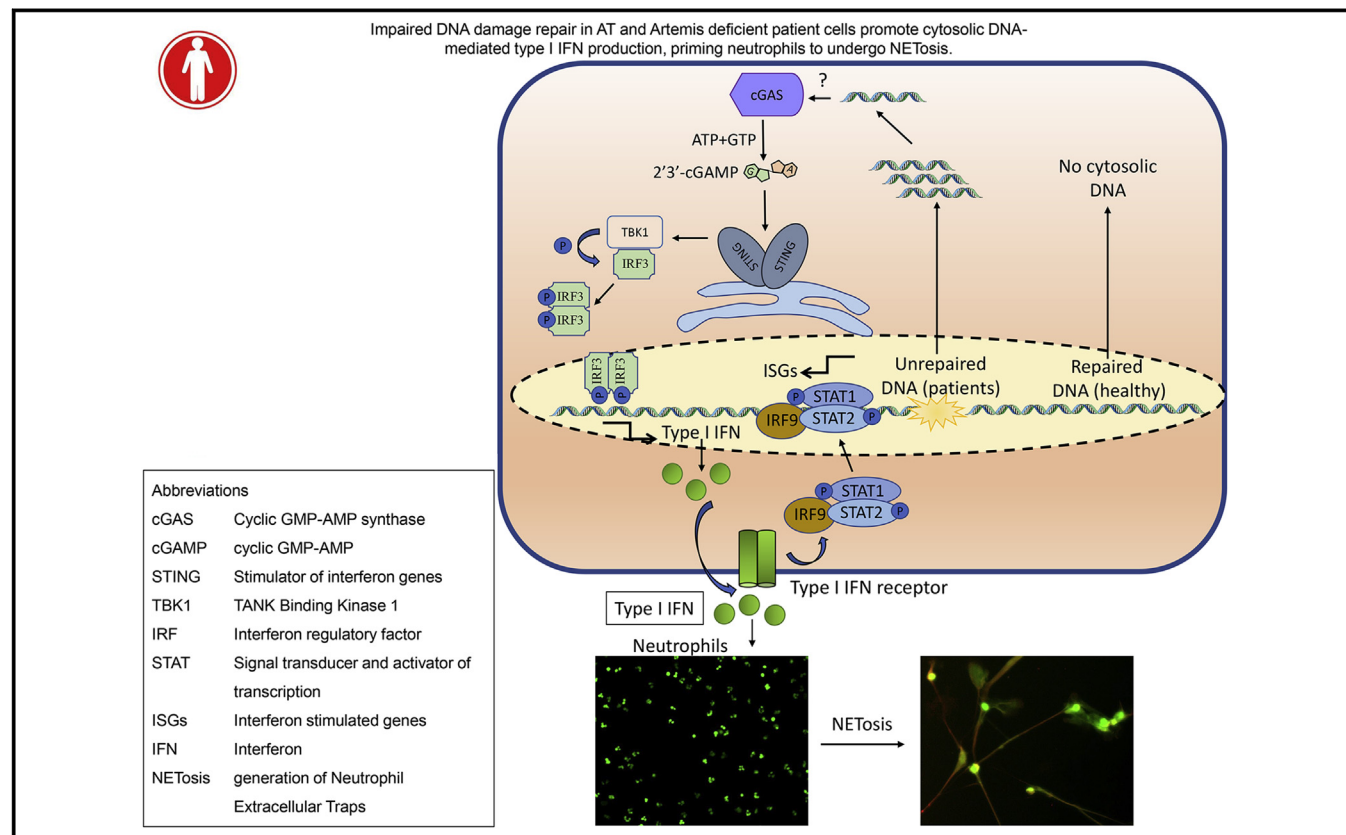


Type I IFN-related NETosis in ataxia telangiectasia and Artemis deficiency



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GRAPHICAL ABSTRACT



Background: Pathological inflammatory syndromes of unknown etiology are commonly observed in ataxia telangiectasia (AT) and Artemis deficiency. Similar inflammatory manifestations also exist in patients with STING-associated vasculopathy in infancy (SAVI).

Objective: We sought to test the hypothesis that the inflammation-associated manifestations observed in patients with AT and Artemis deficiency stem from increased type I IFN signature leading to neutrophil-mediated pathological damage.

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Methods: Cytokine/protein signatures were determined by ELISA, cytometric bead array, or quantitative PCR. Stat1 phosphorylation levels were determined by flow cytometry. DNA species accumulating in the cytosol of patients' cells were quantified microscopically and flow cytometrically. Propensity of isolated polymorphonuclear granulocytes to form neutrophil extracellular traps (NETs) was determined using fluorescence microscopy and picogreen assay. Neutrophil reactive oxygen species levels and mitochondrial stress were assayed using fluorogenic probes, microscopy, and flow cytometry.

Results: Type I and III IFN signatures were elevated in plasma and peripheral blood cells of patients with AT, Artemis deficiency, and SAVI. Chronic IFN production stemmed from the accumulation of DNA in the cytoplasm of AT and Artemis-deficient cells.

Neutrophils isolated from patients spontaneously produced NETs and displayed indicators of oxidative and mitochondrial stress, supportive of their NETotic tendencies. A similar phenomenon was also observed in neutrophils from healthy controls exposed to patient plasma samples or exogenous IFN- α .

Conclusions: Type I IFN-mediated neutrophil activation and NET formation may contribute to inflammatory manifestations observed in patients with AT, Artemis deficiency, and SAVI. Thus, neutrophils represent a promising target to manage inflammatory syndromes in diseases with active type I IFN signature. (J Allergy Clin Immunol 2018;142:246-57.)

Key words: Primary immunodeficiencies, autoinflammation, ataxia telangiectasia, Artemis deficiency, type I IFN, interferonopathy, neutrophil extracellular traps, NETosis

Constitutive type I IFN production in the absence of infection can be detrimental to the host, promoting severe inflammation. In this context, an increasing number of heterogeneous diseases with elevated IFN signatures are being classified under the spectrum of "type I interferonopathies."¹⁻⁴ Dysregulation in nucleic acid removal or recognition is thought to contribute to accumulation of endogenous nucleic acid ligands or constitutive activation of nucleic acid-sensing signaling pathways, culminating in excessive type I IFN production. Accumulating evidence also suggests a link between DNA damage repair mechanisms and type I IFN production. Elevated IFN-stimulated gene signatures were observed in cells exposed to DNA damage.⁵⁻⁹ Of note, DNA damage was demonstrated to stimulate leakage of genome-derived DNA species into the cytosol, thereby inducing the expression of type I IFNs.¹⁰ Interestingly, in patients with ataxia telangiectasia (AT) with loss-of-function mutations in the DNA repair protein ataxia telangiectasia mutated (ATM), unrepaired DNA lesions led to release and accumulation of single-stranded DNA (ssDNA) species into the cytosol, inducing type I IFNs via a cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING)-dependent mechanism.¹¹ AT is a complex inherited multisystem disease characterized by progressive neurodegeneration, combined immunodeficiency, radiosensitivity, and a predisposition to malignancy.¹² Pathological inflammatory and autoimmune syndromes of unknown etiology have been reported in many patients with AT.^{11,13-18} Mutations in the *DCLRE1C* gene encoding Artemis, another DNA double-strand break repair protein further involved in V(D)J recombination during T- and B-cell development,^{19,20} cause immunodeficiency phenotypes ranging from radiosensitive severe combined immunodeficiency

Abbreviations used

AT:	Ataxia telangiectasia
ATM:	Ataxia telangiectasia mutated
dsDNA:	Double-stranded DNA
recIFN- α :	Recombinant IFN- α
SAVI:	STING-associated vasculopathy with onset in infancy
ssDNA:	Single-stranded DNA
NET:	Neutrophil extracellular trap
SLE:	Systemic lupus erythematosus
IP-10:	Interferon-inducible protein 10
ISGs:	Interferon-stimulated genes
ROS:	Reactive oxygen species

to mere antibody deficiency, with frequent autoimmune and inflammatory manifestations.²¹⁻²⁶ It is possible that similar to AT, unrepaired DNA fragments can leak into the cytosol in Artemis-deficient cells and initiate type I IFN production, promoting chronic inflammation. To test this hypothesis, we compared the immune status of patients with AT and Artemis deficiency to that of healthy controls and to that of a patient with a recently identified severe interferonopathy, STING-associated vasculopathy with onset in infancy (SAVI), caused by a gain-of-function mutation in the *TMEM173* gene encoding the adaptor signaling protein STING.²⁷ This syndrome is characterized by neonatal-onset systemic inflammation with cutaneous vasculopathy, skin lesions, and interstitial lung disease.

Similar to some patients with AT and SAVI, unexplained inflammatory skin lesions, erythematous papules, and lung injury (bronchiectasis) are observed in patients with Artemis deficiency,^{14,23,28,29} suggestive of a common underlying immune dysregulation in the pathogenesis of these diseases. In this context, neutrophil extracellular trap (NET) formation and neutrophil-related tissue injury have been linked to the pathophysiology of a broad spectrum of diseases, including systemic lupus erythematosus (SLE),³⁰⁻³⁴ wherein enhanced NET formation was associated with IFN- α signaling.³⁵ Therefore, a possible role of NETs in the chronic inflammatory responses observed in patients with AT, Artemis deficiency, and SAVI was also investigated.

METHODS

Participants

Samples from a patient suspected to have SAVI and his parents were submitted for study after written informed consent had been obtained. The protocol was approved by the institutional review board of Hacettepe University Medical Faculty, Ankara. To detect *TMEM173* gene mutations, exons (3-8) were amplified, followed by bidirectional Sanger sequencing (see the [Methods](#) section in this article's Online Repository at www.jacionline.org). The patient was confirmed to have a de novo N154S mutation in exon V, consistent with a previously defined SAVI phenotype.²⁷

Samples from patients with AT and Artemis deficiency and healthy donors were submitted for study after written informed consent had been obtained. All patients with Artemis deficiency had hypomorphic Artemis mutations (homozygous missense mutation c.194C>T in *DCLRE1C* gene) with minimal residual recombination efficiency, and were of age between 5 and 21 years. Patients with AT were either diagnosed clinically or carried various mutations as reported in [Table E1](#) in this article's Online Repository at www.jacionline.org. Age of patients with AT ranged from 4 to 17 years. Healthy donors' age ranged from 5 to 22 years. The protocol was approved by the institutional review board of Necmettin Erbakan University Medical Faculty, Konya.

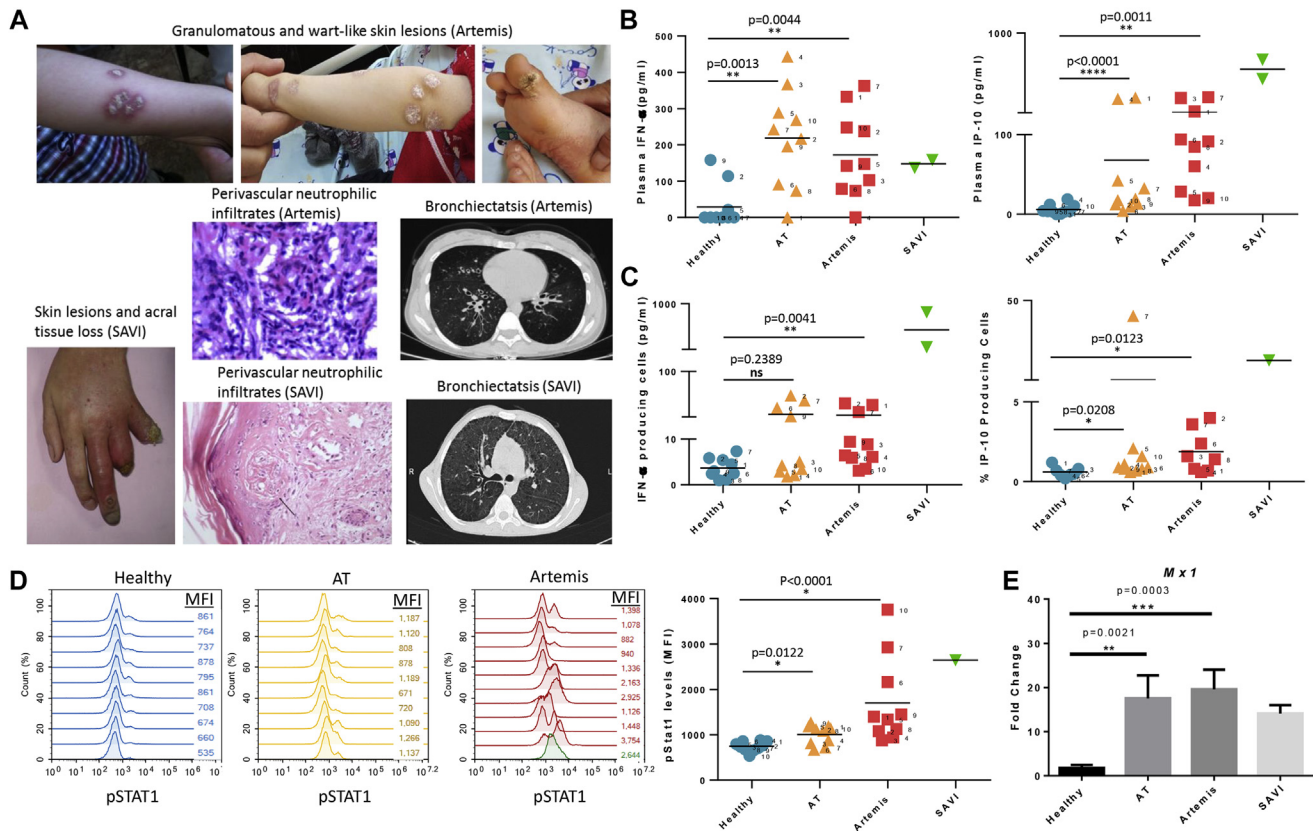


FIG 1. Autoimmune/autoinflammatory features in patients and identifiers of elevated type I IFN signatures. **A**, The autoinflammatory skin manifestations and evidence of interstitial lung disease in patients with Artemis deficiency and SAVI. Vascular inflammation with neutrophilic infiltrates is observed in skin lesion biopsy samples (hematoxylin and eosin, original magnification $\times 400$ [Artemis sample] and $\times 40$ [SAVI sample]). **B**, Increased levels of circulating IFN- α and IP-10 in patients with AT ($n = 10$), Artemis deficiency ($n = 10$) and SAVI ($n = 1$; 2 separate measurements 1 month apart) as compared with healthy subjects ($n = 10$). **C**, PBMCs from patients with AT ($n = 10$ and $n = 10$), Artemis deficiency ($n = 10$ and $n = 8$), and SAVI ($n = 1$; 2 separate measurements 1 month apart) spontaneously secrete IFN- α and intracellularly accumulate more IP-10, respectively, than do healthy control cells ($n = 9$ and $n = 7$). **D**, pSTAT1 levels in PBMCs of healthy subjects (blue) are significantly lower than those observed in patients with AT (orange), Artemis deficiency (red), and SAVI (green) (flow cytometric histograms). MFI values obtained from histograms were also plotted and shown. **E**, Increased expression of IFN-regulated gene *MX1* in patients with AT ($n = 8$) and Artemis deficiency ($n = 9$), as compared with healthy controls ($n = 10$) and with a patient with SAVI ($n = 1$; 2 separate measurements 1 month apart). Data are representative of at least 3 independent experiments. MFI, Mean fluorescence intensity; pSTAT1, phosphorylated STAT1.

Cytokine measurements and functional studies

Circulating and PBMC-secreted or -expressed cytokine/chemokine/protein levels and gene expression analyses were performed according to cytometric bead array, ELISA, or quantitative RT-PCR-based standard procedures and are described in this article's [Methods](#) section. Interferon-inducible protein 10 (IP-10) and phosphorylated Stat1 levels within PBMCs were determined by intracellular cytokine/protein staining and flow cytometry. Cytosolic ssDNA or double-stranded DNA (dsDNA) levels in healthy and patient PBMCs were determined microscopically or flow cytometrically in untreated or ultraviolet light-treated permeabilized cells stained for ssDNA or dsDNA-specific antibodies. Healthy or patient neutrophils were analyzed for NET formation using fluorescence microscopy of samples stained for total DNA, extracellular DNA, and/or myeloperoxidase. Amount of NETs released was quantified using a micrococcal nuclease digestion/picogreen quantitation-based spectrofluorometric assay. Cytosolic and mitochondrial reactive oxygen species (ROS) levels in neutrophils were assessed flow cytometrically using ROS reactive probes Dihydrorhodamine 123 and MitoSox Red, respectively. Neutrophil-associated mitochondrial stress response was assessed using microscopic imaging or flow cytometric analysis of mitochondrial membrane potential-sensitive JC-1 dye-stained

neutrophils. Details of the methods used are provided in this article's [Methods](#) section.

RESULTS

Autoimmune/autoinflammatory manifestations in patients with AT, Artemis deficiency, and SAVI in relation to elevated type I IFN activity

Patients with AT, Artemis deficiency, and SAVI frequently suffer from severe autoimmune/autoinflammatory complications. Skin lesions with neutrophilic infiltrates and interstitial lung disease are commonly observed in patients with Artemis deficiency and in patients with SAVI ([Fig 1, A](#)). In the case of SAVI, the observed inflammation and immunopathology was linked to excessive type I IFN production.²⁷ However, factor(s) instigating chronic inflammation in individuals with AT and Artemis deficiency remain poorly understood. Recent evidence suggests that unrepaired DNA double-strand breaks stimulate

type I IFN production in patients with AT, which may account for the observed chronic inflammatory manifestations.¹¹ Whether spontaneous type I IFN production is also an underlying mechanism contributing to chronic inflammation in patients with Artemis deficiency remained unexplored.

To test the hypothesis that the inflammation-associated manifestations observed in patients with AT and Artemis deficiency may result from an increased type I IFN signature, we compared the immune status of patients with AT and Artemis deficiency with that of healthy controls and with that of a patient with SAVI carrying the N154S (c.461A>G) mutation in exon V of *TMEM173* gene (as a positive control of a known type I interferonopathy; see Fig E1 in this article's Online Repository at www.jacionline.org). All patients with Artemis deficiency harbored identical hypomorphic mutations in exon 3 of *DCLRE1C* gene encoding the protein Artemis (c.194C>T; p.T65I) as previously reported.^{23,24} Patients with AT included in this study received a clinical diagnosis and in 5 patients, the mutation was determined (see Table E1). Clinical characteristics and hematological and serological findings of all patients enrolled in this study are summarized in Tables E1-E3, respectively, in this article's Online Repository at www.jacionline.org.

We observed that the plasma levels of IFN- α (multiple subtypes), IP-10, and IFN- λ 1 (IL-29) were significantly elevated in all patient groups (Fig 1, B; see Fig E2 in this article's Online Repository at www.jacionline.org). There was no increase in circulating levels of other proinflammatory cytokines tested (IL-8, IL-17, IL-1 β , TNF- α and IL-6; Fig E2). Furthermore, PBMCs from patients spontaneously secreted IFN- α 2a and IP-10 into culture supernatants in the absence of any stimulation (Fig 1, C; Fig E3 in this article's Online Repository at www.jacionline.org). When stimulated with the STING ligand 2'3'-cGAMP, AT and Artemis-deficient patient cells produced higher levels of IFN- α 2a and IP-10 than did healthy control cells (see Fig E4 in this article's Online Repository at www.jacionline.org), whereas as expected, the SAVI patient's elevated responses could not be stimulated further with cyclic GMP-AMP (cGAMP).²⁷ Consistent with the elevated type I IFN-associated cytokine/chemokine levels, unstimulated patient cells had significantly higher levels of phosphorylated STAT1 than did cells from healthy controls (Fig 1, D). Stat1 is an important transcription factor that is phosphorylated in response to type I IFN signaling and controls the induction of interferon-stimulated genes (ISGs). Therefore, we also assessed the expression of 2 ISGs in PBMC samples by quantitative RT-PCR and found that the transcription of MX1 (Fig 1, E) and ISG15 (see Fig E5 in this article's Online Repository at www.jacionline.org) was upregulated in patient samples.

These results are of interest because they not only confirm the existence of exaggerated type I IFN signaling in patients with AT but also demonstrate a similar phenomenon in patients with Artemis deficiency 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 patients with SAVI.

DNA spontaneously accumulates in the cytosol of AT and Artemis-deficient patient cells

DNA damage can incite accumulation of cytoplasmic DNA species, thereby activating cytosolic DNA-sensing pathways.⁵⁻¹¹

We therefore assessed cytosolic levels of ssDNA or dsDNA species in healthy and patient PBMCs that were either untreated or exposed to ultraviolet light-induced DNA damage. The extent of spontaneous DNA leakage was consistently higher in untreated patient cells than in cells from healthy controls (Fig 2, A and B, left panels). Ultraviolet light-induced DNA damage stimulated cytosolic DNA release in healthy cells and exaggerated the leakage response in patient cells (Fig 2, B, right panels). Of interest, the staining protocols were first established in THP-1 and TREX-1-deficient THP-1 cells to exclude the possibility that the antibodies would breach the nuclear membrane and interact with nuclear material (see Fig E6 in this article's Online Repository at www.jacionline.org).

To evaluate whether the detected cytosolic DNA was of nuclear and/or mitochondrial origin, cytosolic extracts from 2 patients with AT and 2 patients with Artemis deficiency were prepared.³⁶ Samples were assessed for the absence of mitochondrial and nuclear contamination using the mitochondrial and nuclear markers VDAC and Lamin A/C, respectively, in comparison to a whole cell extract, nuclear extract, and mitochondrial extract prepared from HCT 116 cell line as a positive control of each fraction (Fig 2, C). Cytosolic extracts were then analyzed for nuclear and mitochondrial DNA by quantitative PCR (Fig 2, D). Results demonstrated that patient cytosolic extracts contained DNA of both mitochondrial (103 bp ND5 mitochondrial DNA) and nuclear (133 bp H3 nuclear DNA) origin. Our data suggest that impaired DNA damage repair in AT and Artemis-deficient patient cells promote accumulation of DNA species in cytosol, provoking type I IFN-induced sterile inflammation.

AT, Artemis-deficient and SAVI patient neutrophils spontaneously produce NETs

Increased IFN signature is consistently demonstrated in peripheral blood of patients with SLE.³⁷ Furthermore, neutrophils isolated from patients with SLE exhibit abnormal features such as increased aggregation and a tendency to undergo spontaneous NETosis, suggestive of a link between elevated levels of type I IFNs and neutrophil-mediated disease pathogenesis.³⁸ In fact, one preliminary study has shown that blocking of IFN- α signaling in neutrophils reduced NET formation in murine lupus.³⁵ To date, except for SLE, neutrophil functions in diseases strongly associated with increased type I IFN signature (like SAVI) 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.

Blood polymorphonuclear granulocytes of patients with AT, Artemis deficiency, and SAVI spontaneously released NETs (Fig 3, A; see Fig E7 in this article's Online Repository at www.jacionline.org) visualized as red-stained extracellular DNA (using cell-impermeable dye SYTOX Orange over Hoechst-stained total DNA [blue]). The amount of DNA associated with NETs was also quantified spectrofluorometrically using a micrococcal nuclease/picogreen-based assay. Healthy neutrophils released very low amounts of DNA, whereas AT, Artemis-deficient, and SAVI patient cells released approximately 5-, 4-, and 2.5-fold more NET-associated DNA, respectively (Fig 3, B). The extruded nuclear material was also associated with the neutrophil granular protein myeloperoxidase, indicative of its NETotic origin (Fig 3, C). To further validate the activation

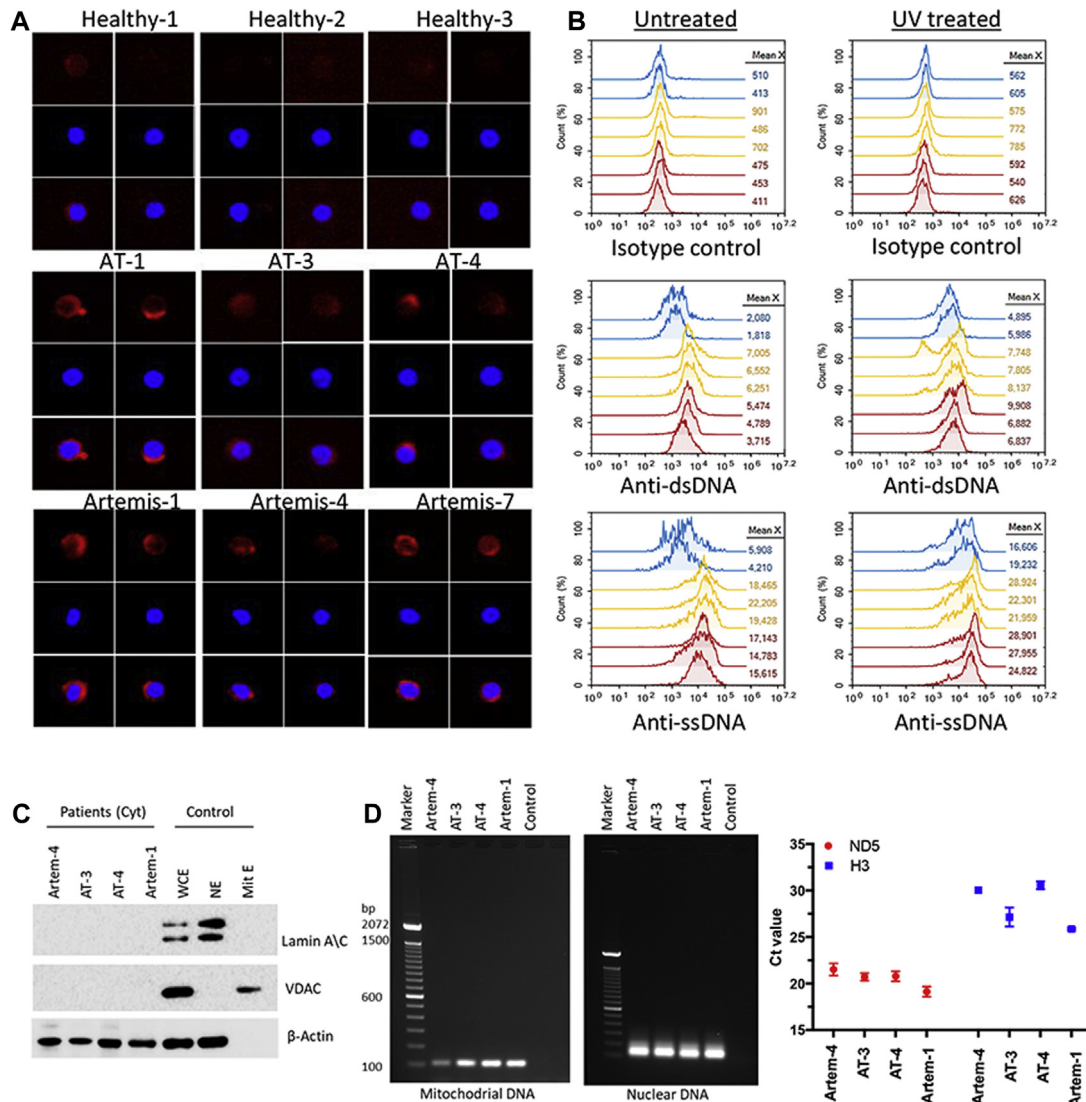


FIG 2. Increased levels of cytosolic ssDNA and dsDNA species in patients with AT and Artemis deficiency. **A**, Immunofluorescence microscopic analysis of cytosolic ssDNA (red) in healthy, AT, and Artemis-deficient patient PBMCs. Nuclei were stained with Hoechst (blue). Each panel represents images from different individuals ($n = 3$). **B**, Flow cytometric analysis of untreated or UV light-treated cells from healthy (blue), AT (orange), or Artemis-deficient (red) patients. Histograms in each plot represent a different individual. Samples stained with isotype control (upper panels), anti-dsDNA (middle), and anti-ssDNA (bottom) are shown. Mean fluorescence intensities are reported in each panel. **C**, Absence of mitochondrial or nuclear contamination in cytosolic extracts prepared from patient cells. Whole cell extract (WCE), nuclear extract (NE), and mitochondrial extract (Mit E) prepared from a control cell line (HCT 116) were included as a positive control of each fraction. **D**, The 103 bp ND5 mitochondrial DNA (left gel image) and the 133 bp H3 nuclear DNA (right gel image) and cycle threshold (Ct) values for ND5 and H3 genes amplified by quantitative PCR (Ct value graph) from cytosolic extracts. The boxes represent mean Ct values and the bars show SDs from 2 independent experiments. Control indicates negative control in quantitative PCR reaction without cytosolic DNA. UV, Ultraviolet.

status of neutrophils in blood, we next determined the concentration of neutrophil elastase in patient versus healthy plasmas. Results showed that patients with AT, Artemis deficiency, and SAVI had significantly higher levels of elastase in their plasma compared with healthy controls (Fig 3, D), suggestive of neutrophil overactivation. Percentage of a distinct subset of low-density, pathogenic granulocytes is frequently elevated in blood of patients with autoimmune/autoinflammatory manifestations.³⁹ We found that in 4 of the 6 tested patients

with AT and Artemis deficiency, the percentage of low-density granulocytes was substantially increased (compared with healthy controls, average low-density granulocyte % was 8- and 7-fold higher in patients with AT and Artemis deficiency, respectively, although this increase was not significant in the AT group; see Fig E8 in this article's Online Repository at www.jacionline.org), suggesting that pathogenic low-density granulocytes could also contribute to chronic inflammation in these patients.

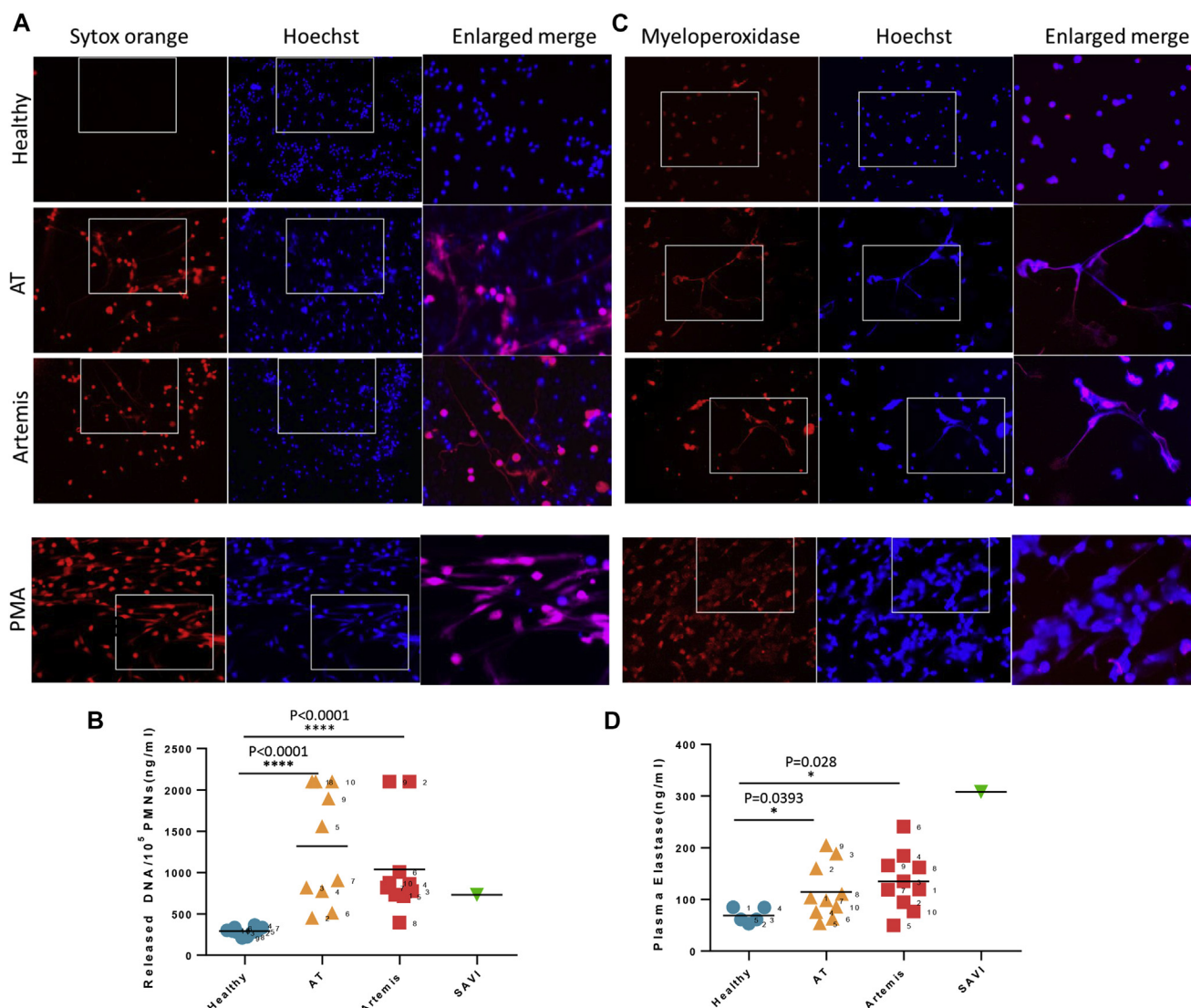


FIG 3. Analysis of spontaneous NET formation in patient neutrophils. **A** and **C**, Patient neutrophils spontaneously produce NETs that are visible in samples stained with Sytox Orange (extracellular DNA), Hoechst (total DNA), and neutrophil myeloperoxidase (Fig 3, C). PMA-stimulated neutrophils served as positive control of NET formation (bottom images). **B**, The quantitation of released NET DNA from patient cells. All patient neutrophils ($n = 10$ for AT and Artemis-deficient and 1 SAVI) released significantly higher levels of DNA into culture supernatants than did healthy control cells ($n = 10$). **D**, Patient plasma samples contained elevated levels of neutrophil activation marker elastase. Data are representative or combined from at least 4 independent experiments.

AT, Artemis-deficient and SAVI patient plasma samples and recombinant IFN- α trigger NET release from healthy neutrophils

Next, to determine whether patient plasma-associated factor(s) contributed to NET formation, neutrophils from healthy donors were cultured in the presence of healthy or patient plasma samples. Results revealed that patient but not healthy plasma samples stimulated healthy neutrophils to undergo NETosis and release their DNA as NETs (Fig 4, A; see Fig E9 in this article's Online Repository at www.jacionline.org). Furthermore, patient plasma-induced NETosis was inhibited in healthy polymorphonuclear cells pretreated with the JAK inhibitor Tofacitinib (see Fig E10 in this article's Online Repository at www.jacionline.org), suggesting that elevated type I IFN-related cytokines/

chemokines may contribute to NET formation in healthy neutrophils. To validate this assumption, healthy neutrophils were treated with either recombinant IFN- α (recIFN- α) or recombinant IFN- γ . Neutrophils exposed to recIFN- α but not to recombinant IFN- γ extruded significantly higher levels of DNA than untreated controls (Fig 4, B) and the extracellular filaments of chromatin were associated with myeloperoxidase (Fig 4, C), suggestive of NETotic death.

Type I IFNs mediate oxidative and mitochondrial stress in neutrophils

ROS production has been reported as an integral part of various NETosis-triggering stimuli.⁴⁰ In AT, chronic oxidative stress and

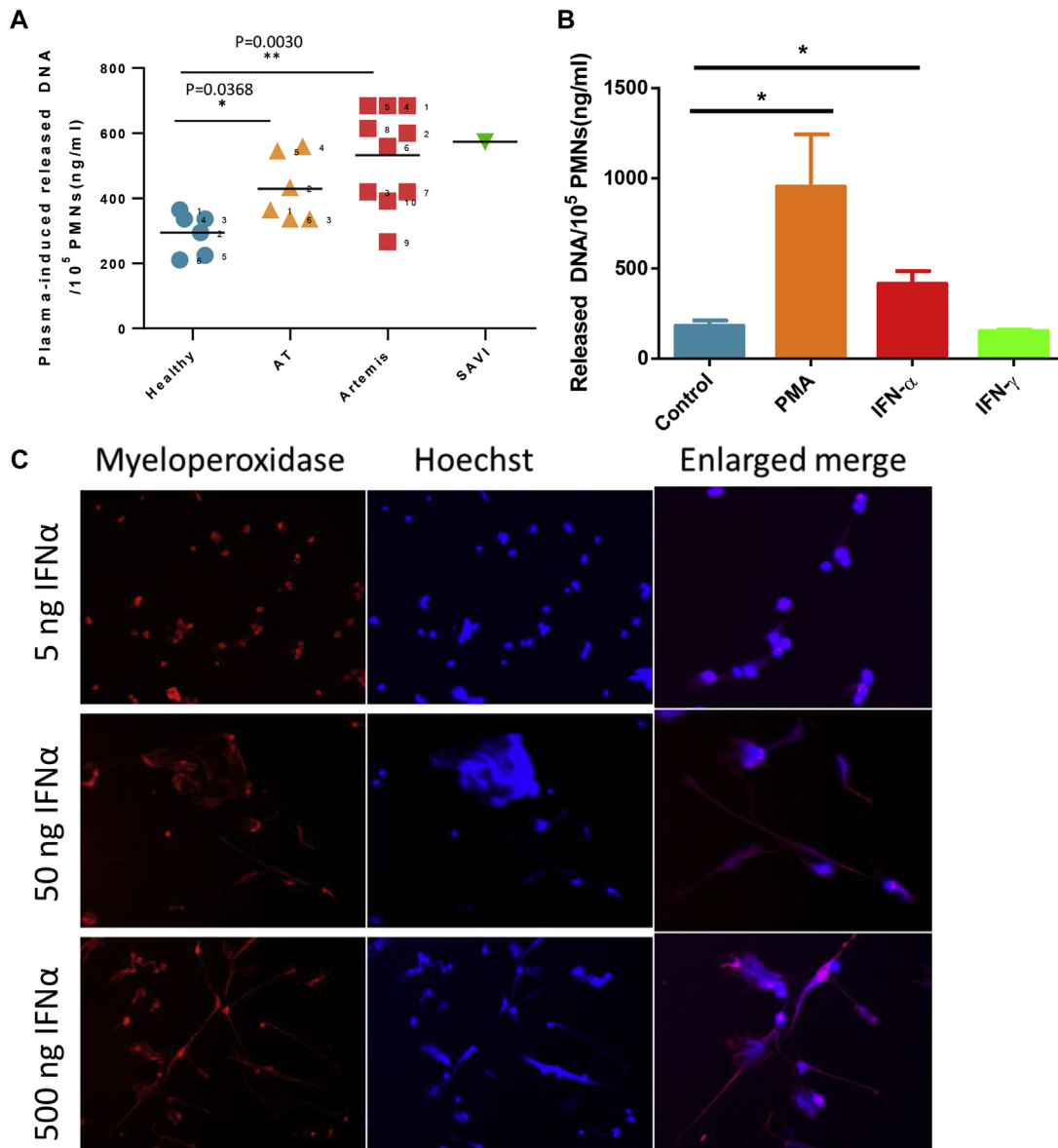


FIG 4. Analysis of patient plasma or recIFN- α -induced NET formation in healthy neutrophils. **A** and **B**, Patient plasma samples and recIFN- α trigger NET formation in healthy neutrophils, whereas healthy plasma or recIFN- γ has no effect. **C**, Healthy neutrophils incubated with various doses of recIFN- α release NETs that stain positive for DNA (blue) and myeloperoxidase (red). Plasma samples from different donors were tested on neutrophils from a single healthy donor. Data are representative of 3 independent experiments.

mitochondrial damage have been implicated as factors underlying disease pathogenesis.⁴¹ We therefore examined the intracellular levels of ROS in patient cells using the cytosolic and mitochondrial ROS indicators Dihydrorhodamine 123 (DHR123) and MitosoxRed, respectively. Freshly isolated neutrophils from patients with AT and Artemis deficiency displayed 2-fold more DHR123 fluorescence than healthy controls (Fig 5, A), indicative of ongoing chronic oxidative stress. Of note, in this experiment, 2 of the 6 patients with Artemis deficiency had recently undergone bone marrow transplantation 6 months earlier (shown as purple lines in histogram plots in Fig 5, A). Although such small sample size and posttransplantation duration are inappropriate to derive definitive conclusions, preliminary evidence suggests that oxidative stress is not

alleviated following hematopoietic stem cell transplantation. Analysis of mitochondrial superoxide anion levels using MitosoxRed revealed no significant differences between patient cells and healthy controls (see Fig E11 in this article's Online Repository at www.jacionline.org). However, AT and Artemis-deficient patient neutrophils exposed to recIFN- α produced 2-fold more mitochondrial superoxide anion than did healthy neutrophils incubated with identical dose of the cytokine (Fig 5, B), suggesting that type I IFNs can exacerbate mitochondrial ROS production and therefore may contribute to mitochondrial damage. To test this hypothesis, JC-1-stained neutrophils from 3 different subjects were incubated without or with recIFN- α for 30 minutes, and mitochondrial health was determined using microscopic images and flow cytometric

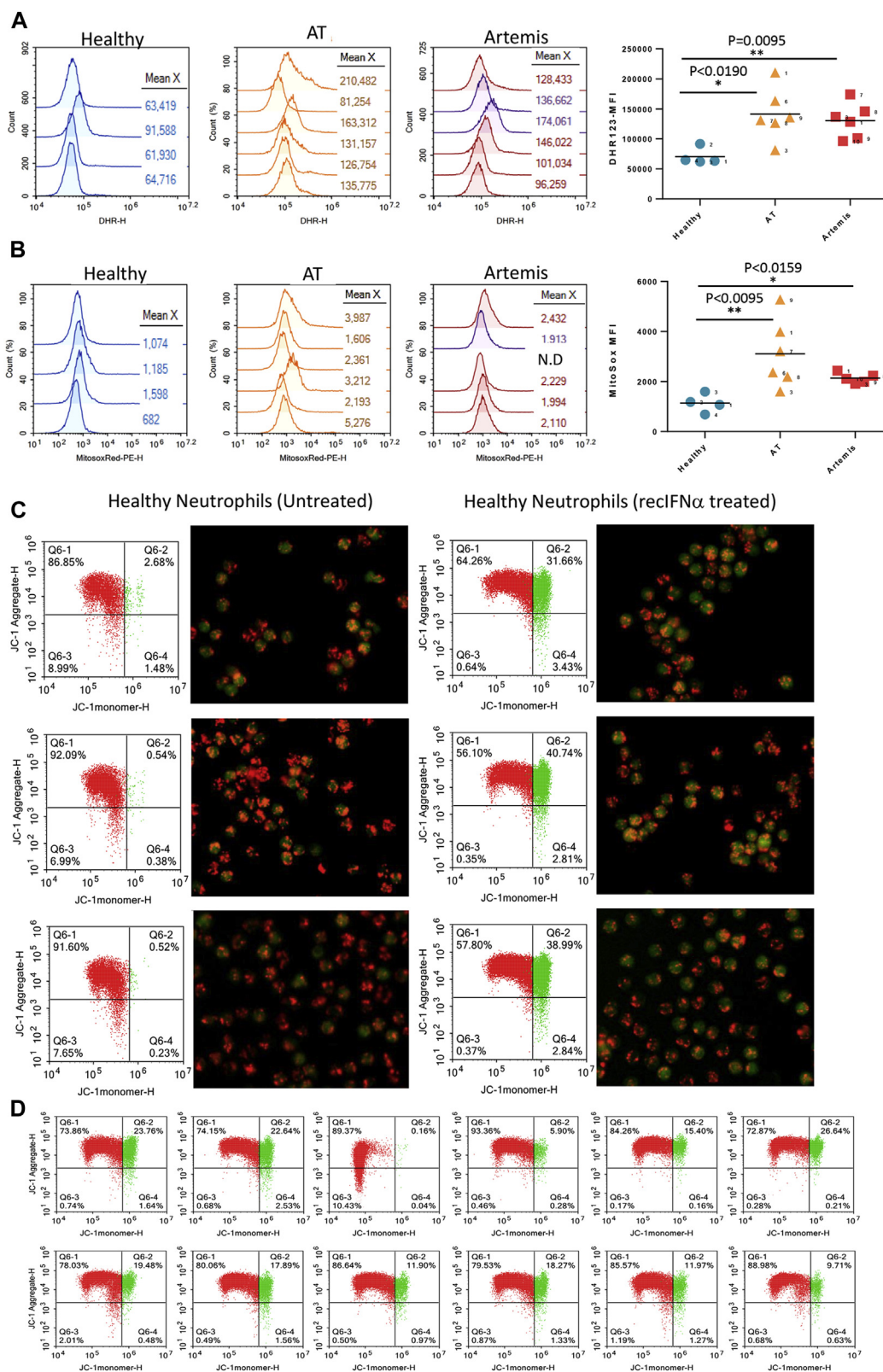


FIG 5. Analysis of cytosolic and mitochondrial ROS levels and measurement of mitochondrial stress in neutrophils. **A** and **B**, Cytosolic (Fig 5, **A**) and mitochondrial (Fig 5, **B**) ROS levels are significantly elevated in unstimulated (Fig 5, **A**) or recIFN- α -stimulated (Fig 5, **B**) AT ($n = 6$) and Artemis-deficient ($n = 6$) patient neutrophils as opposed to healthy controls ($n = 3$). Both flow cytometric individual histograms and plotted mean fluorescence intensity (MFI) values are given. Two of the patients with Artemis deficiency who have received bone marrow transplantation are shown in purple. **C**, Unstimulated neutrophils isolated from healthy individuals ($n = 3$) show low levels of monomeric JC-1 staining (green fluorescence in microscopy images and flow cytometry plots) that is upregulated following exposure to recIFN- α , indicating mitochondrial membrane depolarization and stress. **D**, Unstimulated patient neutrophils displayed JC-1 monomer staining, indicative of ongoing stress response.

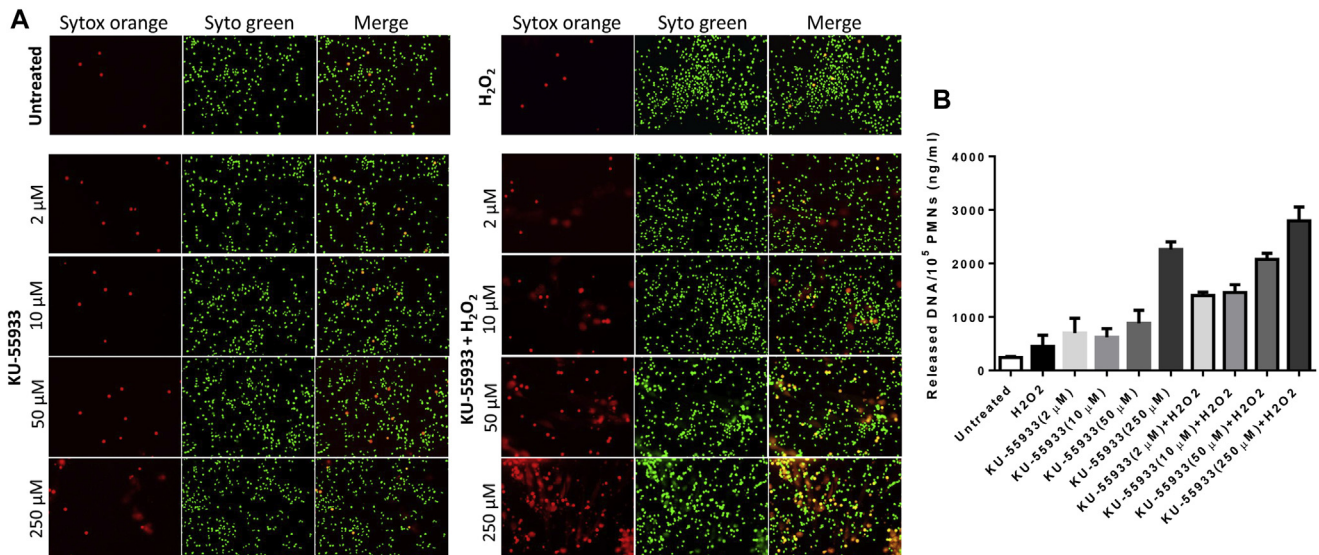


FIG 6. DNA damage in the absence of DNA repair triggers NET formation in healthy neutrophils. **A**, Healthy neutrophils spontaneously produce NETs when exposed to the highest dose of ATM inhibitor (KU-55933; 250 μ M) alone or all doses of the combined inhibitor and H₂O₂ (DNA damage-inducing agent, 400 μ M). NETs are visible in samples stained with Sytox Orange (extracellular DNA) and Syto Green (total DNA). **B**, Quantitation of released NET DNA from treated cells. Results are the average response of 2 healthy donor neutrophils $\pm$ SD.

analysis of cells positive for red JC-1 aggregates (healthy mitochondria) versus green JC-1 monomers (damaged, hypopolarized mitochondria). Healthy neutrophils not exposed to recIFN- α had lower levels of depolarized mitochondria (Fig 5, C). However, within 30 minutes of incubation with recIFN- α , monomeric green JC-1 dye fluorescence intensity was enhanced in approximately 30% to 40% of cells, suggesting that type I IFNs induce mitochondrial ROS (Fig 5, B) and cause mitochondrial membrane depolarization. To examine whether the observed spontaneous ROS (Fig 5, A) and type I IFN-induced exaggerated mitochondrial ROS (Fig 5, B) in patient neutrophils correlated with mitochondrial dysfunction, freshly isolated patient neutrophils were stained with JC-1 and analyzed without exposure to any cytokines. Neutrophils in 5 of the 6 patients with AT and all the 6 patients with Artemis deficiency, including the 2 who had received bone marrow transplantation, exhibited higher percentages of JC-1 green positive neutrophils (Fig 5, D) when compared with healthy controls (Fig 5, C, untreated healthy neutrophils). These results indicate that the ongoing chronic oxidative stress in patient neutrophils and extended exposure to type I IFNs may alter mitochondrial functions in neutrophils and facilitate NET formation.

Our data suggest that type I IFNs contribute to NETosis. Although spontaneous NETosis observed in patient neutrophils may in part depend on elevated type I IFNs, a lack of DNA repair and accumulated DNA damage could potentially represent cell-intrinsic factors exacerbating NETosis. To address this question, healthy neutrophils were left untreated or treated with H₂O₂ alone (DNA damage-inducing agent), KU-55933 alone (ATM inhibitor), or a combination of H₂O₂ and KU-55933. Absence or presence of NETs was evaluated at the end of 5 hours of incubation using fluorescence microscopy and the picogreen assays (Fig 6, A and B, respectively). Data indicated that DNA damage alone (400 μ M H₂O₂) had no measurable effect on healthy neutrophils. However, the highest dose of ATM inhibitor

alone and all doses of the combined inhibitor and H₂O₂ triggered NET formation, suggesting that DNA damage in the absence of repair can initiate NETosis. Collectively, our data advocate for a role of both type I IFNs and DNA damage defect underlying the NETotic phenotype of patient cells.

DISCUSSION

Herein, we showed that AT and Artemis deficiencies share certain features with known interferonopathies as evidenced by high levels of circulating type I and III IFNs, spontaneous secretion of IFN- α , and intracellular production of IP-10 in patient cells, increased phosphorylated STAT1 levels, and elevated expression of ISGs in PBMCs. Our data suggest that in the case of AT and Artemis deficiency, this enhanced type I IFN response most likely stems from defective DNA repair-associated cytosolic DNA accumulation in patient cells, triggering type I IFN secretion through the cGAS-STING cytosolic nucleic acid-sensing pathway.

SAVI and several other autoinflammatory/autoimmune diseases display a unique signature of type I IFN activity, suggesting a common underlying mechanism of clinical pathologies, including systemic inflammation, skin lesions, and pulmonary disease.^{1-4,27} The pathogenic mechanism(s) behind the type I IFN-inflicted tissue damage is unclear. Published work on patients with SLE and adenosine deaminase 2-deficient individuals (a monogenic type I interferonopathy) indicate that neutrophil-mediated mechanisms, and in particular NETs, might be involved in this damage response.^{30-34,42} Whether or not neutrophils contribute to autoinflammatory/autoimmune phenomena in patients with Artemis deficiency (vitiligo, Hashimoto's thyroiditis, juvenile idiopathic arthritis, and granulomatous skin lesions²³) and patients with SAVI remained unexplored. In case of AT, patient neutrophils were shown to overproduce proinflammatory cytokines but whether they formed pathological NETs

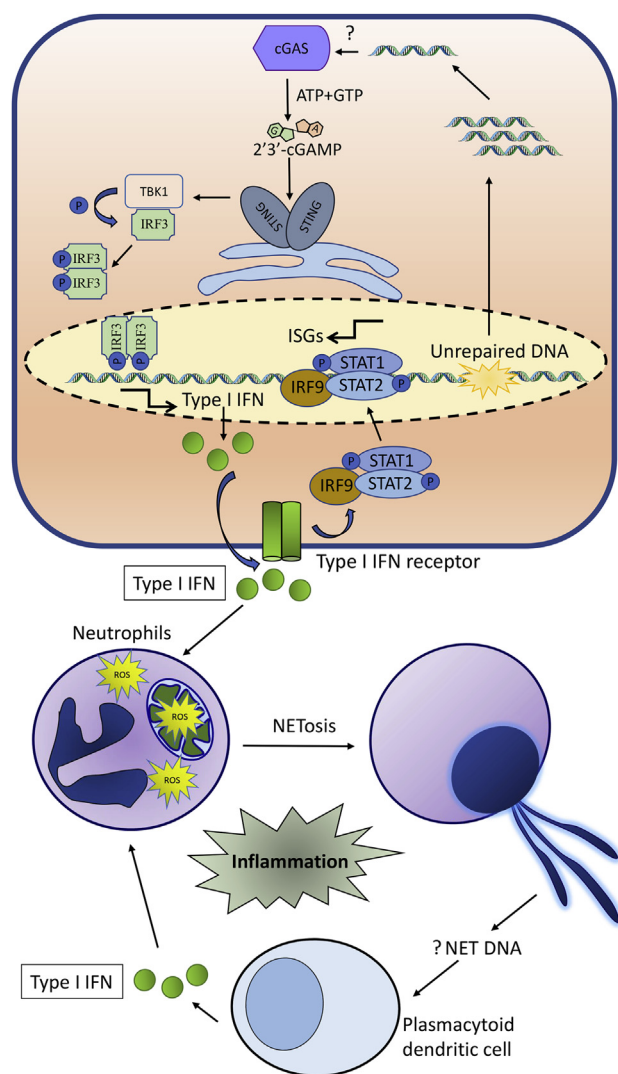


FIG 7. Model of type I IFN-mediated chronic inflammation in patients with AT, Artemis deficiency, and SAVI. In ATM and Artemis-deficient cells, unrepaired DNA fragments leak into the cytosol, activating the nucleic acid-sensing pathways (most probably the cGAS/cGAMP/STING pathway), stimulating TBK1-IRF3-dependent type I IFN production. In cells expressing a gain-of-function mutation in STING (patient with SAVI), the STING pathway is already active, independent of cytosolic DNA and/or 2'3'-cGAMP, resulting in constitutive type I IFN production. Type I IFNs act on cells expressing the type I IFN receptor, stimulating Stat1 and 2 protein phosphorylation-dependent ISG expression. Ongoing oxidative stress in AT and Artemis-deficient patient neutrophils and type I IFN-mediated mitochondrial ROS production and stress primes neutrophils to undergo NETosis. NET-associated DNA may further contribute to type I IFN secretion via plasmacytoid dendritic cells, exacerbating the inflammation.

was not investigated.¹³ Herein, we showed that unlike healthy neutrophils, AT, Artemis-deficient, and SAVI patient neutrophils spontaneously released their DNA in the form of NETs. Furthermore, we established that incubation with patient plasma samples or recIFN- α was sufficient to stimulate NETosis in healthy neutrophils. Exposure to recIFN- α primed neutrophils to produce ROS, an important prerequisite of NETosis. Consistent with the chronic type I IFN signature in AT and Artemis-deficient cells, unprimed patient neutrophils displayed indicators of oxidative and mitochondrial stress responses. Collectively, these

results indicate that the enhanced type I IFN response observed in AT, Artemis deficiency, and SAVI might exert tissue damage through neutrophil-driven chronic processes.

Infection-independent NET formation has been implicated in the pathophysiology of several diseases, including thrombosis,⁴³⁻⁴⁷ autoimmune diseases,^{45,48-53} inflammation,^{38,54-57} type I diabetes,^{58,59} pulmonary diseases,⁶⁰⁻⁶⁴ and fibrosis.⁶⁵ NETotic DNA released from neutrophils is in an oxidized state that acts on plasmacytoid dendritic cells to potentially stimulate more type I IFN production through a TLR9-dependent process.^{30,38} Because our data suggest that IFN- α primes neutrophils to produce ROS and release NET DNA, it is plausible that the released NET DNA might promote further type I IFN production from plasmacytoid dendritic cells, completing a vicious positive feedback cycle of chronic IFN production (Fig 7). Furthermore, our results also indicate that DNA damage in the absence of repair can initiate NETosis, implying that both type I IFNs and DNA damage defect underlie the NETotic phenotype of patient cells. Our study was restricted to patients with Artemis deficiency with hypomorphic mutations in which the mutant protein displayed residual nuclease activity, enabling limited V(D)J recombination and hence a “leaky” severe combined immunodeficiency phenotype. Although this presents as a milder immunodeficiency when compared with patients with DCLRE1C loss-of-function alleles and typical severe combined immunodeficiency phenotype, susceptibility to develop autoimmunity/autoinflammation is also higher.²⁴ Therefore, whether a similar NETotic neutrophil phenotype also exists in patients with Artemis deficiency with loss-of-function mutations remains to be determined.

In summary, our results implicate that neutrophil activation and particularly NET formation may inflict tissue damage in seemingly unrelated diseases with elevated type I IFN signatures. In this context, we propose that drugs that can interfere with ROS production, neutrophil activation, and/or NETosis and therapies blocking IFN signaling could ameliorate organ damage and might be of benefit in the management of sterile inflammatory manifestations of AT, Artemis deficiency, and SAVI. In conclusion, we showed that patients with AT and hypomorphic Artemis mutations have similar characteristics with well-defined interferonopathies, and high levels of type I IFNs may contribute to inflammatory manifestations in such diseases via IFN-mediated neutrophil activation and NET formation.

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Key messages

- Impaired DNA damage repair in AT and Artemis-deficient patient cells promote the accumulation of DNA species in cytosol, provoking type I IFN production.
- Neutrophils isolated from patients spontaneously produced NETs and displayed indicators of oxidative and mitochondrial stress.
- Neutrophils represent a promising target to manage inflammatory syndromes in diseases with active type I IFN signature.

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