore, B-cells from the whole blood was examined in both patient and control groups. All patients had similar percentage of B-cells to healthy controls (Figure 3.16). Besides B-cells, another adaptive immune cells, Tregs, were analyzed by flow cytometer. Although Treg count is increased in other studies, Patient 2,3 and 4 had two fold reduced Treg percentage as compared to control group which might contribute autoimmunity (Figure 3.17). After innate and adaptive immune cell percentages were analyzed, different protein expression levels were investigated in CTLA-4 mutated patients.

CTLA-4 is not the only negative regulator of T-cell survival, PD-L1 is also downregulates T-cell activity. PD-L1 expression levels on CD8⁺ cells, CD4⁺ cells, Treg cells and B-cells of patient and control groups were analyzed to reveal any functional compensation is provided by PD-L1 or not in CTLA-4 mutated patients. Only Patient 4 showed increased levels of PD-L1 on CD8⁺ cells, Treg cells and B-cells as compared to healthy controls (Figure 3.18). High PD-L1 expression might reduce the effect of CTLA-4 mutation in Patient 4 who had normal CD4⁺/CD8⁺ ratio and no LDG population in whole blood as control group. Because CTLA-4 mutation enhances T-cell activation, T-cell receptor (TCR) and CTLA-4 expressions on CD3⁺ T-cells were characterized to see any compensation is provided by reduced TCR expression to minimize the effect of CTLA-4 mutation in patients. All patients showed similar CTLA-4 expression levels to control group while Patient 1 and Patient 4 had reduced TCR expression on their CD3⁺ T-cells (Figure 3.19). Besides enhanced PD-L1 levels, impaired TCR expression may reduce the autoimmune effect of the CTLA-4 mutation in Patient 4. Also, reduced TCR level in Patient 1 might answer the ongoing infection. After surface proteins were investigated, intracellular proteins were also analyzed (Figure 3.20). Interestingly, mTOR and pmTOR levels of patients could not be detected in any patients, and ERK expression levels of patients were similar to healthy controls. Patient 1 and Patient 4 had elevated levels of pAKT as compared to control group. Besides pAKT, Patient 4 had increased p4EBP1 levels which would contribute to compensation of effects of CTLA-4 mutation in Patient 4. However, STAT3, AKT, p4EBP1 and HIF-1 α , which enhances CTLA-4 production, expressions were impaired in all patients as compared to healthy controls. After STAT3 levels of patient were analyzed, the phosphorylation level of STAT3 was investigated after PMA/Ionomycin treatment because functional CTLA-4 facilitates STAT3 phosphorylation. All patients had impaired pSTAT3 levels as expected due to their CTLA-4 mutation which interrupts STAT3 phosphorylation (Figure 3.21).

Immune system alterations in probable mTOR/NF κ B mutation is another subject that was investigated in this thesis. CD4⁺/CD8⁺ ratio of the patient was slightly lower than normal range, but still it was not low enough to indicate presence of any infection (Figure 3.22). Patient had high amount of LDGs in whole blood along with the two-fold increased number of monocytes which can refer autoimmunity (Figure 3.23 and Figure 3.24). Even though pDC percentage was normal in patient, increased Treg cell

population and ten-fold reduced B-cell percentage from whole blood implies impaired adaptive immune system in patient (Figure 3.25, Figure 3.26 and Figure 3.27). After effect of mutation on immune cells were analyzed, innate immune responses of patient were investigated. Surface TLRs mediated proinflammatory IL-6, TNF α and antiviral IFN γ responses were enhanced while STING, AIM2 inflammasome and RIG-I/MAVS mediated proinflammatory and antiviral responses were reduced in patient which could imply increased TLR responses might be caused from impaired cytosolic nucleic acid activity for compensation (Figure 3.28). Besides innate immune response, adaptive immune response of the patient was investigated. After LPS stimulation, pmTOR levels in CD4 $^{+}$ and CD8 $^{+}$ T-cells were higher three-fold in patient which might suggest autoimmunity through exaggerated T-cell response (Figure 3.29). Although B-cell percentage of patient was reduced, basal TNF- α expression from B-cells was higher in patient along with the increased TNF α expression from CD4 $^{+}$ T-cells upon LPS stimulation (Figure 3.30) which supports autoimmune phenotype in the probable mTOR/NF κ B mutation.

In conclusion, our data refer that the complexity of the dysregulation of these deficiencies, and indicate an unappreciated immune functional status of these patients. We affirm that investigation of the innate immune system of these individuals which were perceived as regard to and effecting only adaptive immune system is required for providing more robust therapies to these patients.

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