CHARACTERISATION OF IMMUNE RESPONSES IN TYPE I INTERFERON ASSOCIATED DISEASES

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ABSTRACT

CHARACTERISATION OF IMMUNE RESPONSES IN TYPE I INTERFERON ASSOCIATED DISEASES

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Type-I interferonopathies are a heterogeneous group of diseases arising from deregulation in nucleic acid sensing pathways, leading to constitutive type-I interferon release and pathology. DNA damage, if not repaired can also potentially activate such pathways. To test the hypothesis that 2 different DNA damage repair and immune deficiencies, Ataxia telangiectasia (AT) and Artemis deficiency could suffer from clinical manifestations associated with elevated type I IFN response, we compared the immune status of AT and Artemis deficient patients to healthy controls and to two patients with confirmed type I interferonopathies (one SAVI (STING-associated vasculopathy with onset in infancy) and one TREX-1 deficient patient diagnosed with

Aicardi-Goutières Syndrome (AGS)). Our results demonstrated that type-I and III interferon signatures were elevated in plasma and peripheral blood cells of AT and Artemis deficient patients. Specifically, compared to healthy controls, circulating IP-10 (9- and 14-fold), IFNα (5- and 7-fold) and IFNλ1 (4- and 5-fold) concentrations were significantly higher in AT and Artemis deficient patients, respectively. Moreover, AT and Artemis deficient peripheral blood mononuclear cells (PBMC) secreted ~4- and ~5 fold more IFN-α than healthy controls in the absence of any stimulation. This exaggerated response was still maintained when cells were stimulated with the STING ligand 2'3'-cGAMP (6- and 31-fold more IFNα secretion from AT and Artemis deficient cells). Measurement of phosphorylated STAT1 concentrations within PBMCs revealed elevated levels in AT and Artemis deficient patients with respect to healthy controls. These results suggest that AT and Artemis deficient patients have immune features consistent with elevated type I IFN signature. Next, we hypothesized that aberrant IFN production could activate neutrophils and contribute to tissue damage. Herein, we show for the first time that neutrophils of AT, Artemis deficient and SAVI patients spontaneously produced neutrophil extracellular traps (NETs). Consistent with neutrophil activation, plasma elastase was significantly higher in all tested patient groups suggesting that neutrophils might be contributing to tissue damage in classical and proposed type-I interferonopathies.

Keywords: Type-I interferonopathies, Artemis deficiency, Ataxia Telangiectasia, STING-associated vasculopathy with onset in infancy (SAVI), Aicardi-Goutières Syndrome (AGS)

ÖZ

TİP-1 İNTERFERON İLİŞKİLİ HASTALIKLARDA İMMÜN YANITLARIN KARAKTERİZASYONU

Gül, Ersin

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Nükleik asit duyaçlarındaki bozukluklardan meydana gelen ve sonucunda tip-1 interferon salımı ve immün patolojiye neden olan heterojenik hastalıklar "tip-1 interferonopati" kategorisinde sınıflandırılmaktadır. Onarılmayan DNA hasarının da bu yolakları harekete geçirebeliceği bilinmektedir. Bu çalışmada, DNA hasar onarımı ve immünyetmezlik hastalığı olarak bilinen Ataksi Telaniektazi (AT) ve Artemis defekti hastalıklarında görülen klinik semptompların da yükselmiş tip-1 interferon salımıyla ilişkili olabileceği hipotezimizi test etmek amacıyla, AT ve Artemis eksikliği olan hastların immün yanıtları sağlıklı kontroller ve 2 tane onaylanmış tip-1 interferonopati hastasıyla karşılaştırılmıştır (biri TREX-1 defekti olan Aicardi-

Goutières Sendromu (AGS) ve diğeri SAVI (STING proteini ilişkili erken başlangıçlı vaskülopati)). Sonuçlarımız, AT ve Artemis defektli olan hastların plazmasında ve periferik kan hücrelerinde tip-1 ve tip-3 interferon belirteçlerinin arttığını göstermiştir. Spesifik olarak, AT ve Artemis defektli olan hastaların plazmalarında sırasıyla IP-10 (9 ve 14 kat), IFNα (5 ve 7 kat) ve IFNλ1 (4 ve 5 kat) sitokinlerinin artmış olduğu gözlemlenmiştir. Bunlara ek olarak, AT ve Artemis defektli hastaların periferik kan hücreleri sağlıklılara göre sırasıyla 4 ve 5 kat fazla IFNα salmışlardır. Aşırı IFNα salımının, hücreler STING algaç ligandı 2'3'-cGAMP ile uyarıldığında da devam ettiği gözlemlenmiştir (sırasıyla 6 kat ve 31 kat daha fazla salım.). Periferik kan hücrelerinde fosforlanmış STAT1 molekülü konsantrasyonu da AT ve Artemis defektli hastalarda artış göstermiştir. Bütün bu sonuçlar, AT ve Artemis defektli hastaların immün yanıtlarının artmış tip-1 interferon özelliği gösterdiğine işaret etmektedir. Bir sonraki çalışmada, anormal IFN üretiminin nötrofilleri aktifleştirip doku hasarına katkısı olduğu hipotezini kurduk. Yaptığımız deneylerle, ilk defa, AT, Aretmis defektli, ve SAVI hastalarının nötrofillerinin kendiliğinden NET (nötrofil hücredışı tuzakları) ürettiğini gösterdik. Bu sonuçlarla tutarlı bir şekilde plazmadaki elastaz seviyelerinin yüksek olduğunu gösterdik. Tüm bu sonuçlar, tip-1 interferonpatisi olan hastalarda görülen doku hasarının nedeninin nötrofil aktivitesi olabileceğini destekler niteliktedir.

Anahtar Kelimeler: Tip-I interferonopatiler, Artemis defekti, Ataksi Telanjiektazi, STING-ilişkili erken başlangıçlı vaskülopati (SAVI), Aicardi-Goutières Sendromu (AGS).

To my precious family

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LIST OF ABBREVIATIONS

AGS	Aicardi-Goutières Syndrome
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP Synthase
c-di-GMP	cyclic di guanosine monophosphate
CpG	unmethylated cytosine-phosphate-guanosine motifs
CXCL	CXC chemokine ligand
DAMP	Danger/Damage-associated molecular patterns
DDX41	DEAD box protein 1
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum

IFN	Interferon
IRF	Interferon regulatory factor
IL	Interleukin
IP-10	Interferon-Induced Protein 10
ISG	Interferon Stimulated Gene
JAK	Janus activated Kinase
LGP-2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
MDA-5	Melanoma differentiation associated 5
MFI	Mean florescent intensity
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor-88
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nod-like receptor
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells

PBS	Phosphate Buffered Saline
pDC	Plasmacytoid Dendritic Cells
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Cells
PNPP	p-nitrophenyl phosphate
RA	Rheumatoid Arthritis
RPMI	Roswell Park Memorial Institute
PRR	Pattern Recognition Receptors
RIG-I	Retinoic acid-inducible gene 1
RLR	RIG-like receptor
SAVI	STING-associated vasculopathy with onset in infancy
SLE	Systemic Lupus Erythematous
STAT1	Signal transducer and activator of transcription 1
STING	Stimulator of interferon genes
TBK-1	TANK-binding kinase 1
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
TREX-1	Three prime repair exonuclease 1

CHAPTER 1

INTRODUCTION

1.1.Nucleic Acid Sensors of Innate Immune System

Immune system in vertebrates has evolved to protect the host from invading pathogens. To achieve this, various immune cells work in a complex system and communicate with each other by secreting various cytokines/ chemokines or through cell-cell interactions. Hence, communication between adaptive and innate immune cells provides for the establishment of proper immune responses that would protect the host against pathogens. To prevent the spread of infection and eliminate invading pathogens, invading organisms must first be recognized by the innate immune system which is considered as the first line of defense (Kawai & Akira, 2011). Innate immune system maintains this function by recognizing a group of conserved molecular motifs collectively called as "pathogen-associated molecular patterns (PAMPs)" through a number of germline-encoded receptors known as pattern-recognition receptors (PRRs), a term first proposed by Charles Janeway in 1989 (Janeway, 1989). In this ground-breaking paper, Janeway suggested that PAMPs are unique molecular structures that are conserved and shared by many different pathogens but are absent in the host. Examples include molecules like lipopolysaccharide (LPS), flagellin and peptidoglycan. Although this proposal is valid for many of the PAMPs, recent studies revealed that there are exceptions to this classification such as viral/bacterial nucleic

acids which are not unique to pathogens but are still recognized as PAMPs by the host innate immune system (Medzhitov, 2009). The idea that nucleic acids can be considered as PAMPs facilitated research in innate immunity field and "nucleic acid sensing mechanisms" became a whole new area of study. Nucleic acid sensing mechanisms are critical for development of anti-viral responses and virus eradication since viral genomes (RNA/DNA) and their replication products (RNA/DNA hybrids) can be detected by these sensors (Desmet & Ishii, 2012; Mankan et al., 2014). However, despite the presence of mechanisms that can discriminate self from non-self-nucleic acids, under certain circumstances, nucleic acids of host origin can also engage these sensors, resulting in autoimmune/autoinflammatory diseases (discussed in detail in Section **1.2**) (Ablasser, Hertrich, Waßermann, & Hornung, 2013).

Nucleic acid sensors of innate immune system can be divided into two groups based on their subcellular localization. First group consists of several Toll-like receptors (TLRs) residing in the lumen of endosomes and are mainly found in dendritic cells (DCs), macrophages and B-cells (Blasius & Beutler, 2010). The second group of nucleic acid sensors consists of several cytosolic receptors specific to DNA or RNA (Hornung, 2014a, 2014b; Schlee & Hartmann, 2016). Upon recognition of nucleic acids either in endosomes or in cytosol, both receptor groups initiate a signalling cascade which culminates in synthesis of type-I interferons (IFNs) and/or IL-1β (Platanias, 2005). Following sections introduce basic knowledge on endosomal and cytosolic sensors.

1.1.1. Endosomal Toll-like receptors

PRRs of TLR family capable of recognizing nucleic acids consist of TLR3, TLR7, TLR8, TLR9 and TLR13 (only in mouse) (Guiducci et al., 2010) and (**Figure 1.2**).These receptors carry leucine-rich repeats (LRRs) for PAMP recognition, a transmembrane domain and a cytosolic Toll/IL-1 receptor (TIR) domain for converging signals to downstream adaptors Myd88 or TRIF (Kawai & Akira, 2010).

Endosomal TLRs monitor the vesicle lumen for pathogen derived nucleic acids and signal through adaptor proteins TRIF or Myd88 (Kawai & Akira, 2010). Among these 5 TLRs, only TLR3 signals through TRIF and activates NF-κB and IRF3 whereas the other four signal through Myd88 and activate NF-κB and IRF7. TLR3 recognizes double-stranded RNA (dsRNA), TLR7/8 is specific for single stranded RNA (ssRNA) and TLR9 recognizes unmethylated cytosine-guanosine (CpG)-rich DNA, a common feature of bacterial but not mammalian DNA (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Diebold, Kaisho, Hemmi, Akira, & Sousa, 2004; Hemmi et al., 2000). Upon recognition of their respective ligands and activation of downstream signalling molecules, type-I interferons and pro-inflammatory cytokines are produced and released by immune cells (Kawai & Akira, 2010).

1.1.2. Cytosolic RNA sensors: RIG-I-like receptors (RLRs)

Among the recently identified cytosolic nucleic acid sensors, RIG-I and MDA5 engage cytosolic RNA species (Goubau, Deddouche, & Reis e Sousa, 2013). These are expressed in almost all cells. MDA5 recognizes long dsRNAs (> 300 bp) whereas 5' tri- or di-phosphate containing and/or blunt dsRNA molecules (≥19 bp) bind to and activate RIG-I (Goubau et al., 2014; Hornung, 2014a; Kato et al., 2006; Schlee & Hartmann, 2016; Yoneyama et al., 2004). For both of these receptors, synthetic dsRNA analog polyI:C can be used as a ligand in experimental settings depending on length of the synthetic polyI:C product (Kato et al., 2006). Upon binding to their respective ligands, both RIG-I and MDA5 activate IRF-3 and NF-κB through an adapter protein known as mitochondrial antiviral signalling protein (MAVS) (Peisley, Wu, Yao, Walz, & Hur, 2013). RNA binding triggers a conformational change in the RLR in question, facilitating CARD-domain mediated-oligomerization and signalling through MAVS to activate transcription factors NF-κB and IRF-3, which in turn induce production of pro-inflammatory cytokines and type-I interferons (Q. Sun et al., 2006). Type-I interferons subsequently enhance the expression of several

interferon stimulated genes (ISGs), many of which have direct anti-viral activities, aiding virus clearance (Hornung, 2014a; F. Hou et al., 2011; Schlee & Hartmann, 2016).

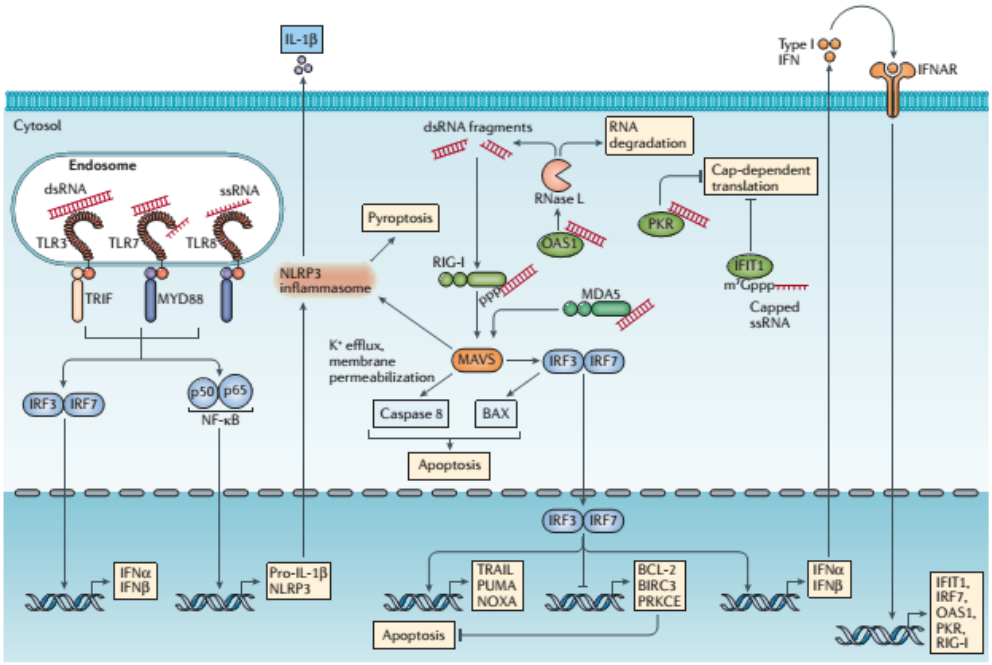


Figure 1.1 RNA sensors of Innate Immune System; adapted from (Schlee & Hartmann, 2016).

1.1.3. Cytosolic DNA sensors

In healthy cells, DNA is strictly contained within the nucleus. Presence of DNA in the cytosol is a potent danger signal. To date, several receptors capable of recognizing cytosolic DNA species have been defined. These are DAI, AIM2, RNA Polymerase III, IFI16, DDX41 and cGAS (**Figure 1.2**) (Hornung, 2014b; Roers, Hiller, & Hornung, 2016; Schlee & Hartmann, 2016). Among the above mentioned cytosolic DNA sensors, cGAS (cyclic GMP-AMP synthase) seems to be the dominant and indispensable receptor for most cell types (Li et al., 2013; L. Sun, Wu, Du, Chen, & Chen, 2013).

Most of these sensors share the same ER-localized signalling adaptor STING (stimulator of IFN genes), a recently identified protein that is essential for type-I interferon production (Ishikawa & Barber, 2008; W. Sun et al., 2009; Zhong et al., 2008). Until the discovery of the cGAS -STING pathway, several other sensors of cytosolic dsDNA s were described although most were either limited to a few specific cell types or had replaceable function. One of these was the DNA-dependent RNA polymerase III, which synthesizes 5'-triphosphate RNA from AT-rich DNA, activating type-I interferon production through the RIG-I-MAVS pathway s (Chiu, MacMillan, & Chen, 2009). Another protein, the DNA-dependent activator of IRFs (DAI), was also proposed as a cytosolic DNA sensor but ensuing studies proved that its function was dispensable in most cell types (Ishii et al., 2008). IFI16, predominantly a nuclear protein, was also found to be involved in cytosolic DNA dependent IRF3 activation and IFN-β production (Unterholzner et al., 2010). However, other groups failed to observe any effect of IFI16 knock down on cytoplasmic DNA-induced IFN-β production (Abe et al., 2013). Similarly, DDX41 was also reported by some groups to be involved in type-I interferon induction but its depletion had very little effect on IFN-β secretion in response to dsDNA in cytosol (E. Lam, Stein, & Falck-Pedersen, 2014; Parvatiyar et al., 2012). As it can be understood from these reports, until the discovery of the cGAS-STING pathway, none of the abovementioned receptors could be considered as the general and non-redundant sensor for cytosolic DNA.

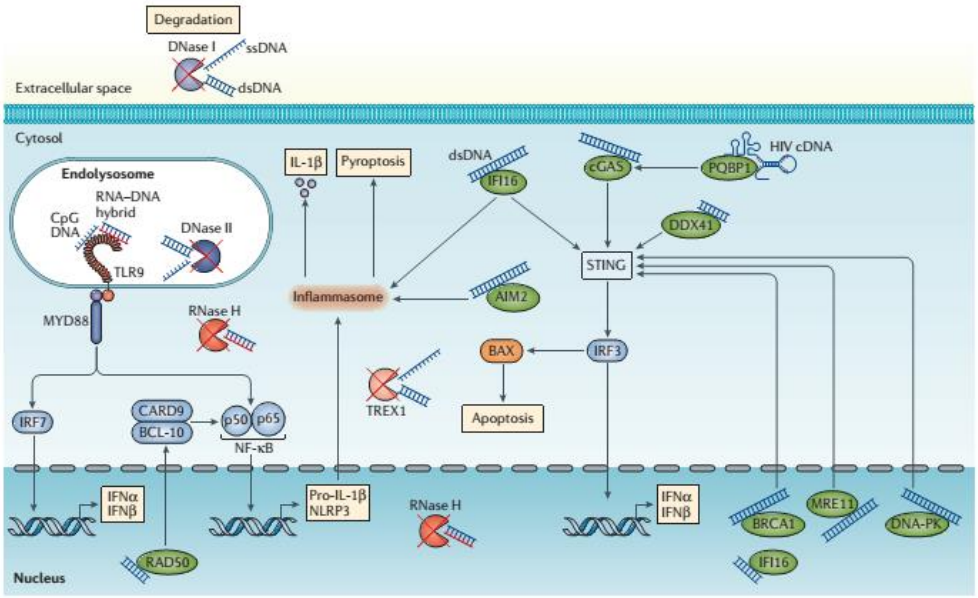


Figure 1.2 DNA receptors of innate immune system; adapted from (Schlee & Hartmann, 2016).

After intensive study in the field of cytosolic DNA sensing, several groups independently identified an important signalling adaptor of type-I interferon pathway named as stimulator of interferon genes (STING, also known as TMEM173, ERIS, MITA) (Ishikawa & Barber, 2008; W. Sun et al., 2009; Zhong et al., 2008). STING is an adaptor protein that resides on endoplasmic reticulum membrane and has diverse tissue distribution which makes it a non-redundant member of nucleic acid recognition (Zhong et al., 2008). Overexpression studies in HEK293T cells which normally do not express detectable amounts of STING demonstrated the role of this adaptor in IRF3 activation and IFN-β induction (Ishikawa & Barber, 2008). Subsequent studies further confirmed STING's role as an IFN-β inducer in MEFs, macrophages and DCs and through knock-out murine studies proved its protective role against herpes simplex virus infections (Ishikawa, Ma, & Barber, 2009). Shortly after the discovery of STING, cyclic GMP-AMP synthase (cGAS) was identified as the major DNA sensor working upstream of STING (L. Sun et al., 2013; J. Wu et al.,

2013). This enzyme is a nucleotidyltransferase found in the cytoplasm. Upon binding to dsDNA, cGAS synthesizes a newly defined second messenger, cyclic GMP-AMP (cGAMP) from ATP and GTP which then binds and activates STING to promote interferon and ISG expression (L. Sun et al., 2013; J. Wu et al., 2013). Discovery of cGAS truly contributed to proper understanding of dsDNA recognition in cytosol. Complete loss of type-I interferon secretion in response to cytosolic dsDNA in cGAS deficient macrophages, fibroblasts, DCs and established cell-lines demonstrated the central role of this enzyme in cytosolic DNA sensing (Li et al., 2013; J. Wu et al., 2013). In addition to its role in dsDNA recognition, cGAS can also bind to retroviral replication elements (RNA:DNA hybrids) (Mankan et al., 2014). Following nucleic acid binding, cGAS dimerizes, triggering synthesis of cGAMP which then binds to STING allowing its translocation to the Golgi complex. There, STING recruits and activates TBK1, resulting in phosphorylation of the transcription factor IRF3 (Ishikawa & Barber, 2008; Saitoh et al., 2009). Phosphorylated, IRF3 homodimers translocate to the nucleus and promote induction of type-I interferons and ISGs. Secreted type-I interferons bind to their respective receptors (IFNAR1 and IFNAR2), triggering their dimerization and downstream signalling through receptor-associated kinases janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). The activated kinases then phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 in cytosol resulting in their heterodimerization and consecutive translocation to the nucleus where they bind to IRF9 to form ISG factor 3 (ISGF3). ISGF3 binds to the interferon stimulated gene (ISG) promoter to further induce expression of type-I interferons and ISGs (Ivashkiv & Donlin, 2014).

In addition to these cytosolic dsDNA sensors, one other important sensor is AIM2. However, AIM2-dsDNA interaction does not lead to type I IFN production but activates the formation of a multi-protein complex called the inflammasome. AIM2 inflammasome activation leads to the generation of active caspase-1 which cleaves pro-inflammatory pro-cytokines IL-1β and IL-18 (Broz & Dixit, 2016) into their active forms. Hence AIM2 inflammasome represents a separate defense pathway and is

essential for host defense against cytosolic bacteria and DNA viruses (Rathinam et al., 2010).

1.2. Contribution of Nucleic Acid Sensing Pathways to Autoimmune/autoinflammatory Diseases

In addition to their role in host defence, nucleic acid sensors have been associated with several autoimmune or autoinflammatory diseases. Ability of these cytosolic nucleic acid sensors to discriminate between self and non-self has its limitations which results in unwarranted immune response (Roers et al., 2016). Studies over the past decade has revealed that endogenous nucleic acids can also be considered as danger signals and trigger an immune response which might be detrimental to the host. Despite their critical role in anti-viral defense, inappropriate secretion of type-I interferons in sterile conditions can cause serious damage to the host. To prevent mounting an immune response against endogenous nucleic acids, immune system has evolved several strategies. One way of doing this is through compartmentalization of endogenous and exogenous nucleic acids (e.g., having TLRs in endosomes and exclusion of nuclear and mitochondrial DNA from cytosol) so that these receptors cannot encounter self RNA/DNA and produce IFNs (Barton & Kagan, 2009; Rongvaux et al., 2014). Another mechanism depends on chemical modification of self-nucleic acids (e.g., presence of viral RNAs without 5' cap or methylation of CpG islands in host genome) so that receptors specifically recognize viral nucleic acids (Kumagai, Takeuchi, & Akira, 2008; Peisley et al., 2013; Rehwinkel, 2010). Finally, host cells use various strategies to eliminate self-nucleic acids (e.g., nuclease functions) derived from dead cells or originating as a result of DNA damage so that they are quickly disposed before triggering an immune response (Nagata, Hanayama, & Kawane, 2010). However, several studies suggest that such strategies are imperfect.

In recent years, a new group of diseases associated with constitutive secretion of type-I interferons were defined and categorized as "type-I interferonopathies" (Y. J. Crow

& Manel, 2015; Y. J. Crow, 2011b). Deregulated type-I interferon production could arise through several mechanisms as summarized in **Figure 1.3**. The following section summarizes mechanisms and pathological signs associated with type-I interferonopathies starting from the classical interferonopathy known as the Aicardi-Goutières Syndrome and extending to many other autoinflammatory diseases which might also be included in this group.

Box 2 | **Type I interferon exposure: too much, for too long, at the wrong time**

There are several possible mechanisms that may lead to the development of a type I interferonopathy:

- Inappropriate stimulation of the type I interferon (IFN) response machinery due to the abnormal accumulation of an endogenous nucleic acid ligand, for example, as a consequence of the lack of a nuclease such as DNA 3′ repair exonuclease 1 (TREX1) or possibly the deoxynucleoside triphosphate triphosphohydrolase SAM domain and HD domain 1 (SAMHD1) (**a**).
- Inappropriate stimulation of the type I IFN response machinery due to a change in the composition of an endogenous nucleic acid ligand, which, for example, could be caused by the retention of ribonucleotides within RNA–DNA hybrids or oxidative damage to DNA[386] (**b**).
- Enhanced sensitivity or ligand-independent (constitutive) activation of nucleic acid receptor signalling to the type I IFN pathway, for example, as in the case of gain-of-function mutations in *IFIH1* (which encodes IFN-induced helicase C domain-containing protein 1) (**c**).
- Enhanced sensitivity or ligand-independent (constitutive) activation of a non-nucleic acid receptor component (for example, an adaptor molecule) of the IFN-induced signalling pathway, such as in the case of mutations in *TMEM173* (which encodes stimulator of interferon genes protein (STING)) (**d**).
- Defective negative regulation of a nucleic acid-dependent type I IFN response, for example, due to mutations in *ISG15* (which encodes IFN-stimulated gene protein 15) (**e**).
- Mutations in other genes involved in non-nucleic-acid-related stimulation or regulation of the type I IFN pathway (including components of the adaptive immune response); for example, possibly exemplified by mutations in *ACP5* (which encodes tartrate-resistant acid phosphatase type 5) (**f**).

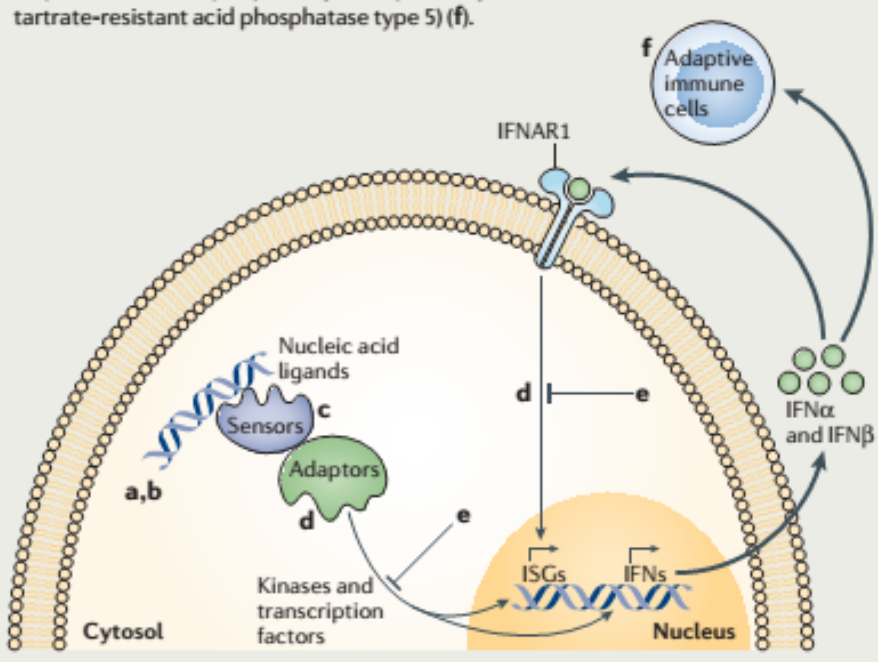


Figure 1.3 Possible mechanisms of Type-I interferonopathies; adapted from (Y. J. Crow & Manel, 2015).

1.2.1. Aicardi-Goutières Syndrome

Aicardi Goutières Syndrome (AGS) is the classical example of type-I interferonopathies and results from mutations in genes (e.g. TREX1, RNASEH2A, RNASEH2C, SAMHD1, ADAR or IFIH1) encoding proteins involved in nucleic acid metabolism (i.e. TREX-1) and signalling (i.e. IFIH1) (Lebon, Meritet, Krivine, & Rozenberg, n.d.). Among the clinical symptoms of AGS, brain damage and granulomatous skin lesions are most common and thought to be related to elevated type-I interferons in cerebrospinal fluid and serum (Lebon et al., 1988). Mutations in genes that are responsible for negative regulation of type I IFN signalling pathway or accumulation of genomic DNA species in cytosol due to inefficient nucleic acid removal cause cell-intrinsic autoinflammation through cGAS-STING-TBK1 pathway (Ablasser et al., 2014; Lebon et al., n.d.; Yang, Lindahl, & Barnes, 2007). Recently, it has been found that retro elements and products of disrupted DNA-damage response might be the source of endogenous nucleic acids initiating type-I IFN response in cytosol (Stetson, Ko, Heidmann, & Medzhitov, 2008; Volkman & Stetson, 2014). TREX-1, one of the mutated proteins in AGS, is a 3' exonuclease responsible for clearance of ssDNA in cytosol, preventing immune activation against endogenous nucleic acids. Its role in developing sterile inflammation with elevated IFN-α/β was confirmed experimentally in mice (Stetson et al., 2008). When deleted, other proteins such as RNase H2 that are critical in negative regulation of nucleic acid sensing can also cause AGS like symptoms in mice (Rabe, 2013). Additionally, ssDNA derived from endogenous retroelements such as long or short interspersed nuclear elements (SINEs and LINEs) and long terminal repeats (LTRs) also accumulate in cytosol of TREX-1 deficient cells (Stetson et al., 2008). In summary, whether from endogenous retroelements or as a result of disrupted DNA-damage response, accumulation of nucleic acid species in cytosol causes activation of multiple signalling pathways involved in anti-viral response and contribute to damage to host tissues.

1.2.2. Systemic Lupus Erythematosus (SLE)

SLE is a rare multifactorial autoimmune disease characterized by disturbance of type-I IFN function and development of autoreactive T and B cells specific to self-DNA and related nuclear proteins. A number of genetic and environmental factors contribute to SLE development which leads to the activation of both innate and adaptive immune systems (Y. J. Crow, 2011a; Y. Deng & Tsao, 2010). Despite the complexity of factors involved in disease development and pathology, several evidence suggest direct involvement of type-I IFNs in pathogenesis of SLE (Banchereau & Pascual, 2006; Theofilopoulos, Baccala, Beutler, & Kono, 2005). For example, patients undergoing IFNα immunotherapy develop SLE-like symptoms (Niewold, 2008). SLE patients reportedly have increased levels of interferon stimulated genes and elevated levels of type-I interferon associated cytokines/chemokines in circulation (Dall'era, Cardarelli, Preston, Witte, & Davis, 2005).Finally, genome wide genetic analyses revealed polymorphisms in genes involved in IFN pathway, suggesting that similar to AGS, SLE might also develop as a result of disturbance in type I IFN pathway (Yasutomo et al., 2001). However, it is important to note that not all SLE patients have deregulated IFN expression. Some SLE subtypes such as chilblain lupus are strongly associated with type I IFN signature and clinical manifestations such as, ulcerating skin lesions are reminiscent of those observed in AGS (Rice et al., 2007; Troedson et al., 2013).

1.2.3. STING Associated Vasculopathy with onset in Infancy (SAVI)

As mentioned in Section **1.1**, response to cytosolic DNA depends on the expression of the adaptor STING, culminating in type I IFN production. Gain-of-function mutations in STING has recently been shown to trigger an interferonopathy characterized by neonatal-onset systemic inflammation with severe cutaneous vasculopathy (STING-associated vasculopathy with onset in infancy, or SAVI) leading to extensive tissue loss and interstitial lung disease (Jeremiah et al., 2014; Liu et al., 2014). Liu et al were the first to show that *de novo* mutations in *TMEM173* encoding STING resulted in

ligand-independent dimerization and constitutive activation of STING (Liu et al., 2014). In accordance with continuous STING activation, authors observed elevated transcription levels of ISGs and interferon and related cytokines/chemokines in peripheral blood cells. Additionally, patient fibroblasts had increased expression of ISGs and up-regulated phosphorylated interferon regulatory factor 3 (IRF3) levels, suggestive of constitutive activation of type-I IFN pathway. Signal transducer and activator of transcription 1 (STAT1) was also constitutively phosphorylated in patient PBMCs, further confirming enhanced IFN activity. Interestingly, patients showed overlapping clinical pathologies with other known interferonopathies (such as AGS) including the presence of vasculitic skin lesions, which might be related to increased IFN-α/β levels in peripheral blood. Although similar findings suggest an overlapping phenotype between SAVI and AGS, there are distinct clinical phenotypes as well. Particularly, in contrast to SAVI patients, lung damage was not reported in AGS patients and unlike AGS, no neurological brain damage was observed in SAVI patients (Y. J. Crow & Casanova, 2014; Lebon et al., n.d.). These phenotypic differences between SAVI and AGS can be explained with various factors contributing to disease development such as differential expression of IFN-related proteins and differential exposure to environmental factors. Nonetheless, SAVI is an important example of type-I interferonopathy and further research on new cases of SAVI might be necessary to reveal a more general phenotype.

1.2.4. Ataxia Telangiectasia (AT) and other possible type-I interferonopathies

In addition to dysregulations in proteins of nucleic acid metabolism (e.g., AGS) and gain of function mutations in proteins of type-I interferon pathway (e.g., SAVI), recent evidence suggests that problems in DNA damage machinery could also account for elevated type-I interferon production. In several studies, cells exposed to DNA-damage showed a profile of increased expression of interferon stimulated genes (Brzostek-Racine, Gordon, Van Scoy, & Reich, 2011; A. R. Lam et al., 2014;

Moschella et al., 2013; Weichselbaum, 2008; Q. Yu et al., 2015). Of note, in these studies, DNA-damage was shown to cause leakage of genomic-DNA species into cytosol, thereby triggering the induction of interferon stimulated genes (ISGs) (Shen et al., 2015). Factors involved in DNA replication and repair such as Rad51 and RPA were recently shown to prevent such escape into cytosol, suggesting a mechanism to guard the cytosol against self-DNA (Wolf et al., 2016).

Interestingly, a recent report demonstrated that in Ataxia Telangiectasia (AT) patients, loss of function mutation in Ataxia-telangiectasia mutated (ATM), an important component of DNA repair machinery, results in spontaneous DNA lesions which subsequently leak to and accumulate in cytosol and cause type-I interferon activity via the cGAS-STING-TBK1 system (**Figure 1.4**) (Härtlova et al., 2015). Fibroblasts from AT patients had increased expression of interferon-related genes (Härtlova et al., 2015). Historically, AT is known as a complex multisystem disease with neurodegenerative manifestations, susceptibility to malignancies, radio sensitivity and a variety of immunodeficiencies and autoimmune syndromes (X. Deng, Ljunggren-Rose, Maas, & Sriram, 2005; Shao et al., 2009; Westbrook & Schiestl, 2010). Existence of autoimmune/autoinflammatory syndromes of unknown etiology suggests involvement of innate immune mechanisms resulting in undesirable sterile inflammation (Drolet et al., 1997; Harbort et al., 2015; McGrath-Morrow et al., 2010). AT patients are susceptible to chronic herpes simplex virus infections and suffer from respiratory bacterial infections, possibly due to defects in adaptive immunity (Kulinski et al., 2012; Schroeder & Zielen, 2014). Curiously, despite these chronic infections related to B-and/or T-cell defects, severe systemic viral infections are uncommon among AT patients, suggestive of possible type-I IFN mediated protection (Nowak-Wegrzyn, Crawford, Winkelstein, Carson, & Lederman, 2004; Schroeder & Zielen, 2014). Collectively, these findings suggest the presence of a common underlying disease etiology in AT and similar DNA-damage repair deficiencies (i.e excessive cytosolic DNA accumulation) causing consecutive type I IFN induction. Therefore, such diseases could in theory be considered as “type-I interferonopathies”.

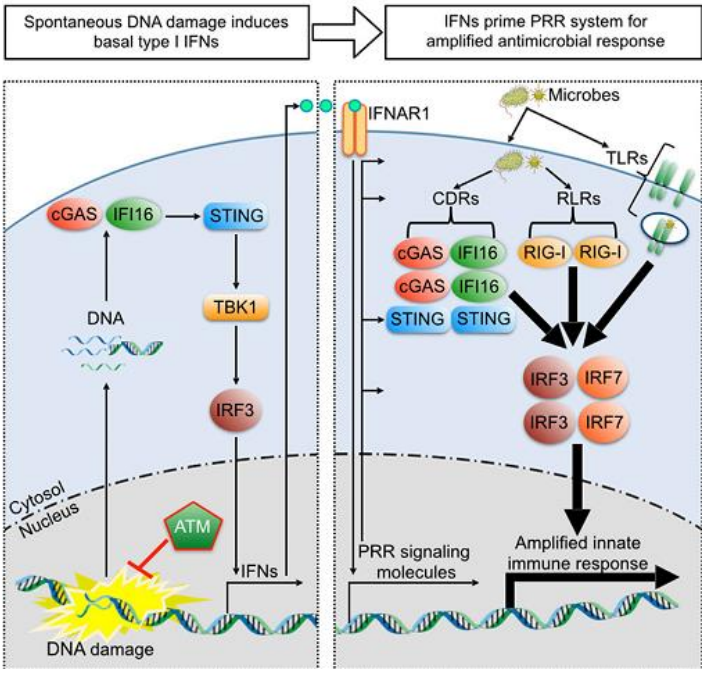


Figure 1.4 Type-I interferon induction through DNA damage in Ataxia Telangiectasia; adapted from (Härtlova et al., 2015).

1.2.5. Artemis Deficiency

Similar to ATM, Artemis, a nuclear protein with endonuclease activity, is also known to be involved in repair of double stranded DNA breaks and function as a DNA repair enzyme in complex with DNA-dependent protein kinase (DNA-PK) (Woodbine, Brunton, Goodarzi, Shibata, & Jeggo, 2011). Mutations in encoding gene *DCLRE1C* causes Artemis Deficiency and related severe combined immunodeficiencies (SCIDs) (Wang et al., 2005). In addition to its role in DNA repair, Artemis is important in V(D)J recombination, the process by which B cell immunoglobulin (IgG) and T-cell receptor (TcR) genes are arranged during development. Correspondingly, Artemis deficient patients are reported to be vulnerable to several bacterial and viral infections (Rooney et al., 2002). Interestingly, similar to some AT and SAVI patients, Artemis deficient patients also show a phenotype characterized by lung injury, unexplained

granulomatous skin lesions and erythematous plaques (Ege et al., 2005; Ijspeert et al., 2011; Liu et al., 2014; Villa, Notarangelo, & Roifman, 2008; Woodbine et al., 2011). Moreover, as a result of impaired DNA-damage repair response, Artemis deficient cells exhibit increased radio sensitivity which is reminiscent of AT (Wang et al., 2005; Woodbine et al., 2011). These findings suggest that Artemis deficient patients might also have a common immune dysregulation similar to type-I interferonopathies. To date, no such study to investigate type-I interferon-Artemis Deficiency interrelation has ever been undertaken.

1.3. Role of Neutrophils and NETosis in Autoimmune/autoinflammatory Diseases

Neutrophils (also known as polymorphonuclear granulocytes or PMNs) are the most abundant circulating leukocytes in the human body (Nauseef & Borregaard, 2014). They are considered as the first line of defence and have vital roles in innate immune system. As terminally differentiated cells, they have a very short lifespan (1-8 h) in circulation although this can extend to 4-5 days in the case of activated neutrophils (Kolaczkowska & Kubes, 2013; Pillay et al., 2010). For targeting of invading pathogens, neutrophils use several strategies including release of granules containing antimicrobial proteins such as neutrophils elastase (NE), myeloperoxidase (MPO) and matrix metalloproteinases (MMPs), phagocytosis of microorganisms in a reactive oxygen species (ROS) dependent manner and trapping of microbes by releasing DNA as a complex meshwork containing antimicrobial histone proteins, granule derived peptides and enzymes termed as neutrophil extracellular traps (NETs) (Bardoel, Kenny, Sollberger, & Zychlinsky, 2014). NETs released by neutrophils can function by immobilization, inactivation and killing of invading microbes and also can contribute to the activation of innate and adaptive systems during inflammation (Brinkmann et al., 2004; Garcia-Romo et al., 2011) . NETosis can be triggered by both microorganisms and sterile stimuli (Bardoel et al., 2014). Molecular mechanisms

behind NETosis needs further investigation to be fully characterized. However, during NET formation the following events are believed to occur: calcium release from ER, PKC activation, ROS generation, NADPH-oxidase assembly which is followed by movement of elastase to the nucleus in a MPO-dependent manner and eventual cleavage of histones to trigger chromatin decondensation. Consequently, membranes surrounding granules and nucleus are decomposed, which elicit mixing of DNA/histones of nucleus with granular content and eventual release of this complex meshwork from the cell (Metzler, Goosmann, Lubojemska, Zychlinsky, & Papayannopoulos, 2014). Having these mechanisms in action, neutrophils are truly powerful killers of immune system. However, in case of autoimmunity/autoinflammation, these features of neutrophils also contribute to tissue damage. Reports suggest that dysregulated activation of neutrophils/NETosis contribute to tissue/organ damage in several systemic autoimmune diseases such as SLE and Rheumatoid arthritis (RA) (Mariana J. Kaplan, 2011; Villanueva et al., 2011). Moreover, inefficient clearance of NETs is shown to exacerbate disease progression and further activate innate immune mechanisms during sterile inflammation (Garcia-Romo et al., 2011).

1.3.1. NETosis in Systemic Lupus Erythematosus (SLE)

Immune cells of SLE patients show deregulated immune activation and this phenomenon is thought to contribute to tissue damage through several ways. One of the effector cells contributing to disease pathogenesis in SLE are the neutrophils. Reports suggest that neutrophils isolated from SLE patients exhibit abnormal features such as formation of aggregates in cell culture media and spontaneous release of NETs which is not the case for neutrophils from healthy individuals (Abramson, Given, Edelson, & Weissmann, 1983; Al-Hadithy, Isenberg, Addison, Goldstone, & Snaith, 1982; Mariana J. Kaplan, 2011; Lindau et al., 2013; Y. Yu & Su, 2013). In fact, a distinct group of polymorphonuclear granulocytes called as low-density granulocytes

(LDGs) were identified in SLE patients' peripheral blood fractions (Denny et al., 2010; Hacbarth & Kajdacsy-Balla, 1986). This sub-group of inflammatory granulocytes are similar to neutrophils but exhibit a higher ability to form NETs, secreting more pro-inflammatory cytokines and possess more toxic content inside their granules (Denny et al., 2010). Studies suggest that even in the absence of positive stimulation (PMA), LDGs release NETs as much as neutrophils stimulated with PMA and NETs from LDGs are found to contain higher levels of antimicrobial peptides such as MPO and LL37 and immune stimulatory dsDNA (Cloke, Munder, Taylor, Müller, & Kropf, 2012; Villanueva et al., 2011). These features make LDGs more dangerous in terms of tissue damage and activation of other immune cells.

Inadequate clearance of NET DNA from periphery as a result of either NET DNA being more resistant to nucleases or presence of anti-DNA antibodies, cause type-I interferon production from plasmacytoid dendritic cells (pDCs) in some patients (L Rönnblom & Pascual, 2008) . DNA-anti-DNA antibody complexes further activate other neutrophils and trigger NETosis (Lande et al., 2011). Recently a report was published regarding type-I IFNs and neutrophil contribution to pathogenesis in SLE (Furumoto et al., 2016). These authors showed that a JAK-STAT inhibitor (tofacitinib), can ameliorate disease manifestations such as decrease in vasculopathy, less skin rashes, less circulating type-I cytokines and less tendency of neutrophils to undergo NETosis in mice (Furumoto et al., 2016). As recent studies indicate, a possible link between type-I IFNs and NETosis does exist and therefore, investigation of neutrophil functions and/or NETosis in pathogenesis of type-I interferonopathies is of great importance. Increased knowledge in mechanisms participating in NETosis may be useful to develop new treatments targeting specific molecules in NET formation as listed in **Figure 1.5.**

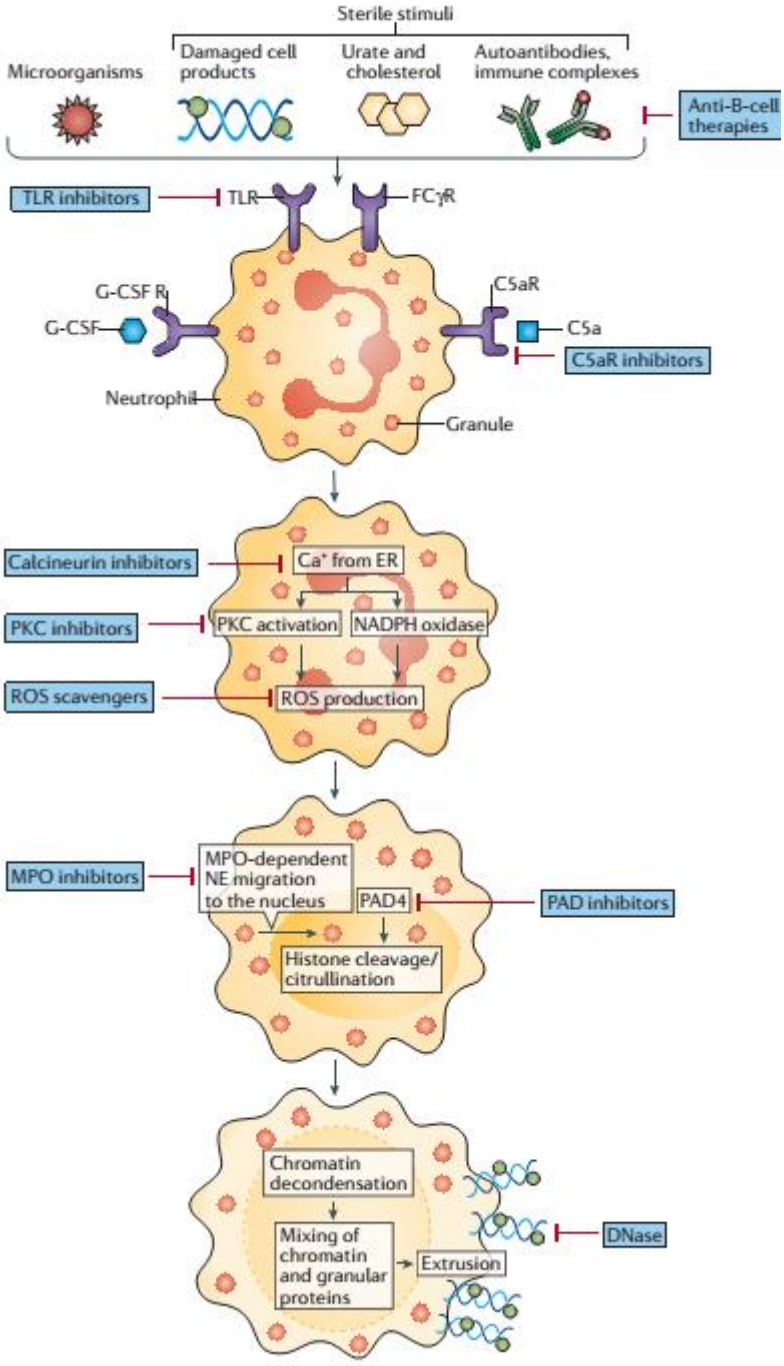


Figure 1.5 NETosis Activation pathways and possible therapeutic targets; adapted from (Gupta & Kaplan, 2016).

1.4. Aim of the Study

Inappropriate induction of type-I IFNs under sterile conditions can be detrimental to the host. A number of diseases with similar immune pathologies are now being classified as "type-I interferonopathies" (Y. J. Crow & Manel, 2015; Y. J. Crow, 2011b). Diseases under this category can share common clinical features such as vasculitic skin lesions (SAVI, AGS), pulmonary diseases (SAVI) and neurological symptoms (AGS) and these are thought to be the result of elevated type-I interferon activity (Y. J. Crow & Manel, 2015; Liu et al., 2014). To date, only one study has shown enhanced type-I IFN signature as a result of unrepaired DNA lesions in AT patients. However, circulating levels of type-I IFN associated cytokines/chemokine were not assessed in these patients (Härtlova et al., 2015). In this study, we hypothesized that as a result of mutations in DNA-damage repair machinery, immune cells from AT and Artemis deficient patients might also exhibit elevated type-I IFN signatures similar to other interferonopathies. To test this hypothesis, we compared the immune response characteristics of AT and Artemis deficient patients with healthy controls and that of a SAVI patient. In some experiments, immune cells isolated from an AGS patient with a confirmed TREX-1 loss of function mutation were also included as a positive control of classical type-I interferonopathy.

Mechanisms leading to type I IFN-mediated tissue damage are not well known. Based on findings from SLE patients pointing to a role of neutrophils in inducing tissue damage, in this thesis we hypothesized and tested the possibility of type I IFN-mediated neutrophil activation and aberrant NETosis involvement in PMNs of AT, Artemis deficient and SAVI patients. To date, interferonopathy-NETosis relationship has never been investigated and our results should be of interest for clinicians and basic scientists working in the field of primary immune deficiencies and/or autoinflammatory diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1.MATERIALS

All reagents, ligands and materials used in this study are listed below.

2.1.1. Reagents

Monoclonal antibody pairs (capture and detection), recombinant standard proteins and alkaline phosphatase conjugated streptavidin (Strep-AP) used for detection of cytokines by Enzyme linked Immunosorbent Assay (ELISA) were purchased from Biolegend (USA) or Mabtech (USA). Substrate for alkaline phosphatase, p-nitrophenyl phosphate sodium salt (PNPP), was purchased Thermo Fisher Scientific, USA. LumiKine human Interferon-α ELISA kit was purchased from Invivogen (USA) with its contents including capture antibody, recombinant standard proteins, Lucia conjugated detection antibody and Luciferase substrate Quanti-Luc. Cytometric Bead Array reagents including all assay buffers, reagents and Flex sets (**Table 2.1**) were purchased from BD Biosciences (USA). Flow Cytometry antibodies (**Table 2.2**) were purchased from Biolegend (USA) and BD Biosciences (USA). Quant-iT Picogreen dsDNA Assay Kit was purchased from ThermoFisher Scientific (USA). dsDNA dyes including Syto Orange, Sytox Green, and NucBlue were purchased from ThermoFisher Scientific (USA).

Table 2.1. CBA Flex Sets

Cytokine Flex Set	Catalog Number	Company
Human IL-1β	558279	BD Biosciences, USA
Human IL-6	558276	BD Biosciences, USA
Human IFN-α	560112	BD Biosciences, USA
Human IP-10	558280	BD Biosciences, USA
Human IL-8	558277	BD Biosciences, USA
Human TNF-α	560112	BD Biosciences, USA

Table 2.2. Florescent dye-conjugated Flow Cytometry Antibodies

Name	Clone	Catalog Number	Company
Alexa Fluor® 488 Anti-Human-pStat1 (pY701)	Clone4a	612596	BD Biosciences, USA
PE Anti-Human-IP10	J034D6	555049	BD Biosciences, USA
FITC Anti-Human CD14	M5E2	557153	Biolegend, USA
PE Anti-Human CD14	M5E2	557154	Biolegend, USA

2.1.2. Ligands Used in Stimulation Experiments

Names, signalling pathway information, catalog numbers, vendors and working concentrations of ligands used in stimulation experiments are listed in **Table 2.3**.

Table 2.3. Ligands used in stimulation experiments

Ligand	Pathway	Catalog #	Company	Concentration
2'-3'cGAMP	**STING**	tlrl-nacga23	Invivogen, (USA)	10 ug/ml
polydA:dT (transfected)*	**AIM2 Inflammasome**	tlrl-patn	Invivogen (USA)	5 ug/ml
HSV60 (transfected)	**cGAS-STING**	tlrl-hsv60n	Invivogen (USA)	5 ug/ml
ISD (transfected)	**cGAS-STING**	tlrl-isdn	Invivogen (USA)	10 ug/ml
PMA	**PKC**	tlrl-pma	Invivogen (USA)	50 ng/ml
Rec IFN-γ	**IFNGR**	-	BOEHRINGER INGELHEIM(Australia)	5-500 ng/ml
Rec IFN-α	**IFNAR-Jak-STAT**	-	Merck (USA)	5-500 ng/ml

*for transfected ligands, Lipofectamine2000 was purchased from ThermoFisher Scientific, USA (0.3 µl reagent was used per 200 µl well in a 96 well flat-bottom plate).

2.1.3. ELISA Kits

Names, vendors and catalog numbers of different ELISA Kits used in experiments are listed in **Table 2.4**.

Table 2.4 ELISA Kits Used for Cytokine Determination

Name	Catalog Number	Company
Human Interferon-α(pan specific)	3425-1A-6	Mabtech, USA
Human IL-1β	3415-1A-6	Mabtech, USA
Human IL-17	433915	Biolegend, USA
Human IP-10	430605	Biolegend, USA
Human IFN-α	luex-hifna	Invivogen, USA
Human IL-29 (IFN-λ1)	Mabtech, USA	Mabtech, USA

2.1.4. Cell Culture media, Buffers and other standard solutions

RPMI 1640 Medium and Tween20 were purchased from Thermo Fisher Scientific, USA. Na-pyruvate, HEPES, Penicillin/Streptomycin, Non-essential amino acids, and Molecular Biology Grade water were purchased from Hyclone, USA. Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), and ACK Lysis Buffer were purchased from Lonza, Switzerland. Normocin, Zeocin and Blasticidin were purchased from

Invivogen (USA). Other buffers, cell culture media and solutions used in this study (i.e. PBS, T-cell Buffer, Blocking Buffer, ELISA Wash Buffer, FACS Buffer, and Nuclease Buffer etc.) are described in **Appendix A**.

2.2. METHODS

2.2.1. Cell Culture

Cell lines of mouse or human origin or primary cells from patient peripheral blood were used in various experiments.

2.2.1.1.Isolation of PBMCs from Peripheral Blood

Blood samples (5-20 ml) from donors were collected into sodium citrate, heparin or EDTA containing vacutainers. In order to isolate peripheral blood mononuclear cells (PBMCs), Lympho-Paque Cell Separation Medium (Genaxxon Bioscience, Germany) was used. Peripheral blood samples were first diluted 1:1 with PBS and then 3 volumes of blood-PBS mixture was slowly layered onto 2 volumes of separation medium and centrifuged for 30 min at 400 x g at room temperature (RT) with "break off" setting. Plasma samples were collected from the upper layer and centrifuged at 2000 x g for 10 min, supernatants were taken into new falcon tubes and stored at -80 ^{o}C for cytometric bead array (CBA) and NETosis assays. Buffy coat between plasma and separation medium which contained mononuclear cells, were carefully collected and transferred into new sterile 50 ml falcon tubes by using a sterile plastic Pasteur pipette (Fisher Scientific, USA). Tubes containing mononuclear cells were then filled with wash medium (RPMI-1640 supplemented with 2% FBS) and centrifuged for 10 min at 400 x g at RT. Supernatants were removed and pellets were washed two more times with wash medium (10 min at 400 x g at RT). Following washing, PBMC pellets were re-suspended in pre-warmed RPMI-1640 containing 10% FBS and cells were counted either by using a Neubauer-improved haemocytometer (Marienfeld, Germany) or by flow cytometry.

2.2.1.2.Isolation of PMNs from Peripheral Blood

Neutrophil polymorphonuclear granulocytes (PMNs) were isolated using 3% dextran/PBS (w/v) sedimentation. Following PBMC isolation as described **in section 2.2.1.1,** separation medium was aspirated until RBC + granulocyte pellets remained. Pellets were then re-suspended in a volume of PBS equal to initial blood volume, and then mixed with an equal volume of 3% dextran. Tubes were incubated in an upright position for 30 min at RT until a clean interface between RBCs and granulocytes was observed. Upper layers (granulocytes) were then collected using a sterile Pasteur pipette into new tubes. Tubes containing granulocytes were then filled with cold PBS and centrifuged for 8 min at 250 x g at 4°C. Supernatants were removed and residual RBCs were lysed by incubation in 2 ml ACK Lysing Buffer (Lonza, Switzerland) for 5 min at RT. Following RBC lysis, pellets were washed with cold PBS two more times (8 min at 250 x g at 4°C). Final pellets containing PMNs were re-suspended in RPMI-1640 supplemented with 5% Oligo FBS and cells were counted using flow cytometer.

2.2.1.3.Cell Counting

For counting of cells using a Neubauer-improved haemocytometer, at the end of the isolation protocols, appropriately diluted PBMCs, PMNs or cell lines were re-suspended in 1 ml growth medium. Then, 20 µl of sample was taken from the original solution, diluted 2-4 x according to cell type and mixed with equal volume of Trypan Blue solution. 20 µl of each sample was applied to the chamber area by capillary action and average number of cells in 1 big square (6.25 nl volume) was counted. Number of cells in 1 ml was calculated using the formula below:

Number of cells/ml = N x 16 x Dilution Factor x 10^4

N: average number of cells in one square

For counting with flow cytometer (BD Accuri C6, USA), 20 µl of samples were taken into 10 mL isotonic solution. Then, 2-3 drops of ZAP-OGLOBIN II Lytic Reagent (Beckman Coulter, USA) was added in order to lyse the remaining RBCs. Samples were acquired in flow cytometer by gating on live cells and excluding dead cells and debris. Number of events in 20 µl was determined using the formula below:

Number of cells/ml = N x 5000 (Dilution Factor) x 500

N: number of events counted in 20 µl diluted sample

2.2.2. Stimulation Experiments

Details of stimulation experiments and protocols for ELISA and CBA are listed in the following sections.

2.2.2.1.Stimulation of PBMCs with Type-I Interferon Inducing Ligands

Isolated PBMCs from patients and healthy blood donors were used in various stimulation experiments. For ELISA and CBA stimulations, 400,000 cells/well were used in 96-well flat-bottom plates. Stimulants were prepared in RPMI-1640 supplemented with 5% FBS according to the concentrations given in **Table 2.3.** Some of the stimulants were delivered to the cytosol by transfection with Lipofectamine2000 (ThermoFisher Scientific, USA) according to the following protocol: Amount of the ligand to be transfected was calculated according to well number and mixed with Lipofectamine2000 (0.3 µl/well) in OptiMEM medium (20-50 µl) and incubated for 5 min to allow for complexation. The mixture was then diluted to its final volume (50µl x number of wells) using RPMI-1640 supplemented with 5% FBS. Un-transfected stimulants were prepared directly in medium, and both transfected and un-transfected ligands were added onto cells (150 µl cells + 50 µl stimulants; 200 µl final volume). After 24 hours incubation at 37°C, plates were spun down by at 300 x g, 5 min and

supernatants (~180 µl) were collected and stored at -20°C until further use for cytokine determination by ELISA and/or CBA.

For stimulation experiments where cells were to be analyzed by flow cytometry, $1x10^6$ cells /ml were used. Stimulations were performed in 15 ml falcon tubes (incubated at a 45°C angle with loose caps in a CO_2 incubator). Ligands were prepared as described above but the Lipofectamine2000 was adjusted to be 2.5 µl/test tube. Samples were incubated for 20 min for pSTAT1 staining or 7 hr for intracellular IP-10 staining (with brefeldin A addition after the initial 5 h of incubation). Cells were fixed, permeabilized, and stained with appropriate fluorochrome conjugated antibodies as described in **Table 2.2**.

2.2.2.2. Determination of Cytokine Levels by Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants that were collected and stored at -20°C following stimulation assays were used to determine cytokine levels in various experiments. Immulon 2HB plates (ThermoFisher, USA) were coated with 50 µl monoclonal capture antibodies in PBS or bicarbonate buffer (4 µg/ml for panIFN-α; 1/200 dilution for IL-17 and IL-29; 1 µg/ml for IFN-α LumiKine) and incubated at 4°C overnight. The following day, content of plates were expelled by inversion and 200 µl blocking buffer (PBS containing 2% BSA) was added to each well. After 2 h of incubation, wells were emptied as before and then plates were washed 5 times with washing buffer (1 X PBS containing 0.05% Tween 20; 5 min incubation for each wash cycle) and rinsed with ddH_2O for three times and blot dried by hitting the plates onto absorbent paper. Cell supernatants (50 µl/well) and 2-fold serially diluted recombinant proteins (50 µl/well in assay buffer, starting from concentrations of 1-20 ng/ml) were added to the wells. After 2 h incubation at RT, plates were washed as previously described and dried. Plates were incubated with biotinylated detection antibody (1:1000 diluted in T-Cell buffer; 50 µl/well) at 4°C overnight. The following day, plates were washed as

described before, and streptavidin-alkaline phosphatase (Strep-AP) was prepared freshly at least 1-2 hours prior to use (1/1000 dilution in PBS) and then added to the wells (50 µl/well). After 1 hour incubation at RT, plates were washed for the last time and developed by addition of 50 µl freshly made PNPP substrate. Colour development was observed and recorded at 405 nm using a Multiskan ELISA Plate Reader, (ThermoFisher Scientific, USA) at 30 min intervals until an S shaped graph developed for recombinant standards. ELISA kits used in different experiments were listed in **Table 2.4.**

For LumiKine ELISA, MaxiSorp white flat-bottom 96-well ELISA plates (Invivogen, USA) were coated with 100 µl/well capture antibody (diluted to 1 mg/ml in coating buffer). Plates were covered with an adhesive seal and incubated at 37°C for 2 h. Content of plates were expelled by inversion and 200 µl blocking buffer (PBS containing 2% BSA) was added to wells. 2 h later, contents of plates were expelled again and plates were washed 3 times with washing buffer (PBS containing 0.05% Tween 20) and blot dried by hitting the plates onto absorbent paper. 100 µl/well samples or 2-fold serially diluted standard recombinant proteins (5 ng/ml to 10 pg/ml) were added to appropriate wells. After 2 h incubation at 37°C, plate contents were expelled and washed as described previously. Then, 100 µl/well of Lucia Conjugated Antibody (diluted to 30 ng/ml in Reagent Diluent) was added to the wells and plates were covered with an adhesive seal for 2 h incubation at 37°C. At the end of the incubation period, plates were washed for the last time, 50 µl/well Quanti-Luc (Invivogen, USA) solution was added and immediately proceeded to reading by adjusting the integration time to the minimum value (0.1-0.5 sec.) using a Synergy HT Microplate Reader (BioTek, China). Relative light units (RLUs) were converted to pg/ml values using 4-parametric non-linear equation.

2.2.2.3. Determination of Cytokine Levels in Cell Culture Supernatants and Plasma by Cytometric Bead Array

Cell culture supernatants were stored at -20°C as described previously. Plasma samples from healthy and patient donors were kept at -80°C until further use for

cytokine determination by CBA. CBA was performed according to manufacturer's instructions, and Flex sets used were listed in (**Table 2.1**). Briefly, standards for each cytokine were reconstituted and serially diluted as in the product manual (10 pg-2500 pg/ml, by 2-fold dilutions). Number of Human Soluble Protein Flex Set Capture Beads were determined before the experiment and mixed vigorously for 10-second by vortexing. Beads were then diluted in Capture Bead Diluent for cell supernatants and Serum Enhancement Buffer for plasma samples in a total volume of 50 µl x number of tests. Prepared capture beads were incubated for 30 min at RT protected from light. Plasma samples were diluted 1:4 in assay buffer provided with the kit. PE detection reagents were also prepared according to number of tests (in a total volume of 50 µl x number of tests) in Detection Reagent Diluent provided with the kit. After preparation of all necessary reagents (capture beads, standard solutions, and detection reagents) assay procedure was followed as such: first 50 µl of cell supernatants or plasma samples were added to the wells of 96-well U bottom plates. Then, 50 µl/well diluted capture beads were added onto samples and incubated for 1h at RT protected from light on a microplate shaker. After 1 h incubation, 50 µl/well mixed PE Detection Reagent was added onto the wells and incubated for 2h at RT protected from light on a microplate shaker. After 2h incubation, plates were centrifuged at 200 x g for 5 minutes and supernatants were aspirated carefully. Remaining pellets were re-suspended in 200 µl wash buffer provided with the kit and acquired on an Accuri C6 Flow Cytometer (BD Biosciences, USA) by using a template as instructed in the manual. Results were analysed using FCAP array software.

2.2.2.4.Flow Cytometric Analysis of Samples

Protocols for flow cytometry analysis including cell fixation, cell surface marker staining, intracellular cytokine staining and pSTAT1 staining are listed in the following sections.

2.2.2.4.1. Cell Fixation

Following stimulation experiments, cells were centrifuged at 250 x g for 5 minutes at RT and supernatants were collected. Remaining cell pellets were fixed using 4 % paraformaldehyde (ThermoFisher Scientific, USA). Briefly, 100 µl fixative was added while vigorously vortexing onto the cells and incubated for 15 min at RT. Cells were then washed with FACS Buffer **(Appendix A)** by addition of 1 ml FACS Buffer and centrifugation at 250 x g for 10 min. Following centrifugation, supernatants were aspirated and cells were re-suspended in FACS buffer for further staining as described in the next section.

2.2.2.4.2. Cell Surface Marker Staining

After fixation, cells were centrifuged and supernatants were discarded. Remaining cell pellets were incubated with 100 µl of 1-10 µg/ml of fluorochrome conjugated antibodies (as detailed in **Table 2.2**) for 30 min at RT protected from light. Following incubation, cells were washed twice with FACS buffer by centrifugation at 300 x g for 10 min. After washing steps, cells were re-suspended in 200 µl PBS and analysed on an Accuri C6 Flow Cytometer.

2.2.2.4.3. Intracellular Cytokine Staining

For intracellular IP-10 staining, cells were treated with relevant stimulants and incubated for 5 h (37ºC, 5% CO_2). 10 µg/ml Brefeldin A (Biolegend, USA) was added to block secretion of IP-10 to the medium and samples were incubated further for 2 h. At the end of this incubation period, cells were fixed as described before and stained for cell surface markers when indicated. Following cell surface staining, cells were permeabilized and stained in 1 µg/ml PE-IP-10 containing permeabilization buffer (ThermoFisher Scientific, USA) for 30 min at RT protected from light. Following 30

min incubation, cells were washed with FACS buffer, re-suspended in 200 µl PBS and analysed by Flow cytometry.

2.2.2.4.4. pSTAT1 Staining

Isolated healthy and patient PBMCs ($1x10^6$/ml) (untreated or treated with recombinant IFN-a (50-500 ng/ml)) were incubated for 20 min and fixed as described previously. Cells were then pelleted and 500 µl ice-cold 95% methanol was added onto pellets while vigorously vortexing. After incubation for 10 min on ice, cells were washed twice with FACS buffer. Pellets were re-suspended in 100 µl FACS buffer containing 3 µg/ml Alexa 488 conjugated anti-human pSTAT1 (**Table 2.2**) and incubated 45 min at RT protected from light. After 2 more washes with FACS buffer, cells were re-suspended in PBS and analysed by Flow Cytometry.

2.2.2.5.Neutrophil Stimulation Experiments

Protocols for NETosis imaging and quantification are given in the following sections.

2.2.2.5.1. Imaging of Neutrophil Extracellular Traps (NETs)

Neutrophils isolated from healthy donors or patients were used in NETosis assays. 80,000 cells/well were seeded onto 96-well flat bottom plates and were left untreated or were treated with PMA as a neutrophil extracellular trap (NET) inducer (50 ng/ml) or with recombinant IFN-α (5-500 ng/ml). In another set of experiments, neutrophils from healthy donors were treated with either healthy or patients plasma samples (20%) and with PMA. Stimulations and incubations were carried out in RPMI-1640 supplemented with 5% Oligo FBS. After 5h incubation at 37°C, cells were stained with 10 µl/well NucBlue (ThermoFisher Scientific, USA), and 5 µM Syto Orange (ThermoFisher Scientific, USA) for 30 min. Samples were then visualized using the

Floid Cell Imaging Station (ThermoFisher Scientific, USA). During imaging, wells were screened carefully and representative images were captured from each well. Images were then analysed using the LSM Image Browser software (Zeiss, Germany).

2.2.2.5.2. Quantification of Neutrophil Extracellular Traps (NETs)

200,000 healthy donor or patient neutrophils were transferred to 96-well flat bottom plates and were left untreated or treated with PMA as a neutrophil extracellular trap (NET) inducer (50 ng/ml) or treated with recombinant IFN-α or remained untreated(5-500 ng/ml).. In another set of experiments, neutrophils from healthy donors were treated with either healthy or patients plasma samples (20%) and with PMA. Stimulations and incubations were done in RPMI-1640 supplemented with 5% Oligo FBS. After 5h incubation at 37°C, samples were treated with 500 mU/ml of micrococcal nuclease (MNase) in 50 µl nuclease buffer for 30 min at 37°C and the reaction was terminated using 5 mM EDTA. Plates were then centrifuged at 200 x g for 5 min and supernatants were collected into new plates and stored at -80°C for quantification with Picogreen. For quantification of released neutrophil extracellular traps (NETs), Quant-iT Picogreen dsDNA Assay Kit (ThermoFisher Scientific, USA) was used according to manufacturer's instructions. Briefly, 100 µl of supernatants were transferred into separate black, opaque, flat-bottomed 96-well plates, and Picogreen reagent was diluted 1:200 in TE Buffer (freshly made). Diluted Picogreen reagent was then added onto supernatants. After 2-5 min incubation at RT in dark, amount of extracellular DNA was quantified using a standard curve based on known concentrations of lambda DNA (0.1-10 µg/ml). Fluorescence values were recorded on a Synergy HT Microplate Reader (BioTek, China) using excitation/emission settings of 485/20 and 525/20.

2.2.3. Statistical Analysis

For statistical analysis, Mann Whitney U-test was used to compare healthy vs. patient groups. For all comparisons, 95% confidence intervals were used and P values of <0.05 were considered significant. All analyses were done with the GraphPad Prism 6.1 (GraphPad Software Inc., USA) software.

2.2.4. Ethics Statement

This study is approved by the Human Research Ethics Committee of Hacettepe University Faculty of Medicine, Necmettin Erbakan University Faculty of Medicine and Cukurova University Faculty of Medicine. All the healthy and patients samples are provided with the consent of donors or their families.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Analysis of Type I and III IFN associated Cytokine/Chemokine Levels in Patient Plasma Samples

Inappropriate type I IFN production in the absence of any infection can promote severe inflammation and is thought to result in immune pathology in an increasing number of diseases that are being classified as “type I interferonopathies” (Y. J. Crow, 2011b). For example, in Aicardi-Goutières (AGS) and STING-associated vasculopathy with onset in infancy (SAVI) patients, vasculitic skin lesions, interstitial lung disease (SAVI) and neurological manifestations (AGS) are commonly observed and these are associated with elevated type I interferon production. To test the hypothesis that the unexplained granulomatous skin lesions and interstitial lung disease observed in Ataxia Telangiectasia (AT) and Artemis deficient patients may also result from an increased type I IFN signature, we compared the immune status of AT and Artemis deficient patients to healthy controls and to a patient with SAVI. In some experiments, samples from an AGS patient with a confirmed TREX-1 loss of function mutation was also included as a positive control of classical type I interferonopathy.

To assess whether AT and Artemis deficient patients displayed increased levels of circulating cytokines and chemokines associated with type-I and III interferon pathways (IP-10, IFN-α, IL-29 (IFN-λ), plasma samples from SAVI, AT, Artemis and

TREX-1 deficient patients and healthy controls were analysed for their presence using CBA or ELISA as described in Sections **2.2.1.1, 2.2.2.2** and **2.2.2.3.** The following sections summarize plasma levels of individual cytokines/chemokines that were measured.

IP-10 is considered as an important signature of type-I interferon related disease and there are several reports showing significantly higher levels of IP-10 in plasma of SAVI, Aicardi Goutières and SLE patients. (Liu et al., 2014; Lit, Wong, Tam, Li, & Lam, 2006; Takanohashi et al., 2013). To date only one study showed that unrepaired DNA lesions in AT patients led to release and accumulation of single stranded DNA species into the cytosol, inducing type I IFN production (Härtlova et al., 2015). However, this study failed to assess circulating levels of Type I IFN-associated chemokines/cytokines in AT patients. Moreover, since direct evidence that AT and related DNA damage repair deficiencies such as Artemis deficiency could indeed be considered as type I interferonopathies is non-existent, we first measured plasma levels of this chemokine in our target patient groups. Results showed that similar to plasma IP-10 levels sampled from a SAVI patient on 4 different occasions and a TREX-1 deficient patient, AT and Artemis patient groups displayed significantly higher levels of circulating IP-10 when compared to healthy donors (**Figure 3.1.a)**.

Healthy donors had very low levels of IP-10 in their plasma (6.1 ± 5.9 pg/ml) whereas the concentration of this chemokine was found to be elevated 9- and 14-fold in AT and Artemis deficient patients, respectively (57.3 ± 59 and 86.6 ± 79.1 pg/ml; $p=0.0005$ and $p<0.0001$). Similar results were obtained with plasma samples collected 6 months after the initial experiment, indicating that the elevated IP-10 levels were not related to any infections but was an indicator of a persistent inflammation (Figure 3.1 b). As expected, the SAVI patient had the highest plasma concentration of IP-10 which remained consistently high when measured on 4 different occasions (~137-fold higher than healthy controls; 827 ±302 pg/ml, **Figure 3.1.a** and **b**). Similarly, TREX-1

deficient patient plasma also yielded high levels of IP-10 (119 pg/ml), confirming previous published results (Takanohashi et al., 2013).

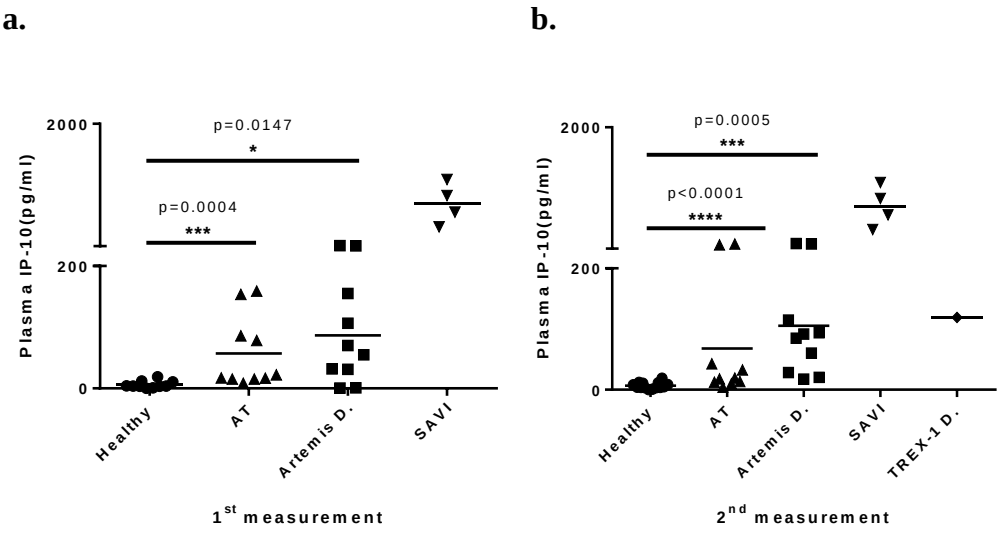


Figure 3.1 AT and Artemis deficient patients have persistently elevated levels of circulating IP-10 suggestive of sterile inflammation.

Patient plasma IP-10 levels were assessed using cytometric bead array as explained in Section **2.2.2.3**. Following flow cytometric analysis of bead samples and standards, pg/ml concentrations were calculated using the FCAP software. Healthy donors, AT and Artemis deficient patient groups consisted of 10 individuals each. **a.** shows the initial levels of IP-10 in AT and Artemis patient plasmas. **b.** shows IP-10 concentrations measured using plasma samples collected 6 months after the initial experiment). Statistical significance was tested using the Mann Whitney U-test. *(*p<0.05, *** p<0.001, **** p<0.0001)*

These findings are of interest as they suggest that similar to SAVI and Aicardi Goutières patients, AT and Artemis deficient patients have dysregulated production of IP-10, suggestive of constitutive activation of interferon signalling pathway.

IFN-α subtypes are made and released by host cells following engagement of endosomal and/or cytosolic nucleic acid sensors and downstream activation of type-I interferon signalling pathways. Pathogen-associated nucleic acids are recognized by Toll-like receptors (TLR) 3, 7/8 and 9 in endosomes, or by cytosolic sensors like cGAS, STING, MDA-5, and RIG-I. like receptors (RLRs), inducing production and secretion of type-I interferons (IFN-α/β) (Hornung et al., 2006; Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003; J. Wu et al., 2013). However, some of these sensors can also be activated by host nucleic acids such as in the case of Aicardi Goutières patients, resulting in sterile inflammation (Ablasser et al., 2014). Previous studies have already shown that patients with SLE and Aicardi Goutières have increased levels of circulating IFN-α (Barth, 2002; M. K. Crow, 2014). These studies also indicate that IFN-α is one of the key cytokines instigating pathology in these patients (Banchereau & Pascual, 2006; Hua, Kirou, Lee, & Crow, 2006). To our knowledge, circulating IFN-α levels were not assessed in SAVI, AT and Artemis deficient patients before. . Therefore in initial experiments, we attempted to measure the plasma levels of this cytokine in patient groups using CBA. However, using this method, we failed to detect IFNα in any of the plasma samples tested.

As mentioned before, interferon alpha has several subtypes (such as IFN-α1, IFN-α2, IFN-α8, IFN-α10, IFN-α14 and IFN-α21), and it is a possibility that the CBA anti-IFN-α Ab was unable to detect the major alpha interferon subtype in our patients' plasma. Of interest, the product sheet of this CBA Kit does not specify the subtype specificity of this capture antibody. Furthermore, several publications comment on the difficulty of detecting IFN-α from plasma samples due to the presence of heterophilic antibodies that interfere with most assay protocols, culminating in false negatives or false positives (Kricka, 1999; Sapin, 2008). This phenomenon might be an influential factor especially if the levels of interferon alpha were already too low. Therefore, to ensure that we could measure a broad spectrum of IFN-α subtypes using a more sensitive approach, we switched to testing using a luciferase based ELISA kit (Human interferon alpha (multiple subtypes) bioluminescent ELISA kit; Invivogen, USA) as

described in Section **2.2.2.2**. Using this method we were able to show that similar to SAVI and Aicardi Goutières patients, AT and Artemis deficient patients had higher concentrations of IFN-α in their plasma (**Figure 3.2**). Whereas healthy donors had very low levels of this cytokine in their plasma (29.45 ± 18.28 pg/ml), AT and Artemis deficient patients had 5- and 7-fold more, IFNα, respectively (157.0 ± 37.34 and 218.8 ± 42.95; p=0.012 and p= 0.0013). SAVI patient had also similar levels of this cytokine with AT and Artemis deficient patients (~5 fold higher than healthy controls, 147.3 ± 10.97) which remained consistently high when measured twice within a 3 month interlude. Aicardi Goutières patient showed the highest levels of IFN-α in the plasma (381.392 pg/ml).

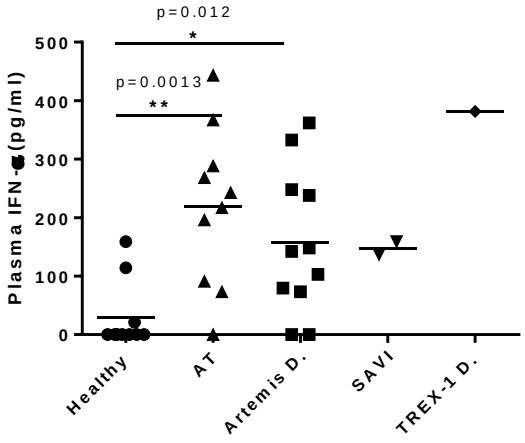


Figure 3.2 AT and Artemis deficient patients have elevated levels of circulating IFNα.

Plasma samples collected from 11 healthy, 10 AT, 10 Artemis deficient, 1 SAVI and 1 Aicardi Goutières patient were assessed by ELISA as explained in Section 2.2.2.2. Data is given as pg/ml and was quantitated based on known concentrations of recIFNα. Statistical significance was tested using the Mann Whitney U-test *(* $p<0.05$, ** $p<0.001$).*

These results further demonstrate that AT and Artemis deficiency are disorders associated with an upregulation of type-I interferon, suggesting that new therapeutic

approaches such as the use of anti-IFN antibodies as in the case of SLE patients might be of benefit (Banchereau & Pascual, 2006; Hua et al., 2006).

Until recently, it was thought that type-I interferons were the only interferon species that are produced in response to viral infections. A new type of interferon species was more recently identified (classified as type-III or IFN-λs) (W. Hou et al., 2009; Sui, Zhou, Chen, Lane, & Imamichi, 2014). Similar to type-I IFNs, type-III IFNs are also induced during viral infections and are important in anti-viral defense. Class III IFNs are produced with slower kinetics and protect mucosal surfaces like the gut and reproductive tract (Odendall & Kagan, 2015; Wack, Terczyńska-Dyla, & Hartmann, 2015). To date, only studies with Lupus patients showed that disease activity correlated with IFN-λ1 levels in the plasma, and stimulation of PBMCs with IFN- λ1 caused increased secretion of IP-10 (Q. Wu, Yang, Lourenco, Sun, & Zhang, 2011). Since type I and III IFNs have similar signalling pathways, we asked whether type-III interferons contributed to the inflammation observed in patients with Ataxia Telangiectasia (AT) and Artemis Deficiency. To address this question we assessed plasma levels of IL-29 (most prominent type III IFN species in human). Results showed that patients indeed had elevated levels of IL-29 (**Figure 3.3**). Although healthy donors also had detectable levels of IL-29 in their plasma (67.93 ± 24.89 pg/ml), circulating concentrations were found to be ~4- and 5-fold increased in AT and Artemis deficient patients (232.4 ± 26.04 and 267.2 ± 37.30 pg/ml; p=0.0017 and p=0.001, respectively) and comparable levels were observed in SAVI patient plasma (282.5 ± 5.5 pg/ml). Similar to IFN-α levels, TREX-1 deficient Aicardi Goutières patient showed the highest levels of IL-29 (~12 fold higher than healthy subjects; 771.5 ± 18.50 pg/ml).

These results are to our knowledge the first to demonstrate type III IFN dysregulation not only in SAVI but also in AT and Artemis deficient patient groups.

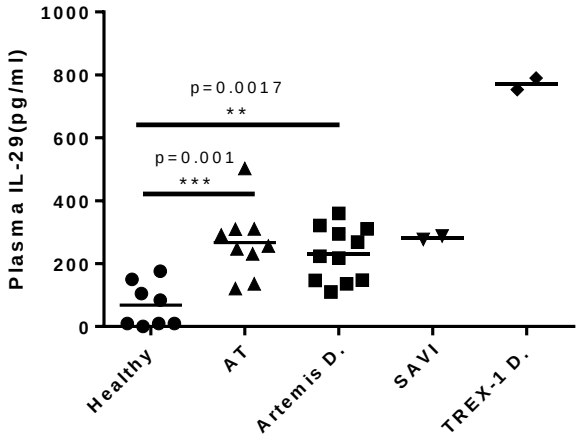


Figure 3.3. 4 AT and Artemis deficient patients have elevated levels of circulating IFNλ1

Plasma samples collected from 8 healthy, 9 AT, 10 Artemis deficient 1 SAVI and 1 Aicardi Goutières patient were assessed by ELISA as explained in Section **2.2.2.2.** Data is given as pg/ml and was quantitated based on known concentrations of recIL-29. Statistical significance was tested using the Mann Whitney U-test *(* $p<0.05$, ** $p<0.001$).*

3.2.Analysis of Pro-inflammatory Cytokine Levels in Patient Plasma Samples

After confirming that all patients tested had elevated type-I and III interferon associated-cytokines in their plasma, we next asked whether classical pro-inflammatory cytokines also contributed to disease pathology. Since type I interferonopathies are associated with elevated type I IFN signature in the absence of dysregulation in other pro-inflammatory cytokines, we hypothesized that circulating levels of these cytokines should be similar to those observed in healthy donors.

Concentration of these "pro-inflammatory" cytokines, are found to be increased during infection or inflammation (Winter et al., 2004). Among these, IL-1β maturation and secretion depends on the formation of a multi-protein complex called as the inflammasome. TNF-α is another important cytokine produced by activated macrophages and other immune cells during inflammation (Dinarello, 2000). IL-6 also contributes to inflammation and plays a pivotal role in initiation of acute phase responses. Concentrations of these cytokines are found to be increased in several auto-inflammatory/auto-immune diseases, such as Familial Mediterranean Fever, Chron's disease and rheumatoid arthritis (Gustot et al., 2005; Koga et al., 2016). To assess whether these patients had any pro-inflammatory cytokine elevation in their plasma, we performed CBA analysis of these cytokines. Data given in **Figure 3.4.a&b** indicates that almost all tested subjects had undetectable levels of TNF-α and IL-1β in their plasma. Although IL-6 was detectable in some of the healthy and patients plasma samples (**Figure 3.4.c**), average values were not significantly different from each other (Healthy: 1.73 ± 2.49; AT: 6.12 ± 19.38; Artemis: 5.72 ± 8.25 pg/ml respectively).

IL-17 is another pro-inflammatory cytokine that is produced mainly by Th-17 type of helper T cells, (Jin & Dong, 2013)**.** It contributes to clearance of extracellular bacterial and fungal (Moschen, Geiger, Krehan, Kaser, & Tilg, 2008). In recent years, studies revealed a role for IL-17 in autoimmune diseases where experiments showed that IL-17 is upregulated in the site of inflammation in autoimmune diseases like Multiple Sclerosis (MS) and Inflammatory Bowel Disease (IBD) (Komiyama et al., 2006; J. J. Yu et al., 2007). In light of such reports we questioned whether IL-17 also contributed to disease pathogenesis in AT, Artemis deficient and SAVI patients. For this, we performed IL-17 ELISA from patient and healthy plasma samples. Results show that IL-17 levels were similar to healthy subjects (**Figure 3.4.d*)***, and no significant differences was observed between tested groups.

Collectively, these results suggest that the inflammation associated symptoms seen in AT and Artemis deficient and SAVI patients are independent of classical pro-inflammatory cytokines but dependent on type-I interferon related cytokines/chemokines.

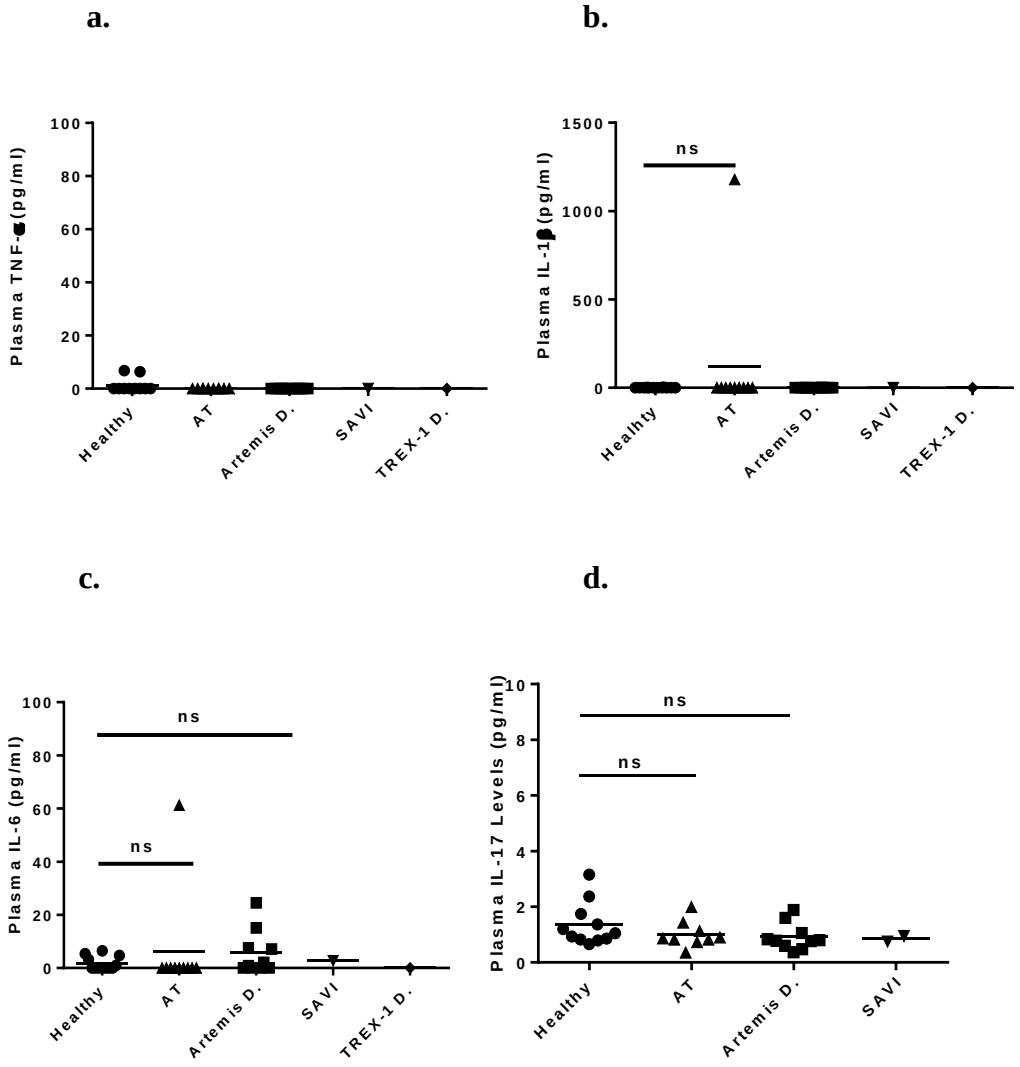


Figure 3.4 Plasma levels of TNF-a, IL-1b, IL-6 and IL-17 were similar in patient groups and healthy controls

TNF-a, IL-1b and IL-6 levels in plasma were assessed using cytometric bead array. IL-17 quantitation was based on ELISA. Healthy donors, AT and Artemis deficient

patient groups consisted of 10 individuals for CBA and 11, 9, and 10 individuals for ELISA. Statistical significance was tested using the Mann Whitney U-test *(p values smaller than 0.05 was considered as significant*).

IL-8 (CXCL-8) is a chemokine that is secreted mainly by activated monocytes but can also be secreted from other leukocytes and mainly functions in neutrophil recruitment (Mukaida, 2003). Reports indicate that even in non-inflamed tissues IL-8 is produced transiently (Boekholdt et al., n.d.). There are reports indicating that type I IFNs inhibit IL-8 expression in a variety of cell types (Benveniste et al., 2016; Oliveira, Sciavolino, Lee, & Vilcek, 1992). Therefore, we also assessed IL-8 levels in AT, Artemis deficient and SAVI patients' plasma by CBA. Results are shown in **Figure 3.5**. Levels of IL-8 were highest in healthy plasma samples (81.27 ± 15.85 pg/ml) which was higher than plasma levels of IL-8 in Artemis deficient and AT patients that had 5-fold (16.48 ± 3.11 pg/ml, p=0.0004) and ~3-fold (26.06 ± 4.452 pg/ml, p=0.0239) lower levels of IL-8 than healthy controls, respectively. Lowest concentrations were observed in the SAVI patient (8.445 ± 1.355 pg/ml) whereas TREX-1 deficient patient had undetectable levels of this cytokine in plasma. These findings suggests that unlike healthy subjects, increased levels of type-I interferons in patients supressed the baseline levels of IL-8 in plasma confirming the inhibitory role of IFNs on IL-8 production (Benveniste et al., 2016; Oliveira et al., 1992).

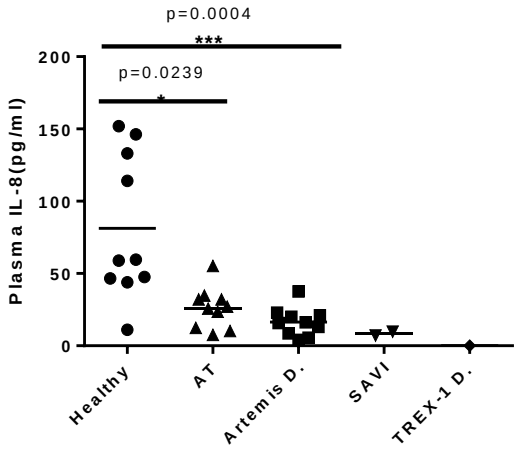


Figure 3.5 Plasma levels of IL-8 is reduced in patient groups

IL-8 levels in plasma were assessed using cytometric bead array as explained in Section **2.2.2.3**. Following flow cytometric analysis of bead samples and standards, pg/ml concentrations were calculated using the FCAP software. Statistical significance was tested using the Mann Whitney U-test *(* $p<0.05$, *** $p<0.001$).*

3.3.Type-I Interferon Associated Immune Responses of Patient PBMCs

Having shown that patient groups had elevated type-I interferons in their plasma, we next asked whether immune cells of these patients also had similar indicators of deregulated type-I interferon signalling. For this, we isolated peripheral mononuclear blood cells (PBMCs) from patients and healthy individuals and analysed the extent of spontaneous cytokine production (IFN-α and IP-10) or STAT1 phosphorylation as signifiers of type I IFN associated signalling pathway activation.

Importance of IFN-α in pathogenesis of interferonopathies has already been discussed. Several reports also indicate increased IFN- α expression and production of from fibroblasts or PBMCs of patients with SLE, Aicardi Goutières and SAVI (Barth, 2002; Jeremiah et al., 2014; Lebon et al., n.d.; Obermoser & Pascual, 2010). To test whether this was also the case in AT and Artemis deficient patients, isolated cells were either stimulated with various ligands (for details, see Section **2.2.2.1** and **Table 2.3**) or left untreated to detect spontaneous cytokine production. Consistent with plasma results, PBMCs from AT, Artemis deficient and SAVI patients spontaneously secreted IFNα whereas no such phenomenon was observed for healthy individuals (**Figure 3.7.a**). Healthy PBMCs secreted negligible levels of IFN- α in the absence of stimulation (3.680 ± 0.7341 pg/ml). In contrast, AT and Artemis deficient patients secreted ~4- and ~5 fold more IFN-α than healthy PBMCs (13.44 ± 3.979 and 15.42 ± 5.956 pg/ml; p=0.2389, p= 0.0041, respectively). As expected, PBMCs isolated from the SAVI patient consistently secreted dramatically high concentrations of IFN-α (~132-fold higher than healthy subjects, 488.3 ± 344.9 pg/ml -measured twice with 3-months interval).

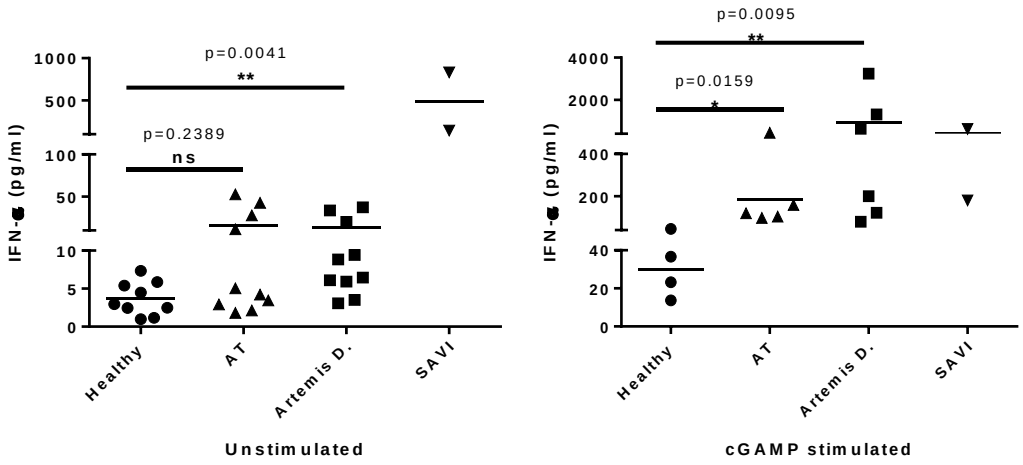


Figure 3.6 Patient PBMCs constitutively secrete IFN-α

PBMCs isolated from patients and healthy donors (2 x 10^6/ml) were incubated with or without cGAMP (2 µg/ml) for 24 h and supernatants were collected and assessed for IFN-α by CBA as explained in Sections **2.2.2.1 and 2.2.2.3.** Following flow cytometric analysis of bead samples and standards, pg/ml concentrations were calculated using the FCAP software. (a) shows spontaneous IFN-α release in the absence of any stimulation and (b) shows cGAMP stimulated release from PBMCs. Statistical significance was determined using Mann-Whitney U-test test. *(* $p<0.05$, ** $p<0.005$)*

Similarly, AT and Artemis deficient patient PBMCs also secreted significantly higher concentrations of IFN-α than healthy controls when stimulated with the STING ligand cGAMP (**Figure 3.7.c**). Specifically, upon stimulation, healthy PBMCs secreted moderate levels of IFN-α (29.94 ± 7.176 pg/ml) whereas AT and Artemis deficient patients secreted 6- and 31-fold more IFN-α (186.4 ± 67.15 and 933.6 ± 500.02 pg/ml; p=0.0159, p=0.0095, respectively). Consistent with published results, SAVI patient constitutively produced very high levels of IFNα, which could not be further boosted with cGAMP stimulation. Since SAVI is caused by a mutation resulting in ligand-independent dimerization and constitutive activation of STING, further activation does not take place in the presence of cGAMP. The enhanced response to cGAMP observed in AT and Artemis deficient patients was specific to type I IFN-associated pathway (as evidenced by higher levels of IFN-α and IP-10 production; **Figure 3.7.a&b**) but not to other signalling pathways affecting pro-inflammatory cytokine production (see **Figure 3.7.b&c*)***.

Collectively, these results suggest that cytokine deregulation in patients is limited to type-I interferon associated cytokines/chemokines. Results also suggest that having been primed, patient PBMCs might respond more robustly to certain viral invaders,

which may account for low frequency of viral infections in AT and Artemis deficient patients despite their ongoing B and/or T cell related immunodeficiencies.

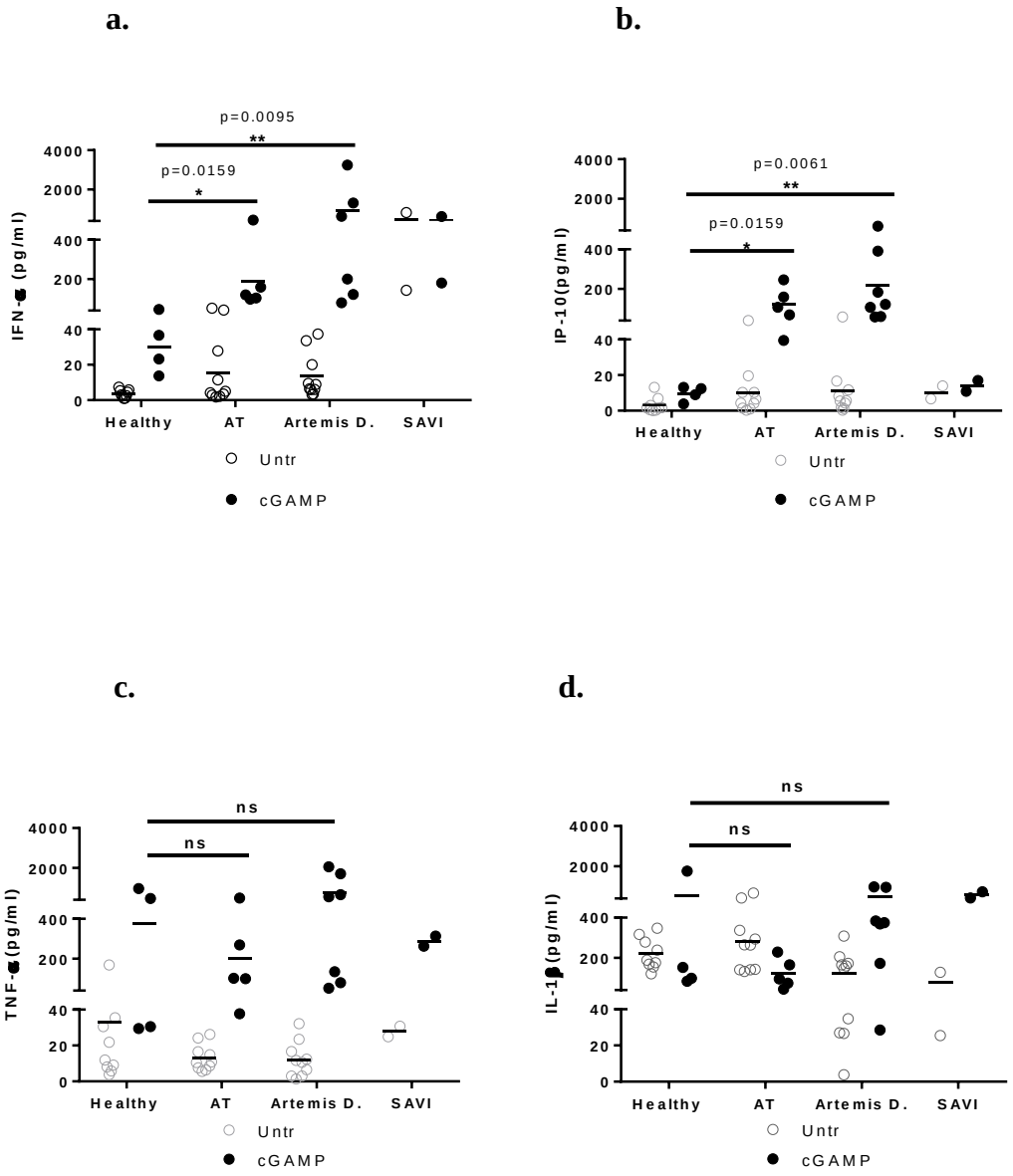


Figure 3.7 Pro-inflammatory cytokine levels were similar in patient and healthy PBMCs

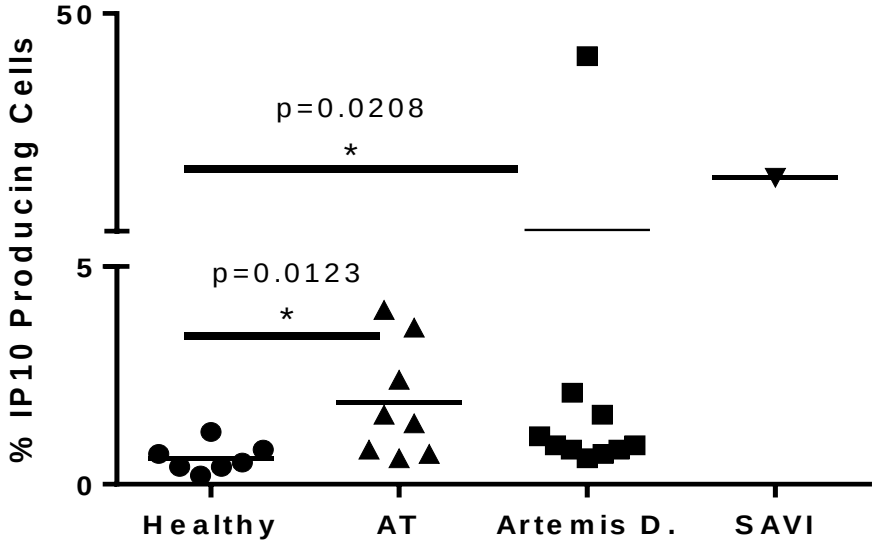
PBMCs isolated from patients and healthy donors (2 x 10^6/ml) were incubated with or without cGAMP (2 µg/ml) for 24 h and supernatants were collected and assessed for

IP-10, TNF-α and IL-1β by CBA as explained in Sections **2.2.2.1 and 2.2.2.3.** Following flow cytometric analysis of bead samples and standards, pg/ml concentrations were calculated using the FCAP software. (a&b&c&d) shows untreated vs cGAMP treated release of IFN-α, IP-10, TNF-α and IL-1β, respectively, from healthy and patient PBMCs. Statistical significance was determined using Mann-Whitney U-test. *(* $p<0.05$, ** $p<0.005$)*

As explained in **Section 3**, patient plasma samples had significantly high levels of IP-10, which we hypothesized was due to constitutive activation of type I IFN-associated pathways. To confirm that patient PBMCs spontaneously produced IP-10, we performed intracellular cytokine staining. Cells were incubated 5 hours with or without stimulation (dsDNA as IP-10 inducer) and were then treated with Brefeldin A (Golgi plug) to prevent IP-10 secretion (see Section 2.2.2.4.3 & 2.2.2.1 for experimental details). Flow cytometric analysis of PBMCs showed that similar to SAVI and TREX-1 deficient patients, percent of IP-10 producing cells were significantly higher in AT and Artemis deficient patients when compared to healthy controls (**Figure 3.7.a&b**; for individual staining results of all patients tested, see **Appendix C**). While average percent of IP-10 producing cells was 0.6 for healthy PBMCs (unstimulated samples), this number was elevated almost 9-fold for Artemis deficient patients (5.07 ± 4.017 %, p=0.0208) and 3-fold for AT patients (1.88 ± 0.407 %, p=0.0123) suggesting that patient PBMCs constitutively produced IP-10. As expected, SAVI patient showed the highest percent of IP-10 producing cells (~27 fold increase when compared to healthy PBMCs; 16.1% of total PBMCs) while TREX-1 deficient patient PBMCs showed 6-fold increase in IP-10 production.

These results further show that peripheral blood cells of tested patients were already in an activated state producing type-I IFNs and associated chemokines like IP-10.

a.



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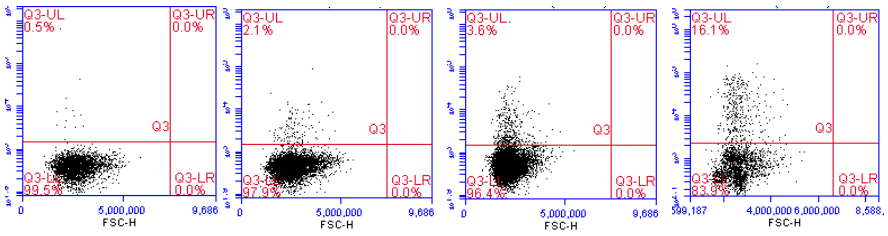


Figure 3.8 AT, Artemis deficient, SAVI and Trex-1 deficient patient PBMCs constitutively produce IP-10

Healthy and patient PBMCs were incubated 7 h (with brefeldin A addition during the last 2 h of incubation) in the absence of any stimulation and cells were then stained for IP-10 and analysed by Flow cytometry. (b) shows gating strategy and representative flow cytometry images of healthy and patient groups. (a) shows percent

of IP-10 producing cells for each individual. Statistical significance was tested using the Mann Whitney U-test. *(* $p<0.05$).*

3.4.Assessment of STAT1 Phosphorylation in Healthy and Patient PBMCs

STAT1 is an important transcription factor that is phosphorylated in response to type-I interferon signalling and controls the induction of interferon stimulated genes (ISGs). (Katze, He, & Gale Jr., 2002). In addition to its role in antiviral defense, reports suggest that in type-I interferon dependent autoinflammatory diseases, constitutive STAT1 phosphorylation contributes to disease progression (Dong, Wang, Zhou, Ma, & Zhang, 2007; Liu et al., 2014). Thus, after establishing that AT and Artemis deficient patients exhibited indicators of elevated type-I signature, we decided to check the status of phosphorylated STAT1 protein in patient cells as a direct signifier of type I IFN-associated pathway activator. Isolated PBMCs were assessed for STAT1 phosphorylation by flow cytometry as explained in Section **2.2.2.4.4.** As can be seen in **Figure 3.9.a&b,** AT and Artemis deficient PBMCs had higher levels of STAT1 phosphorylation when compared to healthy PBMCs, and similar results were obtained for SAVI and TREX-1 deficient patients. Healthy PBMCs showed low levels of STAT1 phosphorylation (735 ± 47.99 MFI) while AT and Artemis deficient patients showed significantly increased levels of pSTAT1 (974.1 ± 66.44 and 1372 ± 205.5 MFI; p=0.0311 and p=0.0004, respectively). Consistent with previous results, SAVI patient showed the highest level of STAT1 phosphorylation (2625.2 MFI), whereas TREX-1 deficient patient's pSTAT1 levels were comparable to those observed in AT and Artemis deficient patients (1333.6 MFI). Of note, cells from selected subjects were also stimulated with recIFNα as a positive control and as expected, this led to a rapid upregulation in pSTAT1 levels (**Figure 3.9.a**) in all tested groups (except the SAVI patient), demonstrating the success of the technique we have used. At the time of this experiment, the SAVI patient was being treated with tofacitinib (a JAK kinase

inhibitor) and thus failed to further upregulate pSTAT1 in response to recIFNα stimulation.

These results are of interest since they not only confirm the existence of exaggerated type I interferon signalling in AT patients but demonstrate a similar phenomenon in Artemis deficient patients for the first time. Furthermore, our results also suggest that these 2 diseases could be classified as "interferonopathies" and could share certain clinical features with SAVI and TREX-1 deficient patients. Another implication of these findings is that certain JAK-Stat inhibitors (such as tofacitinib) might be of benefit in disease management of AT and Artemis deficiency.

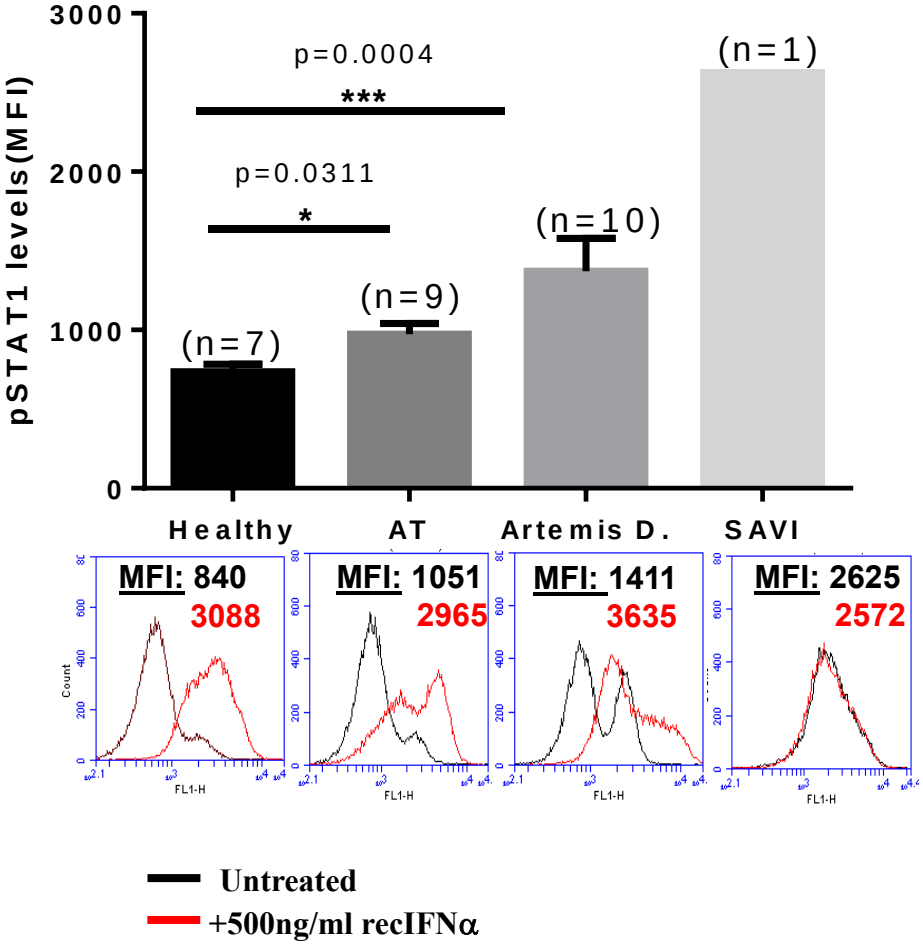


Figure 3.9 Constitutive activation of STAT1 in patient groups

Isolated PBMC (1×10^6 cells/ml) were either treated with rec IFN-a (5-500 ng/ml) or left untreated 20 min. STAT1 phosphorylation was then determined using Flow Cytometry. Figure 3.6.b shows the gating strategy and a representative staining plot of one individual from each group. Black histograms represent unstimulated pStat-1 levels and red histograms indicate levels obtained following stimulation with recIFNα. Numbers above histograms represent mean fluorescence intensities (MFI). **Figure.3.6.a** shows average levels of pSTAT-1 (untreated) STAT1in each group (calculated as mean MFI ± SEM). Numbers above each bar represent number of individuals in each group. Raw data for this graph is presented in **Appendix D**. Statistical significance was tested using Mann Whitney U-test *(* $p<0.05$, *** $p<0.001$).*

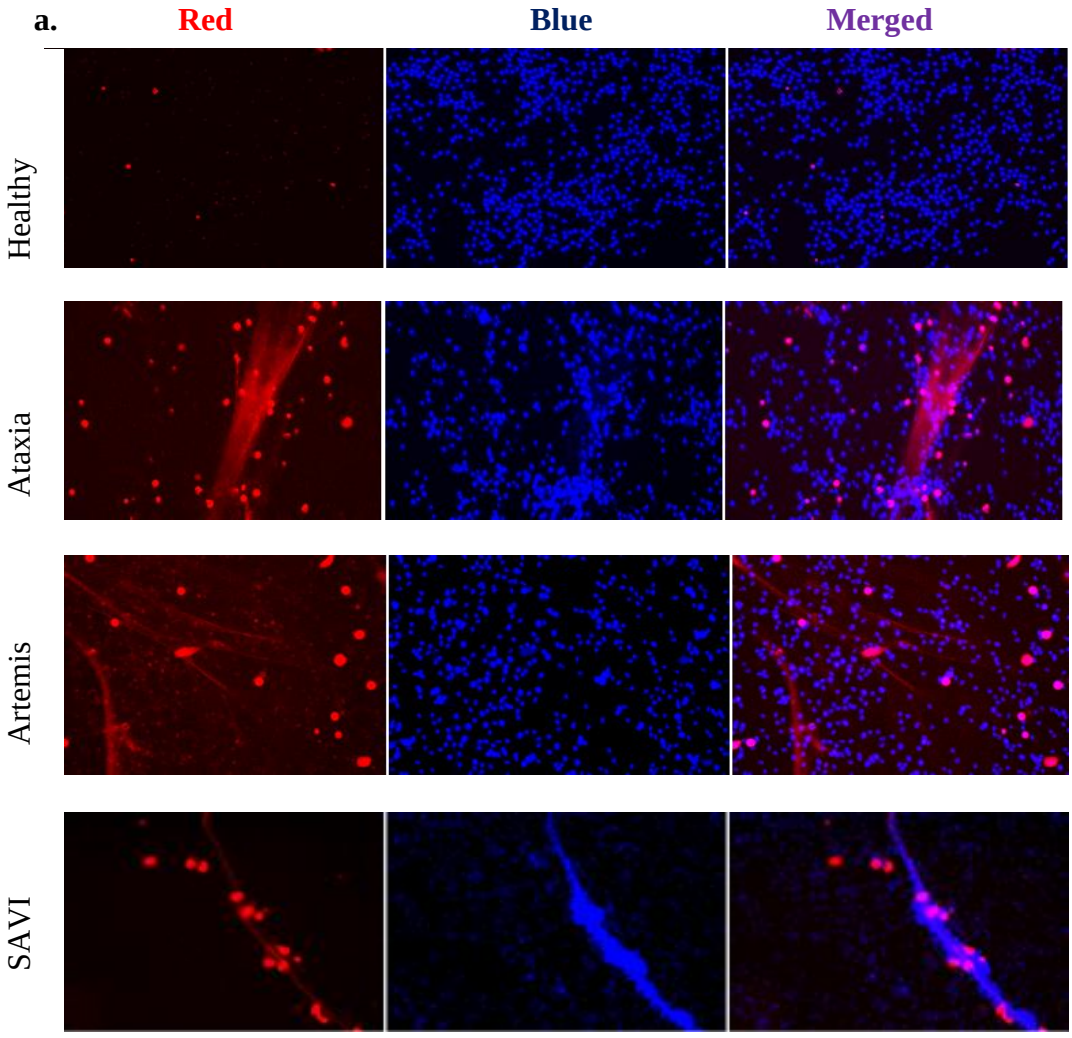
3.5.Analysis of Patient Neutrophils for NETosis Activity

Similar to patients with AGS and SAVI, increased interferon signature is consistently demonstrated in peripheral blood of SLE patients (Bennett et al., 2003; Lars Rönnblom & Alm, 2003). Furthermore, neutrophils isolated from SLE patients exhibit abnormal features such as increased aggregation and a tendency to undergo spontaneous NETosis, suggestive of a link between elevated levels of type I interferons and neutrophil-mediated disease pathogenesis (Abramson et al., 1983; Al-Hadithy et al., 1982; Bennett et al., 2003; Mariana J. Kaplan, 2011; Lindau et al., 2013; Y. Yu & Su, 2013). In fact, one preliminary study has shown that blocking of interferon alpha signalling in neutrophils reduced neutrophil extracellular trap formation in juvenile onset systemic lupus erythematosus (Midgley & Beresford, 2013). To date, neutrophil functions in diseases strongly associated with increased type I interferon signature (like SAVI Aicardi Goutières) have never been tested. Therefore, to assess whether spontaneously activated neutrophils contribute to pathological damage in AT, Artemis deficiency and SAVI, we isolated patient neutrophils and compared their propensity to form NETs with respect to cells obtained from healthy donors.

To test whether patient neutrophils formed NETs, blood polymorphonuclear granulocytes were isolated and then incubated in the absence or presence of PMA (NETosis inducer). NET formation was visualized microscopically (Section **2.2.2.5)** and the amount of DNA associated with NETs was quantified spectrofluorometrically using a micrococcal nuclease/picogreen-based assay (Section ***2.2.2.5.2***). Image analysis of neutrophils isolated from AT, Artemis deficient, and SAVI patients demonstrated spontaneous release of NETs in the absence of any stimulation, whereas no such structures were observed in cultures obtained from healthy subjects (**Figure 3.10.a and Appendix E)**. Of interest, this was not a one-time observation and similar results were obtained when freshly isolated patient cells were re-tested on 3 other occasions. Healthy neutrophils released very low amounts of DNA (290.7 ± 16.9 ng DNA/10^5 PMNs) while AT and Artemis deficient patients released ~5- and 4-fold more NET-associated DNA, respectively (1322 ± 219.6 and 1038 ± 183.9 ng DNA/10^5 PMNs; $p<0.0001$ and $p<0.0001$ respectively). SAVI neutrophils also released higher amounts of DNA (730.67 ng DNA/10^5 PMNs). These results suggest that neutrophil extracellular trap (NET) formation and neutrophil-related tissue injury might play a role in pathophysiology of AT, Artemis deficiency and SAVI. Whether NETosing neutrophils directly contribute to vasculopathy in SAVI patients (Jeremiah et al., 2014; Liu et al., 2014) remains to be determined.

To test whether patient neutrophils released more NETs than healthy controls when stimulated, the experiments were repeated in the presence of NETosis inducer PMA. Indeed PMA stimulation generated significantly more NET associated DNA in patient neutrophils when compared to healthy controls (**Figure 3.10.c and Appendix F**), suggesting that patient neutrophils are already in an activated state and respond more robustly to NET inducers. When stimulated with PMA healthy neutrophils released moderate levels of DNA (851.8 ±103.8 ng DNA/10^5 PMNs) whereas cells from AT and Artemis deficient patients released approximately 2-fold more DNA (1360 ± 182.3 and 1659 ± 107.7 ng DNA/10^5 PMNs, $p=0.153$ and $p= 0.0070$ respectively).

Neutrophils represent the most abundant innate immune cell type armed with potent enzymes and anti-microbial effector molecules (Mariana J Kaplan, 2013; Villanueva et al., 2011). Deregulated neutrophil activation has been shown to drive tissue damage in diseases like SLE and Rheumatoid arthritis (RA). AT, Artemis deficient and SAVI patients share common pathological features such as granulomatous skin lesions and interstitial lung disease which could be the result of improper neutrophil activation. Here, we have shown that neutrophils spontaneously produce NETs which may account for the inflammation-associated tissue damage observed in these patients. We also showed that patient neutrophils responded to PMA (NET inducer) more strongly, providing further proof for the activated state of neutrophils in these diseases.



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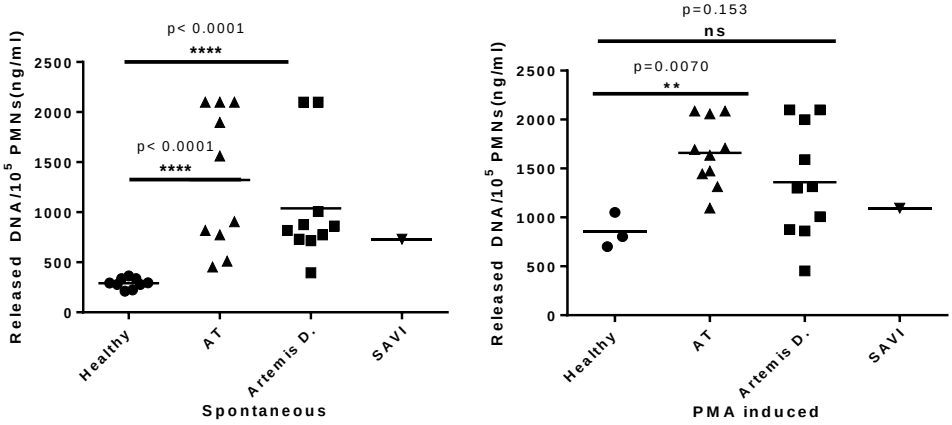


Figure 3.10 Patient neutrophils spontaneously produce NETs

(a) Representative NET images from 1 healthy, AT, Artemis and SAVI patient. (images obtained from other patients are given in **Appendix E**). NETs were imaged 5 h after plating of neutrophils (80,000 cells/200 µl) following staining with Sytox Orange (5 µM) for extracellular DNA and NucBlue (20 µl) for both extracellular and intracellular DNA. (b&c) After 5 h of culture without or with PMA stimulation, cells (500,000/ml) were treated with micrococcal nuclease (500 mU/ml) and supernatants were used for quantitation of released DNA. Statistical significance was tested using Mann Whitney U-test. *(** $p<0.01$).*

During NETosis, neutrophils release their DNA as a complex meshwork containing antimicrobial histone proteins, granule derived peptides and enzymes such as myeloperoxidase (MPO) and neutrophil elastase, and cathepsin G (M J Kaplan & Radic, 2012). To further validate the activation state of neutrophils in blood, we next determined the concentration of neutrophil elastase in patient vs healthy plasmas (see Section **2.2.2.2***)*. Results showed that AT and Artemis deficient patients had significantly higher levels of elastase in their plasma compared to healthy controls (**Figure 3.11).** The patient with SAVI had the highest level of plasma elastase,

suggestive of NETosis or de-granulation associated release. Specifically, plasma elastase levels were found to be ~2-fold increase in Artemis and AT patients (134.6 ± 17.7 and 114.4 ± 16.62 ng/ml; p=0.028 and p=0.0393, respectively) when compared to healthy controls (68.89 ± 6.52 ng/ml). SAVI patient's plasma elastase concentration was the highest among all tested individuals (~4 fold higher than control; 308.31 ng/ml).

These results further support the notion that neutrophils of AT, Artemis deficient and SAVI patients are in an active state due to sustained type-I interferon-mediated sterile inflammation and could indeed be releasing NETs, contributing to pathology.

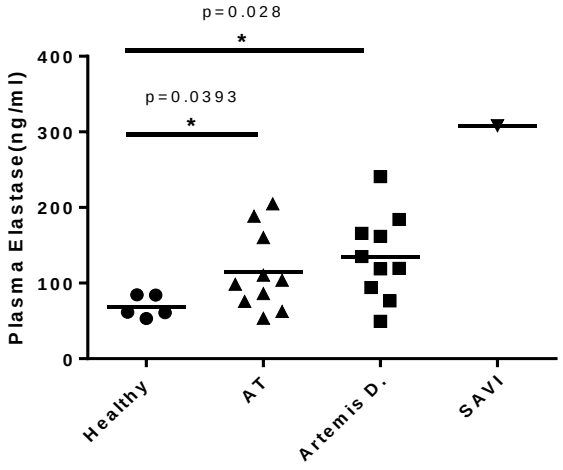
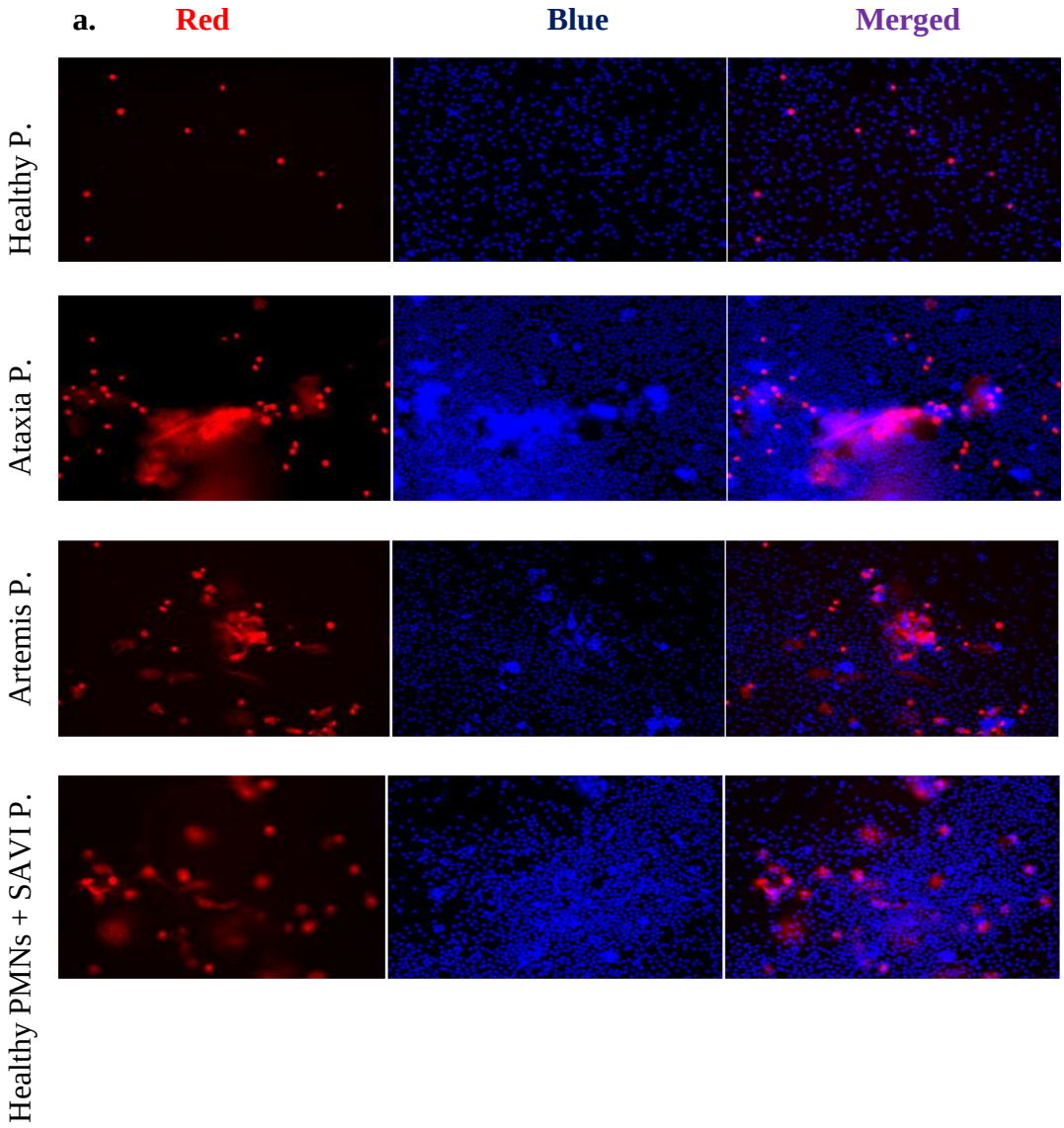


Figure 3.11 Neutrophil elastase levels were found to be elevated in plasma of AT, Artemis deficient and SAVI patients

Elastase levels in plasma were assessed by ELISA as described in Section **2.2.2.2**. Plasma samples from 5 healthy, 10 Artemis and 10 AT and 1 SAVI patients were assessed. Mann Whitney U-test was used for significance determination *(* $p<0.05$).*

After demonstrating that patient neutrophils were spontaneously producing NETs, we next asked whether factors present in patients' plasma were causing neutrophils to

release their DNA. To address this question, plasma samples from patients and healthy subjects were isolated, clarified by centrifugation (2000 x g for 10 min) and neutrophils isolated from healthy subjects were cultured in the presence of 20% of plasma. PMA stimulated samples were used as positive control and NET formation was assessed both as microscopically and quantitatively as before. Results showed that patient but not healthy plasma samples stimulated healthy neutrophils to undergo NETosis and release their DNA as NETs (**Figure 3.12.a and Appendix G).** Healthy plasma induced low levels of DNA release from healthy neutrophils (**Figure 3.12.b**, 294.3 ± 25.94 ng DNA/10^5 PMNs), comparable to levels observed with unstimulated groups (283.5 ± 4.857,**Figure 3.10.b**). In contrast, AT and Artemis deficient patient plasma samples induced 2-fold more DNA release from healthy neutrophils (532.5 ± 46.83 and 429.0 ± 41.58, p=0.0030 and p=0.0349, respectively). Similarly, plasma of SAVI patient also induced substantially more DNA release (**Figure 3.10.b**, 572.913 ng DNA/105 PMNs). These results suggest that elevated type-I interferon related cytokines/chemokines may contribute to NET formation in healthy neutrophils. Considering the fact that patients had elevated plasma levels of IP-10, IFN-α and IFN-λ, these results indicate that type-I/III interferons and associated chemokines might have a role in neutrophil mediated NET release.


a.
Red
Blue
Merged
Healthy P.
Ataxia P.
Artemis P.
Healthy PMNs + SAVI P.

b.

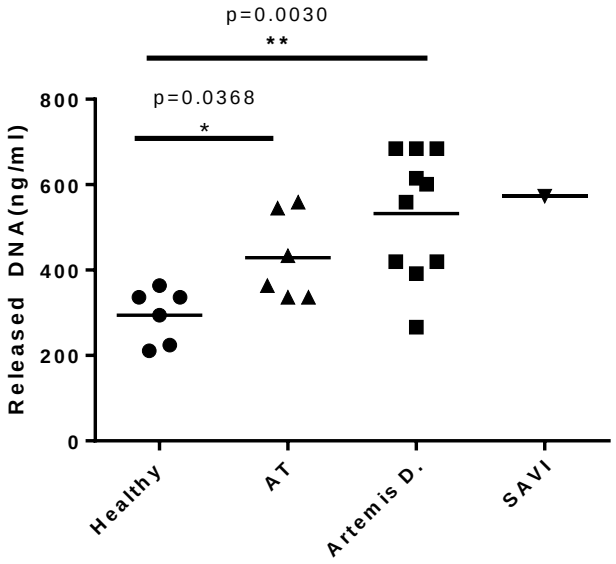


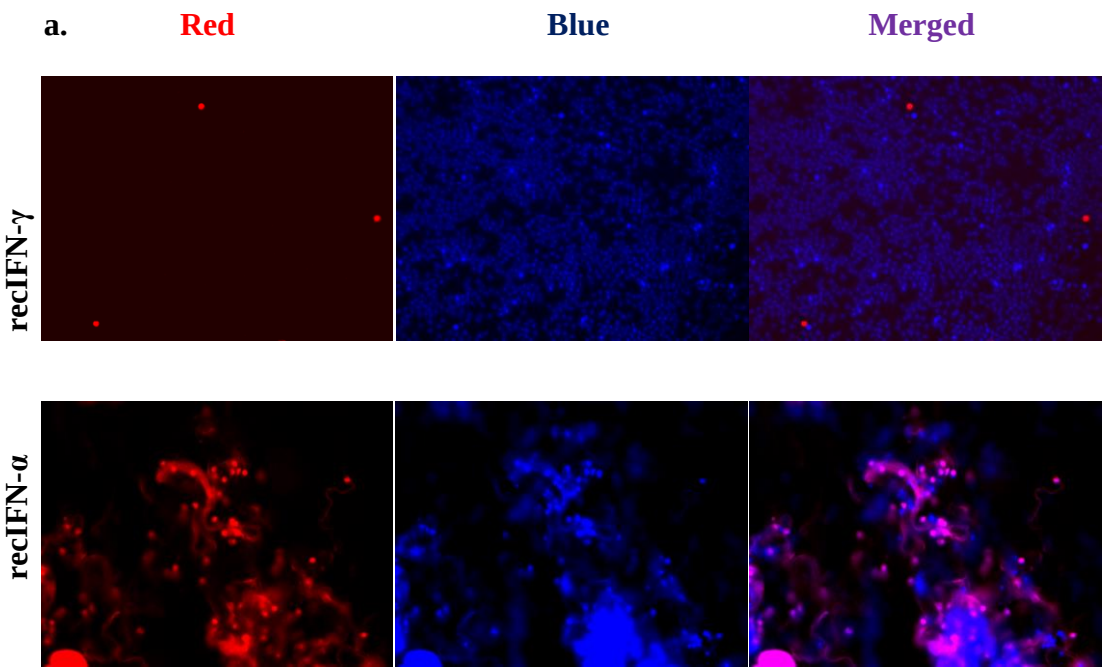
Figure 3.12 Plasma of AT, Artemis deficient and SAVI patients trigger NETosis in healthy neutrophils

(a) Healthy PMNs were incubated with plasma obtained from 5 healthy, 5 AT, 5 Artemis deficient or 1 SAVI patient Microscopy images were taken 5 h later following staining of cells with Sytox orange for extracellular DNA and NucBlue for both extracellular and intracellular DNA. (b) Healthy neutrophils (500,000/ml) incubated for 5 h in the presence of donor plasmas (6 healthy, 10 Artemis deficient, 10 AT and 1 SAVI) were treated with micrococcal nuclease (500 mU/ml) and supernatants were used for quantitation of released DNA. Statistical significance was tested using Mann Whitney U-test *(* $p<0.05$, ** $p<0.01$).*

As previously mentioned, a preliminary study indicated that inhibition of IFN-α signalling in neutrophils from juvenile-onset SLE patients results in reduction of NET formation, suggesting that IFN-α itself enhances NET formation (Midgley & Beresford, 2013). To verify this assumption, we next treated healthy neutrophils with either recombinant interferon alpha or recIFNγ. Neutrophils were isolated as described

before, and left untreated or treated either with PMA (positive control), recIFN- α or IFN-γ (as a negative control). Results showed that recIFN-α but not recIFNγ stimulated healthy neutrophils to undergo NETosis (**Figure 3.13.a**). **Figure3.13.b** demonstrates that as expected, PMA strongly induced the release of DNA (5-fold higher than unstimulated controls, 953.9 ± 290.2 ng DNA/10^5 PMNs). IFN-α treated neutrophils also released significant amounts of NET DNA (415.5 ± 70.2 ng DNA/10^5 PMNs, p=0.0286) compared to unstimulated controls (182.3 ± 29.59 ng DNA/10^5 PMNs) whereas no such phenomenon was observed in IFN-γ treated neutrophils.

These results suggests that as mentioned in Section **3.4**, IFN pathway blockers like JAK-Stat inhibitors (i.e. tofacitinib) might also be effective in inhibiting NETosis in these patients and hence limit tissue damage as in the recently published paper where it effectively ameliorated serum inflammatory cytokine levels and NETosis (Furumoto et al., 2016).



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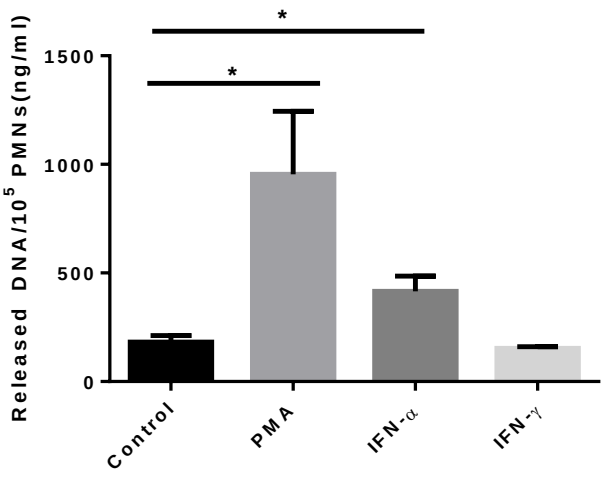

Figure 3.13 recIFN-α stimulates NETosis in healthy neutrophils

Healthy neutrophils were isolated and left untreated or treated either PMA (50 ng/ml), recIFN-α (500 ng/ml) or IFN-γ (100 ng/ml). a. Representative NET images of healthy neutrophils (Red, extracellular DNA; blue, extracellular and intracellular DNA). b. shows quantification of released DNA after indicated treatments. (Results are given as mean ± SEM of three different healthy neutrophils). Mann Whitney U-test was used to test significance (* $p<0.05$).

CHAPTER 4

CONCLUSION

Immune system has various strategies to fight against invading pathogens. Type-I interferon production represents one such strategy and is critical to fight against viral infections. Viral nucleic acid recognition and subsequent type I interferon production depends on the expression of specific pattern recognition receptors and signalling proteins (de Veer et al., 2001; Hua et al., 2006; Schoggins et al., 2011). Extensive research in the field of nucleic acid recognition revealed that in addition to detecting viral infections, loss of negative regulation or gain of function mutations in such signalling pathways can lead to development of autoimmune and autoinflammatory diseases. Type-I interferonopathies are recent example of such diseases in which deregulation of nucleic acid sensing pathways lead to constitutive type-I interferon release, causing pathology. DNA damage, especially if not repaired can also result in activation of these pathways as a result of genomic DNA leakage into cytosol (Härtlova et al., 2015; Raj et al., 2014; Stetson et al., 2008; Yang et al., 2007; Yoshida, Okabe, Kawane, Fukuyama, & Nagata, 2005). To test whether inflammatory skin/organ damage manifestations observed in DNA repair/immuno-deficiency diseases (i.e, AT and Artemis deficiency) could be related to unchecked type-I interferon secretion we compared immune response characteristics of AT and Artemis deficient patients to healthy controls and to known interferonopathy patients (one SAVI and one TREX-1 deficient patient). Our results showed that similar to SAVI and TREX-1 deficient patients, AT and Artemis deficiency was associated with

elevated type-I and III interferon signature (increased concentrations of circulating IP-10, IFN-α and IL-29; **Figure 3.1,3.2** and **3.3**). These results suggest that AT and Artemis deficient patients have deregulated type-I interferon production due to mutations in DNA repair enzymes. To date, only one study demonstrated specific DNA leakage into cytosol in case of ATM deficiency, triggering type-I interferon secretion through cGAS-STING-TBK1 pathway (Härtlova et al., 2015). However in that publication AT plasma samples were not analysed for the presence of type-I interferon-associated cytokines/chemokines. Herein, we expanded these findings and demonstrated for the first time that AT and Artemis deficiencies share certain features with known interferonopathies. Evidence for this comes not only from plasma samples but PBMCs isolated from AT and Artemis deficient patients were also shown to spontaneously secrete IFN-α and IP-10 (**Figure 3.6** and **Figure 3.8**). Moreover, STAT1 protein was constitutively phosphorylated in most patients (**Figure 3.9**). Of note, although not presented in this thesis, qRT-PCR-based work from our lab (conducted by Naz Surucu) also demonstrated elevated expression of 4 ISGs (MX1, IFNβ, OAS and ISG15) in patient but not healthy PBMCs. Therefore, our results contribute to the field of primary immunodeficiencies by establishing that AT and Artemis deficiencies can also be considered as interferonopathies. Such a conclusion should be of interest to clinicians as these results provide an opportunity for adoption of new treatment strategies for these diseases. For example, these patients might benefit from anti-interferon antibody administration or treatment with tofacitinib. How and why type I IFN induction lead to tissue damage is still unknown. Published work on SLE patients indicate that neutrophil-mediated mechanisms might be involved in tissue damage (Mariana J Kaplan, 2013; Villanueva et al., 2011). Similarly, in ADA2 deficiency (another interferonopathy) neutrophils are chronically activated and may be responsible for endothelial damage (Belot et al., 2014). To date, neutrophil involvement in chronic inflammation has never been studied in Artemis deficient and SAVI patients. In AT patients, neutrophils were shown to overproduce pro-inflammatory cytokines but whether or not they formed NETs was not investigated (Harbort et al., 2015). Herein, we showed that unlike healthy neutrophils,

patient neutrophils spontaneously released their DNA in the form of NETs (**Figure 3.10**). Furthermore, we established that incubation with patient plasma samples or recIFNα was sufficient to stimulate NETosis in healthy neutrophils (**Figure 3.12** and **3.13**).

Collectively, these results indicate that elevated type I IFN signature commonly observed in our patient groups might exert tissue damage through neutrophil-driven processes. In this context, we propose that drugs that can interfere with neutrophil activation and or prevent NETosis could ameliorate organ damage and might be of benefit in the management of interferonopathies in general.

CHAPTER 5

FUTURE PERSPECTIVES

These study has a great importance in terms of characterization of type-I interferon signatures in AT and Artemis deficient patients. There are points needs further investigation which would make our suggestion as AT and Artemis deficient patients as type-I interferonopathies and spontaneous NET formation in type-I interferonopathies.

Firstly, to further prove proposed mechanism of type-I interferon induction through cytosolic accumulation of endogenous DNA as a result of impaired DNA damage, investigation of cytosolic DNA species in healthy PBMCs and comparison of this with healthy PBMCs is required. It was already shown in mice BMDMs that $ATM^{-/-}$ cells showed existence of ssDNA species in cytosol a phenomenon not observed in wild type BMDMs (Härtlova et al., 2015). Showing similar findings further in AT and Artemis deficient patient immune cells might be important to support the idea that DNA damage deficiencies cause type-I IFN induction through cytosolic DNA-cGAS-STING pathway.

Secondly, as patients show a lot variability in type-I interferon responses, it might be ideal to compare patients with same molecular defect, same gender and same age.

However, finding these kind of groups with such a rare molecular defect could hardly be possible. What can be done is that evaluating the correlation between levels of IFN-α and related cytokines/chemokines and severity of clinical features in the same group (AT or Artemis deficient groups). By this way, it might give an idea about disease development and relation between different age/gender groups and involvement of different factors in these progression.

Finally, as we suggest that interferon alpha or type-I interferons cause spontaneous NETosis in healthy neutrophils it might be investigative to perform further stimulation experiments with other type-I IFN related cytokines and chemokines such as IFN-β and IP-10. And to prove this argument using anti-IFN antibodies in patient plasma induced NETosis experiments and showing whether they will inhibit patient plasma induced NET release could provide a direct evidence for type-I IFN induced NETosis. Being able to show direct effect of anti-IFN antibodies, might strengthen our suggestion of them as therapeutics. And similarly, usage of tofacitinib on patient PBMCs to see if they will also provide an amelioration of type-I interferon signatures in these cells.

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APPENDICES

APPENDIX A

Recipes for Buffers, Cell Culture Media and Solutions

Blocking Buffer (ELISA): For total 500 mL,

-500 mL PBS (1X)

-20 grams of BSA (2%)

-500 µl Tween20 (0.05%)

BSA is dissolved in PBS totally by using a magnetic stirrer (15-20 min mixing). After preparation buffer is stored at -20°C as aliquots of 50 mL.

Wash Buffer (ELISA): For total 5 lt,

-500 mL PBS (10X)

-2.5 mL Tween20

-4.5 lt ddH2O

T –Cell Buffer (ELISA): For total 500 mL,

-475 mL PBS (1X)

-25 mL FBS (5%)

-250 µl Tween20 (2.5%)

Buffer is stored at -20°C as aliquots of 50 mL.

FACS Buffer: For total of 500 mL,

-500 mL PBS (1X)

-5 g BSA (1%)

-125 mg Na-Azide (0.125%)

Regular RPMI 1640 containing 2%, 5%, 10% FBS: For total 500 mL,

-10 mL, 25 mL or 50 mL FBS (inactivated at 55°C)→ for 2%, 5%, 10% respectively

-5 mL PenStrep (Penicillin/Streptomycin, 50 µg/mL)

-5 mL NaPyruvate (0.11 mg/mL)

- 5 mL HEPES (10 mM)

-5 mL NEAA (non-essential amino acids, diluted 1X from 100X stock)

Final volume is brought to 500 mL by using RPMI-1640 (w/L-Glutamine, w/Phenol Red) from ThermoFisher Scientific, USA. Normocin (100 µg/mL) is used as additional antibiotic against mycoplasma and fungal contaminations.

RPMI 1640 containing 5% Oligo FBS: Similar to regular RPMI with one difference; FBS used in preparation is inactivated at 65°C to further inhibit nuclease activity.

Nuclease Buffer: For Micrococcal Nuclease reaction,

-50 mM Tris-HCl (pH: 8.3)

-10 mM CaCl

APPENDIX B

Plasma CBA Individual Plots

a.

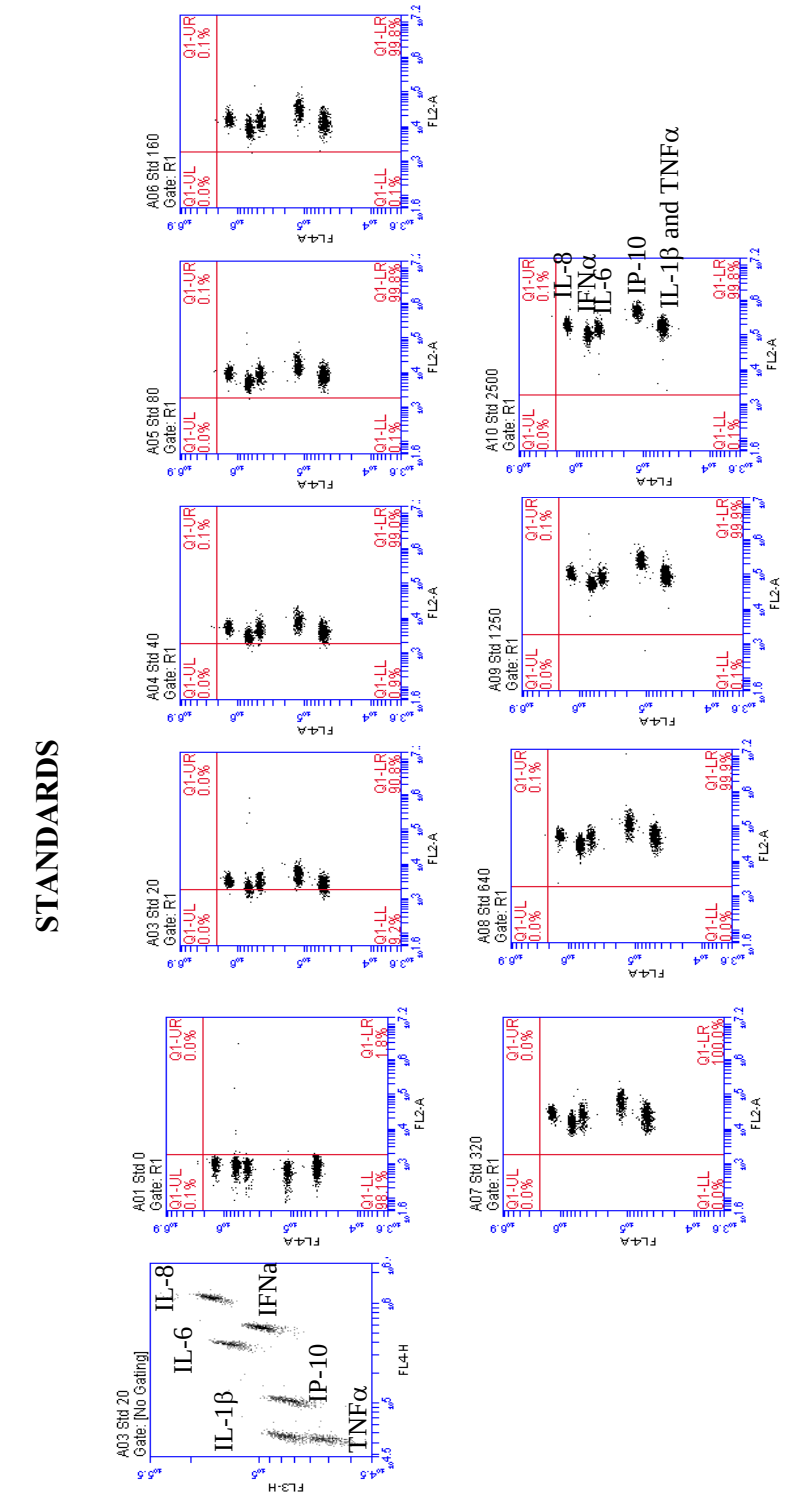
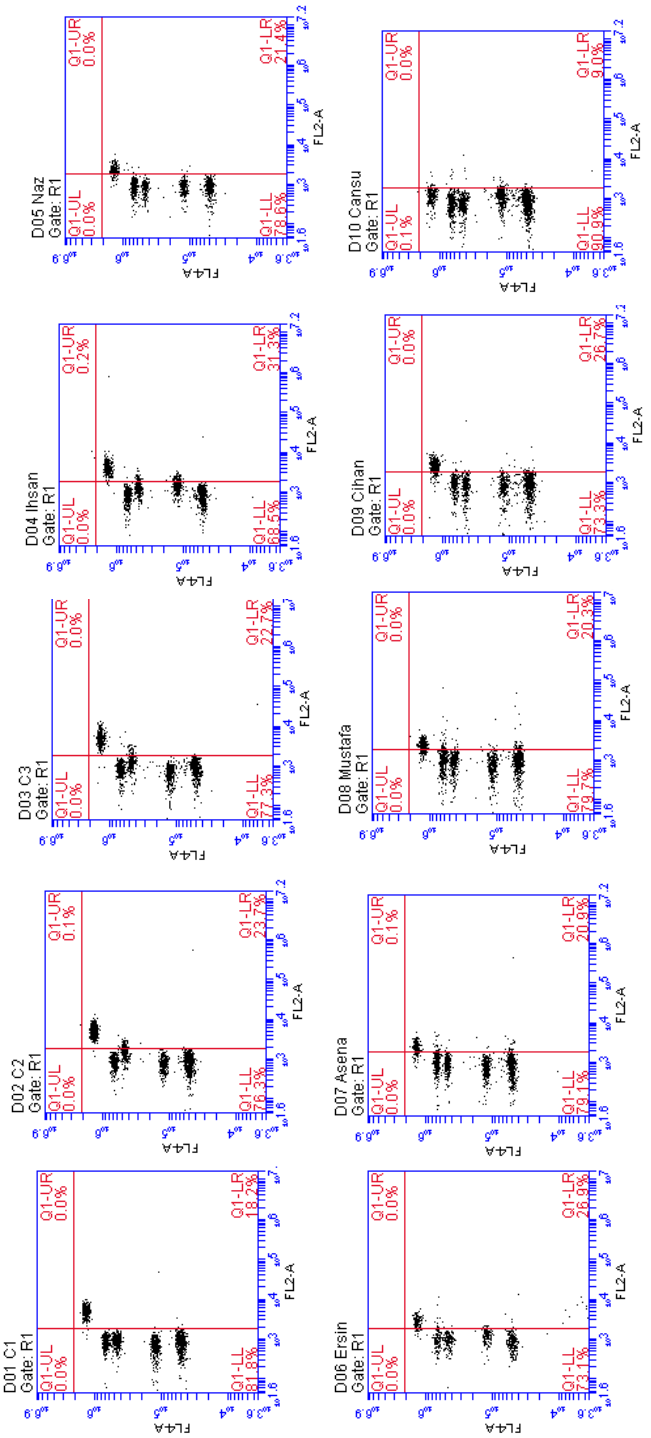


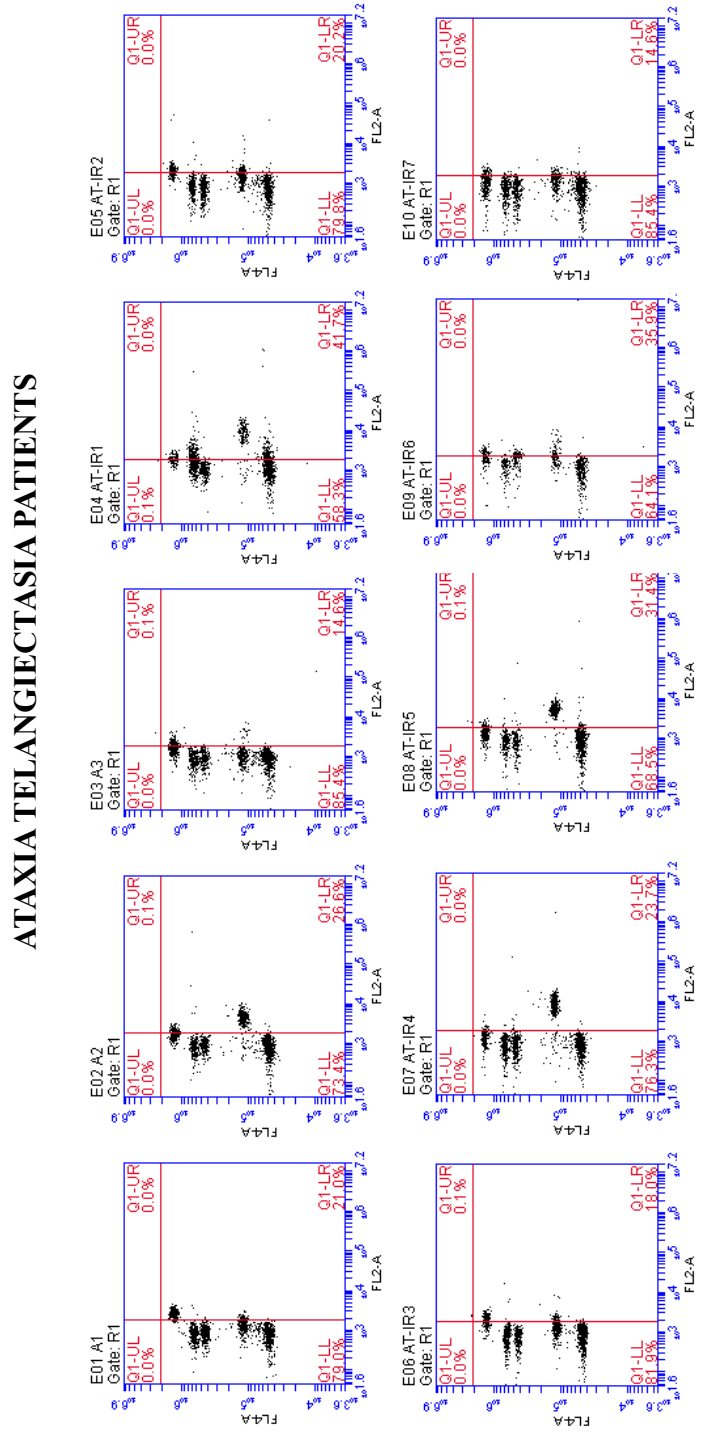
Figure A.1 Individual flow cytometry plots of all the standards and samples of CBA analysis.
(a) shows plots for standards (b) for healthy controls, (c) for AT patients, (d) for Artemis deficient patients, and (e) for SAVI patients

b.

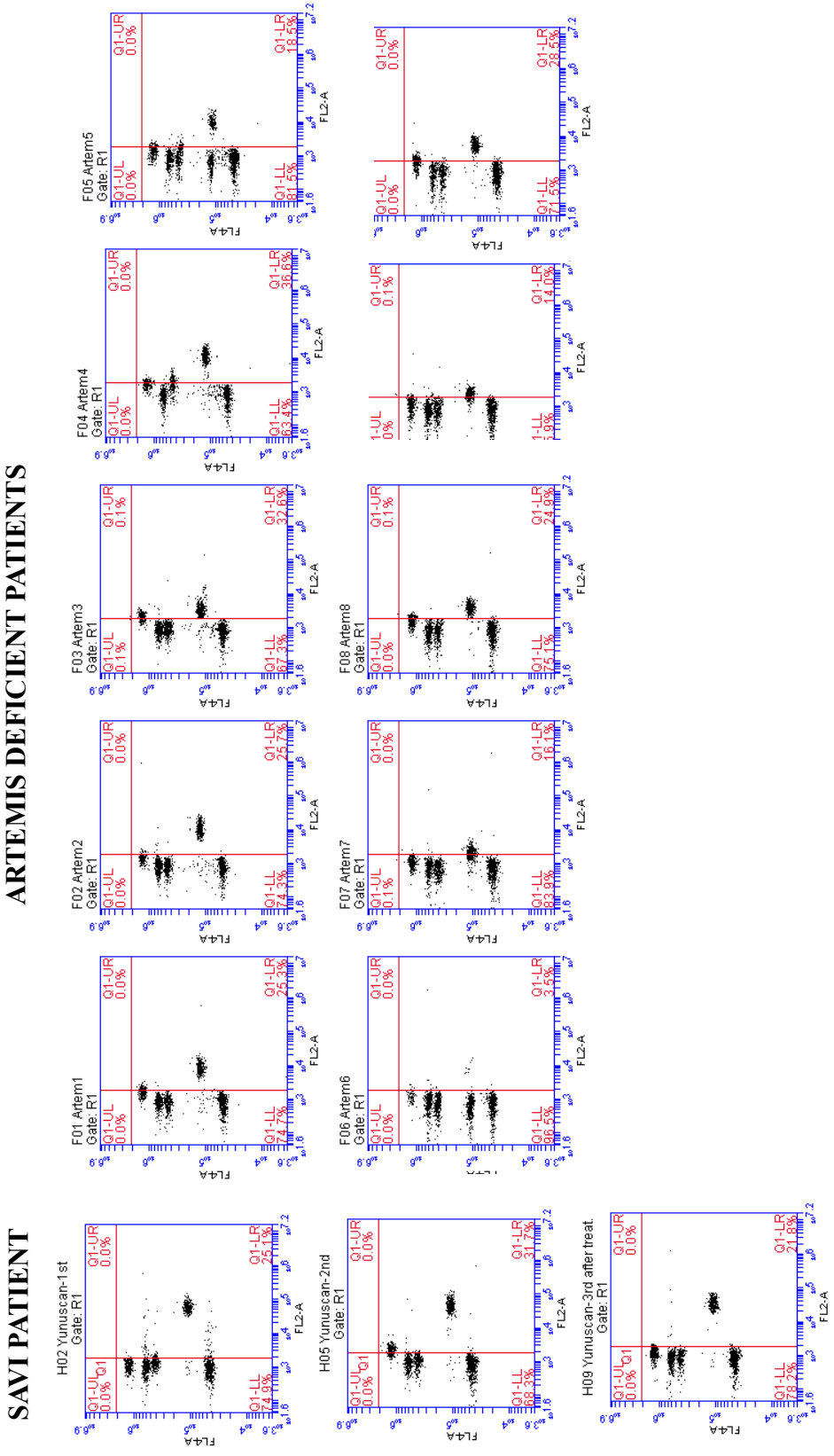
HEALTHY CONTROLS



c.


ATAXIA TELANGIECTASIA PATIENTS
E01 A1
Gate: R1
E02 A2
Gate: R1
E03 A3
Gate: R1
E04 AT-IR1
Gate: R1
E05 AT-IR2
Gate: R1
E06 AT-IR3
Gate: R1
E07 AT-IR4
Gate: R1
E08 AT-IR5
Gate: R1
E09 AT-IR6
Gate: R1
E10 AT-IR7
Gate: R1
FL4-A
FL2-A

d.


ARTEMIS DEFICIENT PATIENTS
SAVI PATIENT
F05 Artem5
Gate: R1
F04 Artem4
Gate: R1
F03 Artem3
Gate: R1
F02 Artem2
Gate: R1
F01 Artem1
Gate: R1
F08 Artem8
Gate: R1
F07 Artem7
Gate: R1
F06 Artem6
Gate: R1
H02 Yunuscan-1st
Gate: R1
H05 Yunuscan-2nd
Gate: R1
H09 Yunuscan-3rd after treat.
Gate: R1
FL4-A
FL2-A

APPENDIX C

Intracellular IP-10 Staining of Patient and Healthy PBMCs

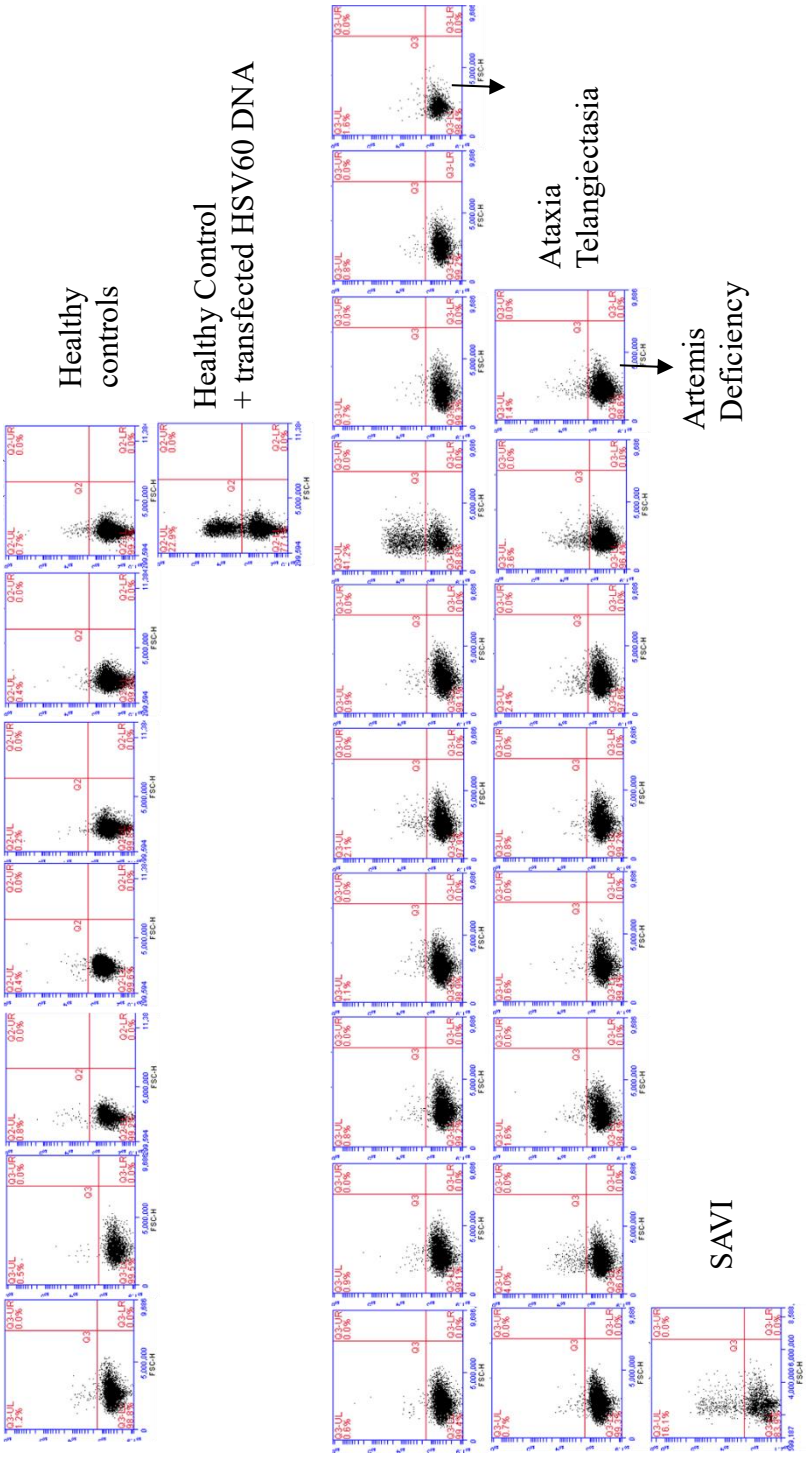


Figure A.2 IP-10 production from healthy and patient PBMCs
Healthy and patient PBMCs were assessed for IP-10 levels by intracellular cytokine staining (ICS) and analyzed by Flow cytometry. Flow cytometry analysis of 7 healthy, 10 AT, 8 Artemis deficient 1 SAVI and 1 dsDNA transfected healthy PBMCs.

APPENDIX D

STAT1 Phosphorylation of Healthy and Patient PBMCs

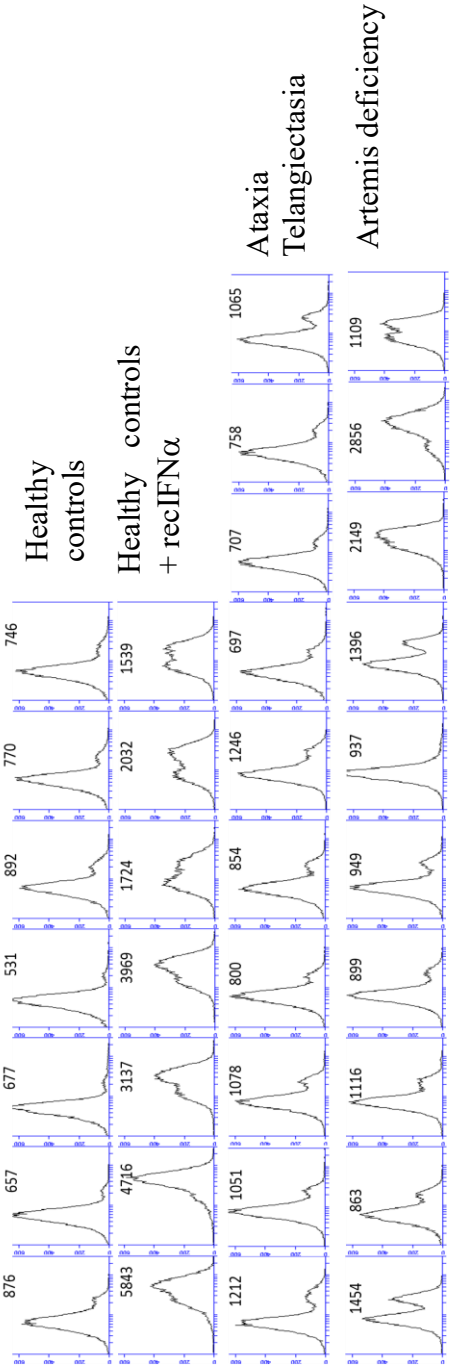


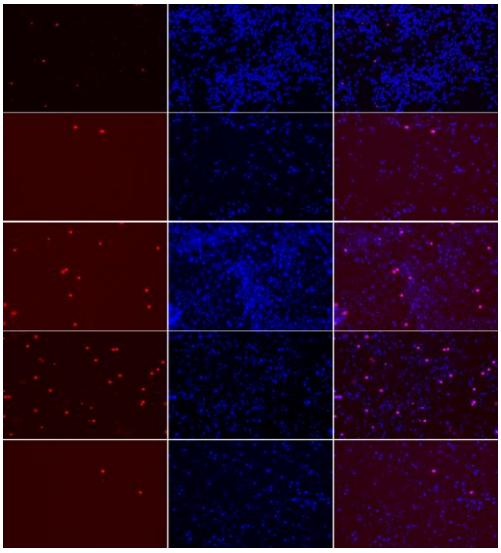
Figure A.3. Spontaneous STAT1 phosphorylation was observed in patients

PBMCs were isolated, and 1×10^6 cells/ml were either treated with rec IFN-a (500ng/ml) or remained untreated. STAT1 phosphorylation was then detected by Flow Cytometry. Flow analysis results of 7 healthy (untreated + recIFN-α treated), 10 AT and Artemis deficient and SAVI patients.

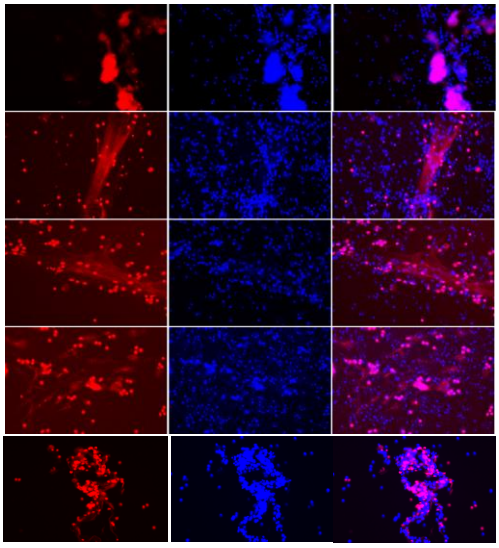
APPENDIX E

Spontaneous NETosis

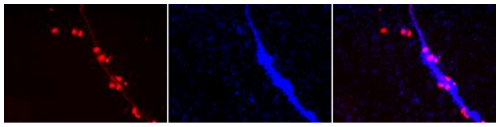
Healthy



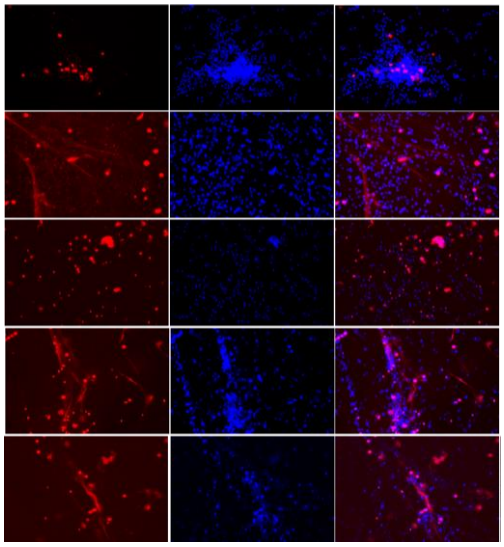
Ataxia Telangiectasia



SAVI



Artemis Deficiency



RED: SYTOX Orange (cell impermeable nucleic acid dye)

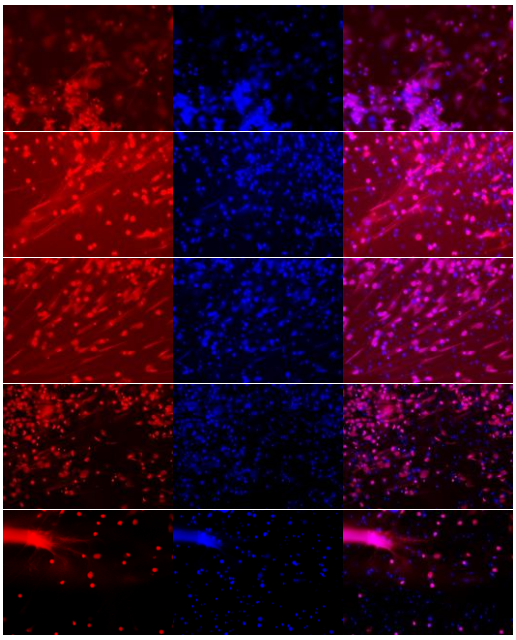
BLUE: NucBlue (cell permeable nucleic acid dye)

Figure A.4. NET images from 5 healthy, 5 AT, 5 Artemis deficient and 1 SAVI patients. Images were taken after 5 h incubation without any treatment. Red represents extracellular nucleic acids, blue represents both extracellular and intracellular nucleic acids. Images were taken by Floid Cell Imaging Station (ThermoFisher Scientific, USA) and analysed using the LSM Image Browser software (Zeiss, Germany).

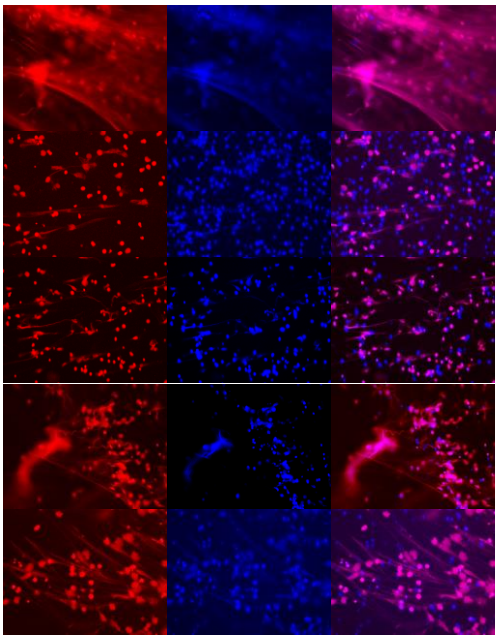
APPENDIX F

PMA Induced NETosis

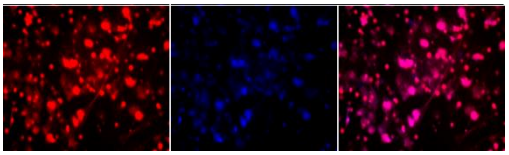
Healthy



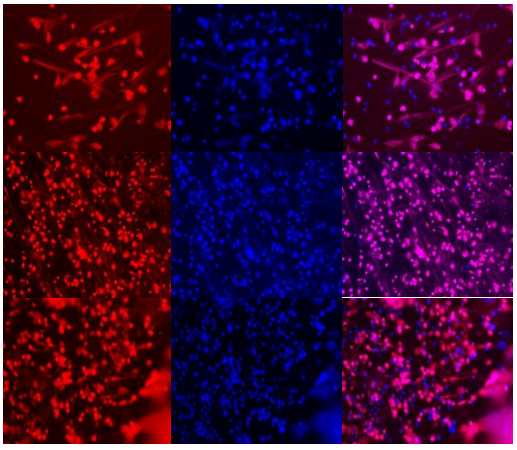
Artemis Deficient



SAVI



Ataxia Telangiectasia



RED: SYTOX Orange (cell impermeable nucleic acid dye)

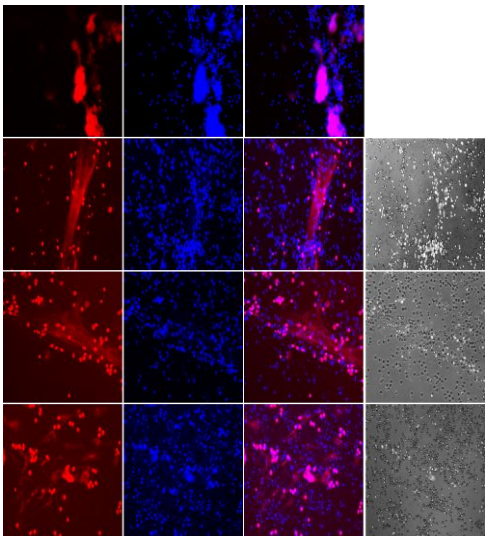
BLUE: NucBlue (cell permeable nucleic acid dye)

Figure A.5. NET images from 5 healthy, 3 AT, 5 Artemis deficient and 1 SAVI patients. Images were taken after 5 h incubation with PMA treatment. Red represents extracellular nucleic acids, blue represents both extracellular and intracellular nucleic acids. Images were taken by Floid Cell Imaging Station (ThermoFisher Scientific, USA) and analysed using the LSM Image Browser software (Zeiss, Germany).

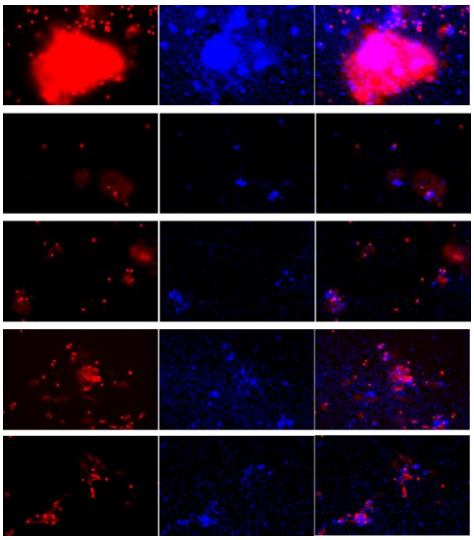
APPENDIX G

Patient Plasma Induced NETosis

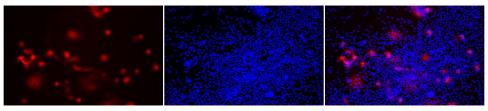
Healthy PMNs + Healthy Plasma



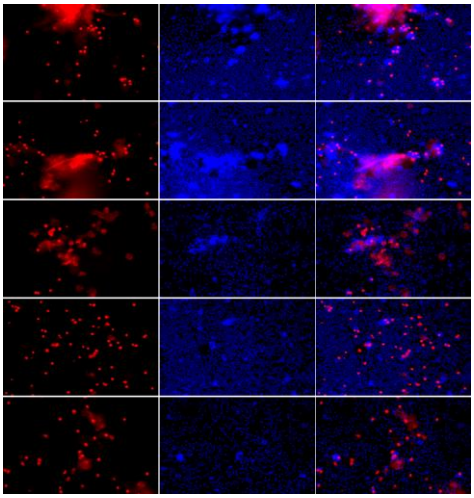
Healthy PMNs + Artemis Plasma



Healthy PMNs + SAVI Plasma



Healthy PMNs + AT Plasma



RED: SYTOX Orange (cell impermeable nucleic acid dye)

BLUE: NucBlue (cell permeable nucleic acid dye)

Figure A.6. NET images from healthy neutrophils incubated with 20% plasma of 5 healthy, 5 AT, 5 Artemis deficient and 1 SAVI patients. Images were taken after 5 h incubation with PMA treatment. Red represents extracellular nucleic acids, blue represents both extracellular and intracellular nucleic acids. Images were taken by Floid Cell Imaging Station (ThermoFisher Scientific, USA) and analysed using the LSM Image Browser software (Zeiss, Germany).

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- M.Sc. Thesis Project: “Functional analysis of cytosolic innate sensors of Myd88 and IRAK4 deficient patients”
- M.Sc. Thesis Project: “screening of immune response characteristics of interferonopathy patients with STING Associated Vasculopathy onset in Infancy (SAVI), Ataxia Telangiectasia, Artemis Deficiency and TREX-1 Deficiency”

PROFESSIONAL EXPERIENCE:

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PUBLICATIONS:

1. Gul E., Eroglu FK, Surucu,N., Yakicier C., Gursel I. , Ozen S., Gursel M. : “Immune Response Characteristics of STING Associated Vasculopathy With Onset In Infancy (SAVI) Patients”, 3rd International Molecular Immunology & Immunogenetics Congress, April 27-30, 2016. Antalya, Turkey (Poster Presentation)

2. E. Gul, Eroglu FK, Yakicier C., Gursel I., Ozen S., Gursel M. : "Type I Interferon Associated Immune Responses In Patients With STING-Associated Vasculopathy With Onset In Infancy", 4th European Congress of Immunology (ECI 2015) in Vienna, Austria (Poster Presentation)

3. Surucu N., **Gul E.**, Gursel I., Gursel M. : "Immunosuppressive effects of synthetic oligodeoxynucleotide A151 on inflammasome activation" 4th European Congress of Immunology (ECI 2015) in Vienna, Austria (Poster Presentation)

4. **Gul E**., Surucu N., Gungor B., Alpdundar E., Gursel I., Gursel M.; "İmmün Baskılayıcı Oligodeoksinükleotid A151'in Tip 1 İnterferonopatilere Yönelik Terapötik Ajan Olarak Etkileri", 23rd Turkish National Congress of Immunology, 2015 26th-30th April 2015, Antalya/TURKEY (Poster Presentation)

5. Surucu N., **Gul E**., Gursel I., Gursel M.; "Immün Baskılayıcı Sentetik Oligodeoksinükleotid A151'in Kontrolsüz Enflamazom Aktivasyonundan Kaynaklı Hastalıklarda Terapötik Ajan Olarak Etkileri", 23rd Turkish National Congress of Immunology, 2015 26th-30th April 2015, Antalya/TURKEY (Poster Presentation)

CONFERENCES & WORKSHOPS ATTENDED:

1. 3rd International Molecular Immunology & Immunogenetics Congress, April 27-30, 2016. Antalya, Turkey
2. 2nd Clinical Immunology Congress, March 31-April 3, 2016. Antalya, Turkey.
3. 4th Europian Congress of Immunology, September 6-9, 2015. Vienna, Austria.
4. 23rd National Immunology Congress, April 26–30, 2015. Antalya, Turkey.
5. Molecular Immunology & Immunogenetics Congress II, April 27-30, 2014. Antalya, Turkey.

SOCIETY MEMBERSHIPS:

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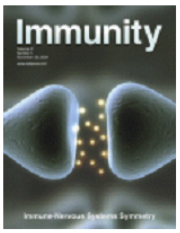

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