

**CHARACTERIZATION OF INNATE AND ADAPTIVE  
IMMUNE RESPONSES OF TWO RARE PRIMARY  
IMMUNE DEFICIENCIES: CTPS1 AND CD55**

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DEFICIENCIES: CTPS1 AND CD55

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September 2019

We certify that we have read this thesis 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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*I dedicate this work to the patients and their  
families....*

# ABSTRACT

## CHARACTERIZATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES OF TWO RARE PRIMARY IMMUNE DEFICIENCIES: CTPS1 AND CD55

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Primary Immune deficiencies (PIDs) are disorders of immune system caused by mutated genes. There are approximately 350 different disorders and each day novel ones are being defined. They can be categorized based on part of immune system harboring mutation; that is, they can be divided into disorders of innate and adaptive immune system. Each of them represents itself distinctly. In that perspective, studies based on characterization of PIDs enable us to comprehend how immune system works. Herein, we characterized innate and adaptive immune responses of two rare immune deficiencies: CTPS1 and CD55 which are novel examples of disorders of adaptive and innate immune system, respectively. CTPS1 is an enzyme functioning in *de novo* synthesis of nucleotide, CTP. Defective CTPS1 enzyme impairs lymphocytes to proliferate, however, other aspects of this deficiency still remain elusive. Since patients are prone to viral infections, we first explored functionality of cytotoxic T-cells through assessing STAT1 phosphorylation levels and expression of activation markers. Even though flow cytometry analyses revealed that CTPS1 deficient CD8<sup>+</sup> T-cells had normal phospho-STAT1 levels, degranulation marker confined to surface of CD8<sup>+</sup> T-cells were found to be elevated. Next, we investigated CD4<sup>+</sup> T-cells with cytokines that are crucial for differentiation and fate. We detected that patient CD4<sup>+</sup> T-cells had low phospho-STAT3 and phospho-STAT5 levels. Then, we checked the cytokine production profiles of CD4<sup>+</sup> T-cells. Data indicated that percentages of IL-17a and IL-

10 secreting cells are reduced in patient whereas Th1 and Th2 signatures were similar to healthy controls. Moreover, IFN- $\alpha$  levels of PBMCs upon TLR3, TLR7 and TLR9 ligand stimulations were found to be similar to healthy responses. Notably, patient had slightly reduced TLR7 and IFI16-STING mediated type II IFN secretion. We further showed that CTPS1 PBMCs had normal IL-12 levels, implying that the reduction in IFN- $\gamma$  was not due to either dysfunction of innate immune cells or by aberrant APC function. Surprisingly, patient PBMCs had higher number of granulocytes and flow cytometry analyses revealed that these granulocytes were CD14<sup>-</sup> CD15<sup>+</sup> low-density granulocytes. This prompted us to assess the NETotic tendencies of CTPS1 neutrophils and we observed via microscopic and spectrofluorometric investigations that they underwent spontaneous NETosis. In the second part of this study, we worked with CD55 deficient patient PBMCs. CD55 is a complement regulatory protein and it inhibits formation of C3-convertase in classical and alternative complement pathways. Thus, patients suffer from aberrant complement activation in its absence as well as severe bowel inflammation and recurrent infections along with nutrient loss leading to malnutrition and growth deprivation. Eculizumab therapy was initiated to these patients in order to neutralize their pathologic C5 levels. We attempted to investigate effect of CD55 deficiency on PRR-complement cross-talk, recurrent infections and checked the contribution of Eculizumab therapy to their immune status. PBMCs of 4 patients, i) before (BT) and ii) after (AT) a single dose of Eculizumab administration were isolated. BT PBMCs had significantly reduced IFN- $\alpha$  and IP-10 secretions upon endosomal (TLR3, TLR7 and TLR9) TLRs and nucleic acid sensors (STING, DAI, RIG-I & MAVS) stimulations. Moreover, single Eculizumab therapy did not alter this innate immune dysfunction. Furthermore, we assessed levels of TNF- $\alpha$ , IL-6 productions from PBMCs stimulated with same ligands and observed that IL-6 but not TNF- $\alpha$  was reduced after PRR stimulations. Next, immunomodulatory effects of CD55 EVs before and after Eculizumab therapy was sought. ELISA results demonstrated that AT EV incubation on CD55<sup>-/-</sup> PBMCs lead to reduced TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  production. Meanwhile, AT EVs increased IL-10 production from patient PBMCs. Lastly, we assessed cytokine levels after healthy PBMCs were incubated with BT and AT EVs and found that PBMCs that incubated with BT EVs had elevated levels of IP-10 cytokine. When taken together, progression of PIDs might have been contributed by extracellular vesicles.

**Keywords:** Primary Immune Deficiencies, CTPS1, CD55, complement, innate immune system, adaptive immune system, Eculizumab, extracellular vesicles.

# ÖZET

## İKİ ADET NADİR GÖRÜLEN PRİMER İMMÜN YETMEZLİKLERİN DOĞAL VE EDİNSEL BAĞIŞIKLIK CEVAPLARININ KARAKTERİZE EDİLMESİ: CTPS1 VE CD55

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Moleküler Biyoloji ve Genetik, Yüksek Lisans

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İmmün sistemde kalıtsal olarak bulunan mutasyonların sonucunda ortaya çıkan hastalıklar, Primer İmmün Yetmezlik (PİY) olarak bilinmektedir. Yaklaşık 350'den fazla PİY tespit edilmiş olmasına rağmen, her geçen gün yeni PİY vakaları tanımlanarak bu liste büyümektedir. PİY'ler, immün sistemi etkiledikleri alanlara göre iki farklı kategoriye ayrılır: Doğal ve edinsel bağışıklık immün yetmezlikleri. Bundan dolayı, her bir immün yetmezlik çeşidinin semptomları birbirinden farklıdır. Bunu göz önünde bulundurarak, PİY üzerine yapılan çalışmalar, immün sistemin nasıl çalıştığını göstermede önemli rol oynamaktadır. Bu çalışmada, iki adet nadir görülen monogenik eksikliğin immün yanıtlarını araştırdık. CTPS1 eksikliği edinsel bağışıklık yetmezliklerinin, CD55 eksikliği ise doğuştan gelen immün yetmezliklerinin en yeni örneklerindedir. CTPS1, sitidin trifosfat (CTP) de novo üretimin yolağında yer alan enzimlerden biridir ve eksikliği lenfositlerin çoğalmasını etkilediği bilinen bir PİY'dir. Fakat, bu hastalığın diğer immün parametreleri nasıl etkilediği hâlâ tam olarak bilinmemektedir. Hastaların viral enfeksiyona yatkınlığı sebebiyle, ilk olarak hastadan izole edilen sitotoksik T-hücrelerin STAT1 fosforlanma seviyelerini ve etkinleşme yüzey belirteç miktarını akan hücre ölçeği ile tespit ettik. STAT1 fosforlanma düzeyi sağlıklı bireylerle aynı düzeyde seyretse de sitotoksik hücrelerin yüzeyinde ifade edilen etkinleşme belirteçlerinin hastada arttığını bulduk. Bunun üzerine, CD4<sup>+</sup> T-hücrelerinin farklılaşmasında önemli rol oynayan

sitokinlerin etkisini arařtırdık. CD4<sup>+</sup> T-hücrelerinin sađlıklı bireylere oranla daha düşük düzeyde STAT3 ve STAT5 fosforlanması gerekleřtirebildiđini tespit ettik. Sonrasında, yardımcı T-hücrelerinden üretilen sitokin seviyelerini inceledik. Sonular, IL-17a ve IL-10 sitokinlerinin seviyelerinin de sađlıklılara kıyasla azalmıř, Th1 ve Th2 yanıtlarının ise benzer seviyelerde olduđunu göstermiřtir. Ayrıca, TLR3, TLR7 ve TLR9 almaları uyarıldıklarında CTPS1 periferik mononükleer hücreleri (PMBCs) tarafından üretilen IFN- $\alpha$  yanıtlarının sađlıklılara göre benzer seviyede olduđunu bulduk. Bunun yanı sıra, hasta PMBC'lerinin TLR7 ve IFI16-STING yolakları uyarıldıđında, tip iki IFN yanıtlarının az miktarda azaldıđını elde ettik. Bu yolakların uyarımı sonucunda üretilen IL-12 yanıtlarının normal seviyelerde olduđunu bulduk. Bu sonular bize, azalmıř IFN- $\gamma$  yanıtlarının dođal bađıřıklık sisteminin düzgün fonksiyon gösterememesinden ya da anormal APC yanıtlarından kaynaklanmadıđını göstermiř oldu. Beklenmedik řekilde, hastanın PMBC'lerinde yüksek miktarda granüllü hücrelerin bulunduđunu gözlemleyince, akan hücre ölçer ile yaptığımız incelemeler bu hücrelerin CD14<sup>-</sup> CD15<sup>+</sup> ifade eden düşük yoğunluklu granüositler olduđunu belirledik. Bu bulgu bizi, hastanın nötrofillerinin NETotik eđilimlerini arařtırmaya itti. Mikroskopik ve spektrofotometrik incelemeler bize CTPS1 eksikliđi bulunan hasta nötrofillerinin spontan NETosiz gerekleřtirdiđini gösterdi. Bu tezin ikinci kısmında, CD55 eksikliđi bulunan hastaların PMBC'leri üzerine alıřmalar yaptık. CD55, kompleman yolađında bulunan bir düzenleyici proteindir. Bu protein, klasik ve alternatif kompleman yolaklarında bulunan C3 Konvertaz'ın etkinliđini düzenlemektedir. Bunun dođal sonucu olarak, CD55 eksikliđi bulunan hastalarda anormal düzeyde kompleman etkinleřmesi gerekleřmektedir. Bununla beraber, hastalarda řiddetli karın iltihabı, sık enfeksiyonlar ve besin kaybı sonucu gerekleřen yetersiz beslenme ve büyüme bozukluđu görölmektedir. Hastalardaki yüksek miktarda seyreden C5a aktif protein seviyelerinin azaltılması amacıyla, Eculizumab adlı ila hastalara verilmeye bařlanmıřtır. Bu nedenle bu alıřmamızda, CD55 eksikliđinin PRR-kompleman apraz konuřması ve tekrarlayan enfeksiyonlar üzerindeki etkisini arařtırmayı amaladık. Bununla beraber, Eculizumab terapisinin immün sistemin üzerine olan etkisini de arařtırdık. Dört hastanın PBCM'leri tek doz Eculizumab i) terapisi öncesi (BT) ve ii) terapisi sonrası (AT) izole edildi. BT PMBC'leri, endozomal TLR (TLR3, TLR7 ve TLR9) ve hücre ii nükleik asit sensör (STING, DAI, RIG-I & MAVS) yolakları uyarıldıđında, sađlıklılara oranla anlamlı derecede düşük seviyelerde IFN- $\alpha$  ve IP-10 üretirlerken, Eculizumab terapisinin, dođal bađıřıklık yanıtlarındaki bozukluđu geri döndürmeye yeterli olmadıđını bulduk. Aynı almalar uyarılıp BT ve AT PMBC'lerinden üretilen TNF- $\alpha$ , ve

IL-6 sitokin miktarları da tayin edildi. Sonular bize sadece IL-6 sitokininin saėlıklılarla karşılaştırıldığında anlamlı derecede düşük seviyelerde üretildiğini gösterdi. CD55 eksikliği bulunan hastalarının hücre dışı keseciklerinin (EVs), immün sistem üzerine etkisini Eculizumab terapisi öncesinde ve sonrasında inceledik. Sonular bize, AT EV'lerinin, CD55 hastalarının PBMC'leri ile beraber bekletilmesi sonucunda, BT EV'lere oranla daha düşük seviyede TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  ürettirdiklerini gösterdi. Aynı zamanda AT EV'lerinin PBMC'lerden üretilen IL-10 miktarını artırdığını de tespit ettik. Son olarak, BT ve AT EV'leri, saėlıklı bireylerden izole edilen PBMC'lerle inkübe edildiğinde, BT EV'lerin, PBMC'lere daha fazla IP-10 salgılattığını tespit ettik. Bütün bu sonular birlikte ele alındığında, EV'lerin, PIY'lerin hastalık sürecine katkıda bulunabileceğini önermektedir.

Anahtar Kelimeler: Primer immün yetmezlik, CTPS1, CD55, kompleman, doėal baėıřıklık sistemi, edinsel baėıřıklık sistemi, Eculizumab, hücre dışı kesecikler.



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## ABBREVIATIONS

ALRs	AIM2-like receptors
AIM2	Absent in Melanoma 2
APC	Antigen Presenting Cell
aHUS	atypical Hemolytic Uremic Syndrome
ATP	Adenosine Triphosphate
AT	After Eculizumab Therapy
BCRs	B-cell Receptors
BT	Before Eculizumab Therapy
C1INH	C1 inhibitor
CHAPLE	Hyperactivation of Complement Angiopathic Thrombosis and Protein Losing Enteropathy
CLRs	C-type Lectin Receptors
CMV	Cytomegalovirus
CTP	Cytidine Triphosphate
CTPS1	Cytidine Triphosphate Synthase 1
CID	Combined Immunodeficiency
DAF	Decay-Accelerating Factor
DAMPs	Damage-Associated Molecular Patterns
DNA	Deoxyribonucleic Acid
dsDNA	Double-Stranded DNA
dsRNA	Double-Stranded RNA
EBV	Epstein-Barr virus
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
FBS	Fetal Bovine Serum
FMF	Familial Mediterranean Fever

GPI	Glycosylphosphatidylinositol
HAE	Hereditary Angioedema
HIV	Human Immunodeficiency Virus
HMBG1	High mobility binding group protein 1
HSCT	Hematopoietic-stem Cell Transplantation
HSV	Herpes Simplex virus
IKK	I $\kappa$ B kinase
iNKT	invariant Natural Killer Cells
IP-10	IFN- $\gamma$ Inducible Protein-10
IRAK	IL-1-receptor associated kinase
IRFs	Interferon Regulatory Factors
IVIG	Intravenous Immunoglobulin
LDNs	Low Density Neutrophils
LGDs	Low Density Granulocytes
LPS	Lipopolysaccharide
LRRs	Leucine Rich Repeats
MAC	Membrane Attack Complex
MAIT	Mucosal Associated invariant T-cells
MAPK	Mitogen-activated protein kinase
MASP	MBL-associated serine proteases
MBL	Mannose-Binding Lectin
MD-2	Myeloid Differentiation protein-2
MDA5	Melanoma differentiation associated 5
MFI	Mean Fluorescent Intensity
MPO	Myeloperoxidase
MVB	Multi vesicular bodies
MyD88	Myeloid differentiation primary response gene 88

NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil Elastase
NETosis	NET formation
NETs	Neutrophil Extracellular Traps
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
PNH	Paroxysmal Nocturnal Hemoglobinuria
poly(dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
poly(I:C)	Polyinosinic-Polycytidylic acid
PIDs	Primary Immune Deficiencies
PRRs	Pathogen Recognition Receptors
RIG-I	Retinoic Acid Inducible Gene I
RLRs	RIG Like Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCID	Severe Combined Immunodeficiency
SLE	Systemic Lupus Erythematosus
ssRNA	Single-Stranded RNA
STING	Stimulator of IFN genes
TANK	TRAF-family member associated NF $\kappa$ B activator
TBK1	Tank Binding Kinase Protein 1

TCC	Terminal Complement Complex
TCRs	T-cell Receptors
Th	T-helper
TLRs	Toll Like Receptors
TNF	Tumor Necrosis Factor
TIR	Toll/IL-1 receptor
TRAF	TNF-receptor associated factor
Tregs	Regulatory T-cells
TRIF	(TIR)-domain-containing adapter-inducing IFN- $\beta$
VZV	Varicella Zoster virus

# 1. INTRODUCTION

## 1.1. IMMUNE SYSTEM

Throughout evolution, all organisms were subjected to threats of pathogenic microorganisms as invaders. Hence, evolutionary parameters selected organisms possessing survival against them. As a consequence of this, complex and unique network of defense responses were evolved which is called as immune system [1].

Although invertebrates have primitive immune responses, as complexity of immune system gets higher, species possessing it become more convoluted in the context of evolutionary order [2]. In that essence, most appreciated immune responses are the ones within class of mammals. Mammalian immune system can be fundamentally divided into i) innate and ii) adaptive arms. These distinct yet interconnected array of responses are composed of special cells, receptors and molecules residing in different layers and levels of physiological barriers [3].

Innate immunity is regarded as first line of defense of the whole immune system. This arm adopted certain levels of strategies, which are anatomic barriers, complement and antimicrobial proteins, innate immune cells, to protect host from invaders [4]. In order to successfully invade, pathogens must pass through anatomic barriers such as skin, intestinal epithelium, oral mucosa, then, they dodge to overcome against complement proteins. If they still prevail, tissue resident innate immune cells can recognize and generate quick responses. Meanwhile, the responses of innate immune cells are achieved through germline encoded receptors that are able to recognize evolutionarily conserved non-self-motifs on pathogens. Even though innate immune cells were used to be considered as having no memory, growing bodies of evidences challenge it; that is, innate immune cells indeed confer a memory through epigenetic reprogramming and 'Trained Immunity' is coined to define this novel aspect of innate immunology field [5–6]. Responses elicited from activated innate immune cells, specifically phagocytes and antigen-presenting cells (APCs), are destined to license adaptive immune cells.

While innate cells provide fast, non-specific immunity, adaptive immunity is established through slow, vast but specific responses against antigenic peptides of invaders. In order to achieve that, two broad categories of adaptive immune system are needed to

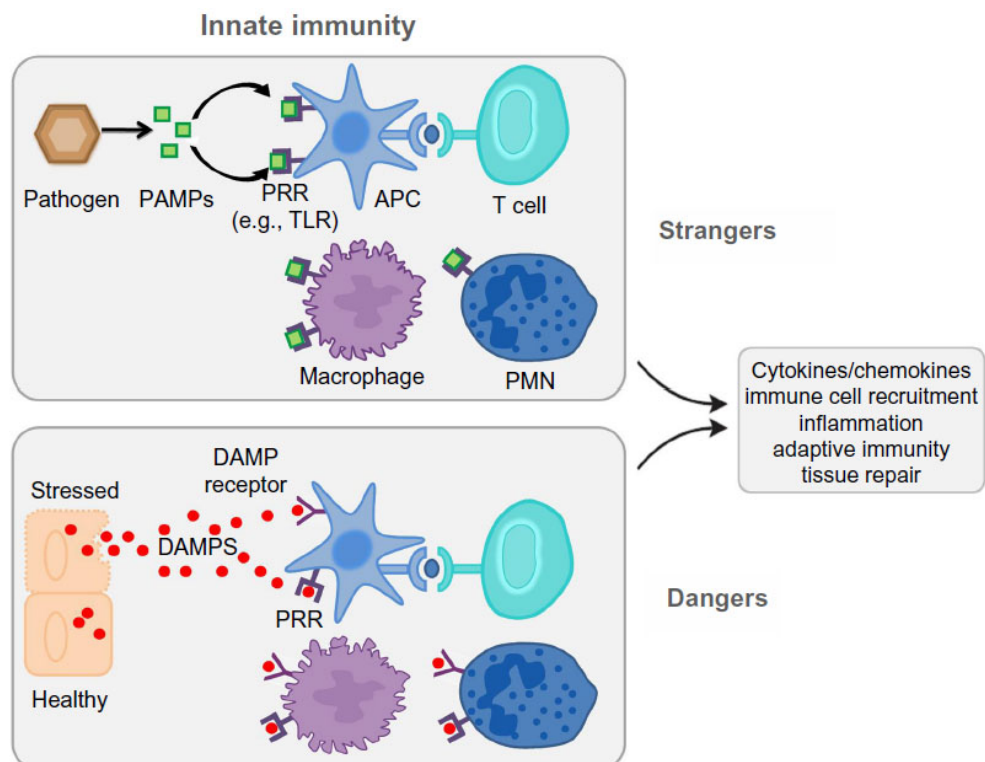
be involved. These are cell-mediated immunity and humoral immunity, comprise from subsets of T-cells and B-cell produced antibodies, respectively [7–8]. Even though all T-cells are primed in secondary lymphoid organs by activated and migrated APCs, their classification is generally divided into CD8<sup>+</sup> and CD4<sup>+</sup> T-cells regarding to their composition of T-cell Receptors (TCRs). CD8<sup>+</sup> T-cells (or cytotoxic T-cells) generate crucial and powerful immune responses in the context of infection of intracellular pathogens and cancer cells [9]. CD4<sup>+</sup> T-cells or helper T-cells (Th), however, are further categorized into subtypes according to their different orchestration of the immune system. As their names imply, they are differentiated into several subtypes; so as to provide assistance to clearance of pathogens. Similarly, B-cells and their subsets, bearing B-cell Receptors (BCRs), are able to be primed through T-helper cells or directly by pathogens per se. Regardless of activation route, they proliferate and differentiate to become plasma cells and secrete specific subtypes of immunoglobulins such as IgA, IgG, IgE [10–11]. Unlike memory mechanism of innate cells, cells of adaptive immunity become quiescent until reinvasion of pathogens and this process is building block of vaccination [12].

### **1.1.1. Innate Immune System**

Tissues of hosts are constantly being received threats from pathogenic and even commensal organisms. In most of the conditions, they reside on external surfaces, barriers of the mammalian tissues. These barriers in frontiers are epithelial tissues that are composed of skin, covering internal linings such as respiratory, urogenital and gastrointestinal tracks of host. Not only they cover surroundings but also, they harbor special cells that are evolved to secrete antimicrobial proteins and peptides. To illustrate, Paneth cells residing within the base of epithelial crypts of small intestines secrete  $\alpha$ -defensins and RegIII $\gamma$ ; furthermore, differentiated keratinocytes in skin produce  $\beta$ -defensins and cathelicidins [13]. These peptides are evolutionarily conserved to kill efficiently and directly the invading pathogens through integrating themselves into membranes of microorganism.

Yet, microorganisms are evolved to penetrate through these barriers. As a result of this, they face another ancient component of innate immunity which is called as complement pathway. The complement system is array of soluble and membrane proteins circulating throughout extracellular fluids and blood within host. Upon an

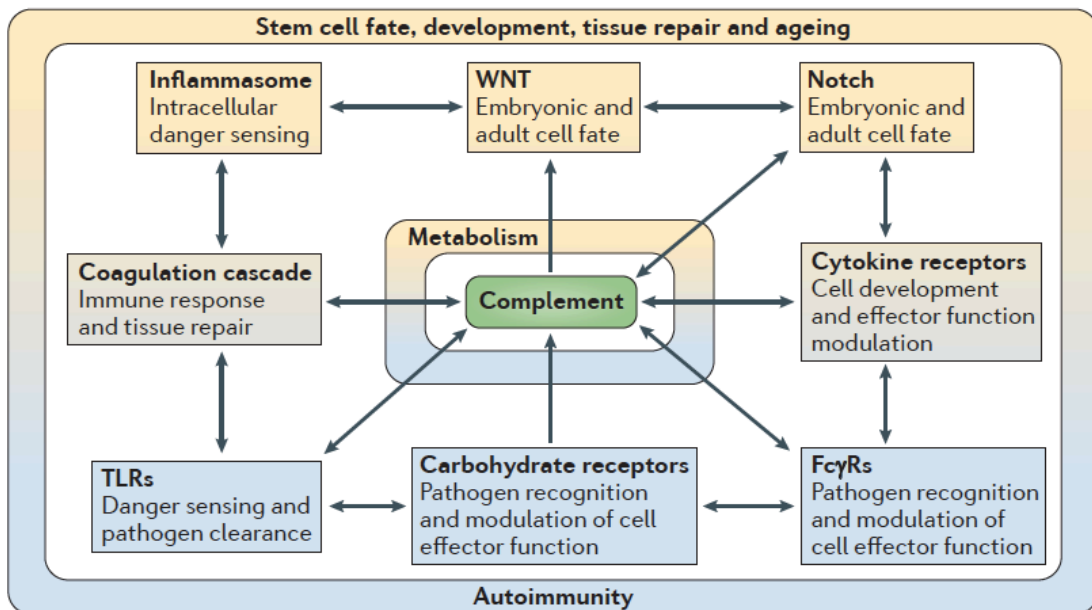
encounter with a pathogen, they get activated and interact with each other to kill and/or facilitate phagocytosis of invaders [14–15]. Nonetheless, pathogens might be equipped to escape complement pathway as well [16]. Then, innate immune cells; for example, monocytes, macrophages, dendritic cells, neutrophils and their receptors, Pathogen Recognition Receptors (PRRs), step in. Innate cells are able to discriminate non-self from self-motifs through 5 distinct PRR families which are: i) Toll-like Receptors (TLRs), ii) Nucleotide Oligomerization Domain (NOD)-like Receptors (NLRs), iii) Retinoic Acid Inducible Gene I (RIG-I) like Receptors (RLRs), iv) C-type Lectin Receptors (CLRs) and v) Absent In Melanoma 2 (AIM2)-like receptors (ALRs) [17–18]. They initiate signals when they sense Pathogen-Associated Molecular Patterns (PAMPs); similarly, they can even recognize sterile inflammatory signals which are Danger-Associated Molecular Patterns (DAMPs) [19]. While PAMPs are component of pathogens such as bacterial LPS, flagellin, unmethylated CpG-rich DNA, host cells, that underwent into necrosis or necroptosis due to stress, release DAMPs such as high mobility binding group protein 1 (HMBG1), DNA, histones, and ATP (Figure 1.1).



**Figure 1.1** Origins of PAMPs, DAMPs and their recognition by innate immunity. (Adopted from [20])

### 1.1.1.1 Complement System

Harboring over then 30 distinct proteins and being discovered 110 years ago, complement system, is one of the most ancient arm of innate immune system [21]. These, membrane bound and soluble proteins get activated upon presence of PAMPs and/or DAMPs and immune complexes (IgM) [22]. Although complement cascade can be activated systemically and locally they all are synthesized as pro-enzymes [22–23]. In order to become active in the cascade they need to be cleaved by other complement proteins. In contrast to common appreciated roles of complements; for example, opsonization, facilitating phagocytosis and direct lysing of pathogens, recent discoveries demonstrated that activated complement proteins are able to cross-talk and influence PRR signaling, especially TLRs, RIG-MAVS and inflammasomes, regulate tissue damage repair in cancer and kidney diseases, influence metabolic activities of cells and modulate responses of adaptive immune cells [21–24, 30] (Figure 1.2).



**Figure 1.2 Cross-talk between complement pathway and physiological systems.** (Adopted from [21])

Systemic complement activation is composed of three distinct cascades: i) Classical, ii) Lectin iii) Alternative pathways. Even though they all lead to same outcome,

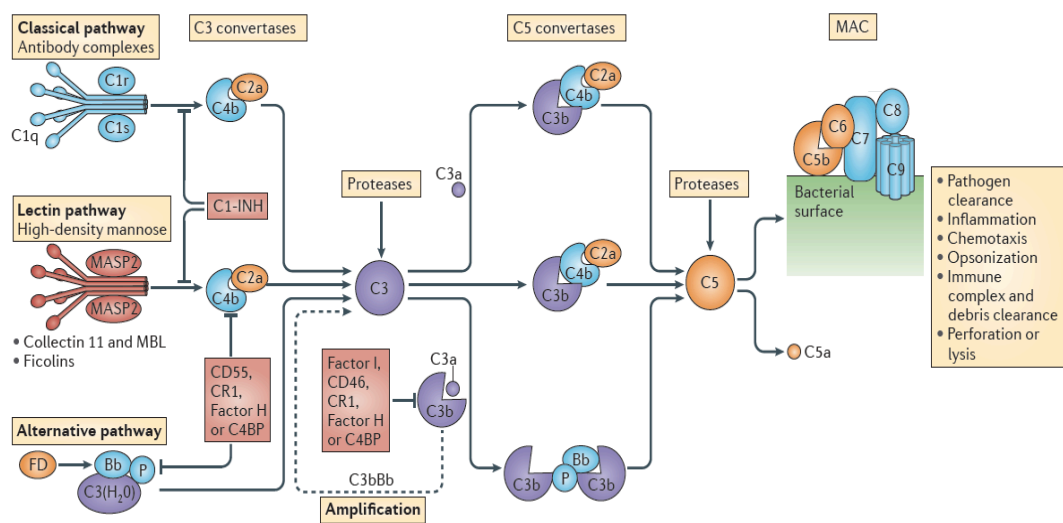


generation of anaphylatoxins alongside Membrane Attack Complex (MAC), each pathway utilizes different combinations of cascade proteins including their respective regulators (Figure 1.3).

Lectin cascade of complement pathway is initiated via oligomeric receptors called mannose-binding lectin (MBL) and Ficolins. While MBLs are evolved to recognize mannose or fucose carbohydrate residues that are only presented on microorganisms, Ficolins recognize acetylated oligosaccharide motifs. Moreover, MBLs and Ficolins form complexes with soluble, inactive MBL-associated serine proteases (MASP) as MASP-1, MASP-2 and MASP-3 circulate throughout blood. Upon binding surface of invaders, MBLs and Ficolins undergo conformational change which in turn activates MASPs. Classical pathway utilizes C1q, C1r and C1s comprising C1 complex. While C1q, functionally and structurally, resembles to MBLs and Ficolins, C1r and C1s can be regarded as MASPs of classical pathway. In contrast to MBLs and Ficolins, C1q recognize pathogens through 3 distinct ways: i) Direct, ii) through C-reactive Protein and iii) constant part of antibodies bound to pathogens. Nevertheless, either activated MASPs or C1r-C1s cleaves C4 complement protein in plasma, eventually leading to generation of C4b and C4a [14–15]. While C4b confined to surface of pathogen, C4a floats in plasma. Then, C4b recruits another cardinal complement protein, C2 and enables its cleavage by MASPs into C2a and C2b. C4bC2a complex is formed and called as C3 convertase or classical C3 convertase. As its name implies, C3 convertase, converts C3 into C3a and C3b. Consequently, C3b remains on (opsonize) pathogen surface by covalent bound and together with C4bC2a, C3b becomes C5 convertase, cleaving soluble C5 protein into C5a and C5b. Meanwhile C3a and C5a becomes one of the anaphylatoxins and contributes to phagocytosis and inflammation. Membrane-bound C5b, however, recruits C6, C7 and C8. C7 undergoes conformational change and inserts its hydrophobic sites into membranes of pathogens while recruiting C8. These series of recruitments eventually lead to incorporation of C9 proteins into membranes. Polymerization of C9 proteins form MAC complex (also called as Terminal Complement Complex (TCC) [31]) which destroy pathogens via loss of intracellular elements and allowing penetration of antimicrobial peptides and lysosomal enzymes into pathogens.

Alternative pathway, rather than directly recognizing the pathogens, is activated by already deposited C3b proteins from alternative and lectin pathway or spontaneous hydrolysis. In the case of former, after factor B binds to cleaved C3b, factor D cleaves

it into membrane bound and soluble forms as Bb and Ba, respectively. C3bBb being alternative C3 convertase contributes to complement pathway. However, in the case of latter, thioester bond of C3 is spontaneously cleaved and form C3(H<sub>2</sub>O). Like C3b, factor B and factor D are able to bind and cleave it, respectively. Therefore, C3(H<sub>2</sub>O)Bb, fluid-phase C3 convertase, is formed. Regardless of way of alternative pathway activation, alternative and fluid-phase C3 convertase require stabilization, which is achieved via factor P.



**Figure 1.3 Activation of systemic complement cascade.** (Adopted from [21])

Even though complement proteins are specific to invading microorganism, it is possible to have activation on host membranes as well. Therefore, soluble, attached to surface of host and membrane integral proteins regulating activation of complement pathways were evolved in different levels (Table 1.1) [31]. To exemplify, C1 inhibitor (C1INH) and Factor H are crucial soluble inhibitors. C1INH displaces serine proteases (C1r/s, MASP2) to reduce activation of classical and lectin pathway. Factor H displace cleaved Bb from C3b of alternative pathway to inhibit the cascade. As a result of being important regulators, deficiency or loss-of-function of C1INH and Factor H leads to hereditary angioedema (HAE) and atypical hemolytic uremic syndrome (aHUS), respectively [32]. In addition to soluble factors, CD55 or decay-accelerating factor (DAF) and CD59 (or

protectin) are regulators that are attached to host cells via Glycosylphosphatidylinositol (GPI) anchor. While DAF binds to C3b to replace Bb from it, protectin inhibits C9 recruitment to C5b678 complex. Like deficiencies of C1NH and Factor H, mutation within the synthesis of GPI tails leads to Paroxysmal Nocturnal Hemoglobinuria (PNH) [33]. Similarly, deficiency of DAF has been identified to have distinct pathophysiology (will be mentioned in Section 1.2.1.3) [34–35]. Last but not least, there are inhibitory factors of complement that are produced by pathogens to avoid complement activation [36–37].

**Table 1.1 Soluble and membrane bound regulators of complement cascade.** (Adopted from [31])

Regulator	Alternative name	Point of action	Ligand	Cell surface binding or expression	Function
<i>Soluble regulators and effectors</i>					
Factor H	None	Alternative pathway	C3b and C3d	Acquired to surface	Cofactor for factor I and acceleration of alternative pathway C3 convertase decay
FHL1	None	Alternative pathway	C3b	Acquired to surface	Cofactor for factor I and acceleration of alternative pathway C3 convertase decay
Properdin	None	Alternative pathway	C3	Binds to apoptotic surfaces	Stabilization of alternative pathway convertases
Carboxypeptidase N	Anaphylatoxin inactivator	Classical pathway and lectin pathway	C3a, C4a and C5a	NA	Inactivation of anaphylatoxins C3a and C5a
C4BP	None	Classical pathway and lectin pathway	C4	Acquired to surface	Cofactor for factor I and acceleration of classical pathway C3 convertase decay
C1q	None	Classical pathway	IgG and IgM immune complexes	Binds to apoptotic surfaces	Activation of the classical pathway
C1INH	None	Classical pathway and lectin pathway	C1r, C1s and MASP2	NA	Blocks serine protease and is a suicide substrate for C1r, C1s, MASP2, coagulation factors and C3b
CFHR1	None	Terminal pathway	C5 convertase and TCC	Acquired to surface	Inhibition of C5 convertase and TCC assembly
<i>Surface bound regulators and effectors</i>					
CR1g	VSIG4	C3	C3b, iC3b and C3c	Macrophages	iC3b-mediated phagocytosis and inhibition of alternative pathway activation
CD46	MCP	C3	C3b and C4b	All cells except erythrocytes	C3 degradation, cofactor for factor I and factor H, and effector for T cell maturation
CD55	DAF	C3	C4b2b and C3bBb	GPI anchor expression by most cell types, including erythrocytes, epithelial cells and endothelial cells	Acceleration of C3 convertase decay
CD59	Protectin	TCC	C8 and TCC	GPI anchor expression by erythrocytes and most nucleated cells, including renal cells	Inhibition of TCC assembly and formation

### **1.1.1.2 Pathogen Recognition Receptors (PRRs)**

#### **1.1.1.2.1 Toll-Like Receptors (TLRs)**

Long before the relationship between immune system and *Toll* gene was identified, it was described as a gene controlling dorso-ventral positioning of embryo throughout development of *Drosophila melanogaster*, fruit fly [38–39]. Then, strikingly, mutations in the product of this developmental gene predispose flies to suffer from gram-positive bacterial and fungal infections [40–42]. Furthermore, homologs of Toll proteins were identified and called as Toll-Like Receptors (TLRs) in humans [43].

Up to date, humans and mice do express 10 and 12 distinct TLRs, respectively [44]. TLRs can be grouped based on their location and adaptor molecules that are used within the intracellular signaling [45]. In the context of former, TLRs are divided into intracellular vesicles and cell surface TLRs. While TLR3, TLR7, TLR8, TLR9 and TLR13 are found within endosomes, TLR1, TLR2, TLR5, TLR6 and TLR11 are found on the surface membranes of cells. However, TLR4, first identified TLR, can be found on plasma membrane and in endosomes [3]. Whereas latter distinguishes TLRs via whether they use Myeloid differentiation primary response gene 88 (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing IFN- $\beta$  (TRIF) adaptor proteins. In that essence, except TLR3, all TLRs use MyD88 adaptor protein. Surprisingly, TLR4 was identified as TLR using both MyD88 and TRIF proteins for its signaling [44].

#### **1.1.1.2.2 TLR Signaling Pathway**

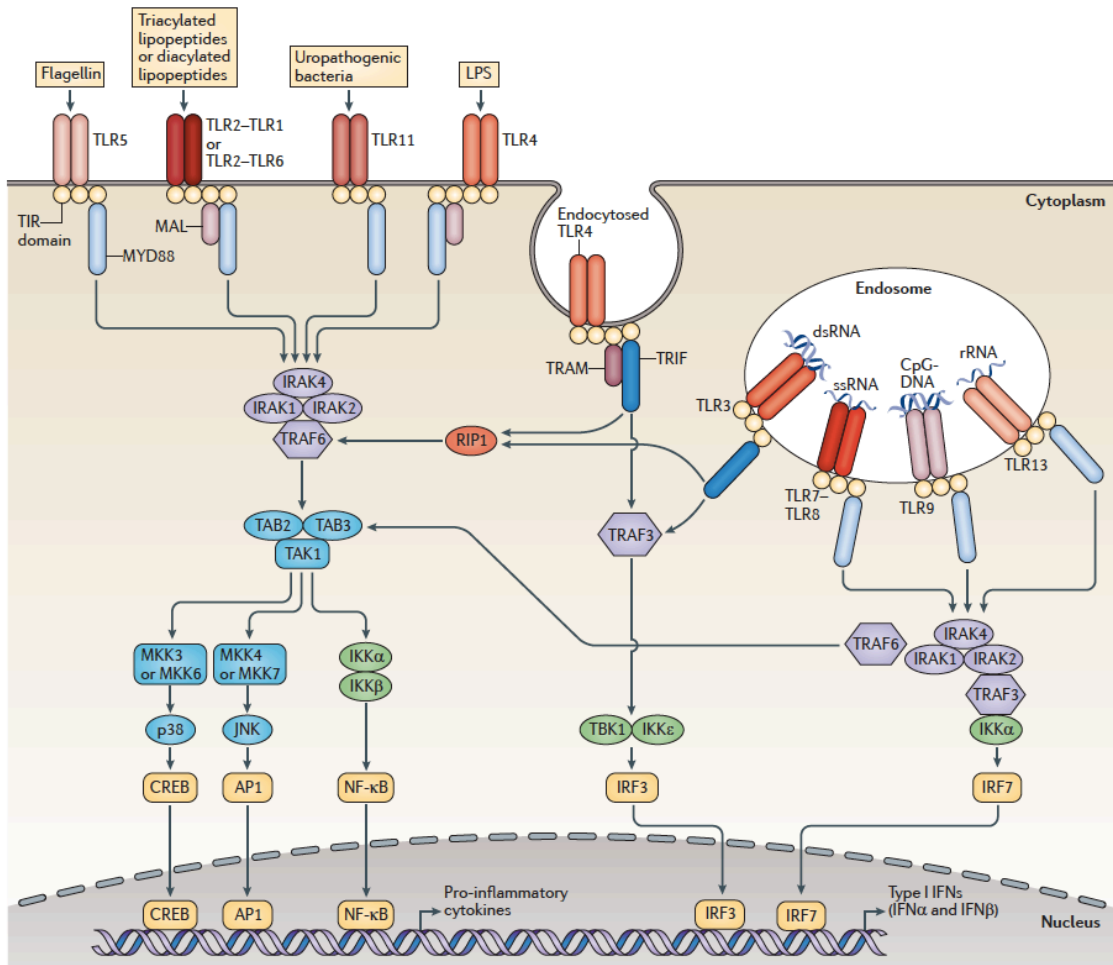
Regardless of localization of receptors and recruited adaptor proteins, all TLRs are composed of three domains: Ectodomain, transmembrane region and cytoplasmic TIR domain [44]. Ectodomains contain leucine rich repeats (LRRs), which are crucial for ligand recognition. TIR domains recruit adaptor proteins such as MyD88 and TRIF and initiates signaling cascades.

These signaling cascades have being extensively studied and delineated (Figure 1.4). Upon dimerization of TLRs, they recruit c or/and TRIF adaptor proteins via their TIR domains. Death domains of recruited MyD88 protein further recruit IL-1-receptor associated kinase 4 (IRAK4) and IRAK1 to establish scaffold protein complex. The formed scaffold, recruits E3 ligase, called as tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and activates it by phosphorylation. Activated TRAF6

along with accessory ubiquitin ligases, establish polyubiquitin scaffold to recruit transforming growth factor- $\beta$ -activated kinase 1 (TAK1) and TAK binding protein1/2 (TAB1/2) adaptor proteins. As a consequence of being recruited, IRAK1 phosphorylates TAK1 and activates it. From activated TAK1, signaling divides into two distinct parts. On one hand, TAK1 activates I $\kappa$ B kinase (IKK) complex. As it names implies, activated IKK complex phosphorylates I $\kappa$ B leading to degradation of I $\kappa$ B and, consequently, enabling NF $\kappa$ B enter into nucleus for pro-inflammatory cytokine production (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ). On the other propagation of signaling, TAK1 activates mitogen-activated protein kinase (MAPK) pathway contributing cytokine production [46].

In addition to pro-inflammatory cytokine production, TLR7 and TLR9 signaling pathway, depending on MyD88 adaptor protein, directly recruits and activates Interferon Regulatory Factor 7 (IRF7) via IRAK1. Phosphorylated and dimerized IRF7s enter nucleus and induce production of type I IFNs, especially IFN- $\alpha$ . [47].

In the contrast to MyD88-dependent signaling, TRIF pathway, that is utilized by TLR3 as well as TLR4, propagates via different proteins. Upon TLR dimerization, TRIF protein is recruited via their TIR domains. TRIF, in turn recruits TRAF3 having E3 ligase activity. This, enzymatic activity ensures formation of polyubiquitin complex. That scaffold activates TRAF-family member-associated NF $\kappa$ B activator (TANK) protein which, in turn, activates Tank-binding Kinase Protein 1 (TBK1). TBK1 phosphorylates transcriptional factor IRF3. Phosphorylated and dimerized IRF3 enters nucleus and initiates production of type I IFNs, especially IFN- $\beta$  [48].



**Figure 1.4 Mammalian TLRs (1-13) recognize different ligands and propagates through different pathways. (Adopted from [45])**

### 1.1.1.2.2.1 Cell Surface Toll-Like Receptors

TLR1, TLR2, TLR4, TLR5 and TLR6, comprise Human Toll-like receptors confined to surface membranes. While TLR1, TLR2 and TLR6 forms two distinct heterodimers upon ligand binding such as TLR1&2 and TLR2&6, TLR4 and TLR5 homodimerize [18]. TLR1&2 and TLR2&6 recognizes lipopeptides: TLR1 and TLR6 enable TLR2 to sense diacyl- and triacyl-lipopeptides, respectively [49]. Moreover, TLR4 and TLR5, which are found on macrophages and dendritic cells, recognize lipopolysaccharide (LPS) and flagellin, respectively.

In addition to homodimerization, TLR4 further requires three accessory proteins such as LPS-binding protein (LPB), Myeloid Differentiation protein-2 (MD2) and CD14. LPB being a plasma soluble protein binds circulating LPS in blood and tissue; then, CD14 recruits LPS bound LBP to TLR4-MD2 complex. As a result of this, MD2 binds active part of LPS; consequently, TLR4 homodimerizes and signaling cascade is activated. TLR5, without using accessory proteins, recognizes flagellin, monomer of flagella [17]. Both LPS and Flagellin induces pro-inflammatory cytokine production via NFκB pathway.

#### **1.1.1.2.2.2 Endosomal Toll-Like Receptors**

Human TLRs residing within the endosomes are TLR3, TLR7, TLR8 and TLR9 [50]. Their common feature is that they are evolved to recognize nucleic acids found in extracellular environment such as RNAs and DNAs. They can bind their corresponding ligands after they fuse with phagocytosed and degraded pathogens or dying infected cells in endosomes.

While TLR3 is confined to macrophages, conventional dendritic cells and intestinal epithelial cells, it senses double stranded RNA (dsRNA) and its analog polyinosinic-polycytidylic acid (poly(I:C)) [51]. Even though TLR3 is specific for dsRNA, strikingly, it was reported that TLR3 is activated upon single stranded RNA (ssRNA) viruses such as West Nile virus, respiratory syncytial virus, and double stranded DNA (dsDNA) viruses (such as herpes simplex virus (HSV)) due to dsRNA production during viral gene transcription [52].

TLR7 (and TLR8 in humans [53]) recognize ssRNA entering endosomes and its synthetic analogs, derivatives of imidazoquinoline (R848, Imiquimod and Resiquimod), and guanine analogs; for example, loxoribine [54, 56]. Moreover, TLR7 has ability to sense some siRNAs as well [57]. While TLR7 is expressed by human plasmacytoid Dendritic Cells (pDCs) and B-cells, TLR8 is confined to DCs, macrophages and neutrophils [58–59].

In addition to be found in pDCs and B-cells, TLR9 is evolved to sense dsDNA, specifically CpG motif containing DNA without methylation since they are 20 times more common in viral or bacterial genome [60]. Therefore, bacteria, dsDNA viruses like Herpes Simplex Virus 1 and 2, protozoa parasites such as *Trypanosoma cruzi* are

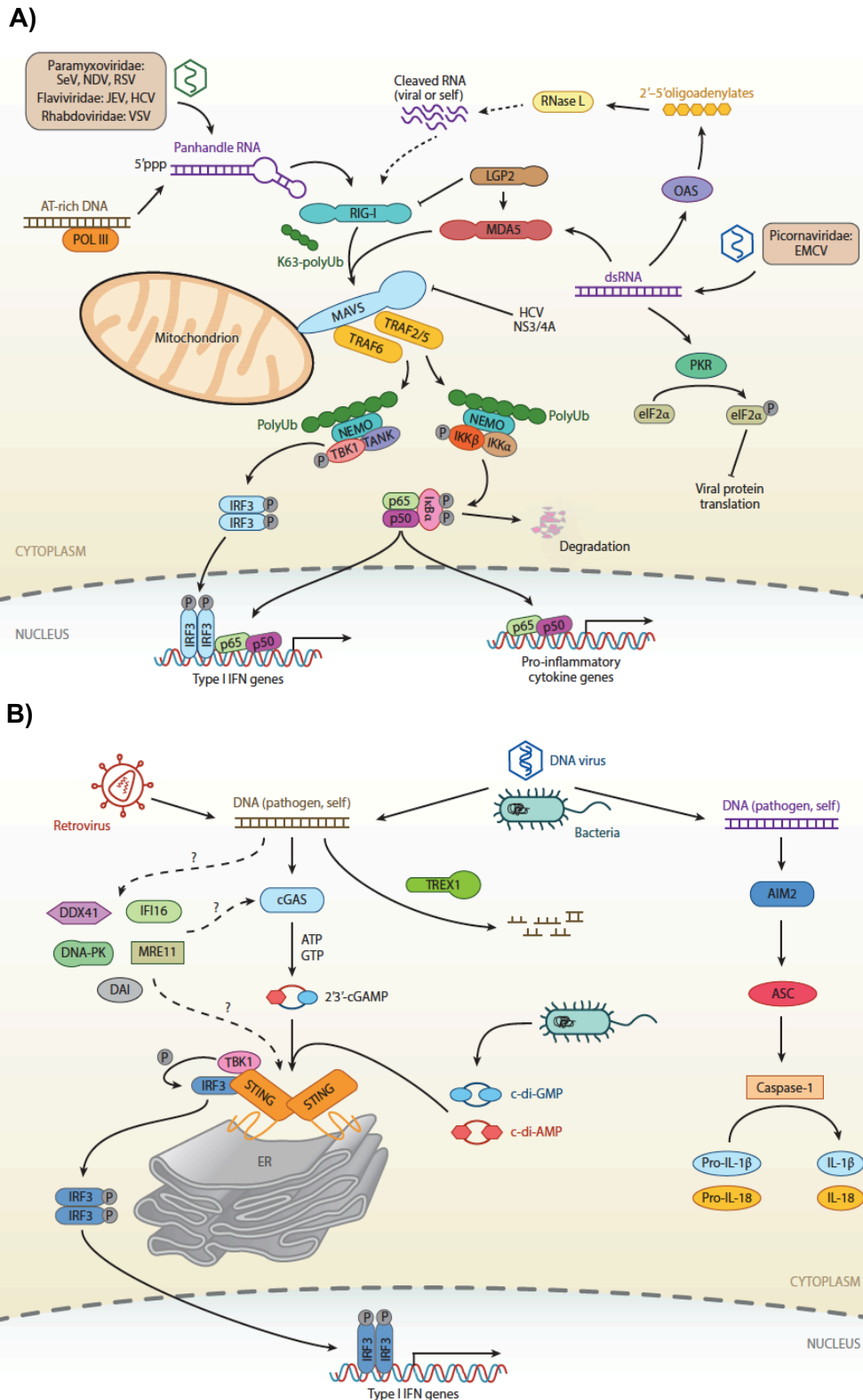
sensed and demonstrated to elicit responses [61–63]. Strikingly, it was reported that malarial DNA along with hemozoin, a malarial pigment, induces strong activation [64]; however, in a previous report, hemozoin *per se* has been already shown to activate TLR9 signaling [65]. Nonetheless, ligand engagement with TLR9 induces MyD88-dependent immune signaling that eventually leading to B-cell proliferation and maturation, copious amount of type I IFN secretion from pDCs [66].

Besides natural DNAs, synthetic oligonucleotides (ODNs) were reported to induce TLR9 activation [67]. Although this effect was attributed to palindromic sequences, in 1995 and 1996, Krieg and Klinman demonstrated that ODN with unmethylated CpG dinucleotide, which is flanked by two 5' purines and two 3' pyrimidines, induces strong activation of innate immune cells [68–69]. Nowadays, three distinct classes of ODNs were determined [70–72]. First, when multiple CpG motifs reside on phosphorothioate backbone, ODN is called 'K' type (called 'B' type as well). Their recognition by TLR9 is achieved within late endosomes and leads to activation of NF $\kappa$ B transcriptional factor. They stimulate B-cells to differentiate and secrete pro-inflammatory cytokines, whereas pDCs were reported to secrete TNF- $\alpha$ . Another class of ODNs were named as D-ODNs (or 'A' type). In contrast to K-ODNs, D-ODNs (also known as A-Type ODN) bear one CpG motif on phosphodiester/phosphorothioate mixed backbone and possess poly-G tail on both 5' and 3' ends. Poly-G tails on both ends of ODN, enable them to form nanoparticle-like structures. As a consequence of their complicated structures, they are recognized in early endosomes. This feature enables them to induce pDCs to secrete massive amounts of type I IFNs. Lastly, mixed type (C-type) ODNs were recently described [72]. As the name implies, it can trigger pDCs to secrete type I IFNs and at the same time leads to maturation of B-cells.

#### **1.1.1.2.3 Cytosolic Nucleic Acid Sensors**

During an infection, it is crucial to detect microorganisms by presence of their nucleic acids in cytosol. As a consequence of this, immune system evolved to bear cytosolic nucleic acid sensors. There are three major sensors of nucleic acids in cytosol: i) RLRs, ii) DNA-dependent activator of interferon regulatory genes and iii) ALRs. While RLRs detects RNA in cytosol (Figure 1.5A), other two detects DNA (Figure 1.5B) [73].





**Figure 1.5 Cytosolic nucleic acid sensors of innate immunity. A) RIG-I, MDA-5, B) STING pathway. (Adopted from [74])**

#### **1.1.1.2.4 RIG-I Like Receptors**

There are three major distinct sensors for RNA in cytosol. They are RIG-I, Melanoma differentiation associated 5 (MDA-5) and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2) [75]. For the scope of this thesis, RIG-I and MDA-5 are merely discussed. Unlike most of the PRRs, their expression is ubiquitous. However, immune signaling varies between cells; to illustrate, even though myeloid cells can respond to cytosolic RNAs, pDCs cannot initiate immune response via RLRs [75].

In order to sense ssRNA, RIG-I differentiates pathogenic RNA from eukaryotic is via sensing modification at 5' end of host ssRNAs [76]. However, there are several microorganisms such as polio virus and hepatitis A, evade this differentiation by transcribing their RNAs within nucleus of host [4]. MDA-5 senses dsRNA, specifically long dsRNAs [77]. Moreover, they can be activated by synthetic compounds such as transfection of p(I:C) and poly(deoxyadenylic-deoxythymidylic) Although former can be recognized directly, latter is recognized by RNA intermediate that is formed by RNA polymerase III due to DNA rich in AT sequence [78].

Upon recognition of corresponding RNAs, autoinhibited conformation is altered; consequently, RIG-I and MDA-5 can bind to mitochondrial antiviral signaling protein (MAVS) on mitochondria. After binding, receptors and MAVS, aggregate and recruit TRAF which propagates signaling towards to activation of NF $\kappa$ B and IRF3. As a result of this, pro-inflammatory cytokines and type I IFNs are produced [79].

#### **1.1.1.2.5 DNA-dependent activator of interferon regulatory genes**

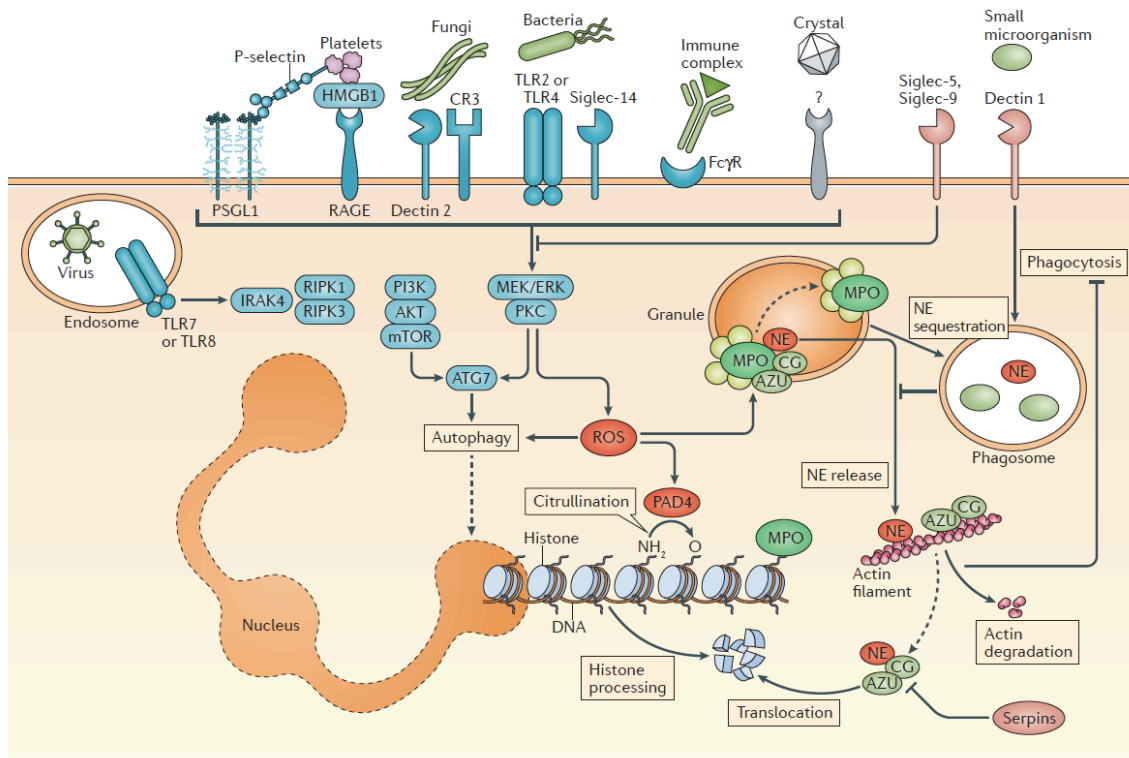
In healthy eukaryotic cells, DNA is strictly confined to nucleus. Therefore, either stress or presence of pathogen can lead to presence of DNA in nucleus. In order to sense and response against them, mammalian immune system is evolved to bear several cytosolic DNA sensors: AIM2, DAI (or ZBP1), RNA polymerase III, IFI16, DDX41 and most recently discovered cGAS [64, 71–72]. However, until the discovery of cGAS [81], none of these sensors were documented to be exclusive; that is, either they are confined to specific cell types or they have redundancy. For example, although IFI16, a protein confined to nucleus, and DAI are reported to sense cytosolic DNA, upon their depletion, cells were still observed to have DNA-dependent immune response [82–84].

As cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS), binds to DNA in cytosol and produces a secondary messenger, cGAMP, from ATP and GTP in cytosol, cGAMP is sensed by an adaptor protein called Stimulator of IFN genes (STING). In addition to natural course of DNA sensing, DNA-dependent activation of STING can be studied by transfecting several product such as 2'3' or 3'3' cGAMP [85], DNA of Herpes Simplex Virus (HSV) [82] and p(dA:dT) [86].

Except AIM2 [87], all cytosolic DNA sensors converge into same Endoplasmic Reticulum localized signaling adaptor, STING. Upon activation, STING proteins dimerize and recruit TBK1 to phosphorylate IRF3. Phosphorylated IRF3 homodimers enter into nucleus and produces type I IFNs. Meanwhile, homodimers of STING activate NF $\kappa$ B signaling to initiate production of pro-inflammatory cytokines as well [84–88]. AIM2 as a member of PYHIN family protein, induces inflammasome formation which eventually lead to maturation and secretion of IL-1 $\beta$  and IL-18 rather than type I IFNs [89].

#### **1.1.1.3 Neutrophils, Low-Density Granulocytes (LDGs) and Neutrophil Extracellular Traps (NETs)**

Being the most abundant immune cell type in peripheral blood circulation puts neutrophils on the frontier barricades of innate immunity [90]; in other words, they are the first cell type that are recruited to inflammatory area [91]. Neutrophils are terminally differentiated cells that are released to circulation from bone marrow; therefore, they have a short-life time in circulation (8-12 hours), however, it has been recently demonstrated that they can live up to 7 days during ongoing inflammatory conditions within tissues [92]. So as to limit the invasion, they have been equipped with three distinct powerful immune mechanisms: i) degranulation of antimicrobial proteins (such as neutrophil elastase (NE), myeloperoxidase (MPO)) in pre-formed granules, ii) phagocytose pathogens into reactive oxygen species (ROS) containing phagosomes and iii) propelling their chromosomes to extracellular environment to form a meshwork of DNA which is called as Neutrophil Extracellular Traps (NETs). [93–95].



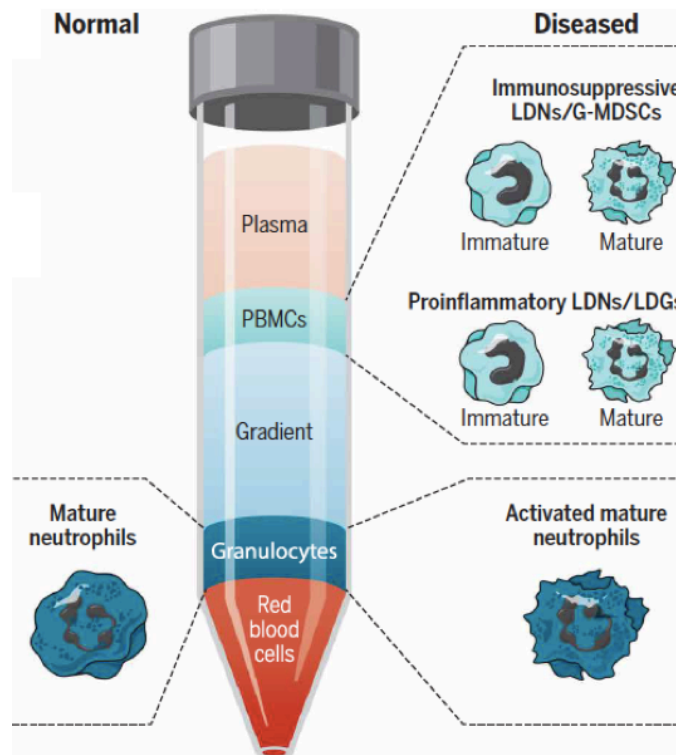
**Figure 1.6 Molecular signaling of NETosis.** (Adopted from [95])

Formation of NETs (NETosis), is a unique subset of regulated necrosis in which invading pathogens can be immobilized, inactivated and killed [96]. Even though it is known that NETs are formed and propelled against certain sterile stimuli (Urate/Cholesterol crystals, autoantibody complexes [97–98]) and microbes (fungi [99], parasites [100], viruses[101]) especially ones that cannot be phagocytosed by neutrophils [102], neither ‘trap or phagocytose’ decision phenomena nor exact mechanism behind NET formation has been elucidated yet [91]. Meanwhile, there are more than one type of NETosis. In addition to regulated death of neutrophils upon stimuli, NETs and proteins in granules can be rapidly propelled against *S. aureus* while neutrophils are still viable and carry on functioning such as phagocytosis [103–104]. Besides non-lytic NETosis, it was shown that there are more than one signaling cascades leading to classical NETosis and, NADPH oxidase-dependent pathway is a well-studied one (Figure 1.6). Upon microbial or sterile stimulation (or mitogenic stimuli: PMA, Concanavalin A and Ionomycin [105]) through receptors increases intracellular levels of  $Ca^{2+}$ . Elevated calcium ion level

activates protein kinase C (PKC) and initiates assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. PKC and NADPH generate ROS, especially hydrogen peroxide. Hydrogen peroxide activates special proteins such as NE, MPO within azurophilic granules. Contents of azurophilic granule is spilled over cytoplasm due to activity of MPO. They gather into nucleus to decondense chromatin via histone processing. While DNA is being released as a meshwork, proteins from granules such as MPO, NE, LL-37, HMGB1 are decorated on them [94–95–102–105–106].

Although NETs are crucial for dissemination of invaders, aberrant NET formation or ineffective NET removal are reported to lead coagulation [107–108], sterile inflammation [109–110], organ/tissue damage [111] and contribute allergy [112], autoimmunity [113–116].

In addition to diverse NETosis mechanisms, several novel subsets of neutrophils have been recently identified [116–120]. They have been named as low-density granulocytes (LDGs) or low-density neutrophils (LDNs) due to fact that they reside within Peripheral Blood Mononuclear Cell (PBMC) fractions after whole bloods were separated by density gradient centrifugation (Figure 1.7) [119–121]. Yet, there is a dilemma on whether LDGs are indeed subsets of neutrophils or modified versions of mature circulating neutrophils in diseased individuals [91]. However, it is certain that these 'subsets' of neutrophils contribute to progression and severity of diseases in which they are harbored. For example, cancer patients, apart from their tumor associated neutrophils (TANs) [122–124], had LDGs with immunosuppressive effects inhibiting T-cell proliferation and functioning [125]. Circulating LDGs in autoimmune diseases like SLE, Psoriasis, Rheumatoid Arthritis [126] are recorded to have increased inflammatory cytokine secretion and NET formation to further exacerbating disease progression.



**Figure 1.7 Subsets of neutrophils and their distribution through whole blood layering during health and disease.** (Adopted from [91])

Moreover, it has been demonstrated that they can enhance activity and effector functions of adaptive immune cells [119–121, 127]. Strikingly, it has been demonstrated that presence of LDGs in HIV infected individuals, correlated strongly with the LDG numbers and HIV severity [128].

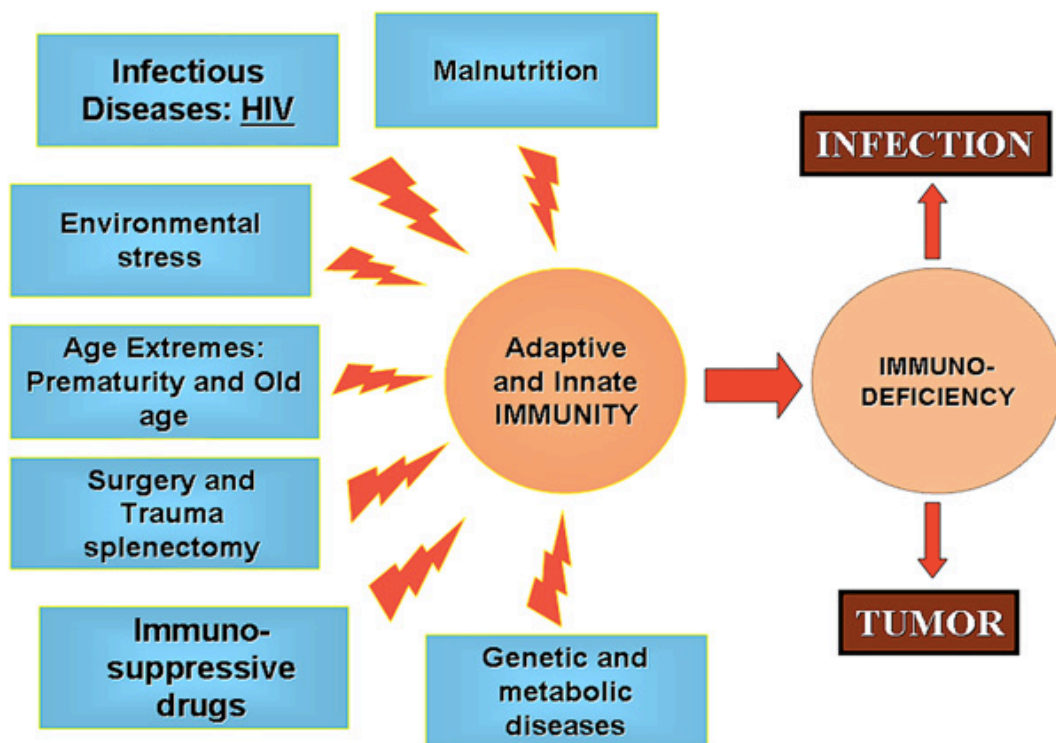
### 1.1.2. IMMUNE DEFICIENCIES

In the course of normal immune responses, pathogenic agents, that have breached the first line of defense barriers, provoke innate immune response, so that it limits the spread of the insult to the surrounding tissues. Professional antigen presenting cells that migrate to the nearest lymph node instruct naive B- and T-cells; consequently, adaptive arm is induced to establish long-lasting and antigen-specific immune response against threats. Alongside with innate immune cells, adaptive immune cells

eliminate threats; moreover, at the end, protective immunity is established as well. Nonetheless, in rare cases, immune responses may fail due to several reasons. These failures of defense mechanisms, are called as Immunodeficiencies [4]. In other words, immunodeficiencies arise when at least one element of or part of immune system cannot function as it is required to be mainly due to mutation(s) in the genes(s). As a consequence of this, infectious microorganisms or internal threats cannot be eliminated or cleared completely.

Defects of immune system are classified into two groups: Primary Immunodeficiency Disorders (hereafter PIDs) and Secondary (or Acquired) Immune Deficiencies [129]. Acquired Immune Deficiencies are consequences of environmental parameters instead of genetic mutations such as but not limited to malnutrition, metabolic diseases, medical therapies or interventions and infectious diseases are considered as general causes of acquired immunodeficiency disorders (Figure 1.8) [130].

Major cause of secondary immune deficiency is malnutrition [131]. Malnutrition is a term to define restricted access to food sources. Such as hypoproteinemia or lankness of micronutrients cause immune system to dysfunction and increase host's susceptibility to internal and external threats [132]. Even though malnutrition is the most common factor leading to secondary immune deficiencies, the most studied one is Human Immunodeficiency Virus (HIV). Although HIV infects cells bearing CD4<sup>+</sup> and CCR5, CXCR4 co-receptors, major replication site of virus is CD4<sup>+</sup> T-cells [133]. As a consequence of this, CD4<sup>+</sup> T-cell levels in blood circulation is reduced to a critical level which leads to increased host susceptibility against opportunistic infections [134].

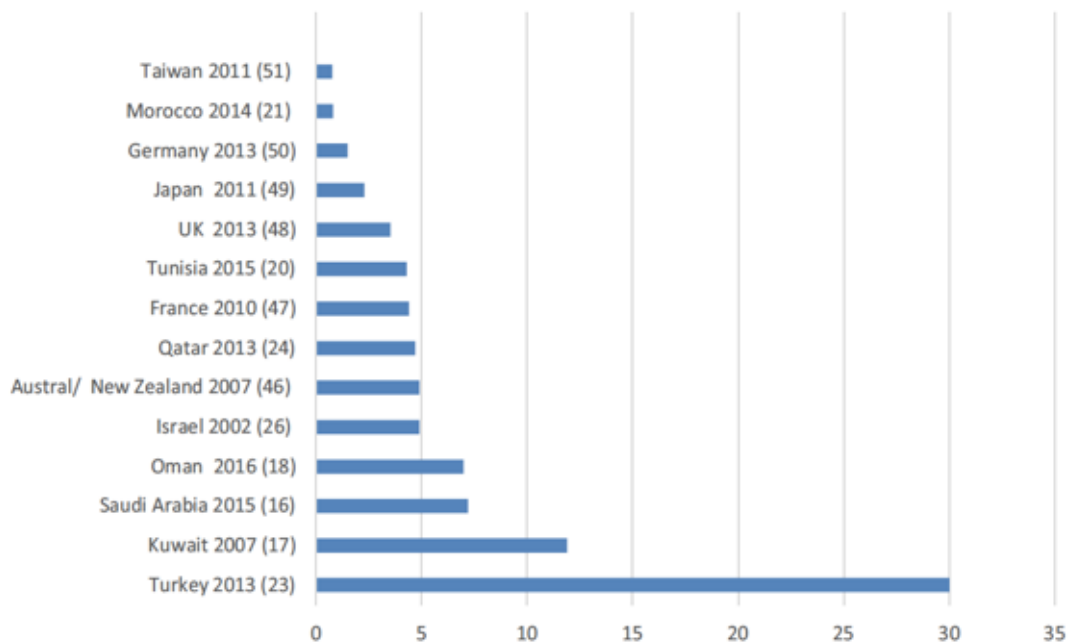


**Figure 1.8 Major factors causing Secondary Immune Deficiencies.** Secondary Immune Deficiencies arise from extrinsic and/or environmental factors. (Adopted from [130])

### 1.1.2.1 Primary Immune Deficiency Disorders

PIDs are congenital disorders in which inherited or *de novo* mutation(s) disrupts immune system functioning or proper development, or both of them. In contrast to being a group of rare disorders, as it is depicted in Figure 1.9, due to genetic basis, it is no surprise that prevalence of PIDs is reported to be high within inbred, consanguineous populations and countries [135].



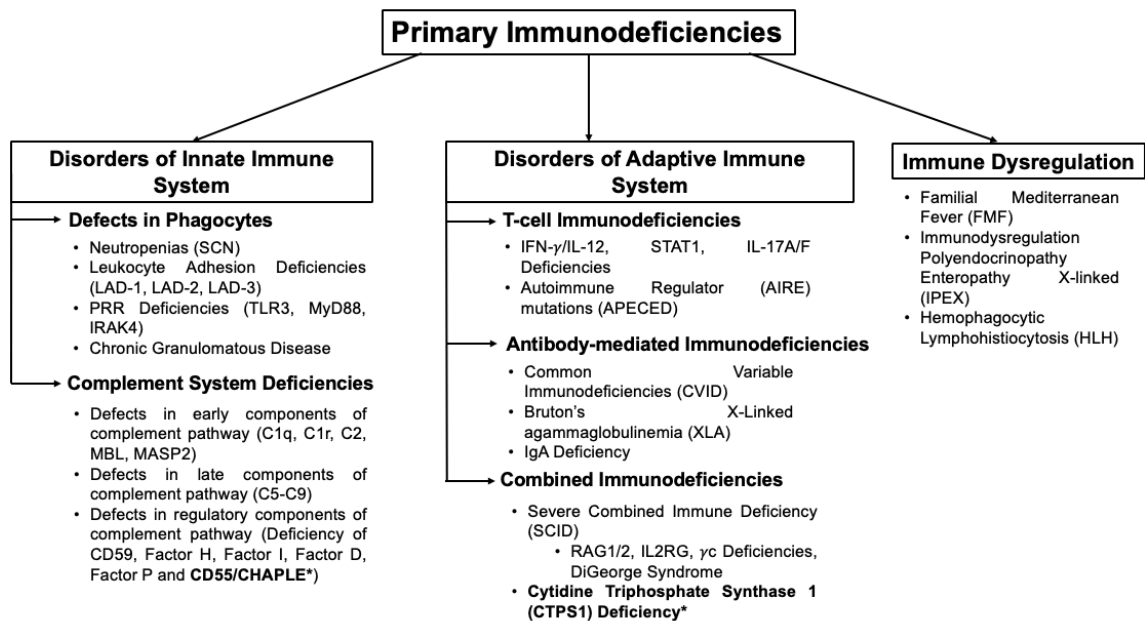


**Figure 1.9 Prevalence (in 100,000) of PIDs in different countries across the world.**  
(Adopted from [135])

Clinical manifestations of PIDs vary in a broad spectrum; to illustrate, even though many deficiencies lead to predisposition to infections, autoimmunity, malignancy as well as excessive inflammation and anatomical abnormalities can be manifested [129, 136–137].

More than 350 deficiencies have been characterized since the first identification of the PID which was agammaglobulinemia [138–139]. Although there is no consensus on classification, identified disorders are generally grouped based on functional part of immune system that is compromised. However, it is important to emphasize that malfunction of a certain part of the immune system may lead to perturbation of others.

PIDs can be classified into two immense categories as: disorder of innate and adaptive immune system (Figure 1.10). Meanwhile, a group of deficiencies, called as Immune Dysregulations, leading to autoimmunity, autoinflammation is included in the PID categorization as well [140].



**Figure 1.10 Classifications and examples of PIDs.** Diseases that are written in bold and indicated with asterisk are topics of this thesis (Compiled from [129–139–143])

Deficiencies within innate immune system can be further grouped into disorders of i) phagocytes and ii) complement system. In the case of phagocytes deficiencies, numbers, adhesion, activation, and killing capabilities of innate immune cells could be reduced or completely malfunctioning. For example, deficiency in neutrophil production, called as neutropenia, can be caused from mutations within *ELA2* gene encoding NE. As a result of this, myelocytes undergo apoptosis during development from promyelocyte to myelocytes [144]. Defects in phagocytes reduce host's capability to eradicate pus-forming bacteria; consequently, granulomas can be presented [142]. Non-functional complement proteins induced deficiencies can be divided into three as defects in early, late and regulatory components of predispose hosts to recurrent infections of encapsulated bacteria and *Neisseria* species; in addition, Systemic Lupus Erythematosus (SLE)-like syndrome can be manifested in certain complement deficiencies [140].

On the contrary, mutations interfering development or functionality of adaptive immune system can be grouped as i) T-cell deficiencies, ii) antibody-mediated deficiencies and iii) combined immune deficiencies (CIDs) [140]. T-cell deficiencies are composed of

defects in receptors or signaling pathways in which T-cell mediated antibody production is not compromised [4]. That is, subsets of T-cells have impaired responses. For example, defects in IL-12/IFN- $\gamma$  cytokines/receptors and IL17 cytokine/receptor manifest as recurrent intracellular bacterial infections and mucocutaneous candidiasis, respectively [140]. In the cases of antibody-mediated deficiencies where it can be due to defects in induction of B- and T-cell responses such as Bruton's X-Linked agammaglobulinemia, hyper-IgM syndrome. Meanwhile, certain impaired genes leading to developmental defects in T- and/or B-cells are termed as CIDs [129]. Most prominent subtype of it is Severe Combined Immunodeficiencies (SCIDs) in which cell-mediated immune responses and T-cell dependent immunoglobulin presence are impaired. Individuals with SCIDs such as RAG1/2, JAK3, IL2RG deficiencies suffer from wide array of pathogens [143].

In the case of third group of deficiencies, immune dysregulations may manifest themselves with autoimmune and inflammatory symptoms without having increased predisposition to pathogenic infections [140]. In those diseases, even though lymphocytes and subsets are present, they are generally dysfunctional which may lead to autoreactivity as well as dysregulation [140]. For instance, Familial Mediterranean Fever (FMF) is an autosomal recessive disease that is resulted from mutations within the gene called Mediterranean Fever (MEFV). MEFV encodes a crucial regulatory protein called pyrin or marenostrin. Even though protein's exact structure and function have not been elucidated yet, it was demonstrated that it inhibits caspase-1 associated inflammasome formation in cells [145]. FMF individuals have mutations leading to overactivation of inflammasome and consequently, sera of patients harbor elevated levels of IL-1 $\beta$  and IL-18 cytokines. Due to role of IL-1 $\beta$  in thermoregulation, patients manifests recurrent fever episodes, renal and dermal complications [146].

As it can be deduced, each classes and sub-classes of deficiencies discretely manifests themselves; thus, by conducting studies on these 'experiments of nature', one can delineate, comprehend normally-functioning immune pathways as well as their redundancy and interactions between other components of the immune system [4, 143–147].

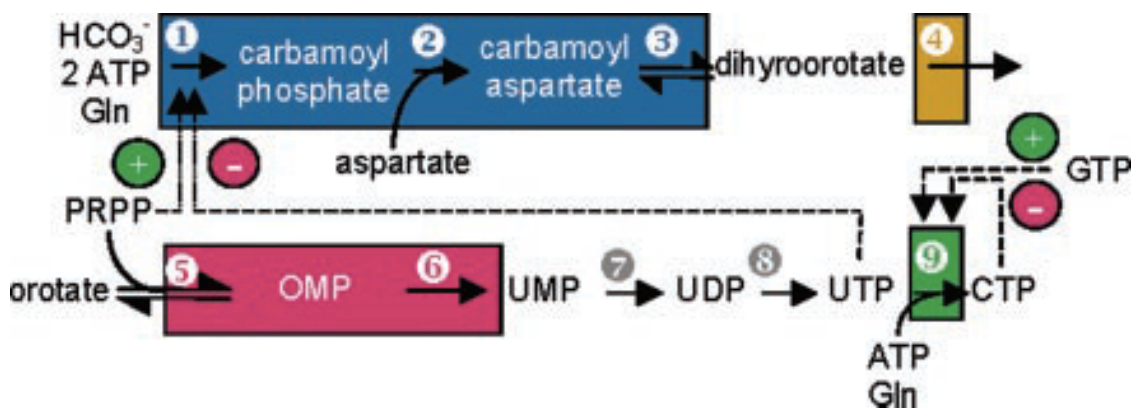
Although certain PIDs can be managed through supportive treatments such as administration of antibiotics, antifungals, intravenous immunoglobulin (IVIG) and

cytokine replacements, others, especially SCIDs, are required to be corrected via hematopoietic-stem cells transplantation (HSCT) due to lethality [140–148].

### 1.1.2.2 Cytidine Triphosphate Synthase 1 (CTPS1) Deficiency

Cytidine Triphosphate Synthase 1 (CTPS1) deficiency has been identified as extremely rare deficiency of immune system in 2014 and it has been further documented in few numbers of articles [149–152]. Common clinical symptoms of CTPS1 deficiency are mild gastrointestinal symptoms, early onset of recurrent and severe infections of Herpes Simplex virus (HSV), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV) and encapsulated bacteria. Moreover, due to EBV infections, individuals predispose to have malignancies, reduced memory B-cell and CD8<sup>+</sup> T-cell numbers [149–152].

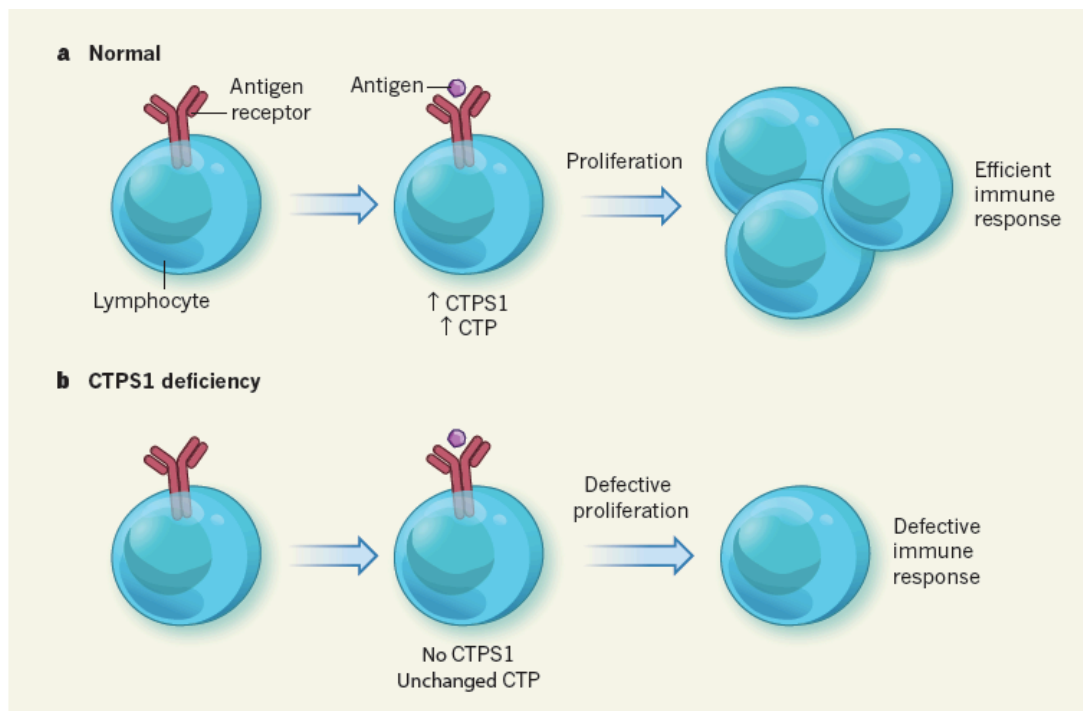
As its name implies, disease results from mutation within a gene encoding CTP synthase 1 enzyme which is one of the key enzymes in *de novo* pyrimidine biosynthesis. In order to synthesize CTP, CTPS1 and CTPS2 (another form of CTPS1) transfer an amide group from Glutamine to Uracil Triphosphate in presence of ATP (Figure 1.11) [153]. However, exact roles of CTPS1 and CTPS2 in nucleic acid biosynthesis pathway have not been elucidated yet [154].



**Figure 1.11 Illustration of mammalian *de novo* pyrimidine synthesis pathway.** The exact positioning of CTPS1 in pathway was indicated by green box number as 9. (Adopted from [153])

As a result of pivotal role in nucleic acid synthesis, proliferation and consequently development of CTPS1 deficient cells even tissues are expected to be impaired. Nevertheless, patients do not manifest physical abnormalities or non-immune symptoms, therefore, it might be interpreted that in some tissues, CTPS2 enzyme is able to compensate deficiency of CTPS1 enzyme [149].

Having CID symptoms lead researchers to reveal consequences of CTPS1 deficiency in adaptive immune system [149]. First and foremost, patients do not have invariant



**Figure 1.12 CTPS1 deficiency results in defective T-cell and B-cell proliferation.** (Adopted from [155])

Natural Killer Cells (iNKT) and Mucosal Associated invariant T-cells (MAIT). In contrast to CTPS2 which is highly expressed in non-activated T-cells, expression of CTPS1 enzyme was determined as low. However, upon CD3 and CD28 co-receptor cross-linking, healthy T-cells immediately express CTPS1 enzyme. CTPS1 deficient T-cells, however, are unable to induce production of CTPS1 enzyme; consequently, they are unable to produce CTP eventually leading to cell cycle arrest in G1 phase. Not only T-cells but also, activation of B-cells upon BCR and TLR9, CD40 engagement in the presence of IL-4 induced resulted in CTPS1 enzyme expression. All in all, proliferation capabilities of T-cells and B-cells were severely impaired upon TCR and BCR

inductions, respectively. That is, responses of T-cells, T-cell dependent and independent B-cell are defective since adaptive immune system normally require proliferation of T- and B-cells (Figure 1.12).

Even though mild cases were reported [150], malignancies, chronic and recurrent infections in CTPS1 deficiency patients are life threatening. Moreover, no cases with prolonged survival has been reported yet [152]. Thus, in two distinct reports, hematopoietic-stem cells transplantation (HSCT) was attempted to cure 13 patients in total [151–152]. Although number of patients that thrived after HSCT is 9, HSCT, right now, is only curative option. Last but not least, since the role of CTPS1 in immune system has been partially revealed, immunosuppression based on CTPS1 enzyme can be promising [149].

### **1.1.2.3 CD55 Deficiency**

One of the most recent complement regulatory protein deficiency was identified in 2017 by Dr. Ahmet Ozen and his colleagues. [34]. From eight consanguineous families, thirteen individuals represented increased visceral thrombosis, overactivation of complement, recurrent infections, protein losing-enteropathy induced gastrointestinal symptoms such malabsorption, abdominal pain and diarrhea. Disease is called CHAPLE syndrome standing for hyperactivation of complement, angiopathic thrombosis and protein-losing enteropathy [34–35]

After genomes of individuals were analyzed via whole-exome sequencing, it has been revealed that patients in cohort bear three distinct rare variants, leading to loss-of-function, in a gene encoding CD55 protein. It has probability of loss-of-function intolerance is 0; in other words, individuals heterozygous for CD55 variants do not present symptoms which are listed above. Therefore, CD55 deficiency is an autosomal recessive disease.

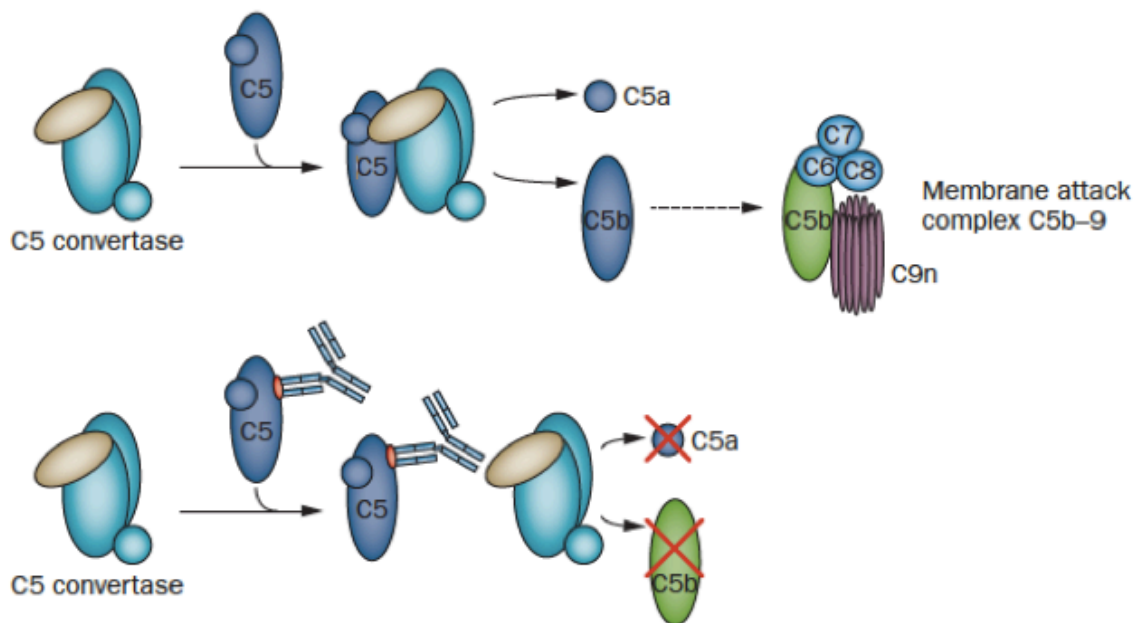
As it was briefly mentioned previously (see section 1.1.1.1), CD55 or DAF, is GPI-anchored plasma membrane protein that was first identified and isolated from human RBCs [156]. Besides membrane bound CD55, plasma soluble can be generated via alternative splicing, the major form of CD55 is membrane bound one [157]. Although expression pattern varies through tissues, stroma, epithelial, glandular and immune

[158] cells, especially granulocytes and monocytes [159]. CD55 play a crucial role in regulation of classical and alternative pathways of complement. It inhibits formation of C3 convertase by destabilizing the C3bBb complex by removing Bb from it. Consequently, hosts cells are vulnerable to deposition of complement proteins and cascade. Moreover, studies reported that CD55 do have complement-independent functions. *In vivo* studies with murine models showed that, mice bearing mutation in *Daf1* gene, homolog of human DAF, have upregulated IFN- $\gamma$ , IL-2 and decreased IL-10 cytokine secretion [160]. Meanwhile, *in vitro* study put forwards that while CD55 co-stimulates CD4+ T-cells through CD97 receptors, it still carries out complement regulation. Upon CD97 and TCR engagement, T-cells have enhanced proliferation, cytokine production and surface activation markers [161].

Due to fundamental role in regulation of complement pathway, as expected, CHAPLE patients suffer from massive amount of complement activation (generation of active components such as C3a, C5a) and consequently, thrombotic events. Thrombosis and thrombosis-induced embolisms are major source of morbidity [35]. Complement and coagulation systems are interrelated; to illustrate, complement effector proteins such as C3a activates platelets via enhancing their aggregation and adhesion which eventually contribute to contact-dependent thrombosis. Whereas C5a upregulates expression of tissue factor which is a crucial element in extrinsic coagulation pathway [162–164]. Interaction between complement and coagulation has been reported in other complement regulatory deficiencies such as aHUS [165] and PNH [166]. Meanwhile, it has been demonstrated that when CHAPLE patients' CD4+ T-cells are incubated with healthy sera, they had increased deposition of inactive C3 fragments C3d and iC3b; however, C3b molecules were not detected since rapid removal of them by other complement regulatory proteins like Factor I. In addition, biopsy from patients' duodenum showed submucosal arteries harbor tremendous amount of MAC formation. Thus, leading to massive cell death and consequently leading to gastrointestinal symptoms. In the context of functions of CD55 other than complement inhibition, researchers found out that CD55 deficient patients' helper T-cells had increased TNF- $\alpha$  yet decreased IL-10 secretion. However, when C5a and C3a receptors on T-cells are inhibited, TNF- $\alpha$  but not IL-10 secretion levels from T-cells are returned to healthy levels. Thus, immunosuppressive role of CD55 is independent from its role in complement pathway. It can be explained by deficient CD55-CD97 receptor-ligand engagement between CD55 bearing APCs and T-cells. Since IL-10 is a pivotal of

immunosuppressive cytokine for systemic [167–168] as well as mucosal immune system [169], dysregulation leading to intestinal, bowel inflammation in CHAPLE patients are caused by insufficient CD97 co-stimulation mediated reduced IL-10 secretion [170–171].

aHUS, PNS [172–173] and other C3 glomerulopathies [174] can be managed through a therapeutic drug called Eculizumab. Eculizumab is a monoclonal recombinant protein binding and consequently, neutralizes C5 proteins floating in plasma. After C5 proteins are neutralized, C5b and C5a cannot be generated through C5 convertases on membranes. Thus, Eculizumab inhibits MAC-mediated cell lysis and alleviates potentiation of inflammation caused by C5a anaphylatoxin. As a result of this, researchers first assessed potential therapeutic value of Eculizumab on T-cells derived from CHAPLE patients [34]. After a successful *in vitro* study, one published [175] and unpublished [35] clinical reports demonstrated clinical manifestations of CHAPLE patients such as thrombosis, protein-losing enteropathy and bowel-movement frequency are faded away even after a Eculizumab dose was given.



**Figure 1.13 Eculizumab: mechanism of action.** (Adopted from [174])



## **1.2. EXTRACELLULAR VESICLES**

After thymocytes of bone marrow chimeras were demonstrated to gather host MHC class molecules in 1981 [176], mechanisms explaining the transportation of membrane proteins between different cells have been extensively studied [177]. One well-studied and appreciated mechanism is secretion of membrane-enclosed vesicles into extracellular environment. Even though secretion of extracellular vesicles (EVs) were previously regarded as a garbage disposal system of cells [178–179], current studies challenge this idea and demonstrate that EVs are evolutionary conserved way of communication between them since secretion of them has been observed in all three domains of life: prokaryotes [180], archaea [181–182], unicellular [183–184] and multicellular eukaryotes [185]. Furthermore, variety of cell types such as tumor cells [186], B-cells [187], neutrophils [178] and DCs [188] are documented to secrete EVs. Thus, it is not a surprise that EVs can be isolated from cultures of bacteria, bodily fluids such as blood, saliva, urine, semen [189].

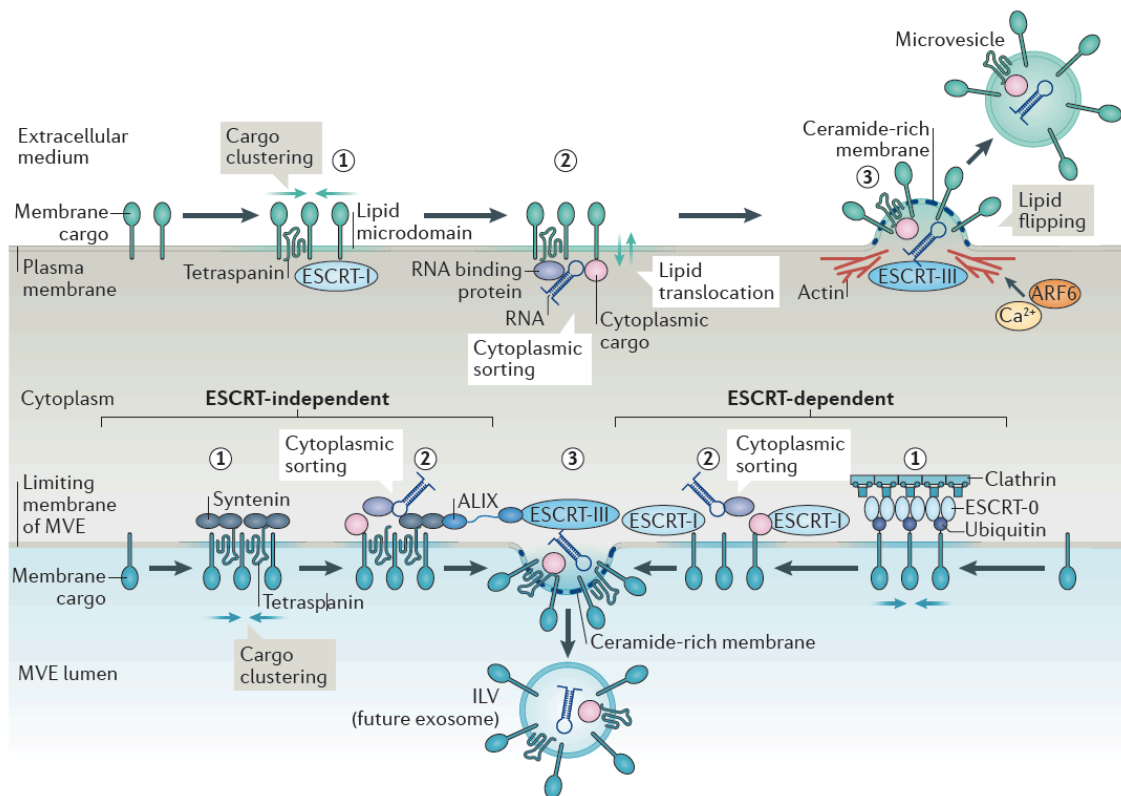
### **1.2.1. Types and biogenesis of EVs**

Extracellular vesicles are lipid enclosed particles carrying molecules from cytosol and plasma membrane such as proteins, lipids, nucleic acids of originating cells. EV term encompasses heterogeneous types of nanoparticles whose sizes varies from 15nm to 50 $\mu$ M. They are divided into three broad categories in the context of sub-cellular origin and size: exosomes, microvesicles and apoptotic bodies [190]. For the scope of this thesis exosomes and microvesicles are discussed solely.

While size of exosomes is between 15 and 100nm, microvesicle' sizes range from 100 to 1000nm. Differences in EV size is a consequence of distinct biogenesis pathways in which they are produced. (Figure 1.14) [191]. Exosomes are intraluminal vesicles that are generated within endocytic system, called multi vesicular bodies (MVB). They can be formed through either endosomal sorting complex required for transport (ESCRT)-dependent or independent mechanisms [190]. Microvesicles bud outward from plasma membrane [185]. Therefore, microvesicles can be released to extracellular environment by merely pinching off. On the contrary, in order to release exosomes into extracellular milieu, MVBs are required to fuse with plasma membrane [189]. Thus,

pace of exosome release is expected to be much more slower compared to microvesicles [190].

Furthermore, there are several parameters discriminating exosomes from microvesicles and *vice versa*. Even though both exosomes and microvesicles bear markers of cells that they have originated from, exosomes carry markers of endosomes; to illustrate tetraspanins, Alix, TSG101, heat-shock proteins, and possess lipid rafts, cholesterol, sphingomyelin and ceramide [192–194]. On the other hand, composition of microvesicles resembles to plasma membrane.



**Figure 1.14 Mechanisms of microvesicle (upper) and exosome (lower) biogenesis.** (Adopted from [190])

### 1.2.2. EVs and their impact on immune system

After EVs are delivered to extracellular environment, they can reach places that are allowed by transportation system of an organism. First, they are needed to be docked at plasma membrane of recipient cells. Then, they can be internalized through several

mechanism [195]. Studies showed that EVs can be taken up by membrane fusion [196], endocytic pathways including phagocytosis [197], receptor-mediated endocytosis [198], clathrin-mediated endocytosis [199], lipid raft-mediated endocytosis [200] and macropinocytosis [199]. Regardless of different EV uptake mechanisms, their protein and RNA content can be utilized by recipient cell. As a consequence of this, they can alter, modulate behavior and responses of recipient cells via cargos that they bear.

EVs can contribute to tuning of certain physiological process in health such as blood coagulation [201], embryo implantation [202], neural cell communication [203]. Meanwhile, their effects have been documented in various diseases such as hemophilia [204], idiopathic thrombocytic purpura [205], autoimmunity [206], atherosclerosis [207] and cancer [208]. Therefore, it is no surprise that EVs have an impact on immune system. *In vitro* and *in vivo* studies demonstrated that EVs do contribute and even stimulate immune responses. Their effects on immune system (both innate and adaptive arms) can be generalized as immunostimulatory and immunosuppressive [209].

First and foremost, EVs are capable of carrying antigens in free form as well as loaded on MHC molecules [210]. So as to reveal antigen transportation, cancer models have been extensively studied. It has been demonstrated that APCs that did not come across with a single cancer cell, can generate immune response against cancer peptides [211]. Thus, it can be interpreted that EVs can carry both surface (such as epidermal growth factor receptor 2, carcinoembryonic antigen) and cytosolic (such tyrosinase related protein 1) antigens [212]. After internalization of EVs by APCs, cargos can be loaded on MHC classes. Consequently, they can be presented to CD8+ and CD4+ T-cells. Not only cancer but also, EVs from host cells that are underwent infections such as *Mycobacterium tuberculosis* [213] and *Toxoplasma gondii* [214] can supply antigens for APCs. Moreover, EVs are documented to present peptides that are loaded on MHC molecules directly to adaptive immune cells rather than through APCs. To illustrate, EVs that are derived from certain APCs such as DCs and B-cells are reported to directly activate CD8+ and CD4+ T-cells via MHCI and MHCII molecules, respectively [215–217]. Furthermore, EVs can stimulate immune cells independently from MHC classes. Studies postulated that Platelet-derived exosomes induces adhesion and proliferation of hematopoietic cells [218]. Moreover, it has been documented that cells that are infected with HIV secrete EVs carrying cGAMP molecules since HIV *per se* is sensed

by cytosolic nucleic acids sensors of host cells. Thus, EVs can alert cells that can be subsequently infected by HIV [217].

Strikingly, certain EVs may exert suppressive effects on immune system. For example, Tregs, which are key immunosuppressive cells of immune system, are known to secrete exosomes bearing CD73 molecules on their surface upon activation through their TCRs [219]. Studies showed that these exosomes repress activities of DCs [220] and proliferation capabilities of CD8+ T-cells [221]. Meanwhile, activated T-cells may secrete EVs that induce apoptosis of other T-cells in proximity through TNF superfamily receptor-ligand interaction [222].

In addition to being secreted from almost all cell types and modulating immune responses, due to cargo that they bear, EVs are started to be investigated whether they can be studied as a potential diagnostic tool for various diseases [223]. One of the most studied cargo in that essence are mRNAs and microRNAs. Plasma and serum samples of cancer [208], cardiovascular [224], neurodegenerative [225] patients can be isolated and their microRNA compositions were differentially characterized. Beside RNA content of EVs, proteomic [226–227] and lipidomic [228] studies are conducted to distinguish disease vs healthy EVs. Lastly, morphology of isolated EVs is diagnostically found to be important, especially in prostate cancer [229].

### **1.3. Subject and Outline of the Thesis**

PIDs are rare disorders resulting from mutated genes within human immune system. They have been primarily recorded in sociologically and economically underdeveloped countries. Even though they can be managed via certain therapeutic interventions, for some cases, only curative option is HSCT. In addition to being regarded as ‘experiments of nature’ and ‘natural knock-outs’, delineation of them enables us to comprehend how components of immune system work and interact under real-life conditions. Thus, it is invaluable to investigate them. Herein, we will attempt to unravel molecular mechanisms of two monogenic and extremely rare PIDs: CTPS1 and CD55 deficiency (or CHAPLE Syndrome). While CTPS1 deficiency is the most recently discovered disorder of adaptive immune system, deficiency of CD55 is a regulatory complement disorder which was characterized in patients from Middle East countries, mostly Turkey. Despite being novel diseases, they have not been gained sufficient

attention. Their investigation and characterization efforts will contribute understanding of mechanisms behind these disorders as well as molecules standalone.

Firstly, since CTPS1 deficient individuals suffer from recurrent viral infections, effect of deficient CTPS1 enzyme on cytotoxic T-cells will be investigated. STAT1 phosphorylation levels of CD8<sup>+</sup> T-cells under IFN- $\beta$  induced, basal conditions will be assessed using flow cytometry. Even though defects in proliferation capabilities were shown, currently, it is not known whether T helper cells are functionally impaired or not. To that end, phosphorylation levels of STAT1, STAT4, STAT5 and STAT6 proteins in CD4<sup>+</sup> T-cells were investigated using flow cytometry after their corresponding receptors will be stimulated or left untreated. In order to further demonstrate effect of deficient CTPS1 enzyme on helper T-cells, cytokine secretions of patient Th1, Th2, Th17 and Treg will be determined by flow cytometry. Then, we will turn our attention to functional consequences of defect CTPS1 enzyme in innate immunity by evaluating responses to PRR stimulations. In that essence, PBMCs from patients and healthy controls will be stimulated with endosomal TLR as well as cytosolic DNA sensor ligands and cytokine levels will be determined using ELISA. Moreover, PRR-mediated responses of adaptive immune cells will be assessed by stimulating TLRs (such as TLR4, TLR5, TLR7 and TLR9) and intracellular DNA sensors of PBMCs. Then, levels of IFN- $\gamma$  and IL-12 cytokines will be evaluated using cytokine ELISA. Next, CTPS1 patient manifests granulomas on skin. Therefore, it is hypothesized that deficient CTPS1 enzyme may lead to autoimmune-like responses. To investigate this, neutrophils from CTPS1 patient and controls will be isolated. Their responses under basal and stimulated conditions will be determined and quantified using live imaging microscopy and spectrofluorometric analysis, respectively. Lastly, CTPS1 plasma derived EVs will be isolated and in order to delineate whether these vesicles contribute to disease pathology EVs from controls and patients will be incubated with allogenic healthy PBMCs and secreted pro-inflammatory and anti-inflammatory cytokine responses will be assessed using ELISA.

CD55 is a complement regulatory protein; in other words, it regulates and inhibits activation of alternative and classical complement cascades. Apart from aberrant complement activation and bowel inflammation, CHAPLE patients suffer from nutrient and micronutrient deficiencies as well as gut pathologies. Here, second part of the thesis will attempt to unearth three crucial questions regarding CD55 deficiency: i) what are the functional consequences of deficient CD55 protein on immune cells, and ii)

How will immune responses be affected after a single Eculizumab therapy, iii) whether single dose of Eculizumab therapy altered the composition of circulating EVs or not. So as to answer those, 4 CHAPLE patients, who are planned to receive Eculizumab therapy, will be included in cohort of this study. PBMCs will be isolated from before and after therapy bloods. Then, they are going to be stimulated with endosomal TLR (TLR3, TLR7 and TLR9) and cytosolic DNA sensor (STING, IFI16) ligands or inflammasome ligands. Inflammatory and anti-inflammatory cytokine responses will be determined by cytokine ELISA. Next, EVs from BT and AT CD55 patients will be isolated. They are going to be incubated with syngeneic BT and AT CHAPLE PBMCs. Culture supernatants will be analyzed to demonstrate how BT and AT EVs alter immunological responses of patients. Human cytokine ELISA will be conducted to detect levels of inflammatory and anti-inflammatory cytokines. To further validate and observe effects of EVs on immune system, healthy PBMCs will be co-incubated with BT or AT EVs and cytokine ELISA will be performed.

## **2. MATERIALS AND METHODS**

### **2.1. Materials**

#### **2.1.1. Cell Culture Media and Buffers**

Fetal Bovine Serum (FBS), RPMI 1640 (with L-Glutamine), Sodium Pyruvate and Penicillin-Streptomycin were purchased from Gibco Life Sciences, USA. Non-Essential Amino Acids, HEPES Buffer were supplied from Biological Industries, Israel. 1X Dulbecco's Phosphate Saline Buffer (DPBS), Cell Culture Grade Water were purchased from ThermoFischer Scientific, USA.

Recipes of complete media were given in Appendix A alongside with non-commercial, hand-made buffers and solutions that were used in this study.

#### **2.1.2. PRR and Cytokine Receptor ligands used in *in vitro* stimulation experiments**

In the following Table 2.1, mitogens, innate sensor ligands, from where they were purchased, brand names, catalogue numbers, working concentrations and their corresponding sensor or signaling pathway names were given.

**Table 2.1.** Mitogens, ligands and cytokines used in this study.

<b>Ligand Name</b>	<b>Brand Name/ (Country)</b>	<b>Catalogue Number</b>	<b>Working Concentration</b>	<b>Receptor/Signaling Pathway</b>
Pam3CSK4	Invivogen (USA)	tlrl-pms	1µg/ml	TLR2&1
p(I:C)	Invivogen (USA)	tlrl-picw	30µg/ml	TLR3
LPS (Isolated from <i>E. coli</i> )	Sigma Aldrich (Germany)	L-2880-100mg	5µg/ml	TLR4
Ultra-Pure Flagellin (Isolated from <i>S. typhimurium</i> )	Invivogen (USA)	tlr-pstfla-5	100ng/ml	TLR5
R848	Enzo Life Sciences (USA)	ALX-420-038-M025	5µg/ml	TLR7
D35-3CG*	Alpha DNA (Canada)	590691	3µM	TLR9
K3*	Alpha DNA (Canada)	539665	1µM	TLR9
2'3'- cGAMP**	Invivogen (USA)	tlrl-cga-23	30µg/ml	STING
HSV-60 Naked**	Invivogen (USA)	tlrl-hsv60n	5µg/ml	cGAS-STING
p(dA:dT)**	Invivogen (USA)	tlrl-patn	5µg/ml	AIM2 Inflammasome & DAI-STING and RIG-I/MAVS
LPS (Isolated from <i>E.coli</i> )**	Sigma Aldrich (Germany)	L-2880-100mg	5µg/ml	Non-Canonical Inflammasome



Ultra-Pure Flagellin (Isolated from <i>S. typhimurium</i> )**	Invivogen (USA)	tlr-pstfla-5	100ng/ml	NLRC4 Inflammasome
Phorbol-12-myristate 13-acetate	Sigma Aldrich (Germany)	P1585	50ng/ml	Protein Kinase C
Ionomycin	Calbiochem (Germany)	407950	1µg/ml	Ca <sup>+2</sup> Channels on Endoplasmic Reticulum
Human Recombinant IL-2	Biolegend (USA)	589104	100ng/ml	IL2R-JAK1/2-STAT5
Human Recombinant IL-4	Biolegend (USA)	574004	200ng/ml	IL4R-JAK3-STAT6
Human Recombinant IL-6	Biolegend (USA)	570802	200ng/ml	IL6R-JAK1/2-STAT3

*\*-Base sequence of D35-3CG CpG ODN: 5' GGtgcacgcatgcaggggGG 3' (synthesized with phosphodiester/phosphorothioate mixed backbone chemistry)*

*-Base sequence of K3 CpG ODN: 5' TCGACTCTCGAGCGTTCTC 3' (synthesized with all phosphorothioate modification)*

*-Uppercase letters indicate bases having phosphorothioate linkages whereas lowercase letters indicate bases with phosphodiester linkages.*

*\*\*-Ligands were transfected by using 0.3µl Lipofectamine 2000® Reagent (ThermoFischer Scientific, USA) per stimulation.*

### **2.1.3. ELISA Reagents and Kits**

Recombinant protein names, where they were purchased, catalogue numbers, working concentrations, clone identities of capturing antibodies, detection antibodies and

recombinant cytokines that were used in ELISA were summarized in **Table 2.2**. Streptavidin-Alkaline Phosphates (SA-ALP, hereafter) were supplied from Mabtech (Sweden).

**Table 2.2.** Antibodies and recombinant proteins used in cytokine ELISA throughout this study.

<b>Recombinant Antibody</b>	<b>Brand Name/ (Country)</b>	<b>Catalogue Number</b>	<b>Clone</b>	<b>Working Concentration</b>
Anti-human IFN- $\alpha$	Mabtech (Sweden)	3425-1A-20	MT1/3/5	4 $\mu$ g/ml
Biotin anti-human IFN- $\alpha$	Mabtech (Sweden)	3425-1A-20	MT2/4/6	1 $\mu$ g/ml
Human Recombinant IFN- $\alpha$	Mabtech (Sweden)	3425-1A-20	NS *	40ng/ml
Anti-human IFN- $\gamma$	Mabtech (Sweden)	3420-1A-20	1-D1K	4 $\mu$ g/ml
Biotin anti-human IFN- $\gamma$	Mabtech (Sweden)	3420-1A-20	7-B6-1	1 $\mu$ g/ml
Human Recombinant IFN- $\gamma$	Mabtech (Sweden)	3420-1A-20	NS	12.5ng/ml
Anti-human IL-1 $\beta$	Mabtech (Sweden)	3416-1A-20	MT175	2 $\mu$ g/ml
Biotin anti-human IL-1 $\beta$	Mabtech (Sweden)	3416-1A-20	7P10	1 $\mu$ g/ml
Human Recombinant IL-1 $\beta$	Mabtech (Sweden)	3416-1A-20	NS	25ng/ml
Anti-human IL-6	Mabtech (Sweden)	3460-1A-20	13A5	0.5 $\mu$ g/ml
Biotin anti-human IL-6	Mabtech (Sweden)	3460-1A-20	39C3	1 $\mu$ g/ml
Human Recombinant IL-6	Mabtech (Sweden)	3460-1A-20	NS	12.5ng/ml
Anti-human IL-8	Mabtech (Sweden)	3560-1A-20	MT8H6	2 $\mu$ g/ml
Biotin anti-human IL-8	Mabtech (Sweden)	3560-1A-20	MT8F19	1 $\mu$ g/ml
Human Recombinant IL-8	Mabtech (Sweden)	3560-1A-20	NS	25ng/ml
Anti-human IL-10	Mabtech (Sweden)	3430-1A-20	9D7	2 $\mu$ g/ml

Biotin anti-human IL-10	Mabtech (Sweden)	3430-1A-20	12G8	1µg/ml
Human Recombinant IL-10	Mabtech (Sweden)	3430-1A-20	NS	8ng/ml
Anti-human IL-12/-23 (p40 specific)	Mabtech (Sweden)	3450-1A-20	MT86/221	2µg/ml
Biotin anti-human IL-12/-23	Mabtech (Sweden)	3450-1A-20	MT618	1µg/ml
Human Recombinant IL-12 (p40 specific)	Mabtech (Sweden)	3450-1A-20	NS	8ng/ml
Anti-human IP-10	BD Biosciences (USA)	555046	4D5/A7/C5	8µg/ml
Biotin anti-human IP-10	BD Biosciences (USA)	555048	6D4/D6/G2	1µg/ml
Human Recombinant IP-10	BD Biosciences (USA)	551130	NS	100ng/ml
Anti-human TNF- $\alpha$	Mabtech (Sweden)	3512-1A-20	MT25C5	2µg/ml
Biotin anti-human TNF- $\alpha$	Mabtech (Sweden)	3512-1A-20	MT20D9	1µg/ml
Human Recombinant TNF- $\alpha$	Mabtech (Sweden)	3512-1A-20	NS	12.5ng/ml

\*NS: Not Specified

#### 2.1.4. Antibodies used in Flow Cytometry

Recombinant antibody names, producer names, catalogue numbers and clones' identities of antibodies that were used during flow cytometry investigations throughout this thesis were indicated in Table 2.3. For alive cell staining, antibodies were diluted in 5% RPMI media, staining of fixed cells or exosome characterizations via bead-based method, antibodies were prepared in FACS Buffer (See Appendix A).

**Table 2.3. Fluorochrome labeled** recombinant antibodies used in flow cytometry analyses throughout this study methods.

<b>Antibody</b>	<b>Brand Name/ (Country)</b>	<b>Catalogue Number</b>	<b>Clone</b>
Anti-human CD3-PE	Biolegend (USA)	300308	HIT3a
Anti-human CD4+-FITC	Biolegend (USA)	300538	RPA-T4
Anti-human CD8a-APC-Cy7	Biolegend (USA)	300926	HIT8a
Anti-human CD9-PE	Biolegend (USA)	312106	HI9a
Anti-human CD14-APC-Cy7	Biolegend (USA)	301820	M5E2
Anti-human CD15-BV650™	Biolegend (USA)	323034	W6D3
Anti-human CD55-PE	Biolegend (USA)	311308	JS11
Anti-human CD80-BV421	Biolegend (USA)	305426	IT2.2
Anti-human IFN $\gamma$ -PerCP/Cy5.5	Biolegend (USA)	506528	B27
Anti-human IL-4-PE/Cy7	Biolegend (USA)	500824	MP4-25D2
Anti-human IL-13-BV650™	Biolegend (USA)	501907	JES10-5A2
Anti-human IL-17a-BV785™	Biolegend (USA)	512338	BL168
Anti-human IL-10-PE	Biolegend (USA)	506804	JES3-19F1
Anti-mouse/rat/human FoxP3- Alexa Fluor®647	Biolegend (USA)	320014	150D
Anti-human HLA-DR-FITC	Biolegend (USA)	307604	L243
Anti-human PD-L1-PE	Biolegend (USA)	329706	29E.2A3
Anti-human pStat3(pY705)- Alexa Fluor® 647	BD Biosciences (USA)	560750	4/P-STAT3
Anti-human pStat5(pY694)- Alexa Fluor® 647	BD Biosciences (USA)	560750	47

Anti-human pStat6(pY641)- Alexa Fluor® 647	BD Biosciences (USA)	560750	18/P-Stat6
Anti-human T-bet - Bv650	BD Biosciences (USA)	564142	O4-46
Anti-human Gata-3 - PE-eFluor 610	Invitrogen (USA)	61-9966-42	TWAJ
Anti-human ROR $\gamma$ T-PE	BD Biosciences (USA)	563081	Q21-559
Mouse IgG1-PE	Biolegend (USA)	400114	MOCP-21

\*NS: Not specified

### 2.1.5. Antibodies used in Western Blotting

All solutions and buffers of SDS-Page/Western Blotting technique in this study was non-commercial and prepared as they were described in Appendix A. All equipments (tanks, cassettes, combs) were supplied from Bio-Rad (USA). In order to determine molecular weight of loaded proteins, Pre-stained protein ladder (ThermoFischer Scientific) was used.

Moreover, antibody names, their producers, clone identities, catalogue numbers, and working dilutions of primary and HRP-conjugated secondary antibodies were listed in Table 2.4.

**Table 2.4.** Features of Western Blot antibodies used throughout the thesis.

Antibody Name	Brand Name/ (Country)	Catalogue Number	Clone	Dilution Factor
CD55	Abcam (UK)	ab133684	EPR6689	1:10000
Alix	Cell Signaling Technology (Germany)	2171	3AG	1:1000
TSG101	Abcam (UK)	ab83	4A10	1:1000
Flotinin-1	Cell Signaling Technology (Germany)	3253S	NS*	1:1000

β-actin	Cell Signaling Technology (Germany)	3700S	8H10D10	1:5000
HRP-conjugated anti-mouse IgG	Cell Signaling Technology (Germany)	7076S	NS	1:10000
HRP-conjugated anti-rabbit IgG	Cell Signaling Technology (Germany)	7074P2	NS	1:10000

## 2.2. Methods

### 2.2.1. Patients and Controls

#### 2.2.1.1 CHAPLE Patients and Controls

Within the study of CD55 Deficiency, 4 CHAPLE patients and their peripheral bloods were recruited from Department of Pediatric Gastroenterology, Gazi University (Ankara, Turkey) and Department of Pediatric Allergy-Immunology, Marmara University (Istanbul, Turkey). Moreover, peripheral blood of each CHAPLE patients was obtained before and after single dose of Eculizumab therapy. Cohort of CHAPLE patients' age (year, mean±SD) was 9±1.3 (year, mean±SD). Time that passed between Eculizumab administration and “after therapy” blood collection was at least one month. Apart from Eculizumab, drugs and therapeutic interventions that patients have being received IVIG, vitamin and micronutrient supplementation, protein-rich diet and serum albumin infusion. Moreover, PCR results indicated that none of patients had underwent Cytomegalovirus (CMV) or EBV-infection. Age of healthy individuals included as control cohort of CHAPLE study was 24±4 (year, mean±SD).

#### 2.2.1.2 CTPS1 Deficient Patient and Controls

Peripheral blood of CTPS1 deficient patient was obtained from Department of Pediatric Immunology, Hacettepe University (Ankara, Turkey) on two separate occasions. Age of CTPS1 patient was 19. Moreover, patient was receiving IVIG and broad-spectrum antibiotics. Total of 4 healthy controls were included in CTPS1 study. Age (year, mean±SD) was: 26±6yrs.

## **2.2.2. Cell Culture**

### **2.2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Whole Blood**

2-30 ml peripheral blood from patients and healthy donors were collected into either K<sub>2</sub>-EDTA or Citrated blood collection tubes (BD Biosciences, USA). Collected bloods were diluted at 1:1 (v/v) ratio with room temperature (RT) 1X DPBS. Diluted bloods were slowly layered on top of lymphocyte separation media (Capricorn Scientific, Germany) at 3:2 (v/v) ratio [230]. Then, samples were centrifuged at 540xg where deacceleration rate was set to zero; in order to preserve formed layers of cell types. After 30 minutes, 4 distinct layers were formed. The upper most layers containing plasmas were collected initially, and they were used to purify EVs which was explained in Section 2.2.7.1. Then, cloudy layers, which reside beneath plasmas and above the lymphocyte separation media, containing PBMCs, were gathered with sterile Pasteur pipette, and washed two times with complete RPMI-1640 medium containing 2% FBS by centrifugation at 540xg for 10 minutes. Washed cells were resuspended in 1 ml of complete RPMI-1640 medium containing 5% FBS (5% RPMI, hereafter). Isolated PBMCs were counted in flow cytometry which was explained in Section 2.2.6.1.

### **2.2.2.2 Purification of Polymorphonuclear Neutrophils from Whole Blood**

After plasmas and PBMCs were isolated as it was explained in Section 2.2.1.1 and lymphocyte media were removed, the undermost, dark-red fractions containing granulocytes and RBCs were completed up to initial volume of diluted blood with RT PBS and slowly mixed in 15 ml falcon tubes [231]. In order to separate neutrophils from RBCs by differences in their densities, 3% (w/v) Dextran (Sigma Aldrich, Germany) was added onto fraction at 1:1 (v/v) ratio and mixed slowly. After incubation for 30-40 minutes in an upright position of tubes, two fractions were formed. Since neutrophils reside within upper, white layer, those were collected with sterile Pasteur pipette and washed twice with RT 1X PBS (for 10 minutes at 300xg). So as to get rid of remaining RBCs, pelleted neutrophils were resuspended in 5 ml of 1X RBC lysis buffer (Biolegend, USA). After incubation at RT for 5 minutes, 35 ml of ice cold 1X PBS was added. Neutrophils were pelleted by centrifugation for 10 minutes at 300xg at +4°C. Then, they were resuspended and washed twice with ice cold 1X PBS. Washed



neutrophils were resuspended in complete RPMI-1640 media containing 2% oligo FBS (2% oligo medium, hereafter) and counted as it was explained in section 2.2.6.1.

### **2.2.3. *In vitro* Stimulation of PBMCs**

Healthy donors and patients PBMCs in 100 $\mu$ l, whose isolation was narrated in section 2.2.1.1, were layered onto 96-well flat bottom plates. Note that concentration of cells that were used in each set of stimulation was indicated in the results section. 50 $\mu$ l stimulants (Table 2.1) or EVs in different amounts (0.6 $\mu$ g, 3 $\mu$ g, 12 $\mu$ g, 15 $\mu$ g), which were prepared in 5% RPMI media were added onto wells [70]. Off note, for transfection, ligands and Lipofectamine 2000<sup>®</sup> were prepared in FBS-free media. Furthermore, PBMC-EV stimulations were conducted in EV-depleted media. Then, whole reaction volume was brought up to 250 $\mu$ l by 5% RPMI media. After 24-36 hours of incubation at 37°C and following cell pelleting at 400xg for 10 minutes, supernatants were collected and used in Cytokine ELISA (Section 2.2.5); in order to detect concentration of secreted cytokines.

### **2.2.4. *In vitro* Assessments of Neutrophil Activities**

#### **2.2.4.1 Visualization of Neutrophil Extracellular Traps (NETs)**

Isolated healthy and patient neutrophils (1x10<sup>5</sup>/ml) were seeded onto 48-well flat bottom plates and they were treated with PMA (50ng/ml) or 2% oligo medium alone for 4 hours at 37°C. Then, nuclear and extracellular DNAs were stained with 0.6mM Syto<sup>™</sup> 16 Green (ThermoFischer Scientific, USA) and 1mM Styox<sup>™</sup> Orange (ThermoFischer Scientific, USA), respectively. Nuclear and extracellular DNAs were visualized, and images were captured with EVOS FL Auto (ThermoFischer Scientific, USA).

#### **2.2.4.2 Quantification Neutrophil Extracellular Traps (NETs)**

Neutrophils (1.6x10<sup>6</sup>/ml) that were isolated from healthy donors and patients, were layered onto 96-well flat bottom plates. They were treated with PMA (50ng/ml) that were isolated from human plasmas (See section 2.2.7.1) or 2% oligo medium for 4 hours at 37°C [231]. After incubation; in order to chop down NETs that were propelled

from neutrophils, cells were incubated with 500µg/ml Micrococcal Nuclease Solution (ThermoFischer Scientific, USA), that was prepared in 1X Nuclease Buffer (See Appendix A), for 30 minutes at 37°C. Then, reaction was stopped by adding 5 mM EDTA solution into wells. Cells were removed by centrifugation at 300xg for 10 minutes and supernatants were collected and stored at -80°C until for further quantification.

100µl of thawed supernatants and 2-fold serially diluted Lambda DNA (ThermoFischer Scientific, USA) as a standard were put into black 96-well flat bottom plates[231]. Then, 100µl of Quant-iT™ PicoGreen™ (ThermoFischer Scientific, USA), which was diluted 1:200 (v/v) in Tris-EDTA Buffer (See Appendix A), was added into each well. After 5 minutes incubation at RT in dark, OD values were measured by microplate reader (Synergy HT Biotek, USA) where excitation and emission spectra were 485/20nm and 528/20nm, respectively. In order to calculate amount of dsDNA in samples, linear standard curve was constructed by using OD values of standards.

#### **2.2.5. Cytokine Enzyme Linked Immunosorbent Assay (Cytokine ELISA)**

All capturing/detection antibodies, recombinant proteins and their working concentrations that were used in ELISA studies were listed in Table 2.2. 2HB immunoplates (SPL Life Sciences, South Korea) were coated with 50µl of coating antibodies, which were prepared in 1X PBS (non-commercial, see Appendix A), for overnight (12-16 hours) at +4°C [232]. Coating antibodies were removed, and wells were blocked with 200µl blocking buffer (See Appendix A) for 2 hours at RT. After plates were washed with washing buffer (See Appendix A) for 5 times and distilled water for 3 times, wells were incubated with either 50µl of supernatants that were collected from stimulated PBMCs (See section 2.2.3) or 2-fold serially diluted 50µl of corresponding recombinant cytokines for overnight at +4°C [233]. Supernatants and standards were removed, and plates were washed as it was narrated previously. Captured cytokines were incubated with 50µl biotinylated antibodies for 2 hours at RT. After removal of biotinylated antibodies and following washing step, plates were incubated with 50µl SA-ALP (1:1000 diluted (v/v) in blocking buffer) for 1 hour at RT. Following washing steps; in order to detect amount of SA-ALP on biotinylated antibodies, 50µl *p*-Nitrophenyl Phosphate (PNPP) solution (ThermoFischer Scientific, USA), was added onto each well. As yellow color develops, in certain time points, OD values at 405nm of each well

were recorded with ELISA Plate Reader (Molecular Devices, USA). In order to calculate concentration of secreted cytokine in supernatants, 4-parametric curves were constructed from OD values of serially diluted recombinant proteins.

## **2.2.6. Flow Cytometry Methods**

### **2.2.6.1 Cell Counting**

20 $\mu$ l of isolated PBMCs, neutrophils or cultured cells, that are suspended in 1ml of corresponding media, was diluted in 5 ml of isotonic solution (Beckmann Coulter, USA). 20 $\mu$ l from that suspension was acquired and analyzed using Novocyte 3000 flow cytometer. Number of the live cells which were determined by FSC and SSC positioning of events by excluding debris and apoptotic cells were gated. To calculate total number of cells per ml media, numbers of events were multiplied with final dilution factor which was calculated below:

$$\text{Isolated Cell Number} = \frac{\text{Counted Event Number}}{20 \text{ (Acquired Volume } (\mu\text{l}))} \times 250 \text{ (Dilution Factor)} \times 1000 \text{ (Dissolved Volume } (\mu\text{l}))$$

### **2.2.6.2 Cell Surface Staining**

To decipher the immune phenotype of cells from whole blood, 100-200 $\mu$ l of whole bloods were incubated with 5 $\mu$ l of fluorochrome labeled antibodies (Table 2.3) for 30 minutes at RT in dark [234]. After incubation, erythrocytes were lysed using 1X RBC lysis buffer (Biolegend, USA) for 20 minutes at RT in dark. Then, RBC lysis buffer was removed via centrifugation for 10 minutes at 500xg. Stained and RBC-deprived cells were resuspended in 5% RPMI Media. Cells were acquired and analyzed using Novocyte 3000 flow cytometer.

For isolated and stimulated (not fixed) PBMCs, cells were stained with 1 $\mu$ g/ml of fluorochrome conjugated antibodies (Table 2.3) prepared in 5% RPMI media for 30 minutes at 4°C in dark [235]. Then, stained cells were washed with 5% RPMI media and resuspended in 5% RPMI media. Cells were acquired and analyzed using Novocyte 3000 flow cytometer. At least 30000 events were acquired for each sample.

### **2.2.6.3 Assessment of STAT Phosphorylation Levels of T-Cells**

To analyze STAT phosphorylation levels, in this study, whole blood (200 $\mu$ l/stimulation) or purified PBMCs (at least  $1 \times 10^6$  cells/stimulation) were used. First, samples were treated with appropriate recombinant cytokines (Table 2.1) or left untreated. After 30 minutes of incubation at 37°C, cells were fixed and RBCs were lysed with 1X FACS lysing solution (BD Biosciences, USA) for 15 minutes at 37°C [236]. In order to get rid of lysing solution, 2 ml cold FACS buffer was added and cells were centrifuged at 600xg for 6 minutes. Cells were permeabilized with ice cold 87% methanol (w/w) on ice for 30 minutes. Then, cells were washed with 2 ml cold FACS buffer twice. They were co-stained with 5 $\mu$ l APC-conjugated anti-human phosphorylated STATs (Table 2.3) along with 1 $\mu$ g/ml of anti-human CD4+ and anti-human CD8+ antibodies (Biolegend, USA) in FACS buffer for 1 hour at RT in dark. Following washing with 2 ml FACS Buffer for three times and resuspending in 150 $\mu$ l-200 $\mu$ l cold FACS buffer, cells were acquired and analyzed immediately using Novocyte 3000 flow cytometer. At least 30000 events were acquired for each sample.

### **2.2.6.4 Intracellular Cytokine Staining (ICS) of T-Cells**

$4 \times 10^5$  healthy and patient PBMCs were layered onto 96 well U bottom plates. Cells were either treated with PMA (50ng/ml) and Ionomycin (1 $\mu$ g/ml) or left untreated for 2 hours at 37°C [237]. In order to stop cytokine secretion, 5 $\mu$ g/ml Brefeldin A (Sigma Aldrich, Germany) was added into wells as a Golgi Stop reagent. After cells were incubated for 4 hours more at 37°C, plates were centrifuged at 400xg for 10 minutes. Supernatants were removed, cells were fixed with 150 $\mu$ l 1X Cytfix Buffer (BD Biosciences, USA) for 20 minutes at RT in dark. After cells were sedimented at 400xg for 10 minutes, they were washed with 250 $\mu$ l FACS buffer and centrifugated at 400xg for 10 minutes. Washing step was carried out once more and cells were permeabilized with 150 $\mu$ l cold 1X Cytoperm Buffer (BD Biosciences, USA) for 15 minutes at RT in dark. In order to remove permeabilization buffer, cells were centrifugated at 400xg for 10 minutes. Then, cells were stained with cell surface markers (1 $\mu$ g/ml) and cytokine antibodies (1 $\mu$ g/ml), which were prepared in FACS buffer, (Table2.3) for 30 minutes at RT in dark. Stained cells were washed twice. After resuspending cells in 150 $\mu$ l-200 $\mu$ l

FACS Buffer, they were analyzed using Novocyte 3000 flow cytometer. At least 30000 events were acquired within CD4<sup>+</sup> gates for each sample.

### **2.2.6.5 Nuclear Transcription Factor Staining of PBMCs**

First, isolated fresh healthy and patient PBMCs were stained with fluorochrome conjugated anti-human CD4<sup>+</sup> (1µg/ml) that was diluted in 5% RPMI for 30 minutes at +4°C [238]. In order to wash out excess fluorochrome-conjugated antibodies, 1 ml FACS Buffer was added into samples and subjected to centrifugation at 400xg for 10 minutes +4°C. Supernatants were removed, cells were dissolved in 100µl 1X True-Nuclear™ solution (Biolegend, USA) which was prepared by diluting 4X True-Nuclear™ Concentrate (Biolegend, USA) with True-Nuclear™ Fix Diluent (Biolegend, USA) at 1:3 (v/v) ratio. After 1 hour of incubation at RT in dark, 1 ml FACS buffer was added onto samples and they were centrifugated at 400xg for 10 minutes. In order to permeabilize fixed cells, 100µl 1X True-Nuclear™ Perm Buffer (Biolegend, USA) was added and cells were immediately centrifugated at 400xg for 10 minutes. Supernatants were discarded and this permeabilizing step was repeated for 3X. After last permeabilization step, cells were resuspended in 1X True-Nuclear™ Perm Buffer containing (1µg/ml) nuclear transcription antibodies (Table 2.3) and incubated for 1 hour at RT in dark. Then cells were washed with 1X True-Nuclear™ Perm Buffer for 3X. After last centrifugation step, cells were dissolved in FACS Buffer and acquired using Novocyte 3000 flow cytometer. At least 30000 events were acquired for each sample.

### **2.2.7. Extracellular Vesicle Isolation and Characterization**

#### **2.2.7.1 Extracellular Vesicle Purification From Human Plasmas**

In order to remove any remnants of cells and debris from isolated plasmas of healthy donors and patients from whole blood following histopaque centrifugation step, as it was explained in Section 2.2.1.1, they were centrifuged at 1500xg for 10 minutes [189]. After supernatants were collected, for long term storage, they were snap frozen in liquid nitrogen for 15 minutes and stored at -80°C until for further use.

One day prior to extracellular vesicle isolation, frozen plasmas were slowly melted for overnight in the fridge at +4°C. After thawing, they were centrifuged at 1500xg for 10 minutes to remove the remaining debris and supernatants were transferred into ultracentrifugation tubes (Beckmann Coulter, USA), they were completed up to 15 ml with cold 1X PBS and centrifuged using an ultracentrifuge (Model: XL-90, Beckmann Coulter, USA) at 10000xg for 10 minutes at +4°C. Supernatants were transferred into new ultracentrifugation tubes for EV pelleting at 100000xg for 90 minutes at +4°C. After, supernatants were discarded, pellets harboring EVs were resuspended in 1X PBS and, centrifugated at 100000xg for 90 minutes at +4°C, one last time. EVs were resuspended in 1X PBS. Amount of purified EVs were determined by concentration of their surface proteins which was explained in Section 2.2.8.

#### **2.2.7.2 Determination of Size and Concentrations of EVs by qNano Gold**

Concentration and distribution of size distribution and zeta-potential of isolated extracellular vesicles from human plasmas were determined by qNano Gold (Izon Science, New Zealand). After EVs were diluted 25x in 1X PBS, they were placed into the stage and analyzed. The concentration vs size plots were obtained from the instrument following analyses.

#### **2.2.7.3 Verification of EV-specific Surface Markers by Bead-based Characterization Method**

For each 100 tests of EV staining, 100µl of Carboxyl modified latex beads (Thermo Fischer Scientific, USA) were allocated [239]. In order to wash beads, 1000µl 1X PBS was added onto them. Then they were pelleted by centrifugation at 13000xg for 10 minutes. This washing step was repeated once again and purified anti-human CD63 antibody (10µg antibody for 100 EV staining) were mixed with these washed latex beads. They were rotated for 30 minutes at RT for rapid conjugation. Afterwards, total volume was completed up to 500µl and the bead-antibody mixture was left on the rotator overnight at RT.

To get rid of excess antibodies, 500µl 1X PBS was added and antibody-conjugated beads were centrifugated at 13000xg for 10 minutes, twice. Next day, non-specific EV binding was prevented by blocking the antibody coated beads with 1X PBS containing

5% BSA for 6 hours at RT. Blocked beads were pelleted and dissolved in 1X PBS containing 1% BSA. For each staining 4µg EV were added into 1µl beads. Like in the case of rapid antibody conjugation process, they were subjected to rotation for 30 minutes at RT without any additional PBS. After 30 minutes of incubation, total volume was completed to 500µl with 1X PBS.

The unbound EVs in solution was removed by adding 500µl 1X PBS and then solution was subjected to centrifugation at 13000xg for 10 minutes. Next, captured EVs were incubated with fluorochrome conjugated antibodies (1µg/ml) or with the proper isotypes controls (Table 2.3) for 1 hour at RT. The excess antibodies were removed by centrifugation as explained earlier. Stained EVs were analyzed using Novocyte 3000 flow cytometer where at least 30000 events were acquired in singlets.

### **2.2.8. Quantification of Protein Concentration by BCA Assay**

Concentration of purified EVs or exosomes or proteins were determined by Pierce™ BCA protein assay kit (ThermoFischer Scientific, USA). 25 µl of samples (EVs or exosomes were 5X diluted in 1X PBS) and 25µl of 2-fold serially diluted BSA standards ranging from 2000 µg/ml to 31.25 µg/ml, were transferred onto 96-well flat bottom plates. 200µl of working reagent, which was prepared by 1 volume of Solution A and 50 volume of Solution B, was mixed with EVs or exosomes and standards. After incubation at 37°C for 30 minutes, OD values at 562 nm were measured by microplate reader (Synergy HT). By using linear standard curve which was constructed by BSA standards, concentration of EVs or exosomes and proteins were calculated.

### **2.2.9. SDS-Page and Western Blot**

#### **2.2.9.1 Protein Isolation from Cell Lysates and EVs/Exosomes**

Cell lysates (at least  $5 \times 10^6$  cells) were suspended within 500µl RIPA Buffer (Appendix A). They were incubated on ice for 20 minutes (vortexed for 15 seconds at every 5 minutes) which was followed by centrifugation at 12000xg for 20 minutes at +4°C. Supernatants were collected, and BCA kit was used to determine protein concentration.

Similarly, 1 volume of isolated EVs or exosomes (at least 100µg) were mixed with 1 volume of RIPA buffer. Mixtures were sonicated (VibraCell, Sonics, USA) for total 15

minutes. Mixtures were subjected to vortex for 15 seconds at the end of each 5 minutes of sonication. After that, they were incubated on ice for 20 minutes (vortexed for 15 seconds at every 5 minutes). Then, they were centrifuged at 12000xg for 20 minutes at +4°C. Concentration of proteins in collected supernatants were determined by Pierce™ BCA kit (section 2.2.8).

### **2.2.9.2 Denaturation of Proteins and SDS-Page Running**

In order to denature proteins, samples were mixed with 4X loading dye (Appendix A) where total volume was completed up to 45µl by cell culture grade water [240]. Then, mixtures were subjected to heat (+95°C) for 5 minutes. Denatured samples were incubated on ice until they were loaded in wells of gel.

Throughout this thesis, concentration of stacking and separating gels were prepared as 5% and 7%, respectively (See Appendix A). Upon solidification of gels, they were transferred into Tank (Bio-Rad, Germany) which was filled with 1X running buffer (See Appendix A). Denatured protein samples were loaded into each well (for cell line lysates: 10µg/lane; for EVs or exosomes: 30µg/lane in 40µl). Afterwards, 60V power was supplied until samples passed stacking gel. Then, running was carried out with 100V until loading dyes reached at the very end of SDS-gel.

### **2.2.9.3 SDS-Page to and PDVF Membrane Transfer**

In this study, all transfer procedures were performed using wet-transfer method. Briefly, for each transfer, 4 Whatman papers (GE Healthcare, USA) and one 0.45µm PDVF membranes (ThermoFischer Scientific, USA) along with the SDS gel were properly positioned in a transfer cassette [241]. Transfer cassette was put into tank that was filled with cold 1X transfer buffer (Appendix A). Transfers were carried out at 100V for 2 hours on ice.

### **2.2.9.4 Blotting and Imaging**

To validate proteins that were properly transferred into membranes, PDVF membranes were incubated with Ponceau S (See Appendix A) until color formation was observed. Then, membranes were washed with PBS-T (See Appendix A) for 5 times. PDVF



membranes were blocked with blocking buffer (See Appendix A) for 2 hours at RT. Membranes were incubated with desired primary antibodies (Table 2.4) which were prepared in antibody dilution buffer (See Appendix A) for 14 hours at +4°C. After incubation, membranes were washed 5X followed by incubation with corresponding HRP-conjugated secondary antibodies (Table 2.4) for 2 hours at RT. To remove excess antibodies, membranes were washed 5X and they were stored in PBS-T until they were visualized. For protein detection, membranes were incubated with Amersham ECL Prime Western Blotting Reagent (GE Healthcare, USA) and visualized under Amersham Imager 600 (GE Healthcare, USA).

#### **2.2.10. Statistical Analyses**

All statistical analyses were carried out with GrapPad Prism 6 Software (USA). Statistical significances in CHAPLE study were determined by Kruskal-Wallis H test and Student's t-test.  $p \leq 0.05$  was used as threshold for significance.

## **3. RESULTS**

### **3.1. Characterization of Immune Responses of CTPS1 Deficiency**

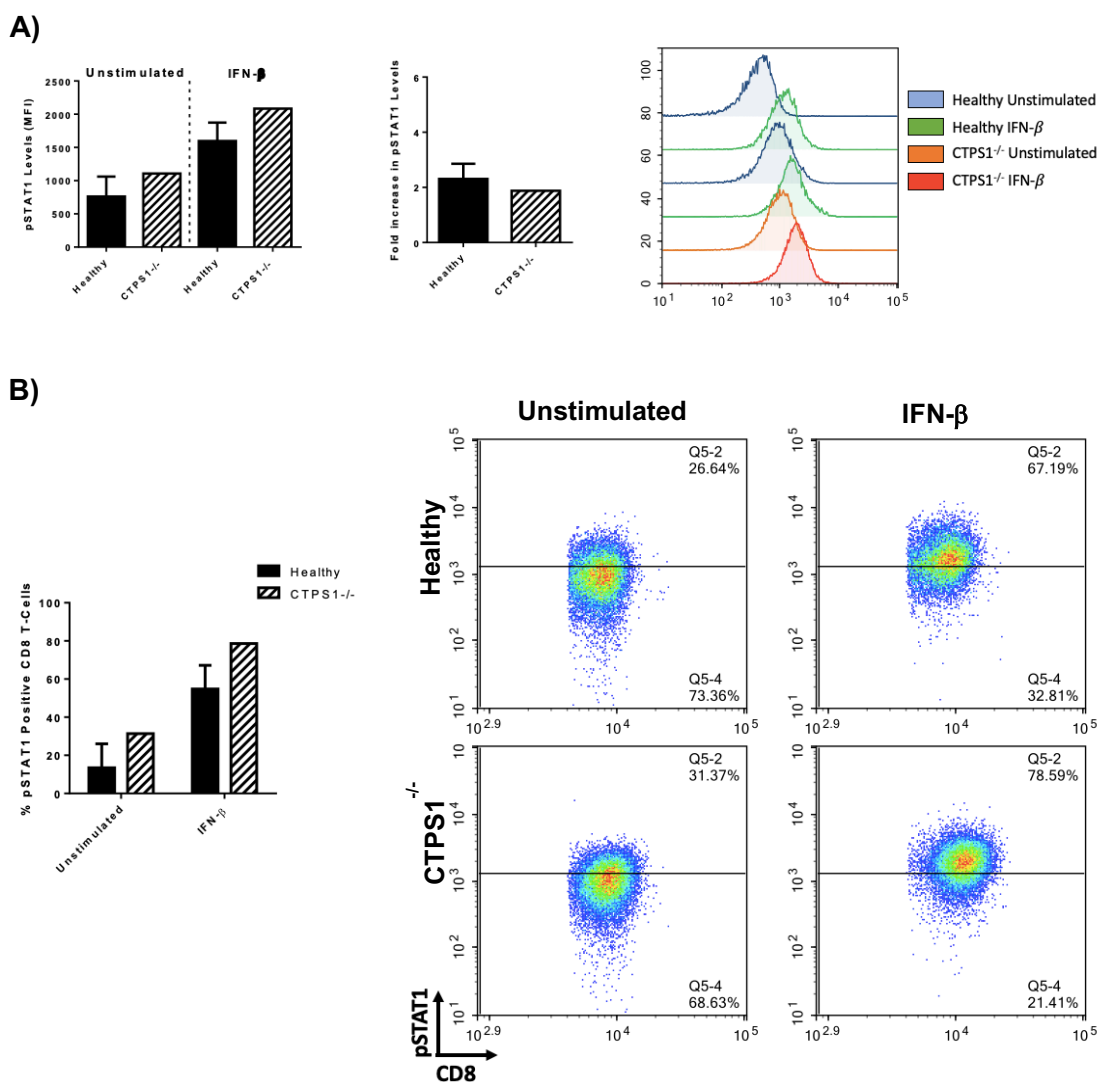
#### **3.1.1. Functional studies of adaptive immune cells of CTPS1 deficient patient**

##### **3.1.1.1 CTPS1 deficient patient CD8<sup>+</sup> T-cells had normal STAT1 phosphorylation level but increased CD107a expression on their surface**

Deficiency of CTPS1 enzyme in human leukocytes render adaptive immune system to fail, eventually leading to combined immunodeficiency; consequently, predisposing individuals to suffer from severe, chronic viral infections alongside with recurrent bacterial infections [149]. In order to check manifestation of altered immune response such as persistent chronic viral infection and cellular sign of CID in the patient, CD4<sup>+</sup>:CD8<sup>+</sup> T-cell ratio was determined. Off note, healthy individuals normally display ~1.5-2 ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T-cell levels [242]. Flow cytometric analysis revealed that CD4<sup>+</sup>/CD8<sup>+</sup> ratio of healthy controls (n=2) was as expected ~1.93 whereas it was ~0.43 for the CTPS1 patient (Appendix B1.1, left panel). To ensure that this was not an experimental error, we analyzed CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios following multiple blood donations at different times and we observed that within 3 months intervals consistent with the first results the patient PBMCs had reciprocal CD4<sup>+</sup>/CD8<sup>+</sup> ratio of healthy individuals (n=2) vs patient was ca. 4.6 fold vs ca. 0.47 fold (Appendix B1.1, right panel).

Indication of chronic viral infection lead us to speculate indirect role of CTPS1 enzyme within the context of host's viral responses. During viral infection, type I IFNs secreted from APCs are known to influence effector function of several cell types especially cytotoxic T-cells [243]. To this end, type I IFN signaling pathway within CD8<sup>+</sup> T-cells were investigated by assessing STAT1 phosphorylation (*i.e.* pSTAT1) levels. Flow cytometric study revealed that CD8<sup>+</sup> T-cells of CTPS1<sup>-/-</sup> PBMCs had similar pSTAT1 levels under basal conditions (Figure 3.1A). Healthy and patient cells were incubated with IFN- $\beta$ ; in order to activate JAK-STAT pathway through its receptor. As it was illustrated in Figure 3.1A, upon activation of IFN- $\beta$  receptor, both controls and patient

had similar STAT1 phosphorylation levels. Meanwhile, percent of phosphorylated STAT1 bearing cytotoxic T-cells were compared as well (Figure 3.1B). As it can be examined in Figure 3.1B, only 13.49% of healthy CD8<sup>+</sup> T-cells have phosphorylated STAT1 protein whereas CTPS1 patient's PBMCs had 31.37% CD8<sup>+</sup> T-cells with pSTAT1 protein under basal conditions. Moreover, upon receptor stimulation, percentage of healthy and patient CD8<sup>+</sup> T-cells having pSTAT1 elevated to 54.78% and 78.59%, respectively.

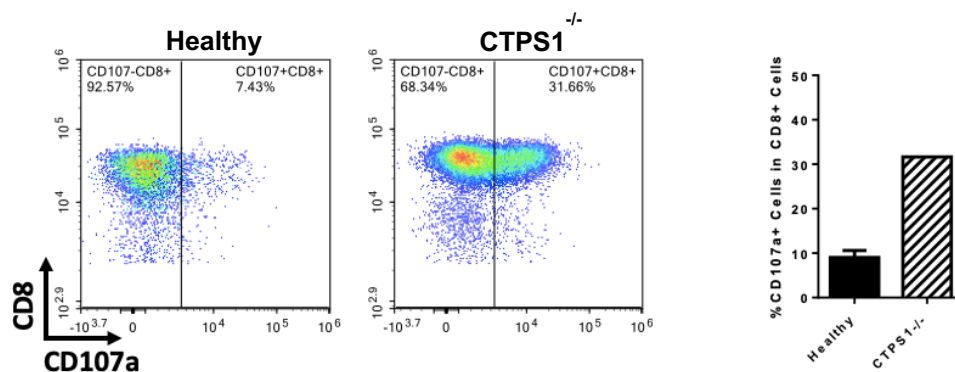


**Figure 3.1 STAT1 phosphorylation levels of control and patient CD8<sup>+</sup> T-Cells in response to IFN-β stimulation.** 200μl whole blood of healthy controls (n=2) and patient were either left untreated or treated with IFN-β (100ng/ml) for 30 minutes. Then, cells were

fixed, permeabilized and stained with fluorochrome labeled anti-CD3, anti-CD8<sup>+</sup> and anti-pSTAT1 protein. **A)** Bar graph that represents mean fluorescent intensity levels (MFI) of pSTAT1 of unstimulated or IFN- $\beta$  stimulated CD8<sup>+</sup> T-cells gated in CD3 events (left panel) which were evaluated from flow-cytometry histograms (right panel). **B)** Bar graph that represents percentages of pSTAT1 CD8<sup>+</sup> T-cells (left panel) which were evaluated from representative flow cytometer density plots (right panels). For each analysis,  $4 \times 10^4$  cells were acquired within CD8<sup>+</sup> gate.

Although there was a variation within healthy control group CD8<sup>+</sup> T-cell percentages CTPS1<sup>-/-</sup> might have slightly increased pSTAT1 levels in CD8<sup>+</sup> T-cells. Nevertheless, this data implied that absence of CTPS1 enzyme does not influence IFN- $\beta$  induced STAT1 phosphorylation in cytotoxic T-cells.

Having normal STAT1 phosphorylation levels within CD8<sup>+</sup> T-cell population upon IFN- $\beta$  stimulation lead us to explore effector function of cytotoxic T-cells. CD107a surface expression on CD8<sup>+</sup> T-cells at basal level was evaluated since CD107a is a protein residing within the granules of NK, CD8<sup>+</sup> T-cells and upon activation and following degranulation CD107a is expressed on the surface of cytotoxic T-cells and is a reminiscent of activation and degranulation status. As it can be observed from Figure 3.2, percentages of CD107a expressing healthy and patient CD3<sup>+</sup> CD8<sup>+</sup> cells were 7.43 and 31.66, respectively. However, it is important to note that CD107a expression on surface may not be directly correlated with effector function since it depends on expression and activity of perforin and granzyme B molecules as well [244].



**Figure 3.2 CD107a surface expression of unstimulated CD8<sup>+</sup> T-cells.** Whole bloods of healthy donors (n=2) and patient were stained with anti-CD45, anti-CD3, anti-CD8<sup>+</sup>, anti-CD107a and were analyzed using flow cytometer. Representative density plots (left and

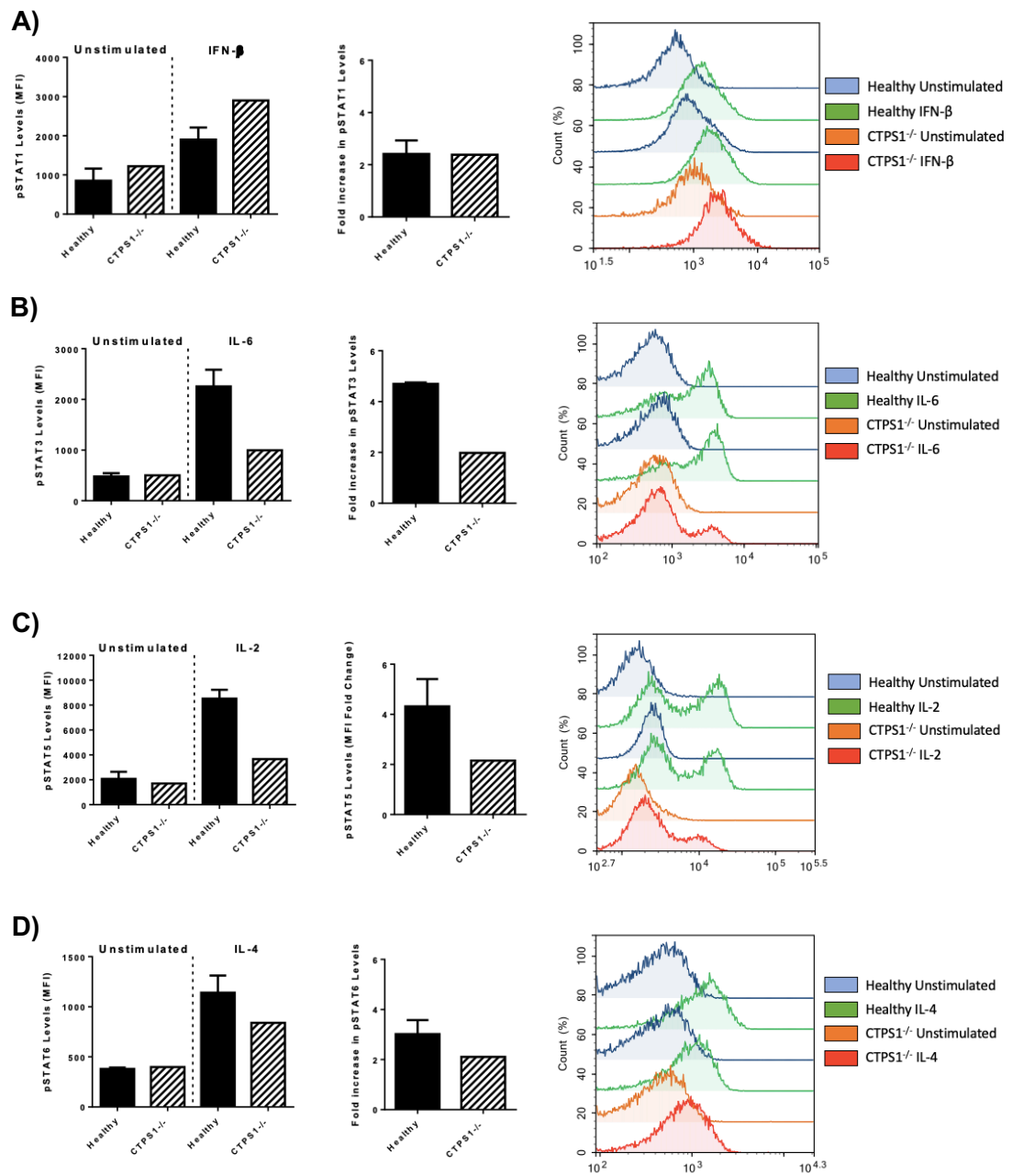
middle panels) and bar graph (right panel) show CD3<sup>+</sup>CD8<sup>+</sup>CD107a<sup>+</sup> cell percentages. Cells were gated in lymphocytes and singlets by forward and side scattering.

In conclusion, normal pSTAT1 levels and elevated CD107a surface expression implied that chronic viral infections in CTPS1 deficient patient might not be related to activation and effector function of cytotoxic T-cells.

### **3.1.1.2 Assessment of STAT phosphorylation levels within patient CD4<sup>+</sup> T-cells revealed reduced STAT3 and STAT5 phosphorylation**

Although CD8<sup>+</sup> T-cells are major subtypes of T-cells to kill infected cells, they require help of CD4<sup>+</sup> T-cells; thus, they are called T-helper cells as well. Through antigens presented on MHCs of APCs, T helpers are activated to further amplify cytotoxic T-cell dependent functions. Moreover, CD4<sup>+</sup> T-cells are grouped into distinct subtypes having different effector function. In order to properly differentiate into T helper subtypes, naive CD4<sup>+</sup> T-cells require environmental clues alongside with TCR and co-stimulatory receptor engagement. These environmental clues are provided by antigen-presenting cells, which are activated by pathogens, as cytokines. Through intracellular JAK-STAT pathway of cytokine receptors, cytokines determine the fate of primed CD4<sup>+</sup> T-cells.

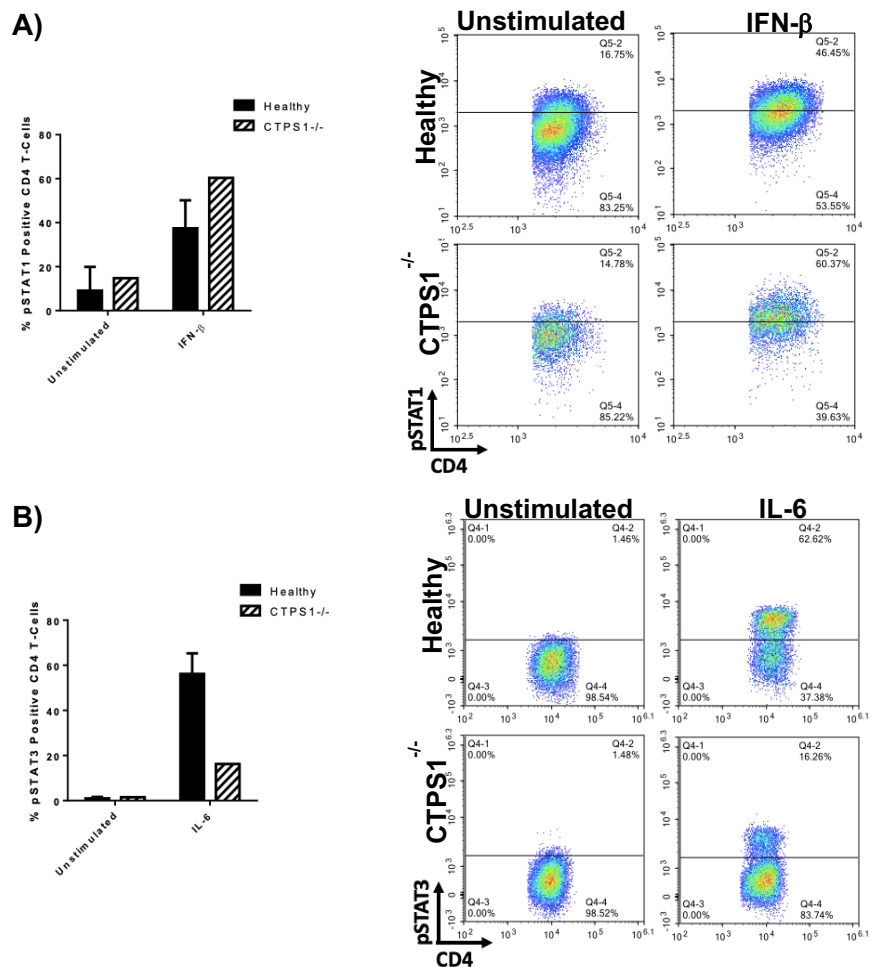
It was attempted to explore contribution of helper T-cells to immunodeficiency of CTPS1 following CD8<sup>+</sup> involvement was sought. To that end, cells from healthy controls and patient were incubated with four major fate-determining cytokines: IFN- $\beta$ , IL-6, IL-4 and IL-2. Then, cells were fixed, permeabilized and stained against CD4<sup>+</sup>, pSTAT1, pSTAT3, pSTAT5, pSTAT6 proteins and evaluated using flow-cytometer. Baseline or IFN- $\beta$  treated conditions, both healthy controls and CTPS1<sup>-/-</sup> had similar STAT1 phosphorylation levels within the CD4<sup>+</sup> T-cells (Figure 3.3A). Figure 3.3B demonstrated that IL-6 led to >2-fold STAT3 phosphorylation in control T-cells compared to CTPS1<sup>-/-</sup> cells. However, healthy and patient STAT6 protein phosphorylation levels were not similar following IL-4 incubation. Patient cells had almost 25% lesser phosphorylation compared to healthy controls (Figure 3.3C). Strikingly, IL-2 treatment elevated MFI levels of pSTAT5 to 8500 $\pm$ 500 (Mean $\pm$ Sd, n=2) and 3070 (Mean, n=1) in healthy controls and CTPS1<sup>-/-</sup>, respectively (Figure 3.3D). That is, phosphorylation of STAT5 in patient was 2.5-fold less when compared to healthy STAT5 phosphorylation.

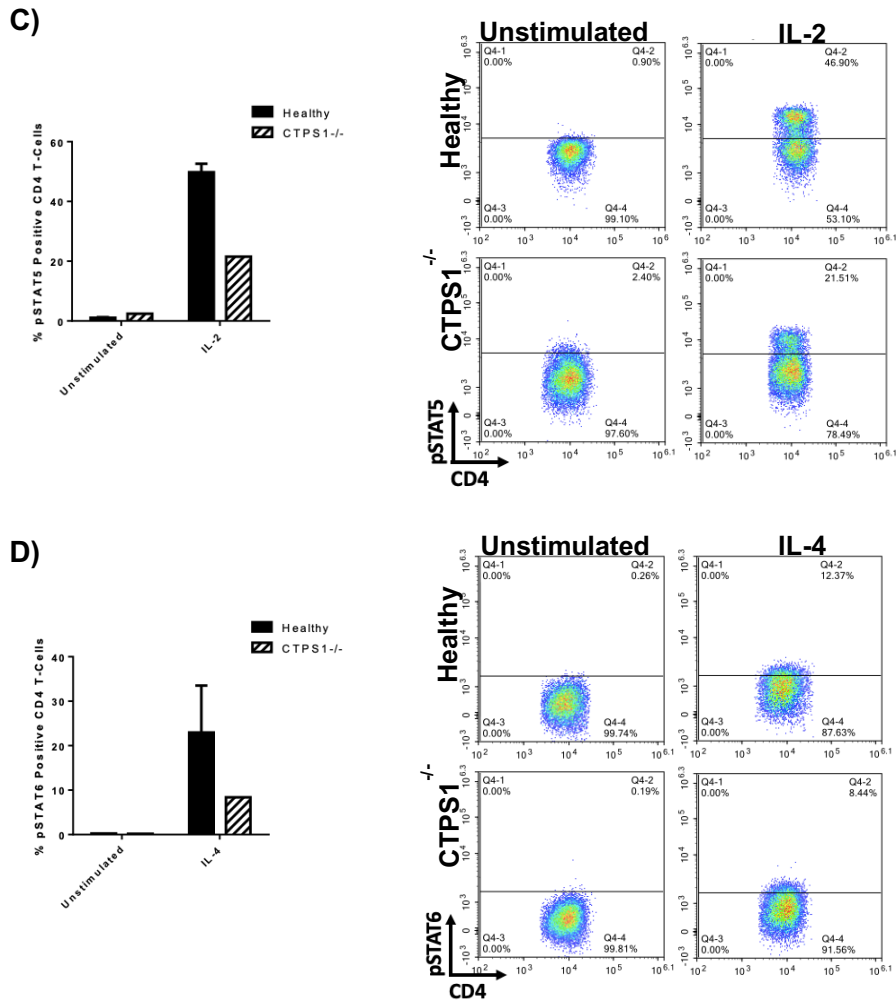


**Figure 3.3 STAT phosphorylation levels of CD4<sup>+</sup> T-Cells of healthy donors and CTPS1 patient.** 200 $\mu$ l whole bloods of healthy individuals (n=2) and patient were either left untreated or treated with IFN- $\beta$  (100ng/ml); IL-6 (200ng/ml); IL-2 (100ng/ml); IL4 (200ng/ml) for 30 minutes. Then, cells were fixed, permeabilized and stained for anti-CD4<sup>+</sup> and anti-pSTAT1, anti-pSTAT3, anti-pSTAT5, anti-pSTAT6 proteins. **A)** STAT1, **B)** STAT3, **C)** STAT5 and **D)** STAT6 phosphorylation levels of CD4<sup>+</sup> T-cells were evaluated using Novocyte 3000 flow cytometer. Mean Fluorescent Intensity (MFI) of pSTAT proteins (left panels), fold of increase in pSTAT levels (middle panels) were demonstrated in bar graphs,

while representative histograms were presented in right panels. For each analysis,  $4 \times 10^4$  cells were acquired in CD4<sup>+</sup> gates.

Meanwhile, percent of phosphorylated STAT protein bearing CD4<sup>+</sup> T-cells were assessed and compared as well. Flow cytometric studies revealed that percentages of healthy control and patient CD4<sup>+</sup> T-cells positive for pSTAT1 proteins were  $37.46 \pm 12.72\%$  (Mean $\pm$ SD, n=2) and 60.37%, respectively (Figure 3.4A). On the contrary, as it can be seen from Figure 3.4B, IL-6 induced pSTAT3 bearing CD4<sup>+</sup> T-cell percentages were approximately  $\sim 3.5X$  less in patient PBMC (16.26%) compared to healthy controls ( $56.16 \pm 9.13\%$ ; Mean $\pm$ SD, n=2). Like in the case of STAT1, percentages of pSTAT6<sup>+</sup> CD4<sup>+</sup> T-cells were similar in control and patient cells (Figure 3.4C). Lastly, patient CD4<sup>+</sup> T-cells had 2.3-fold less phosphorylated STAT5 proteins compared to healthy controls (Figure 3.4D).





**Figure 3.4 Percentages of pSTAT positive CD4<sup>+</sup> T-cells from healthy and CTPS1<sup>-/-</sup> individuals following cytokine incubations. A) pSTAT1<sup>+</sup>, B) pSTAT3<sup>+</sup>, C) pSTAT5<sup>+</sup>, D) pSTAT6<sup>+</sup> CD4<sup>+</sup> T-cells percentages were evaluated by flow cytometry utilization. Bar graphs demonstrate percentages of unstimulated or stimulated CD4<sup>+</sup> T-cells in left panels. In right panels, representative density plots of one healthy and patient CD4<sup>+</sup> T-cells were stationed.**

Collectively, these results implied that CTPS1 deficient CD4<sup>+</sup> T-cells had impaired STAT3 and STAT5 but normal STAT1 or STAT6 phosphorylation when corresponding cytokines were added in the culture media. However, it is worthwhile to mention that control groups during assessment of STAT1 and STAT6 phosphorylation did have



significant variation. We feel that more healthy controls should be added to better demonstrate the healthy levels of these pSTATs.

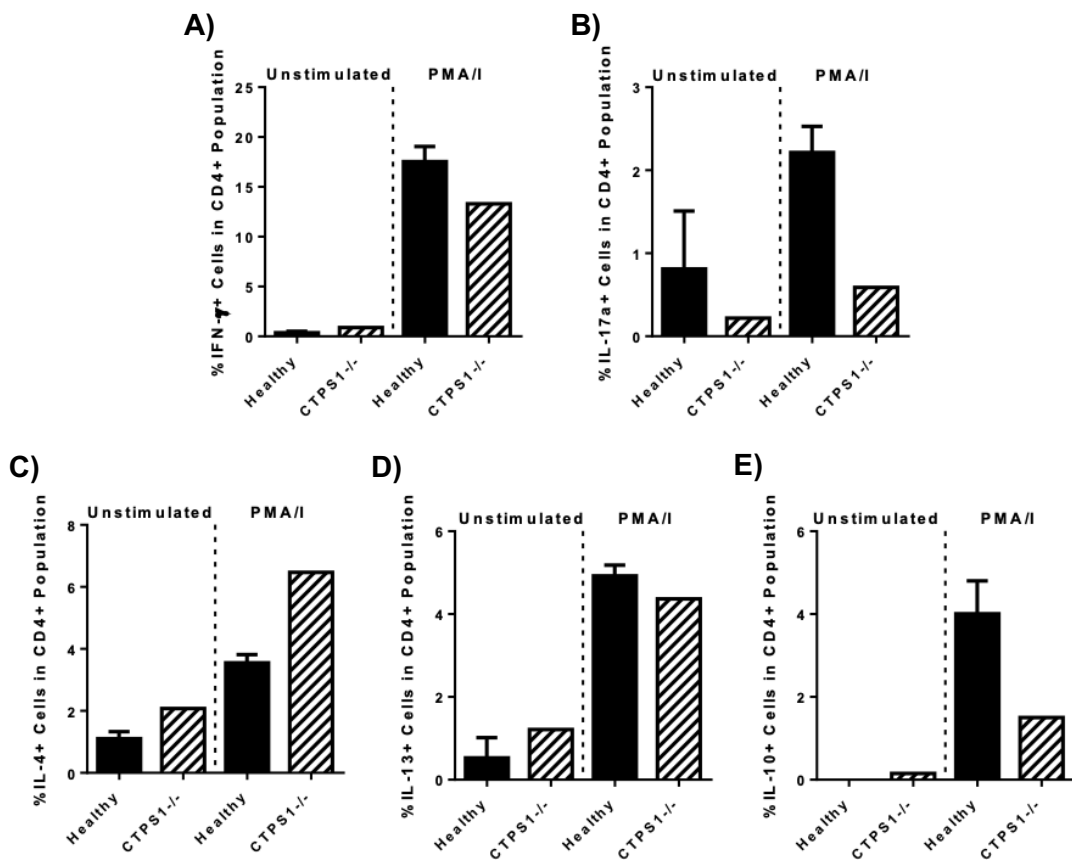
### **3.1.1.3 Intracellular cytokine staining of patient T-helper cells demonstrated impaired Th17, Treg signatures**

STAT phosphorylation results (Section 3.1.1.2) lead us to hypothesize that impaired CTPS1 enzyme might render certain subtypes of CD4<sup>+</sup> T-cells to dysfunction, specifically Th17 and Tregs due to reduced STAT3 and STAT5 phosphorylation, respectively. In order to unravel this, isolated PBMCs from healthy controls and patient were incubated with PMA and Ionomycin mimicking T-cell priming, bypassing TCR and CD28 stimulation, directly activating intracellular pathways; consequently, forcing CD4<sup>+</sup> T-cells to produce and secrete cytokines. After cells were fixed, permeabilized and stained, percent of each cytokine producing T-cells was assessed using flow cytometer (Figure 3.5 and Appendix B1.2)

As Figure 3.5A-B illustrated, even though IFN- $\gamma$  producing CD4<sup>+</sup> T-cell percentages of controls ( $17.52 \pm 2.15\%$ ) and patient ( $13.3\%$ ) were similar, intracellular IL-17a staining revealed that patient blood cells had  $\sim 3.75$ -fold less Th17 cells in circulation compared to healthy controls (control and patient IL-17a producing CD4<sup>+</sup> T-cells were  $2.22 \pm 0.44\%$  and  $0.59\%$ , respectively). Previously in addition to pSTAT3, we observed that pSTAT5 was reduced in CTPS1 patient. To confirm that this reduction affects Treg function and frequency we investigated T-regulatory cell population in CTPS1 patient. Therefore, intracellular IL-10 levels within cells were stained and analyzed using flow cytometer. As it can be seen in Figure 3.5C, while IL-10 producing cell percentage for the healthy donors was  $4.00 \pm 1.12\%$ , it was only  $1.50\%$  in patient. In other words, CTPS1<sup>-/-</sup> IL-10 producing CD4<sup>+</sup> T-regulatory cells in the circulation were almost 2.7-fold less compared to healthy controls.

Furthermore, Th2 responses were also sought. Primarily, IL-4 producing and secreting cell percentages were determined. As Figure 3.5D depicts, PMA and Ionomycin stimulation lead to  $3.5 \pm 0.3\%$  of healthy CD4<sup>+</sup> T-cells to secrete IL-4. Unexpectedly, IL-4 producing T-cells was  $6.47\%$  in patient PBMCs upon PMA and ionomycin stimulation. That is, IL-4 producing cells in CTPS1<sup>-/-</sup> circulation was 3-fold more compared to healthy controls. It is important to note that this result is conflicting with the previous

pSTAT6 staining findings (see Figure 3.3 and 3.4). Finally, IL-13 which is another Th2-related cytokine was assessed using flow cytometer, (Figure 3.5E). In contrast to IL-4, IL-13 producing T-helper cell numbers were similar between controls and patient PBMCs ( $4.92 \pm 0.36\%$  and  $4.37\%$ , respectively).



**Figure 3.5 Cytokine production profiles of CD4<sup>+</sup> T-cell in response to PMA/Ionomycin stimulation.** Healthy (n=2) and CTPS1<sup>-/-</sup> PBMCs ( $1.6 \times 10^6/\text{ml}$ ) were either left untreated or stimulated with PMA/Ionomycin (50ng/ml,  $1 \mu\text{g}/\text{ml}$ , respectively) for 2 hours. Then Brefeldin A was added, and stimulations were continued for another 4 hours. Cells were fixed, permeabilized and stained with anti-CD4<sup>+</sup>, anti-IFN- $\gamma$ , anti-IL-17a, anti-IL-4, anti-IL-13 and anti-IL-10 then they were evaluated in flow cytometry. **A)** IFN- $\gamma$ , **B)** IL-17a, **C)** IL-4, **D)** IL-13 and **E)** IL-10 cytokine producing CD4<sup>+</sup> T-cell percentages were represented in bar graphs. For each analysis,  $3 \times 10^4$  cells were acquired in CD4<sup>+</sup> gates.

Next, it was attempted to determine circulating T-helper cell subtypes. Thus, PBMCs were stained against CD4<sup>+</sup> T-cell specific cardinal transcription factors. Among several TF tested, only T-reg associated transcription factor, FoxP3 staining was successful stained and it further confirmed 2-fold less IL-10 producing CD4<sup>+</sup> T-cells in CTPS1 deficient individual compared to healthy controls (Appendix B1.3).

When these results are taken together, considering STAT phosphorylation, intracellular cytokine staining and Foxp3 findings revealed that CTPS1 patient might have i) impaired Th17 activation and /or response, thus, could lead to biased Th1/Th17 balance in addition to ii) T-regulatory dependent dysregulation.

### **3.1.2. Functional studies of innate immune cells of CTPS1 deficient patient**

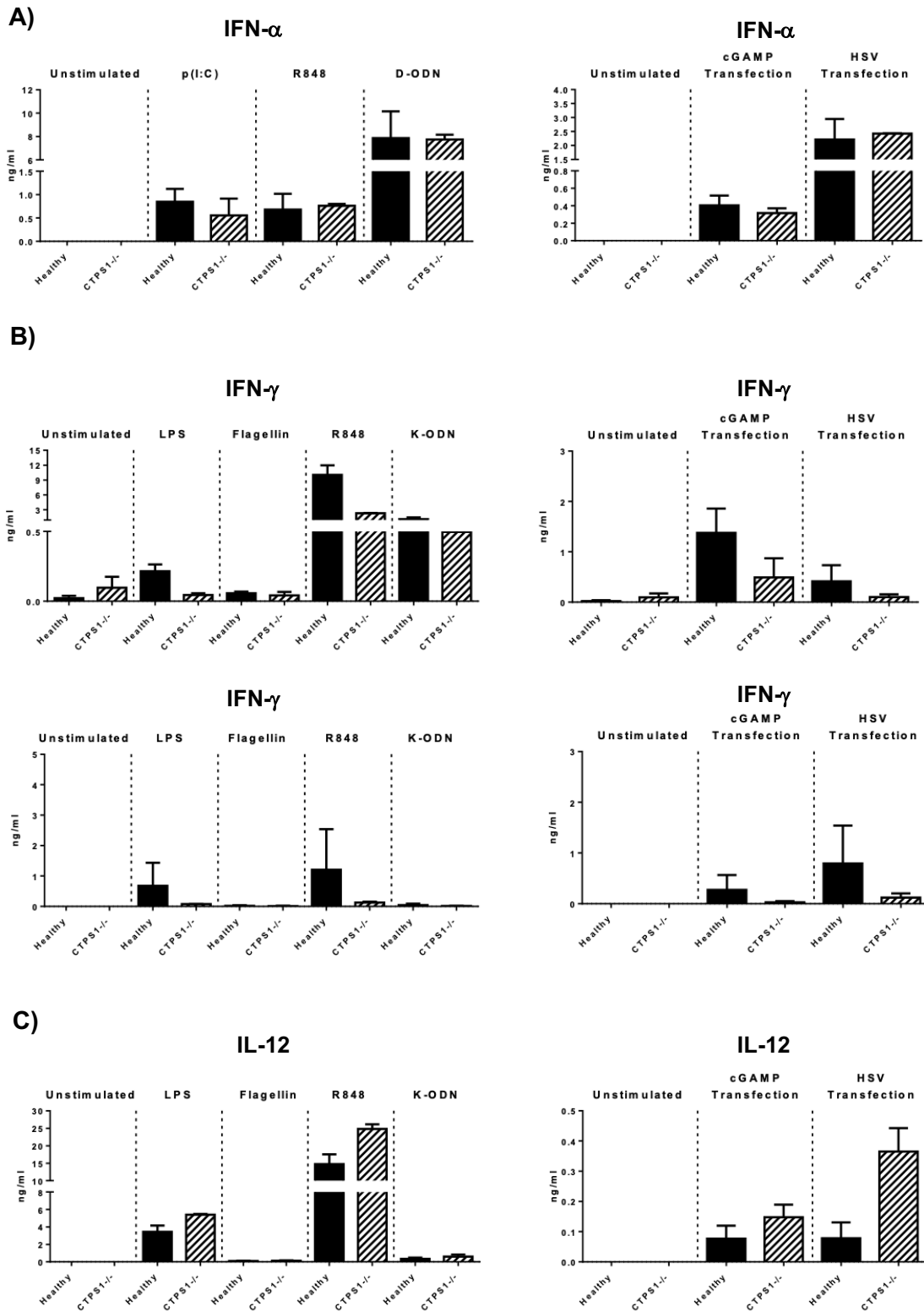
Although adaptive immune responses of CTPS1 deficiency have been attempted to be explored, immune dysregulation of the innate arm of immunity was not fully investigated. Therefore, through series of experiments, it was aimed to establish the impact of CTPS1 enzyme deficiency on innate cells.

#### **3.1.2.1 Responses of CTPS1 PBMCs in the context of PRR ligand stimulations**

##### **3.1.2.1.1 IFN- $\gamma$ but not IFN- $\alpha$ secretion from CTPS1 deficient PBMCs is reduced upon TLR and Cytosolic ligand stimulation**

As it was mentioned previously, one of the cardinal cytokines that are produced and utilized during viral infections are type I IFNs. They are produced from variety of cells; consequently, act on their receptors to modulate immune responses against viruses. First, it was aimed to checked whether chronic viral infections are due to distorted type I IFN secretion from immune cells. In that regard, PRRs involved in virus detection and leading to IFN- $\alpha$  secretion were stimulated. Cytokine ELISA results from those treated cell supernatants were demonstrated that responses of endosomal TLRs and intracellular DNA-dependent cytosolic sensor were not altered in CTPS1<sup>-/-</sup> cells (Figure 3.6A). Then, responses of NK, NKT, iNKT alongside with CD8<sup>+</sup> T-cells were

investigated by assessing IFN- $\gamma$  secretion upon PRR-ligand engagement. As is was illustrated in Figure 3.6B (upper panel), strikingly, type II IFN secretion upon TLR7 induction was ca. 5-fold lower in patient. Moreover, reduced IFN- $\gamma$  responses in patient were present in TLR9 (2-fold) and STING (1.75-fold) stimulations, albeit to a lesser extent. To prove that these results were reliable, patient PBMCs were isolated on a different day and were stimulated and responses were checked again. Although responses of healthy PBMCs resulted in enormous amount of deviation, TLR7, TLR9 and STING induced IFN- $\gamma$  production were still lower in CTPS1 deficient patient (Figure 3.6B, lower panel). IFN- $\gamma$  secreting cells upon PRR stimulations require IL-12 from APCs. Next, whether reduced IFN- $\gamma$  production and secretion resulted from lower percentages of cell types destined to produce it or APCs per se had impaired IL-12 production was sought. As it was illustrated in Figure 3.6C, apart from 4.2-fold more IL-12 secretion via IFI16 mediated STING induction in patient PBMCs, other PRR responses were found to be similar in patient compared to healthy cells, implying that IL12 is not an issue for the observed reduced IFN- $\gamma$  levels.



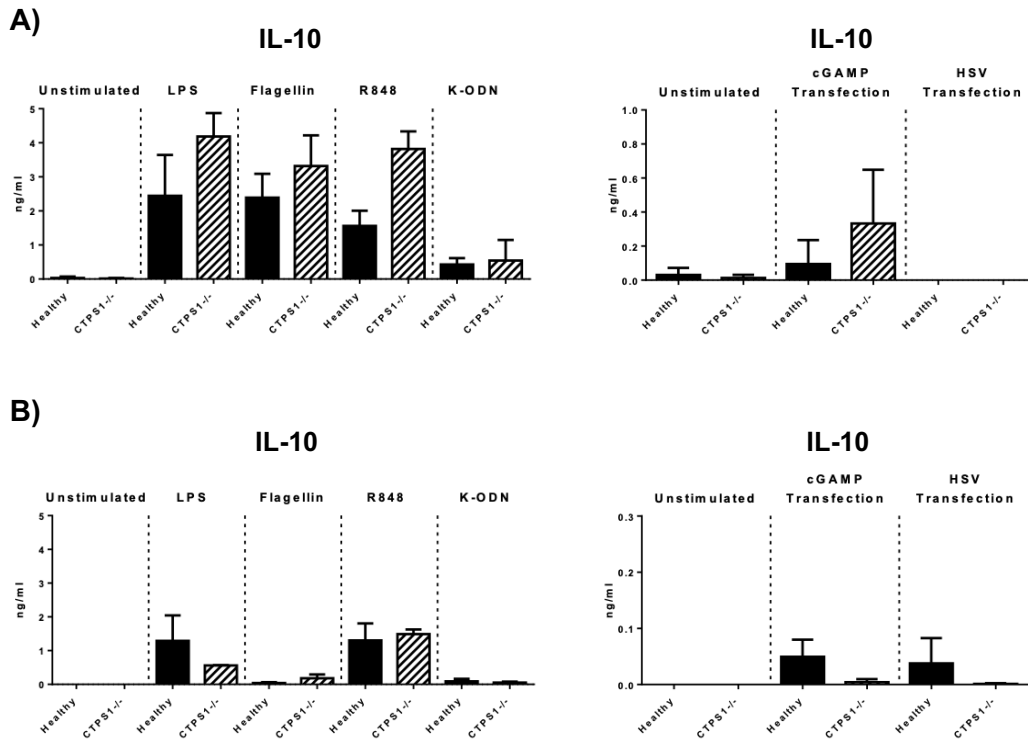
**Figure 3.6 IFN- $\alpha$ , IFN- $\gamma$  and IL-12 cytokine production profiles from healthy and patient PBMCs upon multiple PRR ligand stimulations.** Healthy (n=2) and patient PBMCs ( $8 \times 10^5$ /ml) were stimulated with TLR3 (p(I:C):  $30 \mu\text{g/ml}$ ), TLR4 (LPS:  $5 \mu\text{g/ml}$ ), TLR5 (Flagellin:  $100 \text{ng/ml}$ ), TLR7 (R848:  $5 \mu\text{g/ml}$ ), TLR9 (D35-3CG:  $3 \mu\text{M}$ ; K3:  $1 \mu\text{M}$ ), STING (2'3' cGAMP:  $30 \mu\text{g/ml}$ ), IFI6-STING (HSV:  $5 \mu\text{g/ml}$ ) ligands for 24 hours. Supernatants were collected and secreted IFN- $\alpha$  (**A**), IFN- $\gamma$  (Run-1: Upper panel, Run-2: Lower panel) (**B**) and IL-12 (**C**) levels were assessed by cytokine ELISA. Stimulations were run in duplicates.

Moreover, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  pro-inflammatory cytokine secretion upon TLR and intracellular PRR stimulations were assessed as well. Even though HSV transfection lead to approximately 3-fold more IL-1 $\beta$  production in CTPS1<sup>-/-</sup> PBMCs (Appendix B1.4A, upper panel), this could not be replicated when tested for the second time (Appendix B1.4A, lower panel). Meanwhile, no differences in IL-6 and TNF- $\alpha$  production levels were detected between healthy and patient PBMCs upon PRR engagements (Appendix B1.4B-C).

To conclude, results demonstrated that even though IFN- $\alpha$ , IL-6 and TNF- $\alpha$  secretion levels have not been altered in CTPS1<sup>-/-</sup> cells, PRR mediated IFN- $\gamma$  production was significantly lower in the patient and it is not a result of impaired IL-12 production by APCs in PBMCs.

#### **3.1.2.1.2 Healthy and CTPS1 PBMCs have similar IL-10 cytokine secretion levels upon PRRs engagements**

As it was demonstrated previously (see Figure 3.5 and Appendix B1.3), CTPS1 patient had reduced Treg numbers in circulation; consequently, Treg dependent immune dysregulation could arise. Therefore, it was plausible to ask whether innate immune cells also contributed to the observed dysregulation of anti-inflammatory response. To that end, isolated healthy and patient PBMCs were stimulated with surface or endosomal TLR as well as intracellular danger sensor ligands; then, amount of secreted IL-10 was determined by ELISA. As it is seen in Figure 3.7A (left panel), only TLR7 mediated IL-10 secretion was found to be elevated (~2.5-fold) in patient. Unfortunately, this trend was not reproduced when we assayed for the second time. In other words, patient and healthy PBMCs had similar anti-inflammatory cytokine secretion levels (Figure 3.7B).



**Figure 3.7 Effect of PRR engagement on healthy and CTPS1<sup>-/-</sup> PBMCs in the context of IL-10 secretion.** Health (n=2) and patient PBMCs ( $8 \times 10^5$ /ml) isolated from first (A) and second (B) blood drawing events were stimulated with TLR4 (LPS:  $5 \mu\text{g/ml}$ ), TLR5 (Flagellin:  $100 \text{ng/ml}$ ), TLR7 (R848:  $5 \mu\text{g/ml}$ ), TLR9 (K3:  $1 \mu\text{M}$ ) ligands (left panels); STING (2'3' cGAMP:  $30 \mu\text{g/ml}$ ), IFI6-STING (HSV:  $5 \mu\text{g/ml}$ ) ligands (right panels) for 24 hours. Supernatants were collected and secreted IL-10 levels were assessed by cytokine ELISA. Stimulations were run in duplicates.

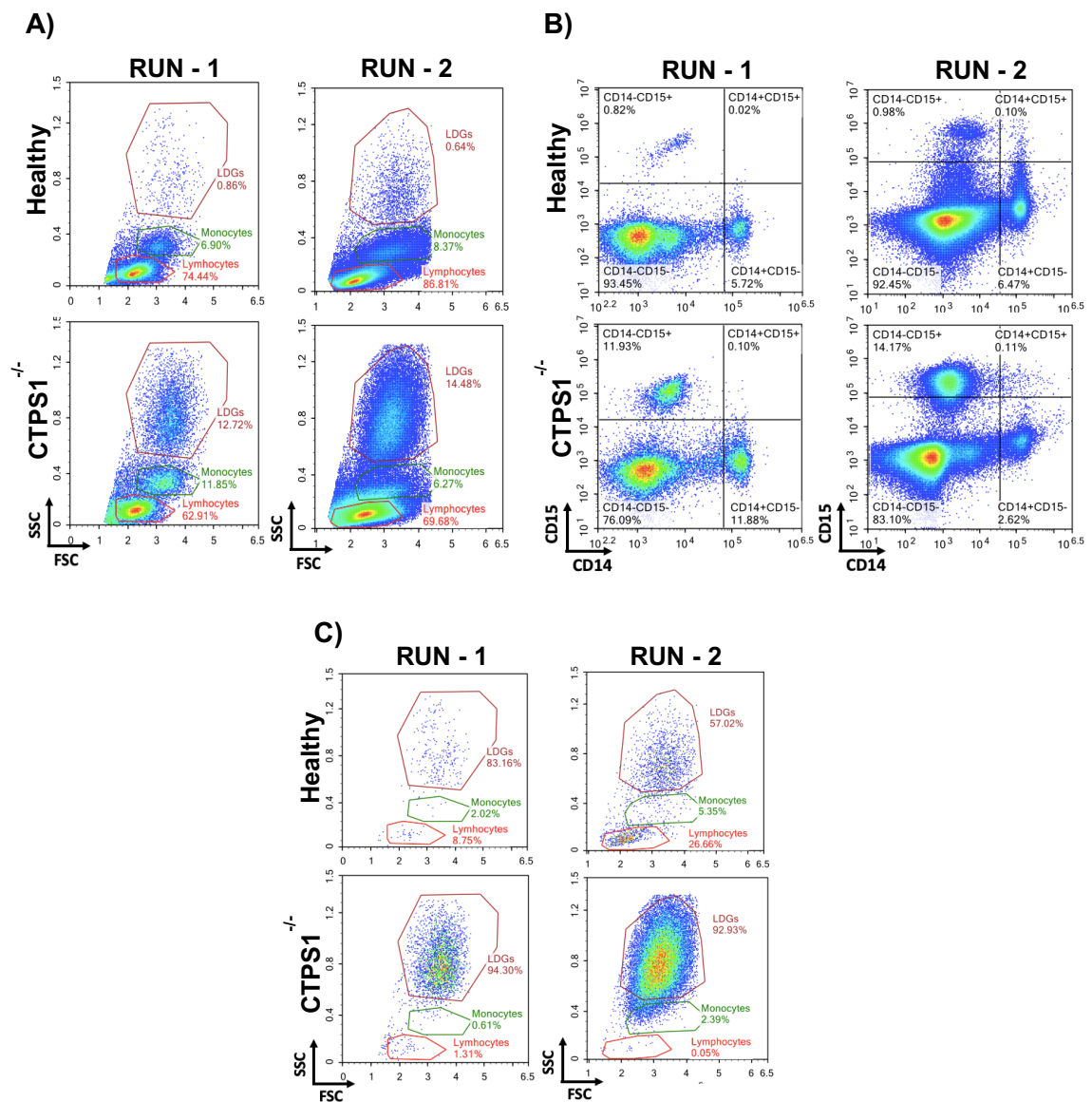
In conclusion, results from anti-inflammatory aspect implied that defect in CTPS1 enzyme might not lead to reduced anti-inflammatory capacities of innate immune cells upon PRR stimulation.

### 3.1.2.2 CTPS1 deficient patient had Low-Density Granulocytes (LGDs) in peripheral blood

While PBMCs were being isolated from whole blood, strikingly, it was observed that CTPS1 patient had immense number of granulocytes in their PBMC fraction (Figure 3.8A). Thus, we speculated that these cells might be Low-Density Granulocytes

(LDGs). In order to confirm presence of and quantitate LDGs and also differentiate them from monocytes, PBMCs were stained with CD14 and CD15 surface markers and analyzed using flow cytometer. It is important to note that, while human monocytes are characterized by their CD14<sup>+</sup> CD15<sup>-</sup> surface protein expressions, LDGs are defined to express CD14<sup>-</sup> CD15<sup>+</sup> on their surface [119]. As it can be seen from Figure 3.8B, flow cytometry analyses revealed that, patient had 12-15% CD14<sup>-</sup> CD15<sup>+</sup> population whereas healthy donors had only 1-2% of CD14<sup>-</sup> CD15<sup>+</sup> cells. To further confirm LDG population, CD14<sup>-</sup> CD15<sup>+</sup> cells were backgated in FSC versus SSC density plots to demonstrate that they are indeed reside in granulocyte fraction of PBMCs (Figure 3.8C); meanwhile, they were stained with CD66b which is also one of surface markers of neutrophils (Appendix B1.5).





**Figure 3.8 Identification of Low-Density Granulocytes within patient's PBMCs fraction.** LDGs within healthy (Upper panels) and patient (lower panels) PBMCs was revealed by Forward and Side Scattering (A) and the dual staining with anti-CD14, anti-CD15 (B); CD14<sup>-</sup>CD15<sup>+</sup> events were backgated in forward vs Side Scattering density plots (C).

Even though LDGs were observed in CTPS1<sup>-/-</sup> PBMCs, healthy controls do have low percentages of CD14<sup>-</sup>CD15<sup>+</sup> granulocytes which can be explained by residual neutrophils that had not passed through PBMC and sucrose layers or they were contaminating degranulated neutrophils in the PBMC fraction.

Overall, CTPS1 patient had low-density granulocytes in their circulation. This might explain the granulomas on the skin that patient had since LDGs in autoimmune patients like SLE are known to have increased NETotic tendencies and secrete inflammatory cytokines and consequently contribute to an ongoing severe inflammation.

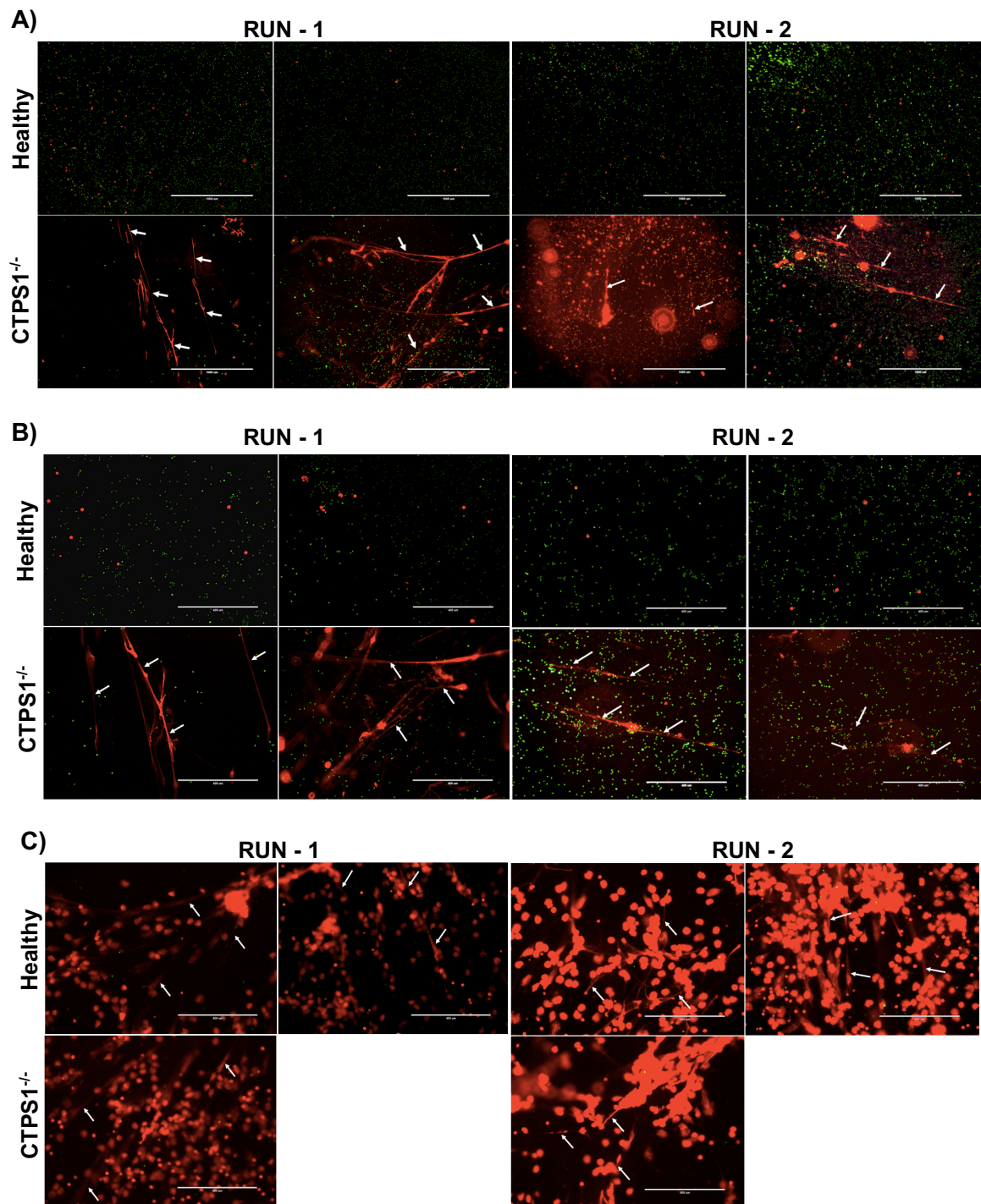
### **3.1.2.3 Investigation of Neutrophils in CTPS1 deficiency**

Presence of circulating Low-density granulocytes in CTPS1<sup>-/-</sup> led us to check immunological activities of patient neutrophils since LDGs have pro-inflammatory phenotype and they are coupled with increased activities of neutrophils in autoimmune diseases and cancer [119].

#### **3.1.2.3.1 Neutrophils of CTPS1 deficient patient had higher but non-significant spontaneous NETotic tendencies**

In order to demonstrate NET formation under basal and stimulated conditions, isolated healthy and patient neutrophils were treated with PMA, which is a known inducer of NETosis, or left alone. After 4 hours of incubation, intracellular and extracellular dsDNAs were stained and NET-like DNA mesh formation was visualized under fluorescent microscopy (Figure 3.9).

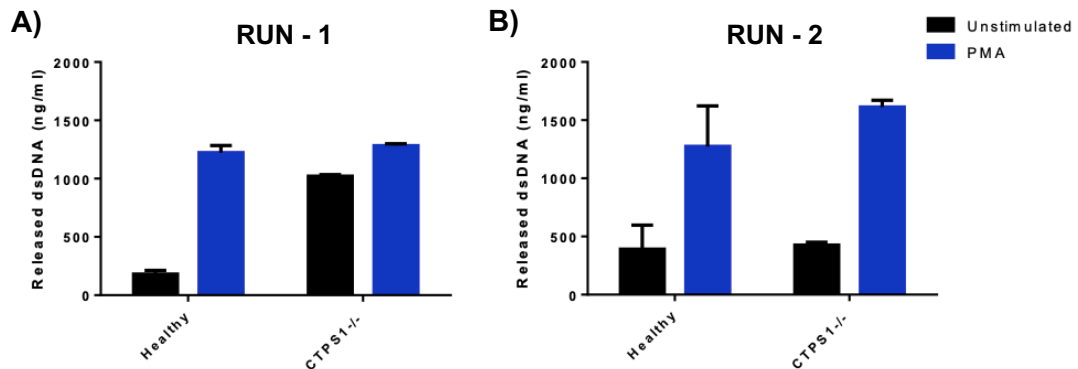
Under unstimulated conditions, as expected, healthy neutrophils did not have NET-like DNA formation in extracellular space. However, strikingly, images of neutrophils isolated from CTPS1<sup>-/-</sup> patient had spontaneous NET release without any stimulation (Figure 3.9A-B). In order to confirm spontaneous NETosis is happening and is independent of any persisting infection or effect of any drug therapy, neutrophils were isolated again from the same patient 3 months after the first blood donation. Consistently, NET formation was observed, albeit to lesser extent when analyzed in the second investigation, (Figure 3.9A-B). Meanwhile, PMA-stimulated neutrophils were assessed as well; in order to check whether patient neutrophils had increased or decreased dsDNA release under stimulated condition mimicking infectious microorganism. As Figure 3.9C demonstrated, both CTPS1<sup>-/-</sup> and healthy neutrophils expelled similar level of NET-like dsDNA to the extracellular environment following stimulation.



**Figure 3.9 Representative immunofluorescent images of healthy and CTPS1<sup>-/-</sup> neutrophils undergoing NETosis. A-B)** Neutrophils ( $10^6/\text{ml}$ ) were left untreated; **C)** stimulated with PMA (50ng/ml) for 4 hours. In order to visualize NET formation (Indicated by white arrows), intracellular DNA (Green) and extracellular DNA (Red) were stained with

Syto16 Green and Sytox Orange, respectively. **A)** 4X (Scale bars represent: 1000 $\mu$ m); **B-C)** 10X (Scale bars represent: 400 $\mu$ m) images were taken.

After NETs were visualized, quantity of released dsDNA from neutrophils under unstimulated and PMA-stimulated conditions were assessed via fluorometric investigations from supernatants which were treated and stained with non-specific nuclease (Micrococcal Nuclease) and dsDNA-binding Picogreen, respectively. Results indicated that, healthy neutrophils expelled 181 $\pm$ 30 ng/ml (Mean $\pm$ SD, n=2) dsDNA under unstimulated condition; on the contrary, patient neutrophils released 1021 $\pm$ 12 ng/ml (Mean $\pm$ SD, n=1) dsDNA into extracellular space (Figure 3.10A). In other words, CTPS1 deficient neutrophils released 6-fold more dsDNA compared to healthy individuals under no stimulation conditions. When these measurements were repeated 3 months later, the results were somewhat different than our first observation. While CTPS1-deficient neutrophils expelled 426 $\pm$ 25 ng/ml, healthy neutrophils released 390 $\pm$ 206 ng/ml of dsDNA to extracellular space (Figure 3.10B). Off the note, PMA-induced dsDNA release was not found to be different between healthy and patient neutrophils in two different occasions.



**Figure 3.10 Quantification of released dsDNA from neutrophils.** From **(A)** first **(B)** second blood gathering events, isolated healthy (n=2) and patient neutrophils (1.6 $\times$ 10<sup>6</sup>/ml) were stimulated with PMA (50ng/ml) or left untreated. After 4 hours of stimulation, from supernatants, Micrococcal Nuclease Assay was performed. Experiment was run in duplicates.

When taken together, images and spectrofluorometric analyses demonstrated that patient neutrophils showed spontaneous NETosis. Even though only CTPS1<sup>-/-</sup> neutrophils had NET-like DNA formation, quantification demonstrated that both healthy and patient neutrophils secreted similar amounts of dsDNA when investigated for a second time.

### **3.1.3. Effects of CTPS1 deficient EVs on immune system**

Exosomes and microparticles generally referred to as extracellular vesicles (EVs hereafter) have attracted much interest because of their diverse functions, or their potential utilization either as disease biomarkers or emerging potential for therapeutic exploitation. Understanding the pathophysiological relevance of EVs as well as their therapeutic and diagnostic potential to revert dysregulation and/or severity of diseases is critical. In this thesis, in addition to cellular aspects of CTPS1 enzyme deficiency, contribution of EVs to disease pathogenesis were studied.

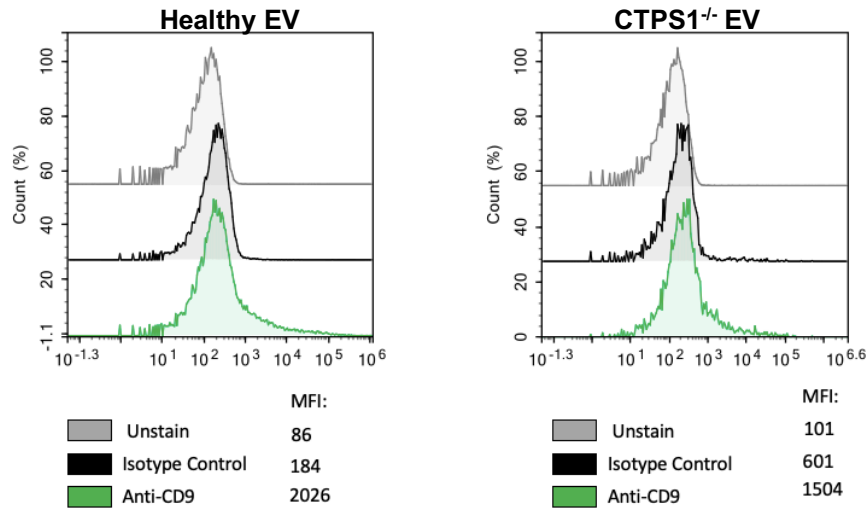
#### **3.1.3.1 CTPS1 and healthy EVs have similar effect on healthy PBMCs**

Plasmas were isolated from whole blood and then they were used to purify extracellular vesicles. EVs were isolated from 2 distinct healthy controls; meanwhile, patient EVs were isolated from CTPS1-deficient blood plasmas on 2 occasions and were named as CTPS1 - 1, CTPS1 - 2, respectively.

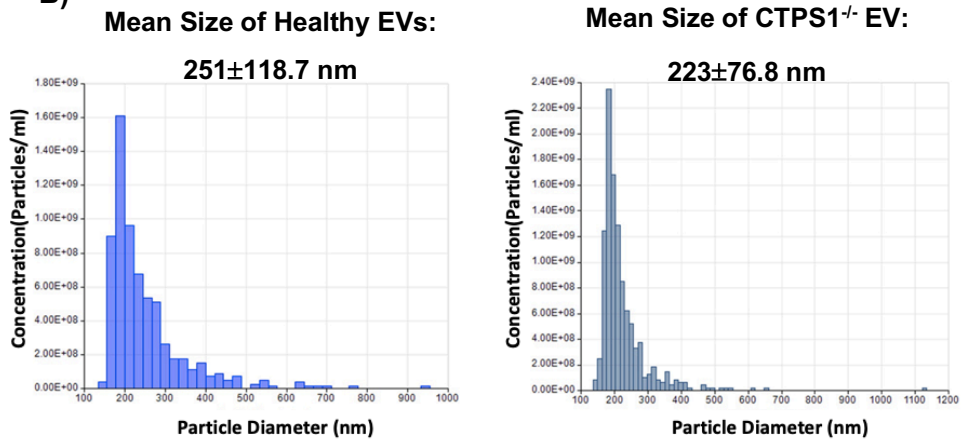
Preliminary assays included to confirm that isolated EVs were indeed exosomes, therefore, purified exosome samples were investigated by the use i) bead-based flow cytometric assay and ii) size distribution using qNano device.

Initial findings also implicated that as expected, EVs that were captured with CD63 antibody coated beads, expressed several exosome-specific markers, such as CD9 tetraspanin protein (Figure 3.11B). qNano gold measurement further confirmed that distribution of EVs were within the expected size range [190]. While healthy EVs had 251±118.7 nm average hydrated size, patient EVs were 223±76.8 nm (Figure 3.11C)

**A)**

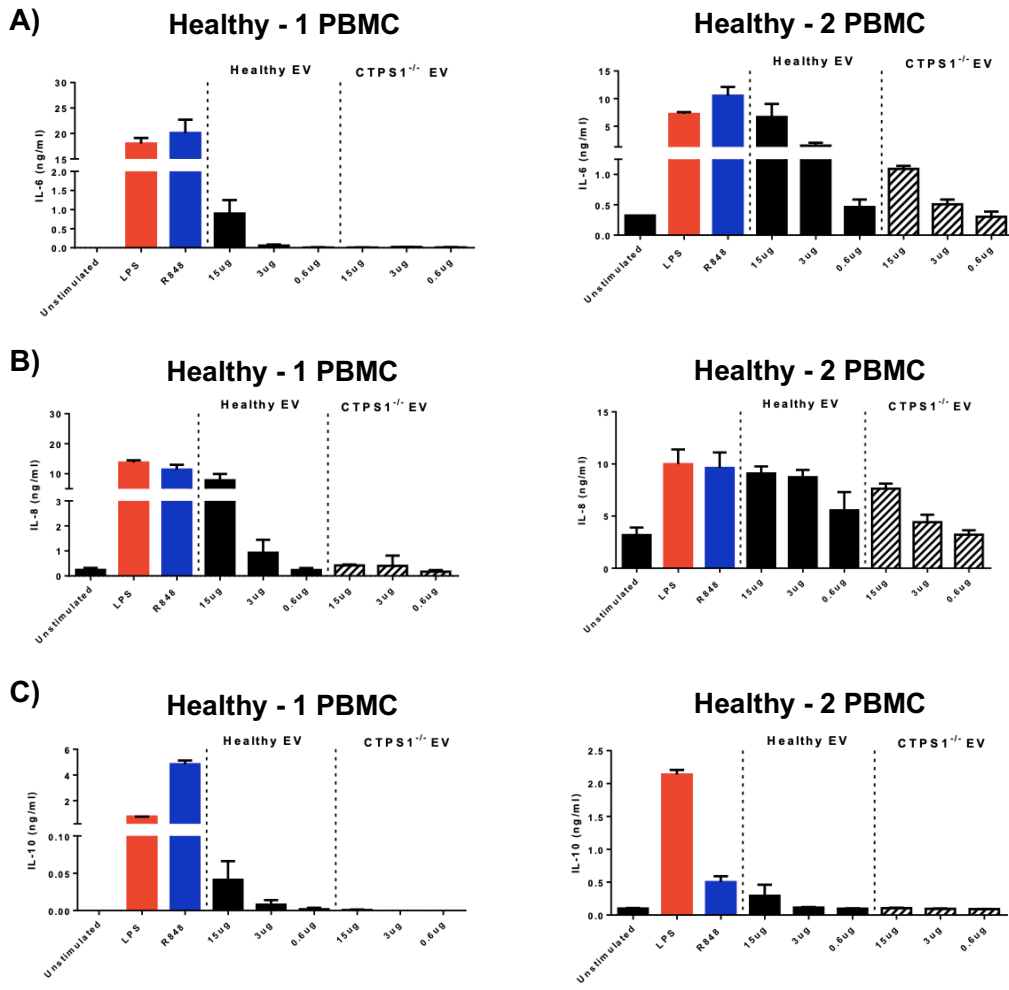


**B)**



**Figure 3.11 Verification of extracellular vesicle isolation from control and CTPS1<sup>-/-</sup> plasmas. A)** Healthy (left panel) and patient (right panel) EVs were captured by Anti-CD63 coated latex beads and stained against CD9, in the presence of proper isotype control. MFI levels of CD9 were analyzed using flow cytometer and represented as histogram plots. **B)** Isolated EVs were analyzed in qEV Nano Gold instrument. Plots represent concentration and diameter of particles from healthy (left panel) and patient (right panel) EVs.

After isolation and confirmation of EVs, immunomodulatory activities of CTPS1 deficient patient EVs were pursued. In these assays, healthy PBMCs were incubated with three doses (15 $\mu$ g, 3 $\mu$ g and 0.6 $\mu$ g) of allogenic healthy EVs or CTPS1<sup>-/-</sup> EVs for 36 hours in culture; then, pro-inflammatory cytokines such as IL-6, IL-8 and anti-inflammatory cytokine, IL-10 secretion profiles were investigated using cytokine ELISA. As positive controls for cytokine secretion cells were treated with TLR4 and TLR7 ligands. As it was illustrated in Figure 3.12A, on two independent occasions CTPS1<sup>-/-</sup> EVs in a dose dependent manner induced less IL-6 secretion from healthy PBMCs compared to healthy EVs. At the highest dose of EVs that was incubated with PBMCs, there were ~8-fold less IL6 production by CTPS1-deficient EVs compared to healthy EVs. Similarly, patient EVs lead to a ~20-fold less IL-8 production from healthy PBMCs (Figure 3.12B, left panel). The cytokine inductive capacities of these EVs were different on different healthy PBMCs, as expected. For example, IL8 secretion was much higher on healthy 2 PBMCs the difference was only seen at the lowest dose tested such as 3 $\mu$ g (Figure 3.12B, right panel). Surprisingly, neither healthy nor patient EV stimulations led to IL-10 production, as it can be seen in Figure 3.12C.

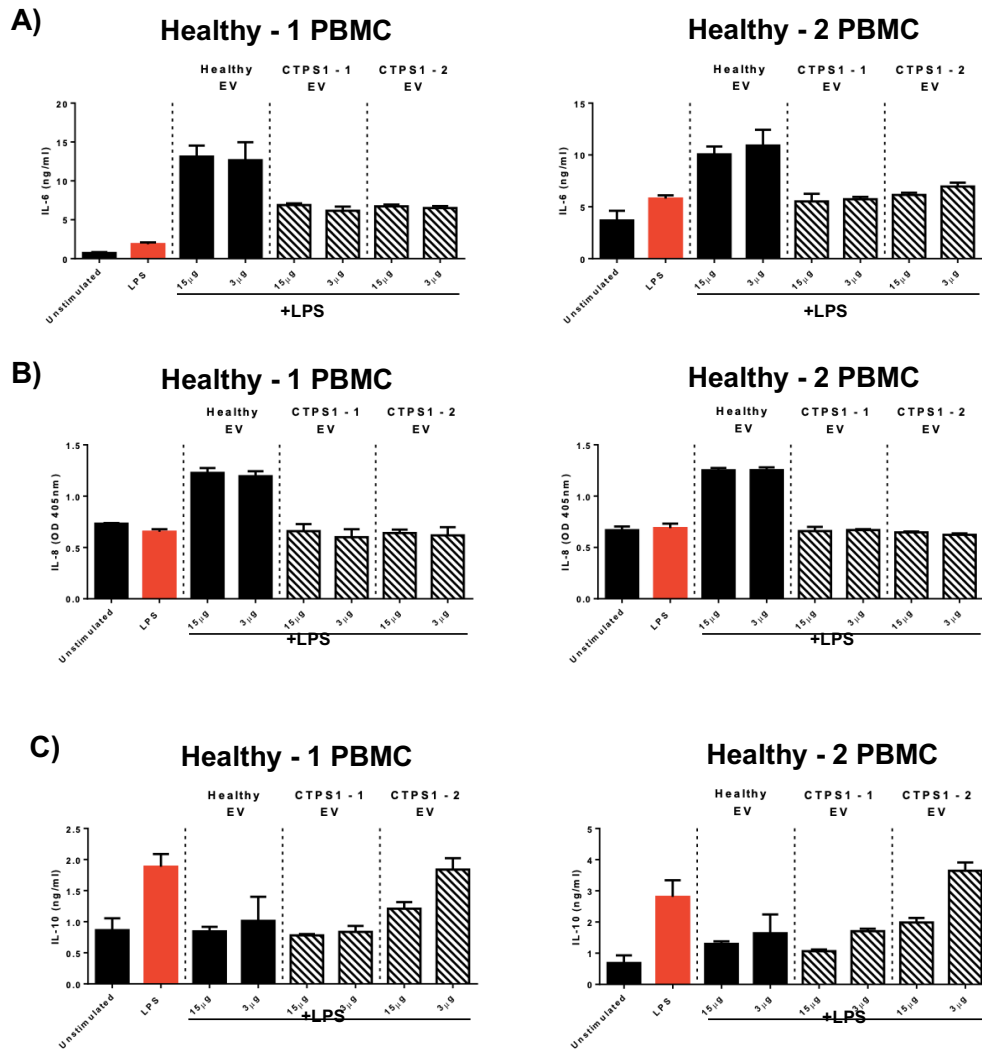


**Figure 3.12 Differential activation capacities of healthy and CTPS1-deficient patient plasma-derived EVs on healthy PBMCs.** Two control PBMCs ( $1.2 \times 10^6/\text{ml}$ ) were stimulated with three doses ( $15\mu\text{g}$ ,  $3\mu\text{g}$  and  $0.6\mu\text{g}$ ) of allogenic healthy ( $n=2$ ) and CTPS1 - 2 EVs. After 36 hours, supernatants were collected. **A)** IL-6, **B)** IL-8, and **C)** IL-10 secretions from the supernatants were determined using cytokine ELISA. Experiment was conducted in duplicates.

Since CTPS1<sup>-/-</sup>-derived EVs induced less pro-inflammatory cytokine secretion compared to healthy EVs, it was hypothesized that CTPS1<sup>-/-</sup> EVs might have immunosuppressive effect on immune system. Therefore, in another experiment the ability of EVs to down-regulate TLR ligand mediated immune activation was sought.



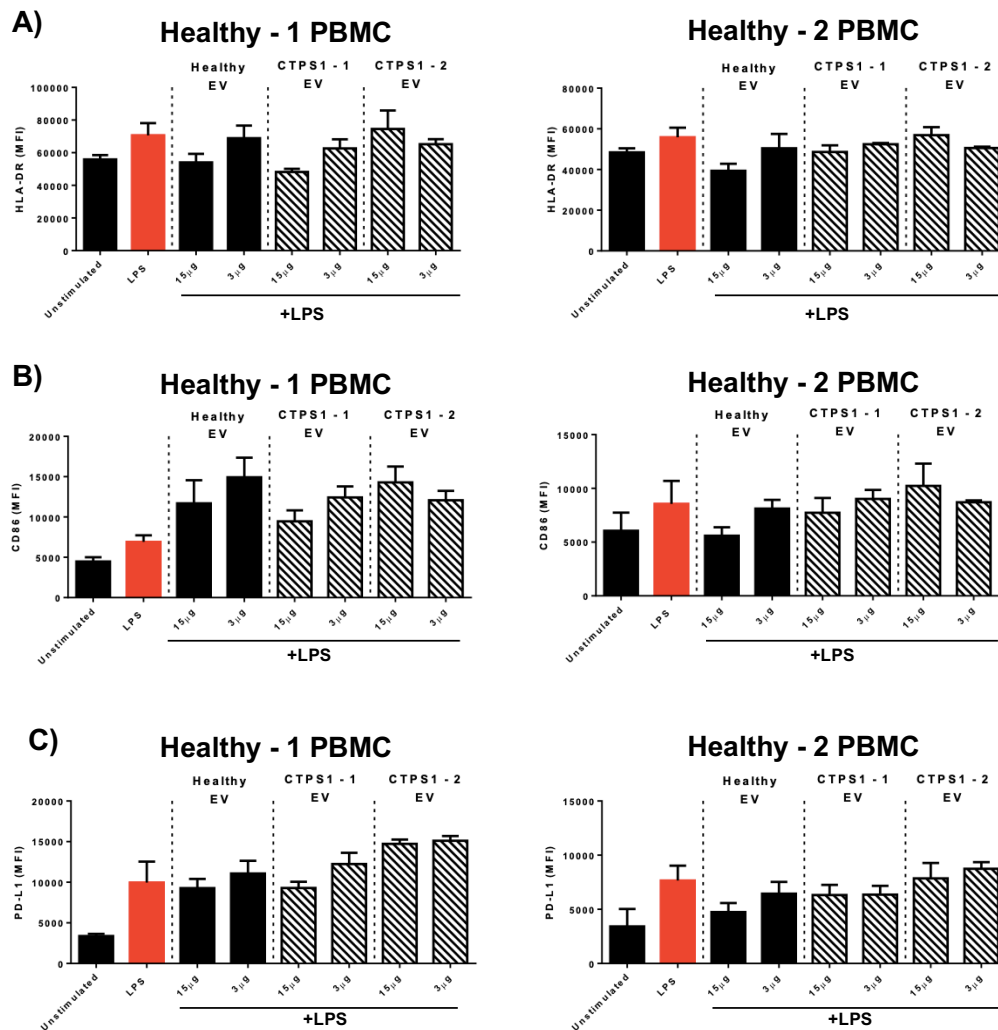
Two healthy control PBMCs were isolated and pre-treated with two doses (15 $\mu$ g and 3 $\mu$ g) of allogenic healthy or patient EVs for 36 hours. Then, cells were stimulated with TLR4 ligand, LPS, for 24 hours. Figure 3.13A illustrates cytokine ELISA from stimulated cell supernatants and it revealed that CTPS1<sup>-/-</sup> EVs induced 2-fold less IL-6 secretion from control PBMCs and this effect can be observed in patient EVs isolated from two different occasions, implying that this effect is reproducible, and consistently suppresses LPS mediated IL6 production from PBMCs. Even though patient EV pre-treated PBMCs secreted less IL-6 compared to PBMCs that were pre-treated with control EVs, patient EVs were failed to reduce IL-6 production in comparison to only LPS treated cells. Apart from healthy EV pre-treatment, stimulated along with unstimulated PBMCs had similar IL-8 secretion levels. That is, at basal conditions, control PBMCs increased IL-8 secretion and it cannot be further enhanced by even LPS stimulation. Off note, healthy EV pre-treated PBMCs enhanced IL-8 secretion which was not seen with CTPS1 EV pre-incubation (Figure 3.13B). Lastly, PBMCs incubated with patient EVs had similar IL-10 secretion levels when compared to control EV pre-treated PBMCs. Interestingly, both control and patient EV pre-treatments reduced anti-inflammatory cytokine secretion compared to TLR4 treated group (Figure 3.13C).



**Figure 3.13 Differential activation capacities of healthy and CTPS1-deficient patient plasma-derived EVs on healthy PBMCs.** Control healthy PBMCs ( $n=2$ ) ( $1.2 \times 10^6/\text{ml}$ ) were pre-treated with two doses ( $15\mu\text{g}$ ,  $3\mu\text{g}$ ) of allogenic healthy ( $n=2$ ) and CTPS1 EVs. After 36 hours, cells were stimulated with LPS ( $100\text{ng}/\text{ml}$ ) for 24 hours. Then, supernatants were collected and by cytokine ELISA **A)** IL-6, **B)** IL-8 and **C)** IL-10 cytokine production levels were determined. Experiments were conducted in duplicates.

Upon TLR stimulations, antigen presenting cells not only produce cytokines, but also migrate through secondary lymphoid organs to license adaptive immune cells. In order to activate as well as regulate them, they increase expression of MHC class II (HLA-DR), co-stimulatory molecules (CD80 and CD86) as well as inhibitory molecules (PD-L1, PD-L2) expression for restoring the immune homeostasis [245]. Therefore, expression of surface molecules such as HLA-DR, CD86 and PD-L1 on EV pre-treated

and LPS stimulated PBMCs were checked. From Figure 3.14A, healthy PBMCs that were pre-incubated with high dose control EVs had almost 1.5-fold less surface expression of HLA-DR compared to LPS alone stimulation. Moreover, pre-treatment with CTPS1 EVs, had same effect on HLA-DR expression. However, CTPS1 EVs from different blood collection time did not alter HLA-DR expression following LPS stimulation (Figure 3.14A). This data implicated that the therapy dependent alteration of circulating EVs might be happening and the pathological nature of these EVs could depend on the ongoing therapy and duration. Lastly, while pre-treatment with CTPS1 - 2 EVs altered, elevated, PD-L1 expression on the surface of healthy 1 PBMCs, this trend was not reproduced in healthy 2 PBMCs (Figure 3.14C), suggesting the individual to individual variation is also a critical element of these results.



**Figure 3.14** Effect of CTPS1<sup>-/-</sup> EV pre-treatment on surface expression of HLA-DR, CD86 and PD-L1 molecules from healthy donor PBMCs. After healthy control cells (n=2) were pre-treated with EVs and stimulated with LPS (100ng/ml) for 36 and 24 hours, respectively, they were assessed to check the expression levels of surface markers using flow cytometer. Cells were stained against HLA-DR, CD86 and PD-L1 molecules. **A)** HLA-DR, **B)** CD86 and **C)** PD-L1 expression levels were calculated as Mean Fluorescent Intensity. Cells were gated in PBMCs and singlets. Experiments were conducted in duplicates.

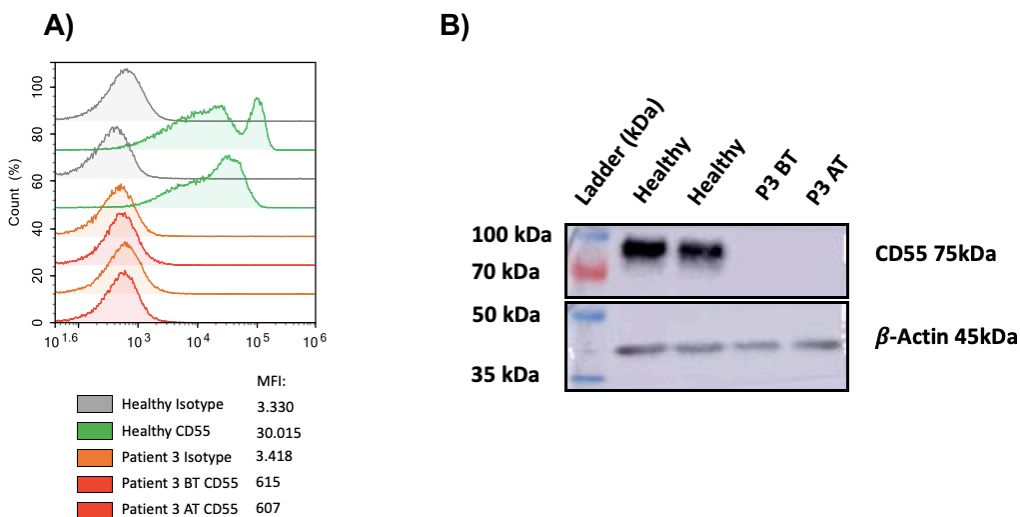
To conclude, CTPS1 EVs pre-treatment reduced IL-6 production. Yet they failed to alter the expression of cardinal activation and inhibition surface molecules. It is impossible to deduce insurmountable effect of CTPS1<sup>-/-</sup> EVs on immune system by these sets of

experiments. More patients and multiple readings using same healthy as well as different donor PBMCs are required to draw a convincing conclusion regarding the immunomodulatory nature of CTPS1-deficient plasma derived EVs.

### 3.2. Characterization of CD55 Deficiency before and after Eculizumab therapy

In the second part of this project recently identified complement system deficiency (Figure 1.10), CHAPLE or CD55 deficient patient PBMCs were studied. One of the unique properties of this disease is that all identified patients were from Middle East countries, especially Turkish origin families [34]. Responses of Healthy and CD55 deficient PBMCs upon PRR ligand engagement are different.

Absence of CD55 protein in patient PBMCs and following Eculizumab therapy it does not rectify the expression of CD55 molecule was checked by flow cytometry and immunoblotting techniques (Figure 3.15 left and right panel, respectively) from patient PBMCs either before therapy (BT) or after therapy (AT). Healthy PBMCs were used as control blood.

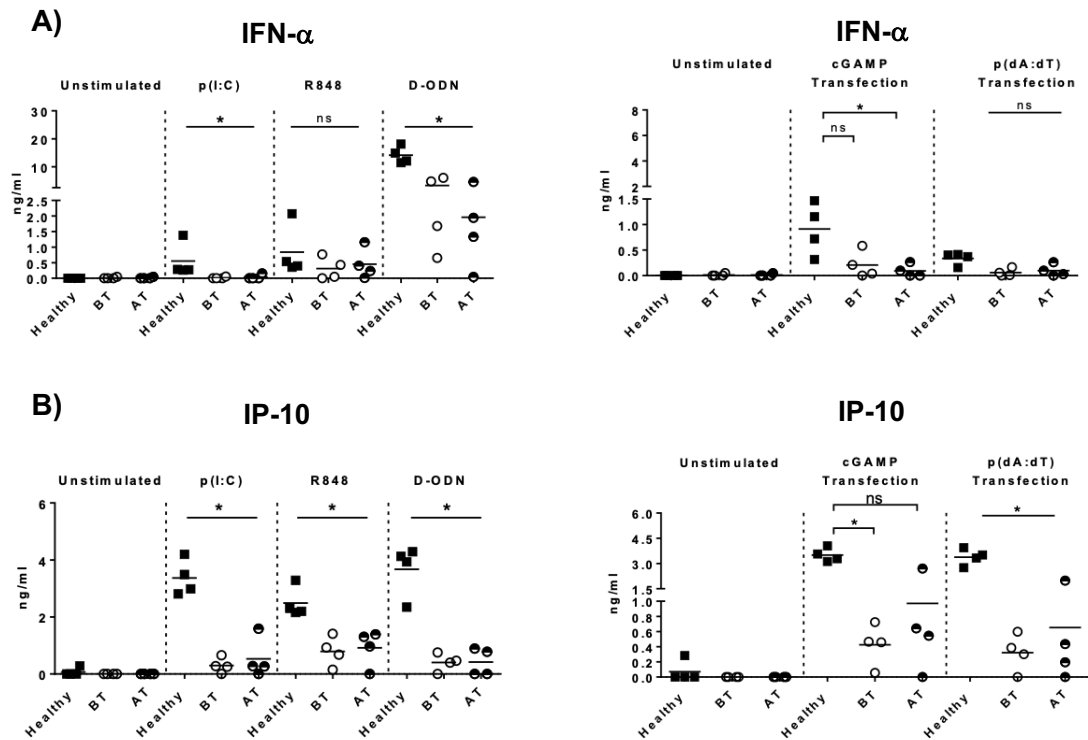


**Figure 3.15 Confirmation of the absence of CD55 protein in patient cells by flow cytometry and immunoblotting assays. A)** After healthy (n=2) and patient (BT and AT) PBMCs were stained against anti-CD55 and its appropriate isotype control, they were analyzed using flow cytometer and represented as histogram plots. **B)** 15 $\mu$ g protein from healthy (n=2), BT and AT PBMC lysates were loaded and investigated by immunoblotting. Gel images illustrates CD55 expression levels in healthy and patients at the top panel and the loading control  $\beta$ -actin at the bottom panel.

Figure 3.15 A and B clearly demonstrated that patient PBMCs did not express CD55 molecules and the protein expression was not altered following therapy. As it was reported in the original study these patients present exacerbated inflammatory conditions; therefore, the underlying cause were aimed to be investigated. Even though CD55 *per se* is a regulatory protein within early complement pathway, CHAPLE patients were reported to suffer from recurrent respiratory track and EBV infections [34]. That is, deficiency in complement regulatory protein predisposes host to frequent infections which could be related to aberrant functioning of the innate immune cells. To understand whether dysregulation in innate cells could be related to CD55 absence, patient PBMCs before and after Eculizumab therapy were stimulated with several PRRs ligands including endosomal TLRs (such as TLR3, TLR7 and TLR9) and intracellular nucleic acid ligands (such as STING, DAI and RIG-I). Then, cytokine production from culture supernatants was determined by ELISA. Figure 3.16 depicts IFN- $\alpha$  and IP-10 responses of healthy, BT and AT PBMCs. As it was described previously, IFN- $\alpha$ , as a member of type I IFNs, is produced through IRF7 involving PRR signaling; in order to reduce viral burden in the host. Furthermore, secreted type I IFNs as well as IFN- $\gamma$  induce production of IP-10 chemokine to attract and promote T-cells to the infection site, especially Th1 cells [230].

In Figure 3.16A (left panel), BT and AT PBMCs that were treated with TLR3, and TLR9 ligands had significantly reduced IFN- $\alpha$  production compared to healthy PBMCs. Meanwhile, even though STING induced IFN- $\alpha$  production was lower in both BT and AT PBMCs, only reduced responses of AT PBMCs were found to be significant (Figure 3.16A, right panel). Off note, TLR7 and DAI-STING & RIG-MAVS inductions were slightly, but non-significantly reduced in both BT and AT PBMCs compared to control responses. When, IP-10 secretion in response to same ligand engagements were assessed and as it was illustrated in Figure 3.16B revealed that all three endosomal TLRs induced significantly higher IP-10 secretion in healthy individual PBMCs compared

to BT and AT PBMCs of CD55-deficient patient. Moreover, DNA-dependent IRF activation was assessed in the context of IP-10 secretion. Although CD55<sup>-/-</sup> PBMCs had lower responses, regardless of therapy, only insignificant reduction in IP-10 production was cGAMP stimulated AT PBMCs (Figure 3.16B, right panel).



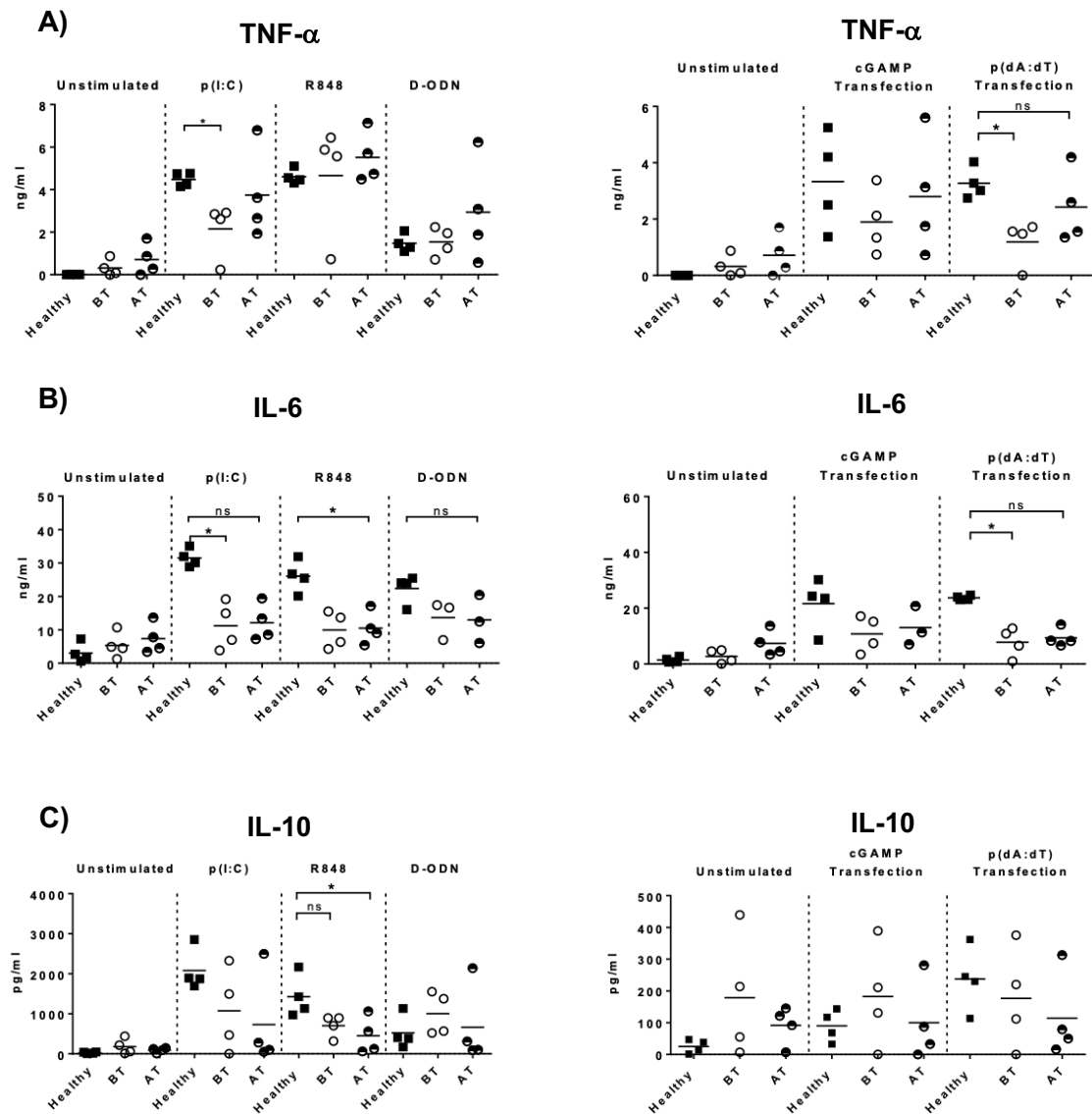
**Figure 3.16 Production of IFN- $\alpha$  and IP-10 cytokines from healthy, BT and AT of CHAPLE (CD55<sup>-/-</sup>) patient PBMCs upon stimulation with various PRR ligands.** Isolated healthy, BT and AT PBMCs (N=4, for each group;  $1.6 \times 10^6$ /ml) were stimulated with TLR3 (p(I:C):  $30 \mu\text{g/ml}$ ), TLR7 (R848:  $1 \mu\text{g/ml}$ ), D-type TLR9 (D35-3CG:  $3 \mu\text{M}$ ) ligands (left panels); STING (2'3' cGAMP:  $30 \mu\text{g/ml}$ ), DAI-STING & RIG-I/MAVS & AIM2 Inflammasome (p(dA:dT):  $5 \mu\text{g/ml}$ ) ligands (right panels) for 24 hours. In order to assess levels of secreted IFN- $\alpha$  (A) and IP-10 (B), supernatants were analyzed using human cytokine ELISA. Experiments were conducted in duplicates. Kruskal-Wallis test and Dunn's multiple comparison correction were performed between healthy individuals and patient groups for each ligand stimulation. (ns: non-significant; \* $p \leq 0.05$ ).

In addition to viral associated responses (type I IFN and IP-10), PRR-mediated pro-inflammatory cytokine production was investigated since cross-talk between PRRs and complement receptors during the aberrant complement activation may contribute wide

range of pathogenesis [246]. Moreover, it was important to point out anti-inflammatory response in the context of innate immune cells since patients suffer from aberrant inflammation and T-cell dependent dysregulation.

Therefore, from supernatants of stimulated BT and AT CHAPLE PBMCs, cytokine ELISA was performed to determine levels of pro-inflammatory TNF- $\alpha$ , IL-6 and anti-inflammatory IL-10 cytokines. As it can be observed in Figure 3.17A, apart from TLR3 and DAI-STING and/or RIG-I-MAVS induction, healthy and BT, AT CD55<sup>-/-</sup> PBMCs had similar responses. One striking observation from this study is the following: After single Eculizumab injection, AT PBMCs responded positively to several PRR ligations and showed an upward movement by secreting higher amounts of TNF- $\alpha$ . In other words, to all stimulations TNF- $\alpha$  secretion from BT but not AT PBMCs were significantly lower compared to healthy controls and to AT PBMCs. This implied that single eculizumab injection upregulated pro-inflammatory response of these patients. When IL-6 production levels were investigated PRR induced IL-6 production from either BT or from AT PBMCs were indistinguishably similar to each other and yet compared to healthy controls these responses were significantly lower implying that single Eculizumab injection does not restore IL-6 secretions from patients towards normal levels (Figure 3.17B). Strikingly as it was depicted in Figure 3.17C, PRR stimulations led to substantially lower levels of IL-10 secretion from AT PBMCs compared to BT PBMCs. Similarly, these responses were much less than normal healthy individual IL-10 levels. Eculizumab injection reduces suppressor or inhibitory cells to secrete immunomodulatory cytokine such as IL-10 implying that AT PBMCs are more TH1-biased compared to BT PBMCs. This idea is supported by the unstimulated IL-10 levels of these PBMCs. Eculizumab injection induced a downward trend of the BT IL-10 response (compare the left part of the right panel levels) (Figure 3.17C, right panel).





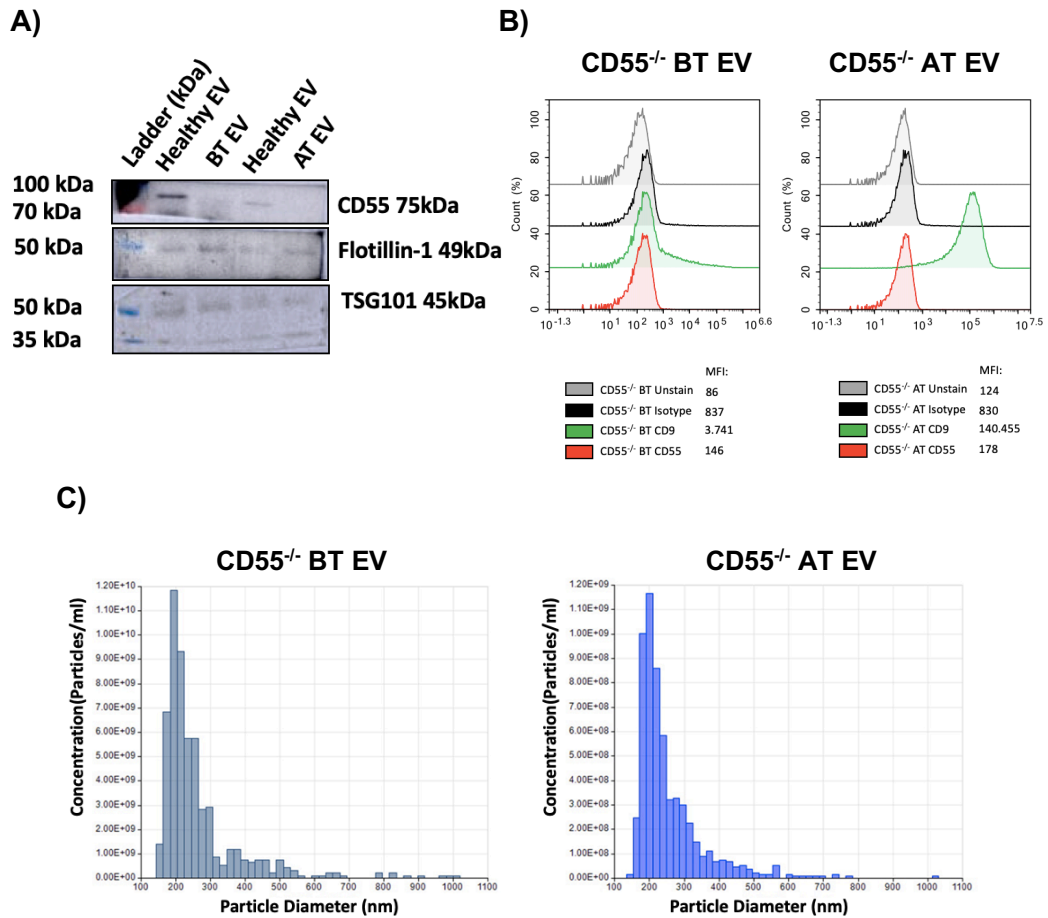
**Figure 3.17** Effect of PRR stimulations on pro-inflammatory and anti-inflammatory cytokine secretion profiles of healthy, BT or AT of CHAPLE (CD55<sup>-/-</sup>) patient PBMCs upon stimulation with various PRR ligands. Isolated healthy, BT and AT PBMCs (n=4, for each group;  $1.6 \times 10^6$ /ml) were stimulated with TLR3 (p(I:C):  $30 \mu\text{g/ml}$ ), TLR7 (R848:  $1 \mu\text{g/ml}$ ), D-type TLR9 (D35-3CG:  $3 \mu\text{M}$ ) ligands (left panels); STING (2'3' cGAMP:  $30 \mu\text{g/ml}$ ), DAI-STING & RIG-I/MAVS & AIM2 Inflammasome (p(dA:dT):  $5 \mu\text{g/ml}$ ) ligands (right panels) for 24 hours. Supernatants were collected to determine TNF- $\alpha$  (A), IL-6 (B) and IL-10 (C) cytokine levels by ELISA. Experiments were conducted in duplicates. Kruskal-Wallis test and Dunn's multiple comparison correction were performed between healthy individuals and patients for each ligand stimulation. (ns: non-significant; \* $p \leq 0.05$ ).

Taken together, these results implied that CHAPLE patients had altered innate immune responses and they were merely restored by a single dose of Eculizumab therapy. But in general, the Eculizumab injection initiates a restorative cascade with regard to innate immune cell functioning.

### **3.2.1. EV-dependent immune modification of CD55 deficient PBMCs before and after Eculizumab therapy**

Next, it was intended to investigate immunological aspect of patient EVs before and after Eculizumab therapy. From the perspective of this thesis it was crucial to establish whether a single dose of Eculizumab administration altered the concentration, composition, or the pathogenic nature or the cargo content of these circulating EVs in patients. In order to clarify these issues, EVs were isolated from both before and after therapy CD55<sup>-/-</sup> patient plasmas and validated by i) western blot, ii) bead-based flow-cytometric analysis technique and iii) size distribution quantification (Figure 3.18).

As it was illustrated in Figure 3.18A, western blot gel images demonstrated that isolated particles bear cardinal EV markers such as Flotillin-1 and TSG101. Furthermore, as it is illustrated in Figure 3.18B, it was successfully demonstrated that isolated EVs as expected, expressed either CD63 or CD9 proteins on their surface: following flow cytometry evaluation. Of special interest, as expected, both western gel images and bead-based EV characterization assays once again validated that similar to the case seen for immune cells, CD55 protein cannot be detected on EVs that was purified from BT or AT patient plasmas (Figure 3.18A-B). Lastly, using qNano Gold instrument through nanoparticle tracking analyses approach the size ranges of CHAPLE EVs were determined to be within the expected range (Figure 3.18C). The hydrated sizes of BT and AT EVs were 264±125 nm and 254±96 nm (Mean±SD), respectively.

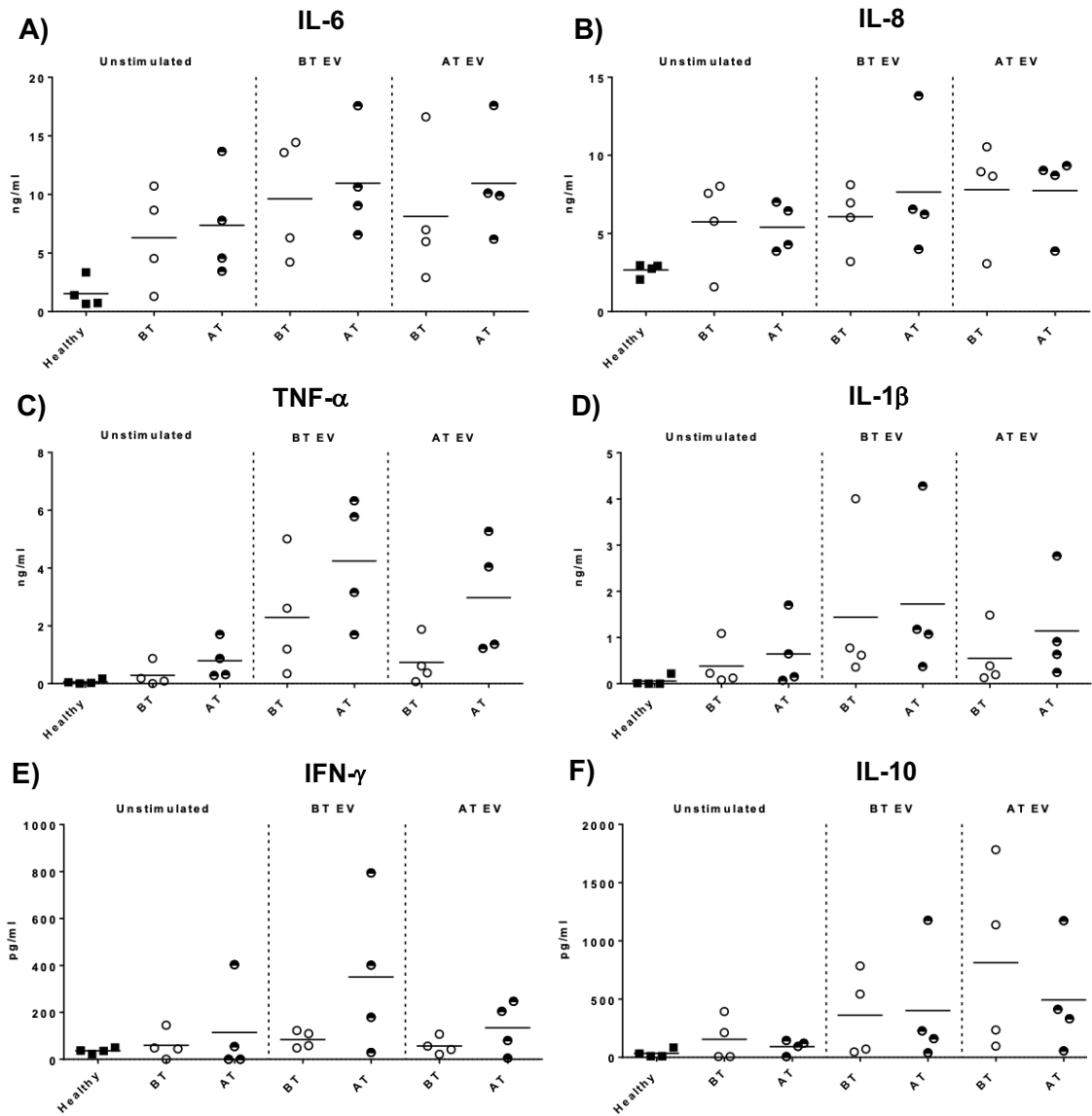


**Figure 3.18 Verification of extracellular vesicle isolation from control and BT or AT CHAPLE (CD55<sup>-/-</sup>) patients. A)** 30 $\mu$ g protein of each isolated EVs were loaded into western blot lanes. Gel images show presence of EV markers (i.e. Flotillin-1 and TSG101) and absence of CD55 protein. **B)** Isolated BT and AT CHAPLE EVs were captured by CD63 coated beads. Then, they were stained against anti-CD9 and anti-CD55 and their proper isotype controls. Following flow cytometric analyses, representative histogram plots in MFI values of each surface markers are shown. **C)** Concentration vs size distribution plots of BT and AT EVs were determined by qNano Gold instrument. Results are average of 3 independent measurements run in triplicates.

Although single dose Eculizumab therapy slightly altered the innate immune responses of PBMCs (see section 3.2.1), it was postulated that may be pathophysiologic nature of the BT EVs could be alleviated much faster following Eculizumab therapy. To this end, isolated BT and AT EVs were incubated with syngeneic BT or AT PBMCs, hence,

contribution of EVs to healing process of disease alongside effect of Eculizumab on the innate immune cells could be evaluated.

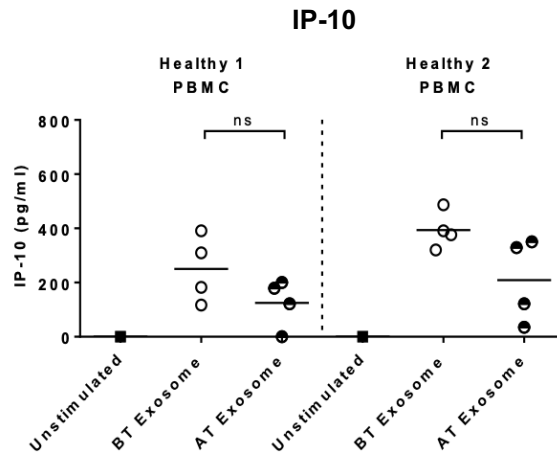
Figure 3.19A-B revealed that patient BT and AT PBMCs under unstimulated state had elevated secretion of IL-6 and IL-8 (4.5-fold and 2-fold, respectively) compared to healthy PBMCs. Unfortunately, secretion of pro-inflammatory IL-6 and IL-8 cytokines were not altered upon syngeneic BT or AT EVs treatment. Unlike IL-6 and IL-8, production of other pro-inflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , were higher in syngeneic BT EV incubation compared to syngeneic AT EV stimulation. In other words, AT EV were of much immunosuppressive character compared to BT EVs and incubation with AT EVs reduced production of TNF- $\alpha$  and IL-1 $\beta$  cytokines to basal levels (Figure 3. 19C-D). Furthermore, IFN- $\gamma$  levels were assessed from EV stimulated PBMCs and ELISA results demonstrated that while AT PBMCs that were incubated with syngeneic BT EVs had 2.6-fold more type II IFN production compared to incubation with syngeneic AT EVs, neither BT nor AT EV incubation was able to alter cytokine secretion from BT PBMCs (Figure 3.19E). Lastly, anti-inflammatory responses were attempted to be unraveled since AT EV incubated PBMCs were observed to had reduced inflammatory cytokine secretion. Figure 3.19F revealed that syngeneic BT EVs induced BT PBMCs to secrete 2.4-fold more IL-10 compared to their basal levels. Meanwhile, AT EV incubation with BT PBMCs further lead to 2.3-fold more anti-inflammatory cytokine secretion when compared to BT EV stimulation. Furthermore, even though BT EV incubated AT PBMCs secreted 4-fold more IL-10 compared to their basal levels, AT EV stimulation did not have remarkable effect on IL-10 production of AT PBMCs.



**Figure 3.19 Cytokine secretion profiles of before or after therapy EVs on BT or AT CD55 deficient patient PBMCs.** BT and AT CD55 deficient PBMCs ( $n=4$ , for each group;  $1 \times 10^6$ /ml) were incubated with syngeneic BT or AT EVs ( $12 \mu\text{g}$ ) for 36 hours. Supernatants were collected and secreted IL-6 (A), IL-8 (B), IL-1 $\beta$  (C), TNF- $\alpha$  (D), IFN- $\gamma$  (E) and IL-10 (F) were determined using human cytokine ELISA. Experiments were performed in duplicates.

Next, by another sets of experiments, cytokine secretion profile of healthy controls PBMCs, that were incubated with BT or AT EVs, were determined (Figure 3.20). Although AT EV incubated healthy PBMCs secreted 2-fold less IP-10 compared to

induction mediated by BT EVs (Figure 3.20), other cytokines were unable to be detected (data not shown).



**Figure 3.20 Induction profiles of EVs of CHAPLE patients (BT or AT) from healthy PBMCs.** Healthy control PBMCs ( $n=2$ ,  $1.6 \times 10^6/\text{ml}$ ) were stimulated with BT or AT CHAPLE EVs ( $12 \mu\text{g}/\text{stimulation}$ ,  $n=4$ ) for 36 hours. Supernatants were collected and IP-10 levels were assessed by cytokine ELISA. Experiments were conducted in duplicates. t-test was performed between healthy individuals and patients for each stimulation. (ns: no significance).

To conclude, results implied that even after a single dose of Eculizumab, EV dependent immune activation of CD55 PBMCs displayed non-significant but substantially lesser pathogenic responses. In other words, AT but not BT EVs mediate less severe inflammatory response on patients PBMCs.

## 4. DISCUSSION

Mutations within the genomes of organisms are raw material for evolution. As they accumulate, it may lead to changes in structural and/or functional aspects of proteins. Thus, certain mutations may confer advantage against versatile environments for populations. Presence of complex systems are great examples for this mechanism. For example, immune system confers resistance against other species and consequently, increases survival capacity of organisms [2]. Having been composed of different arms and cell types makes each elements of immune system indispensable for generation of collective and efficient responses against invaders. Therefore, disruption within any crucial component of immune system may manifest itself as aberrant responses. These inefficient responses leading to increased susceptibility to infections, malignancies and dysregulations are called as immune deficiencies [140]. Besides being rare disorders of immune system, one of the major reasons of immune deficiencies is, surprisingly, mutations disrupting generation of proper immune responses. While they might arise as *de novo*, many cases are due to inbreeding, consanguineous marriage within human populations since it elevates chance of having homozygous deleterious or recessive genes. These poor or absence immune responses are collectively referred as primary immune deficiencies [247].

Identification and characterization of PIDs enable to delineate, comprehend and appreciate how immune system works and redundancy of its components; meanwhile, it may lead to generation of novel therapeutic applications for many other diseases. Like other aspects of immune system studies, PIDs can be investigated by generation of animal models via ENU mutagenesis or knock-out/knock-in techniques. However, they are insufficient to replicate normal course and progression of PIDs since animal models are under highly controlled environment and inbred populations. Thus, they can only mimic them to certain extent [147]. Therefore, characterization of PIDs through utilization of human subjects makes invaluable approach.

First of all, deficiency of CTPS1 enzyme, residing within crucial part of nucleotide synthesis pathway, is the most recently discovered PID that primarily distorts adequate functioning of adaptive immunity [143] (figure 1.10). As it can be interpreted from literature, it was reported that there are only handful cases of CTPS1 deficiency [149–

152]. On one hand, first report, identifying the disease, focused on effects of CTPS1 enzyme defect on adaptive immunity and proliferation capabilities of lymphocytes. On the other hand, rest of the findings attempt to cure the deficiency via hematopoietic stem cell transplantation. Secondly, CD55 deficiency, another lately identified PID, classified as complement regulatory protein deficiency, under the innate immune system disorders [34–35] (Figure 1.10). Compared to other regulatory complement deficiencies such as aHUS and PNH, CHAPLE manifests itself quite distinctly: aberrant complement activation, thrombosis in arteries, bowel inflammation induced protein-losing enteropathy. Report, published in 2017, demonstrated molecular effects of loss-of-function in CD55 protein. T-cells are required be engaged by CD55 through its receptor, CD97; otherwise, anti-inflammatory capacities of adaptive immune cells are rendered to be impaired. Moreover, Eculizumab was proven to be effectively reduces increased complement activation on immune cells as well as clinically [175]. Not only these two novel PIDs are rare, but also current literature and findings are penurious. In this thesis, we have aimed to unearth the unidentified innate and adaptive cellular as well as EV aspects of CTPS1 and CD55 deficiencies. It is crucial to point out that, to our knowledge, this is the first study trying to unravel contribution of EVs to the immunopathology of PIDs.

In that essence, first stunning finding from our experiments was evidences suggesting the presence of low-density granulocytes within the PBMC layers of CTPS1 deficient patient (Figure 3.8 and Appendix Figure B6.5). Moreover, their presence was reproducibly confirmed; that is, this was not a one-time event or consequence of experimental error. LDGs may have either suppressive or stimulatory effects on immune system, depending on the context of disease they have been found (Figure 1.7). In order to further confirm that they are indeed LDGs and characterize their immunomodulatory effect, we aimed to isolate LDGs via fluorescent-activated cell sorting. Yet our desperate isolation attempts were huge unsuccess due to several parameters. Nevertheless, it was important to demonstrate the presence of LDGs in CTPS1 because even though presence of LDGs was reported in cancer [248], autoimmune [119] and even asthma patients [249], to our knowledge, this is the first evidence in the literature implying that PIDs harbor LDGs. However, there are reports that bacterial [250] and viral [251] infections may lead to presence of LDGs in circulation. Thus, it is plausible to ask whether infection mediated pathology or enduring inflammation caused by impaired adaptive immune system is the reason why LDGs



are present in patient. In either way, LDGs in CTPS1 patient may contribute to ongoing inflammation via secreting inflammatory cytokines and undergoing NETosis [126]. Moreover, results (Figure 3.9 and 3.10), in which patient neutrophils had spontaneous NETosis, are implication for their chronically involvement in deficiency. In addition to elevated spontaneous NETosis, inflammatory cytokine secretions and their contribution to vascular damage was studied in certain PIDs such as Adenosine Deaminase 2 (ADA2) deficiency [252], Artemis deficiency and Ataxia Telangiectasia [231–253–254]. They demonstrated that aberrant neutrophil activity in these patients are due to spontaneous type I IFN production from peripheral cells and consequently, elevated levels of type I IFNs in their plasmas; however, CTPS1 PBMCs results (Figure 3.6A) did not observe neither spontaneous nor elevated IFN- $\alpha$  production upon PRR engagement. Unfortunately, we did not check levels of type I IFNs and IP-10 in plasma. Another possible explanation for spontaneous NETosis is recurrent viral infections which cannot be cleared due to defect adaptive immune cells. Certain viral [255–256] as well as bacterial infections [257–258] are recorded to induce NETosis [259]. Due to limitations (restricted sample size, inability to acquire multiple readings in different time points) of this study, we are unable to resolve questions regarding LDGs and spontaneous NETosis.

After discovery of normal IFN- $\alpha$  production levels from PBMCs (Figure 3.6A), we were relentlessly trying to unearth molecular consequences leading to increased susceptibility to viral infections. To that end, effect of PRRs on IFN- $\gamma$  productions were investigated. Stimulated APCs secrete IL-12 and IL-18 which eventually induce type II IFN secretion from innate (NK, NKT and iNKT) [260–261] and adaptive cells (CD4<sup>+</sup> Th1 cells and CD8<sup>+</sup> T-cells). Even though results from two distinct time points were inconsistent, data revealed that IFN- $\gamma$  levels from PBMCs were found to be slightly reduced. However, this was not a result of reduced IL-12 production from APCs (Figure 3.6C). Even though this phenomenon can be explained by absence of iNKT, invariant T-cells in CTPS1 deficiency [149], as we reported they still harbor major IFN- $\gamma$  producing cells such as CD8 T-cells and Th1 Cells with normal functionality. In conclusion, we demonstrated that innate immunity may not contribute to recurrence of viral infections.

Our results regarding CTPS1 deficiency also demonstrated that patient might suffer from immune dysregulation (Figure 3.3, Figure 3.4, Figure 3.5 and Appendix B6.3).

One of flow cytometry analyses (Figure 3.5) showed that patient had severely impaired IL-10 production from CD4<sup>+</sup> T-cells upon forced activation. There are numerous subsets of CD4<sup>+</sup> T-cells that are able to produce IL-10. It has been documented that Th1 cells [262–263], Th2 cells [264] and even Th17 cells [265] are able to secrete master immune regulator cytokine, under certain conditions. However, apart from them, major IL-10 producing T-cell subsets are Foxp3<sup>+</sup> and Foxp3<sup>-</sup> (or Tr1) T-cells. Although latter is formed after chronic stimulation of CD4<sup>+</sup> T-cells in the presence of IL-10 [266–267], former is fundamental for immune homeostasis [268–269]. Therefore, low percentages of Foxp3<sup>+</sup> expressing T-cells and IL-10 production may indicate Treg-dependent immune dysregulation. None of the exiting reports investigated levels of cytokine production from CD4<sup>+</sup> T-cells. In addition, we have demonstrated that phosphorylation of STAT5 protein upon IL-2 receptor stimulation is impaired in patient helper T-cells (Figure 3.3, Figure 3.4). IL-2 is a crucial cytokine for proliferation and differentiation of already primed T-cells [270] as well as development of FoxP3<sup>+</sup> Tregs in thymus and boosting the functioning of them [271–273]. Even though it was demonstrated that defect in CTPS1 enzyme renders proliferation capability of adaptive immune cells to impaired, our STAT5 phosphorylation results put forward that IL-2 receptor signaling cascade required for proliferation, differentiation may be disturbed as well. Moreover, Low levels of pSTAT5 might be attributed to decreased ability to upregulate CD25 molecules in CTPS1 patient cells [149]. Because it is known that after T-cells are stimulated via MHC and co-stimulatory molecules, they i) secrete IL-2 and ii) upregulate gamma chain of IL-2 receptor, CD25. Since we were unable to differentiate naïve CD4<sup>+</sup> from activated ones, effect of low levels of pSTAT5 may be caused by low CD25 surface expression on already primed T-cells. In order to resolve these, pSTAT5 levels can be further investigated after CTPS1 enzyme is reconstituted in CD4<sup>+</sup> T-cells. Since adaptive immune cell derived anti-inflammatory capacities were altered, we assessed IL-10 secretion from innate immune cells (Figure 3.7). In contrast to adaptive immune responses, anti-inflammatory responses of innate cells were not affected from CTPS1 enzyme deficiency. To summarize, dysregulation of immune system in CTPS1 patient may arise from improper functioning in adaptive immunity.

We also reported that helper T-cells of patient had reduced STAT3 phosphorylation and IL-17 secretion but normal STAT1 phosphorylation and IFN- $\gamma$  secretion leading to biased Th1/Th17 balance favoring Th1 (Figure 3.3, 3.4 and 3.5). Th17 cells stimulating stromal and epithelial cells via IL-17 and IL-22, respectively [274]. By stimulation of

stromal cells, they are indirect inducers of granulopoiesis and neutrophil recruitment through secreting cytokines. Thus, it may clarify why patient had low number of neutrophils (CTPS1:  $\sim 8 \times 10^5$  cells from 5ml whole blood). However, it does not explain why neutrophils were already activated and underwent spontaneous NETosis.

We have tried to investigate immunomodulatory nature of CTPS1 plasma-derived EVs on immune system. We conducted two distinct experimental sets up. First, we assessed direct effects EVs on PBMCs by solely co-incubating them. CTPS1 EVs induced less IL-6 and IL-8 compared to healthy EVs (Figure 3.12). Then, we incubated cells with EVs before stimulating their TLR4 cascade. While cytokine results demonstrated CTPS1 EVs induced low levels of IL-6 production, neither surface activation nor suppression markers were found to be altered (Figure 3.13, 3.14). To conclude, we were unable to come up with a plausible description for their immunomodulatory nature. Therefore, besides functional assays, cargos such as proteins, miRNAs, mRNAs that are circulating within CTPS1 EVs should be identified and characterized in near future.

In the second part of this thesis, we have prompted to investigate impact of deficiency of CD55 molecule on innate immune system, especially PRRs and answer in what aspects responses of immune system of CHAPLE patients can be altered after a single dose of Eculizumab. To that end, whole bloods of 4 distinct CD55 deficient patients were collected and PBMCs were isolated before and after a single dose of Eculizumab given. Our findings (Figure 3.16A-B) indicated that CD55 deficient patient have abnormal, altered innate immune response in the context of endosomal TLRs (TLR3, TLR7 and TLR9) and intracellular nucleic acid sensors (STING, DAI-STING & RIG-I). As it can be interpreted that effect of CD55 molecule is independent from MyD88 signaling since same effect were observed in all endosomal TLR response. Meanwhile, same effect was observed in intracellular nucleic acid sensing. Thus, our results might imply that both IRF3 and IRF7 signaling is affected by CD55 deficiency. To our attention, no reports have investigated that DAF *per se* regulates endosomal TLR signaling in the context of type I IFN. These results may explain why patients suffer from recurrent infections associated with hypogammaglobulinemia even though percentages of major immune cell subsets were within the expected range [34]. Same set of experiments vaguely demonstrated that, the innate immune dysfunction was merely restored by Eculizumab injection. Therefore, our data might implicate that PRR-mediated anti-viral responses might not be effected by C5aR-PRR cross-talking since

Eculizumab, being neutralizer of C5 protein in plasma, dampens the production of C5a [24–174]. However, we were unable to present any data to explain the effect of absence of CD55 on viral responses.

In same context and experimental set up, other part of PRR signaling cascade, NF $\kappa$ B, was in our central investigation: pro-inflammatory cytokine responses of CHAPLE PBMCs. It (Figure 3.17A-B) demonstrated that while IL-6 secretion levels upon PRR engagements were reduced in both BT and AT PBMCs, apart from TLR3 response of BT PBMCs, TNF- $\alpha$  responses were normal and affected by neither CD55 absence nor Eculizumab therapy. Our findings contradict with literature; that is, in one study conducted in DAF<sup>-/-</sup> mice model, demonstrated that TLR4, TLR2&6 and TLR9 mediated pro-inflammatory cytokine such as IL-1 $\beta$ , IL-6, TNF- $\alpha$  secretions were elevated compared to wild-type mice [275]. However, this raises another question whether mice and human have different mechanism of cross-talking between PRRs and complement receptors. Lastly, cytokine ELISA results demonstrated only TLR7 stimulated PBMCs have immune dysregulation (Figure 3.17). Overall, it is not possible to interpret exact effect of CD55 on PRR-mediated NF $\kappa$ B signaling cascade since not only responses of pro-inflammatory cytokine are differentially secreted as well as our findings contradict with current literature.

Growing bodies of evidences demonstrated that EVs play imperative roles in progression of diseases; however, to our knowledge, this study is the first one demonstrating direct effect of EVs, isolated from a complement disorder, on immune cells. We demonstrated that AT EV incubated PBMCs had reduced secretion of TNF- $\alpha$ , IL-1 $\beta$  but not IL-6 and IL-8 cytokines compared to BT EV incubation (Figure 3.19A-D). Although we could not explain, the reason why differential pro-inflammatory cytokine secretion might be caused by composition of AT EVs and/or CHAPLE PBMCs had already been primed to secrete high levels of IL-6 and IL-8 cytokines at a naive state and it cannot be further downregulated. Likewise, secretion of type II IFN from AT incubated PBMCs were decreased (Figure 3.19E). Strikingly, AT EVs increased secretion of anti-inflammatory cytokine, IL-10, from BT and to lesser extent AT PBMCs (Figure 3.19F). It would have been more compelling if we had further demonstrated immunomodulatory aspect of AT EVs by means of changes in surface expressions of activation and inhibitory markers. Next, we further demonstrated inflammatory biased immunomodulatory effect of BT EVs through incubating BT and AT EVs with two

healthy PBMCs. Unfortunately, only detectable cytokine was IP-10 and it further strengthened previous observation. That is, AT EVs are less immunostimulatory. Overall, even a single dose of Eculizumab injection alters composition of EVs in a way that they might exert their immunomodulatory effects by potentiating IL-10 secretion from PBMCs which eventually moderates inflammatory cytokine profile.

In addition to our characterization attempts, we were unable to conduct certain experiments since sample size and amount of cell, plasma that can be isolated from peripheral blood are major restrictions. Thus, we propose that there are several prospective future experiments to be conducted regarding CHAPLE disease: i) therapeutic application of exosomes or EVs as standalone inhibitors for complement cascade, ii) determine whether EVs derived from CHAPLE patients contribute to coagulation, iii) investigation, identification of plasma-derived BT and AT EVs via proteomics studies, iv) is there any advantage of bearing null CD55 protein against parasitic infections. First of all, we hypothesized that EVs or exosomes overexpressing CD55 protein on themselves may be used as a novel therapeutic agent rather than Eculizumab therapy. Although we did not present preliminary data regarding this aspect of utilization of EVs, sets of experiments demonstrated that when PBMCs were incubated with CD55-expressing exosome in the presence of sera, deposition of inactive or cleaved components of the complement system on cells was increased. Meanwhile, exosome incubation did not alter MAC-mediated cell lysis. These preliminary data will need to be worked on and optimized for possible application of exosomes in the therapeutics of CHAPLE disease. Secondly, as it was mentioned previously, CHAPLE patients suffer from severe thrombosis in arteries; meanwhile, studies showed that exosomes, microparticles and even apoptotic bodies contribute to the thrombosis and coagulation pathway [276–278]. Third, amounts of EVs left after experiments that we narrated above were tied to our hands to carry out proteomics study. As novel patients are identified, the composition of their circulating EVs is desperately needed to be lightened. Lastly, there are reports indicating that the absence of CD55 protein impairs malaria parasites to adhere to the surface of host erythrocytes [279–282]. Since the distribution of CD55 deficiency is mostly concentrated in Middle East countries in which Leishmaniasis is common [283–284], it will be exciting to answer whether null CD55 protein in patients confers resistance to Leishmaniasis or not.

Collectively, not only we demonstrated several novel outcomes for both CTPS1 and CD55 deficiencies but also this is the first study focusing on investigation of PID EVs.

Our results suggest that while presence of LDGs, neutrophils with spontaneous NETosis, impaired STAT5 phosphorylation along with reduced IL-10 secretion from helper T-cells in CTPS1 deficiency contribute ongoing inflammation, impaired phosphorylation of STAT3, reduced levels of IL-17a secretion contribute dysfunction of adaptive immune system. Moreover, predisposition to viral infections is not contributed by cytotoxic T-cells. In the context of CHAPLE syndrome, we have unraveled that patients had impaired anti-viral PRR responses which cannot be altered by Eculizumab therapy. Last but not least, Eculizumab therapy altered the immunomodulatory effect of CD55 EVs.

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## 6. APPENDICES

### 6.1. Appendix A

#### Cell Culture Media and Buffers Recipes

##### RPMI-1640 Media

- 500 ml RPMI-1640 Media (with L-Glutamine at a final concentration 2mM)
  - For **2%**: 10.64 ml; **5%**: 27.47ml; **10%**: 58.14 ml FBS
- (For Regular Media, FBS was heat-inactivated for 1 hour at 55°C, for Oligo Media, FBS was heat-inactivated for 1 hour at 65°C. Moreover, in order to prepare EV depleted media, heat-inactivated FBS were subjected to centrifugation at 100000xg for 5 hours.)
- For **2%**: 5.32 ml; **5%**: 5.5 ml; **10%**: 5.8 ml HEPES (10mM)
  - For **2%**: 5.32 ml; **5%**: 5.5 ml; **10%**: 5.8 ml Non-Essential Amino Acids (1X from 100X Stock Solution)
  - For **2%**: 5.32 ml; **5%**: 5.5 ml; **10%**: 5.8 ml Na-Pyruvate (110µg/ml)
  - For **2%**: 5.32 ml; **5%**: 5.5 ml; **10%**: 5.8 ml Penicillin/Streptomycin (50µg/ml)

##### 10X Phosphate Buffered Saline (PBS) (For ELISA Studies and FACS Buffer)

- 80 grams NaCl
- 2 grams KCl
- 15.2 grams Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O
- 2 grams KH<sub>2</sub>PO<sub>4</sub>
- Completed up to 1 liter with distilled H<sub>2</sub>O

pH was adjusted to 6.8, then autoclaved.

In order to prepare 1X PBS, 10X PBS was diluted with distilled water. pH was adjusted to 7.4, then autoclaved.

### **Blocking Buffer (ELISA)**

- 25 grams Bovine Serum Albumin (5%)
- 250  $\mu$ L Tween20 (0.025%)
- Completed up to 500 mL with 1X PBS

First, BSA was dissolved in certain amount of 1X PBS (less than 500 ml), then Tween20 was added and solution was mixed until BSA was completely dissolved. Completed up to 500 ml and aliquoted, stored at -20°C until they were used.

### **Wash Buffer (ELISA)**

- 4.5 L distilled H<sub>2</sub>O
- 0.5 L 10X PBS
- 2.5 mL Tween20

Wash buffer was prepared freshly prior to each usage.

### **Fluorescent Activated Cell Sorting (FACS) Buffer (Flow Cytometry)**

- 5 g BSA
- 250 mg NaN<sub>3</sub>
- Completed up to 500 ml with 1X PBS.

Prepared buffer was stored at +4°C.

### **1X Tris-EDTA Buffer (Micrococcal Nuclease Reaction)**

- 10 mM Tris (pH 8.0)
- 1 mM EDTA
- Completed up to 100 ml with Cell Culture Grade H<sub>2</sub>O.

It was stored at +4°C.

### **10X Nuclease Buffer (Micrococcal Nuclease Reaction)**

- 10 mM CaCl<sub>2</sub>
- 50 mM Tris-HCl
- Completed up to 100 ml with Cell Culture Grade H<sub>2</sub>O.

pH was adjusted to  $\cong$ 8.3. 10X Nuclease Buffer was stored at +4°C. 1X Nuclease buffer was prepared with diluting 10X Nuclease Buffer with Tris-EDTA Buffer.

### **10X Running Buffer (SDS Page-Western Blot)**

- 144 g Glycine
- 30 g Tris-Base
- 10 g SDS

Complete up to 1 liter with distilled water. 1X Running Buffer was prepared with diluting 10X buffer with distilled water.

### **10X Transfer Buffer (SDS Page-Western Blot)**

- 144 g Glycine
- 30 g Tris-Base

Complete up to 1 liter with distilled water, store at +4°C.

1X Transfer Buffer was prepared prior to each use: 700 ml distilled water, 200 ml Methanol, 100 ml 10X Transfer Buffer. Stored at -20°C.

### **Ponceau S Staining Solution (SDS Page-Western Blot)**

- 1 g Ponceau S
- 15 ml Acetic Acid

Complete up to 500 ml with distilled water.

**Blocking Buffer (SDS Page-Western Blot)**

- 2.5 g Milk powder

- 50 µl Tween20

Completed up to 50 ml with 1X PBS.

**PBS-T Buffer (SDS Page-Western Blot)**

- 999 ml 1X PBS

- 1 ml Tween20

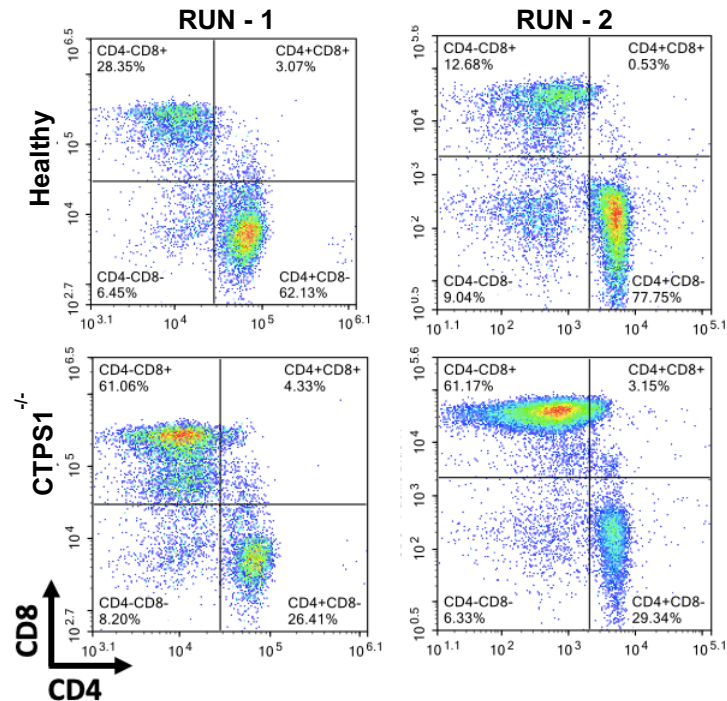
**Antibody Dilution Buffer (SDS Page-Western Blot)**

- 2.5 g Milk Powder

- 25 µl Tween20

Completed up to 50 ml with 1X PBS.

## 6.2. Appendix B



**Figure B1.1** Immunophenotyping of circulating lymphocytes revealed CTPS1<sup>-/-</sup> patient had inverted CD4<sup>+</sup>/CD8<sup>+</sup> ratio. Whole blood samples of healthy donors (n=2) and patient were stained with anti-CD45, anti-CD3, anti-CD4, anti-CD8 in two separate blood collection events. CD4<sup>+</sup> and CD8<sup>+</sup> T-Cells, gated in CD3<sup>+</sup> and singlets by forwards and side scattering, were assessed in density-plots using flow-cytometer.

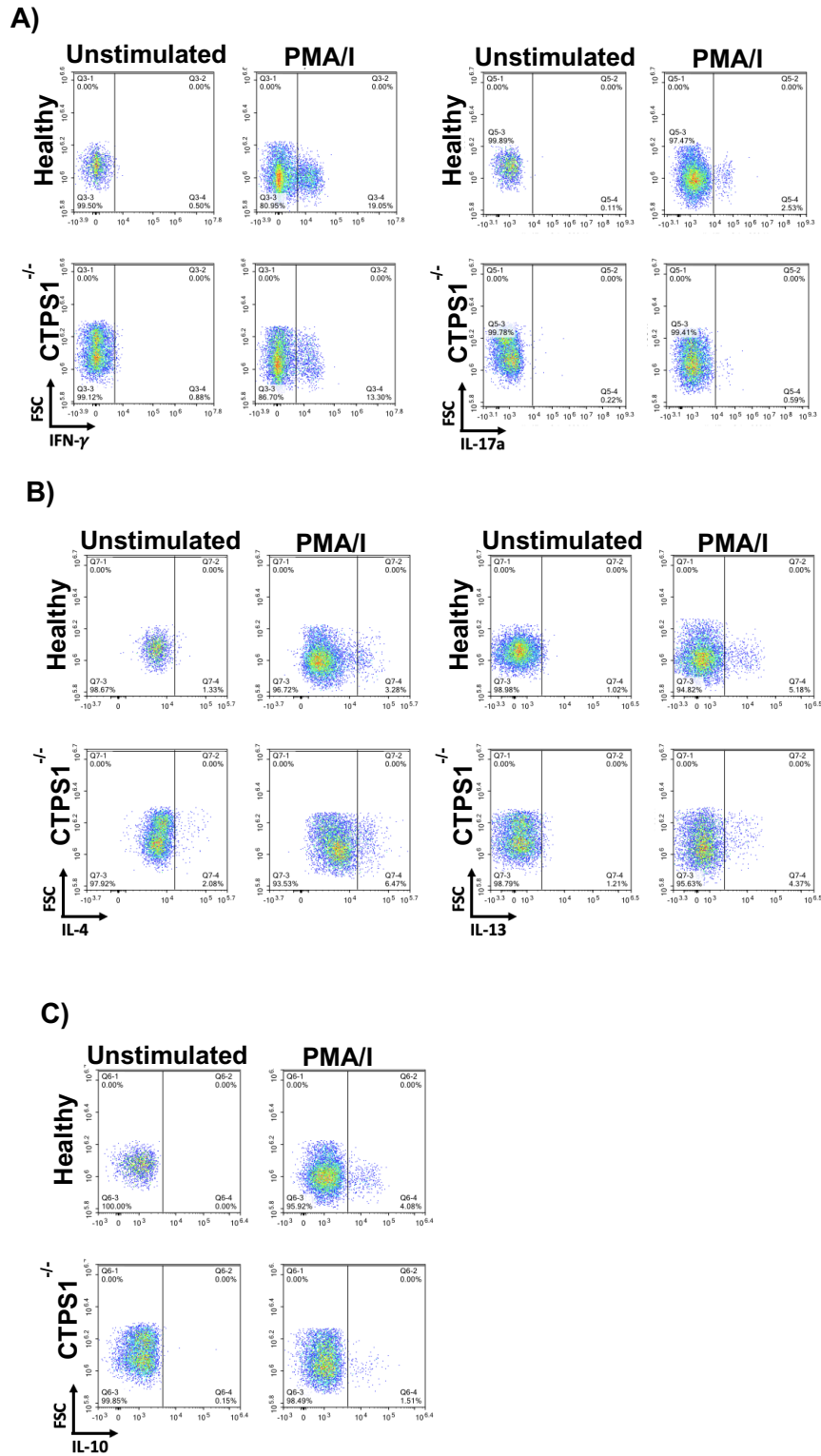
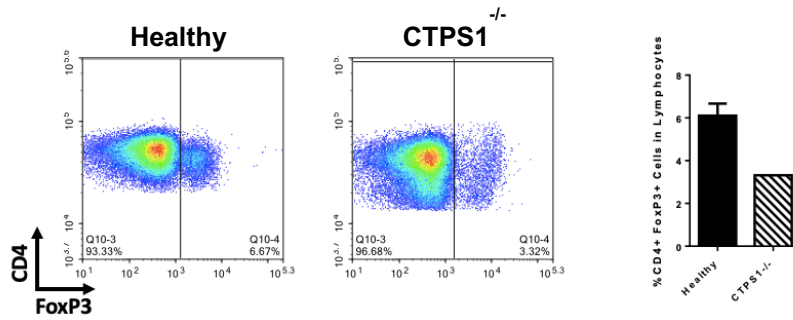
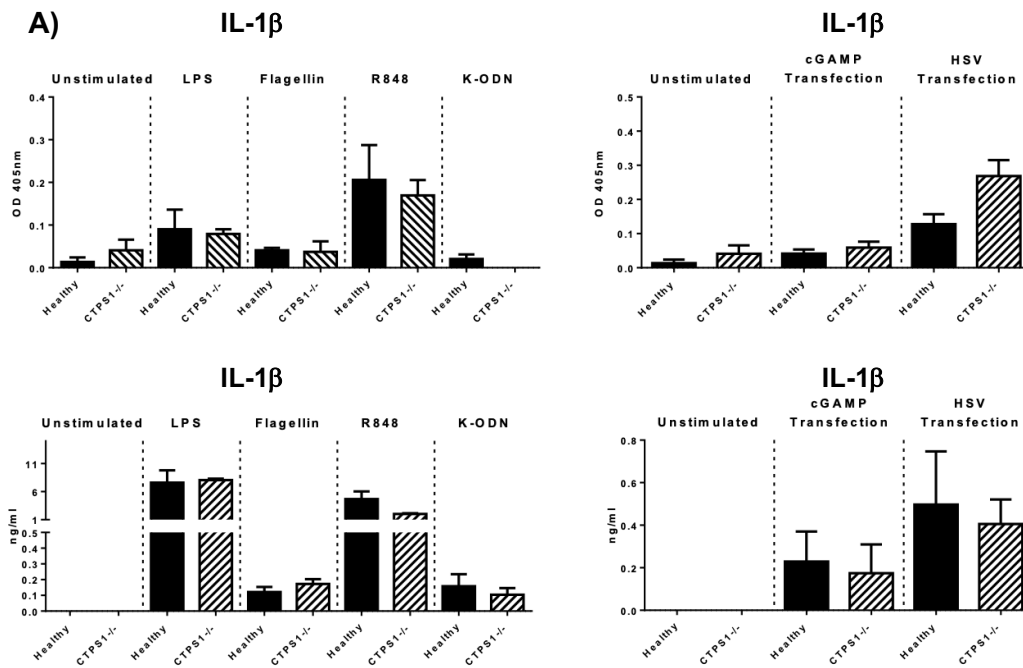


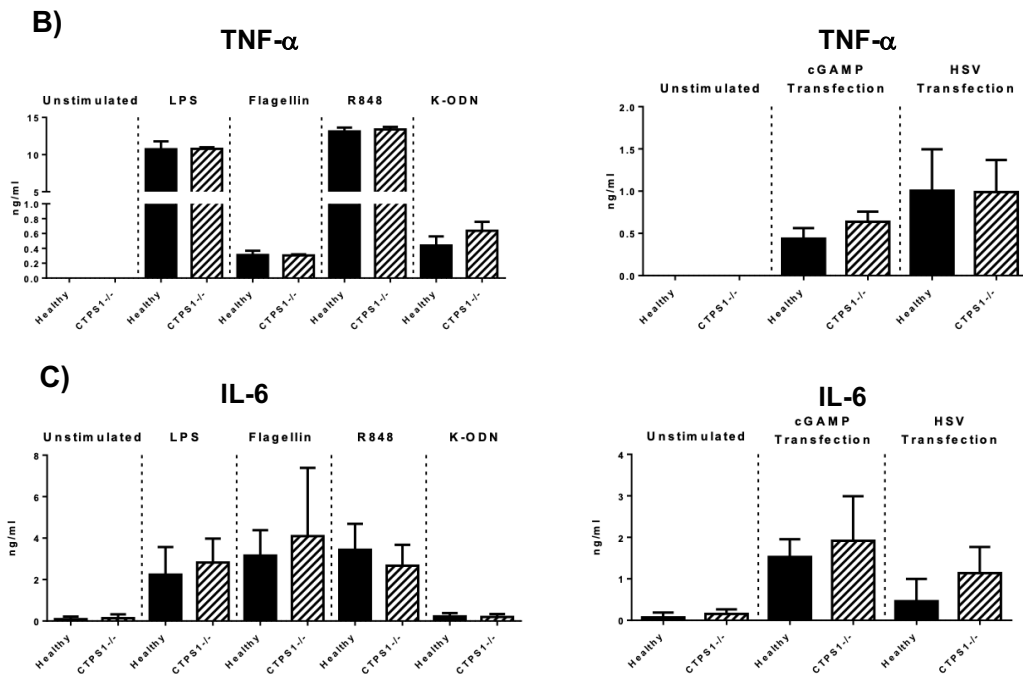
Figure B1.2 Cytokine production of healthy and CTPS1<sup>-/-</sup> patient CD4<sup>+</sup> T-cells in response to PMA/Ionomycin stimulation. Healthy and patient PBMCs (1.6x10<sup>6</sup>/ml) were

left untreated or stimulated with PMA/Ionomycin (50ng/ml, 1 $\mu$ g/ml) for 6 hours in the presence of Brefeldin A. Density plots indicate **A**) IFN- $\gamma$  (left panel), IL-17a (right panel), **B**) IL-4 (left panel), IL-13 (right panel) and **C**) IL-10 producing CD4<sup>+</sup> T-cell percentages which were determined with flow-cytometry.

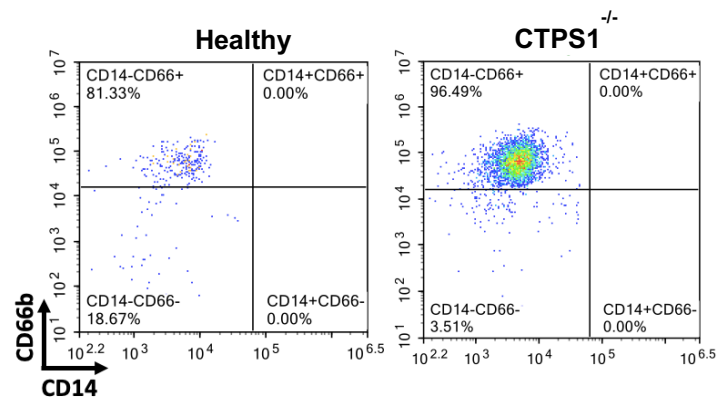


**Figure B1.3 Transcription factor staining revealed reduced Foxp3<sup>+</sup> CD4<sup>+</sup> T-cell numbers in CTPS1<sup>-/-</sup> patient circulation.** After control (n=2) and patient PBMCs isolated from whole blood of patient and donors, cells were stained with anti-CD4, anti-FoxP3. Percentages of transcription factor expressions were evaluated from density-plots (left) using flow cytometry and AVE $\pm$ SD levels are represented in bar graphs (right).





**Figure B.4 Pro-inflammatory cytokine production from healthy and patient PBMCs upon PRR ligand stimulations.** Healthy (n=2) and patient PBMCs ( $8 \times 10^5$ /ml) were stimulated with TLR4 (LPS:  $5 \mu\text{g/ml}$ ), TLR5 (Flagellin:  $100 \text{ng/ml}$ ), TLR7 (R848:  $5 \mu\text{g/ml}$ ), TLR9 (K3:  $1 \mu\text{M}$ ), STING (2'3' cGAMP:  $30 \mu\text{g/ml}$ ), IFI6-STING (HSV:  $5 \mu\text{g/ml}$ ) ligands for 24 hours. Supernatants were collected and secreted IFN- $\alpha$  IL-1 $\beta$  (Run-1: Upper panel, Run-2: Lower panel) (A), TNF- $\alpha$  (B) and IL-6 (C) levels were assessed by cytokine ELISA. Stimulations were run in duplicates.





**Figure B1.5 Confirmation of Low-Density Granulocytes within patient's PBMCs fraction.** Density plots represent LDGs within healthy (Left panel) and patient (right panel) PBMCs was confirmed by dual staining with anti-CD14, antiCD66b via flow cytometry.

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