



## A novel *ATM* mutation associated with elevated atypical lymphocyte populations, hyper-IgM, and cutaneous granulomas

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### ABSTRACT

Ataxia-Telangiectasia (AT) is an immunodeficiency most often associated with T cell abnormalities. We describe a patient with a hyper-IgM phenotype and immune cell abnormalities that suggest a distinct clinical phenotype. Significant B cell abnormalities with increased unswitched memory B cells, decreased naive transitional B cells, and an elevated frequency of CD19<sup>+</sup>CD38<sup>lo</sup>CD27<sup>-</sup>CD10<sup>-</sup>CD21<sup>-/low</sup> B cells expressing high levels of T-bet and Fas were demonstrated. The B cells were hyporesponsive to *in vitro* stimulation through the B cell receptor, Toll like receptors (TLR) 7 and 9, and CD40. T cell homeostasis was also disturbed with a significant increase in  $\gamma\delta$  T cells, circulating T follicular helper cells (Tfh), and decreased numbers of T regulatory cells. The *ATM* mutations in this patient are posited to have resulted in the perturbations in the frequencies and distributions of B and T cell subsets, resulting in the phenotype in this patient.

**Key messages:** A novel mutation creating a premature stop codon and a nonsense mutation in the *ATM* gene are postulated to have resulted in the unique clinical picture characterized by abnormal B and T cell populations, lymphocyte subset dysfunction, granuloma formation, and a hyper-IgM phenotype.

**Capsule summary:** A patient presented with ataxia-telangiectasia, cutaneous granulomas, and a hyper-IgM phenotype; a novel combination of mutations in the *ATM* gene was associated with abnormal distributions, frequencies, and function of T and B lymphocyte subsets.

### 1. Introduction

Ataxia-Telangiectasia (AT) is caused by mutations in the *ATM* gene located on chromosome 11q22-23. AT is characterized by ataxia, neurologic decline, oculocutaneous telangiectasia, and a wide range of immune function abnormalities [1–4]. One subset of patients exhibits a hyper-IgM phenotype with an abnormal B-cell profile [5]. Patients with the hyper-IgM phenotype and low serum levels of IgG and IgA have been associated with more severe disease and an increased risk of malignancy [1]. The underlying relationships between AT and hypogammaglobulinemia and the hyper-IgM phenotype have not been well delineated. It is known that a significant percentage of AT patients, but not all, show diverse abnormalities in B and T cell homeostasis primarily marked by low circulating B and T cell numbers [6,7]. The presentation of oculocutaneous manifestations may appear later in childhood so that consideration of AT in the context of hyper-IgM may

be delayed [5]. Elevated serum alpha-fetoprotein levels, rearrangement of chromosomes 7 and/or 14, and radiosensitivity establish the diagnosis of AT [1]. There have been a number of reports of patients with AT who develop severe and often progressive cutaneous granulomatous lesions, some with the hyper-IgM phenotype [8]. The relationship between these granulomatous lesions and AT is unclear but has been thought to reflect the underlying immunodeficiency. Persistence of rubella vaccine virus in cutaneous lesions in AT patients as well as other primary immunodeficiency diseases is now well documented, perhaps serving as an antigenic trigger of the granulomas [5,9,10]. To date, there have been no distinguishing genetic features that align AT with this unique hyper-IgM phenotype or presence of cutaneous granulomas. We present a patient with AT, a novel mutation in *ATM*, cutaneous granulomas, a hyper-IgM phenotype, and extensive B- and T-cell abnormalities that may define this subgroup of AT patients.

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### 1.1. Case presentation

A 5 year-old female presented for evaluation of concern for immunodeficiency because of frequent upper respiratory tract illnesses. None required hospitalization and were presumed to be viral. She was found to have an ataxic gait, some speech delay, and mild ocular and ear pinna telangiectasia. In addition, she had an ulcerative rash initially present on her left upper and right lower extremity. Initial blood work showed elevated  $\alpha$ -fetoprotein levels (50 ng/mL), elevated serum IgM (719 mg/dL), and low IgG (< 75 mg/dL) and IgA (0.9 mg/dL). Skin biopsies of the granulomatous lesions showed lymphoid hyperplasia with granuloma-like formation and lymphoplasmacytic infiltrate with macrophages. A repeat skin biopsy showed pseudoepithomatous hyperplasia with underlying non-necrotizing granulomatous inflammation. Mycobacterial, fungal, and bacterial cultures were negative, and repeat cultures performed on follow-up visits were negative for mycobacterial infection as well. She had no improvement in her skin lesions despite initiation of Ig replacement, topical and systemic corticosteroids, clarithromycin, azithromycin, and topical anti-fungal and calcineurin inhibitor therapy. Vaccine-strain rubella virus was identified in the granulomatous tissue by immunofluorescence staining [10]. Progression of the granulomatous lesions was arrested along with improvement following initiation of anti-TNF $\alpha$  therapy. Both parents were healthy. Over the course of her 2-year follow-up, repeated assessments by polymerase chain reactions for respiratory viral pathogens or Epstein-Barr, cytomegalovirus, Herpes simplex and parvovirus were negative.

## 2. Methods

### 2.1. Genetic analysis

Next generation sequencing was used to identify the mutations in the *ATM* gene. A panel of 345 genes was designed based on reports of the relationship of mutations in those genes to primary immune deficiencies [11,12]. Exon coordinates for all exons  $\pm$  50 base pairs resulted in 4360 targets with a region of interest (ROI) of ca. 2 Mb. These coordinates were submitted to Agilent (Santa Clara, CA) for synthesis of RNA capture nucleotides (Sure Select QXT custom library kit). Genomic DNA was isolated from whole blood and enriched for targets using the custom capture kit according to manufacturer's directions. Sequencing was performed on a MiSeq (MiSeq, San Diego, CA). Initially, sequence data were analyzed for variants using NextGENe ([softgenetics.com](http://softgenetics.com)), and mutations confirmed by Sanger sequencing of targeted PCR amplicons.

### 2.2. Serum cytokine analysis

The patient's serum was analyzed for various cytokines by ELISA on two separate occasions.

### 2.3. Flow cytometry

B cells were purified from the patient's peripheral blood by positive selection using CD20 magnetic beads (Miltenyi Biotec, Cambridge, MA). CD4<sup>+</sup> T cells were isolated from the peripheral blood of research subjects using the EasySep human CD4<sup>+</sup> T cell enrichment kit (STEMCELL Technologies, Cambridge, MA). The following antibodies were used for flow cytometric staining: CD19 APC-Cy7, CD27 PerCP-Cy5.5, CD10 PE-Cy7, CD21 V450, CD69 PE, CD86 APC, FAS Alexa 647, CD4 APC-Cy7, CD127 PerCP-Cy5.5, CD45RO Alexa 700, CXCR5 PerCP-Cy5.5, PD-1 PE-Cy7, ICOS APC (all from Biolegend, San Diego, CA), CD3 eFluor 605NC (from eBioscience, San Diego, CA), and CD21 BD Horizon V450 (BD). Intracellular staining for FOXP3 Alexa 488 (eBioscience) and T-bet PE was performed using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) in accordance

with the manufacturer's directions.

### 2.4. Mitogen and antigen stimulated proliferation assays

Patient's peripheral blood mononuclear cells (PBMC) were isolated following gradient centrifugation and cultured for 3 days with mitogen (phytohemagglutinin, Concanavalin A (Con A) and pokeweed mitogen) or for 5 days with antigen (tetanus toxoid, *C. albicans*) as described using age-matched controls [13].

### 2.5. B cell activation assay

B cells were purified from the peripheral blood by positive selection using CD20 magnetic beads (Miltenyi Biotec) and plated for 48 h at 150,000–200,000 cells per well in a 96-well plate in RPMI containing 10% FBS alone or RPMI + 10% FBS and either 2  $\mu$ g/mL polyclonal F(ab')<sub>2</sub> rabbit anti-human IgM (Jackson ImmunoResearch, West Grove, PA), multimeric, soluble, 0.05  $\mu$ g/mL recombinant-human CD40L (Alexis Biochemicals, San Diego, CA), 2  $\mu$ g/mL gardiquimod (TLR7 agonist; Invitrogen, Waltham, MA), or 0.5  $\mu$ g/mL CpG (TLR9 agonist; Invitrogen).

### 2.6. In-vitro regulatory T cell (Treg) suppression assay

CD4<sup>+</sup> T cells were enriched using the EasySep Human CD4<sup>+</sup> T cell enrichment kit (STEMCELL Technologies). CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup> Tregs and CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> responder T cells (Tresp) were sorted by fluorescence activated cell sorting (FACS) and then labeled with 5  $\mu$ M of CFSE using the CellTrace CFSE Cell Proliferation kit (Invitrogen). Treg and Tresp cells were co-cultured at a 1:1 ratio in the presence of beads loaded with anti-CD2, anti-CD3, and anti-CD28 (Human Treg Suppression Inspector; Miltenyi Biotec). On day 4.5, proliferation of the Tresp cells was analyzed by flow cytometry measuring CFSE dilution.

## 3. Results

### 3.1. Identification of *ATM* mutations

Next generation sequencing revealed two pathogenic mutations in the *ATM* gene, a novel mutation creating a premature stop codon [c.237delA,(p.Lys79Asnfs370)] (inherited from the mother) and a nonsense mutation [c.3372C > G, (p.Tyr124Ter)] (inherited from the father).

### 3.2. Immune evaluation

The results of initial peripheral blood lymphocyte evaluation are summarized in Table 1. Over a 2-year follow-up, these numbers remained relatively unchanged. Compared to laboratory controls, absolute numbers of CD3<sup>+</sup> T cells were slightly low; CD8<sup>+</sup> T cell numbers were low and  $\gamma\delta$  T cell numbers were slightly elevated as previously reported in AT [14]. Proliferative responses of PBMC to mitogens (PHA, Con A, PWM) were reduced to roughly half of the age-matched control responses; the response to tetanus was normal whereas the response to *C. albicans* was absent. Serum cytokine analyses demonstrated elevations in levels of TNF $\alpha$  on two occasions (14.5 and 16.7 pg/mL) and IL-10 (7.0 and 7.9 pg/mL); levels of IFN- $\gamma$ , IL-2, IL-5, IL-6, and IL-12 were below the limits of detection.

### 3.3. An altered B cell compartment in the AT patient

Though the number of total CD19<sup>+</sup>CD20<sup>+</sup> B cells was within the normal range, we examined the frequencies of various subtypes of B cells as perturbations of the distribution of B cell subset frequencies has been reported in patients with other *ATM* mutations [6]. The frequencies of total CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>-</sup> naive and CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>+</sup>

**Table 1**  
Lymphocyte Studies.

	Patient	Reference Range
WBC	5200 cells/ $\mu$ L	4.0–12.0
Absolute Lymph Count	2122	1.0–4.4
CD8 <sup>+</sup>	119 cells/ $\mu$ L (5.6%) <sup>a</sup>	490–1300
CD4 <sup>+</sup>	963 cells/ $\mu$ L (45.4%)	700–2200
CD3 <sup>+</sup>	1133 cells/ $\mu$ L (53.4%) <sup>a</sup>	1400–3700
CD19 <sup>+</sup>	526 cells/ $\mu$ L (24.8%)	390–1400
CD16 <sup>+</sup> /56 <sup>+</sup>	429 cells/ $\mu$ L (20.2%)	130–720
CD4 <sup>+</sup> /CD45RA <sup>+</sup>	13.0% T cells	9.5–41.9%
CD4 <sup>+</sup> /CD45RO <sup>+</sup>	84.0% T cells	16.5–42.2%
TCR/ $\alpha\beta$	93.2% T cells	> 85%
TCR/ $\gamma\delta$	8.4% T cells <sup>a</sup>	0.5–6%
IgD <sup>+</sup> /CD27 <sup>+</sup>	54.0% CD20 <sup>+</sup> cells <sup>a</sup>	4.9–14.2%
IgD <sup>-</sup> /CD27 <sup>+</sup>	3.1% CD20 <sup>+</sup> cells	2.9–9.2%
CD38 <sup>hi</sup> /IgM <sup>hi</sup>	0.2% CD20 <sup>+</sup> cells	0.3–9.2% of B cells
CD38 <sup>hi</sup> /IgM <sup>lo</sup>	0.4% CD20 <sup>+</sup> cells	0.1–4.7% of B cells

<sup>a</sup> Denotes abnormal value.

conventional memory B cells were similar to that of age-matched healthy controls (reference value  $76.6 \pm 7.4\%$  and  $18.9 \pm 7.1\%$  for naive and conventional memory B cells, respectively [15] (Fig. 1A). Within the memory B cell compartment however, there was a marked increased frequency of unswitched CD27<sup>+</sup>IgD<sup>+</sup> memory B cells (54%) compared to age-matched healthy controls (reference value: 8.3%, range 4.8–16.1% [16]. The CD27<sup>+</sup>IgD<sup>-</sup> switched memory proportion (3.1%) was within the reference range (0.9–9.3%) [16] (Table 1). Among the naive B cells, the proportion of CD19<sup>+</sup>CD27<sup>-</sup>CD21<sup>-</sup>CD10<sup>+</sup>IgM<sup>hi</sup> transitional B cells that newly emigrated from the bone marrow (BM) was found to be diminished to 1.1% of the naive B cell compartment (age-matched reference value 5.8%, range 3.4–9.0%, [16] (Fig. 1B).

While the occurrence of CD19<sup>+</sup>CD38<sup>lo</sup>CD27<sup>-</sup>CD10<sup>-</sup>CD21<sup>-/low</sup> (referred henceforth as CD21<sup>-/low</sup>) B cells is rare compared to conventional CD19<sup>+</sup>CD27<sup>-</sup>CD10<sup>-</sup>CD21<sup>+</sup> mature naive B cells, we found an elevated frequency of CD21<sup>-/low</sup> B cells (40%) in the AT patient (Fig. 1C). The frequency of this subset of B cells in the parents was similar to that in controls (< 3.5%). This marked elevation in the patient is in agreement with the increased proportion of CD21<sup>-/low</sup> B cells previously described in some AT patients with other *ATM* mutations and also reported in Freiburg class 1A CVID patients [6,17–19]. In CVID patients and murine models where CD21<sup>-/low</sup> B cell frequency is elevated, gene microarray data revealed that these cells express high levels of *TBET* and *FAS* [17,20]. We similarly found elevated B cell expression of T-bet and Fas in this AT patient with a high CD21<sup>-/low</sup> B cell frequency compared to levels in healthy donor control B cells processed simultaneously (Fig. 1D and E).

In addition to differences at the gene and protein expression levels in CD21<sup>-/low</sup> B cells compared to conventional CD21<sup>+</sup> mature naive B cells, functional studies reported CD21<sup>-/low</sup> B cells to be hyporesponsive to stimulation *in vitro* [17,21]. We assessed the responses of these B cells after activation through the B cell receptor (BCR), TLR7 and TLR9, and CD40 as previously described [17,21]. We found decreased B cell activation in response to all of these stimuli as evidenced by a lower percentage of B cells expressing the activation markers CD69 and CD86 relative to the healthy control samples processed in parallel (Fig. 2A and B).

### 3.4. Dysregulated T cell populations in peripheral blood

Along with perturbed B cell population frequencies and activation in the patient, we also found alterations in the T cell compartment. There was a decreased frequency of total CD3<sup>+</sup> cells but there appeared to be a normal absolute number of CD3<sup>+</sup>CD4<sup>+</sup> T cells (Table 1). There was also a decreased proportion of naive

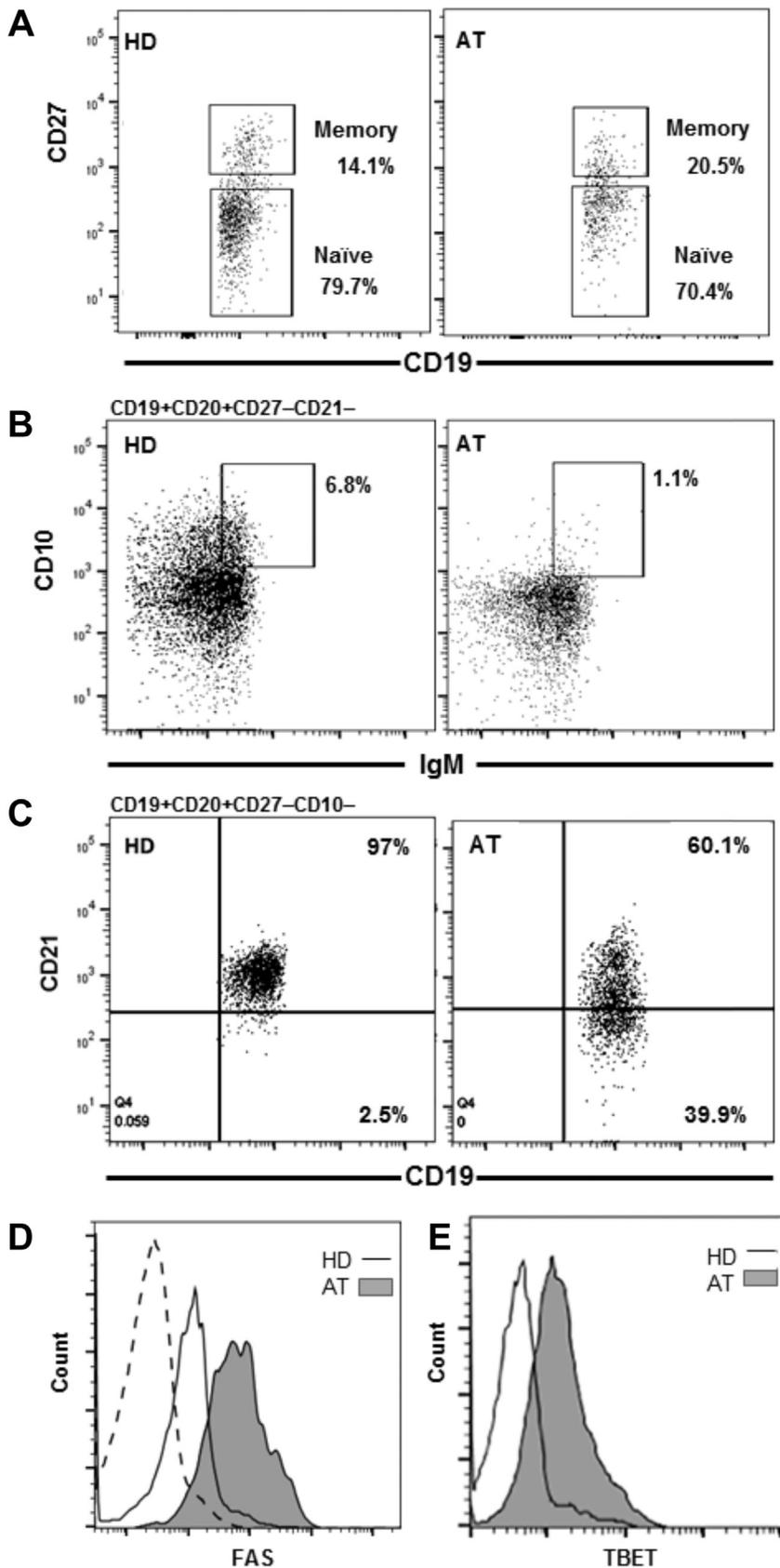
CD3<sup>+</sup>CD4<sup>+</sup>CD45RO<sup>-</sup>CD62L<sup>+</sup> T cells (7.8%) (Fig. 3A) compared to age-matched healthy controls (reference median: 65.1%, range: 55.6–75.8% [16,22]). Conversely, there was a striking increase in the CD3<sup>+</sup>CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cell compartment frequency (89.8%) compared to the reference value in healthy donors (median [22]: 29%, range: 22.1–36.6%) (Fig. 3A). This appeared to be largely attributed to the increased proportion of CD3<sup>+</sup>CD4<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>-</sup> effector memory T cells (62.7%) (Fig. 3A).

Given the presence of elevated serum IgM and decreased levels of other immunoglobulin isotypes, we examined T follicular helper (Tfh) cells as these play an important role in class-switch recombination (CSR) [23]. While Tfh cells are typically found in secondary lymphoid organs, peripheral blood is the most readily accessible tissue for human immunology studies. A population of CXCR5<sup>+</sup>PD-1<sup>hi</sup>ICOS<sup>+</sup>CD4<sup>+</sup> T cells circulating in the peripheral blood shares similar functional characteristics to secondary lymphoid Tfh cells, including the ability to help naive B cells produce antibodies of different isotypes [24,25]. This population was thus referred to as circulating Tfh (cTfh) cells. The average frequency of cTfh cells in a cohort of healthy controls has been reported at around 4% with the 90th percentile below 10% [21]. We found that the cTfh cell frequency in the patient was 5-fold higher (19%) than the average for healthy donors (Fig. 3B). ICOS expression is important for Tfh cell development and function in the germinal centers (GCs) of secondary lymphoid organs [26]. Despite differences in the frequency of cTfh cells, ICOS expression levels in the AT patient cTfh cells were similar to those in the healthy donor cTfh cells processed in parallel (Fig. 3C).

Decreased frequencies of Tregs have been reported in CD40L- and AID-deficient patients who display a hyper-IgM phenotype and suffer from impaired CSR [27]. Given the hyper-IgM phenotype in the patient, we examined Treg numbers and function. Similar to CD40L- and AID-deficient patients, we found a decreased Treg frequency (1.1%) compared to healthy donor controls processed simultaneously and to the age-matched reference value (median: 5.4%, range: 2.3–7.7% [16]) (Fig. 3D). In addition to decreased numbers of Tregs, AID-deficient patients also have decreased Treg suppressive capacity [28]. The AT patient described here exhibited strikingly elevated CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cell frequencies which is also seen in IPEX syndrome patients where there are mutations in the *FOXP3* transcription factor important for Treg function [29]. Observing a decreased frequency of Treg in this AT patient and in light of functional Treg impairment in other conditions with shared immunophenotypes, we evaluated Treg function and found that the suppressive capacity of Tregs was not impaired as Tregs from this patient were capable of suppressing both autologous and heterologous (from healthy donor controls) Tresp cells (Fig. 3E). However, despite intact functional capacity, decreased Treg numbers may have resulted in a similar outcome as decreased Treg function.

## 4. Discussion

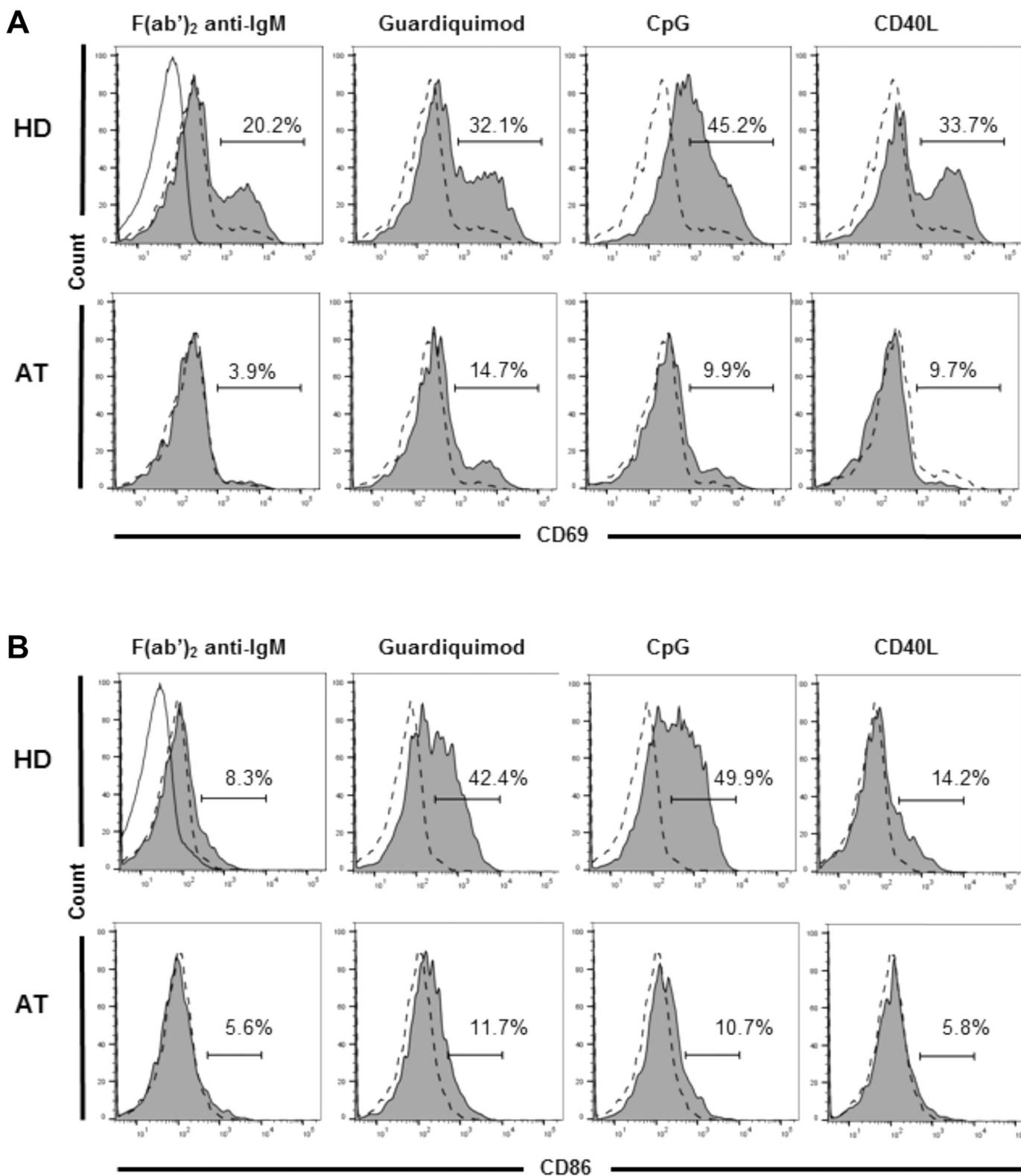
AT is a complex disorder that not only encompasses oculocutaneous telangiectasias or the progressive neurodegeneration leading to cerebellar ataxia, but also immune dysregulation, which is present in about 2 out of every 3 AT patients [1]. The disease is heterogeneous in the variability of the clinical manifestations and constellation of symptoms [1,3,4,6]. We report here the clinical phenotype and immunologic consequences of two mutations in *ATM*, the previously reported c.3372C > G, (p.Tyr124Ter) nonsense mutation and a novel c.237delA, (p.Lys79Asnfs370) mutation encoding a premature stop codon. *ATM* is important in recognizing, stabilizing, and mediating repair of double-strand breaks (DSB) in DNA. Thus, it is a key player in the V(D)J gene recombination process involved in generating a diverse repertoire of functional BCRs/immunoglobulins and T cell receptors (TCRs) [30–32]. Furthermore, *ATM* plays a role in cell-cycle checkpoint regulation, and its dysfunction also results in continued DNA replication in the presence of unrepaired DSB leading to genomic instability and, as a result,



**Fig. 1.** *ATM* mutations result in altered new emigrant and mature naive B cell population frequencies. A) B cells isolated from PBMC by anti-CD20 magnetic bead separation from a healthy donor (HD) control sample processed in parallel with the AT patient sample show similar frequencies of the CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>+</sup> memory and CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>-</sup> naive B cell compartments. B) Despite similar overall naive B cell compartment frequencies, the naive CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>-</sup>CD21<sup>-</sup>CD10<sup>+</sup>IgM<sup>hi</sup> new emigrant B cell population transitioning from the bone marrow is decreased in the AT patient. C) Conversely, the mature naive B cell compartment in the AT patient contains an elevated frequency of atypical CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>-</sup>CD10<sup>-</sup>CD21<sup>-/low</sup> B cells. D) This compartment in the AT patient exhibits elevated Fas expression and E) is enriched in TBET<sup>+</sup> cells compared to lower Fas and TBET expression in the HD control (dashed histogram represents isotype control for both Fas and TBET).

increased cell death [30]. As V(D)J recombination in developing B cells takes place in the BM, the decreased frequency of transitional B cells emigrating from the BM into the peripheral blood in the setting of these *ATM* mutations may be attributed to increased BM B cell apoptosis as a

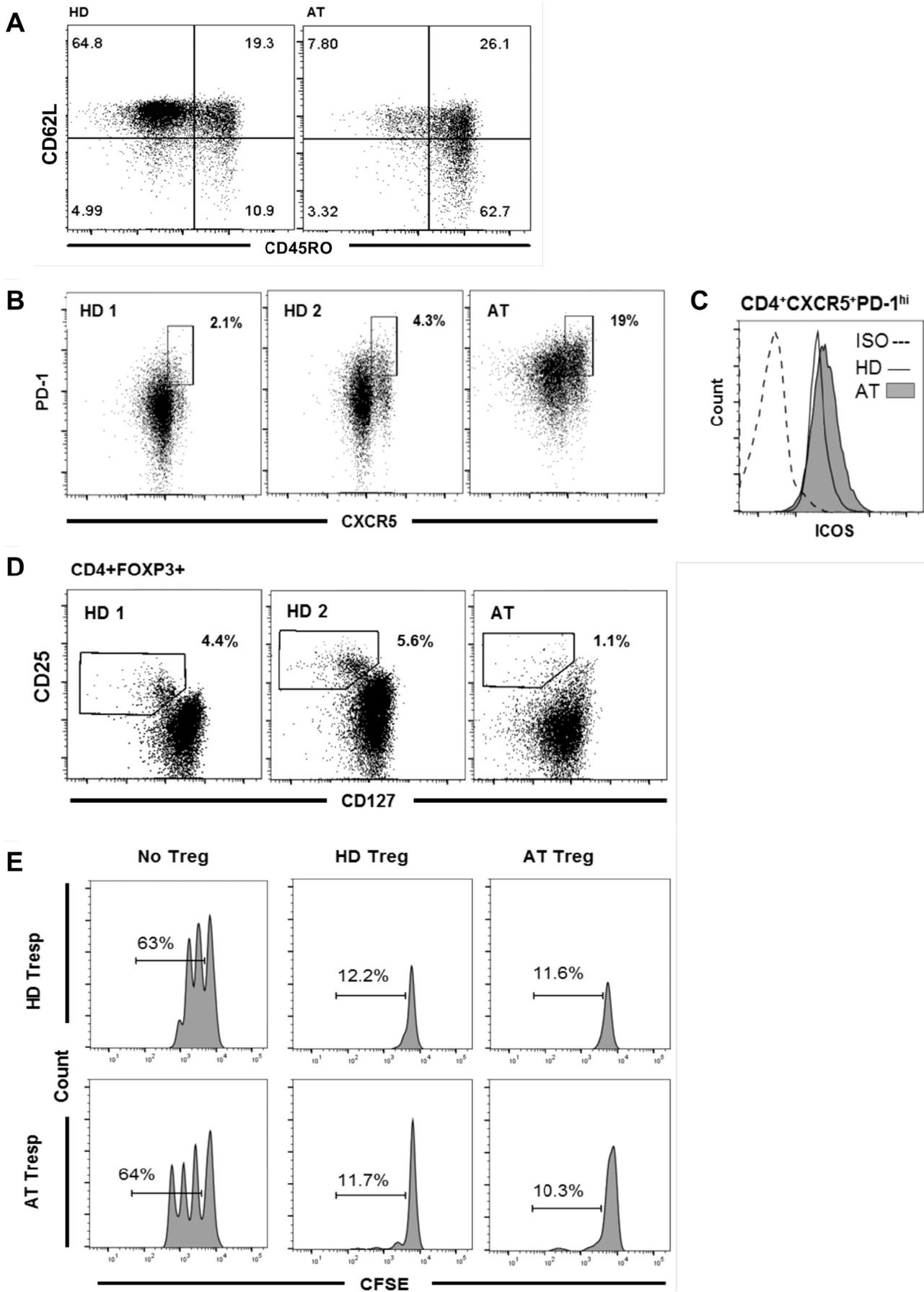
consequence of unrepaired DNA DSB during BCR gene recombination and resultant increased genomic instability. Similarly, the decreased naive T cell population frequency is likely also attributable to defects in DSB repair during TCR V(D)J recombination and consequent decreased



**Fig. 2.** *ATM* mutations result in defects in *in vitro* B cell activation. A) Purified CD19<sup>+</sup>CD20<sup>+</sup>CD27<sup>-</sup> naive B cells from a representative HD (top) and the AT patient (bottom) show decreased expression of the activated B cell marker CD69 in the AT patient after 48 h of stimulation with the specified B cell activating agents (Solid line with unshaded area in F(ab')<sub>2</sub> anti-IgM panel represents isotype control for all panels; dotted line with unshaded area in all panels represents unstimulated HD or AT B cells; shaded area in all panels represents HD or AT B cells after stimulation). B) Similar B cell activation assay for expression of the activated B cell marker CD86 shows lower CD86 levels in the AT patient.

viable T-cell output from the thymus [7]. The observed decreased transitional B cell and naive T cell frequencies are likely directly related to these mutations (and not to secondary effects of the disease such as

poor nutrition or increased infection) as these findings have been demonstrated in *Atm*<sup>-/-</sup> mouse models and also in AT patients with other *ATM* mutations, particularly those in the AT subgroup with



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**Fig. 3.** Altered T cell compartments in the setting of the *ATM* mutations. A) There are fewer naive CD4<sup>+</sup>CD62L<sup>+</sup>CD45RO<sup>-</sup> T cells in the AT patient. Conversely, there is an elevated memory T cell compartment (CD45RO<sup>+</sup>) in the AT patient compared to the HD control. There is an increase in the central memory (CD62L<sup>+</sup>CD45RO<sup>+</sup>) compartment, and the increased memory T cell frequency is particularly evident in the effector memory (CD62L<sup>-</sup>CD45RO<sup>+</sup>) T cell population. B) Purified CD4<sup>+</sup> T cells from two representative HD and the AT patient show expansion of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>hi</sup> circulating Tfh (cTfh) cells in the peripheral blood of the AT patient. C) Despite an increase in the frequency of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>hi</sup> cTfh cells, the *ATM* mutations do not affect levels of ICOS, which is important for Tfh cell differentiation and function. (ISO = isotype control). D) Purified CD4<sup>+</sup> T cells from two representative HD and the AT patient show a decreased frequency of CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Tregs in the peripheral blood of the AT patient. E) Despite this, Treg suppressive capacity is preserved as shown in representative histograms of Treg-mediated suppression of autologous and heterologous CFSE-labeled Tresp cell proliferation on day 4.5 from the AT patient and a HD control. Percentages of Tresp cells undergoing division.

hypogammaglobulinemia [6,7,32].

In the B cell compartment, the mutations in *ATM* described here were associated with abnormal development of CD21<sup>-/low</sup> B cells expressing high levels of T-bet and FAS. Of note, the parents, each carriers of one of the mutations, showed no increase in this population of B cells. Increased numbers of CD21<sup>-/low</sup> B cells have been reported in several conditions from CVID to infections such as hepatitis C, HIV, and malaria and also in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and Sjogren syndrome [17,18,33,34]. In addition, animal models of autoimmunity display elevated frequencies of CD21<sup>-/low</sup> B cells [20]. CD21<sup>-/low</sup> B cells have also been reported in AT patients with other *ATM* mutations where the highest proportions are noted among AT patients with hypogammaglobulinemia as in the current patient [6]. The description of CD21<sup>-/low</sup> B cells is as heterogeneous as the diseases in which they have been found with some reports describing them as unresponsive to stimulation *in vitro* while others describe them as more similar to exhausted tissue memory B cells or innate-like B cells [18,19,35]. In terms of similarity to other primary immunodeficiency states, the mutations in *ATM* described here were associated with the hyporesponsive, pro-apoptotic phenotype that has been described in CVID patients [17]. Elevated levels of CD21<sup>-/low</sup> cells with high T-bet expression in the B cell compartment was unexpected and intriguing. T-bet expression in B cells has recently been associated with autoimmune diseases in animal models [37]. Elevated frequency of T-bet-expressing B cells and the presence of IgG anti-chromatin antibodies were also reported in SLE patients [38]. This presence of T-bet expressing B cells in *ATM* patients has not been reported. To date, with limited screening, we have not detected any autoantibodies in this patient, including IgG anti-chromatin or anti-nuclear autoantibodies. Given her age, we cannot rule out the potential auto-reactive nature of T-bet-expressing B cells at a later time.

It remains to be further investigated whether there is any connection between T-bet expression in B cells and the high Tfh counts in this patient. One possibility is that the elevated frequency of Tfh leads to higher levels of IL-21 production which in turn can influence T-bet expression in B cells as has been recently demonstrated [39]. While the exact nature and function of this atypical subgroup of CD21<sup>-/low</sup>T-bet<sup>+</sup> B cells is the subject of ongoing research, it is apparent that they are a marker of dysregulated immunity from a variety of causes. Whether they are a driver or consequence of an abnormal immune system remains to be fully clarified, and further examination of *ATM* in the development of these cells may help elucidate their origin and role.

The hyper-IgM hypogammaglobulinemic phenotype of this patient may be a direct sequela of these novel mutations in *ATM* as CSR is impaired in *ATM* deficiency [40]. CSR occurs in the light zone of GCs of secondary lymphoid organs where Tfh cells provide signals to B cells and mediate isotype switching, which requires DNA DSB and gene rearrangement [23]. Given the role of Tfh cells in CSR, it is intriguing that an increased frequency of cTfh cells is observed in the presence of mutations in *ATM*. Interestingly, there is also increased frequency of cTfh cells in patients deficient in AID, a key enzyme induced in B cells by GC Tfh cells to mediate CSR [28]. Of note, *ATM* deficiency also impairs AID activation and function in CSR [36]. Thus, there appears to be an association between key enzymes involved in CSR and cTfh cell frequency, and *ATM* acts at multiple critical points in the CSR pathway. The exact mechanism for the increase in cTfh cells in the setting of *ATM*

deficiency is unclear; however, it is thought to be from impaired B and T cell interactions and defects in multiple successful GC reactions [6].

Another clinical phenotype in this AT patient that is potentially related to the increase in cTfh cells is the presence of noninfectious, non-caseating cutaneous granulomas. Significantly higher percentages of cTfh cells were shown in CVID patients with granulomatous skin disease compared to CVID patients without granulomas despite similar frequencies of overall CD4<sup>+</sup> T cells [41]. In addition, as in the patient discussed here, CVID patients with elevated cTfh cell frequencies had a concomitantly decreased proportion of Tregs [21]. This inverse relationship between cTfh cells and Tregs was also demonstrated in hyper-IgM, AID-deficient patients [28]. In further support of a relationship between cTfh cells and Tregs, IPEX syndrome patients, LRBA-deficient, and CTLA-4-deficient patients lacking functional Tregs also exhibited an elevated cTfh cell percentage [29,42]. Thus, finding this inverse relationship in the setting of the *ATM* mutations described here supports observations in other primary immunodeficiencies that share similar immunologic phenotypes of elevated CD21<sup>-/low</sup> B cells and defects in CSR, and suggests a potential role for this imbalance in the pathogenesis of cutaneous granulomas. As in the patient presented here, descriptions of other AT patients with cutaneous granulomas report a common phenotype of hyper-IgM with hypogammaglobulinemia of other isotypes and decreased frequency of naive CD4<sup>+</sup> T cells but increased T cells bearing  $\gamma\delta$  TCRs [1,8,39,43,44]. This immunophenotype is different from that seen in AT patients without cutaneous granulomas [8].

In terms of a trigger for granuloma formation in this patient, vaccine-strain rubella virus DNA has been reported to persist in the granulomas of patients with AT. It is suggested that the viral DNA serves as a continuous antigen trigger leading to macrophage and T cell activation; however, the exact causal mechanism by which this occurs needs further investigation [9,10]. Another postulated theory for the increased granulomatous skin disease in AT patients with a hyper-IgM, hypogammaglobulinemic phenotype is related to the elevated frequency of  $\gamma\delta$  T cells, which are a major source of IFN- $\gamma$  in granulomatous disease [8]. IFN- $\gamma$  stimulates macrophage granuloma formation, and individuals with altered IFN- $\gamma$  signaling cannot form granulomas in response to mycobacterial infections [45]. In this patient, at least at the time point assessed, the IFN- $\gamma$  level was within normal limits pointing to an alternative mechanism for cytokine stimulation of macrophage activation for granuloma formation. The decreased Treg frequency observed in this patient and others with primary immunodeficiency and cutaneous granulomas may account for increased T effector cell frequency and production of other pro-inflammatory cytokines important in granuloma formation and maintenance, such as TNF $\alpha$ . In addition,  $\gamma\delta$  T cells are also a known source of TNF $\alpha$  [46,47]. Furthermore, in addition to its elevation in diseases like sarcoidosis, TNF $\alpha$  may play a role in cutaneous granulomas in immunodeficiency as seen in granulomatous CVID [48] and anti-TNF $\alpha$  therapy has been used to treat granulomatous skin disease in both CVID and AT [49,50]. Given the elevated TNF $\alpha$  levels in this patient, anti-TNF $\alpha$  therapy was initiated and appeared helpful in reducing the size and progression of the cutaneous granulomas.

## 5. Conclusion

The mutations in *ATM* described here add to the growing understanding of the heterogeneity in degree and complex nature of the immunodeficiency seen in patients with AT. It is unclear why this variability exists among AT patients, but further examination and categorization of novel mutations in *ATM* may help clarify this issue. The mutations presented here resulted in perturbations in frequencies and distributions of normal and atypical B and T cell subsets, which can explain some immunologic aspects of the clinical phenotype of this patient. Our findings confirm observations in other AT patients with different *ATM* mutations and also in patients with other primary immunodeficiencies that share similar clinical and immunologic phenotypes. The clinical phenotype of cutaneous granulomas, hyper-IgM, and hypogammaglobulinemia helps to subcategorize the type of AT resulting from these identified mutations. This hyper-IgM subtype of AT is associated with worse disease outcome and higher lymphoid malignancy risk when combined with low IgA and IgG. Thus, further understanding of the mechanistic role of these novel mutations may have prognostic and therapeutic implications for those likely to have a more clinically severe disease course than AT patients with mutations linked to milder presentations. The information gained from examination of the immunologic effects of these novel mutations will be helpful for further understanding of the role of *ATM* in normal and pathologic immune system development and function.

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