

INVESTIGATION OF IMMUNE IMPAIRMENT STATUS IN PRIMARY IMMUNE DEFICIENCY AND SPINAL CORD INJURY

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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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ABSTRACT

Investigation of Immune Impairment Status in Primary Immune Deficiency and Spinal Cord Injury

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Primary Immunodeficiencies (PIDs) are raised by immune impairment causing disorders. In the patients carrying deficiency in any part of immune system, various defects and symptoms are observed. Generally, abnormality against microbial agents are seen in patients and different therapies are applied depending on defect. That's why it is crucial to characterize how immune system works in PIDs for better understating of disease and treatment options. In this study, we investigated two different mutations (CD27 and STAT1 GOF) and their effects on innate and adaptive arm of immune system.

A trans-membrane receptor, CD27, is a one of the leukocyte differentiation markers for T, B and NK cells. It is involved in the activation of these cells by binding to the co-stimulatory molecule CD70 and mediates cell survival. In CD27 deficiency, loss of T cell dependent B cell responses and antibody production along with T cell dysfunction are mainly seen. Herein, immunological phenotypes of two patients with genetic CD27 deficiency were described. CD27 expression of the PBMCs, CD3⁺ T and CD19⁺ B cells obtained from the patients were absent in patients. Frequency of CD19⁺/CD24^{hi}/CD38^{hi} transitional B cells were higher compared to healthy controls. Reversed CD4⁺/CD8⁺ T cell ratio was observed in one patient. After IFN- β stimulation, the phosphorylation level of STAT1 in CD4⁺ T cells was increased in CD27^{-/-} patients as compared to healthy group. There was no difference in phospho-STAT3 levels between CD27^{-/-} and healthy donor CD4⁺ T cells upon IL-6 stimulation. However, decreased STAT5 phosphorylation levels were observed upon IL-2 stimulation of CD4⁺ T cells of CD27^{-/-}. When PBMCs from healthy and CD27 deficient patients were stimulated with PMA/Ionomycin and analyzed for cytokine secretions, increased IFN- γ , IL-4 and IL-17a secretion were investigated. Through activation of different TLR and inflammasome pathways, normal IFN- γ and IL-1 β responses were observed while increased TNF- α and IL-10 was present in patients. Results suggest that patients' both humoral and cellular immune responses could be impaired in CD27 deficiency.

STAT1 is a member of the STAT protein family whose mutations can cause gain-of-function (GOF) or loss of function (LOF). Recurrent widespread infections are common in both mutations. In this study, the responses of innate and adaptive immune systems of 6 patients from different hospitals known to have STAT1 GOF were examined. To determine the STAT1 phosphorylation and dephosphorylation levels, cells were treated with IFN- β and CD4⁺ T cell phosphorylation levels were analyzed by flow cytometry. Th1 and Th17 responses were examined since imbalanced Th1 and Th17 response is known in STAT1 GOF patients. To identify defects in innate immunity, cytokine ELISA was performed through different PRR ligands. For neutrophil activities, cells were examined as untreated and treated form by microscopically and fluorometrically. As a result, it was found that p-STAT1 levels and p-STAT1 positive CD4⁺ T cells of all patients were higher than healthy controls which approves STAT1 GOF mutation in patients. Although the STAT1 phosphorylation level was high, it was determined that IFN- γ released due to Th1 response was low in patients. Th17 response were low in patients which is supported by current infection of Chronic Mucocutaneous Candidiasis. Type II interferon responses of the patients to the ligands of the endosomal and inflammatory pathways were lower than healthy controls while normal IL-12 secretion was observed. Some patients had increased NETotic activities at the basal levels supported by spontaneous NET formation, while some patients were not able to induce NETosis through PMA stimulation. Impaired immune status was observed in STAT1 GOF patients as well.

Spinal cord injury (SCI) is damage occurring in the spinal cord and nerves within the spinal canal that causes completely or partially loss of sensation around injury site. Since uncontrolled immune response occurs at the post-traumatic injury site in SCI patients and this situation increases the damage caused by the lesion, it is important to examine the immune system responses in these patients. In this study, we tried to determine how innate and adaptive immune system responses of SCI patients with and without infection differ. Percentages of T, B, NK cells and pDCs were determined from whole blood. Reversed CD4/CD8 ratio was observed in two patients. Increased Treg and pDCs cell percentage was seen in patients while B and NK cell percentages are different from patient to patient. Released IFN- γ , IL-17 and IL-10 levels were determined by flow cytometry upon PMA/Ionomycin. Normal Th17, increased Th1 and Treg was investigated in SCI patients. NETotic activities were examined microscopically and fluorometrically and via ROS production. Although NETotic activities of neutrophils of all patients were similar in healthy subjects, fluorometric results showed that some patients had more NETotic activities compared to healthy controls. It was also found that patients produced lower amounts of ROS as a result of incubation with PMA. These results suggest that SCI patients become more susceptible to infection due to the low response to the pathogen despite increased neutrophil activity and the immunosuppressive effect of the amount of IL-10 caused by excess Treg activity.

Keywords: CD27, STAT1 GOF, Spinal Cord Injury, Primary Immune Deficiency, Innate and Adaptive Immunity



ÖZET

Birincil Bağışıklık Yetersizliği ve Omurilik Yaralanmasında Bağışıklık Bozukluğu Durumunun Araştırılması

Birincil İmmün Yetmezlikler (PID'ler), bağışıklık sisteminin herhangi bir kısmında sorunlara neden olan kalıtsal bozukluklardan kaynaklanır. Mutasyonlara bağlı olarak hastalarda birbirinde farklı belirtiler görülebilir. Genellikle hastalarda mikrobiyal ajanlara karşı cevaplarda anormallik görülürken, immün sistemde olan defekte göre her hastaya farklı tedaviler uygulanmaktadır. Bu nedenle, hastalığı ve tedavi seçeneklerini daha iyi anlamak için bağışıklık sisteminin PID'lerde nasıl çalıştığını karakterize etmek çok önemlidir. Bu çalışmada, iki farklı mutasyonu (CD27 ve STAT1 GOF) ve bunların bağışıklık sisteminin doğuştan ve adaptif kolu üzerindeki etkilerini araştırdık.

Bir trans-membran reseptörü olan CD27, T, B ve NK hücreleri için lökosit farklılaşma belirteçlerinden biridir. Ortak uyarıcı molekül CD70'e bağlanarak bu hücrelerin aktivasyonunda yer alır ve hücre hayatta kalmasına aracılık eder. CD27 eksikliğinde, esas olarak T hücresine bağlı B hücresi yanıtlarının kaybı ve T hücre disfonksiyonu ile birlikte antikor üretimi görülür. Burada, genetik CD27 eksikliği olan iki hastanın immünolojik fenotipleri tanımlanmıştır. Hastalardan elde edilen PBMC'lerin, CD3⁺T ve CD19⁺B hücrelerinin CD27 ekspresyonu yoktu. CD19⁺/CD24^{hi} / CD38^{hi} geçiş B hücrelerinin sıklığı, sağlıklı hücrelere göre daha yüksekti. Bir hastada ters CD4⁺/CD8⁺T hücre oranı gözlemlendi. IFN- β uyarımından sonra, CD4⁺T hücrelerinde STAT1'in fosforilasyon seviyesi, sağlıklı gruba göre CD27^{-/-} hastalarda artmıştır. IL-6 uyarımı üzerine CD27^{-/-} ve sağlıklı donör CD4⁺T hücreleri arasında fosfo-STAT3 seviyelerinde bir fark yoktu. Bununla birlikte, CD27^{-/-}'nin CD4⁺T hücrelerinin IL-2 stimülasyonu üzerine azalmış STAT5 fosforilasyon seviyeleri gözlemlendi. Sağlıklı ve CD27 eksikliği olan hastalardan alınan PBMC'ler PMA / İonomisin ile uyarıldığında ve sitokin salınımları açısından analiz edildiğinde, artmış IFN- γ , IL-4 ve IL-17a salımı gözlemlendi. Farklı TLR ve enflamasyon yollarının aktivasyonu yoluyla, hastalarda normal IFN- γ ve IL-1 β tepkileri gözlenirken, artmış TNF- α ve IL-10 mevcuttu. Sonuçlar, hastaların hem humoral hem de hücrel immün tepkilerinin CD27 eksikliğinde bozulabileceğini göstermektedir.

STAT1, mutasyonları işlev kazanımı (GOF) veya işlev kaybına (LOF) neden olabilen STAT protein ailesinin bir üyesidir. Her iki mutasyonda da tekrarlayan enfeksiyonlar yaygındır. Bu çalışmada STAT1 GOF olduğu bilinen farklı hastanelerden 6 hastanın doğuştan ve adaptif bağışıklık sistemlerinin yanıtları incelenmiştir. Hücrelerin STAT1 fosforilasyon ve defosforilasyon seviyelerini

belirlemek için, IFN- β tedavisi ile CD4⁺T hücresi fosforilasyon seviyeleri akış sitometrisi ile analiz edildi. STAT1 GOF hastalarında dengesiz Th1 ve Th17 yanıtı bilindiği için Th1 ve Th17 yanıtları incelendi. Doğuştan gelen bağışıklıktaki kusurları belirlemek için sitokin ELISA, farklı PRR ligandları aracılığıyla gerçekleştirildi. Nötrofil aktiviteleri için hücreler uyarılmış ve uyarılmamış olarak mikroskopik ve florometrik yöntemlerle incelendi. Sonuç olarak, tüm hastaların p-STAT1 düzeyleri ve p-STAT1 pozitif CD4⁺T hücrelerinin, hastalarda STAT1 GOF mutasyonunu onaylayan sağlıklı kontrollere göre daha yüksek olduğu bulundu. STAT1 fosforilasyon seviyesi yüksek olmasına rağmen hastalarda Th1 cevabına bağlı olarak salınan IFN- γ 'nın düşük olduğu belirlendi. Mevcut Kronik Mukokutanöz Kandidiyaz enfeksiyonu ile desteklenen hastalarda Th17 yanıtı da düşüktü. Hastaların endozomal ve enflamatuvar yolların ligandlarına tip II interferon tepkileri sağlıklı kontrollere göre daha düşük iken normal IL-12 sekresyonu gözlemlendi. Bazı hastalarda bazal düzeyde artmış NETotik aktiviteler, spontane NET oluşumu görülürken bazıları PMA stimülasyonu yoluyla NETozu indükleyemedi. STAT1 GOF hastalarında da bozulmuş bağışıklık durumu gözlemlendi.

Omurilik yaralanması (SCI), omurilikte ve omurilik kanalındaki sinirlerde meydana gelen ve yaralanma bölgesinde tamamen veya kısmen his kaybına neden olan hasardır. SCI hastalarında travma sonrası yaralanma bölgesinde kontrolsüz immün yanıt oluştuğu ve bu durum lezyonun neden olduğu hasarı artırdığı için bu hastalarda immün sistem yanıtlarının incelenmesi önemlidir. Bu çalışmada, enfeksiyonlu ve enfeksiyonsuz SCI hastalarının doğuştan gelen ve uyarlanabilir bağışıklık sistemi yanıtlarının ne kadar farklı olduğunu belirlemeye çalıştık. T, B, NK hücreleri ve pDC'lerin yüzdeleri tam kandan belirlendi. İki hastada ters CD4 / CD8 oranı gözlemlendi. Hastalarda artmış Treg ve pDCs hücre yüzdesi görülürken, B ve NK hücre yüzdeleri hastadan hastaya farklı olduğu belirlendi. Salınan IFN- γ , IL-17 ve IL-10 seviyeleri, PMA / İonomisin üzerinde akış sitometrisi ile belirlendi. SCI hastalarında normal Th17, artmış Th1 ve Treg yanıtlarının olduğu belirlendi. NETotik aktiviteleri mikroskopik ve florometrik olarak ve ROS üretimi yoluyla incelendi. Sağlıklı deneklerde tüm hastaların nötrofillerinin NETotik aktiviteleri benzer olmasına rağmen, florometrik sonuçlar bazı hastaların sağlıklı kontrollerden daha fazla NETotik aktiviteye bazal seviyede sahip olduğunu gösterdi. Ayrıca hastaların PMA ile inkübasyon sonucunda daha düşük miktarlarda ROS ürettikleri de bulundu. Bu sonuçlar, SCI hastalarının, artan nötrofil aktivitesine ve aşırı Treg aktivitesinin neden olduğu IL-10 miktarının immünosupresif etkisine rağmen patojene düşük yanıt nedeniyle enfeksiyona daha duyarlı hale geldiğini göstermektedir.

Anahtar Kelimeler: CD27, STAT1 GOF, Omurilik Yaralanması, Birincil İmmün Yetmezlik, Doğuştan ve Adaptif Bağışıklık

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DEDICATION

To my precious family...

(written in blood and tears)



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ABBREVIATIONS

ALRs	AIM2-like receptors
AIM2	Absent in Melanoma 2
APC	Antigen Presenting Cells
ATP	Adenosine Triphosphate
BCRs	B-cell Receptors
CMC	Chronic Mucocutaneous Candidiasis
DAMPs	Damage Associated Molecular Patterns
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
FBS	Fetal Bovine Serum
IKK	I κ B kinase
IP-10	IFN- γ Inducible Protein-10
IRAK	IL-1 receptor associated kinase
IRFs	Interferon Regulatory Factors
ISG	Interferon Stimulated Genes
LBP	Lipopolysaccharide Binding Protein
LPG2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
LRRs	Leucine Rich Repeats
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial Antiviral Signaling Protein
MD-2	Myeloid Differentiation protein-2
MDA5	Melanoma differentiation associated 5
MFI	Mean Fluorescent Intensity
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene 88
NE	Neutrophil elastase
NETosis	NET formation

NETs	Neutrophil Extracellular Traps
NFκB	Nuclear Factor Kappa B
NK cells	Natural Killer Cells
NLRs	NOD like receptors
NOD	Nucleotide Oligomerization Domain
ODNs	Oligodeoxynucleotides
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
pDCs	Plasmacytoid Dendritic Cells
PKC	Protein Kinase C
poly (dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
poly (I:C)	Polyinosinic-Polycytidylic acid
PIDs	Primer Immune Deficiencies
PRRs	Pathogen Recognition Receptors
RIG-I	Retinoic Acid Inducible Gene I
RLRs	RIG Like Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCI	Spinal Cord Injury
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
TCR	T-cell Receptor
Th	T-helper
TLRs	Toll Like Receptors
TNF	Tumor Necrosis Factor
TIR	Toll/IL-1 receptor
TRAF	TNF receptor associated factor
Treg	Regulatory T cells
TRIF	TIR domain containing adapter inducing IFNβ

CHAPTER 1

INTRODUCTION

1.1 Immune System

All species have been exposed to pathogens that forms treat for host cell during evolution. Depending on this, species develop survival mechanisms by evolutionary criteria. That's why special and complicate defense network named as immune system had been evolved. Immune system become more complicated in the sense of mechanism, as going from invertebrates to mammals depending on evolution. While invertebrates have the simplest version of immune mechanism, mammals have the most complicated and unique mechanism classified as innate and adaptive immunity and composed of receptors, special cells and various layers and different stages of barriers [1].

The first line of protection for entire immune system is known as innate immunity consisting of complement system, physical barriers and innate immune cells. Invading pathogens first need to move across physical barriers (skin, mucosa), then complement system to efficiently penetrate. If they cannot be recognized and eliminated through first layer, innate immune cells resident in tissue cause rapid response. Basic and conserved patterns present in pathogen can be detected by germline encoded receptors within immune cells. Although innate immune cells were accepted as without memory, evidences showed that antigen presenting cells (APCs) from innate immunity are key for crosslink between adaptive immunity. While quick and antigen independent immunity is observed in innate immunity, adaptive immunity requires antigen specific slow response. Adaptive immunity can be divided into two arms: cell mediated immunity achieved by T-cell and humoral immunity formed by B cells. T cells are classified with regard to T cell receptors (TCRs) into $CD8^+$ T and $CD4^+$ T cells. Efficient immune response against pathogens is generally generated by $CD8^+$ T (cytotoxic cells) cells while $CD4^+$ T cells (helper cells) are responsible for balance of immunity. Depending on pathogen, $CD4^+$ T cells are diversified into subtypes (Th1, Th2, Th17 and Treg) and help immune cells

for efficient immune response. B cells containing B cell receptors (BCRs) is also important for immune response since depending on activation with pathogen or CD4⁺ T cells, they are able to transform into plasma cells that produces immunoglobulins [2,3].

1.1.1 Innate Immunity

The innate immune system is able to identify and eliminate various invading pathogens within short time before any damage is caused. In order to avoid entry of pathogens, mucosal and epithelial barriers prevent invasion of pathogen prior to innate immune response. In the first line of protection, antimicrobial protein and peptides are secreted by phagocytes as well as epithelial cells to cause damage in membranes or cell walls of fungi and bacteria [4]. Second layer of innate immunity is complementary system consisting of plasma proteins. Through complement proteins, invading pathogens are labelled to phagocytosed and lysed by immune cells [5]. Even though, these chemical and physical barriers are effective against many pathogens, still some of them can avoid these mechanisms of defense. In this instance, pattern recognition receptors (PRRs) located in immune cells take role for recognition of pathogen associated molecular patterns (PAMPs) found on invading pathogen and danger- associated molecular patterns (DAMPs) derived from damage of cells. Recognition of PAMPs and DAMPs by PRRs can lead to directly killing of pathogen. In addition to direct destruction, PRRs also triggers secretion of cytokines, chemokines and stimulatory molecules to induce adaptive immune response [6].

1.1.1.1 Pattern Recognition Receptor (PRRs)

The importance of Toll like receptors was firstly came across with the essential role for *Drosophila* dorsal-ventral pattern development [7]. Then it was identified that aside from dorsal-ventral pattern development, Toll like receptors in *Drosophila* was important for antifungal responses [8]. From then, the role of Toll like receptors in immunity was mainly studied in both mammals and insects. Mammalian homolog of Toll like receptor from *Drosophila* was firstly discovered and termed as TLR4 [9], then following studies revealed structurally related receptors currently known as TLRs. Other than TLRs, several distinct PRRs are present in mammals

comprising intracellular DNA sensors, AIM-2 like receptor (ALRs), RIG-I like receptors (RLRs) and Nod like receptors (NLRs) [10].

1.1.1.1.1 Toll Like Receptors (TLRs)

Activation of innate immune system by pathogens are mediated through recognition of pathogen associated molecular patterns (PAMPs) with Toll like receptors. Pattern recognition by TLRs induces innate immune cell activation, afterwards adaptive immunity. Signaling of TLRs are mediated either with MyD88 dependent or TRIF-dependent.

Currently, 13 TLR types are known in mammals where humans only have ten of them (TLR1-10) but mice have twelve of them (TLR1-10,11-13). Recognition pattern of each TLRs are different from each other and they can recognize PAMPs from various pathogens like parasite, virus, bacteria and fungi [11].

Each TLR consists three structural parts: ectodomain (containing leucine rich repeats LRR), transmembrane domain and cytoplasmic Toll/Il-1 receptor (TIR) domain. Recognition of DAMPs through TLR is initiated by interaction with ectodomain to pathogen structure. Then, TIR domain containing adaptors are recruited to TLRs and signaling pathways like IRFs (Interferon Regulatory Factors) and NFκB (Nuclear Factor κ B) is activated for interferon, chemokine and cytokine secretion [10].

1.1.1.1.2 Cellular Localization of TLRs

Toll like receptors are localized either in cell surface or within intracellular vesicles depending on which pattern they recognize. Generally, cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) are able to recognize membrane components of microbes whereas intracellular vesicle resident TLRs (TLR3, TLR7, TLR8, TLR9) can identify nucleic acid that are obtained from uptake of pathogen by cells [12].

1.1.1.1.3 Cell Surface TLRs

1.1.1.1.3.1 TLR1, TLR2 and TLR6

Diverse pathogens (virus, bacteria, fungi, parasite) can be recognized by TLR2 according as its ability to interact with other TLRs. TLR2 heterodimerizes with TLR1 or TLR6 for molecular pattern recognition. TLR1-2 can recognize triacyl-lipopeptides coming from Gram negative bacteria whereas TLR1-2 is able to recognize diacyl-lipopeptides from mycoplasma [13]. Aside from immune cells, non-immune cells like endothelial, epithelial cells and fibroblasts express TLR2 in their cell surface [14].

1.1.1.1.3.2 TLR4

Gram negative bacteria outer membrane, LPS (lipopolysaccharide), is major pathogen associated molecular pattern recognized by TLR4. LPS recognition by TLR4 requires myeloid differentiation protein-2 (MD2), CD14 and LPS-binding protein (LBP) involvement. LPS first binds LBP, then LBP interacts with LRRs (Leucine Rich Repeat) containing CD14 and LPS-LBP-CD14 is delivered to TLR4-MD2. Through LPS binding, two signaling pathways Myd88 dependent and TRIF dependent are activated [15].

1.1.1.1.3.3 TLR5

Bacterial flagella component, flagellin, is recognized by TLR5 found on dendritic cells and macrophages. TLR5 expressing cells are generally found in body parts prone to bacteria invasion and infection like digestive-respiratory-urinary tract [14]. Activation of TLR5 induces epithelial cells for chemokine secretion and leukocyte invasion to infection site.

1.1.1.1.4 Endosomal TLRs

1.1.1.1.4.1 TLR3

Recognition of viral dsRNA is achieved by TLR3. Immune cells including NK cells, B cells, DCs and macrophages and non-immune cells like epithelial cells, endothelial cells and fibroblast express TLR3 [16,17].

Other than dsRNA, TLR3 is able to recognize ssRNA viruses like West Nile and dsDNA viruses like HSV during dsRNA transcription [18].

1.1.1.1.4.2 TLR7/8

Viral ssRNA is detected with endosomal TLR7 and TLR8 receptors. Through receptor mediated endocytosis, viral entry into cell is occurred and virus reaches to phagolysosomes. In phagolysosomes viral coat protein is hydrolyzed due to acidic environment and disruptive enzymes and exposed viral RNA is recognized with TLRs. Since RNase activity in plasma, ssRNA from host cell cannot be recognized and entered into phagolysosomes [19]. Expression of TLR7 is seen in pDCs and B cells while TLR8 is expressed in macrophages, neutrophils and DCs [20, 21].

1.1.1.1.4.3 TLR9

Bacterial dsDNA is recognized by TLR9 depending on its abundant unmethylated CpG motif. TLR9 ligand involvement initiates MyD88 dependent signaling pathway that contributes to type I IFN production by pDCs and B cell fate. Synthetic oligonucleotides (ODNs) have been identified as TLR agonist. In 90s, Krieg and Klinman showed that oligonucleotide flanking between purine and pyrimidines and containing unmethylated CpG dinucleotide can be able to induce cytokine secretion from immune cells. Three distinct type of ODNs are known currently: K-type, D-type and mixed type. Each type has unique recognition patterns and cytokine production. D-ODN type consists of phosphonothioate and phosphodiester backbone, single CpG motif and poly-G tail in both ends. Recognition of D-ODN occurs in early endosomes and leads to induction of type I IFN from pDCs. K-type consists of only phosphonothioate backbone and several CpG motifs. Recognition of K-ODN type occurs in late endosomes and leads to induction of proinflammatory cytokine from B cells and TNF- α from pDCs. Mixed ODNs consists of only

phosphonothioate backbone and leads to both B cell and pDC stimulation [22,23,24,25].

TLR	Localization	Pathogen-PAMP	Induced Signaling Pathway	Agonist
TLR1-2	Cell surface	Bacteria- triacylipopeptide	MyD88	PGN
TLR2-6	Cell surface	Mycoplasma- diacylipopeptide	MyD88	Zymosan, β -glucan
TLR3	Endosome	Virus- dsRNA	TRIF	p(I:C), polyU
TLR4	Cell surface	Bacteria- lipopolysaccharide	MyD88 and TRIF	LPS
TLR5	Cell surface	Bacteria-flagellin	MyD88	
TLR7/8	Endosome	Virus-ssRNA	MyD88	R848 Imiquimod Resiquimod
TLR9	Endosome	Virus, bacteria, parasite- dsDNA	MyD88	CpG ODN

Table 1.1 Mammalian TLR receptors, their localizations, recognized patterns from pathogen and activated signaling pathways

1.1.1.1.5 TLR Signaling Pathway

1.1.1.1.5.1 MyD88 Dependent Pathway

In mammals, activation of all TLRs except TLR3 activates signaling pathways through recruitment of adaptor protein MyD88. Other than TLR1-2-6, TLR4 and TLR5, MyD88 is directly recruited to TLRs. Recruitment of MyD88 to TLR1-2-6, TLR4 and TLR5 requires adaptor protein known as MAL [26]. After recruitment of MyD88, IL-1 receptor associated kinase 4 (IRAK4) and IRAK1 are recruited via death domains of MyD88 for scaffold protein formation. Tumor necrosis factor (TNF) receptor associated factor (TRAF6) is recruited to scaffold complex work as E3 ligase that is activated through phosphorylation. Activation of TRAF6 leads to polyubiquitin scaffold establishment depending on ubiquitin ligases and recruits transforming growth factor β activated kinase 1 (TAK1) and binding proteins (TAB1/2). Activation of TAK1 occurs with phosphorylation of it by IRAK1. Signaling pathway from this point splits into two pathways. From one source, degradation of I κ B by IKK complex phosphorylation starting with TAK1 activation results in entering of NF κ B into nucleus to induce pro-inflammatory cytokine production. From other hand, mitogen activated protein kinase (MAPK) is activated through TAK1 for cytokine secretion [27]. Type I interferon secretion from TLR7 and TLR9 activation requires recruitment of interferon regulatory factor 7 (IRF7) by MyD88 and IRAK1. Direct recruitment results in phosphorylation subsequently dimerization of IRF7 into nucleus to produce IFN- α [14].

1.1.1.1.5.2 TRIF Dependent Pathway

TRIF dependent pathway works different from MyD88 dependent pathway. Together ligand association with TLR3 or TLR4, receptor dimerization and recruitment of TRIF proteins happen. Then, TRAF3 (E3 ligase) is recruited to TLR-TRIF complex for polyubiquitin complex formation. Activation of TRAF-family member associated NF κ B activator (TANK) leads to activation of Tank binding kinase protein 1 (TBK1) which causes phosphorylation of IRF3. Type I interferon secretion happens depending on phosphorylation, dimerization and entrance of IRF3 to nucleus [28].

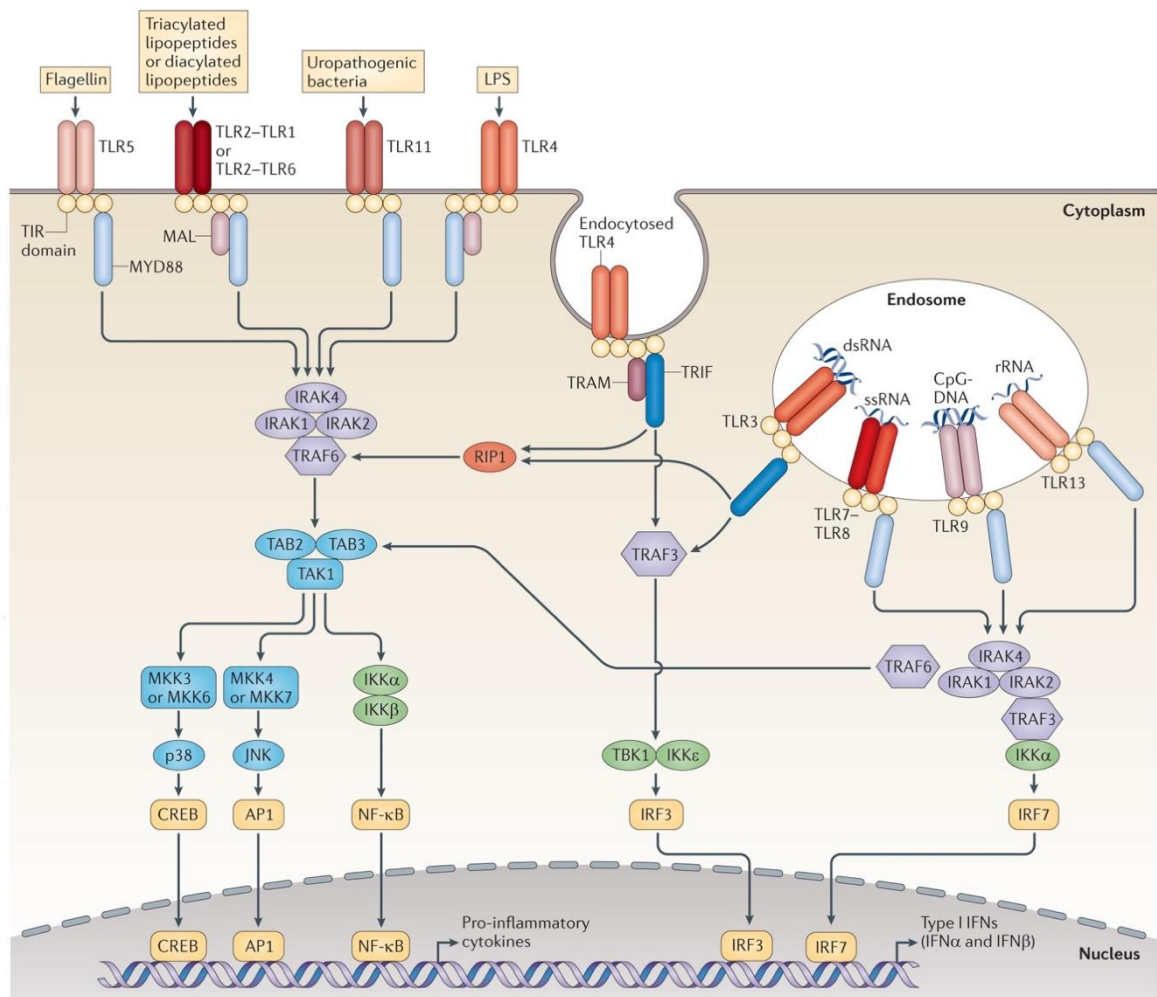


Figure 1.1 Detailed mammalian TLR signaling pathway adopted from [29]

1.1.1.2 Cytosolic Nucleic Acid Sensors

Nucleic acids coming from pathogens during infection are detected with cytosolic nucleic acid sensors including RIG-I like receptors (RLRs), AIM2 like receptors (ALRs) and DNA dependent activators for interferon regulation. Detection of RNAs is performed by RLRs whereas ALRs and DNA dependent activators are evolved to detect DNA [30].

1.1.1.2.1 RIG-I like Receptors

Non-self RNA recognition in cytosol is actualized with RIG-I, LPG2 (laboratory of genetics and physiology 2) and MDA-5 (Melanoma differentiation associated 5). Recognition of pathogen ssRNA by RIG-I depends on 5' end of host ssRNA since differentiation requires sensitization of modifications at that end. dsRNA from pathogen is recognize through MDA5 whom also activated by p(I:C) transfection. Recognition of pathogen by MDA5 requires sensing AT rich DNA sequence. Both MDA5 and RIG-I is activated upon pathogen RNA recognition and interact with

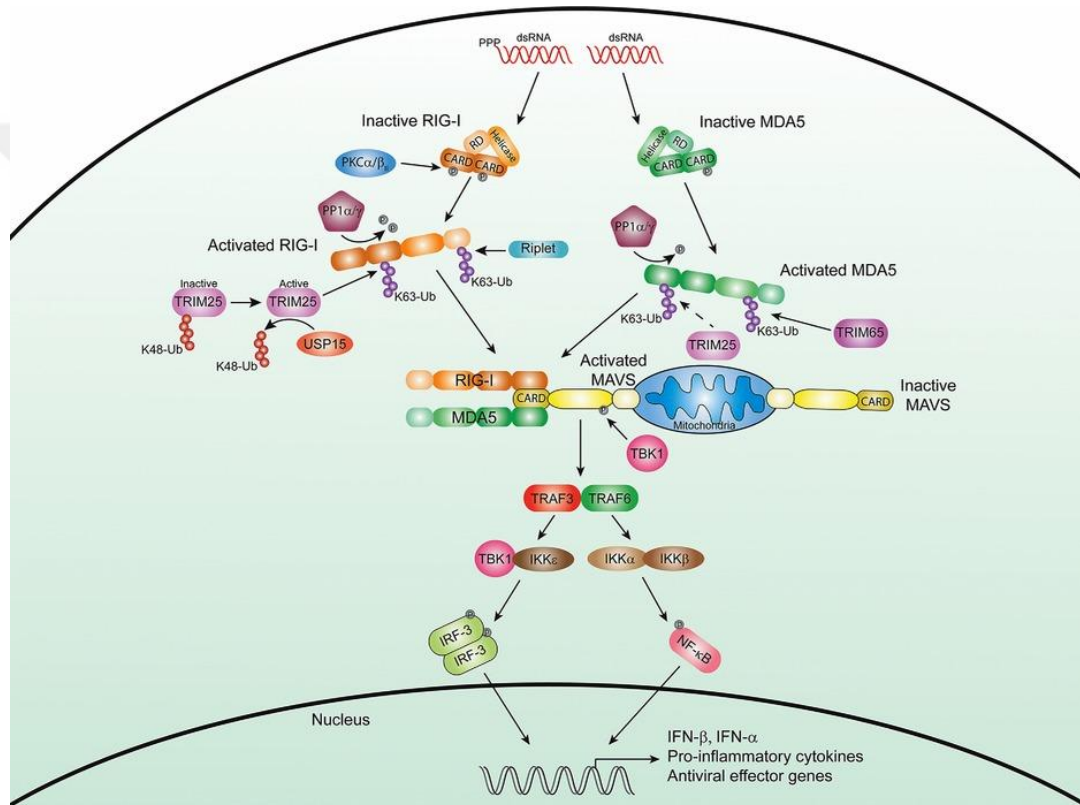


Figure 1.2 RIG-I and MDA5 dependent signaling pathways adopted from [33]

mitochondrial antiviral signaling protein (MAVS) to recruit TRAF for production of type I IFN and proinflammatory cytokines through IRF3 and NFκb activation [31,32].

1.1.1.2.2 Activation of Interferon Regulatory Genes by DNA Dependent Activator

Presence of DNA in cytosol is the danger signal for cells since eukaryotic DNA is captive in nucleus. Recognition of DNA in cytosol is performed with DNA sensors AIM2, IFI16, cGAS and DAI. DNA from pathogen is recognize through cGAS-STING whom also activated by p(dA:dT), HSV and 2'3 cGAMP transfection. cGAS (cGAMP synthase) binding to cytosolic DNA initiates formation of cGAMP (secondary messenger) from cytosolic GTP and ATP. Secondary messenger then is recognized with STING (stimulator of IFN genes) that results in dimerization of STING proteins and recruitment of TBK1 that phosphorylates IRF3. Phosphorylation of IRF3 leads to entrance to nucleus and production of type I interferon while activation of NFκB and secretion of pro-inflammatory cytokines is initiated with STING homodimers [34].

All cytosolic DNA sensors except for AIM2 requires signaling adaptor STING resident in endoplasmic reticulum. Rather than type I IFN secretion, AIM2 causes inflammation formation and IL-1β together with IL-18 secretion [35].

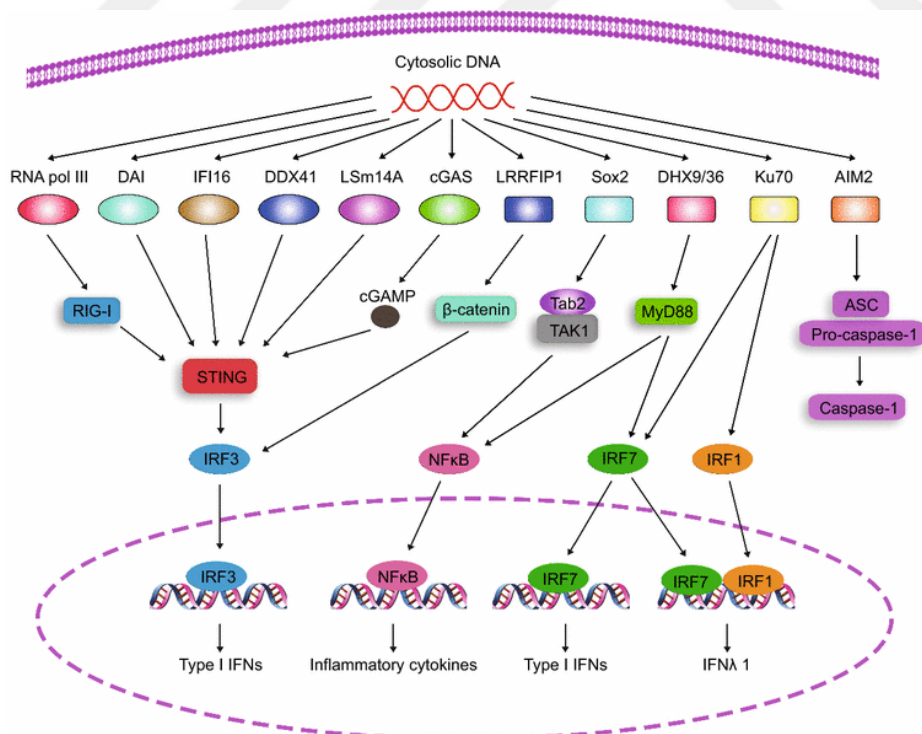


Figure 1.3 Cytosolic DNA recognition with cytosolic acid sensors adopted from [36]

1.1.1.3 Neutrophils and Neutrophil Extracellular Traps (NETs)

In peripheral blood, neutrophils produced from bone marrow are the mostly present immune cells that are the first protection barrier and firstly coming cells to in inflammation sites. Even though in inflammatory situations they can survive for 7 days, neutrophils have short time life span ranging from 8 hours to 12 hours. Protection mechanisms for neutrophils are mainly divided into three: forming meshwork of DNA known as neutrophil extracellular traps (NETs) into microenvironment, phagocytosis of invading pathogens into phagosomes with reaction oxygen species and antimicrobial protein degranulation.

Through one way of programmed cell death, NETosis, pathogens are neutralized and killed by neutrophils. Signaling mechanism behind formation of NETosis has not been completely defined. NETosis can be divided into 2 separates signaling as NETosis and non-lytic NETosis. In NETosis, through pathogen recognition, nuclear envelop is disassembled, membrane is ruptured and NETs are released into environment. In non-lytic NETosis live cells directly degranulate and expulse nuclear chromatin into environment [37].

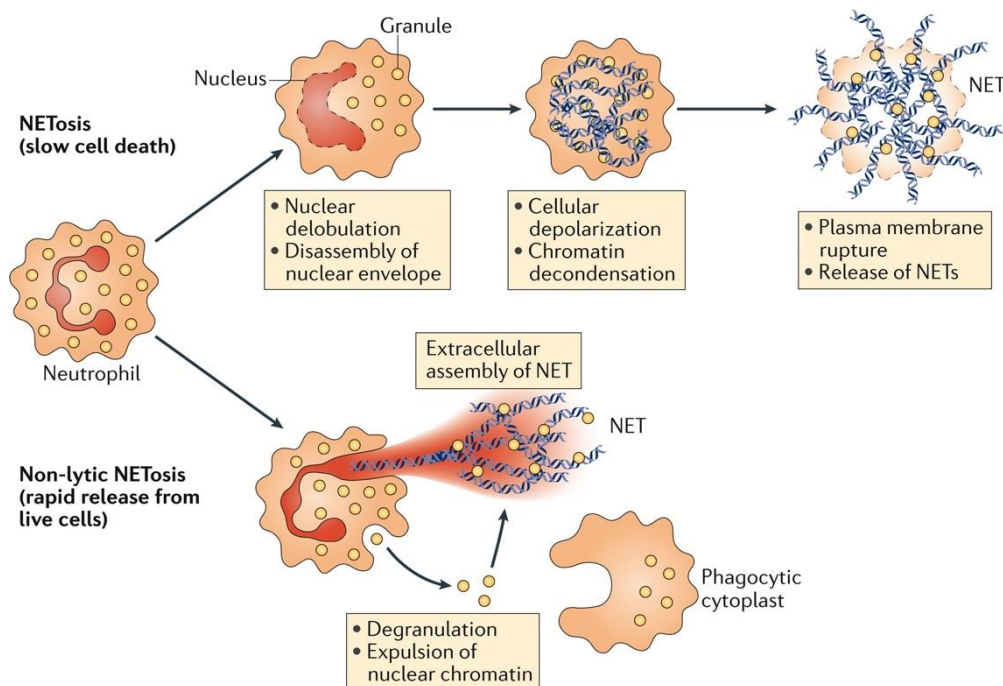


Figure 1.4 Types of NETosis adopted from [37]

Change in Ca^{+2} levels within environment through either sterile or microbial stimuli recognition by receptors leads to activation of Protein Kinase C (PKC) and formation of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase. With ROS (Reactive Oxygen Species) formation azurophilic granules containing proteins of NE (Neutrophil elastase) and MPO (Myeloperoxidase) are activated and inside of granules are released into cytoplasm. Histone modifications decondense chromatin and granules release DNA [38,39].

Even if net formation is important for elimination of invading pathogens, excessive formation of dsDNA into environment from neutrophils could cause inflammation, allergy, tissue damage and autoimmunity [40].

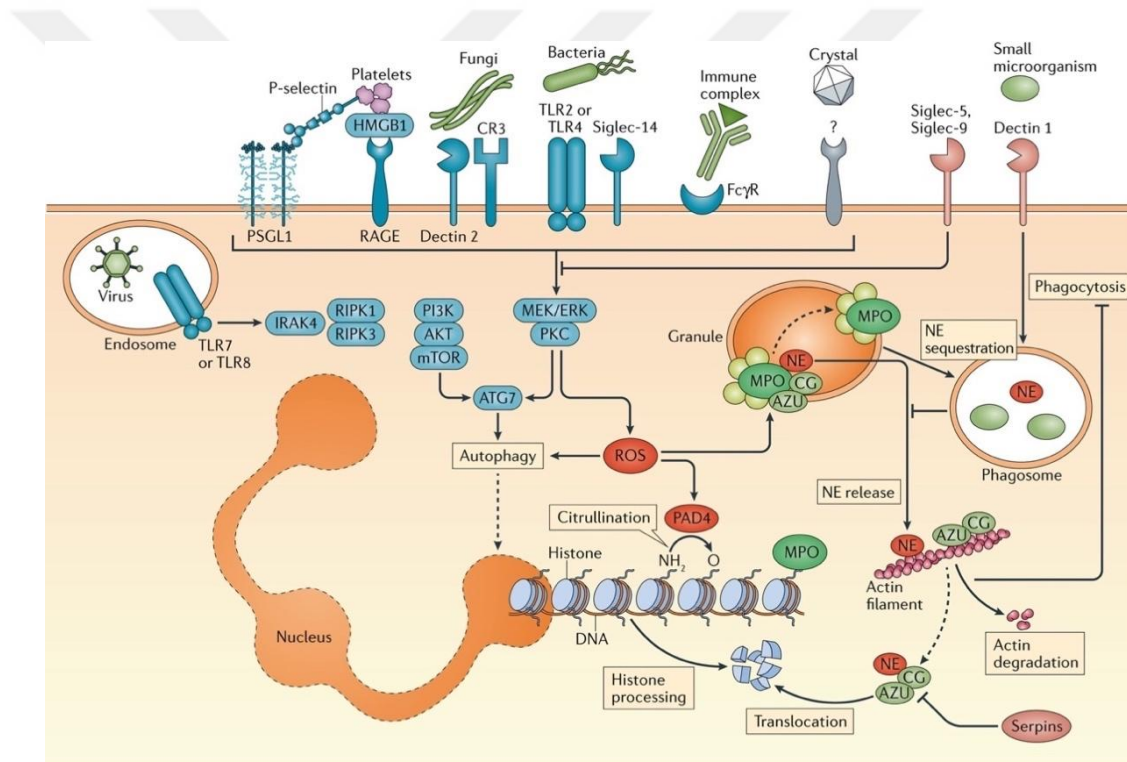


Figure 1.5 Signaling pathway of NETosis adopted from [37]

1.1.2 Immunodeficiency

In healthy immune response, innate immunity is evoked by pathogen invasion that has ability to overcome first defense line. Through innate immune cells, proper response against pathogen is given for limiting spread of invaders. Besides innate immune response, antigen specific response from T and B cells that are activated via antigen presentation is induced as adaptive response against pathogens. However, depending on different defects and mutations in any part of immune system, defense mechanism against pathogen can fail. Failure in immunity is defined as immunodeficiency and characterized by impairment of clearance and elimination of internal or external threats [41].

Failure and defects in immune system are categorized into two depending on reason for deficiency. While primer immune deficiency depends on mutation of immunologically related genes, acquired or secondary immune deficiency requires environmental factors including therapy and malnutrition [42].

1.1.2.1 Primary Immune Deficiency

Primary Immune Deficiencies (PIDs) embrace several and different genetic abnormalities in both innate and adaptive immune responses. With the help of improved detection strategies and laboratory methods, various and unique PIDs has been defined. People with PIDs are generally prone to infectious diseases and have susceptibleness for non-infectious complications like autoimmunity, allergy and cancer.

Primary immune deficiencies are divided into two classes as monogenic and polygenic immunodeficiencies. Monogenic immunodeficiencies are resulted from mutations in genes and causes immunological tolerance that results in development of autoimmunity. Polygenic immunodeficiencies are defined with heterogeneous clinical symptoms with complex pathophysiology [43].

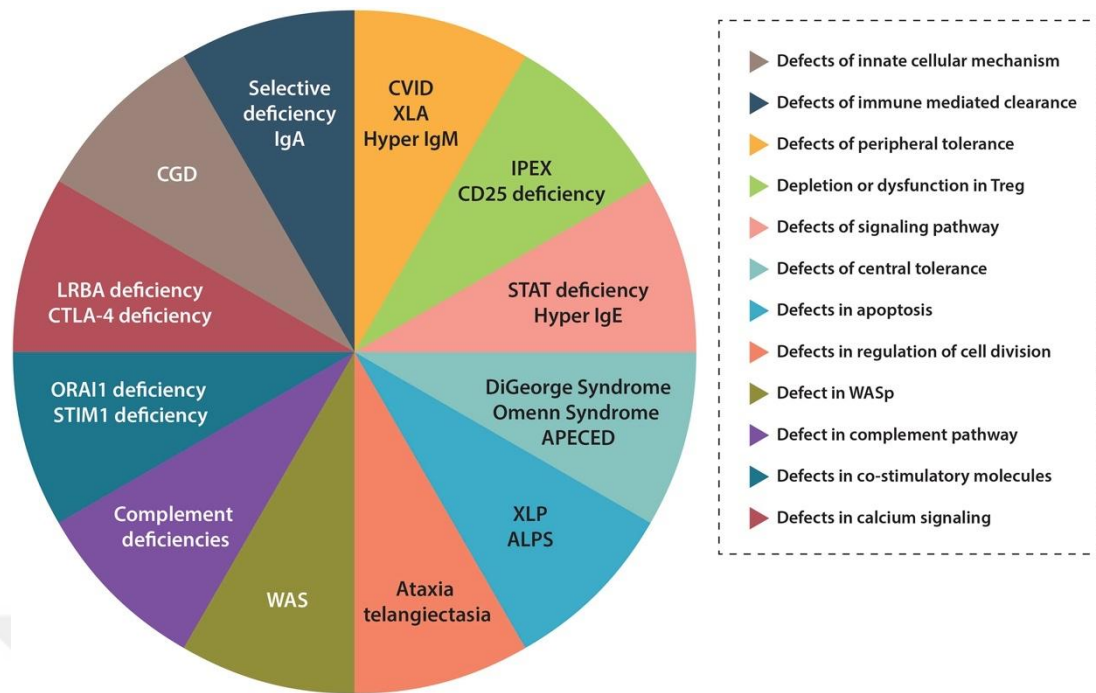


Figure 1.6 Different types of Primary Immune Deficiencies and their corresponding genetic defects adopted from [43]

1.1.2.1.1 CD27 and Deficiency

Transmembrane receptor CD27 belongs to TNF receptors and crucial for T/B and NK cell differentiation. Activated cells and naïve/memory T cells express CD27. Activation of T/B and NK cell requires CD27 as well as costimulatory signal, CD70 interaction. Interaction of CD27 with its ligand is key regulatory signal for function and survival of T cells (by decreasing apoptosis through expressing antiapoptotic proteins), differentiation of B cells into plasma cells and function of NK cells. With help of CD27-CD70 interaction, adaptor proteins like TRAF2 and TRAF5 are recruited for activation of NF κ b or JNK (c-Jun N terminal kinase) pathways [3,44]. Several studies suggested that interaction of CD27 with CD70 favors not cause Th1 CD4⁺ T cell subtype development by IL-12 independent way [45].

Deficiency in CD27 results in immunodeficiency where persistent viral infections like EBV (Epstein Barr Virus), abnormal antibody functions and hypogammaglobulinemia are seen mostly [46].

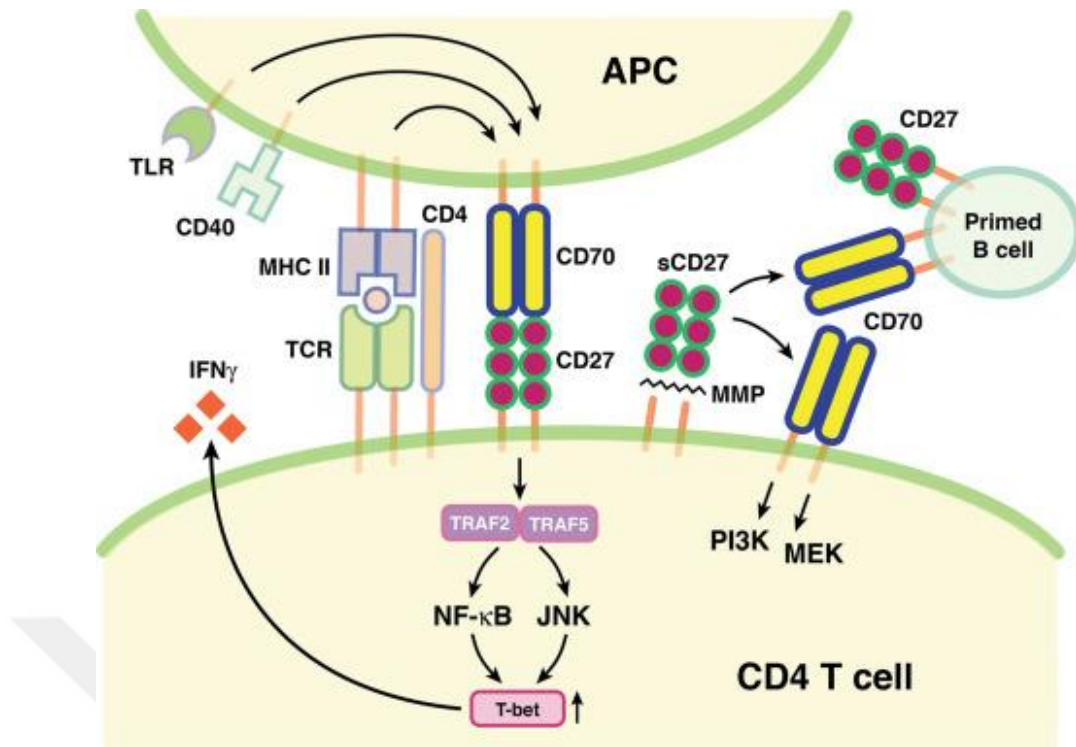


Figure 1.7 CD27-CD70 signaling through immune cells adopted from [47]

1.1.2.1.2 Signal Transducer and Activator of Transcription (STAT1) and Deficiency

Interferons are important cytokines responsible for protection from viral infections. Three different types of interferons are present: type I IFN (IFN α , IFN β), type II IFN (IFN γ) and type III IFN (IFN λ). Engagement of interferons with interferon receptors activate signaling pathways for interferon secretion. Transcription of interferon stimulated genes (ISG) are induced by both type I and type II interferon interaction with receptor following Janus kinase (JAK) and TYK2 activation and phosphorylation. Activation of kinases leads to recruitment of STAT1 proteins to docking site, subsequently causes phosphorylation and translocation of STAT1 proteins into nucleus for induction of interferon genes [49].

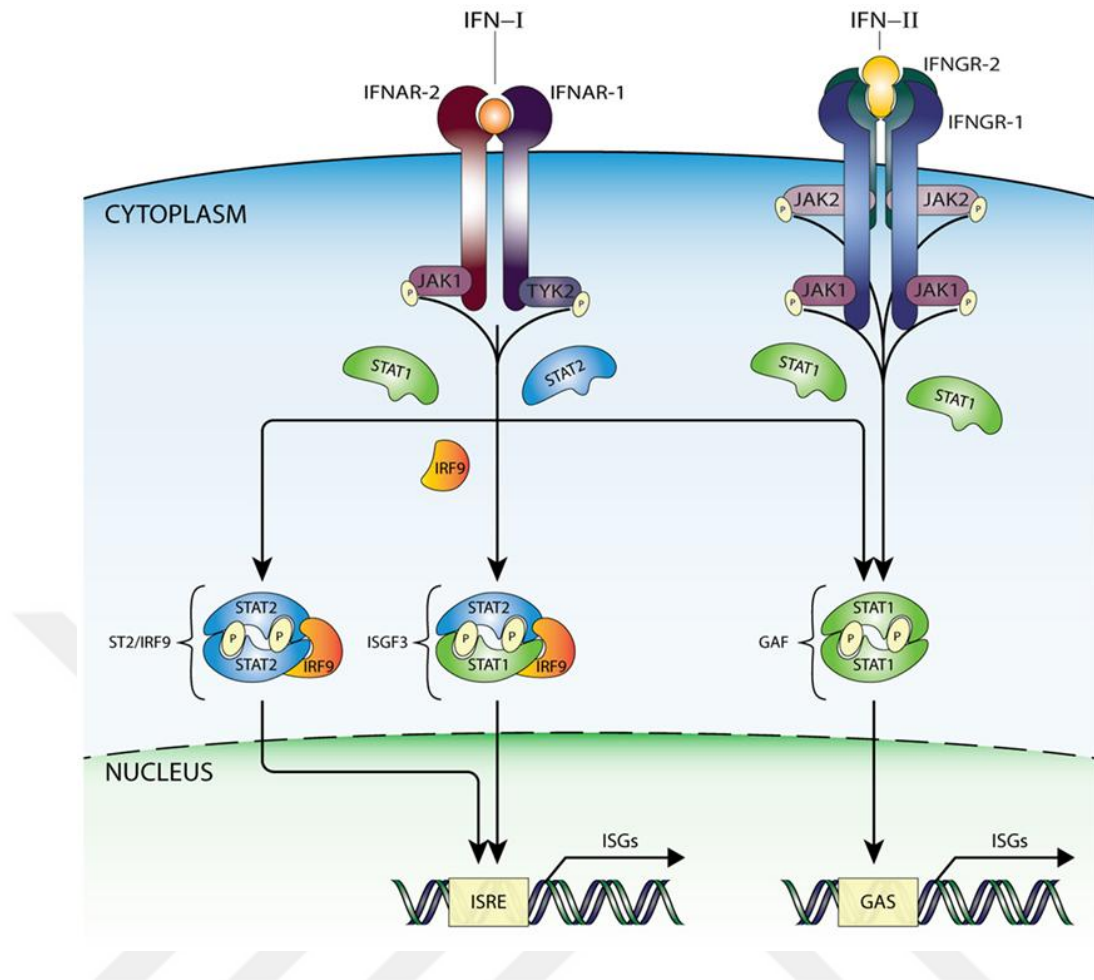


Figure 1.8 IFN and STAT signaling adopted from [48]

Naïve $CD4^{+}$ T cells are divided into different effector T helper as well as regulatory cells based on existence of antigen stimulation signal obtained from APCs. These helper and regulatory cells have distinct characterization distinguished via different transcription factors, cytokines and surface markers. Fate of $CD4^{+}$ T cell subtypes depends on TCR signaling and cytokines present in environment. According as cytokines, JAK-STAT signaling is activated and different STAT proteins are phosphorylated and induces different gene expression what determines also Th cell subtype. Each subtype has different function range from humoral immunity to cell immunity [50].

Mutation in STAT1 that leads to gain of function causes viral, bacterial and fungal especially chronic mucocutaneous candidiasis (CMC) infections in patients. Increased STAT1 phosphorylation and delay in dephosphorylation are the basic characterization of STAT1 GOF mutations. However, patients show variety of clinical aspects due to association of mutation both with innate and adaptive immunity [51]. Relationship between STAT1, STAT3 and CMC infection had been widely studied. STAT1 GOF mutation causes impairment of gene transcription depending STAT3 pathway, but it does not affect dimerization, trans-localization and phosphorylation of STAT3 which subsequently results in decreased IL-17a secretion and CMC infection [51,52].

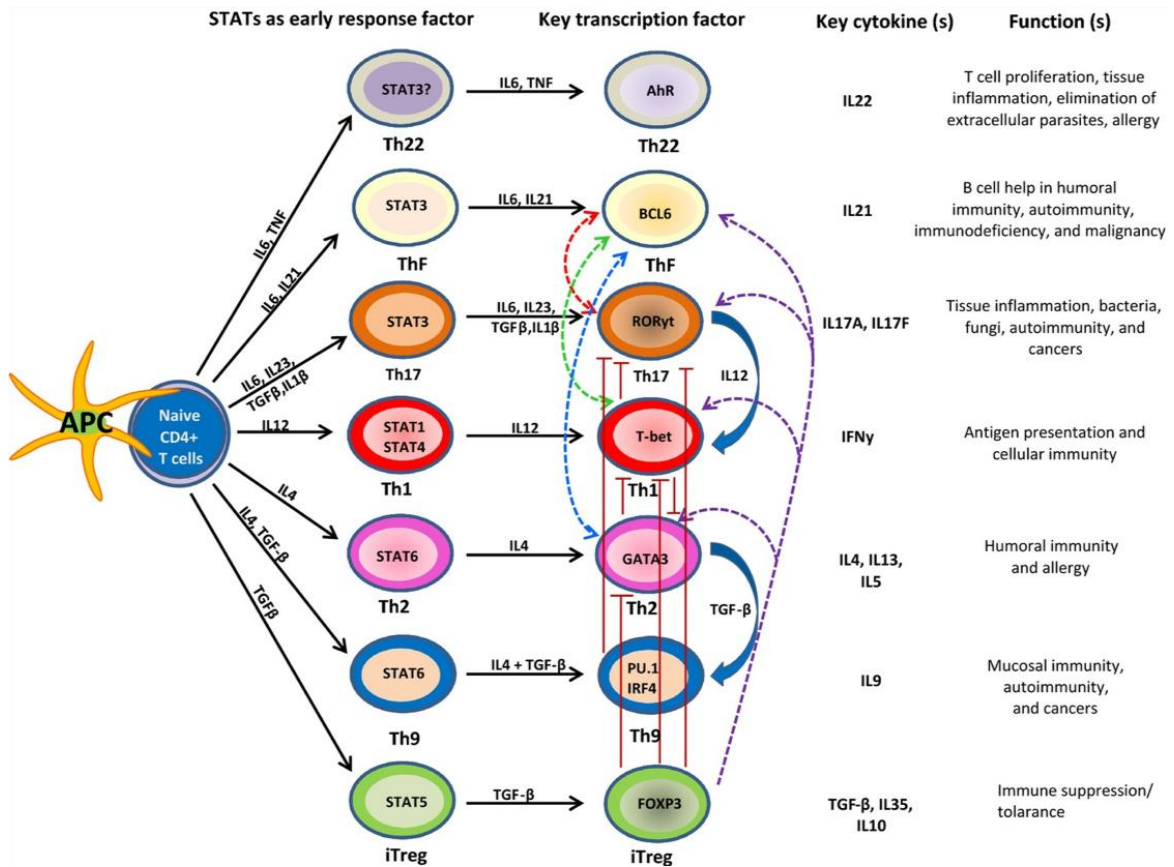


Figure 1.9 Helper T cell subsets adopted from [53]

1.1.3 Spinal Cord Injury

Spinal cord injury (SCI) is damage to the spinal cord and nerves in the spinal canal, causing permanent or partial loss of sensation under the injury site. Uncontrolled immune response occurs at the post traumatic injury site that increases the damage caused by lesion. Release of post injury necrosis products activates microglia, induces inflammatory cytokine secretion and infiltration of immune cells. Increase of chemoattractant and immune cells into injury site can sustain inflammation for months of after injury. Other than neuroinflammation, autoantibody production from antigen presentation of destructed neural tissues has effect on SCI patient's recovery period [54]. Even though SCI patients have inflammatory situations, impaired immunity that causes infection and immunosuppression are also associated with injury. Previous studies show us that patients with spinal cord damage have impairment in function of immunological cells including lymphocytes, macrophages and neutrophils [55]. Studies done in mice suggested that SCI causes impairment of CD8⁺ T cells during viral infection and upregulation of immunomodulatory pathways containing Tregs [56].

Astrocytes within central nervous system are important part of immune system in SCI since they are able to recruit immune cells to injury site. They are able to induce chemokines (especially MCP-1), recruit macrophages and neutrophils to injury site and promotes macrophage differentiation [58].

Neutrophils are important for SCI since inhibition of pro-inflammatory activity of them within injury site leads for recovery. Activation of neutrophils in injury side can cause elevated levels of Myeloperoxidase (MPO) and increased host tissue damage [59].

Both damage and recovery of SCI depends on macrophages as well. While M1 type macrophages are associated with damage to SCI, M2 type macrophages are linked to recovery. Excessive TNF- α secretion from M1 macrophages can cause increased inflammatory response and elevated level of neuropathic pain [60].

In short, immune cells can be either beneficial or detrimental for injury site in SCI patients.

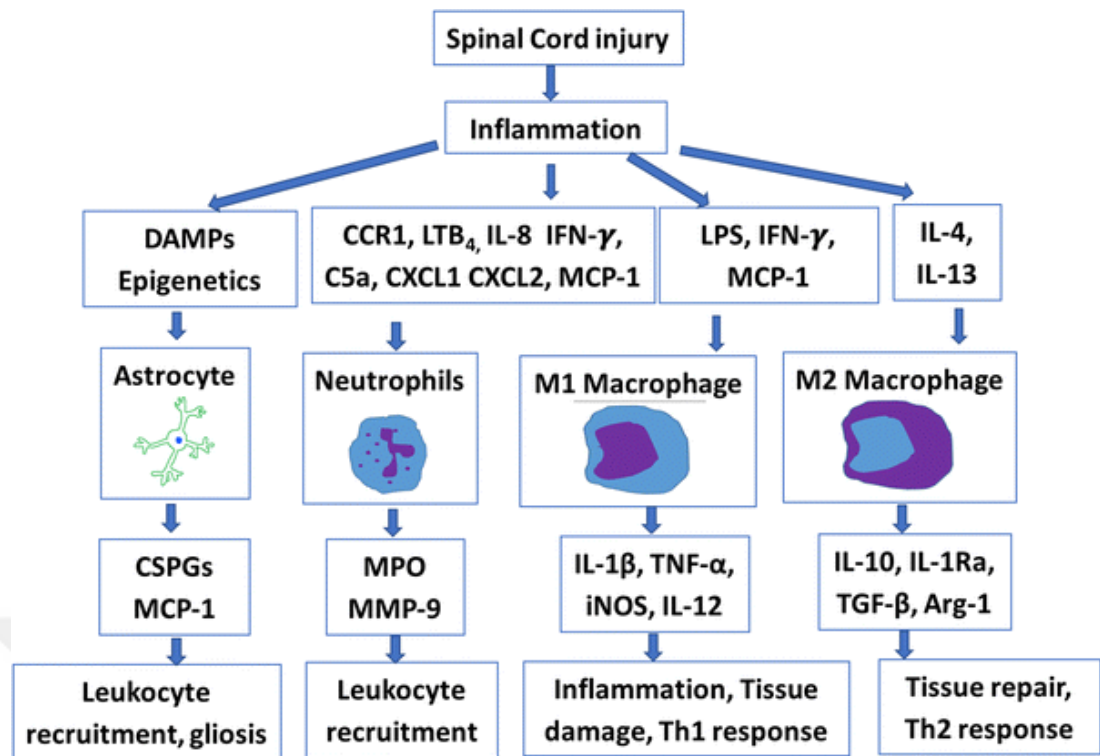


Figure 1.10 Immunological activities in Spinal Cord Patients adopted from [57]

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Culture and Solutions

2.1.1.1 Cell Culture

Fetal bovine serum (FBS) (Gibco Life Sciences, USA), HEPES Buffer (Biological Industries, Israel), RPMI-1640 with L-Glutamine (Gibco Life Sciences, USA), Non-Essential Amino Acids (Biological Industries, Israel), Sodium Pyruvate (Gibco Life Sciences, USA), Penicillin-Streptomycin (Gibco Life Sciences, USA), 1X Dulbecco's Phosphate Saline Buffer (DPBS) (Biological Industries, Israel), Ultrapure Cell Culture Water (Bio-sera, USA)

2.1.1.1.1 Cell Culture Media Contents

Fetal Bovine Serum:

Heat inactivated at 55°C for 1 hour for Regular Medium preparation

Heat inactivated at 65°C for 1 hour for Oligo Medium preparation

2% RPMI-1640 Medium:

- 10.64 ml FBS (inactivated at 55°C for 1 hour)
- 5.32 ml HEPES (10 mM)
- 5.32 ml non-essential amino acids
- 5.32 ml Na-Pyruvate (110 µg/ml)
- 5.32 ml Penicillin /Streptomycin (50 µg/ml)
- 500 ml RPMI-1640 with L-Glutamine (final concentration 2 mM)

5% RPMI-1640 Medium:

- 27.47 ml FBS (inactivated at 55°C for 1 hour)
- 5.5 ml HEPES (10 mM)
- 5.5 ml non-essential amino acids
- 5.5 ml Na-Pyruvate (110 µg/ml)
- 5.5 ml Penicillin /Streptomycin (50 µg/ml)
- 500 ml RPMI-1640 with L-Glutamine (final concentration 2 mM)

2% Oligo Medium:

- 0.96 ml FBS (inactivated at 65°C for 1 hour)
- 0.48 ml HEPES (10 mM)
- 0.48 ml non-essential amino acids
- 0.48 ml Na-Pyruvate (110 µg/ml)
- 0.48 ml Penicillin /Streptomycin (50 µg/ml)
- 50 ml RPMI-1640 with L-Glutamine (final concentration 2 mM)

2.1.1.1.2 Solutions and Contents

10X Phosphate Buffered Saline (10X PBS):

For ELISA wash buffer and FACS buffer preparation

- 80 grams NaCl
- 2 grams KCl
- 15.2 grams Na₂HPO₄·2H₂O
- 2 grams KH₂PO₄
- Completed to 1 liter with dH₂O
- Adjusted pH: 6.8
- Autoclaved
- Stored at room temperature

1X PBS:

- 10X PBS was diluted with dH₂O
- Adjusted pH: 7.2-7.4
- Autoclaved
- Stored at room temperature

Fluorescent Activated Cell Sorting (FACS) Buffer:

- 5 grams Bovine Serum Albumin (BSA)
- 250 milligrams Sodium azide
- Completed to 500 ml with 1X PBS
- Stored at +4°C

Blocking Buffer:

For ELISA usage

- 250 grams (5%) Bovine Serum Albumin (BSA)
- 250 µL (0.025%) Tween20
- Completed to 500 ml with 1X PBS
- Stored at -20°C

Wash Buffer:

For ELISA usage

- 4.5-liter dH₂O
- 0.5-liter 10X PBS
- 2.5 ml Tween20
- Prepared prior to usage

3% Dextran Solution:

For Neutrophil isolation

- 3 grams Dextran from Leuconostoc mesenteroides
- 100 ml ultrapure cell culture water
- Prepared prior to usage

1X Tris-EDTA Buffer:

For Micrococcal nuclease reaction

- 1 mM EDTA
- 10 mM Tris (pH:8)
- Completed to 100 ml with cell culture grade H₂O
- Stored at room temperature

10X Nuclease Buffer:

For Micrococcal nuclease reaction

- 50 mM Tris-HCl
- 10 mM CaCl₂
- Completed to 100 ml cell culture grade H₂O
- Adjusted pH:8.3
- Stored at +4°C

1X Nuclease Buffer:

10X nuclease buffer diluted with 1X Tris-EDTA buffer

2.1.2 Cytokine Receptor and PRR ligands for *in vitro* Stimulation

Ligands, their corresponding receptor/signaling pathways, working concentrations, brand and catalogue numbers are given in the Table 2.1.

LIGAND	TARGET RECEPTOR SIGNALING PATHWAY	BRAND NAME - COUNTRY	CATALOGUE NUMBER	WORKING CONCENTRATION
Pam3CSK4	TLR1/2	Invivogen - USA	tlrl-pms	1 µg/ml
p (I:C)	TLR3	Invivogen - USA	tlrl-picw	30 µg/ml
LPS (isolated from <i>E. coli</i>)	TLR4	Sigma Aldrich -Germany	L-2880-100mg	5 µg/ml
Flagellin (isolated from <i>S. typhimurium</i>)	TLR5	Invivogen - USA	tlr-pstfla-5	100 ng/ml
R848	TLR7	Enzo Life Sciences -	ALX-420-038- M025	5 µg/ml

		USA		
D35-3CG* ¹	TLR9	Alpha DNA- Canada	590691	3 µM
K3* ²	TLR9	Alpha DNA- Canada	539665	1 µM
2'3'-cGAMP*	STING	Invivogen - USA	tlrl-cga-23	30 µg/ml
HSV60*	cGAS-STING	Invivogen- USA	tlrl-hsv60n	5 µg/ml
P (dA: dT)*	AIM2, RIG- I/MAVS, DAI-STING	Invivogen - USA	tlrl-patn	5 µg/ml
LPS*	Non- canonical inflammasom e	Sigma Aldrich -Germany	L-2880-100mg	5 µg/ml
Flagellin*	NLRC4	Invivogen - USA	tlr-pstfla-5	100 ng/ml
ATP	NLRP3	Invivogen - USA	tlrl-atpl	100 µg/ml
Nigericin	NLRP3	Invivogen- USA	tlrl-nig	2 µg/ml
Phorbol-12- myristate 13- acetate (PMA)	PKC	Sigma Aldrich -Germany	P1585	50 ng/ml
Ionomycin	Endoplasmic reticulum Ca ⁺² channels	Calbiochem - Germany	407950	1 µg/ml
Human Recombinant IFN-β	IFNAR- JAK1/2- STAT1	Biolegend- USA	592702	100 ng/ml

Human Recombinant IL-6	IL6R- JAK1/2- STAT3	Biolegend - USA	570802	200 ng/ml
Human Recombinant IL-2	IL2R- JAK1/2- STAT5	Biolegend - USA	589104	100 ng/ml

*¹ D35-3CG CpG ODN sequence: 5' GGtgcacgatgcaggggGG 3' (contains phosphodiester (linkage shown with upper case) / phosphonothioate (linkage shown with lower case) mixes backbone)

*² K3 CpG ODN sequence: 5' TCGACTCTCGAGCGTTCTC 3' (contains phosphonothioate (linkage shown with lower case) backbone)

•Transfection was performed with these ligands by using 0.3 µl per stimulation Lipofectamine 2000 reagent (ThermoFischer Scientific, USA).

Table 2.1 Recombinant proteins, ligands and mitogens used in stimulation experiments.

2.1.3 ELISA Antibodies

Antibodies used in ELISA experiments, brand names, clones, catalogue numbers and working concentrations are given in Table 2.2.

RECOMBINANT ANTIBODY	BRAND NAME-COUNTRY	CATALOGUE NUMBER	CLONE	WORKING CONCENTRATION
Anti-human IL-1 β	Mabtech - Sweden	3416-1A-20	MT175	2 μ g/ml
Biotin anti human IL-1 β	Mabtech - Sweden	3416-1A-20	7NP10	1 μ g/ml
Human recombinant IL-1 β	Mabtech - Sweden	3416-1A-20	-	25 ng/ml
Anti-human IL-4	Mabtech - Sweden	3410-1A-20	IL4-I	1 μ g/ml
Biotin anti human IL-4	Mabtech - Sweden	3410-1A-20	IL4-II	1 μ g/ml
Human recombinant IL-4	Mabtech - Sweden	3410-1A-20	-	12.5 ng/ml
Anti-human IL-10	Mabtech - Sweden	3430-1A-20	9D7	2 μ g/ml
Biotin anti human IL-10	Mabtech - Sweden	3430-1A-20	12G8	1 μ g/ml
Human recombinant IL-10	Mabtech - Sweden	3430-1A-20	-	8 ng/ml
Anti-human IL-12/23 (p40 specific)	Mabtech - Sweden	3450-1A-20	MT86/221	2 μ g/ml
Biotin anti human IL-12/23 (p40 specific)	Mabtech - Sweden	3450-1A-20	MT618	1 μ g/ml
Human recombinant IL-12/23 (p40	Mabtech - Sweden	3450-1A-20	-	8 ng/ml

specific)				
Anti-human IL-17a	Mabtech - Sweden	3520-1A-20	MT44.6	2 µg/ml
Biotin anti human IL-17a	Mabtech - Sweden	3520-1A-20	MT504	0.5 µg/ml
Human recombinant IL-17a	Mabtech - Sweden	3520-1A-20	-	20 ng/ml
Anti-human IL-22	Mabtech - Sweden	3475-1A-20	MT12A3	2 µg/ml
Biotin anti human IL-22	Mabtech - Sweden	3475-1A-20	MT7B27	0.5 µg/ml
Human recombinant IL-22	Mabtech - Sweden	3475-1A-20	-	20 ng/ml
Anti-human IP-10	BD Biosciences - USA	555046	4D5/A7/C5	8 µg/ml
Biotin anti human IP-10	BD Biosciences - USA	555048	6D4/D6/G2	1 µg/ml
Human recombinant IP-10	BD Biosciences - USA	551130	-	100 ng/ml
Anti-human IFN-α	Mabtech - Sweden	3425-1A-20	MT1/3/5	4 µg/ml
Biotin anti human IFN-α	Mabtech - Sweden	3425-1A-20	MT2/4/6	1 µg/ml
Human recombinant IFN-α	Mabtech - Sweden	3425-1A-20	-	40 ng/ml
Anti-human IFN-γ	Mabtech -	3420-1A-20	1-D1K	4 µg/ml

	Sweden			
Biotin anti human IFN- γ	Mabtech - Sweden	3420-1A-20	7-B6-1	1 μ g/ml
Human recombinant IFN- γ	Mabtech - Sweden	3420-1A-20	-	12.5 ng/ml
Anti-human TNF- α	Mabtech - Sweden	3512-1A-20	MT25C5	2 μ g/ml
Biotin anti human TNF- α	Mabtech - Sweden	3512-1A-20	M520D9	1 μ g/ml
Human recombinant TNF- α	Mabtech - Sweden	3512-1A-20	-	12.5 ng/ml
Streptavidin- Alkaline Phosphates (SA- ALP)	Mabtech - Sweden	3310-8-1000	-	1:1000 diluted

Table 2.2 ELISA reagent used in experiments.

2.1.4 Flow Cytometry Antibodies

Fluorochrome conjugated antibodies used in experiments requiring Flow Cytometry, brand names, clones and catalogue numbers are given in Table 2.3.

ANTIBODY	FLUOROCHROME	BRAND	CLONE	CATALOGUE
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	CONJUGATE	NAME- COUNTRY		NUMBER
Anti-human CD19	PE	Biolegend -USA	HIB19	302208
Anti-human CD21	PE	Biolegend -USA	Bu32	354904
Anti-human CD38	PE	Biolegend -USA	HIT2	556604
Anti-human CD127	PE	Biolegend -USA	A019D5	351340
Anti-human IFN- γ	PE	Becton Dickinson (BD) - USA	4S. B3	554552
Anti-human CD24	APC	Biolegend -USA	ML5	311118
Anti-human CD123	APC	Biolegend -USA	6H6	306012
Anti-human IL-13	APC	Biolegend -USA	JES10-5A2	501907
Anti- human pSTAT1 (pY701)	Alexa Fluor 647	BD-USA	4a	560750
Anti- human pSTAT3 (pY705)	Alexa Fluor 647	BD-USA	4/P-STAT3	560750
Anti- human pSTAT5 (pY694)	Alexa Fluor 647	BD-USA	47	560750
Anti-human CD45RO	PE Texas Red	Biolegend -USA	UCHL1	304248
Anti-human CD4	FITC	Biolegend -USA	RPA-T4	300538

Anti-human CD19	FITC	Biolegend -USA	HIB19	302206
Anti-human CD303	FITC	Biolegend -USA	201A	354208
Anti- human IFN- γ	FITC	BD-USA	B27	552887
Anti-human CD56	BV785	Biolegend -USA	5.1H11	362550
Anti-human IL17A	BV785	Biolegend -USA	BL168	512338
Anti-human IgD	BV785	Biolegend -USA	IA6-2	3482421
Anti-human CD123	PerCP/Cy5.5	Biolegend-USA	6H6	306015
Anti-human IL-6	PerCP/Cy5.5	Biolegend-USA	MQ2-13A5	501117
Anti-human IL-10	PerCP/Cy5.5	Biolegend-USA	JES3-9D7	501417
Anti- human IFN- γ	PerCP/Cy5.5	Biolegend-USA	4S. B3	502525
Anti-human CD8	APC/Cy7	Biolegend -USA	SK1	344714
Anti-human CD25	APC/Cy7	Biolegend -USA	BC96	302614
Anti-human CD8	APC/Cy7	Biolegend -USA	SK1	344718
Anti-human CD27	BV421	Biolegend-USA	O323	302824
Anti-human IL-10	eFluor 450	eBiosciences	JES3-9D7	48-7108-42
Anti-human CD45	PerCP	Biolegend	2D-1	368506

Anti-human CD45	APC	Biolegend	2D-1	368512
Anti-human CD3	APC	Biolegend	SK7	344811
Anti-human CD3	BV605	Biolegend	SK7	344835
Anti-human CD3	PE	Biolegend	SK7	344805

Table 2.3 Fluorochrome conjugated flow cytometry antibodies.



2.2 Methods

2.2.1 Patients and Healthy Controls

2.2.1.1 CD27 Deficient (CD27^{-/-}) and Controls

Peripheral blood of two CD27 deficient patients were received from Hacettepe University, Department of Immunology (Ankara, Turkey). Two healthy age matched controls were studied with CD27 deficient patients.

2.2.1.2 STAT1 Gain of Function Containing Patients and Controls

Peripheral blood of three STAT1 GOF containing patients were obtained from Hacettepe University, Department of Immunology (Ankara, Turkey). Besides these patients, three more STAT1 GOF containing patients were received from Ankara University. Four healthy age matched controls were included in the STAT1 GOF study.

2.2.1.3 Spinal Cord Injured Patients and Controls

Peripheral blood samples of six Spinal Cord Injured patients were obtained from S.B.U. Veterans Physical Therapy and Rehabilitation Training and Research Hospital, Physical Medicine and Rehabilitation Department (Ankara, Turkey). Patients were categorized depending on their ongoing infection severity. Two healthy age-controlled controls were obtained for study. Patients are coded as #1 and #2 are less prone to infections compared to others.

2.2.2 Cell Culture

2.2.2.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation from Whole Blood

Peripheral blood (varying volumes, 2-10 ml) was received either in citrated or EDTA blood collection tubes (BD Biosciences, USA). The blood samples were first transferred into falcon tubes and diluted with 1X DPBS (@ RT) as 1:1 (v/v) ratio. Diluted blood samples were layered on lymphocyte separation media (Capricorn Scientific, Germany) as 1.5:1 (v/v) ratio. Centrifugation was conducted at 540xg for 30 minutes at 21°C. The key part in the centrifuge step was setting up deceleration to zero for the purpose of keeping formed layers intact. After centrifuge four distinct layers were observed. Plasma was layered into the top part whereas red blood cells

(RBCs) and granulocytes were layered into the bottom part. In between these, lymphocyte separation media and PBMCs (cloudy appearance) were placed. From these layers PBMCs were collected with sterile Pasteur pipette, transferred into falcon and wash with 2% FBS containing RPMI-1640 medium at 540xg for 10 minutes. If cells were used for experiments, pellet was resuspended in 1 ml 5% FBS containing RPMI-1640 medium and total cell counts were determined using a flow cytometer. Otherwise, pellet was resuspended with 1 ml FBS, counted and frozen in the presence of 10% DMSO.

2.2.2.2 Polymorphonuclear Neutrophil Isolation from Whole Blood

After PBMCs and plasmas were collected from the donor samples, the bottom layer containing RBCs and granulocytes were left for the isolation of neutrophils. Dulbecco's PBS (kept at RT) was added into blood cells and granulocytes containing red fraction matching the initial volume of diluted blood and tubes were mixed slowly. Then to separate neutrophils from RBCs using density differences, 3% (w/v) Dextran from *Leuconostoc mesenteroides* (Sigma Aldrich, Germany) was added into tubes as 1:1 (v/v) ratio and tubes were rotated slowly for 2 minutes. Tubes were incubated at RT for 40 minutes in an upright position and left for fraction formation with gravity. Neutrophils were layered at the top part while RBCs were placed at the bottom part of the separation tubes, because of density. The top part containing neutrophils were collected with sterile Pasteur pipette, transferred into falcon tubes and washed with two times warm 1X DPBS at 300xg for 10 minutes. To eliminate residual RBCs, neutrophils were resuspended with 5 ml 1X RBC lysis buffer (Biolegend, USA) and incubated for 5 minutes at RT. Then, neutrophils were washed third times with ice cold 1X DPBS at 300xg for 10 minutes at 4°C. Pelleted neutrophils were resuspended with 1 ml 2% oligo FBS containing RPMI-1640 media, then counted using flow cytometer and freshly used for experiments [62].

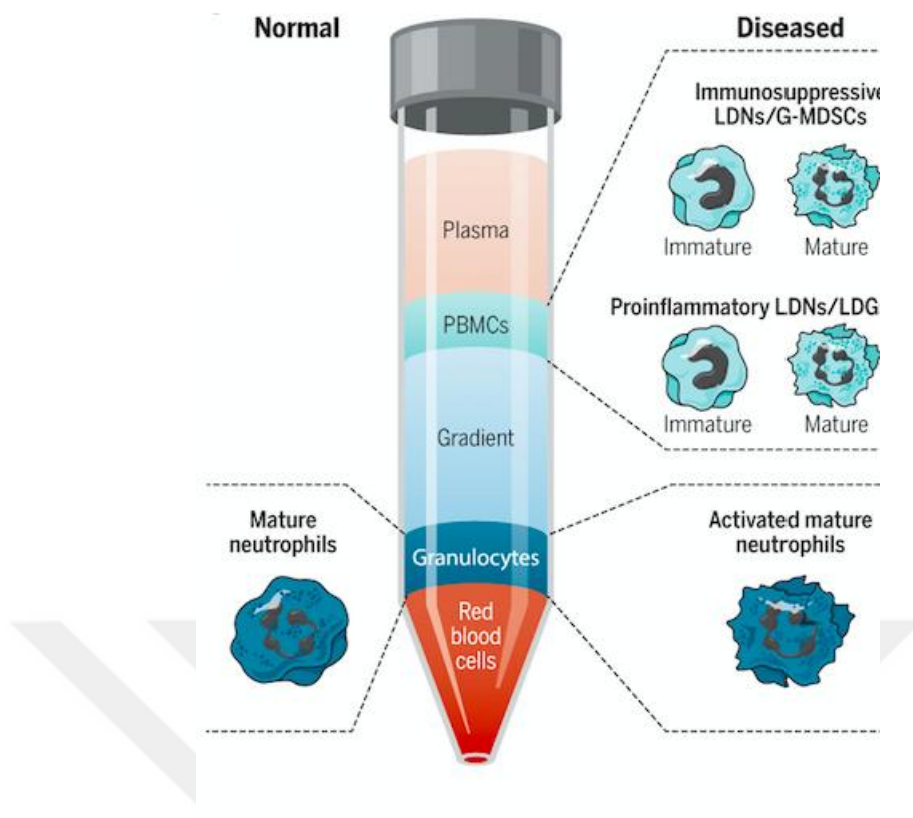


Figure 2.1 Different layers of isolated cells from whole blood in disease and normal states adopted from [61]

2.2.3 Assessments of Neutrophil Activity

2.2.3.1 Neutrophil Extracellular Traps (NETs) Visualization

Neutrophils that were isolated from patients and healthy control samples were seeded ($1 \times 10^5/\text{ml}$) into flat bottom 48-well plate for visualization of NETs. Cells were treated with PMA (50 ng/ml) or left untreated at 37°C for 4 hours. Following incubation, nuclear DNA was stained with 0.6 mM SytoTM 16 Green (ThermoFischer, USA) while extracellular DNA was stained with 1 mM SytoxTM Orange (ThermoFischer, USA) for 10 minutes at 37°C . Images of nuclear and extracellular DNAs were visualized and captured using EVOS FL Auto live imaging system (ThermoFischer, USA).

2.2.3.2 Detection of Respiratory Burst of Neutrophils

Neutrophils that was isolated from patients and healthy controls were seeded (8×10^5 /ml) into 1.5 ml Eppendorf for detection of respiratory burst. Cells were treated with PMA (50 ng/ml) or left untreated at 37°C for 4 hours. Then, DHR123-FITC dye (1:8000 diluted) was added and incubated for 15 minutes at 37°C. Respiratory burst of neutrophils were detected using flow cytometry as reading 40.000 event for each sample in FITC channel.

2.2.3.3 Neutrophil Extracellular Traps (NETs) Quantification

1.6×10^6 /ml patients and healthy controls` neutrophils that were seeded into flat bottom 96-well plates were treated with PMA (50ng/ml) or left for untreated at 37°C for 4 hours. Following treatment, 500 mu/ml Micrococcal Nuclease (ThermoFischer, USA) was prepared with 1X Nuclease Buffer and neutrophils were incubated with nuclease at 37°C for 30 minutes for cleaving expelled neutrophils-specific NETs. 5 mM EDTA was prepared with ultrapure cell culture water and added onto neutrophils to stop cleavage reaction. For collection of supernatants, cells were centrifuged for 10 minutes at 300xg and supernatants were saved into new plates and stored at -80°C for quantification of NETs.

Supernatants were taken out from -80°C right before quantification assay. Lambda DNA (ThermoFischer, USA) was prepared in Tris-EDTA buffer and starting from 2000ng/ml concentration, it was 2-fold diluted with Tris-EDTA during the construction of standard curve studies. 100 µl of supernatants and diluted standards were added into black flat bottom 96-well plate. Qanti-iT™ Picogreen™ (ThermoFischer, USA) was diluted 1:200 ratio with Tris-EDTA buffer, 100 µl of this was added onto samples and incubated at dark for 5 minutes. Microplate reader (Synergy HT Biotek, USA) was used to measure OD values by setting up excitation spectra at 485/20 nm and emission spectra at 528/20 nm. The linear portion of the standard curve was established and amount of released dsDNA was calculated [63].

2.2.4 In vitro Stimulation of PBMCs

PBMCs of healthy controls and patients were seeded into flat bottom 96-well plate in 100 μ l. For PRR stimulation 8×10^5 cells/ml PBMCs were layered. Stimulants were prepared in 5% FBS containing RPMI-1640 media and distributed into wells as 50 μ l/well. For transfected ligands, Lipofectamine 2000 was added with FBS free media as per manufacturers protocol. Volume of each reaction was completed to 250 μ l with 5% FBS containing RPMI-1640 media and cells were left for incubation at 37°C for overnight. Supernatants were collected by removing cells with 400xg for 10 minutes centrifugation and stored at -20°C for Cytokine ELISA.

2.2.5 Cytokine Enzyme Linked Immunosorbent Assay (Cytokine ELISA)

Coating antibodies were prepared in 1X DPBS and 2HB immunoplates (SPL Life Sciences, South Korea) were coated as 50 μ l/well antibody and incubated at 4°C for overnight (16-18 hours) or room temperature for 4 hours. Next day, plates were blocked with 200 μ l/well blocking buffer at room temperature for 2 hours. After blocking, plates were washed with a wash buffer 4x times and rinsed with dH₂O 2x times. Supernatants coming from stimulated PBMCs and recombinant proteins that were serially diluted 2-fold were added to plates as 50 μ l/well and incubated at 4°C overnight. Following incubation with standards and samples, plates were washed with wash buffer 4x and rinsed with dH₂O 2x. Biotinylated antibodies were diluted (1:1000) with blocking buffer added as 50 μ l/well and incubated at RT for 2 hours. Plates were washed with a wash buffer 4x and rinsed with dH₂O 2x and phosphatase-streptavidin (1:1000 diluted with blocking buffer) was layered as 50 μ l/well and plates were incubated at RT for 1 hours. Next, p-nitrophenyl phosphate-PNPP (ThermoFischer, USA) substrates were added as 50 μ l/well and optical density values were measured at 405 nm by using ELISA plate reader (Molecular Devices, USA). Concentration of released cytokines were calculated from 4 parametric S-shape standard curves formed by standard recombinant protein concentrations.

2.2.6 Flow Cytometry

2.2.6.1 Cell Counting

To count cells, 20 μ l of neutrophil or PBMCs from 1 ml suspensions were pipetted out and added into 5 ml diluent (isotonic solution, Beckmann Coulter, USA). Using Novocyte 3000 flow cytometer, 20 μ l portion of sample in isotonic solution was analyzed. Live cells were gated by positioning SSC and FSC and eliminating debris and live cells were calculated multiplying counted events per μ l by the dilution factor cell volume.

2.2.6.2 Cell Surface Marker Staining from Whole Blood

Fluorochrome conjugated flow antibodies were vortexed and 5 μ l of each antibody was added into FACS tubes. On top of antibodies 100 μ l of blood samples from patients and controls were pipetted, vortexed and incubated at dark for 30 minutes. After incubation, 2 ml 1X RBC lysis buffer was transferred to the tubes in order to lyse erythrocytes. The solution was vortexed and incubated for 15 minutes at dark. RBC lysis buffer was removed with centrifugation at 600xg for 10 minutes. Stained cells were resuspended in either FACS buffer or 5% FBS containing RPMI-1640 and analyzed with flow cytometer (Novocyte 3000, Agilent Technologies, USA).

2.2.6.3 Cell Surface Staining of PBMCs

Fluorochrome-labelled antibodies were prepared in 5% FBS containing RPMI-1640 by adjusting final concentration to 1 μ g/ml and stimulated/unstimulated PBMCs were stained at 4°C for 30 minutes in dark. To remove unbound antibodies, cells were washed with 5% FBS containing RPMI-1640 two times at 600xg for 10 minutes. Cells were resuspended in culture medium and analyzed by acquiring 30.000 events per sample with Novocyte 3000 flow cytometer.

2.2.6.4 Determination of Phosphorylation Levels of CD4⁺ & CD8⁺ T Cells

STAT phosphorylation of T cells was determined using either 200 µl/ stimulation of whole blood cells or 1x10⁶ cell/stimulation of PBMCs. Samples were stimulated with proper recombinant cytokines or left for untreated and incubated for 30 minutes at 37°C. To lyse RBCs and fix cells, 1X FACS lysing solution (BD Biosciences, USA) was added and cells were stimulated at 37°C for 15 minutes. Next, to remove lysing solution, 2 ml cold FACS buffer was added and cells were centrifuged at 600xg for 10 minutes. Permeabilization of cells were performed by adding cold 87% methanol and incubating them on ice for 30 minutes. Cells were then washed 2x with 2 ml cold FACS buffer at 600xg for 10 minutes. Cells were stained with pSTAT and CD4/CD8 specific fluorochrome-labelled antibodies in FACS buffer at dark/RT for 1 hour. Anti-human pSTATs-APC were used as 5 µl per sample while anti-human CD4/CD8 conjugated antibodies were used as 1 µg/ml. After incubation, cells were washed with 2 ml cold FACS buffer at 600xg for 10 minutes at 4°C, resuspended in 200 µl FACS buffer and analyzed as 30.000 event per sample with flow cytometry (Novocyte 3000).

2.2.6.5 Intracellular Cytokine Staining (ICS) of CD4⁺ Cells

Patient and healthy PBMCs were seeded into U bottom 96-well as 4x10⁵/well. Cells were incubated with PMA (50 ng/ml) and Ionomycin (1 µg/ml) at 37°C for 2 hours or left untreated. To prevent cytokine secretion from cells, 5 µg/ml Brefeldin A (Sigma Aldrich, Germany) was included and incubated for 4 more hours at 37°C. To pellet cells, samples were centrifuged for 10 minutes at 400xg and supernatants were aspirated. Cytofix Buffer (150 µl, BD Biosciences, USA) was added into each well to fix cells and samples were incubated at room temperature for 20 minutes in dark. First cells were pelleted at 400xg for 10 minutes, then washed two times with 250 µl/well FACS buffer for 10 minutes at 400xg. 150 µl cold Cytoperm Buffer (BD Biosciences, USA) was added into each well for permeabilization samples were incubated at room temperature for 15 minutes in dark. For removal of permeabilization buffer, cells were pelleted for 10 minutes at 400xg. Cell surface markers and cytokine antibodies were prepared in FACS buffer and used as 1 µg/ml.

Cells were stained with dyes at RT for 30 minutes in dark. Samples were washed twice

with 250 μ l/well FACS buffer for 10 minutes at 400xg, resuspended in 200 μ l FACS buffer and analyzed with flow cytometry Novocyte 3000 as 30.000 events in CD4⁺ cell gate [64].

CHAPTER 3

RESULTS

3.1 Characterization of Innate and Adaptive Immune Responses in CD27 Deficiency (CD27^{-/-})

3.1.1 Determination of Immune Cell Percentages in Whole Blood

3.1.1.1 Absence of CD27 surface expression in PBMCs, CD3⁺ and CD19⁺ cells of patients was confirmed

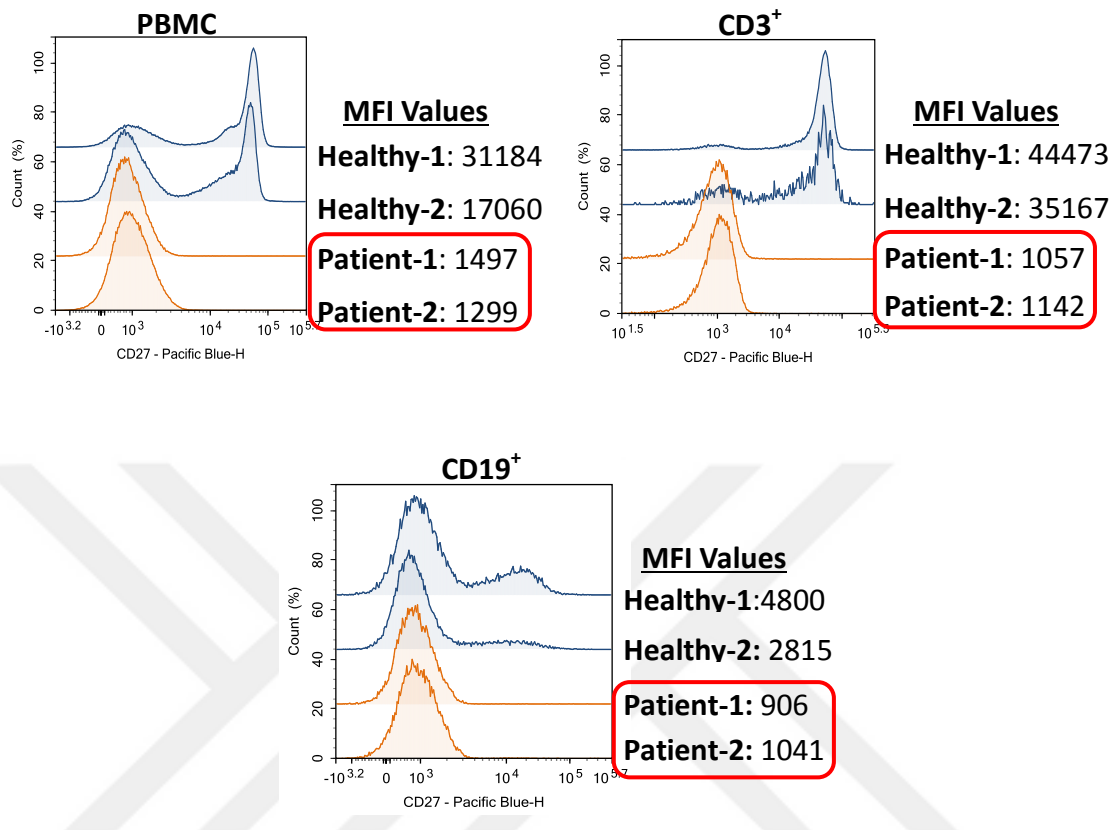


Figure 3.1 CD27 surface expression of PBMCs, CD3⁺ and CD19⁺ cells from CD27 deficient patients and healthy controls. Whole blood from patients and healthy subjects were stained with anti-human CD27, anti-human CD3 and anti-human CD19 fluorochrome conjugated antibodies and analyzed by flow cytometry. Gating was performed from first forward and side scattering of lymphocytes, then singlets. Plots show mean fluorescence intensity of CD27.

Since CD27 deficiency is engaged with impaired T and B cell functions, first we determined whether patients are absent in terms of CD27 expression in their T and B cells. Whole blood from patients and healthy controls were stained with anti-human CD27, anti-human CD45 (PBMC), anti-human CD3 (T cell) and anti-human CD19 (B cell) fluorochrome conjugated antibodies and analyzed with flow cytometry for surface expression of CD27. Both patients had absence of CD27 expression in their PBMCs, CD3⁺ T cells and CD19⁺ B cells (**Figure 3.1**).

3.1.1.2 Reversed CD4/CD8 ratio was observed in one of the patients

CD4/CD8 ratio is the indication of impaired immune system. Normally, in healthy blood it should be greater than 1 but, through infection, immunodeficiency and autoimmune diseases, it is inverted [65]. Since CD27 patients are prone to infections and deficiency is related to T cell function and survival, we aimed to determine CD4⁺ and CD8⁺ T cell percentages in the whole blood. Whole bloods from patients and healthies were stained with anti-human CD3, anti-human CD45, anti-human CD4 and anti-human CD8 labelled antibodies and analyzed with flow cytometry. Healthy controls had normal CD4/CD8 ratio (range between 1.8-2.6), whereas one of CD27 deficient patient showed reversed CD4/CD8 ratio (CD4⁺/CD8⁺: 0.2) referring either immunodeficiency and infection status (**Figure 3.2**).

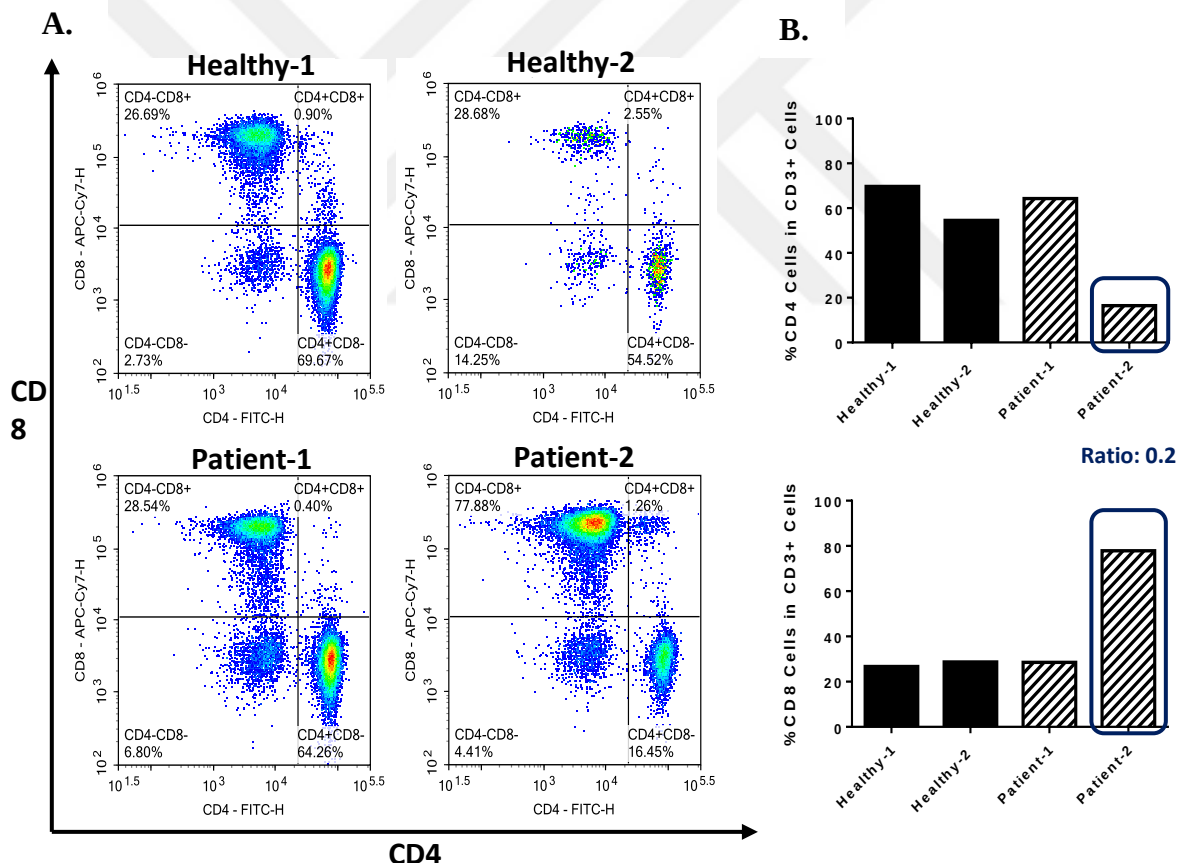


Figure 3.2 CD4 and CD8 surface expression of CD45⁺CD3⁺ cells. The whole blood of healthy controls and CD27 deficient patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, anti-human CD4 and anti-human CD8 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺CD3⁺ CD4⁺- and

CD8⁺ cells. **B.** Representative bar graphs of CD4⁺ and CD8⁺ cell percentages in CD3⁺ cells.

3.1.1.3 Elevated B cell, Naïve B cell and Transitional B cell were identified in CD27^{-/-} patients

CD27 is an important transmembrane receptor for B cell response and differentiation [66]. Thus, before functional studies, first percentages of B cell subsets were analyzed. Whole blood from healthy controls and patients were stained with anti-human CD45, anti-human CD19 (for B cell), anti-human CD27 (for naïve B cell), anti-human IgD (for naïve B cell), anti-human CD24 (for transitional B cell) and anti-human CD38 (for transitional B cell). Both patients had elevated CD24⁺⁺CD38⁺⁺ transitional B cells (4-fold more) compared to healthy controls (**Figure 3.5**) while Patient-1 had slightly elevated B cell (**Figure 3.3**) and Patient-2 had increased naïve B cells (2.4 fold) (**Figure 3.4**).

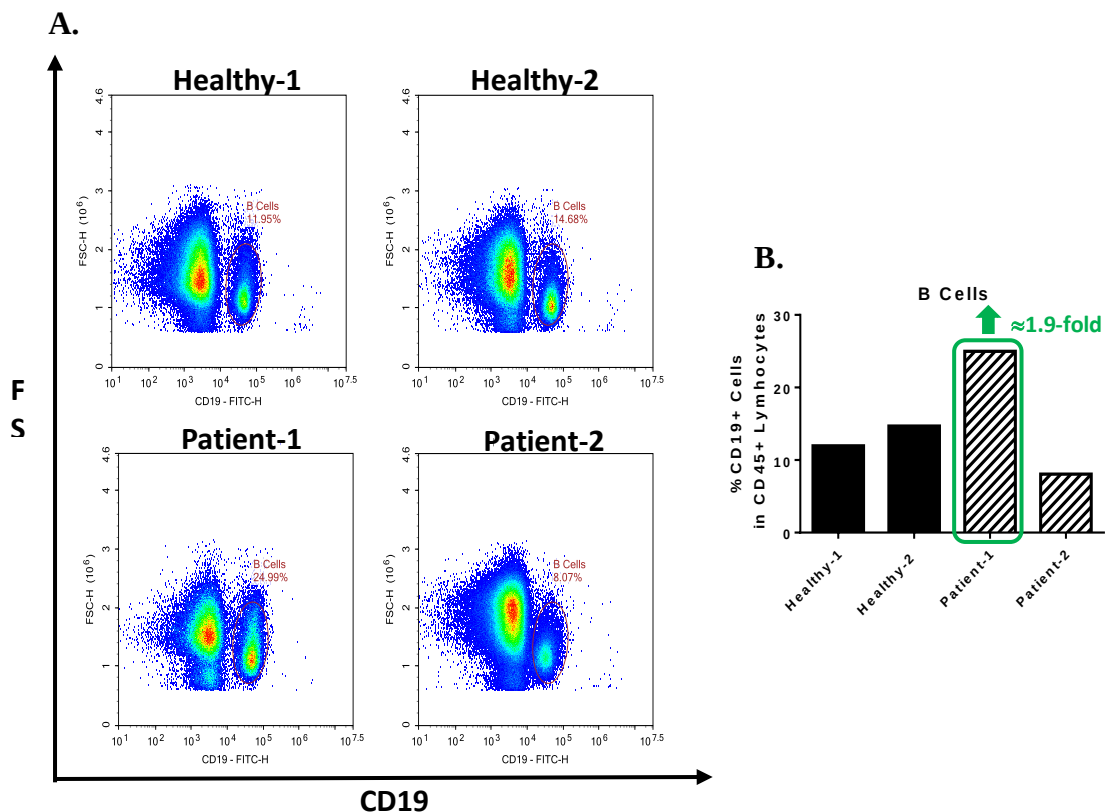


Figure 3.3 CD19 surface expression of CD45⁺CD3⁻ cells. The whole blood of healthy controls and CD27 deficient patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, and anti-human CD19 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺CD3⁻ CD19⁺ cells. **B.** Representative bar graphs of cell percentages of CD19⁺ cell percentages in CD45⁺ lymphocytes for healthy controls and patients.

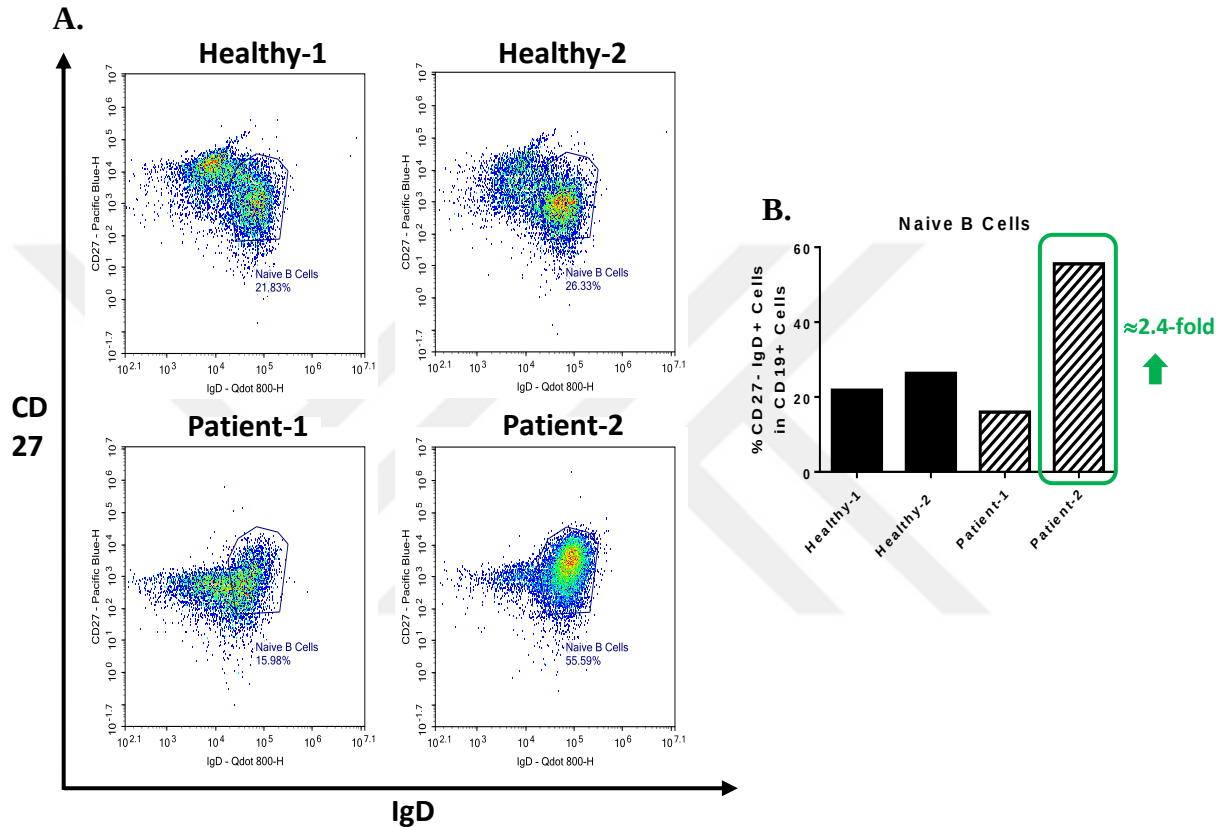


Figure 3.4 CD19 and IgD surface expression of CD27⁻CD3⁻ cells. The whole blood of healthy controls and CD27 deficient patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, anti-human CD27, anti-human CD19 and anti-human IgD antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺CD3⁻ CD27⁻CD19⁺IgD⁺ cells. **B.** Representative bar graphs of CD27⁻IgD⁺ cell percentages in CD19⁺ cells for healthy controls and patients.

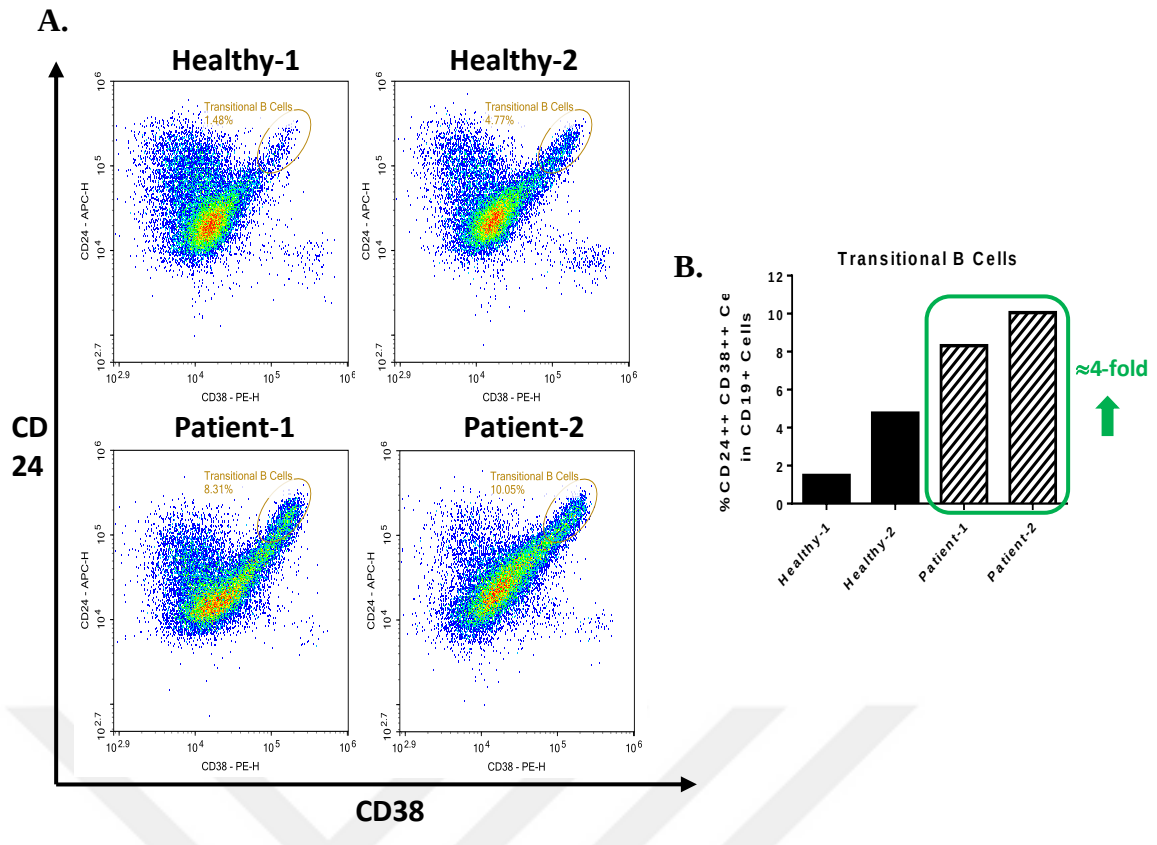


Figure 3.5 CD24 and CD38 surface expression of CD19⁺. The whole blood of healthy controls and CD27 deficient patients (100 μ l) were stained with fluorochrome conjugated anti-human CD45, anti-human CD27, anti-human CD19, anti-human CD24 and CD38 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺ CD19⁺CD24⁺⁺CD38⁺⁺ cells. **B.** Representative bar graphs of CD24⁺⁺CD38⁺⁺ cell percentages in CD19⁺ cells for healthy controls and patients.

3.1.2 Assessment of STAT Phosphorylation Levels for CD4⁺ T Cell Subset Activities

3.1.2.1 Increased STAT1 phosphorylation upon IFN- β treatment was observed in CD27 deficient patient

To explore role of CD4⁺ T cell subsets in CD27 deficiency, whole blood from patients and controls treated with IFN- β and analyzed with flow cytometry after fixation, permeabilization and staining with anti-human CD4 and anti-human pSTAT1. Untreated healthy and patients' cells had similar STAT1 phosphorylation level in CD4⁺ T cells while upon stimulation CD27 deficient patients showed increased pSTAT1 levels and pSTAT1⁺% in CD4⁺ T cells compared to healthy subject (**Figure 3.6 and Figure 3.7**).

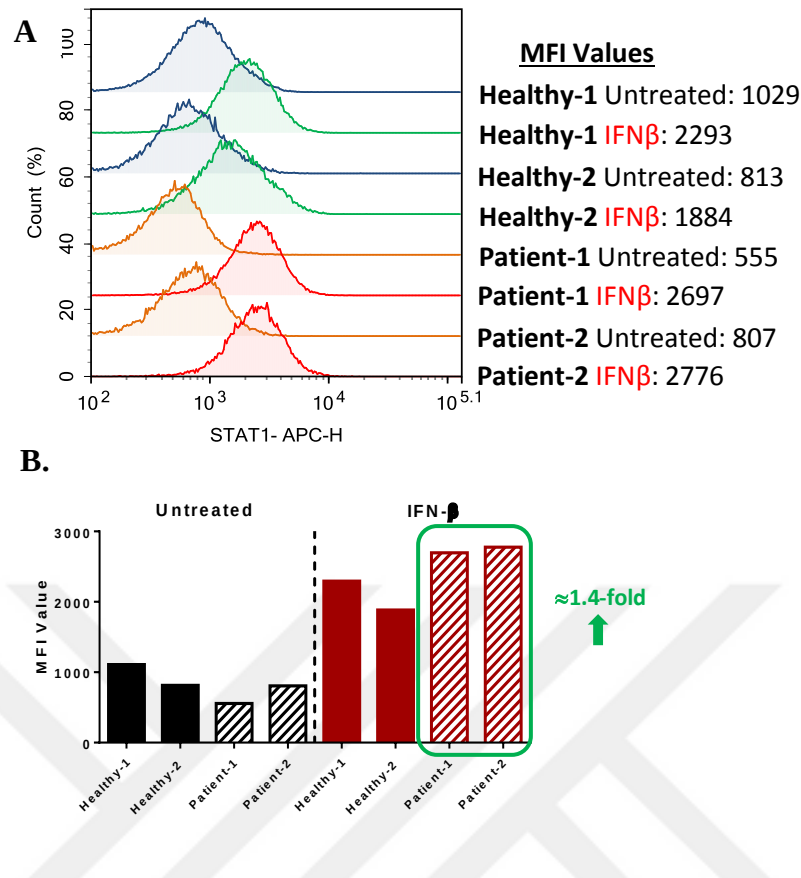


Figure 3.6 STAT1 phosphorylation levels of CD27 deficient patients and healthy controls' CD4⁺ T Cells. Patients and healthy controls' whole blood (200 μ l) were treated with IFN- β (100 ng/ml) or left untreated over 30 minutes. Fixation, permeabilization and co-staining of anti-human CD4 and anti-human pSTAT1 were performed and phosphorylation levels of pSTAT1 proteins were analyzed by flow cytometry by acquiring 4×10^4 cells within CD4⁺ cell gate. **A.** Mean fluorescence intensity plot of pSTAT1 proteins. **B.** Representative bar graph.

3.1.2.2 Normal STAT3 but decreased STAT5 phosphorylation was determined in CD27 deficient patients

Depending on crosstalk between STAT proteins, we analyzed STAT3 (important for Th17 response) and STAT5 (key regulator of Treg response) phosphorylation through IL-6 and IL-2 treatment, respectively. Whole blood from patients and healthy subjects were incubated with recombinant proteins and fixed, permeabilized, stained with anti-human CD4, anti-human pSTAT3 and anti-human pSTAT5. STAT3 phosphorylation levels of CD4⁺ T cells and pSTAT3+/% in CD4⁺ cells from patients were similar to healthy controls (**Figure 3.8 and Figure 3.9**). Yet,

decreased STAT5 phosphorylation was observed in CD27 deficient patients (**Figure 3.10**).



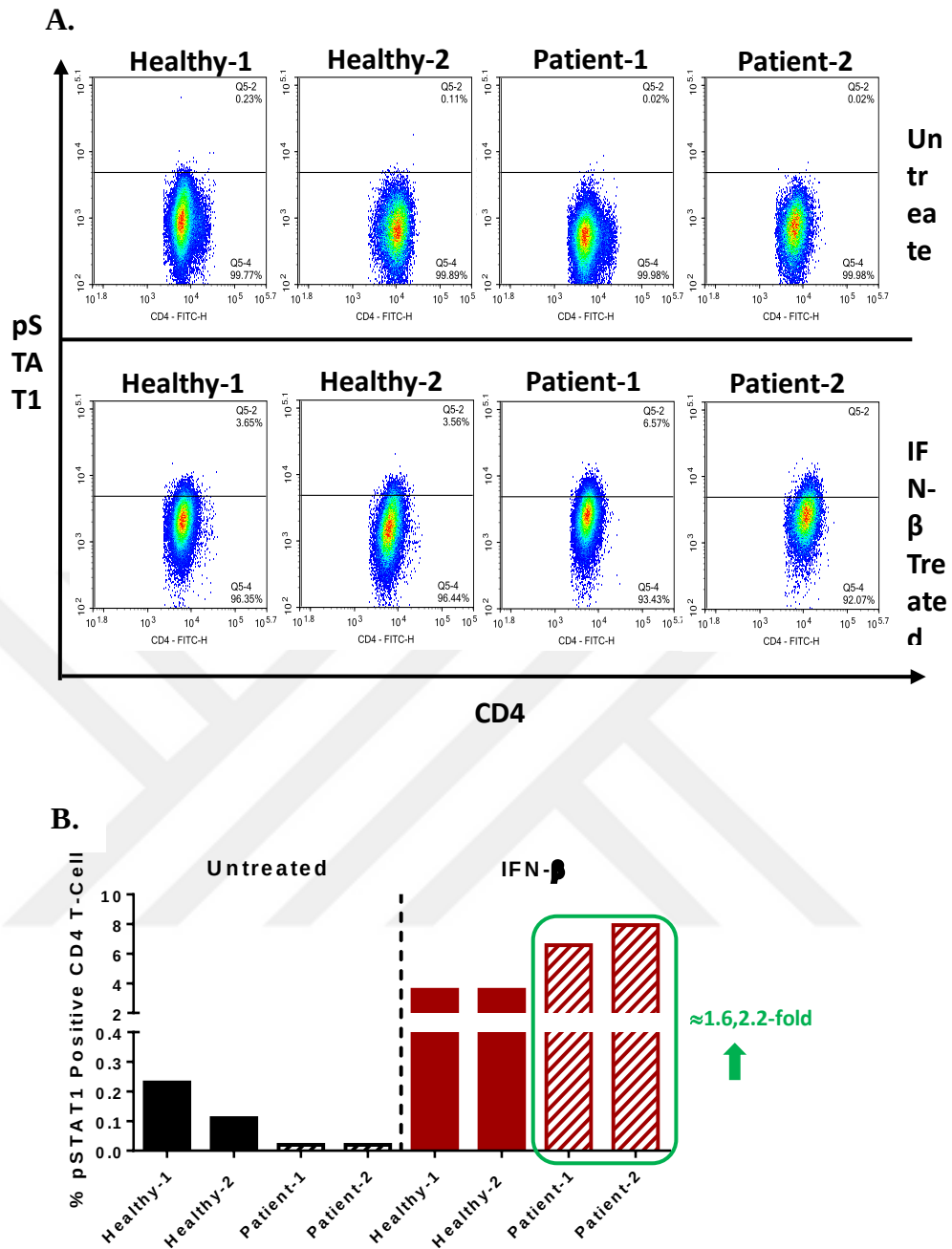


Figure 3.7 Percentage of pSTAT1⁺ cells within CD4⁺ T cells from CD27 deficient patients and healthy controls after IFN-β stimulation. A. Density plots of pSTAT1 proteins within CD4⁺ T cells analyzed by flow cytometry. **B.** Representative bar graph.

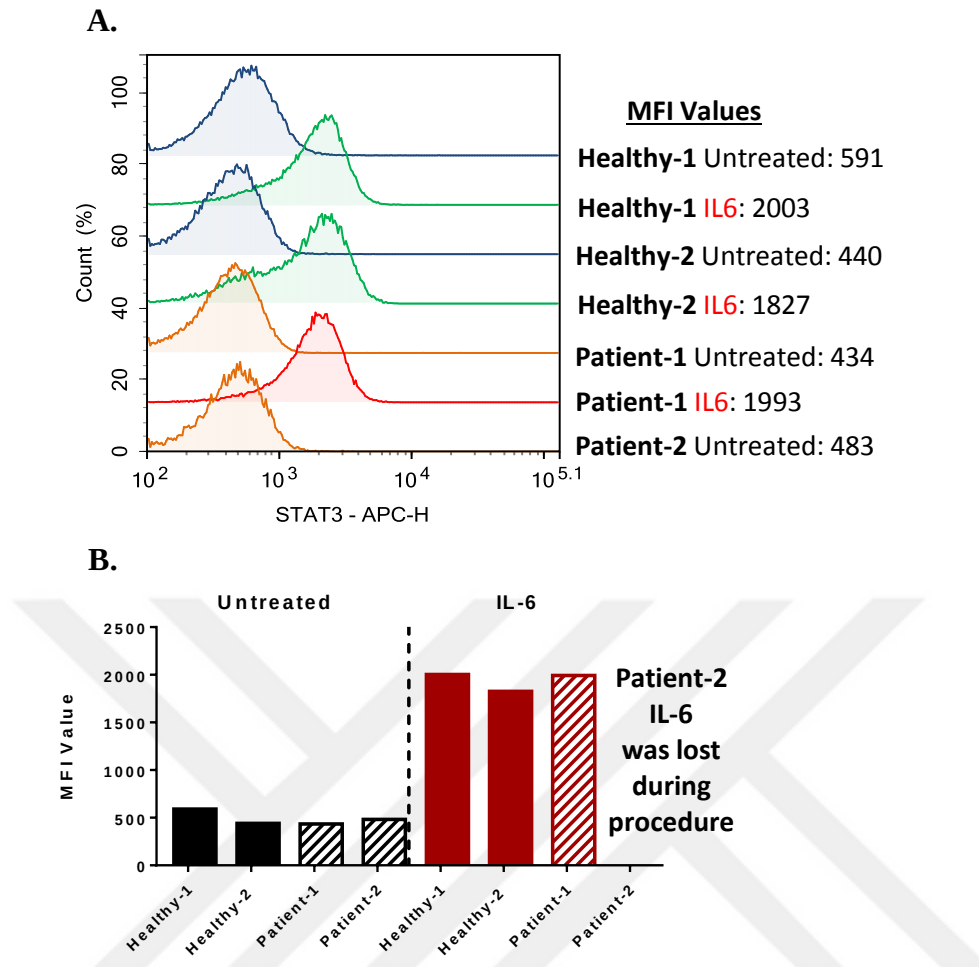


Figure 3.8 STAT3 phosphorylation levels of CD27 deficient patients and healthy controls' CD4⁺ T Cells. Patients and healthy controls' whole blood (200 μ l) were treated with IL-6 (200 ng/ml) or left untreated over 30 minutes. Fixation, permeabilization and co-staining of anti-human CD4 and anti-human pSTAT3 were performed and phosphorylation levels of pSTAT1 proteins were analyzed by flow cytometry by acquiring 4×10^4 cells within CD4⁺ cell gate. **A.** Mean fluorescence intensity plot of pSTAT3 proteins. **B.** Representative bar graph.

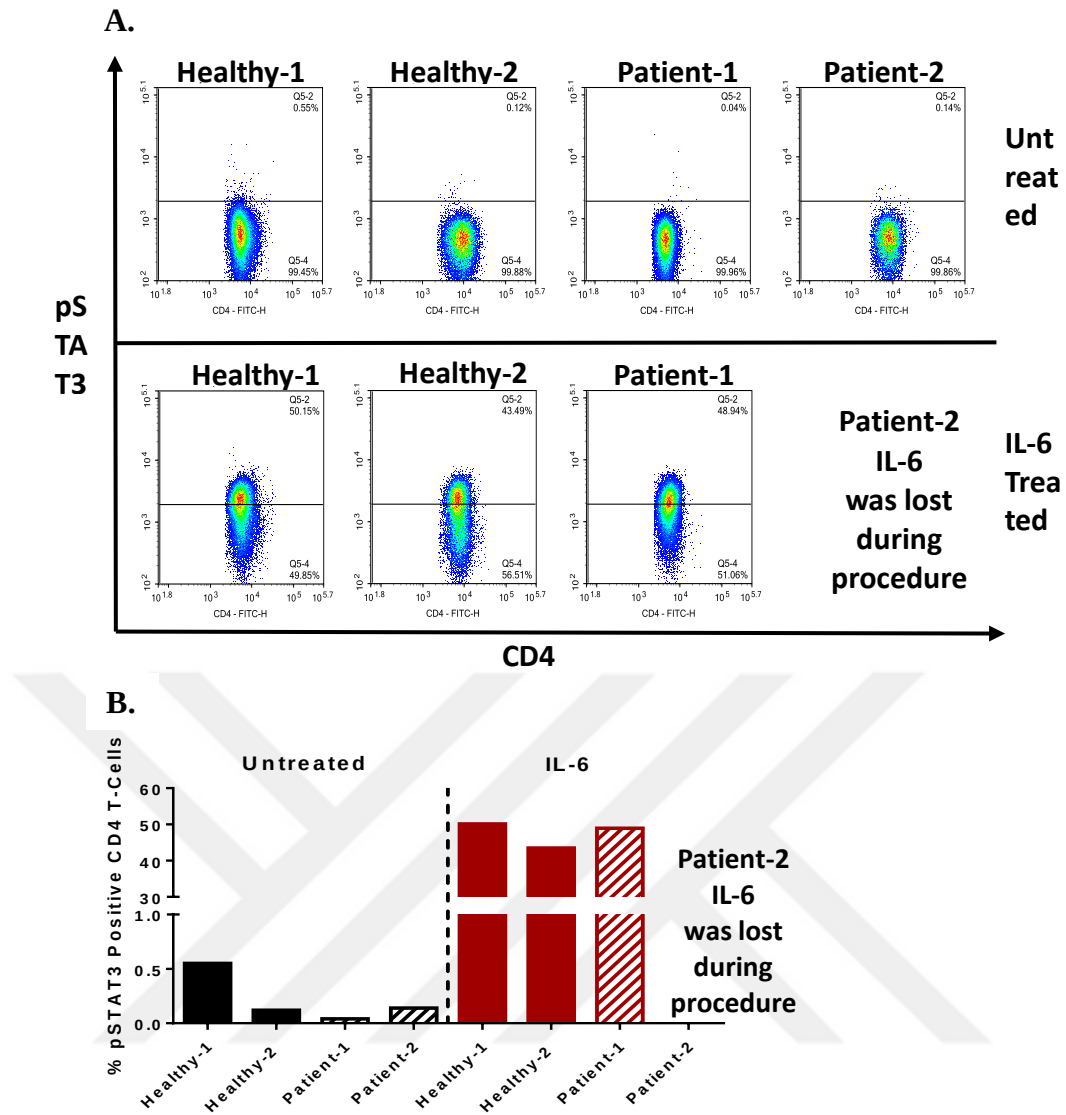
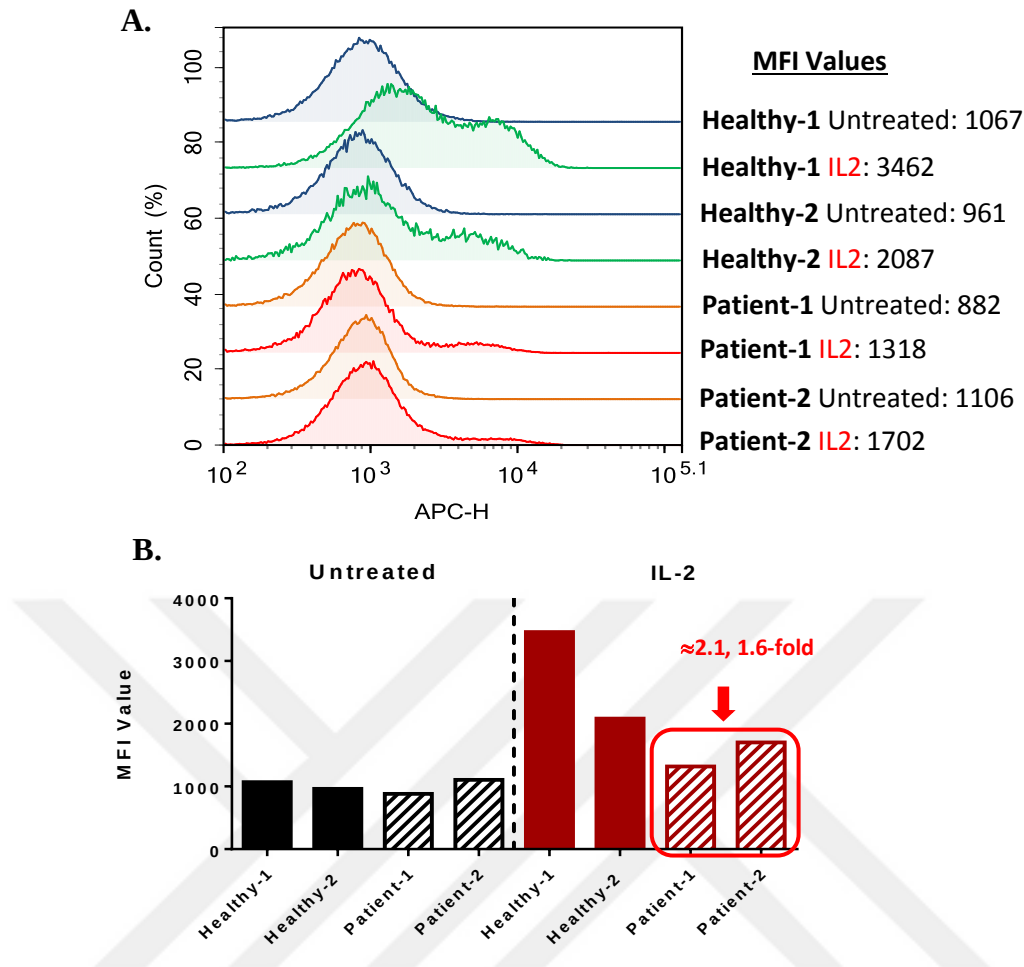


Figure 3.9 Percentage of pSTAT3⁺ cells within CD4⁺ T cells from CD27 deficient patients and healthy controls after IL-6 stimulation. A. Density plots of pSTAT3 proteins within CD4⁺ T cells analyzed by flow cytometry. **B.** Representative bar graph.



3.1.3 Investigation of Innate Immune Responses in CD27 Deficiency

Effect of CD27 deficiency in adaptive immune responses were mostly studied, but possible dysregulations in innate arm was not investigated. That's why we aimed to study innate responses of PBMCs from patients through PRR stimulations and cytokine responses.

Figure 3.10 STAT5 phosphorylation levels of CD27 deficient patients and healthy controls' CD4⁺ T Cells. Patients and healthy controls whole blood (200 μ l) were treated with IL-2 (100 ng/ml) or left untreated over 30 minutes. Fixation, permeabilization and co-staining of anti-human CD4 and anti-human pSTAT5 were performed and phosphorylation levels of pSTAT5 proteins were analyzed by flow cytometry by acquiring 4×10^4 cells within CD4⁺ cell gate. **A.** Mean fluorescence intensity plot of pSTAT5 proteins. **B.** Representative bar graph.

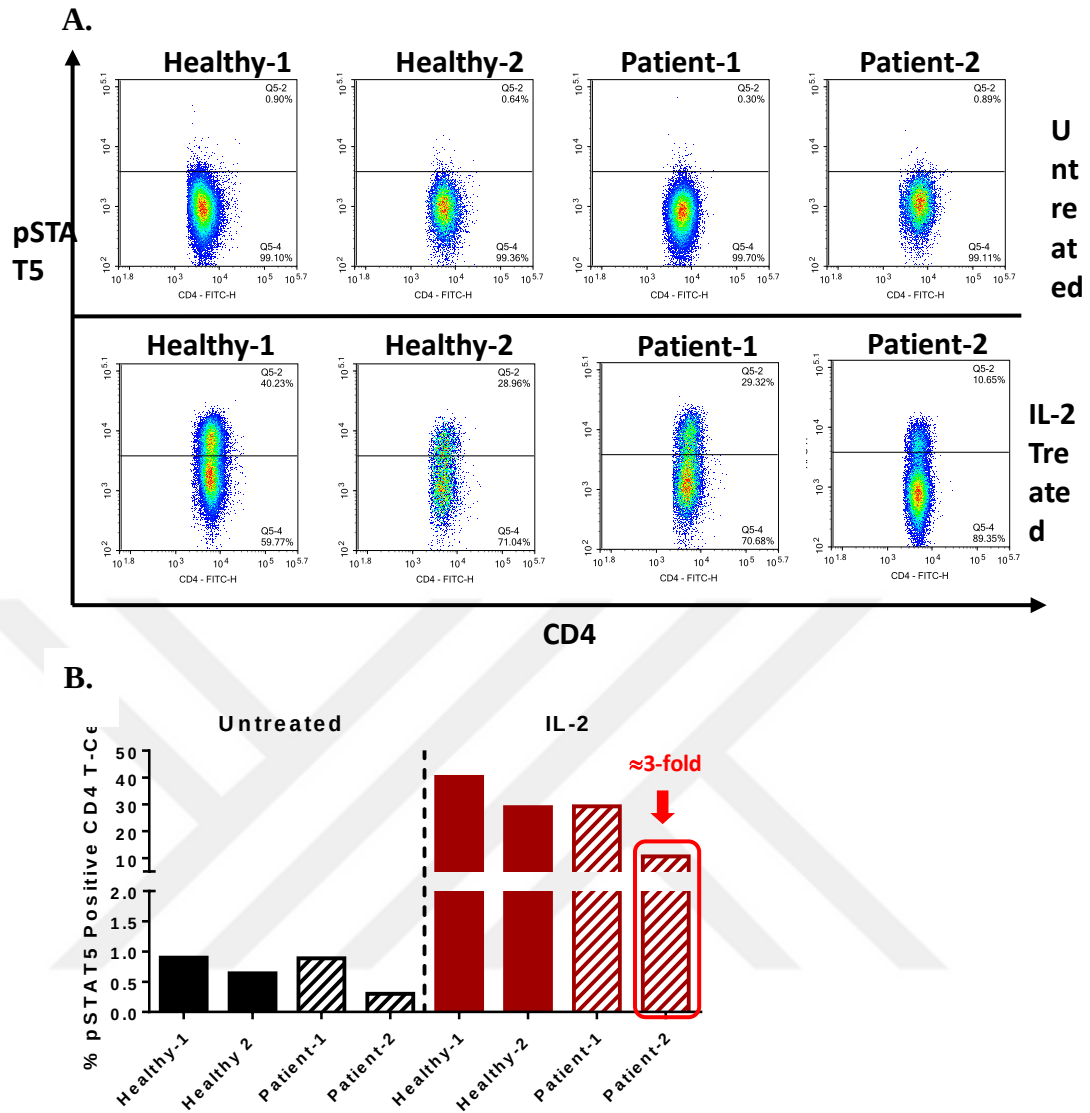


Figure 3.11 Percentage of pSTAT5⁺ cells within CD4⁺ T cells from CD27 deficient patients and healthy controls after IL-2 stimulation. A. Density plots of pSTAT5 proteins within CD4⁺ T cells analyzed by flow cytometry. **B.** Representative bar graph.

3.1.3.1 CD27 deficient patients had various IFN- α responses

Type I IFNs are produced from variety of cells from engagement of infectious DNA and RNA through TLRs and PRRs. It is the one of the most important cytokines produce during viral infections. Since CD27 deficient patients are prone to viral infections like EBV, we aimed to controlled type I IFN secretion from immune cells. To mimic infection, TLR3 (p(I:C)), TLR7/8 (R848), TLR9 (D-ODN), STING (2'3'-cGAMP), cGAS-STING (HSV60) and AIM-RIG-I/MAVS (p (dA: dT) ligands were given to PBMCs from patients and healthy controls and cytokine levels were assessed with ELISA. Patients had similar responses to healthy controls for endosomal TLRs and intracellular DNA-dependent cytosolic sensors. However, Patient-1 showed slightly increased response to TLR9 engagement while decreased response to

P(dA:dT) treatment (**Figure 3.12**).

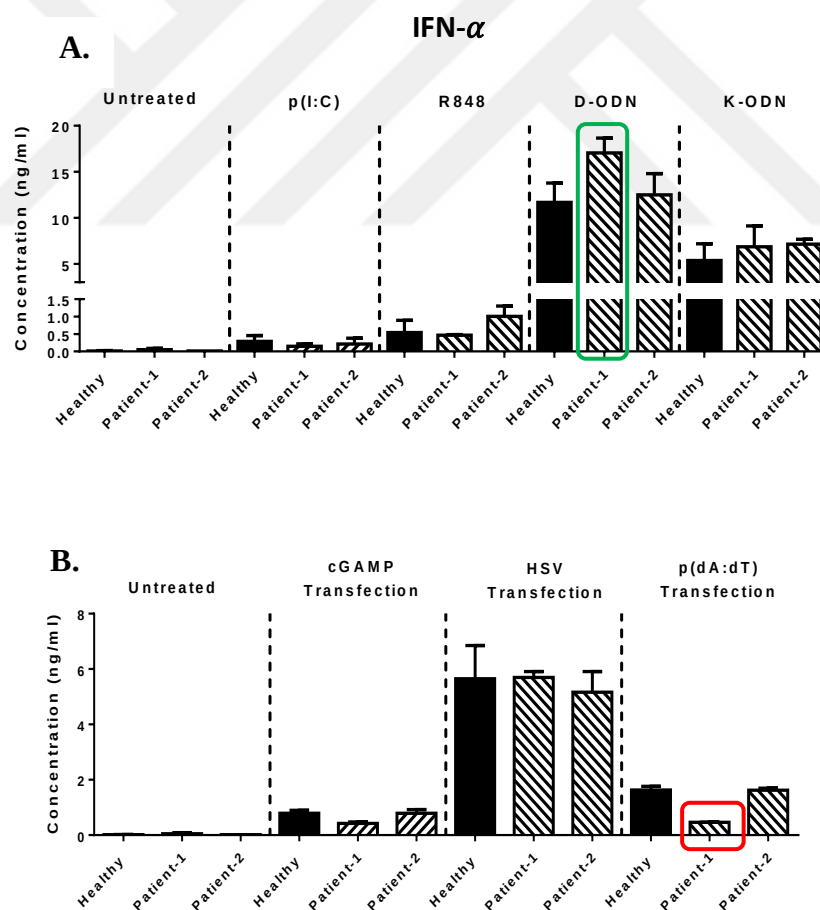


Figure 3.12 IFN- α cytokine production profile of PBMCs from CD27 deficient patients and healthy controls (n=2) upon PRR stimulations for 24 hours.

Patients and healthy controls' PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (K and D ODN, 3 μ M and 1 μ M), STING (2'3' cGAMP, 30 μ g/ml), IFI16-STING (HSV, 5 μ g/ml) and AIM2 inflammasome, RIG-I/MAVS (p(dA:dT), μ g/ml) for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- α levels from TLR stimulation. **B.** IFN- α levels from inflammasome pathway stimulation.

3.1.3.2 CD27 deficient patients had normal type II IFN responses in context of endosomal and intracellular TLRs, PRRs and inflammasome pathways

To determine responses from CD8⁺ T cells, NK and NKT cells, PBMCs from patients and healthy controls were treated with TLR1/2 (Pam3CSK4), TLR3 (p(I:C), TLR4 (LPS), TLR5 (Flagellin), TLR7/8 (R848), TLR9 (K-D ODN) and inflammasome pathways ligands and cytokine secretion was determined with ELISA. No difference was observed between patients and healthies for surface and endosomal TLRs as well as inflammasome pathways (**Figure 3.13**).

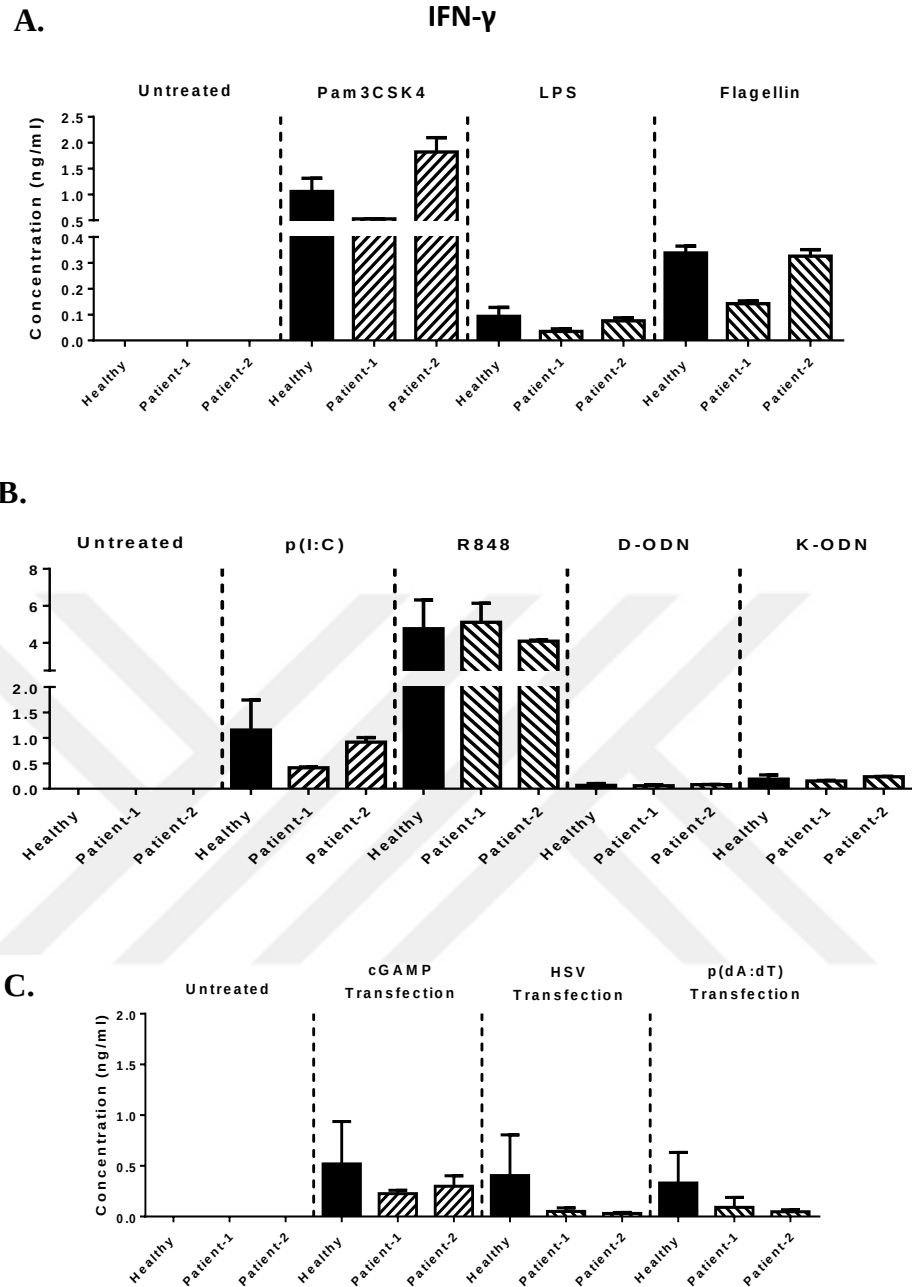


Figure 3.13 IFN- γ cytokine production profile of PBMCs from CD27 deficient patients and healthy controls (n=2) upon PRR stimulations for 24 hours.

Patients and healthy controls PMBCs were stimulated with TLR1/2 (Pam3CSK4, 1 μ g/ml), TLR3 (p(I:C), 30 μ g/ml), TLR4 (LPS, 5 μ g/ml), TLR5 (Flagellin, 100 ng/ml), TLR7 (R848, 5 μ g/ml), TLR9 (K and D ODN, 3 μ M and 1 μ M), STING (2'3' cGAMP, 30 μ g/ml), IFI16-STING (HSV, 5 μ g/ml) and AIM2 inflammasome, RIG-I/MAVS (p(dA:dT), 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- γ levels from surface TLR stimulation. **B.** IFN- γ levels from endosomal TLR stimulation. **C.** IFN- γ levels from inflammasome pathway stimulation.

3.1.3.3 CD27 deficient patients had normal IL-1 β responses, but elevated TNF- α responses from endosomal TLR and inflammasome pathways were observed

Pro-inflammatory cytokine secretion from cells through TLR and PRR stimulations were revealed that IL-1 β production levels of patients' PBMCs were similar to healthy controls (**Figure 3.14**). However, only Patient-2 showed increased TNF- α secretion when stimulated with TLR4, TLR5, TLR7, STING and non-canonical inflammasome pathways were triggered (**Figure 3.15**).

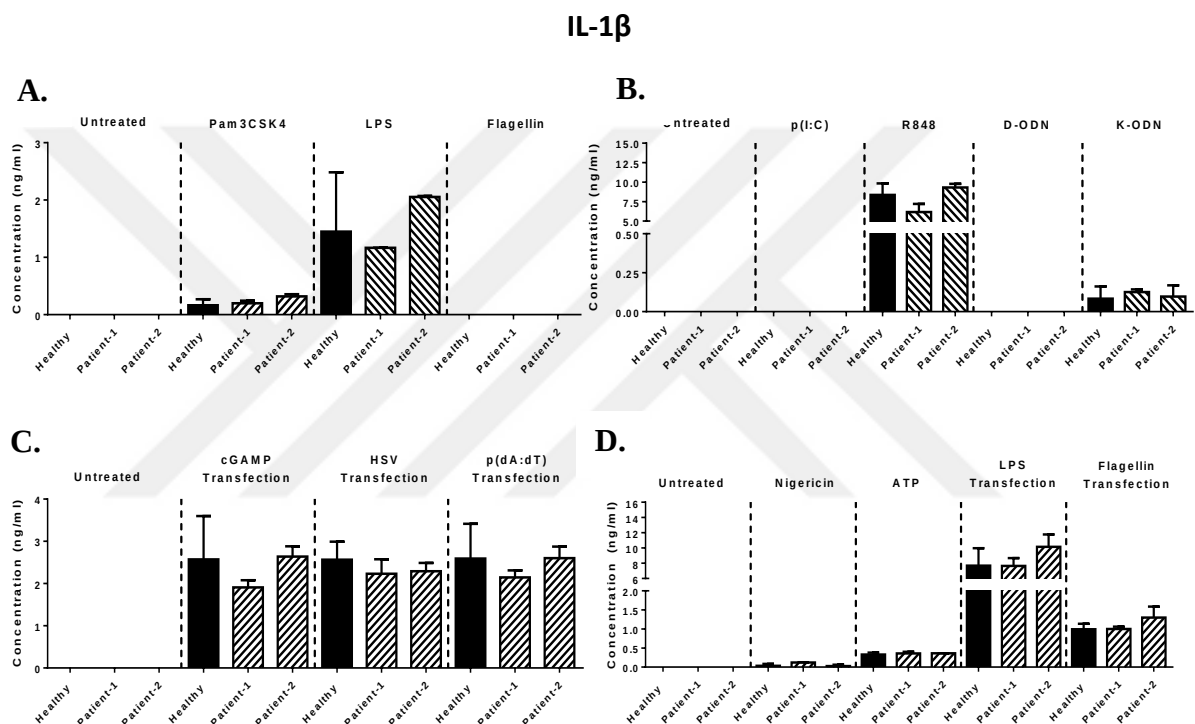


Figure 3.14 IL-1 β cytokine production profile of PBMCs from CD27 deficient patients and healthy controls (n=2) upon PRR stimulations for 24 hours.

Patients and healthy controls PMBCs were stimulated with TLR1/2 (Pam3CSK4, 1 μ g/ml), TLR3 (p(I:C), 30 μ g/ml), TLR4 (LPS, 5 μ g/ml), TLR5 (Flagellin, 100 ng/ml), TLR7 (R848, 5 μ g/ml), TLR9 (K and D ODN, 3 μ M and 1 μ M), STING (2'3' cGAMP, 30 μ g/ml), IFI16-STING (HSV, 5 μ g/ml) and AIM2 inflammasome, RIG-I/MAVS (p(dA:dT), 5 μ g/ml) Nigericin/ATP (NLRP3, 2 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IL-1 β levels from surface TLR stimulation. **B.** IL-1 β levels from endosomal TLR stimulation. **C-D.** IL-1 β levels from inflammasome pathway stimulation.

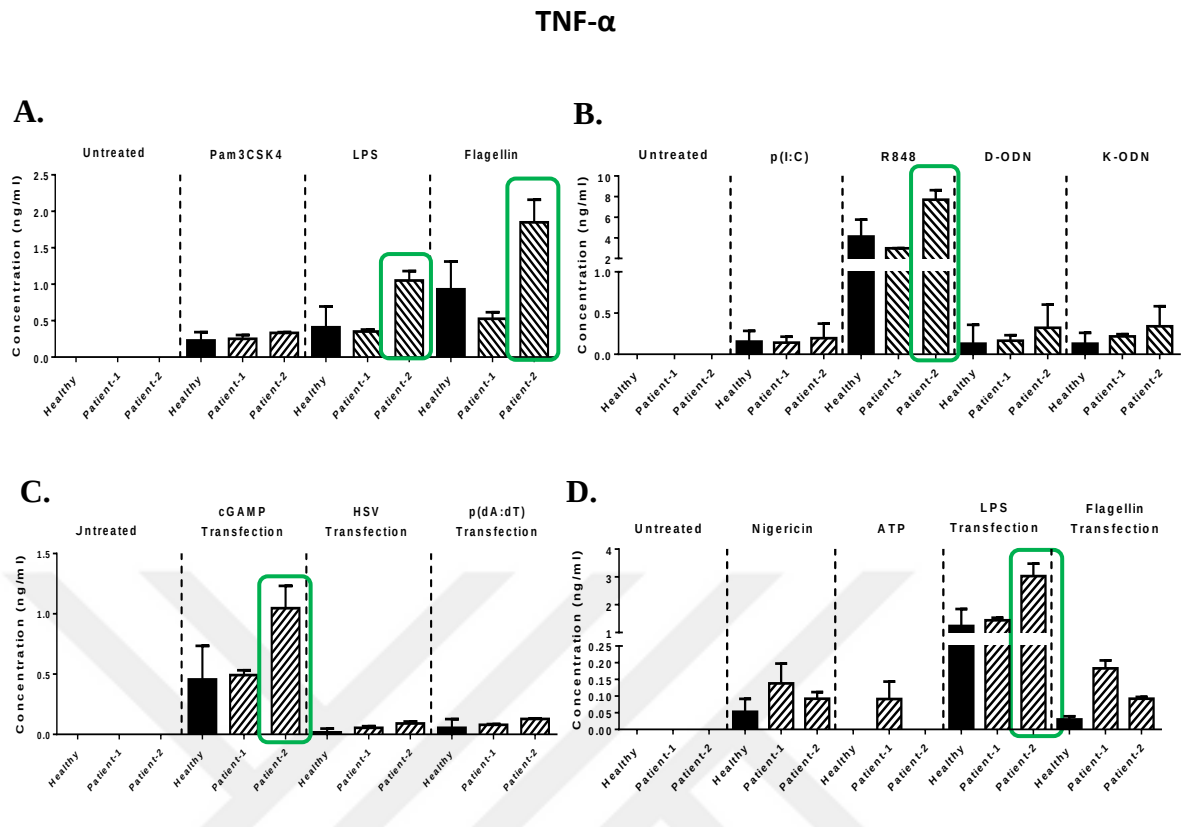


Figure 3.15 TNF- α cytokine production profile of PBMCs from CD27 deficient patients and healthy controls (n=2) upon PRR stimulations for 24 hours.

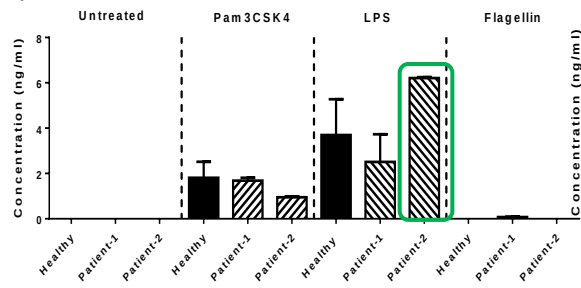
Patients and healthy controls PMBCs were stimulated with TLR1/2 (Pam3CSK4, 1 μ g/ml), TLR3 (p(I:C), 30 μ g/ml), TLR4 (LPS, 5 μ g/ml), TLR5 (Flagellin, 100 ng/ml), TLR7 (R848, 5 μ g/ml), TLR9 (K and D ODN, 3 μ M and 1 μ M), STING (2'3' cGAMP, 30 μ g/ml), IFI16-STING (HSV, 5 μ g/ml) and AIM2 inflammasome, RIG-I/MAVS (p(dA:dT), 5 μ g/ml) Nigericin/ATP (NLRP3, 2 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** TNF- α levels from surface TLR stimulation. **B.** TNF- α levels from endosomal TLR stimulation. **C-D.** TNF- α levels from inflammasome pathway stimulation.

3.1.3.4 Normal IL-10 secretion was observed upon inflammasome pathway stimulations, increased response was seen in TLR4 and TLR9 activation

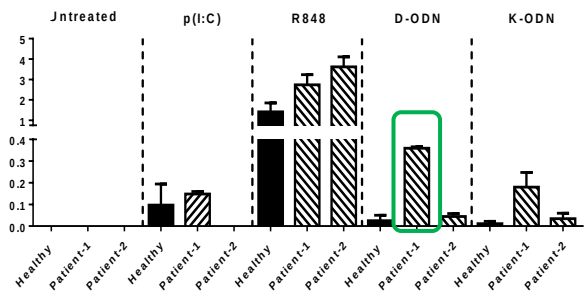
Anti-inflammatory responses of PBMCs were investigated through TLR and intracellular danger signals. Even though upon inflammasome pathway stimulations no differences were observed in IL-10 secretion, elevated immune response was detected with TLR4 (in Patient-2) and TLR9 activation (in Patient-1) (**Figure 3.16**).

IL-10

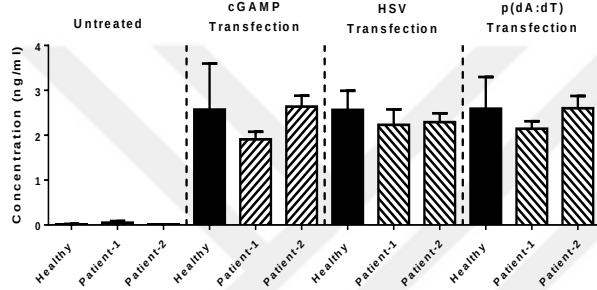
A.



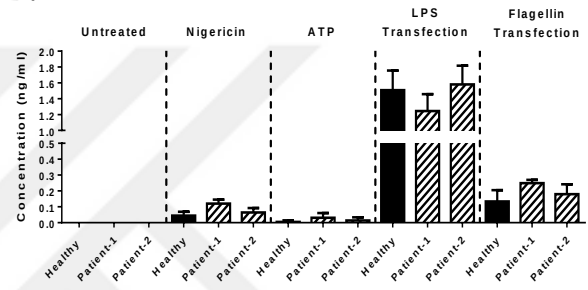
B.



C.

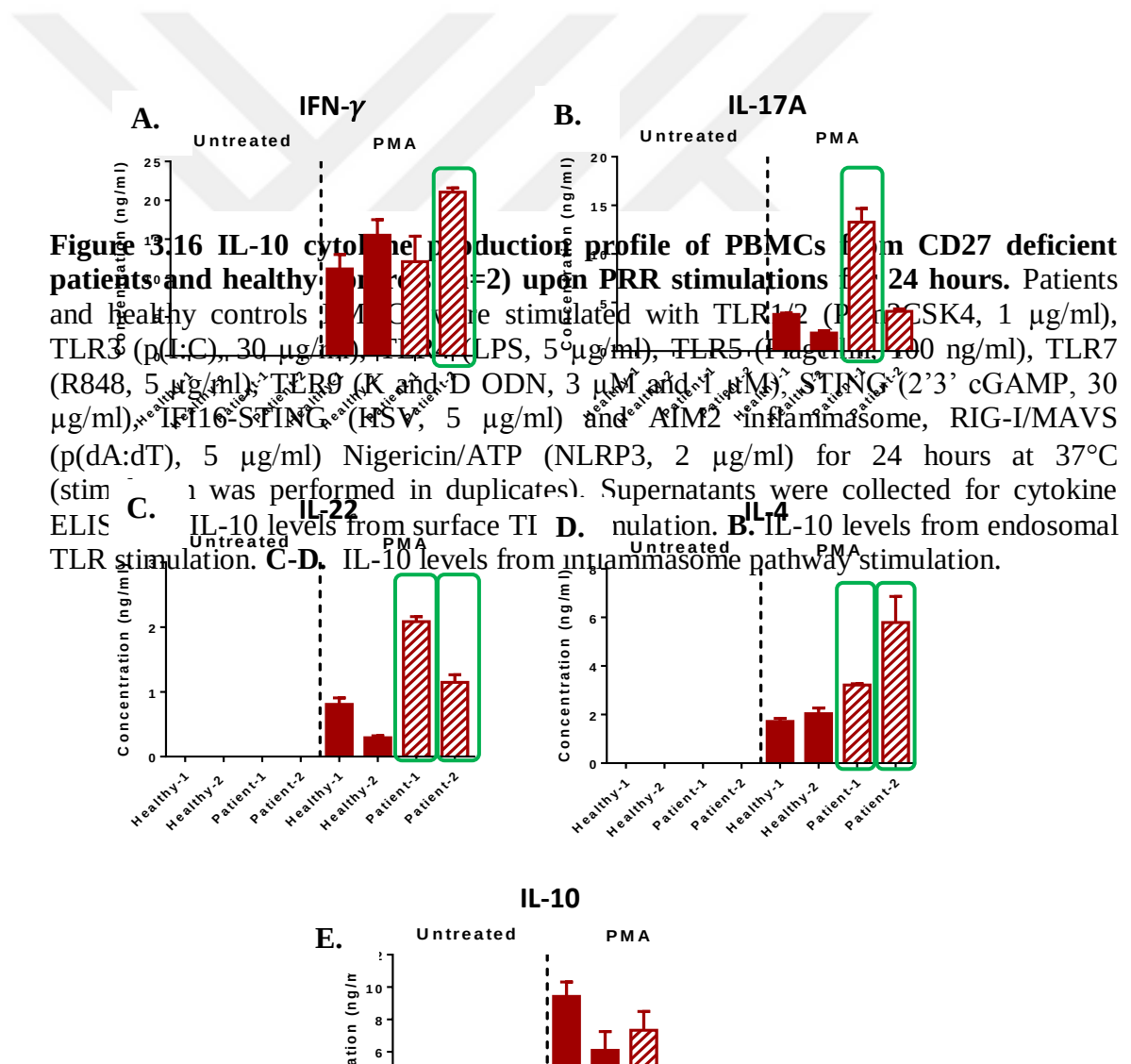


D.



3.1.3.5 Increased IFN- γ , IL-4 and IL-17a, decreased IL-10 responses were observed in patients upon PMA/I stimulations of PBMCs

To activate specifically T cells within PBMCs, cells from patients and controls were treated with PMA/I for 24 hours and cytokine production profiles were investigated with ELISA. Increased IFN- γ response from especially CD8⁺ T cells was observed in Patient-1 (**Figure 3.17-A**). Both CD27 deficient patients had increased IL-17a and IL-22 response from NK, NKT and T cells (**Figure 3.17-B,C**) and IL-4 secretion from Th2 subset CD4⁺ T cells (**Figure 3.17-D**). Patients had decreased IL-10 responses upon PMA treatment that was expected since decreased STAT5 phosphorylation was observed in patients (**Figure 3.17-E**).



3.1.4 Investigation of Neutrophil Activities in CD27 Deficiency

Since in autoimmune diseases neutrophil activities are increased, immunological activities of from neutrophils CD27 deficient patients were investigated.

3.1.4.1 CD27 deficient patients had normal NETotic tendencies and respiratory burst

To investigate NETosis under basal levels and stimulated conditions by fluorometric and microscopic methods, neutrophils isolated from patients and healthy controls were either treated with PMA for 4 hours or left for untreated. Following 4-hour stimulation, both extracellular and intracellular double stranded DNAs were stained to visualized with fluorescent microscopy.

Untreated neutrophils from both patients and healthy controls, spontaneous protruded NET-like formation was not observed. PMA stimulated neutrophils were shown similar tendencies between healthy controls and patients. Patients' neutrophils were able to induce dsDNA formation to extracellular environment when microorganism infection was mimicked with PMA stimulation (**Figure 3.18-A**).

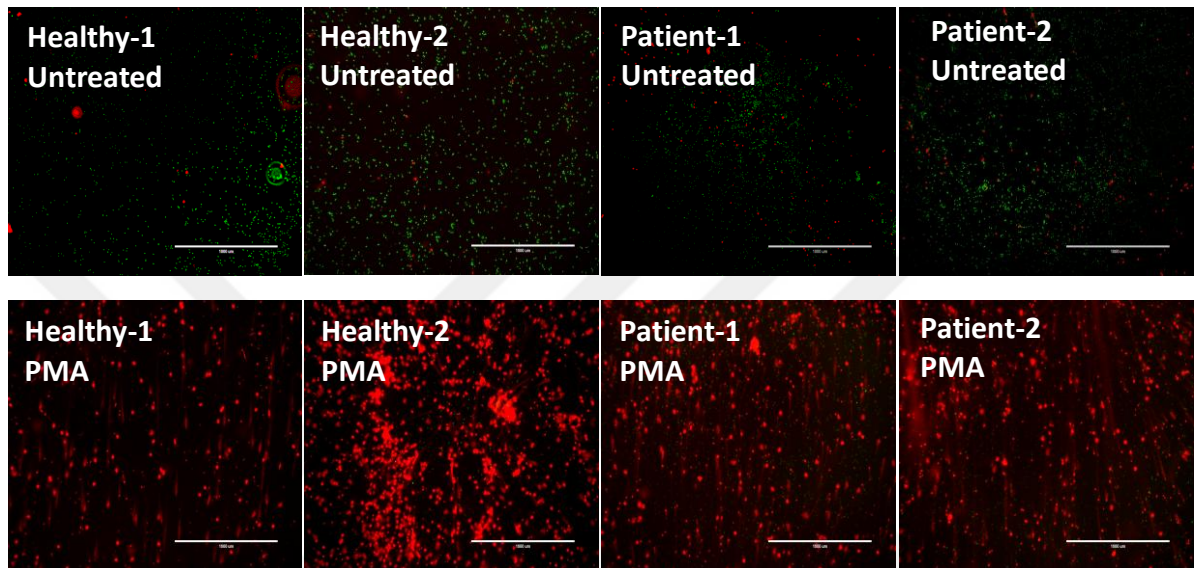
To quantify released dsDNA from untreated and PMA treated healthy and patient neutrophils, fluorometric investigation was performed with supernatants from neutrophil stimulations. Released dsDNA was first treated with Micrococcal Nuclease (non-specific) and then stained with dsDNA binding dye Picogreen. Both healthy controls and patients' neutrophils expelled 300-500 ng/ml dsDNA to extracellular space when unstimulated. With PMA treatment, no difference in the released dsDNA was observed between healthies and patients. Both were able to induce NET-like formation in the range 900-1200 ng/ml (**Figure 3.18-B**).

Analysis of respiratory burst abilities of neutrophils was performed with Dihydrorhodamine 123 (DHR123)-FITC conjugated antibody that is used as reactive oxygen species (ROS). Since stimulated neutrophils generate reactive oxygen species through NADPH oxidase (NOX2) activation, it is an efficient way to determine neutrophil activation through mimicking infection and analyzing ROS formation. Neutrophils isolated from patients and healthy controls were stimulated with PMA or left for untreated for 4 hours. Following treatment with PMA, they were stained with DHR123-FITC at 37°C for 15 minutes and analyzed by flow

cytometry. No difference was observed in untreated and PMA treated neutrophils of healthies and patients.

When all considered, no abnormalities were observed in patients' neutrophil activities (**Figure 3.19**).

A.



B.

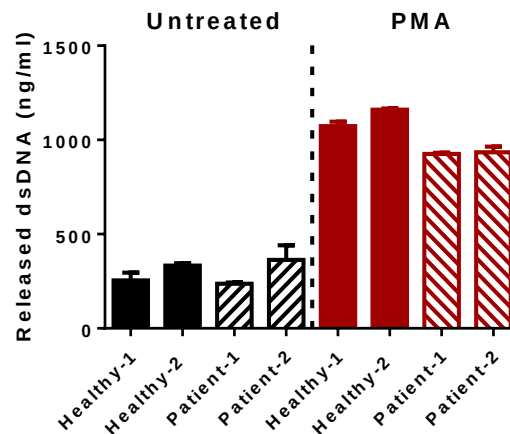


Figure 3.18 Microscopic and fluorometric analysis of CD27 deficient patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left untreated for 4 hours and NET formation was analyzed by staining extracellular DNA (represented in red) with SytoxOrange and nuclear DNA (represented in green) with Syto16Green. Quantification of released dsDNA was determined by micrococcal nuclease analysis. **A.** Representative immunofluorescent images of neutrophils captured at 4X. **B.** Quantification of released dsDNA.

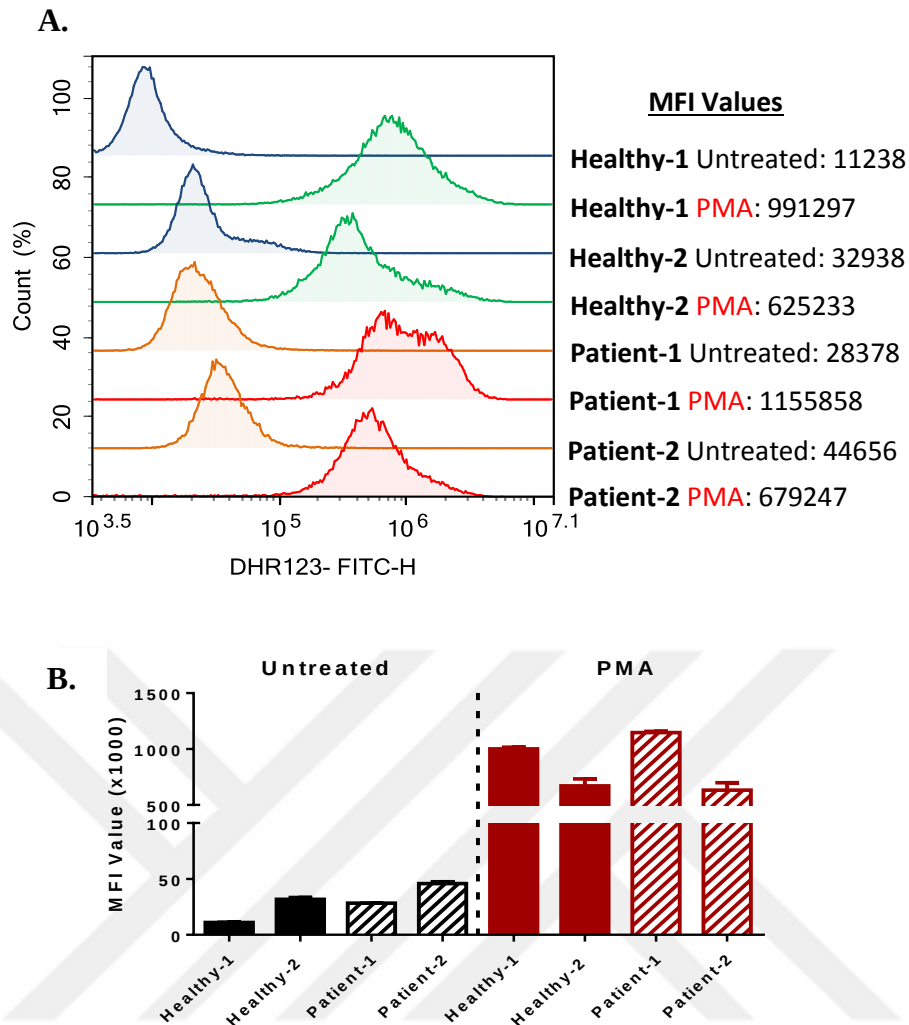


Figure 3.19 Determination of respiratory burst of CD27 deficient patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left for untreated for 4 hours and respiratory burst was analyzed by staining neutrophils with DHR123. **A.** Mean fluorescence intensity plot of DHR123⁺ neutrophils. **B.** Representative bar graph.

3.2 Characterization of Innate and Adaptive Immune Responses in

STAT1 GOF Patients (PART-I)

3.2.1 Determination of STAT1 Phosphorylation and De-phosphorylation

3.2.1.1 STAT1 GOF patients had increased STAT1 phosphorylation and late de-phosphorylation

Since STAT1 GOF disease is mainly characterized as hyperphosphorylation and delayed dephosphorylation [67], we first aimed to check STAT1 phosphorylation levels of patients. Whole blood from STAT1 GOF patients and healthy controls were stimulated with type I IFN and phosphorylation and dephosphorylation of STAT1 levels of CD4⁺ T cells were assessed by flow cytometry analysis. Hyperphosphorylation of STAT1(3-fold increase in patients) and delayed and not complete dephosphorylation were observed in all STAT1 GOF patients compared to healthy controls (**Figure 3.20**). In addition, increased pSTAT1⁺ cell percentage (2-fold) within CD4⁺ T cell gate was also observed STAT1 GOF patients (**Figure 3.21**).

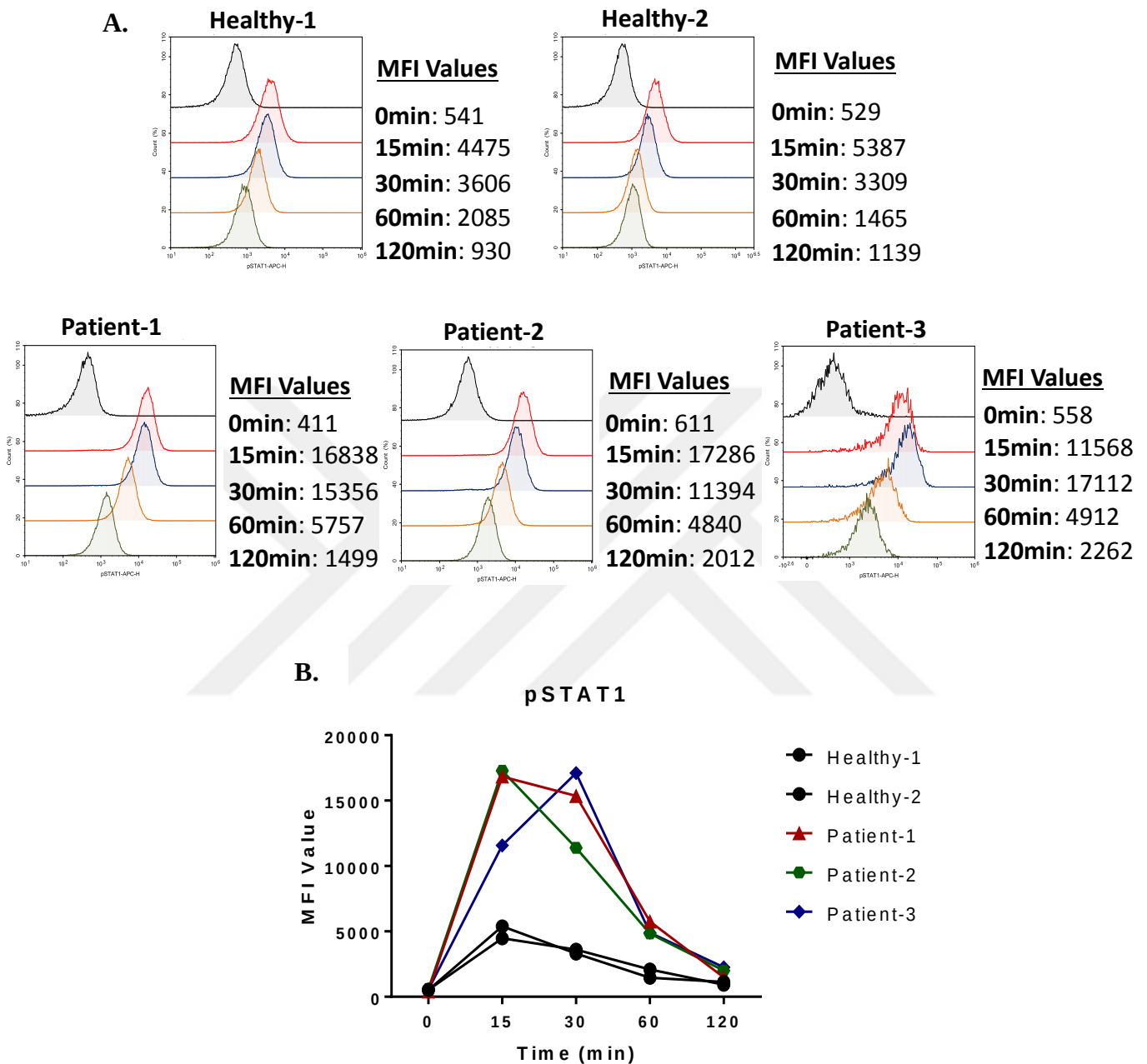
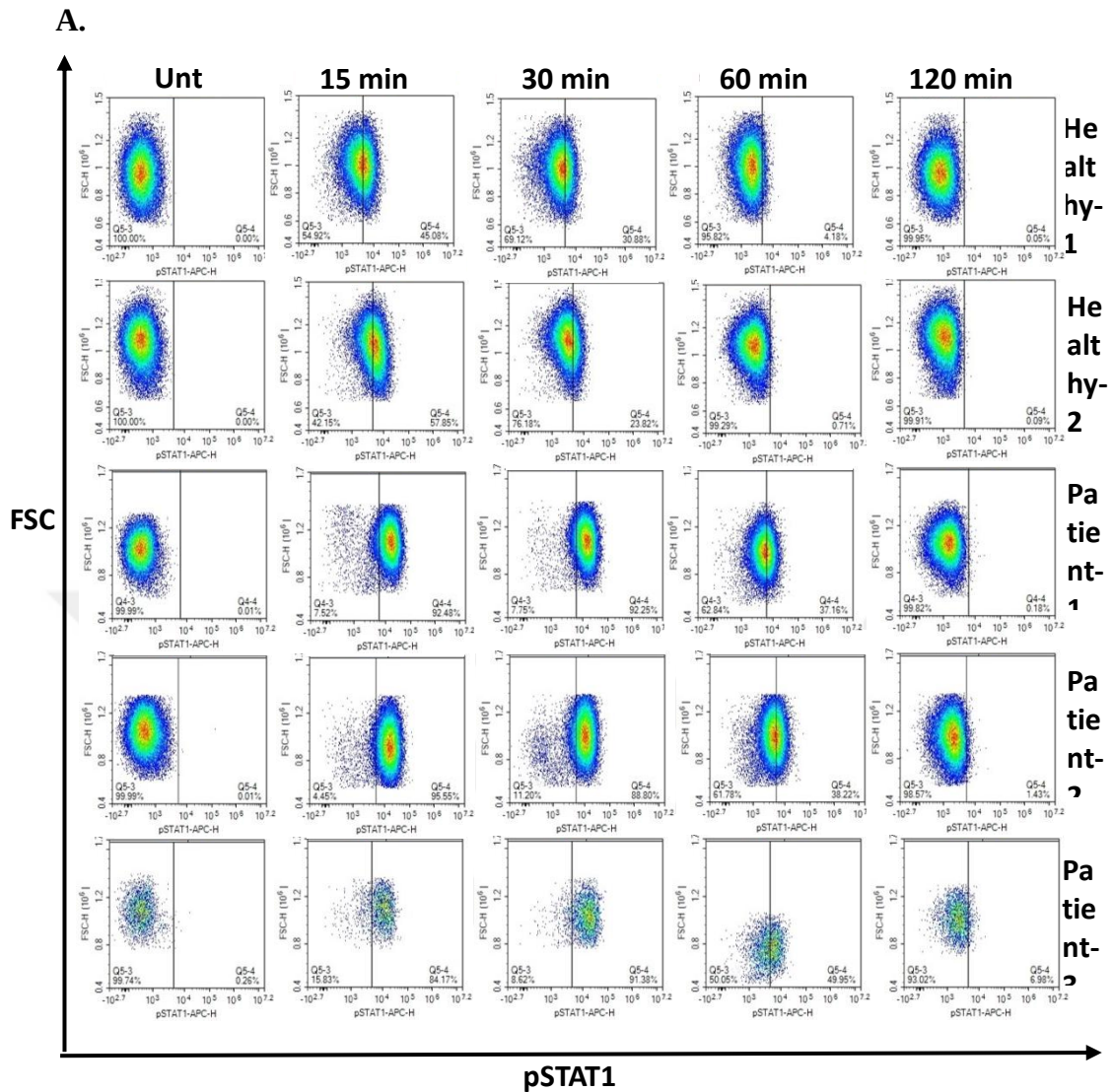


Figure 3.20 STAT1 phosphorylation levels of STAT1 GOF patients and healthy controls' CD4⁺ T Cells. Patients and healthy controls' whole blood (200 μ l) were treated with IFN- β (100 ng/ml) or left untreated over 15, 30, 60 and 120 minutes. Fixation, permeabilization and co-staining of anti-human CD4 and anti-human pSTAT1 were performed and phosphorylation levels of pSTAT1 proteins were analyzed by flow cytometry by acquiring 4×10^4 cells within CD4⁺ cell gate. **A.** Mean fluorescence intensity plot of pSTAT1 proteins. **B.** Representative line graph.



B. pSTAT1 positive CD4⁺ T Cells

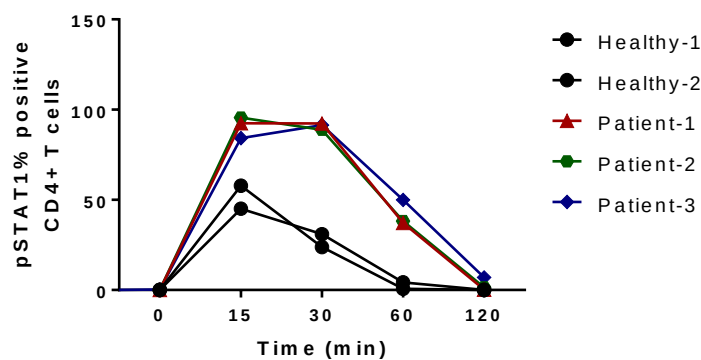
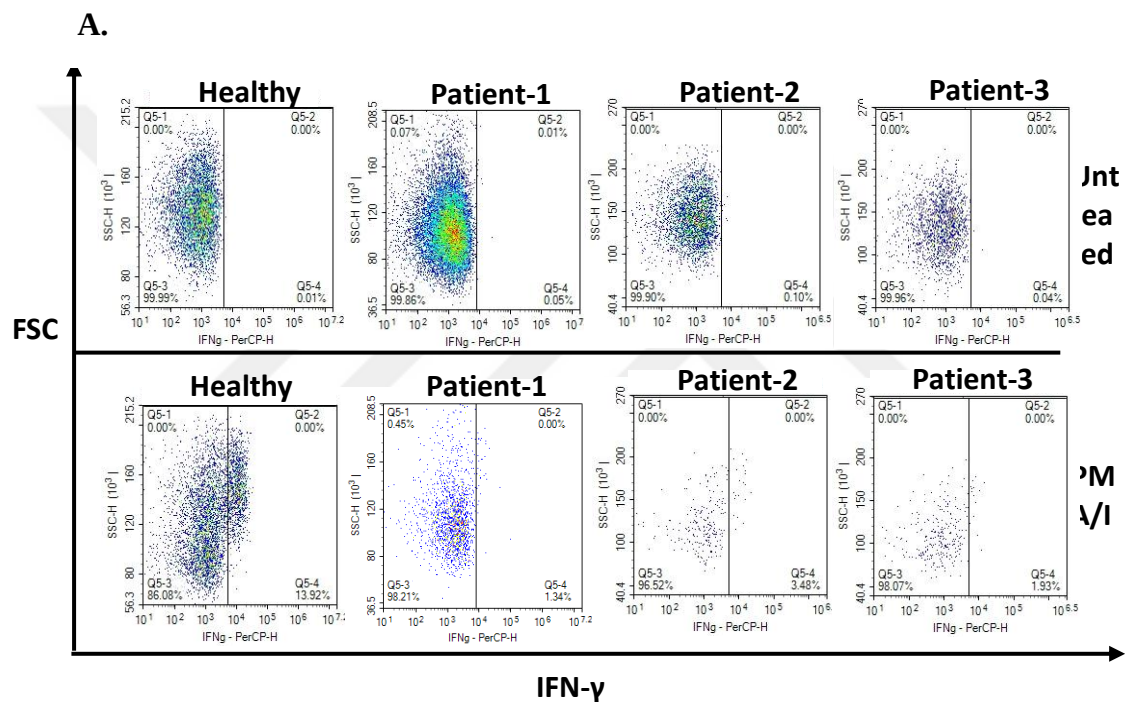


Figure 3.21 Percentage of pSTAT1⁺ cells within CD4⁺ T cells from STAT1 GOF patients and healthy controls after IFN- β stimulation. A. Density plots of pSTAT1 proteins within CD4⁺ T cells analyzed by flow cytometry. **B.** Representative line graph.

3.2.2 Investigation of Th1 and Th17 Balance

3.2.2.1 Decreased IFN- γ and IL-17a responses were observed in STAT1 GOF patients upon PMA/I treatment

STAT1 GOF deficiency is generally engaged with chronic mucocutaneous candidiasis and impaired Th17 response [68]. Therefore, we investigated Th1 and Th17 responses in STAT1 GOF patients. Even though increased STAT1 phosphorylation is present, STAT1 GOF patients had $\cong$ 4- fold decreased IFN- γ response upon PMA (Figure 3.22). As expected, Th17 response was impaired in all STAT1 patients ($\cong$ 2-fold less than controls) (Figure 3.23).



B.

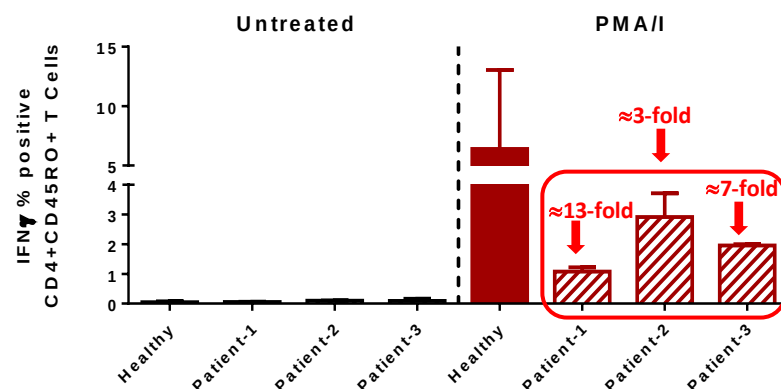


Figure 3.22 Th1 cytokine responses of STAT1 GOF patients and healthy controls (n=2) upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4 and anti-human IFN- γ were performed and analyzed by flow cytometry evaluating 3×10^4 cells within CD4⁺ T cell gate. **A.** Density plots of IFN- γ in PBMCs of healthy controls and patients. **B.** Representative bar graph.

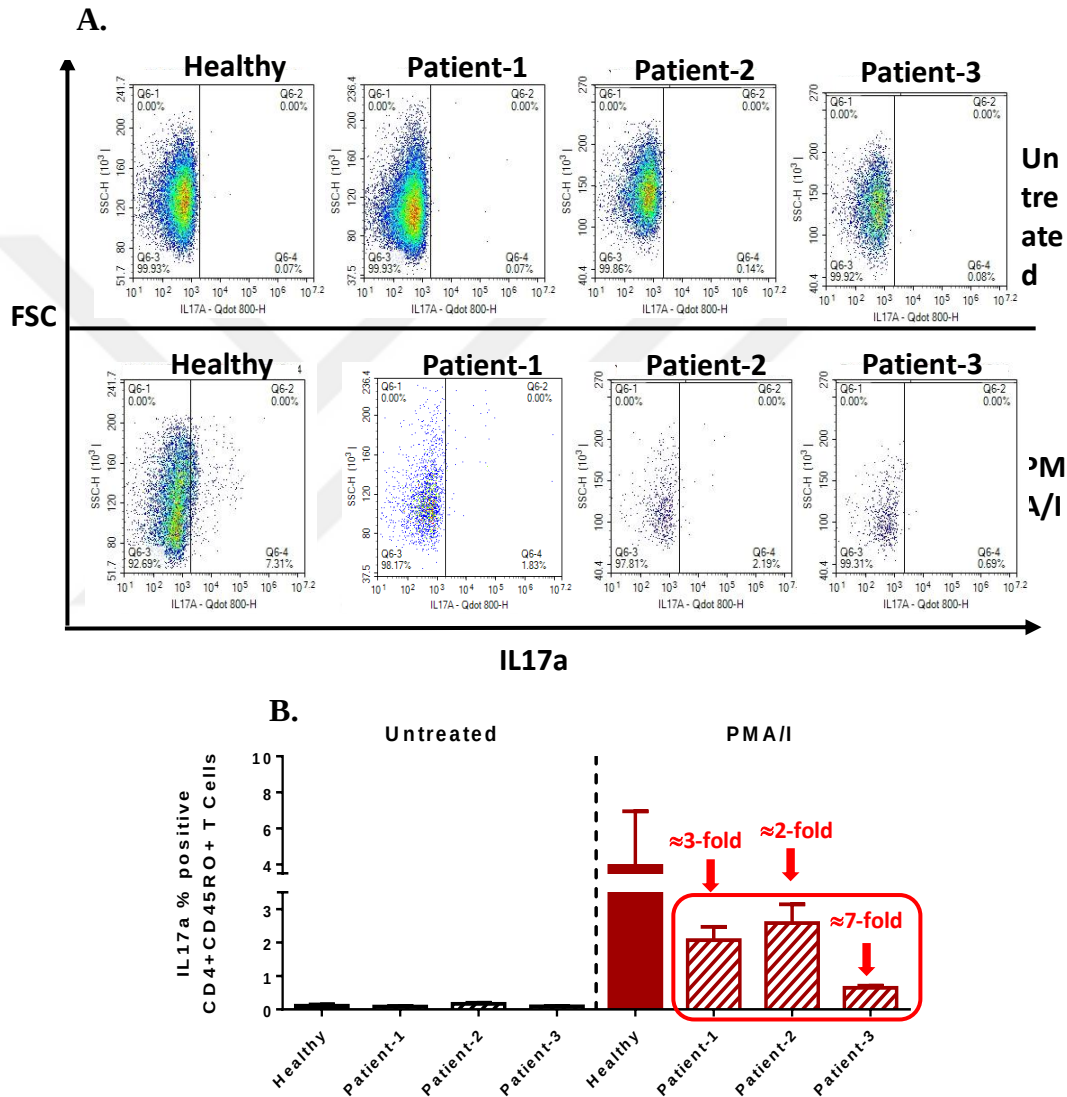
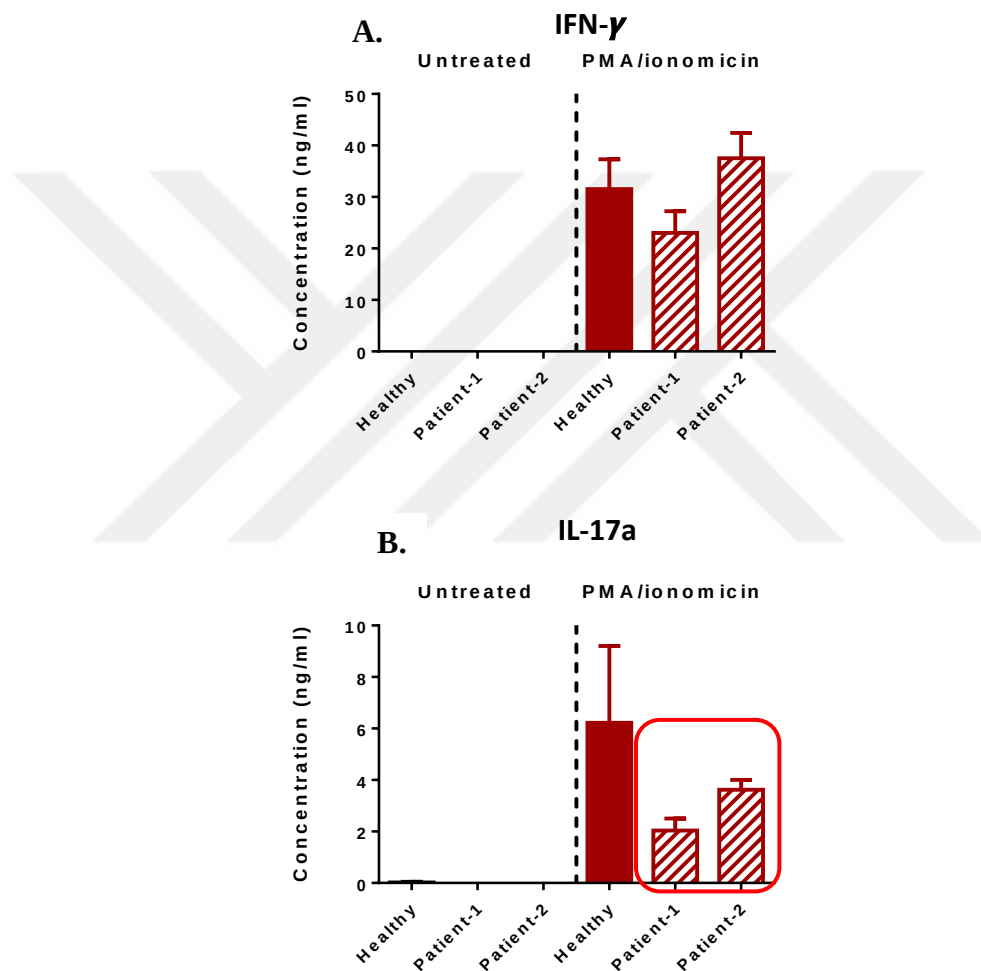


Figure 3.23 Th17 cytokine responses of STAT1 GOF patients and healthy controls (n=2) upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4 and anti-human IL-17a were performed and analyzed by flow cytometry evaluating 3×10^4 cells within CD4⁺ T cell gate. **A.** Density plots of IL-17a in PBMCs of healthy controls and patients. **B.** Representative bar graph

3.2.3 Analysis of Cytokine Secretion Profile upon PRR Stimulations of PBMCs

3.2.3.1 Normal IFN- γ but decreased IL-17a secretion was determined in STAT1 GOF patients

To determine innate arm of abnormalities in STAT1 GOF patients, PBMCs from healthy controls and patients were stimulated with different TLR and PRR ligands



and cytokine secretion profiles of cells were analyzed by ELISA. Upon PMA/I treatment

T cells, NK and NKT cells of patients were able to induce IFN- γ secretion while IL-17a secretion from these cells was impaired. STAT1 GOF patients had 3-fold

Figure 3.24 IFN- γ and IL-17a cytokine production of PBMCs from STAT1 GOF patients and healthy controls upon PMA/I treatment for 24 hours.

Patients and healthy controls PMBCs were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- γ , **B.** IL-17a levels.

decreased IL-17a secretion compared to healthy controls (**Figure 3.24**).

3.2.3.2 STAT1 GOF patients had normal Type I IFN responses

Since STAT1 GOF patients have impaired immune responses against infections, we tried to determine whether this predisposition is coming from dysregulation in type I IFN immune response. Infection of cells were mimicked through stimulation of PBMCs through endosomal TLR and PRR ligands. Normal IFN- α secretion was observed in STAT1 GOF patients (**Figure 3.25**).

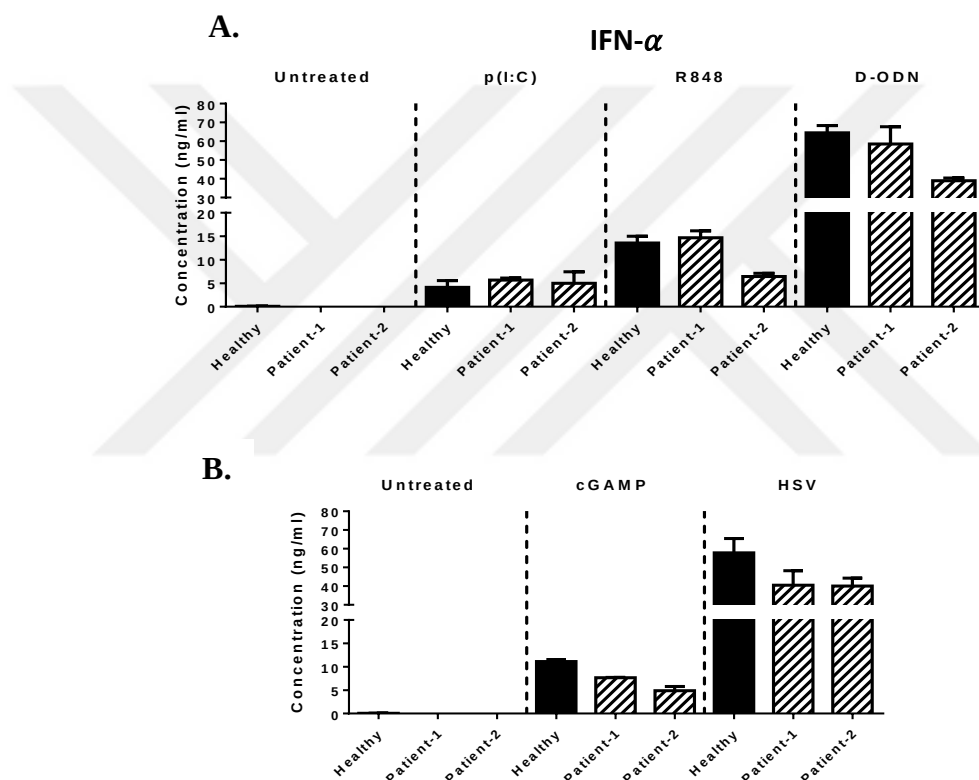


Figure 3.25 IFN- α cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours.

Patients and healthy controls PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (D ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- α levels from TLR stimulation. **B.** IFN- α levels from inflammasome pathway stimulation

3.2.3.3 Decreased IFN- γ but mostly normal IL-12 levels were observed in STAT1 GOF patients

STAT1 GOF patients had normal levels of endosomal TLR induced IL-12 levels while type II secretion was found to be decreased for endosomal TLR and inflammasome pathway trigger. Both CD27 deficient patient had low levels of IL-12 against stimulation of STING while Patient-1 had high, Patient-2 had decreased level for IFI16 pathway activation. Normal IL-12 but decreased IFN- γ levels force us to think about dysregulation of IL-12 induced Th1 differentiation.

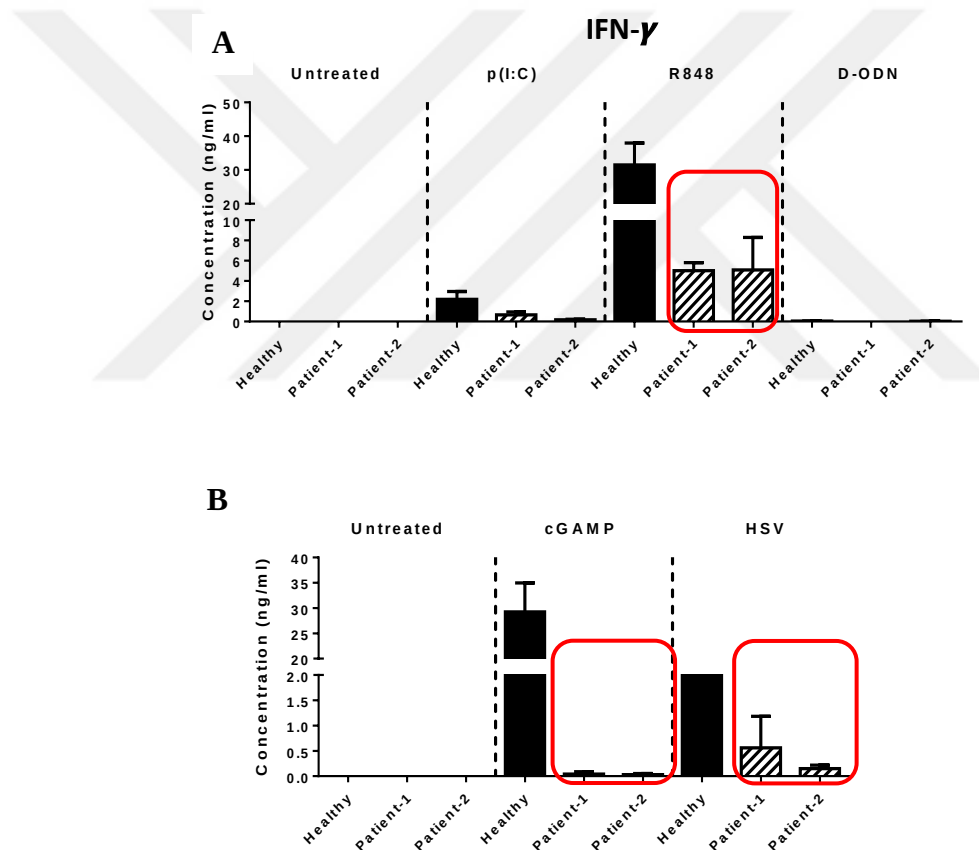


Figure 3.26 IFN- γ cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (D ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.**IFN- γ levels from TLR stimulation. **B.** IFN- γ

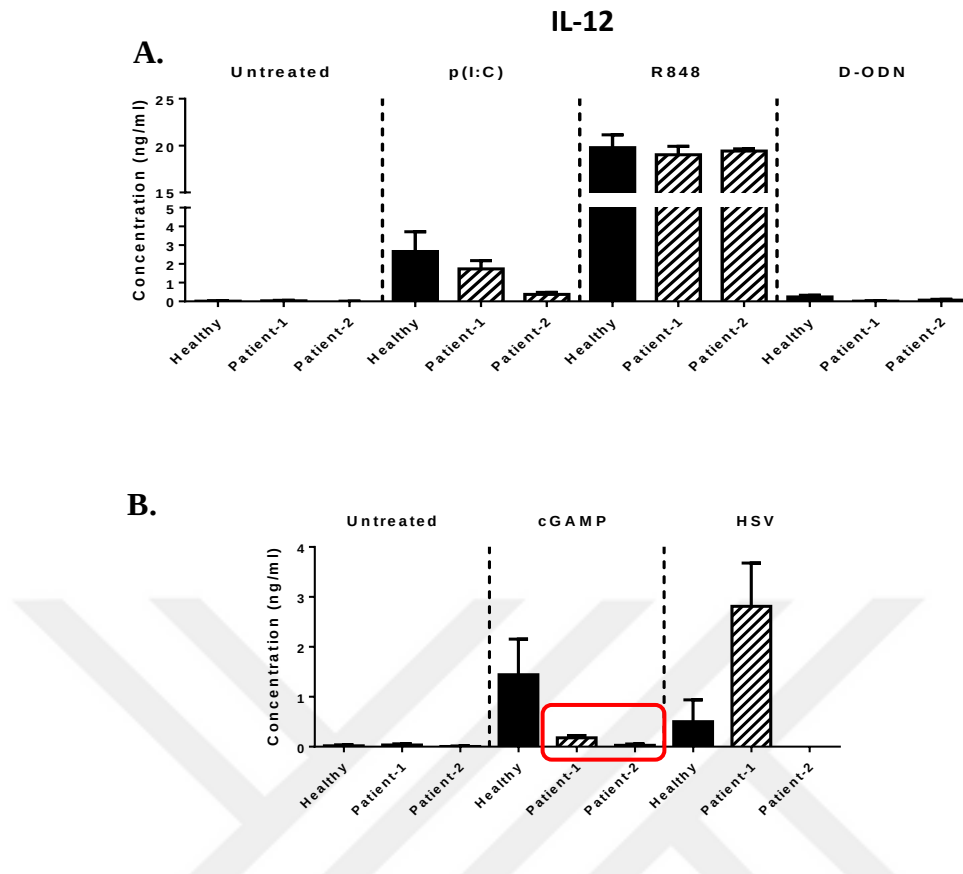


Figure 3.27 Figure 25: IL-12 cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (D ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IL-12 levels from TLR stimulation. **B.** IL-12 levels from inflammasome pathway stimulation.

3.2.4 Investigation of Neutrophil Activities in STAT1 GOF Patients

3.2.4.1 Microscopically decreased NET-like formation was determined in STAT1 GOF patient upon PMA treatment

Even though innate and adaptive responses of STAT1 GOF patients were mainly studied, there is no information regarding to neutrophil activities. For this reason, we decided to work on neutrophil activities of STAT1 GOF patients in terms of NET formation and respiratory burst capabilities. At basal level, microscopically healthy controls and patients did not have protruded NET-like formations. But, upon PMA treatment, neutrophils coming from Patient-3 were not able to induce dsDNA into

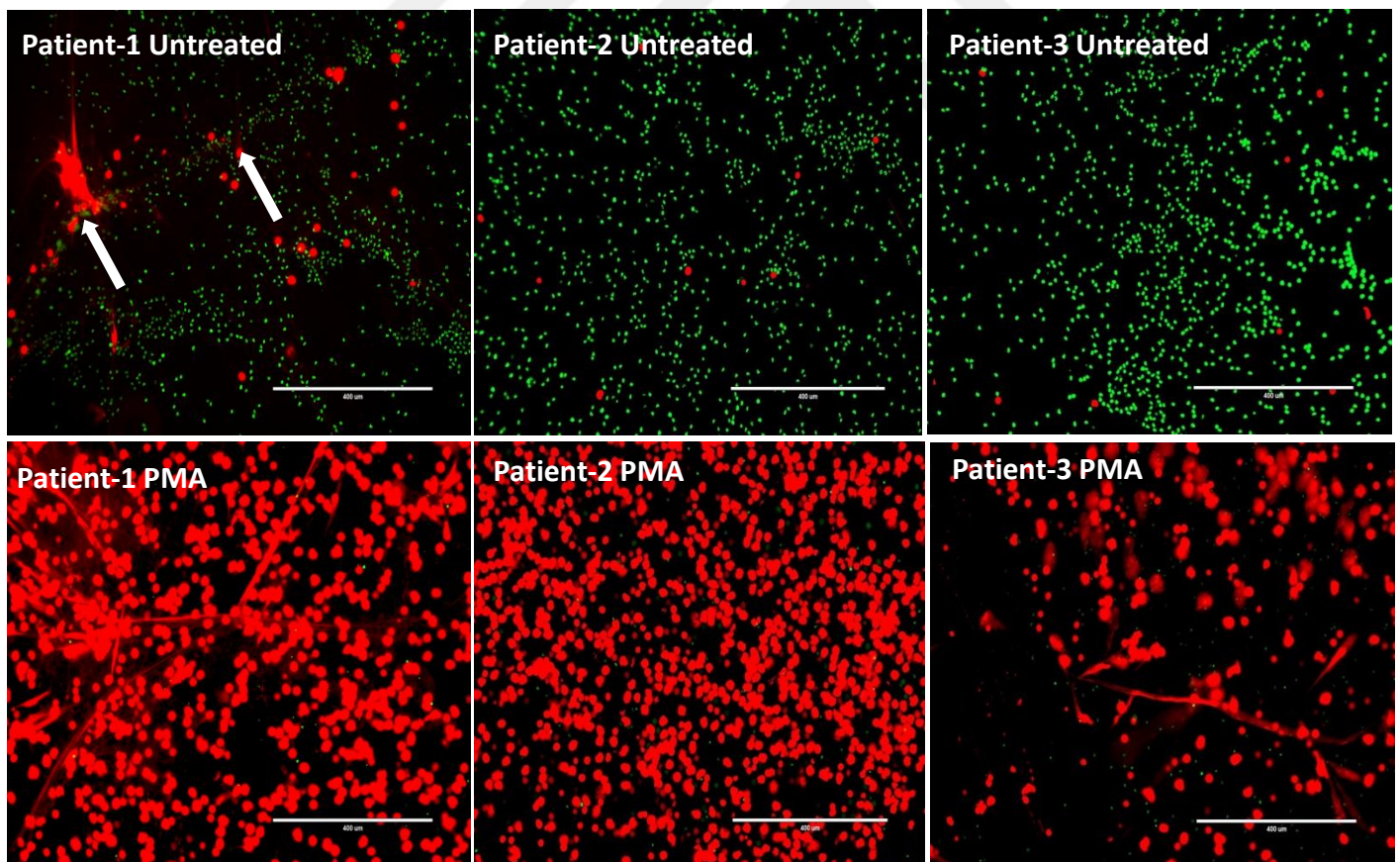
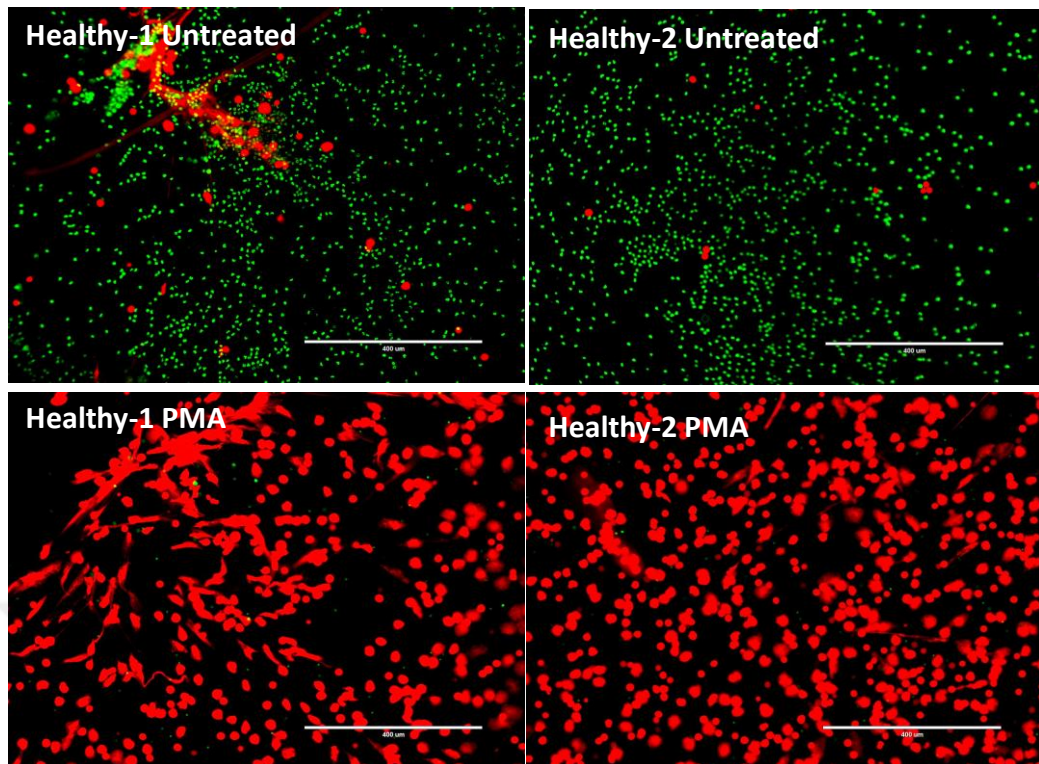
extracellular space compared to healthy controls both fluorometrically and microscopically (**Figure 3.28**).

3.2.4.2 Normal respiratory burst was observed in untreated neutrophils of STAT1 GOF patient

Out of three, only Patient-1 had higher capacity to have respiratory burst at basal level. All STAT1 GOF patients had normal level of respiratory burst upon PMA treatment (**Figure 3.29**).



A.



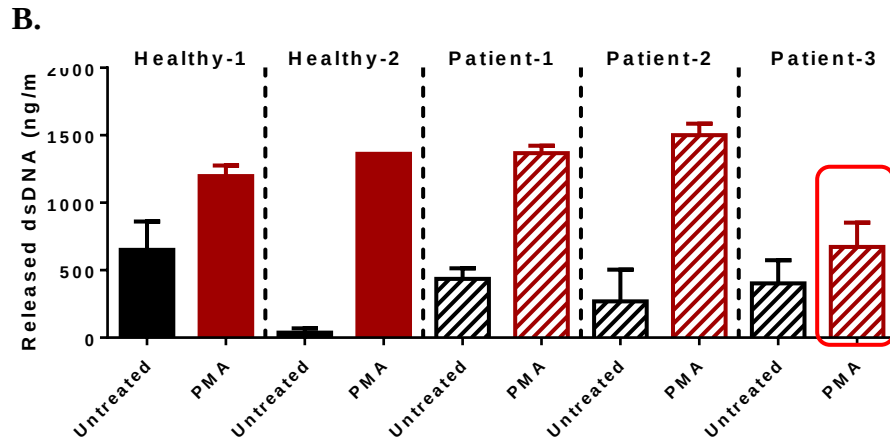


Figure 3.28 Microscopic and fluorometric analysis of STAT1 GOF patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50 ng/ml) or left untreated for 4 hours and NET formation was analyzed by staining extracellular DNA (represented in red) with SytoxOrange and nuclear DNA (represented in green) with Syto16Green. Quantification of released dsDNA was determined by micrococcal nuclease analysis. **A.** Representative immunofluorescent images of neutrophils pictures captured at 4X. **B.** Quantification of released dsDNA.

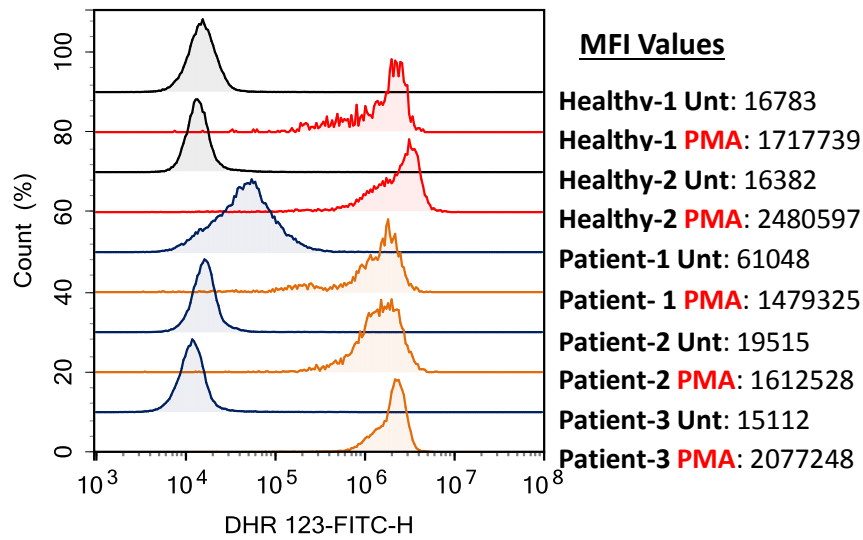
3.3 Characterization of Innate and Adaptive Immune Responses in STAT1 GOF Patients (PART-II)

3.3.1 Determination of Percentages of Immune Cells in Whole Blood

3.3.1.1 Reversed CD4/CD8 ratio was observed in STAT1 GOF patients

CD4⁺/CD8⁺ cell ratio was ranged between 1.5 to 3 for healthy controls. All STAT1 GOF patients had reversed ratio (Patient-1:0.6, Patient-2: 1, Patient-3: 0.5) (**Figure 3.30**).

A.



B.

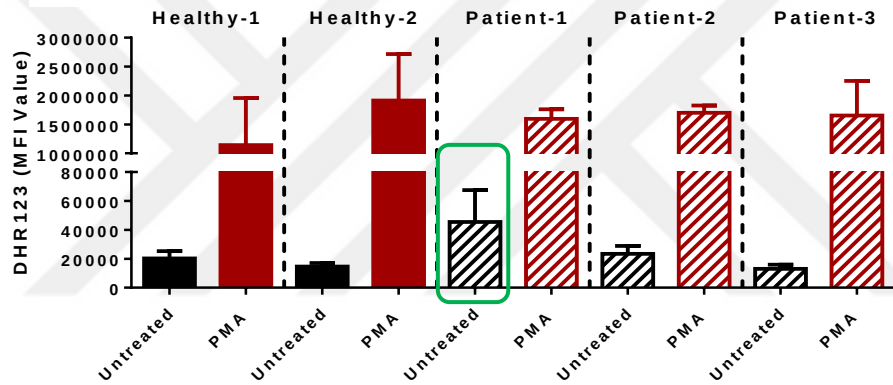


Figure 3.29 Determination of respiratory burst of STAT1 GOF patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left untreated for 4 hours and respiratory burst was analyzed by staining neutrophils with DHR123. **A.** Mean fluorescence intensity plot of DHR123⁺ neutrophils. **B.** Representative bar graph.

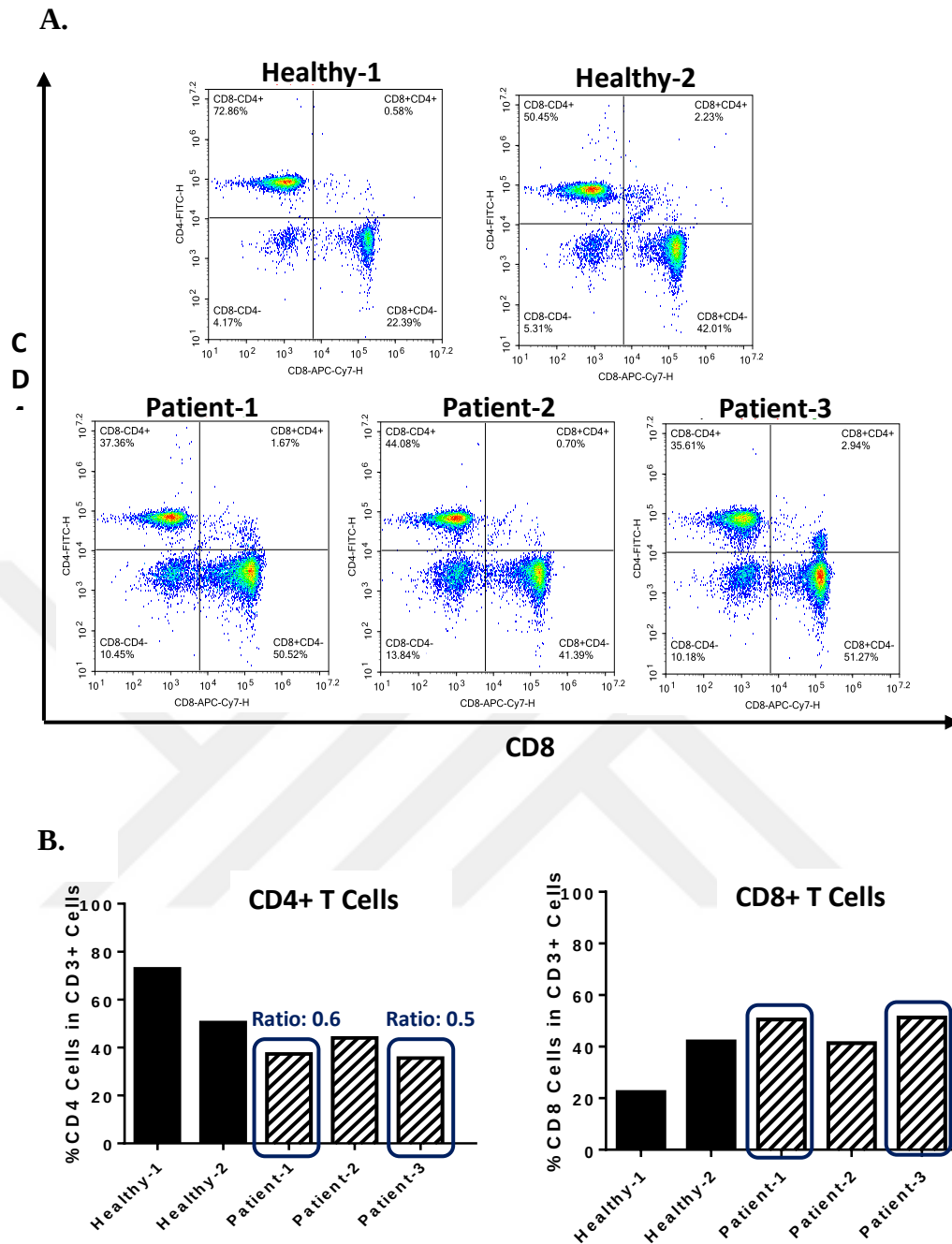


Figure 3.30 CD4 and CD8 surface expression of $CD45^+CD3^+$ cells. The whole blood of healthy controls (100 μ l) and STAT1 GOF patients were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, anti-human CD4 and anti-human CD8 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of $CD45^+CD3^+$ $CD4^{+/-}$ and $CD8^{+/-}$ cells. **B.** Representative bar graphs of $CD4^+$ and $CD8^+$ cell percentages in $CD3^+$ cells for healthy controls and patients.

3.3.1.2 STAT1 GOF patients had decreased Treg percentage

Whole blood from patients and healthy controls were stained with anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD25 anti-human CD127 for analysis of Treg cell percentage by flow cytometry. Patient-1 and Patient-3 had 2.5-fold decreased Treg cells compared to healthy subjects (**Figure 3.31**).

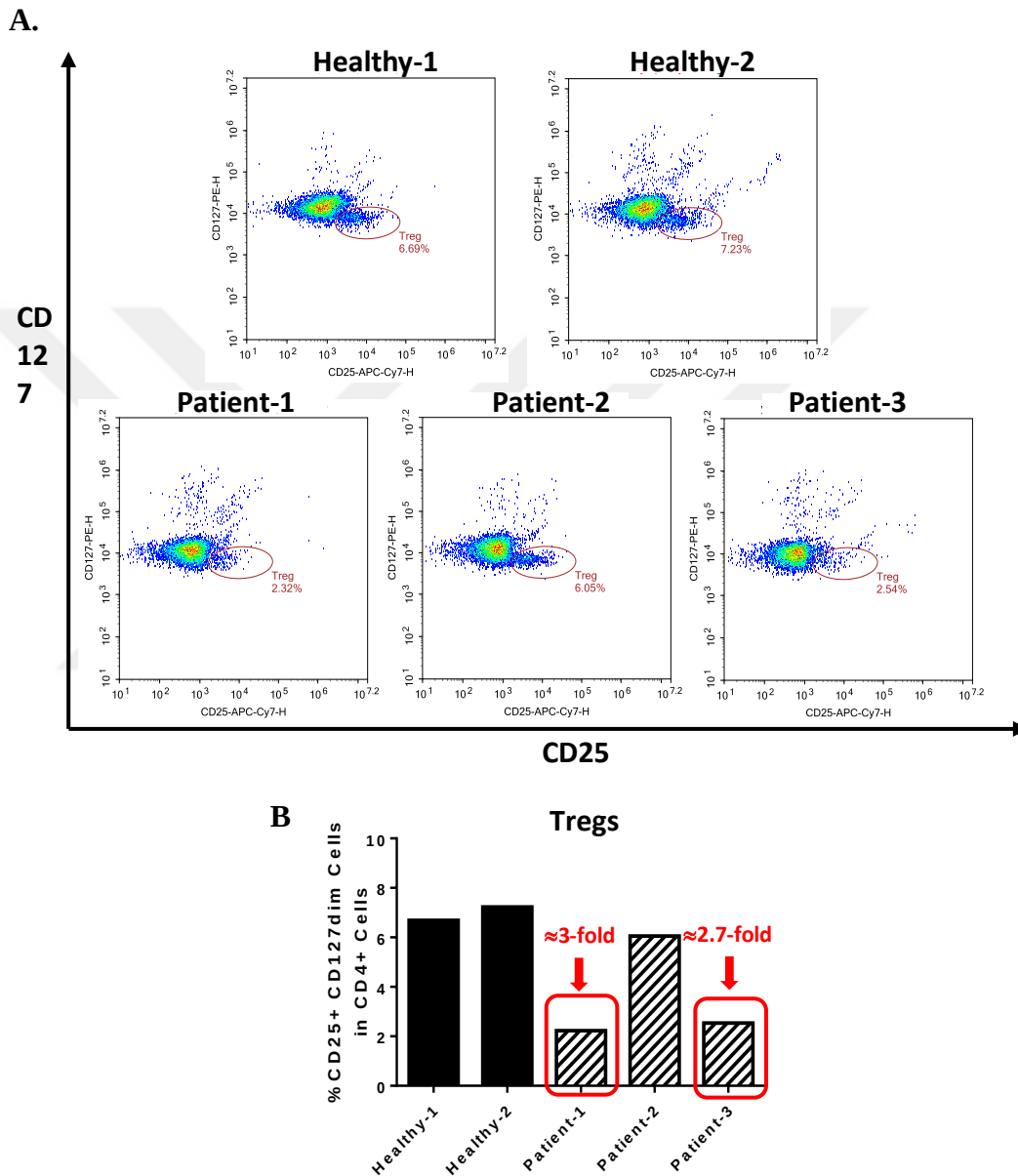


Figure 3.31 CD25 and CD127 surface expression of CD45⁺CD3⁺CD4⁺ cells. The whole blood of healthy controls (100 μ l) and STAT1 GOF patients were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. A. Density plots of CD45⁺CD3⁺ CD4⁺ CD25⁺ CD127^{dim} cells. B. Representative bar graphs of CD25⁺CD127^{dim} cell percentages in CD4⁺ cells for healthy and patients.

3.3.1.3 Patients had different B cell percentages in whole blood

B cell percentage showed difference patient to patient. Patient-2 had similar level of B cells compared to healthy controls, Patient-1 had 5-fold decreased whereas Patient-3 had 2-fold increased B cells in whole blood (**Figure 3.32**).

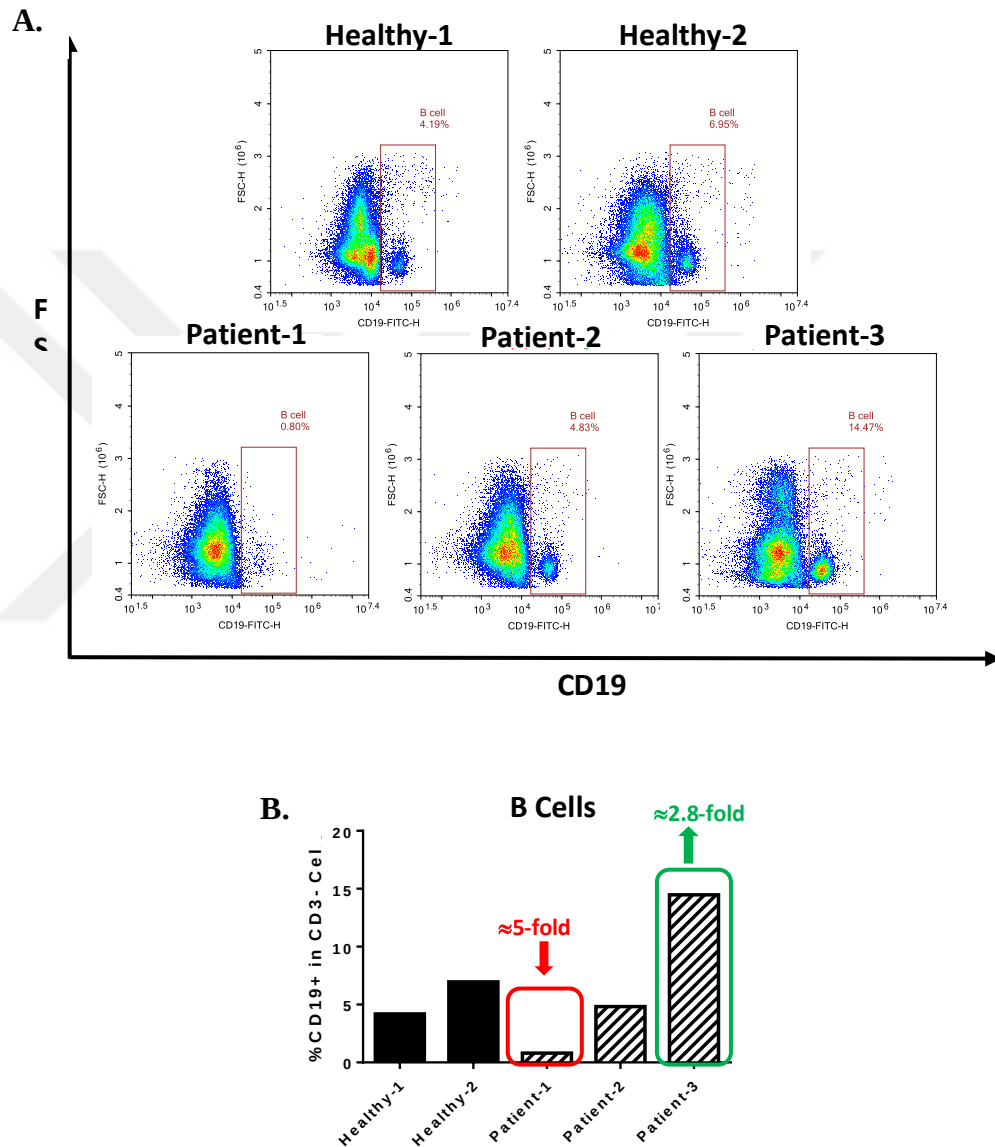


Figure 3.32 CD19 expression of CD45⁺CD3⁻ cells. The whole blood of healthy controls and STAT1 GOF patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti-human CD45, and anti-human CD19 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. A. Density plots of CD45⁺CD3⁻ CD19⁺ cells. B. Representative bar graphs of CD19⁺CD3⁻ cell percentages for healthy and patients.

3.3.1.4 Decreased Memory B cell was investigated in STAT1 GOF patients

Since it is known that STAT1 GOF patients had low number of memory B cells, we also checked memory B cell circulation in whole blood. All STAT1 GOF patients had almost none memory B cell in circulating (**Figure 3.33**).

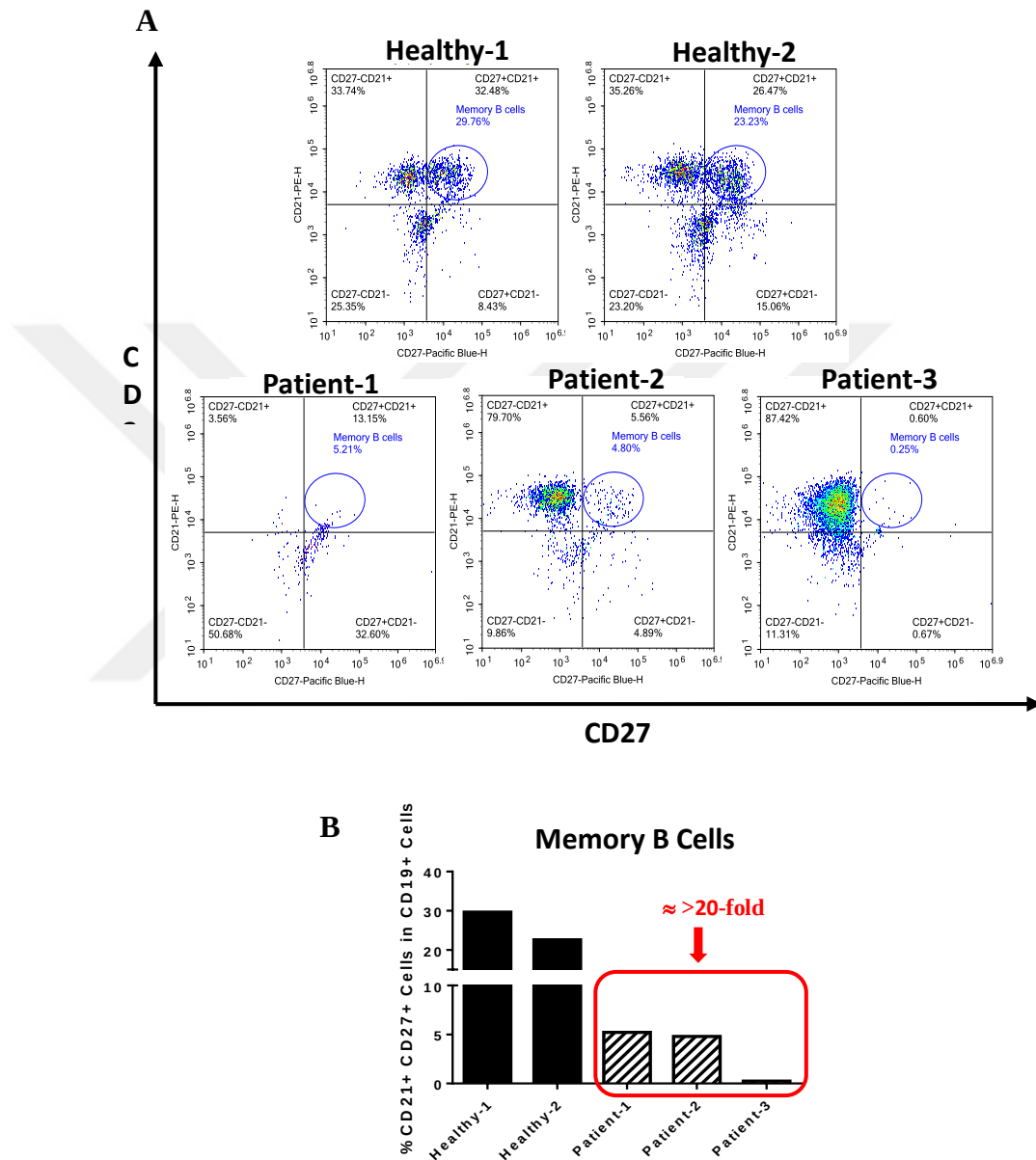


Figure 3.33 CD21 and CD27 expression of CD45⁺CD19⁺ cells. The whole blood of healthy controls and STAT1 GOF patients (100 μ l) were stained with fluorochrome conjugated anti-human CD45, anti-human CD19, anti-human CD21 and anti-human CD27 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. A. Density plots of CD45⁺CD19⁺CD21⁺CD27⁺ cells. B. Representative bar graphs of CD21⁺CD27⁺ cell percentages in CD19⁺ cells for healthy controls and patients.

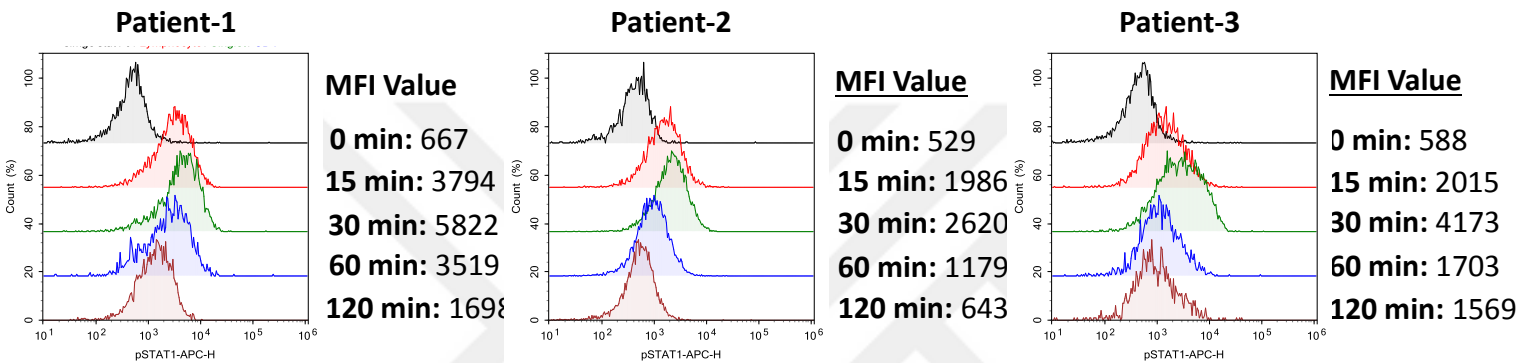
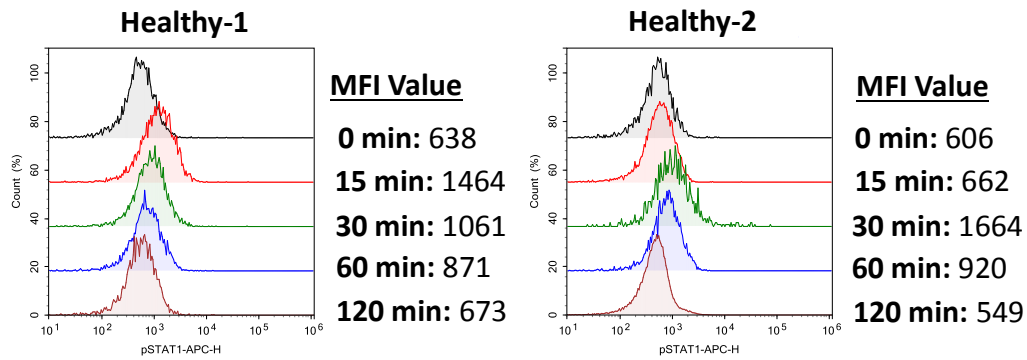
3.3.2 Determination of STAT1 Phosphorylation and De-phosphorylation

3.3.2.1 STAT1 GOF patients had increased STAT1 phosphorylation and late de-phosphorylation

Hyperphosphorylation of STAT1 and delay in dephosphorylation were observed in STAT1 GOF patients as expected (**Figure 3.34**). Also, percentage of pSTAT1 positive CD4⁺ T cell was 3-fold high in patients compared to healthies (**Figure 3.35**).



A.



B.

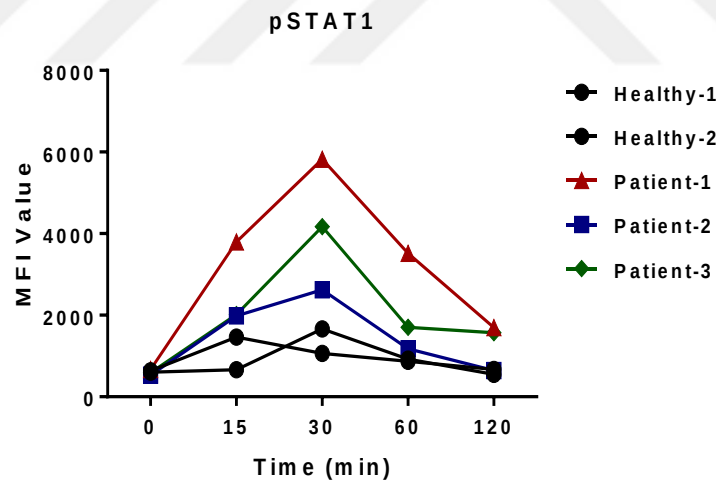


Figure 3.34 STAT1 phosphorylation levels of STAT1 GOF patients and healthy controls' CD4⁺ T Cells. Patients and healthy controls' whole blood (200 μ l) were treated with IFN- β (100 ng/ml) or left untreated over 15, 30, 60 and 120 minutes. Fixation, permeabilization and co-staining of anti-human CD4 and anti-human pSTAT1 were performed and phosphorylation levels of pSTAT1 proteins were analyzed by flow cytometry by acquiring 4×10^4 cells within CD4⁺ cell gate. **A.** Mean fluorescence intensity plot of pSTAT1 proteins. **B.** Representative line graph.

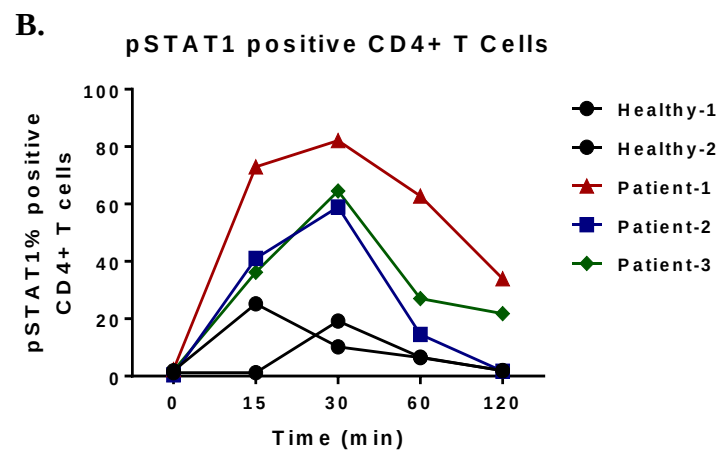
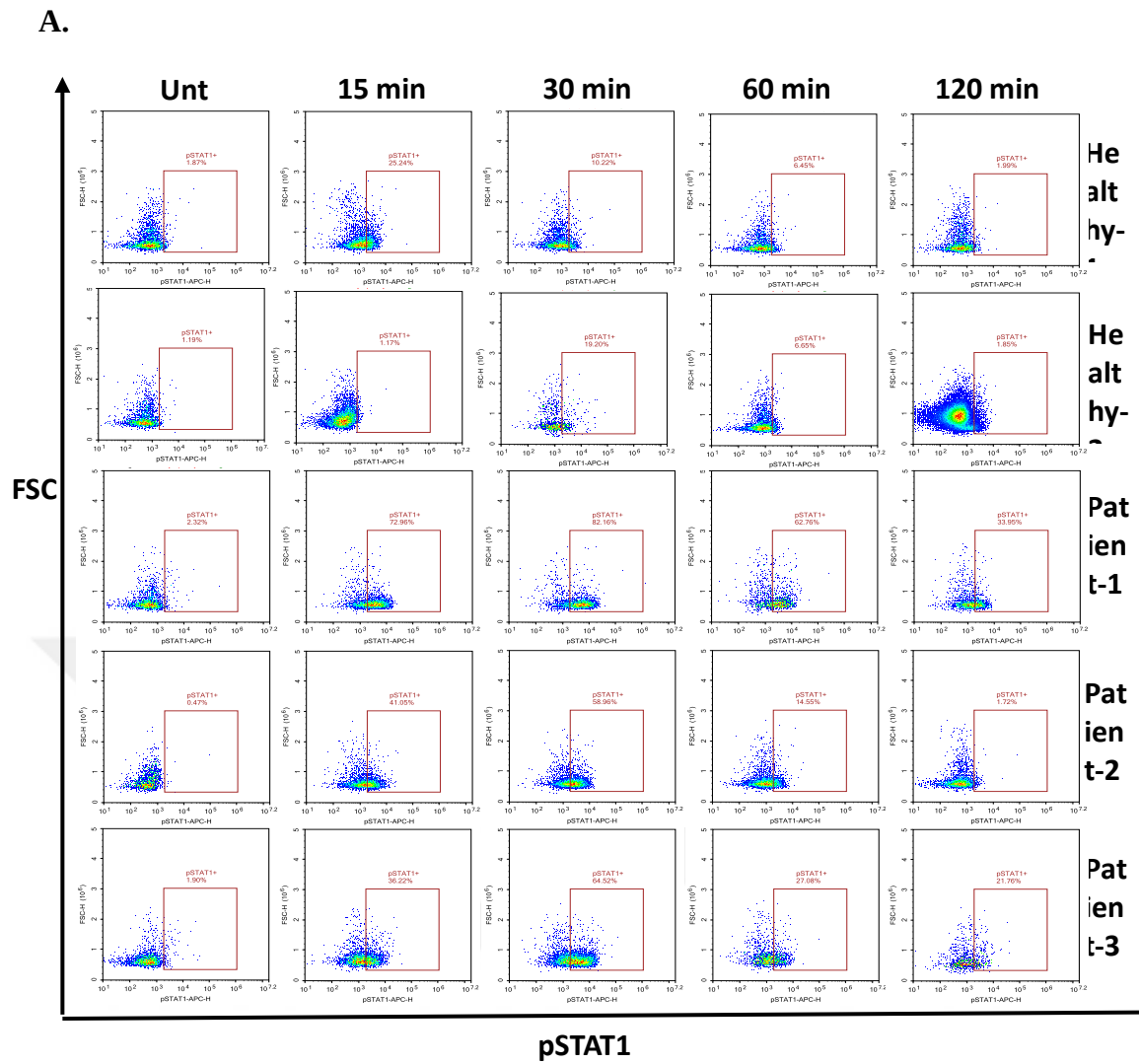


Figure 3.35 Percentage of pSTAT1⁺ cells within CD4⁺ T cells from STAT1 GOF patients and healthy controls after IFN- β stimulation. A. Density plots of pSTAT1 proteins within CD4⁺ T cells analyzed by flow cytometry. **B.** Representative line graph.

3.3.3 Investigation of Th Subset Responses

3.3.3.1 Normal Th1 response was observed STAT1 GOF patients except one

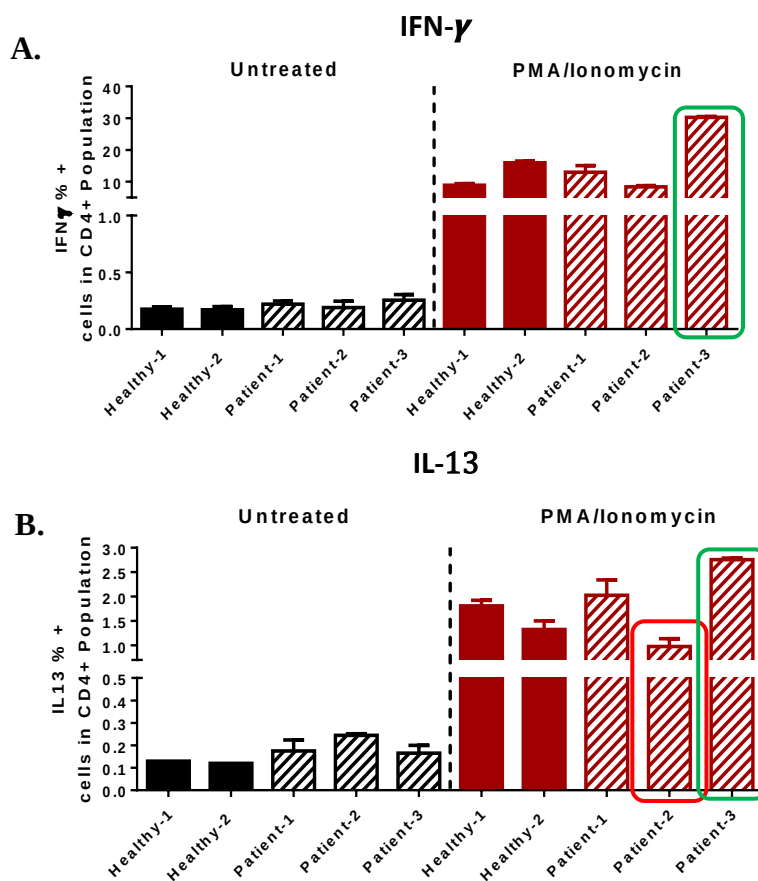
Although all patients had elevated STAT1 phosphorylation levels, IFN- γ secretion from CD4⁺ T cells were similar to healthy controls except for Patient-3. Elevated Th1 response upon PMA stimulation was observed in CD4⁺ T cells of Patient-3 (**Figure 3.36-A**).

3.3.3.2 Th2 and Th17 responses showed difference patient to patient

Decreased IL-17a secretion from CD4⁺ T cells was observed in Patient-2 and Patient-3 as the situation in STAT1 GOF patients while increased Th17 response was seen in Patient-1 (**Figure 3.36-C**). Th2 responses of CD4⁺ T cells also showed variety in patient to patient. Patient-1 and 2 had normal Th2 activity and IL-13 secretion but, Patient-3 had increased activity (**Figure 3.36-B**).

3.3.3.3 Elevated Treg response was investigated in STAT1 GOF patients

IL-10 secretion from Treg cells upon PMA/I treatment was increased in Patient-1 and Patient-3 compared to healthy controls (**Figure 3.36-D**).



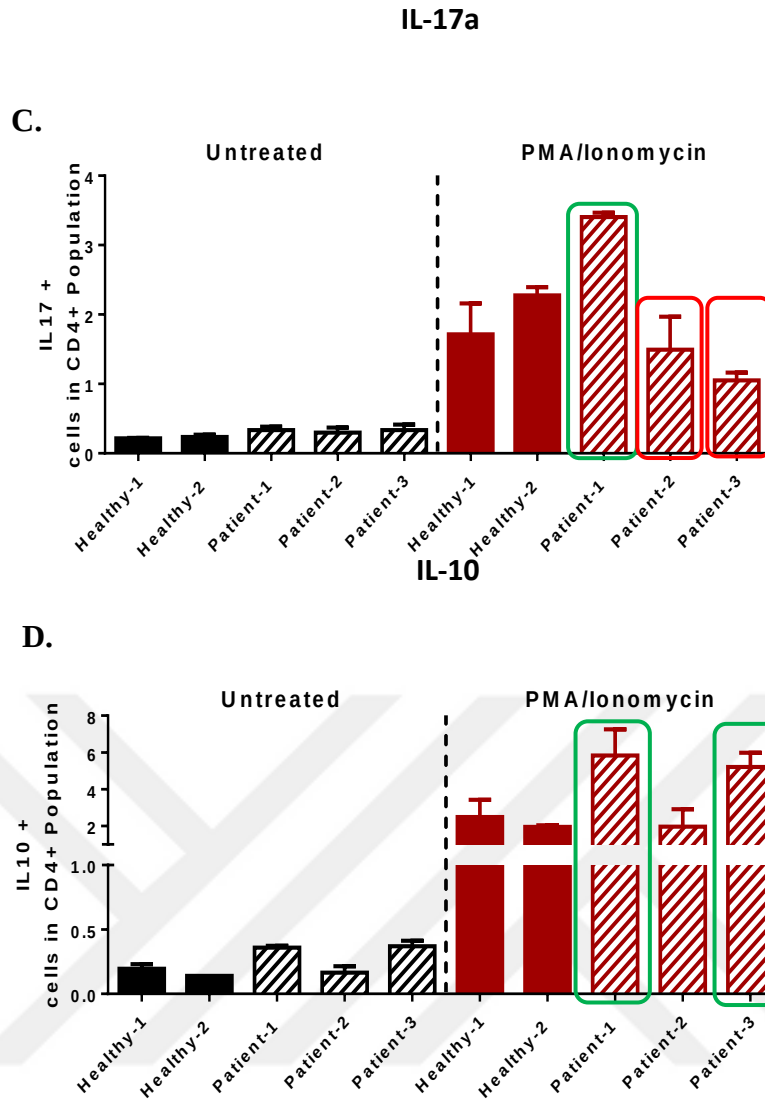


Figure 3.36 CD4⁺ T cell cytokine responses of STAT1 GOF patients and healthy controls (n=2) upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 µg/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4, anti-human IFN-γ, anti-human IL-13, anti-human IL-6, anti-human IL-17a and anti-human IL-10 were performed and analyzed by flow cytometry evaluating 3x10⁴ cells within CD4⁺ T cell gate. **A.** Th1, **B.** Th2, **C.** Th17 and **D.** Treg responses.

3.3.4 Analysis of cytokine secretion profile upon PRR and PMA/Ionomycin stimulations of PBMCs

3.3.4.1 Decreased IFN-γ and IL-17a secretion was determined in STAT1 GOF patients

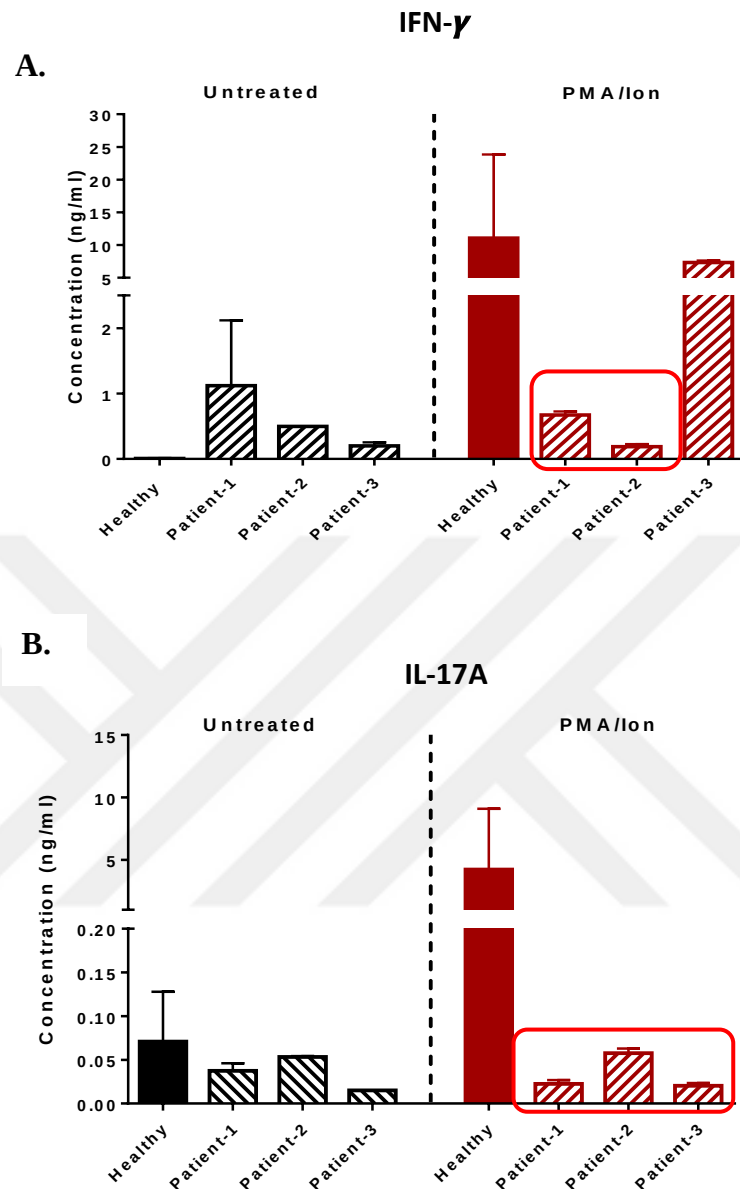


Figure 3.37 IFN- γ and IL-17a cytokine production of PBMCs from STAT1 GOF patients and healthy controls upon PMA/I treatment for 24 hours. Patients and healthy controls PMBCs were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- γ , **B.** IL-17a levels.

All STAT1 GOF patients had impaired NK, NKT and CD8⁺ T cell dependent IL-17a secretion. Also, upon PMA treatment STAT1 GOF patients had low IFN- γ secretion (Figure 3.37).

3.3.4.2 Increased proinflammatory IL-1 β response was observed in STAT1 GOF patients upon TLR and PRR stimulation

Endosomal TLR dependent IL-1 β secretion was overactivated in STAT1 GOF patients which lead us to think about overactive macrophage, T and B cells. In addition, Patient-1 and Patient-3 had increased IL-1 β levels because of IFI16 dependent inflammasome pathway activation (Figure 3.38).

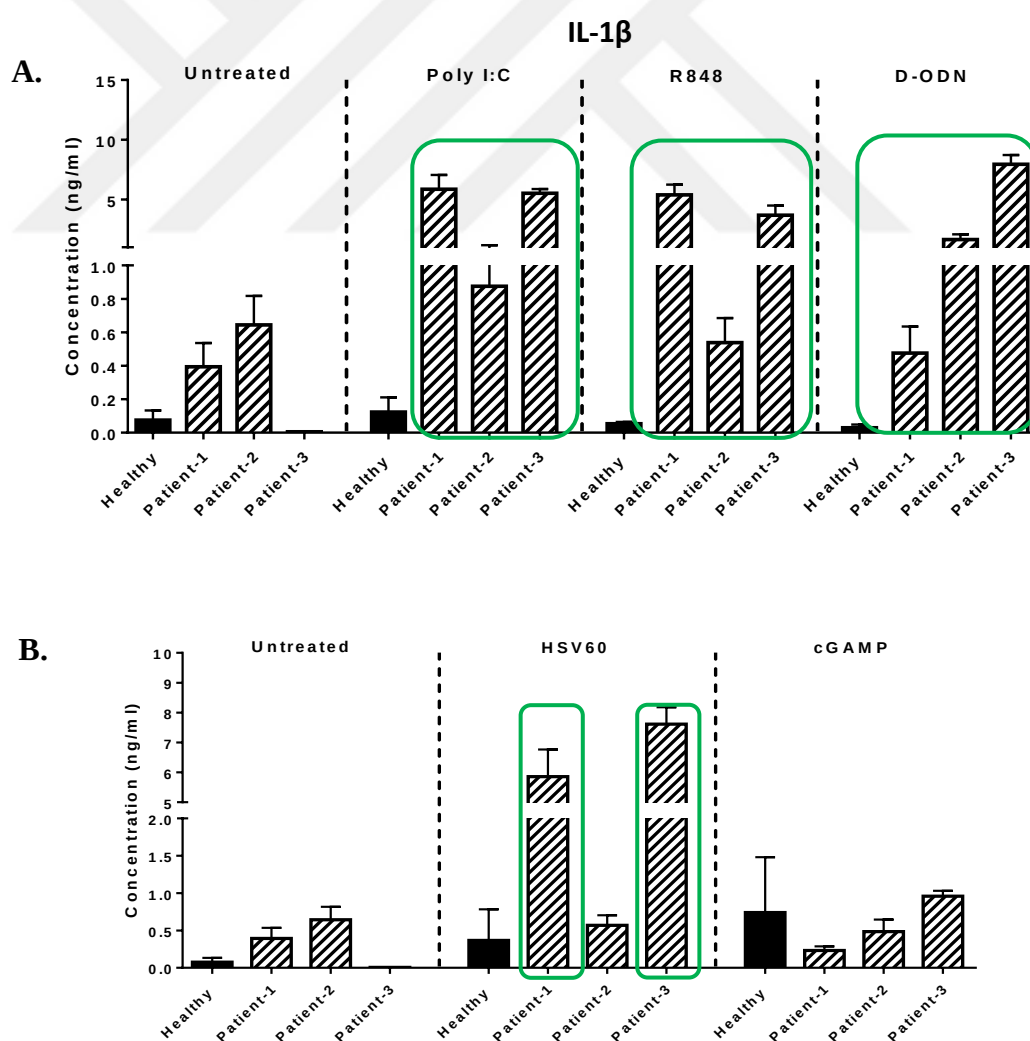


Figure 3.38 IL-1 β cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (D ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IL-1 β levels from TLR stimulation. **B.** IL-1 β levels from inflammasome pathway stimulation.

3.3.4.3 Increased IFN- γ and IL-12 upon IFI16-NLRP3 pathway activation, decreased IFN- γ and IL-12 responses upon STING pathway stimulation were observed in STAT1 GOF patients

STAT1 GOF patients had high IFI16 dependent inflammasome activity whereas low STING dependent activity for IFN- γ (**Figure 3.39**). Patient-3 had overactive IL-12 secretion from endosomal TLR and IFI16 dependent inflammasome pathway. Patient-1 and Patient-2 had decreased STING activity (**Figure 3.40**).

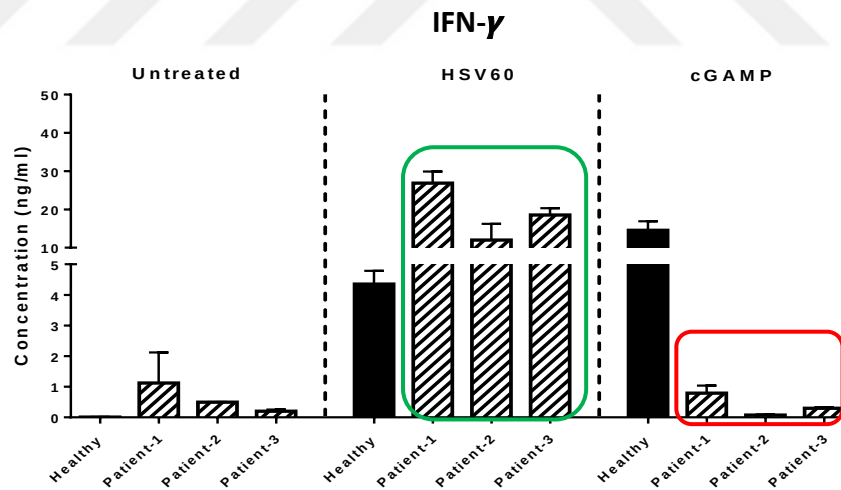


Figure 3.39 IFN- γ cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA.

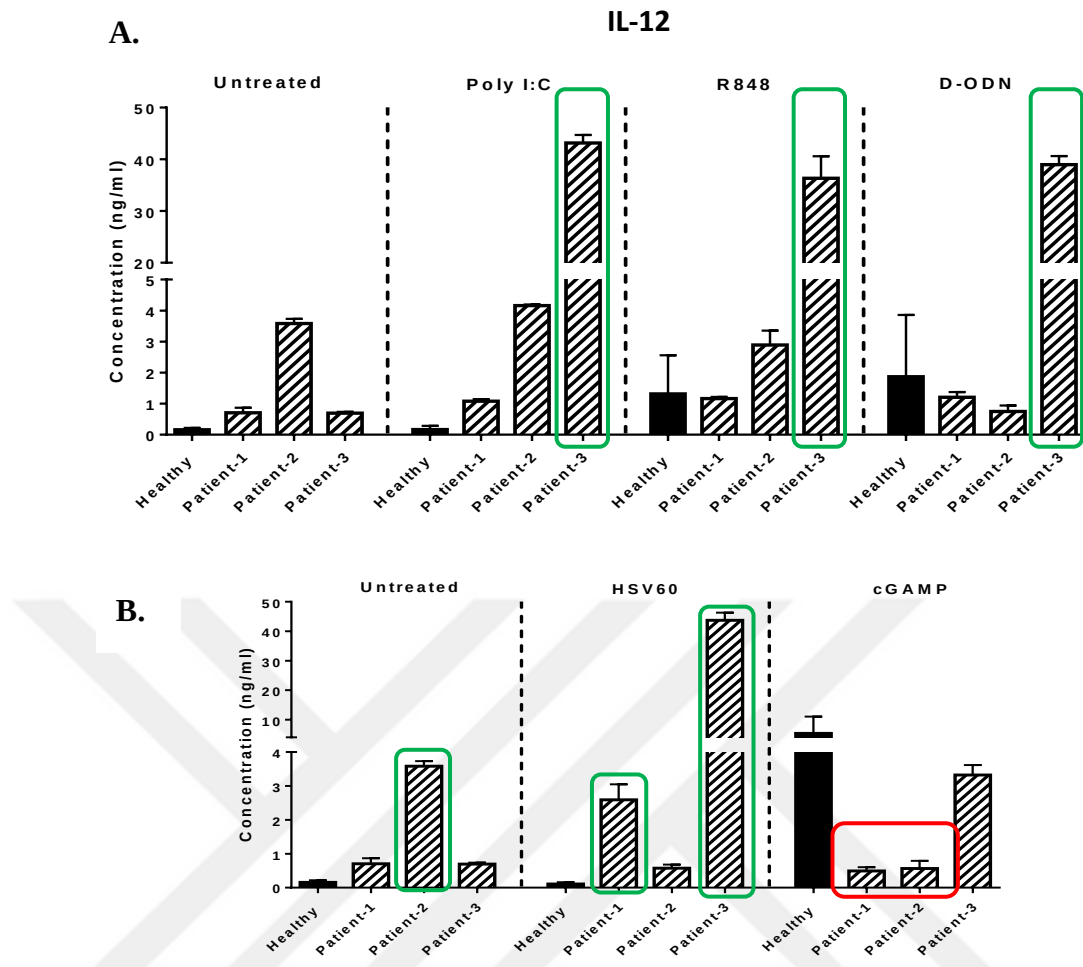


Figure 3.40 IL-12 cytokine production profile of PBMCs from STAT1 GOF patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR3 (p(I:C), 30 μ g/ml), TLR7 (R848, 5 μ g/ml), TLR9 (D ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IL-12 levels from TLR stimulation. **B.** IL-12 levels from inflammasome pathway stimulation.

3.3.5 Investigation of Neutrophil activities in STAT1 GOF patients

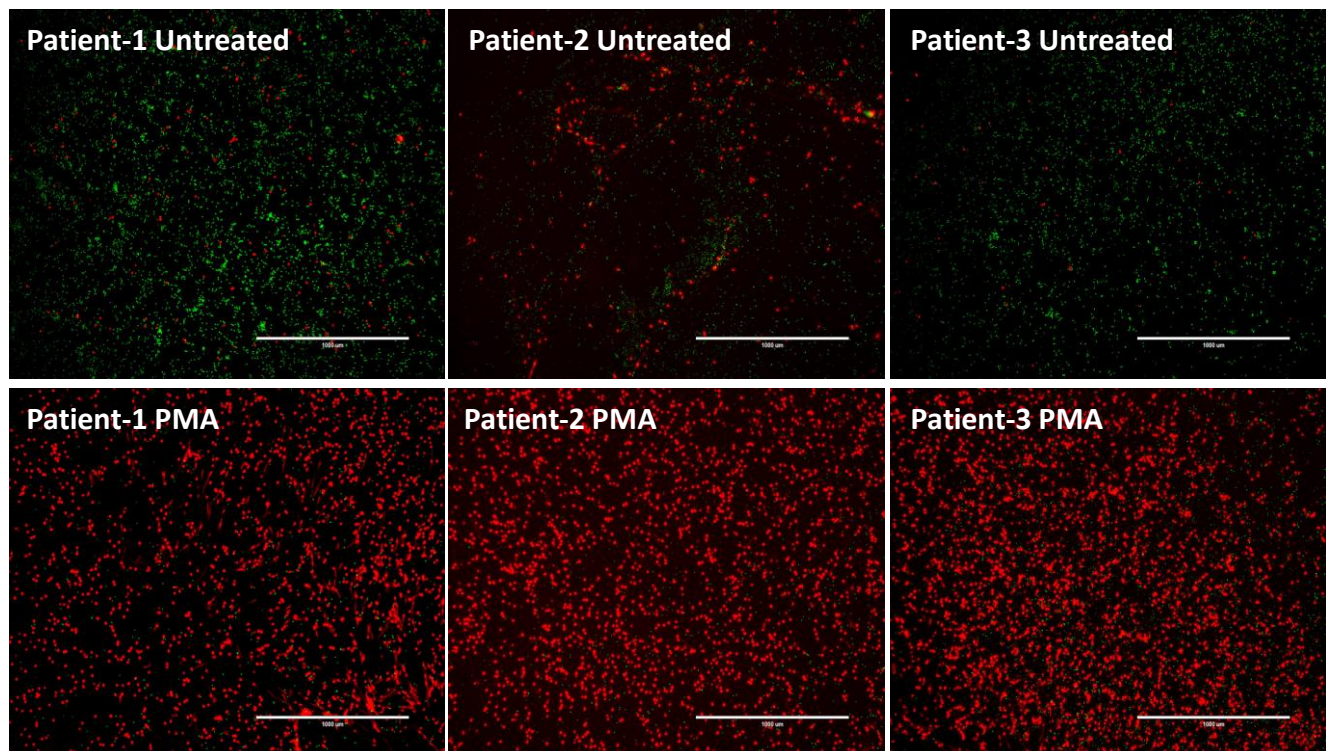
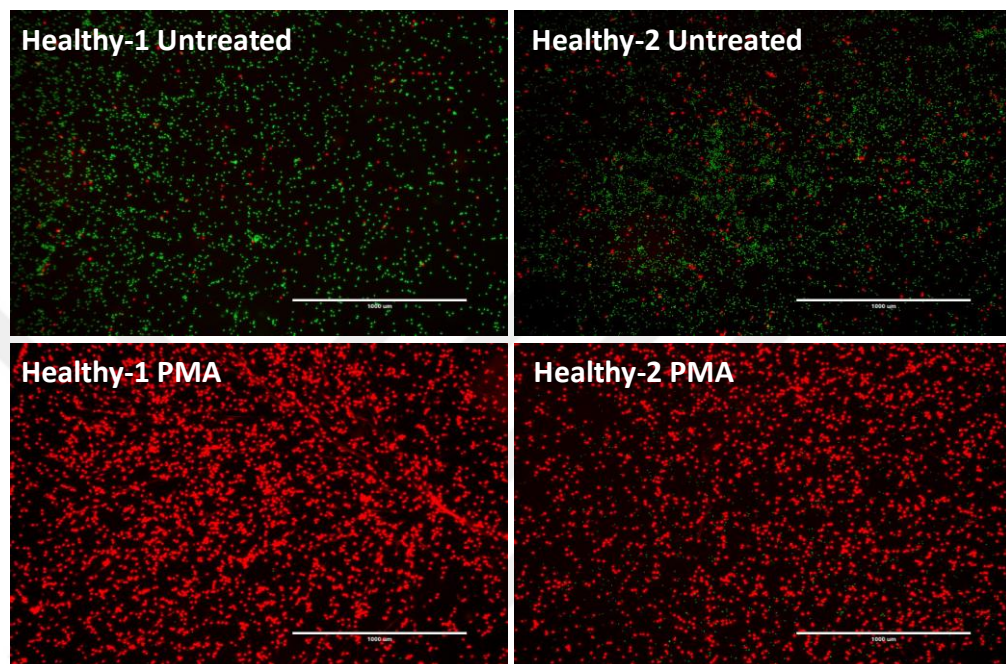
3.3.5.1 Microscopically normal, fluorometrically decreased NET-like formation was determined in STAT1 GOF patient upon PMA treatment

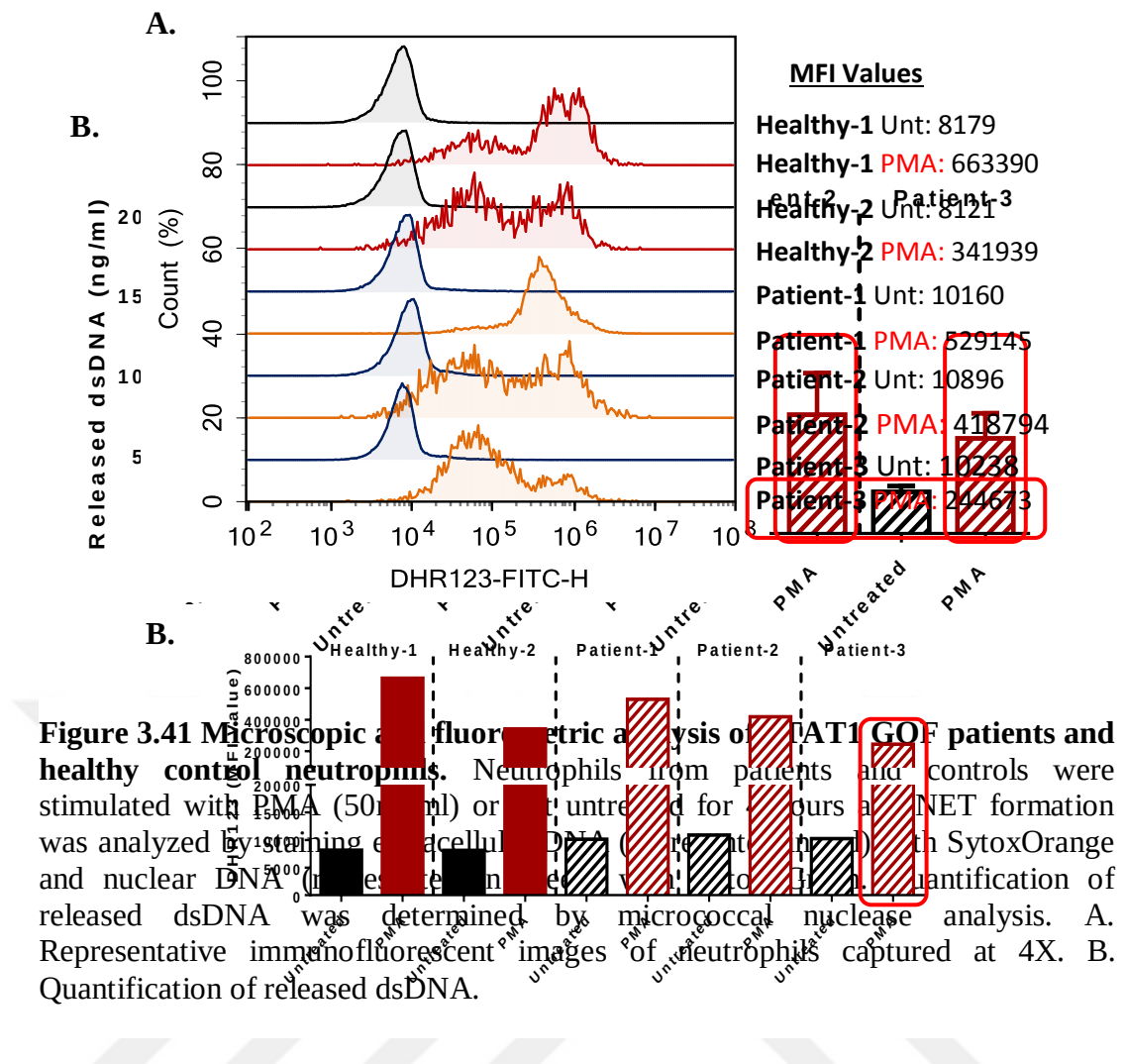
Neutrophils from patients and healthy controls were not differ at basal level and upon PMA stimulation when we analyzed with fluorescence microscope. However, out of three, two patients' neutrophils were not able to induce NET like formation under PMA stimulation that could be reason for exhausted neutrophils (**Figure 3.41**).

3.3.5.2 Normal respiratory burst was observed in untreated neutrophils of STAT1 GOF patient

Reactive oxygen productions from neutrophils upon mimicking infection was similar in patients compared to healthy controls (**Figure 3.42**).

A.





3.4 Characterization of Innate and Adaptive Immune Responses in Spinal Cord Injured Patients

3.4.1 Determination of percentages of immune cells in whole blood

3.4.1.1 Low density granulocytes were observed in SCI patients

Spinal cord injured patients have increased susceptibility to infections because of impaired signaling pathways in immune system. Patients generally had low number of NK, macrophages, neutrophils and T cells and shows respiratory and urinary infections. Thence, first we aimed to investigate distribution of immune cells in whole blood.

Figure 3.42 Determination of respiratory burst of STAT1 GOF patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left untreated for 4 hours and respiratory burst was analyzed by staining neutrophils with DHR123. **A.** Mean fluorescence intensity plot of DHR123⁺ neutrophils. **B.** Representative bar graph.

It is known that low density granulocytes are contributing to severe inflammation through increased NETotic activity of neutrophils and increased inflammatory cytokine productions [69]. Our attention was caught when large and granular cells in isolated PBMC fractions were investigated by flow cytometry. Two of SCI (Patient-3,6) patients had LDGs compared to healthy controls (**Figure 3.43**).

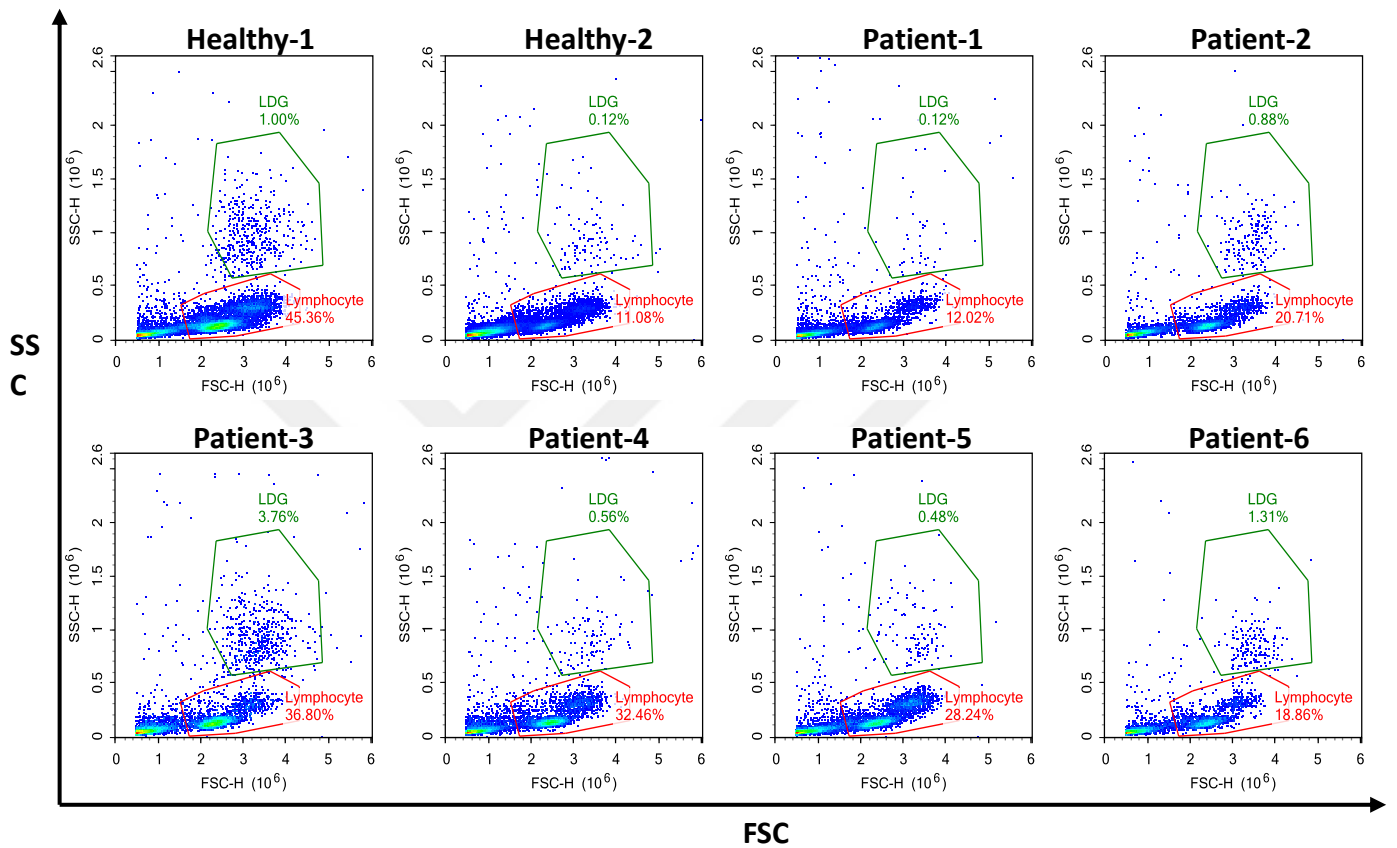


Figure 3.43 Determination of low-density granulocytes of Spinal Cord Injured (SCI) patients and healthy controls by analyzing PBMC fractions with flow cytometry.

3.4.1.2 Reversed CD4/CD8 ratio was observed in patients

Healthy controls had CD4⁺/CD8⁺ ratio ranging from 1.3-1.7, out of six SCI patients two (Patient-1,4) of them had reversed ratio (≤ 0.9) (**Figure 3.44**).

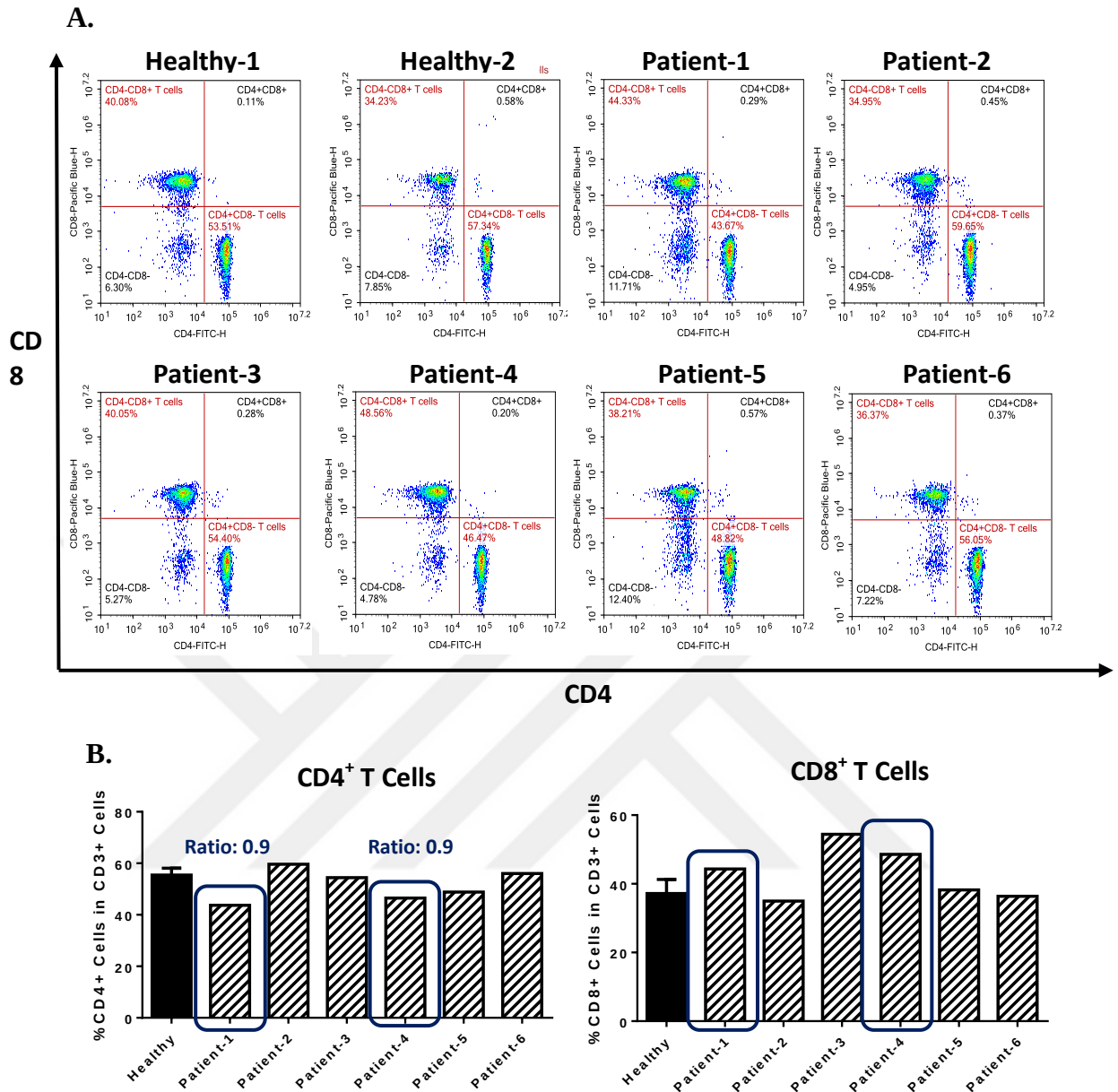


Figure 3.44 CD4 and CD8 surface expression of CD45⁺CD3⁺ cells. The whole blood of healthy controls and SCI patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, anti-human CD4 and anti-human CD8 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺CD3⁺ CD4⁺ and CD8⁺ cells. **B.** Representative bar graphs of CD4⁺ and CD8⁺ cell percentages in CD3⁺ cells for healthy controls and patients.

3.4.1.3 B and NK cell percentages were different patient to patient

Decreased lymphocyte and NK cell function is observed in most SCI patients. To determine whether our patients had low percentage of B and NK cell in circulation,



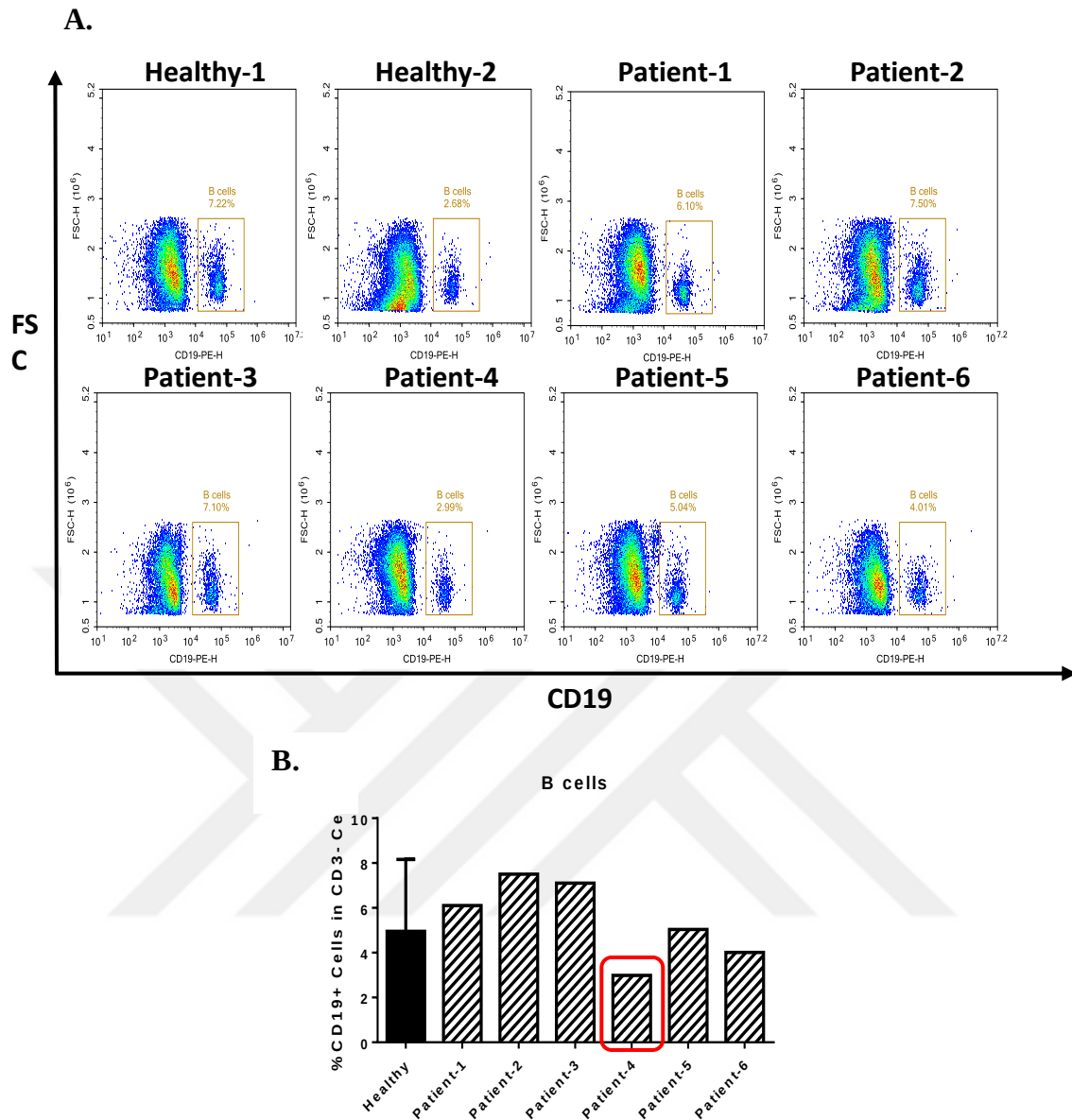


Figure 3.45 CD19 expression of CD45⁺CD3⁻ cells. The whole blood of healthy controls and SCI patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, and anti-human CD19 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺CD3⁻ CD19⁺ cells. **B.** Representative bar graphs of CD19⁺ cell percentages in CD3⁻ cells for healthy controls and patients.

whole blood from patients and healthy controls were stained with anti-human CD3,

anti-human CD19 and anti-human CD56, analyzed by flow cytometry. B and NK cell percentage in whole blood was various from patient to patients. Patient-1,3,6 had low whereas Patient-4,5 had elevated NK cells compared to healthies (**Figure 3.46**). Normal B cell percentage was observed in SCI patients except for Patient-4 **A.** had decreased cell percentage (**Figure 3.45**).

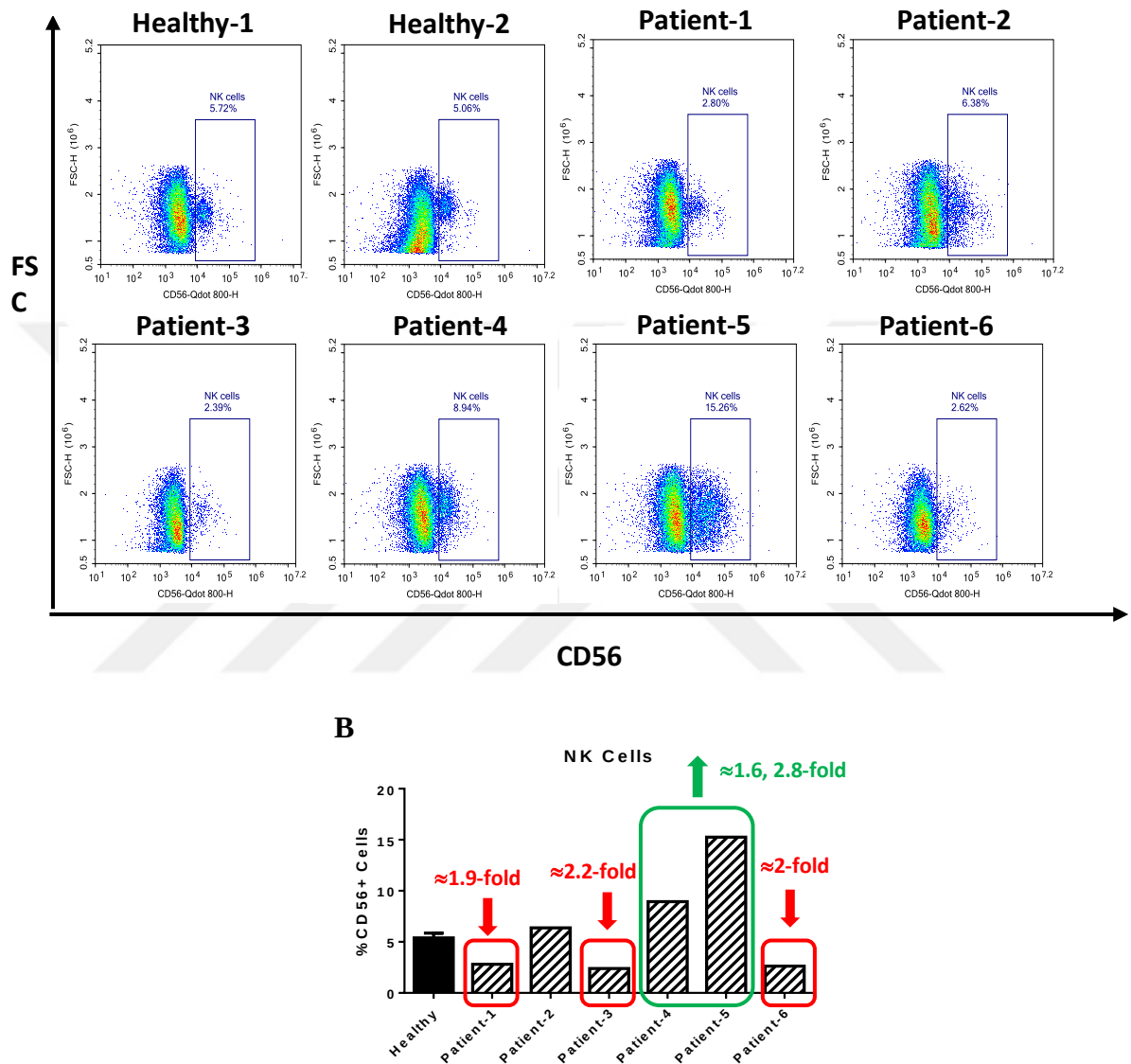


Figure 3.46 CD56 expression of CD3⁺ cells. The whole blood of healthy controls and SCI patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti- human CD45, and anti-human CD19 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD3⁺ CD56⁺ cells. **B.** Representative bar graphs of cell percentages.

3.4.1.4 SCI patients had increased Treg percentage

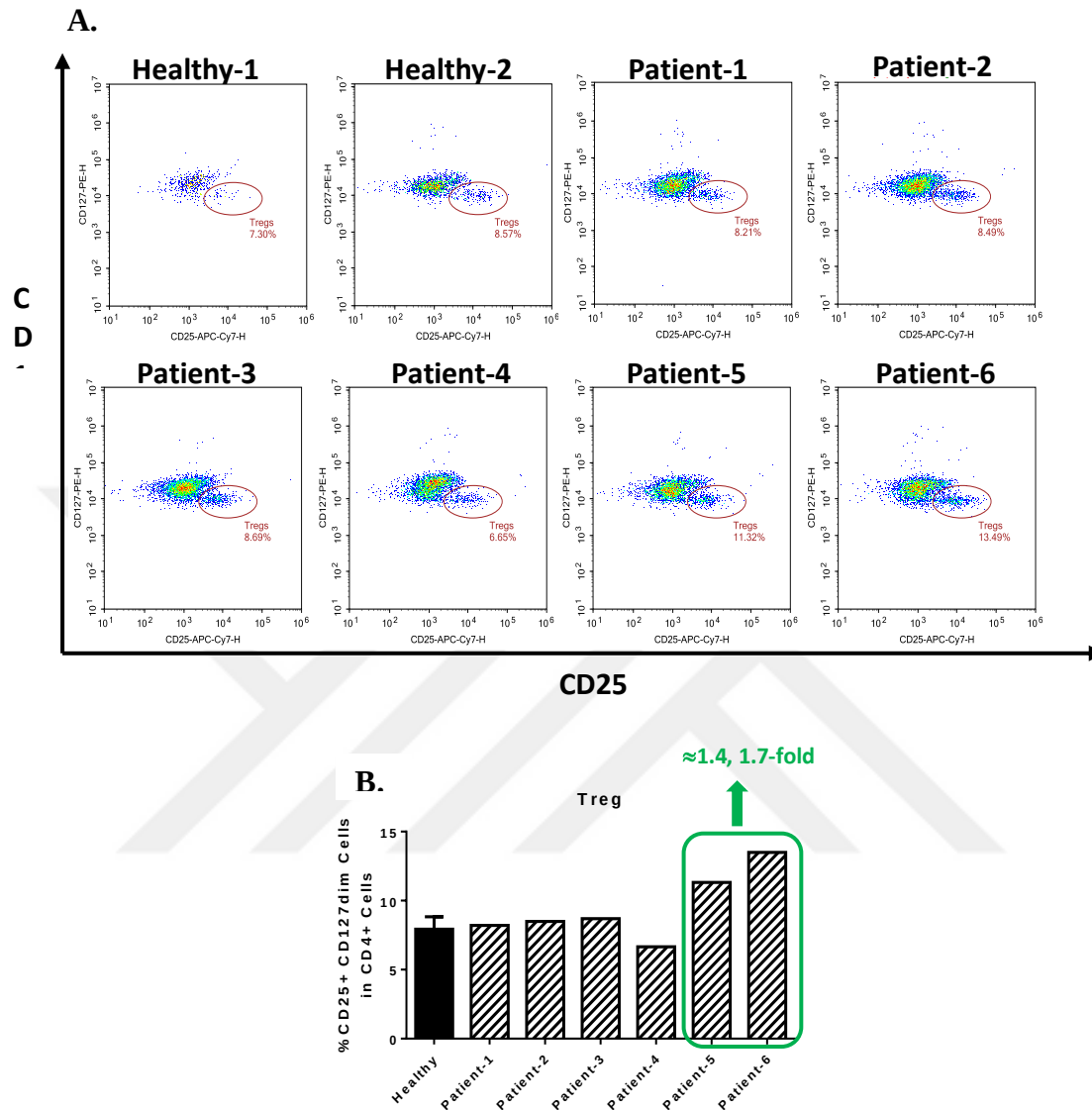


Figure 3.47 CD25 and CD127 surface expression of $CD45^+CD3^+CD4^+$ cells. The whole blood of healthy controls and SCI patients (100 μ l) were stained with fluorochrome conjugated anti-human CD3, anti-human CD45, anti-human CD4, anti-human CD25 and anti-human CD127 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of $CD45^+CD3^+CD4^+CD25^+CD127^{dim}$ cells. **B.** Representative bar graphs of $CD25^+CD127^{dim}$ cell percentages in $CD4^+$ cells for healthy and patients.

Increased $CD25^+CD127^{dim}$ Treg cells were observed in Patient-5,6 that could be the reason for immune suppression and increased infection (**Figure 3.27**).

3.4.1.5 Elevated pDCs percentage was investigated in some SCI patients

Plasmacytoid dendritic cells (pDCs) are important for type I IFN secretion during viral infections and are resided less than 0.2% in circulation whole blood. Three patients (Patient-1,4,5) out of six had increased number (2-4 fold more) of pDCs in

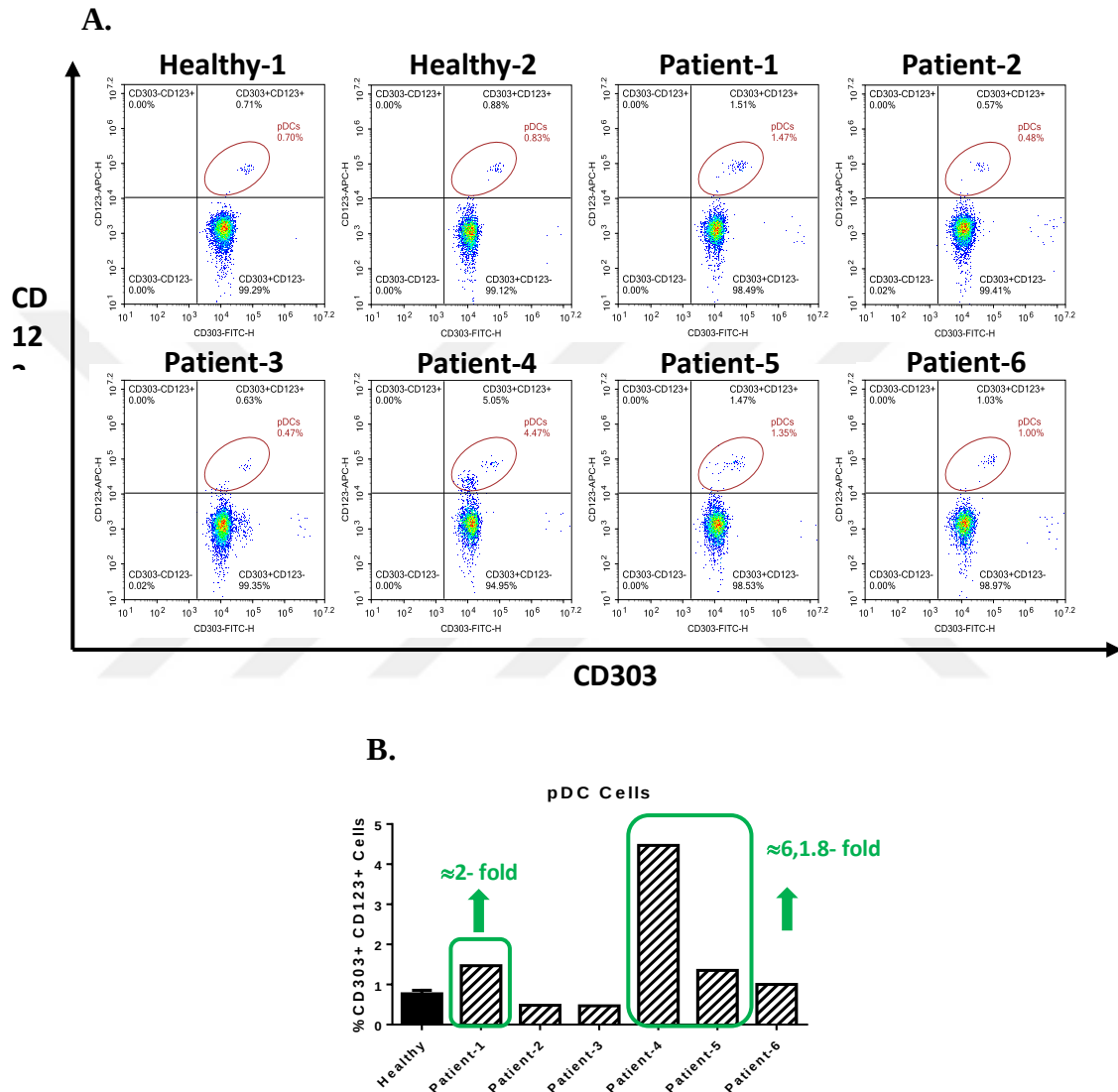


Figure 3.48 CD123 and CD303 surface expression of CD45⁺ cells. The whole blood of healthy controls and SCI deficient patients (100 μ l) were stained with fluorochrome conjugated anti-human CD45, anti-human CD123 and anti- human CD303 antibodies. Gating was performed from first forward and side scattering of lymphocytes, then singlets. **A.** Density plots of CD45⁺ CD123⁺CD303⁺cells. **B.** Representative bar graphs of CD303⁺CD123⁺ cell percentages in CD45⁺ cells for healthy controls and patients. their blood compared to healthy controls (**Figure 3.48**).

3.4.2 Investigation of Th subset responses

3.4.2.1 Patients had normal Th1 responses except one

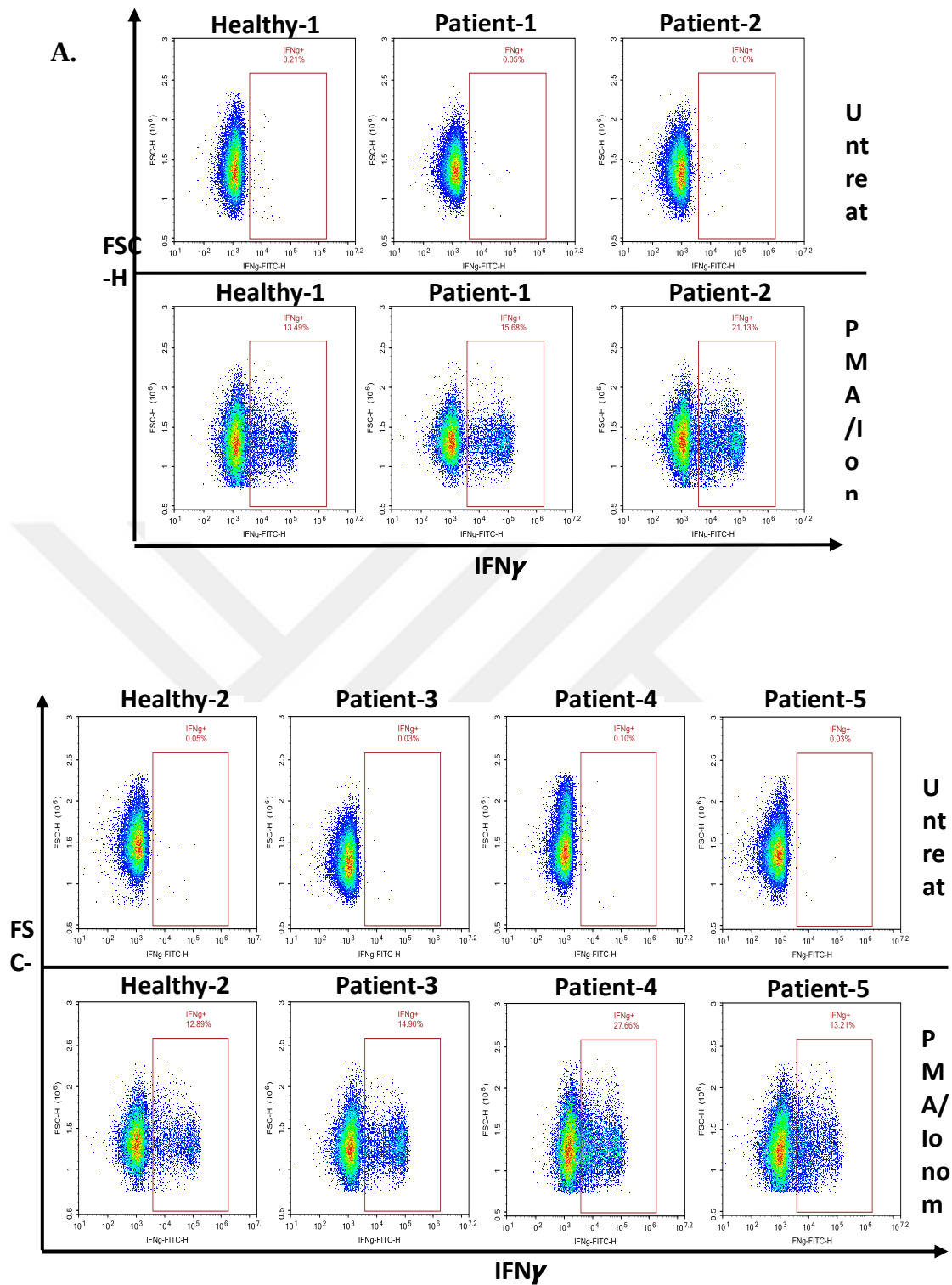
Since SCI patients had dysregulation in lymphocyte function, we decided to check CD4⁺ T cell responses together with PMA/I stimulation and intracellular cytokine staining. Normal IFN- γ secretion from CD4⁺ T cell of SCI patients were observed except for Patient-4. Elevated Th1 response together with increased IFN- γ secretion was present in Patient-4 (**Figure 3.49**).

3.4.2.2 Normal Th17 response was observed in SCI patients

Normal IL-17a secretion from CD4⁺ T cell of SCI patients were observed compared to healthy controls (**Figure 3.50**).

3.4.2.3 Elevated Treg response was investigated in SCI patients

Out of six patients two of them (Patient-2,6) had elevated Treg response depending on IL-10 secretion. However, IL-10 secretion from PBMCs of Patient-2,6 were normal (**Figure 3.51**).



3.4.3 Analysis of cytokine secretion profile upon PRR stimulations of PBMCs

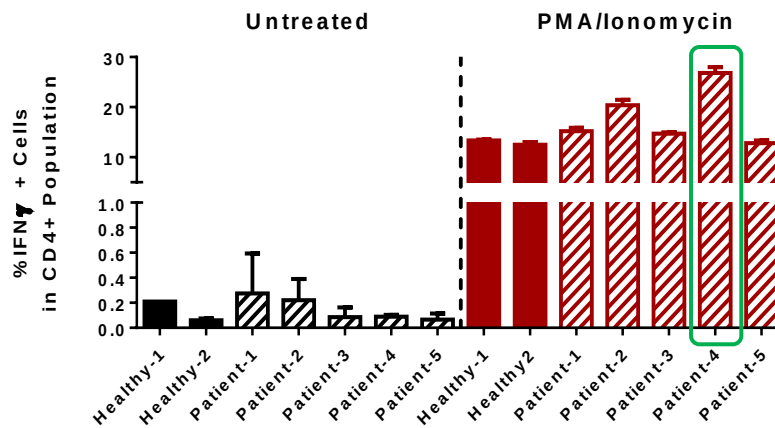
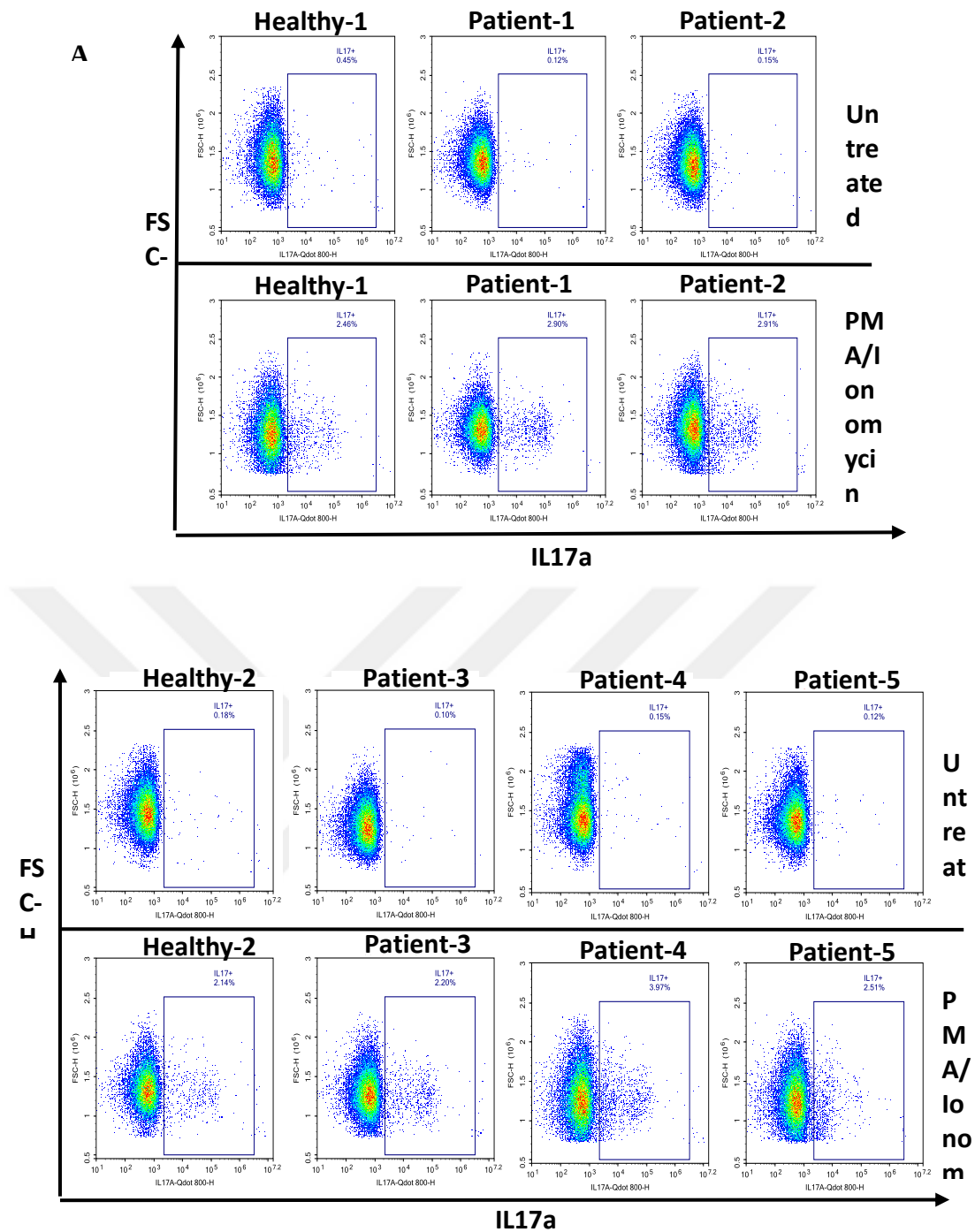
B

Figure 3.49 CD4⁺ T cell cytokine responses of SCI patients and healthy controls upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 µg/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4 and anti-human IFN-γ were performed and analyzed by flow cytometry evaluating 3x10⁴ cells within CD4⁺ T cell gate. **A.** Density plot of Th1 responses of healthy and patient PBMCs. **B.** Representative bar graph.

3.4.3.1 Impaired Type II IFN, IL-12 and IP-10 responses were observed in SCI patients

Both IL-12 and IFN-γ levels were decreased in SCI patients upon TLR4, TLR7, STING and IFI16 pathways activation (**Figure 3.52 and 3.53**). It was not depended on CD4⁺ T cell response since all SCI patients were able to induce IFN-γ secretion from CD4⁺ T cells.

The IP-10 chemokine produced as a result of interferon signal is also important mediator in terms of showing type and severity of immune response. It is used to express how severely the response will develop in Th1 direction. We analyzed IP-10 secretion from patient and healthy controls PBMCs upon TLR and PRR ligands and cytokine ELISA. All SCI patients showed impaired STING and IFI16 dependent inflammasome responses for IP-10 whereas only two patients (Patient-2,6) had impaired TLR9 response (**Figure 3.55**).



3.4.3.2 Elevated IL-10 response was observed in patients

Increased IL-10 levels were observed in patients (Patient-4,5) against TLR4 stimulation. These patients did not have elevated Treg responses from CD4⁺ T cells. Probably source for IL-10 for Patient-4,5 was macrophages and DCs (**Figure 3.54**).

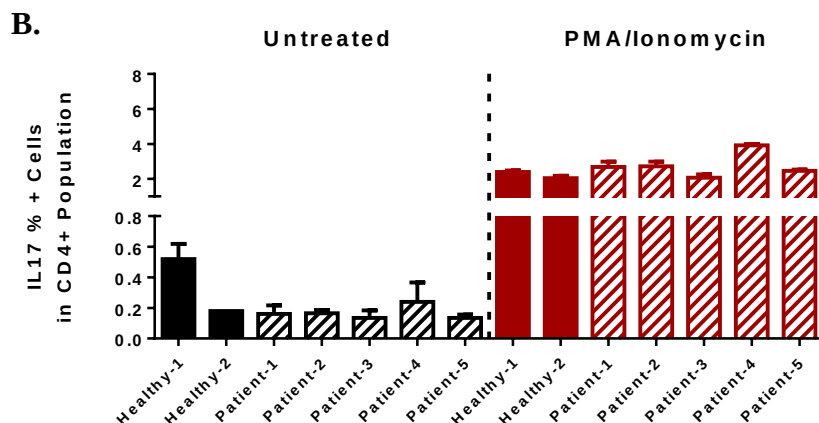
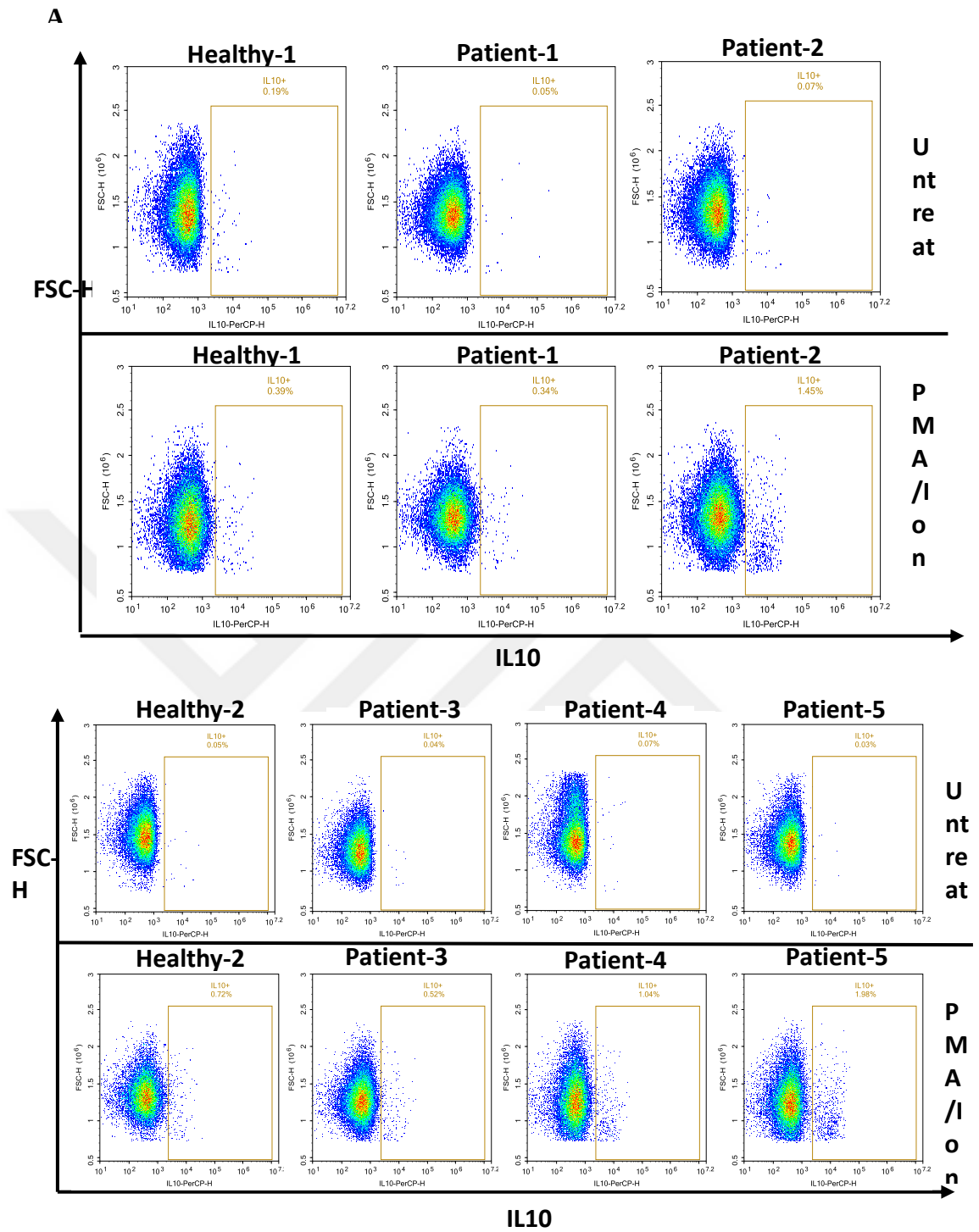


Figure 3.50 CD4⁺ T cell cytokine responses of SCI patients and healthy controls upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4 and anti-human IL-17a were performed and analyzed by flow cytometry evaluating 3×10^4 cells within CD4⁺ T cell gate. **A.** Density plot of Th17 responses of healthy and patient PBMCs. **B.** Representative bar graph.

3.4.4 Investigation of Neutrophil activities in SCI patients

3.4.4.1 Microscopically normal, fluorometrically decreased NET-like formation was determined in SCI patient upon PMA treatment

No difference was observed in terms of NETosis under fluorescence microscope, while micrococcal nuclease assay showed that two patients (Patient-3,4) had spontaneous NET- like formation at basal level. Upon PMA treatment, neutrophils of three patients (Patient-2,5,6) out of six were not able to induce dsDNA into extracellular space (**Figure 3.56**).



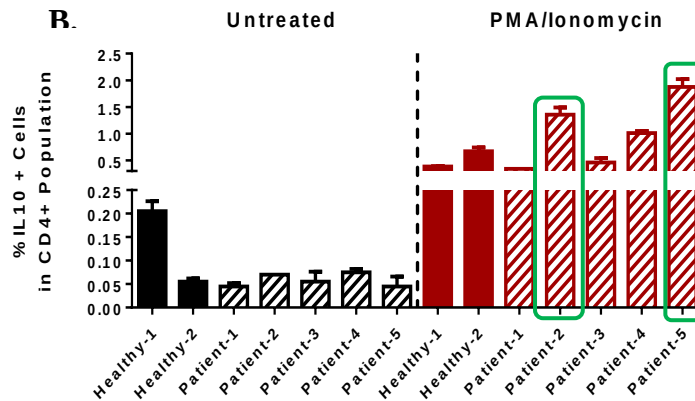


Figure 3.51 CD4⁺ T cell cytokine responses of SCI patients and healthy controls upon PMA/I treatment. PBMCs from patients and healthies were stimulated with PMA (50ng/ml) and Ionomycin (1 μ g/ml) for 2 hours, following 4 hours Brefeldin A treatment. Fixation, permeabilization and staining of cells with anti-human CD4 and anti-human IL-10 were performed and analyzed by flow cytometry evaluating 3×10^4 cells within CD4⁺ T cell gate. **A.** Density plot of Treg responses of healthy and patient PBMCs. **B.** Representative bar graph.

3.4.4.2 Decreased respiratory burst was observed in PMA treated neutrophils of SCI patient

Decreased reactive oxygen species formation was observed in SCI patients (Patient-3,4,5) when neutrophils were stimulated with PMA and stained with DHR123-FITC. These suggested us that SCI patients became more susceptible to infection due to low response to pathogen despite increased neutrophil activity at basal level (**Figure 3.57**).

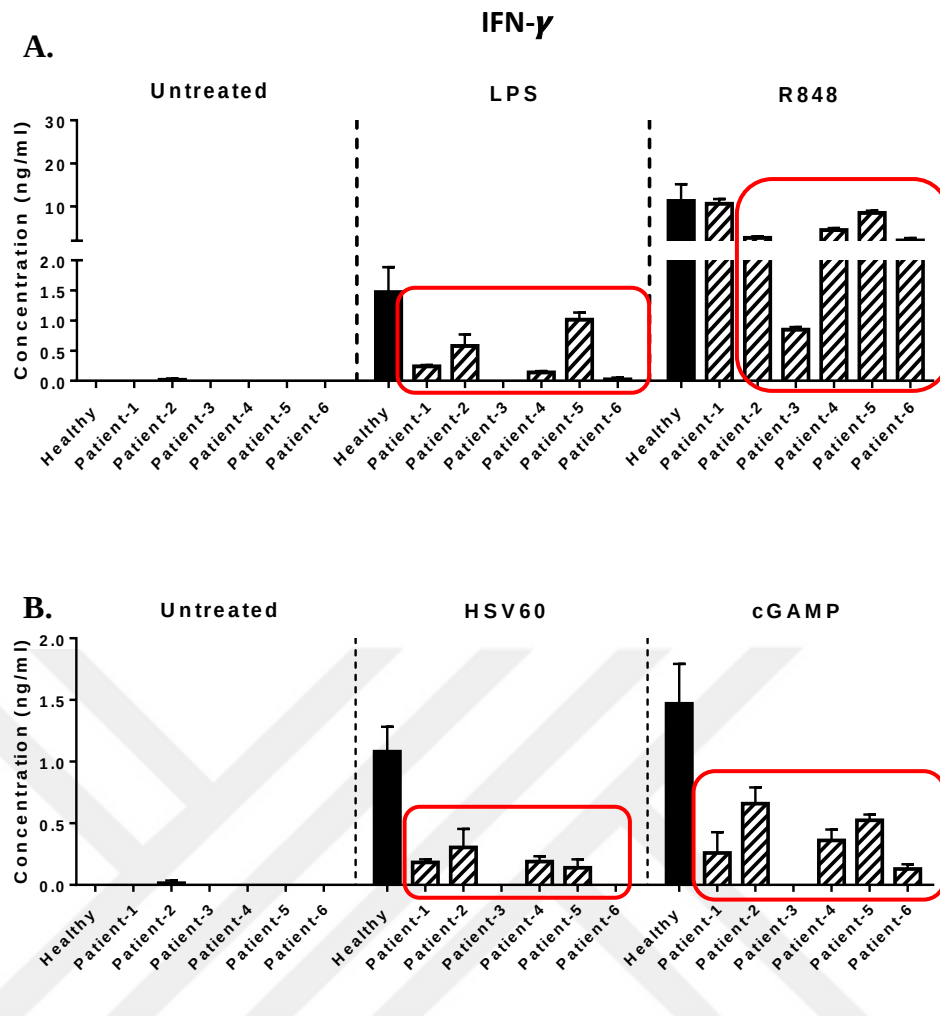


Figure 3.52 IFN- γ cytokine production profile of PBMCs from SCI patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR4 (LPS, 5 μ g/ml), TLR7 (R848, 5 μ g/ml), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IFN- γ levels from TLR stimulation. **B.** IFN- γ levels from inflammasome pathway stimulation.

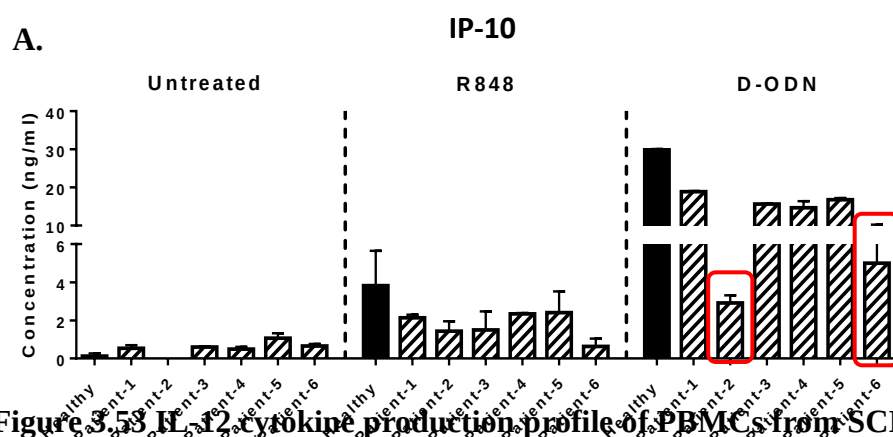
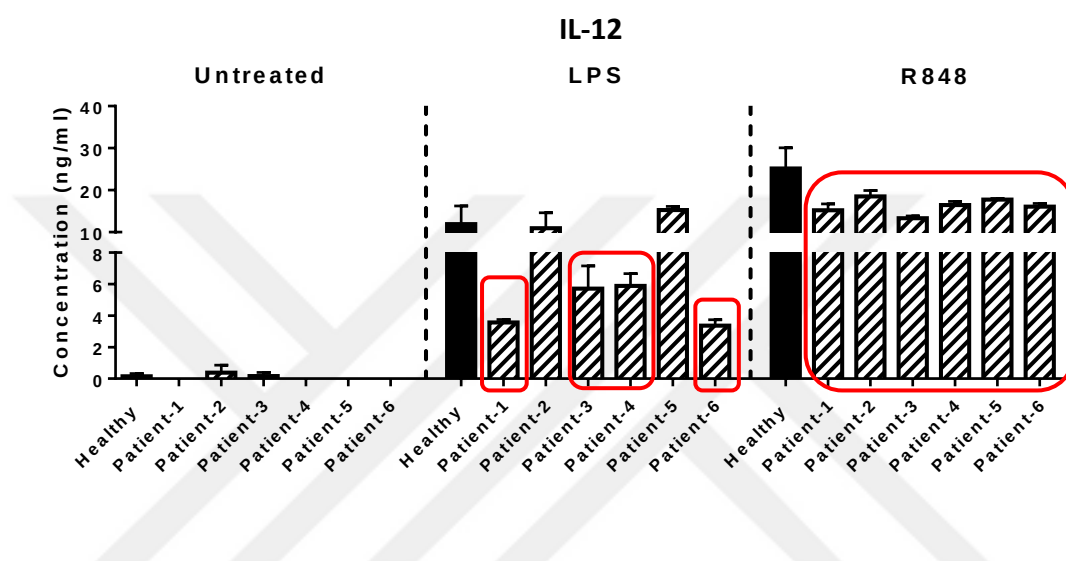


Figure 3.53 IL-12 cytokine production profile of PBMCs from SCI patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR4 (LPS, 5 μ g/ml), TLR7 (R848, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA.

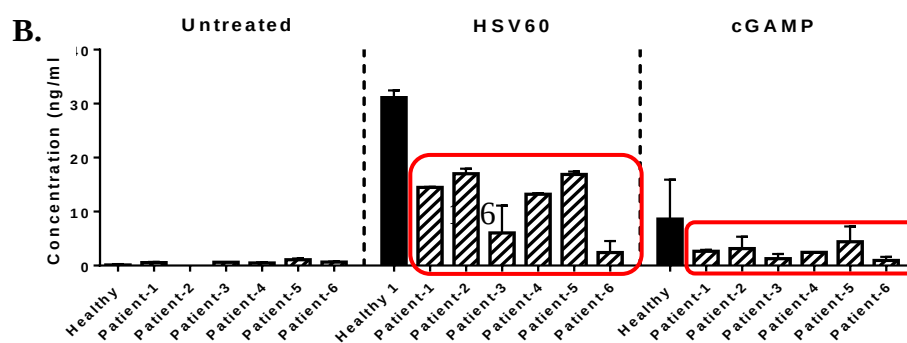


Figure 3.55 IP-10 cytokine production profile of PBMCs from SCI patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR7 (R848, 5 μ g/ml), TLR9 (D-ODN, 3 μ M), STING (2'3' cGAMP, 30 μ g/ml) and IFI16-STING (HSV, 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA. **A.** IP-10 levels from TLR stimulation. **B.** IP-10 levels from inflammasome pathway stimulation.

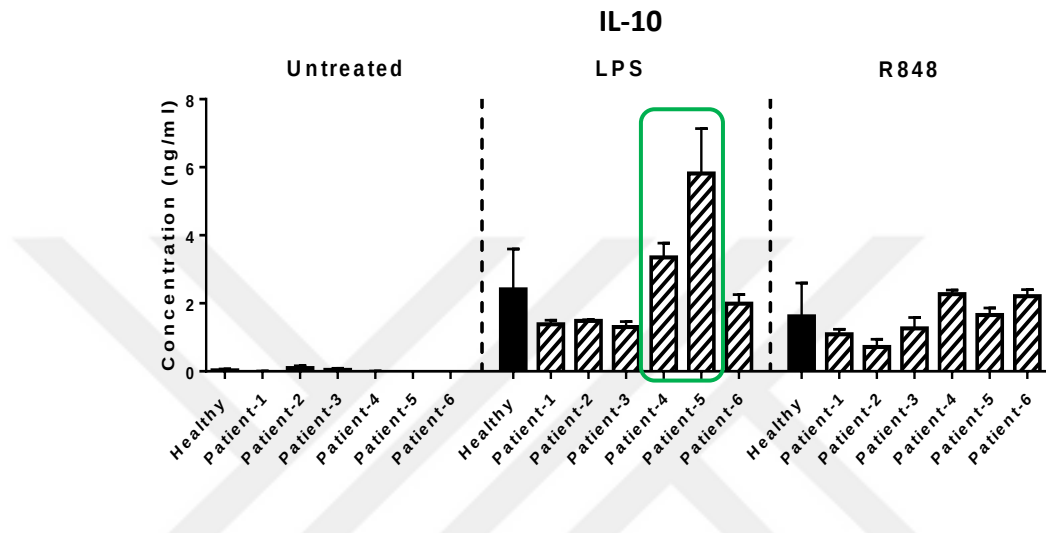
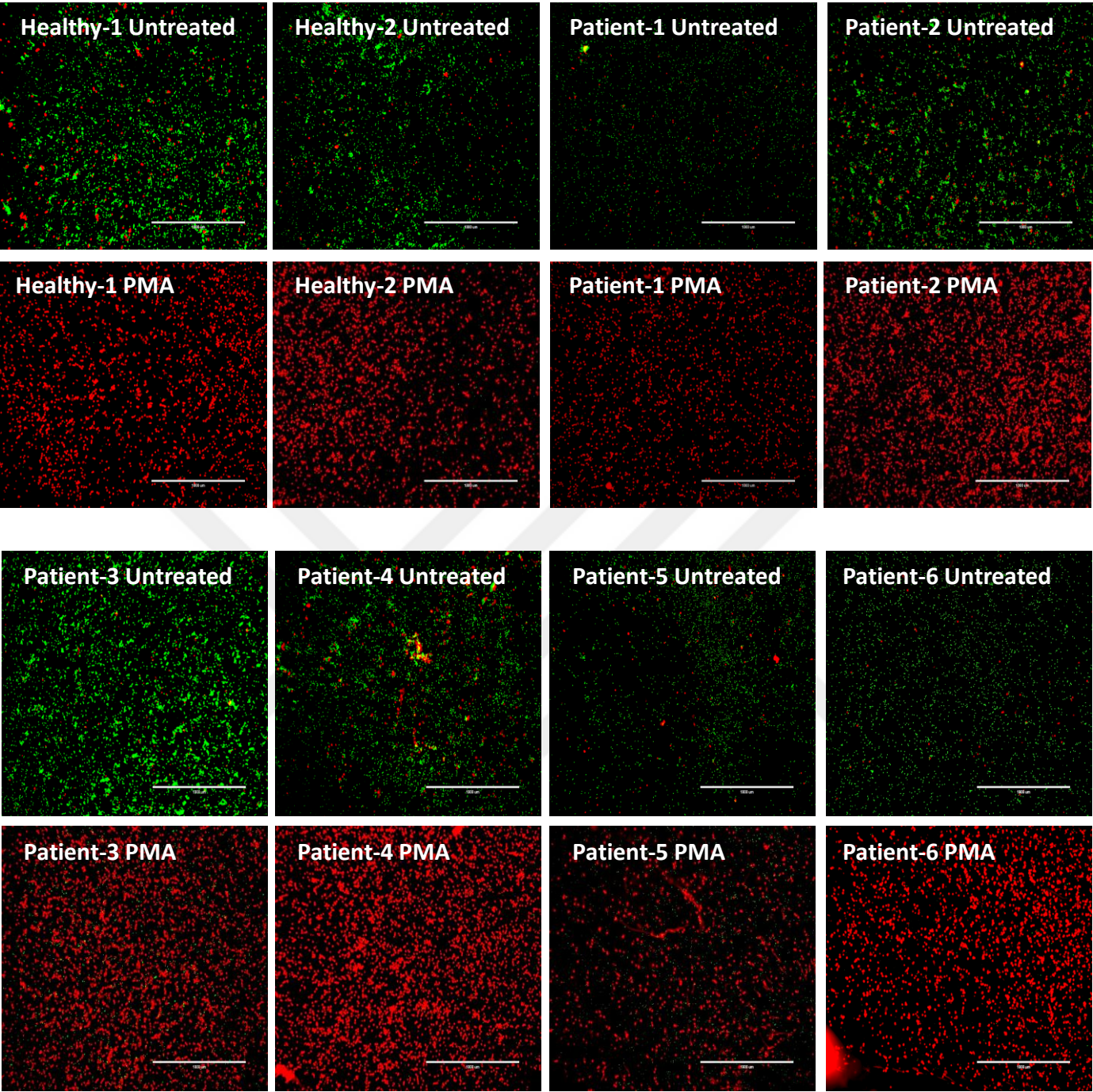


Figure 3.54 IL-10 cytokine production profile of PBMCs from SCI patients and healthy controls (n=2) upon PRR stimulations for 24 hours. Patients and healthy controls PMBCs were stimulated with TLR4 (LPS, 5 μ g/ml) and TLR7 (R848 5 μ g/ml) ligands for 24 hours at 37°C (stimulation was performed in duplicates). Supernatants were collected for cytokine ELISA.

A.



B.

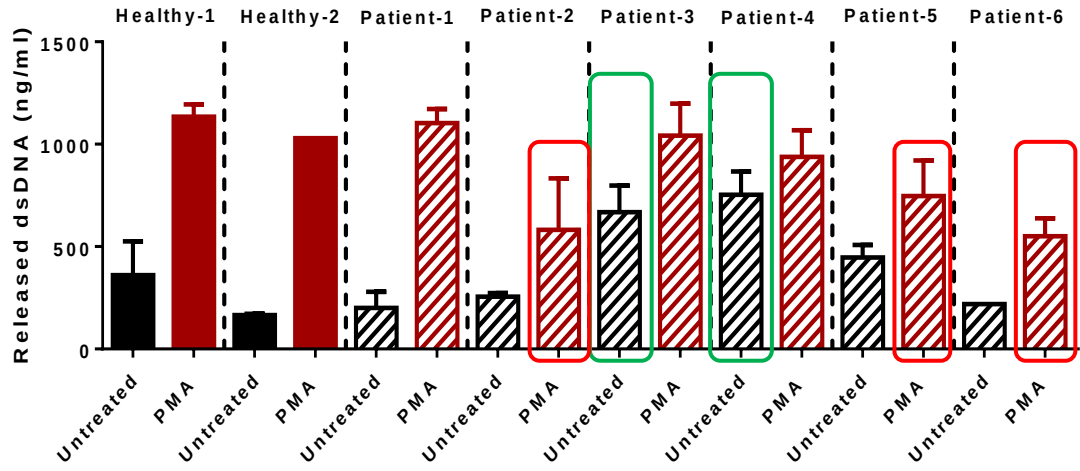
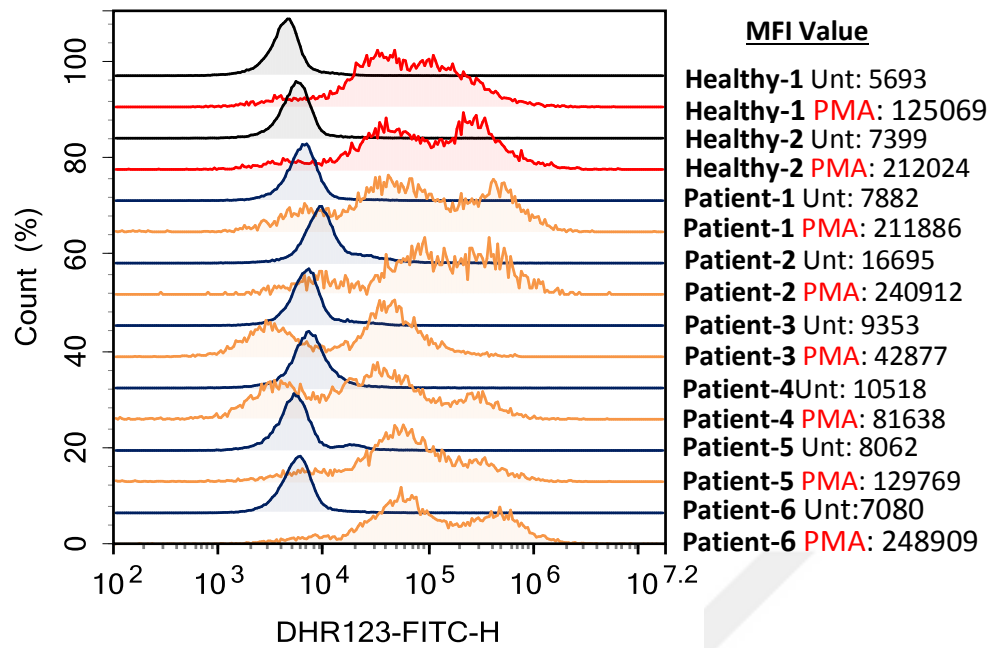


Figure 3.56 Microscopic and fluorometric analysis of SCI patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left untreated for 4 hours and NET formation was analyzed by staining extracellular DNA (represented in red) with SytoxOrange and nuclear DNA (represented in green) with Syto16Green. Quantification of released dsDNA was determined by micrococcal nuclease analysis. **A.** Representative immunofluorescent images of neutrophils captured at 4X. **B.** Quantification of released dsDNA.

A.



B.

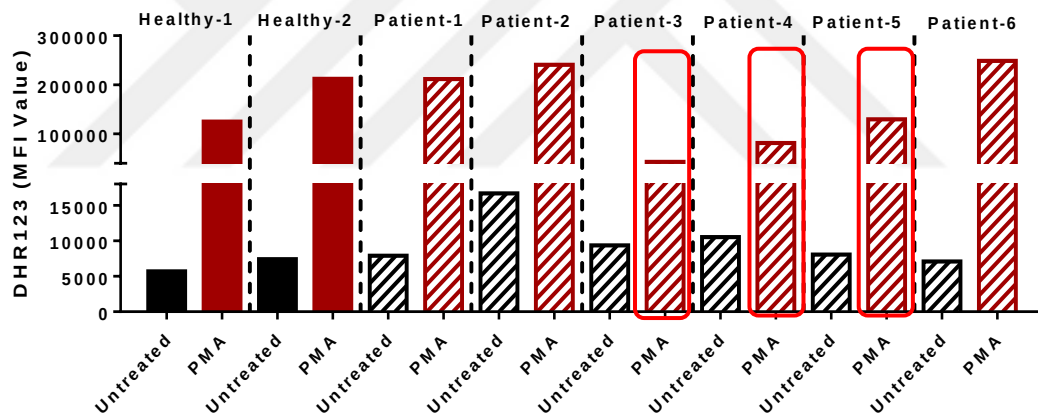


Figure 3.57 Determination of respiratory burst of SCI patients and healthy control neutrophils. Neutrophils from patients and controls were stimulated with PMA (50ng/ml) or left untreated for 4 hours and respiratory burst was analyzed by staining neutrophils with DHR123. **A.** Mean fluorescence intensity plot of DHR123⁺ neutrophils. **B.** Representative bar graph.

CHAPTER 4

DISCUSSION

Evolution depends on mutations among genomes of organisms. Functional and structural properties of proteins may change depending on mutation accumulation and may favor populations against environmental changes. Mammals have developed improved complex systems including immune system throughout evolutionary development to increase survival capacity. Efficient and collective immune response against pathogens are crucial for people. Changing in any part of this complex system of immunity can disrupt responses and leads to immunological changes including increased infections, immunodeficiencies and malignancies [70]. Immunodeficiencies are generally occurred due to de novo mutations that arises from consanguineous marriages and causes genetic alterations. It is crucial to find changes of immune responses behind primary immune deficient patients since limited replication of diseases in animal models.

CD27 is the one of the members of TNF receptor family that has role on T cell differentiation, activation and B cell responses. Its interaction with ligand, CD70, is detrimental for survival of antigen specific CD8⁺ T cells, B cell response and memory. Deficiency in CD27 membrane receptor mainly causes loss of T cell dependent B cell responses following both cellular and humoral immune system [71]. In this thesis, immunological phenotypes of two patients with genetic CD27 deficiency were described. Absence of CD27 receptor was confirmed in CD45⁺, CD19⁺ and CD3⁺ cells of patients. In one patient reversed CD4/CD8 ratio was determined which could be depend on either immunodeficiency or infection status. Change in percentages of B cell types in circulating blood was different in patient to patient. Transitional B cells are intermediate B cells that acts as a bridge in between immature cells in bone marrow and mature cells in spleen. Negative selection with autoantibodies is mediated by transitional B cells and maturation of B cells in secondary lymphoid organs are occurred [72, 73]. Increased transitional B cell percentage in whole blood was observed in CD27^{-/-} patients.

As deficiency in CD27 affects T cell responses, phosphorylation of STAT proteins and cytokine production from them was analyzed through recombinant protein stimulation and flow analysis. After IFN- β stimulation, the phosphorylation level of STAT1 in CD4⁺ T cells was increased in CD27-deficient patients as compared to healthy group. Patients with CD27 deficiency are susceptible for viral infections due to impaired antigen T cell response, IFN- γ secretion from PRRs on PBMCs also were investigated. IFN- γ secretion from innate cells including iNKT, NK and NKT were normal but, due to increased STAT1 phosphorylation elevated IFN- γ from adaptive cells including CD4⁺ and CD8⁺ T cells were observed. While no difference in STAT3 phosphorylation levels induced by IL-6 was observed, decreased STAT5 phosphorylation induced by IL-2 was seen in patients. Differentiation and proliferation of primed T cells requires IL-2. Impaired differentiation of T cells in CD27^{-/-} may also cause by decreased phosphorylation of STAT5 protein and its signaling pathway. In addition to these, even though patients had normal STAT3 phosphorylation levels, increased STAT1 phosphorylation may damage balance in Th1/Th17 that favors Th1 response. Overall, abnormality in Th subsets may be the reason for immune impairment in CD27 deficiency. Beside adaptive immunity, innate immune responses of patients were studied through activation of different TLR and inflammasome pathways. Normal IFN- γ and IL-1 β responses were observed from PBMCs while increased TNF- α and IL-10 was present in patients. Results suggest that patients' both humoral and cellular immune responses could be impaired.

STAT1 is a member of the STAT protein family whose mutations can cause gain-of-function (GOF) or loss of function (LOF). Recurrent widespread infections are common in both mutations. In this study, another primary immune deficiency arises from gain of mutation in STAT1 gene was investigated. Main characterization of STAT1 GOF mutation is defined as hyper phosphorylation in STAT1 and delay in de-phosphorylation. To determine the STAT1 phosphorylation and dephosphorylation levels of cells, with IFN- β treatment CD4⁺ T cell phosphorylation levels were analyzed by flow cytometry. All STAT1 GOF defined patients had increased STAT1 phosphorylation levels and decreased and delay in dephosphorylation which confirms deficiency. Imbalance in Th1 and Th17 responses were observed in patients that results in fungal infections depending on

low IL-17a levels [74]. Although the STAT1 phosphorylation level was high, it was determined that IFN- γ released due to Th1 response was low in patients as well as IL-17a released due to Th17 response. To identify defects in innate immunity, cytokine ELISA was performed through different PRR ligands. It was observed that the IFN- γ responses of the patients to the ligands of the endosomal and inflammatory pathways were lower than healthy controls while they have normal level of IL-12 levels. Normal IL-12 levels and elevated IFN- γ levels could be caused by defect in IL-12 induced Th1 differentiation. In addition, increased NETotic activities at the basal level and spontaneous NET formation as well as decreased NETotic activity via PMA stimulations was investigated in patients. Increased STAT1 phosphorylation, imbalanced Th1/Th17 balance and impaired regulation in NETotic activity could be reason for immune dysregulation in STAT1 GOF patients.

Uncontrolled immune responses and impairment in immunological mechanisms are seen in SCI patients. In both acute or chronic spinal cord injury, inflammation is present but, reasons behind them are not fully defined. Determining immunological abnormalities in SCI patients are important for new approaches for therapy options [75]. In this study, we tried to determine how innate and adaptive immune system responses of SCI patients with and without infection differ. Reversed CD4/CD8 ratio was observed in SCI patients supports rehospitalization due to current infections. Increased Treg and pDCs cell percentage was seen in patients while B and NK cell percentages are different from patient to patient pointing out that different immunological activities. When we investigated adaptive immune activity, we found that while SCI patients showed normal Th1 responses, Th17 and Treg responses were elevated in patients. Imbalance Th1/Th17 activity that favors Th17 and increased immunosuppressive activity of Tregs could be the reason for susceptibility to infection of SCI patients. Although NETotic activities of neutrophils of all patients were similar in healthy subjects, fluorometric results showed that some patients had more NETotic activities than healthy controls. These results suggest that SCI patients become more susceptible to infection due to the low response to the pathogen despite increased neutrophil activity and the immunosuppressive effect of the amount of IL-10 caused by excess Treg activity.

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