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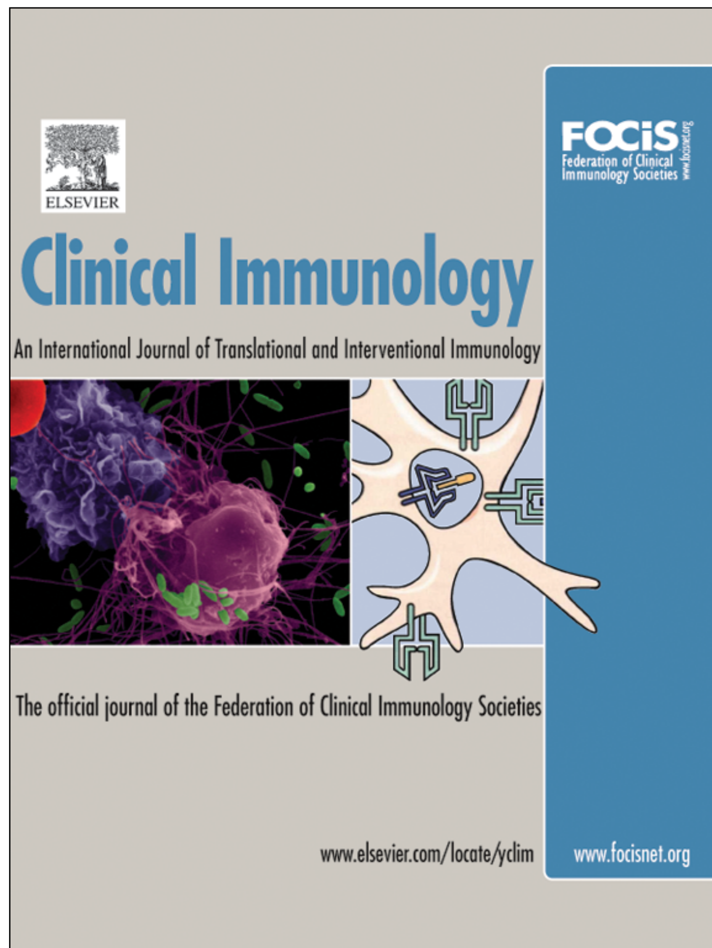

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Clinical Immunology 175 (2017) 16–25

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Abnormal cell-clearance and accumulation of autophagic vesicles in lymphocytes from patients affected with Ataxia-Teleangiectasia

Roberta D'Assante [a,1], Anna Fusco [a,1], Loredana Palamaro [a], Elena Polishchuk [b], Roman Polishchuk [b], Gabriella Bianchino [c], Vitina Grieco [c], Maria Rosaria Prencipe [a], Andrea Ballabio [a,b], Claudio Pignata [a,*]

[a] *Department of Translational Medical Sciences, Federico II University, Naples, Italy*
[b] *Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy*
[c] *Laboratory of Clinical Research and Advanced Diagnostics, IRCCS Referral Cancer Center of Basilicata, Rionero in Vulture, Potenza, Italy*

A R T I C L E I N F O

Article history:
Received 20 April 2016
Received in revised form 17 October 2016
accepted with revision 28 November 2016
Available online 30 November 2016

Keywords:
Ataxia-Teleangiectasia
Autophagosomes
Autophagy
Autophagosome-lysosome fusion
Cell-clearance

A B S T R A C T

Ataxia-Teleangiectasia (A-T) is a neurodegenerative disorder due to mutations in ATM gene. ATM in the nucleus ensures DNA repair, while its role in the cytosol is still poorly clarified. Abnormal autophagy has been documented in other neurodegenerative disorders, thus we evaluated whether alteration in this process may be involved in the pathogenesis of A-T by analyzing the autophagic vesicles and the genes implicated in the different stages of autophagy. Through transmission electron microscopy (TEM) and immunofluorescence analysis we observed an accumulation of APs associated with a LC3 puncta pattern, and a reduced number of ALs. We also documented an increased expression of genes involved in AP and lysosome biogenesis and function, and a decrease of *Vps18* expression, involved in their vesicular trafficking and fusion. mTORC1-controlled proteins were hyperphosphorylated in A-T, in keeping with an increased mTOR inhibitory influence of autophagy. Betamethasone is able to promote the degradation of SQSTM1, a biomarker of autophagy. Collectively, our results indicate that in cells from A-T patients, the APs maturation is active, while the fusion between APs and lysosomes is inappropriate, thus implying abnormalities in the cell-clearance process. We also documented a positive effect of Betamethasone on molecules implicated in autophagosome degradation.

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1. Introduction

Ataxia-Teleangiectasia (A-T) (MIM #208900) is a rare recessive disorder characterized by cerebellar neurodegeneration with the loss of Purkinje cells, oculocutaneous teleangiectasia, susceptibility to cancer, growth retardation, diabetes mellitus and immunodeficiency [1–3]. Recently, in a few clinical trials it has been documented that a short-term treatment with glucocorticoids (GCs) is able to partially rescue the A-T neurological phenotype and lymphocyte proliferation even though the mechanism of action has not yet been defined [4–7]. The improvement of cerebellar symptoms during steroid treatment was inversely correlated with the severity of cerebellar atrophy [6]. It has recently been documented that Dexamethasone *in vitro* leads to the skipping of mutations upstream of nucleotide residue 8450 of *Ataxia Telangiectasia Mutated* (*ATM*) coding sequence, resulting in a new ATM variant with the complete kinase domain, which was shown to be likely active [8].

A-T is caused by mutations in the *ATM* gene encoding a serine/threonine protein kinase involved in cell cycle control and repair of DNA double-strand breaks [9,10]. Evidence exists that ATM exerts additional functions in the cytoplasm independent of its role in the DNA damage response [11], such as participation in the autophagy pathway [12]. To date, many studies have been performed to identify the pathogenic mechanism responsible for the disease, mainly focusing on the nuclear activity of ATM protein. Thus, the pleiotropic aspects of the phenotype have only partially been clarified.

To date, A-T remains an incurable disease that leads relentlessly to death around the third decade of life [13].

Autophagy alterations have been implicated in several chronic nervous system disorders, such as proteinopathies (Alzheimer's, Parkinson's, Huntington's diseases) and acute brain injuries [14], whose hallmarks are organelle damage, synaptic dysfunction and neuronal degeneration. Autophagy is a constitutive lysosomal catabolic process during which, cytoplasmic components, damaged proteins and entire organelles are degraded and recycled to generate building blocks for anabolic processes. Autophagy, known originally as an adaptive response to nutrient deprivation in mitotic cells, including lymphocytes, is now recognized as an arbiter of neuronal survival and homeostasis in that neurons are post-mitotic cells, which require effective protein degradation to prevent accumulation of toxic aggregates. Reactive oxygen species (ROS)

* Corresponding author at: Department of Translational Medical Sciences, Federico II University, Via S. Pansini 5, 80131 Naples, Italy.
E-mail address: pignata@unina.it (C. Pignata).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.clim.2016.11.015
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generation from dysfunctional mitochondria, as documented in cells from A-T patients, is a potent trigger of autophagy, which acts to clean up damaged organelles [15]. It has been demonstrated that in the presence of elevated ROS levels, the activation of the serin/threonine kinase ATM in the cytoplasm leads to activation of LKB1 tumor suppressor gene, which, in turn, phosphorylates and activates AMP protein kinase (AMPK) [16]. AMPK regulates several metabolic processes and activates Tuberous sclerosis complex 2 (TSC2), which participates in energy sensing and growth factor signaling [17]. TSC2, by inhibiting the GTPase Ras homolog enriched in brain (Rheb), is able to repress the mechanistic target of rapamycin (mTOR) kinase, a key regulator of the protein synthesis and cell growth, thus leading to the activation of autophagy [18]. The repression of mTOR complex 1 (mTORC1) signaling results in the absence of phosphorylation of p70 ribosome S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1), direct targets of the mTOR kinase, involved in protein synthesis and survival.

Thus, a direct effect of ATM-mediated inhibition of mTORC1 is the activation of autophagy, a dynamic process, which includes the initiation, formation, maturation and degradation of autophagosomes (APs). The lipidation of the microtubule-associated protein 1 light chain 3 (LC3) molecule, through the conjugation of phosphatidylethanolamine (PE) converting the cytosolic LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), is essential for autophagy induction, and thus for APs visualization by transmission electron microscopy (TEM). The lipidated LC3-II form may also be visualized under fluorescence microscope in that it shows a punctate staining pattern and by western blot in that it has faster electrophoretic mobility compared with diffused LC3-I form [19]. An increase of LC3-II form may result from either an enhancement of AP biogenesis or inhibition of AP degradation, or may be due by other mechanisms [19]. p62/sequestosome (SQSTM1), is a further biomarker widely used to evaluate the appropriateness of autophagic activity. It can bind LC3 protein to promote the recruitment of unwanted material into autophagosomes and the subsequent degradation through autolysosomes. When a normal autophagic flux occurs, p62, as substrate of autophagy itself, is degraded [20]. Thus, increased LC3-II and decreased SQSTM1 levels are indicative of an appropriate autophagic activity whereas increased SQSTM1 levels reveal defective autophagy [20].

Recently, genes involved in autophagosomal/lysosomal biogenesis and lysosomal functionality, belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network, have been described as crucial for a proper cell clearance through an autophagic process [21] during catabolic conditions. In particular, the expression of the UV Radiation Resistance Associated (*UVRAG*) gene plays a pivotal role in the first phase of the process, which consists in AP biogenesis and formation [22,23]. To ensure a proper degradation of unwanted material and thus cell clearance, the APs must fuse to the lysosomes that contain several active hydrolases, such as β-glucosidase (GBA), β-glucuronidase (GUS) and cathepsins (CST) [21]. The fusion process depends on the intracellular positioning of lysosomes in that, only the lysosomes that are transported along microtubules in the perinuclear area, thanks to kinesins (such as KIF2A and KIF1B-β and the monomeric GTPase ARL8B), are able to fuse with APs [24]. Moreover, Vacuolar Protein Sorting 11 and 18 (*Vps11* and *Vps18*) protein products are also involved in vesicular trafficking to allow the encounter between APs and lysosomes, resulting in their fusion [25]. Eventually, the formation of autolysosomes (ALs), whose content is degraded by lysosomal enzymes and recycled, leads to preserved cellular homeostasis.

In the present work, we evaluated whether abnormal autophagy may be involved in the pathogenesis of A-T by analyzing the autophagic vesicles and the genes implicated in the different stages of autophagy process.

Through *in vitro* experiments using freshly isolated lymphocytes from A-T patients, we found that in A-T lymphocytes, under resting conditions, there is an accumulation of APs, associated with a high expression of genes involved in the process of biogenesis and function of APs and lysosomes. The addition *in vitro* of Betamethasone helps decrease SQSTM1 levels, which is associated to autophagy progression. Moreover, a reduced expression of *Vps18* and *Vps11* molecules, involved in the vesicular trafficking and fusion between APs and lysosomes, was also found. Taken together, our results indicate that the stage of fusion between APs and lysosomes represents a limiting step, which leads to AP accumulation and inappropriate cell clearance and trafficking in A-T.

2. Materials and methods

2.1. Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from A-T patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Lymphoblastoid B-cell lines (BCLs) were generated by Epstein Barr Virus (EBV) immortalization of patients' and healthy donors' PBMCs using standard procedures, and were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, California), 2 mmol L-glutamine (Gibco, Carlsbad, California), 50 g/ml gentamycine (Gibco, Carlsbad, California), 10% penicillin-streptomycin (Lonza, Verviers, Belgium), and cultured at 37 °C, 5% CO_2. Serum starvation was induced incubating the cells in medium without FBS for 2 h. All the experiments were approved by the Ethical Committee “Comitato Etico per le Attività Biomediche Carlo Romano” of the Federico II University of Naples.

2.2. Transmission electron microscopy

PBMCs obtained from A-T patients and healthy donors were washed in PBS and centrifuged to obtain a visible pellet. Cells were than fixed with a 1% glutaraldehyde and 0.2 M Hepes. After dehydration, from each sample thin sections were cut with a Leica EM UC7 ultramicrotome and further investigated using a FEI Tecnai-12 (FEI, Eindhoven, The Netherlands) electron microscope equipped with a Veletta CCD camera for digital image acquisition at different magnifications.

Autophagosomes were identified on the basis of their ultrastructural morphology. These vesicles have a double membrane usually visible as two membrane bilayers, which contain cytosol and/or morphologically intact organelles. Autolysosomes were identified on the basis of their characteristic single limiting membrane, containing unwanted cytoplasmic material and/or organelles at various stages of degradation [26].

2.3. Western blotting

Total lysates were obtained from lymphoblastoid B-cell lines of A-T patients and healthy donors. The cells were pre-treated or not with 80 nM of Betamethasone (BMZ) for 2 h, 0.2 mM H_2O_2 for 30 min, or 100 nM Bafilomycin A1 (BAFA1) (Sigma-Aldrich, St. Louis, MO) for 1 h, washed with ice-cold PBS (Lonza, Walkersville, MD) and lysed in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadatum (Na_3VO_4), 5 μg/ml leupeptin and 5 μg/ml aprotinin on ice for 45 min. Fractioned cell lysates were obtained from lymphoblastoid B-cell lines generated by EBV immortalization of PBMCs from A-T patients and healthy donors. The cells were treated with 0.2 mM H_2O_2 for 15, 30 or 60 min, washed with ice-cold PBS and resuspended in Buffer A (10 mM Hepes/KOH pH 7.9, 1.5 mM $MgCl_2$, 10 m MKCl, 0.5 mM Dithiothreitol (DTT) pH 7.9, 0.5 mM PMSF, 0.5 mM Na_3VO_4, 5 μg/ml leupeptin and 5 μg/ml aprotinin) on ice for 10 min. Cells were centrifuged to collect the supernatant containing the cytosolic fraction. The pellet was resuspended in Buffer C (20 mM Hepes/KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM Na_3VO_4, 5 μg/ml leupeptin and 5 μg/ml aprotinin) on ice for 20 min. Cells were centrifuged to collect the supernatant containing the nuclear fraction. The

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cell lysates were stored at −80 °C. Protein concentration was determined by Bio-Rad Protein Assay, based on Bradford's method. Proteins for LC3, SQSTM1, p-S6K, p-S6 and p-4EBP1 were separated on 4–12% Novex NuPAGE SDS-PAGE gels (Invitrogen, Carlsbad, CA), while proteins for p-mTOR and mTOR were separated on 5% SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes (Sigma-Aldrich, St. Louis, MO). The membranes were then washed three times in wash buffer, blocked and incubated with the specific primary antibodies for LC3 (Biorbyt, Cambridge, UK), SQSTM1 (Fitzgerald Industries International, MA, USA), p-S6K (Thr389) (Cell Signaling Technology), p-S6 (Ser 235/236) (Cell Signaling Technology), p-4EBP1 (Thr37/46) (Cell Signaling Technology, MA, USA), p-mTOR (Ser2448) (Merk Millipore, Germany), m-TOR (Santa Cruz Biotechnology, TX, USA) or Tubulin (Biorbyt, Cambridge, UK). Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked antibodies. ECL reagent (Bio-Rad, Woodinville, WA, USA) was used as detection system for visualization. Densitometric analysis was performed using ImageJ software.

2.4. Real-time quantitative reverse transcriptase PCR analysis

Total RNA was extracted from ATM-deficient and control PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA), and Phase-lock gel columns (Eppendorf, Germany) according to the manufacturer's instructions. RNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The qPCR reactions were performed in duplicate. The amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler 480 (Roche Applied Systems, Germany). Cycling conditions comprised an initial denaturation at 94 °C for 5 min, a phase of annealing/extension specific for each gene. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. Primers used are listed in Table 1. Results are mean ± standard error (SE) of 2 repeated experiments and each gene expression was normalized to β-actin as housekeeping gene. The relative transcript abundance was represented as −DCt = (Ct gene − Ct reference) and the relative changes in gene expression was analyzed using the 2^{-DDCt} method [27].

2.5. Fluorescence microscopy

PBMCs obtained from A-T patients and healthy donors were washed with PBS (Lonza, Walkersville, MD) and spotted on sterile coverslips through Shandon CytoSpin III Cytocentrifuge. Each spot was delimited with the DakoPen (Dako, Denmark). Each section was blocked with normal goat serum before staining and then treated with a mix of 1:100 LC3 (Biorbyt, Cambridge, UK) and 1:100 lysosome-associated membrane protein (LAMP) (Biorbyt, Cambridge, UK) antibodies. Appropriate anti-rabbit or anti-mouse peroxidase-linked secondary antibodies were used. Nuclear counterstain was visualized with DAPI (4′,6-diamidino-2-phenylindole, 0.05 mg/ml (Vector Laboratories, CA, USA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

2.6. Statistics

GraphPad Prism software was used for data analysis. The Student's *t*-test was used to analyze the statistical significance of differences. The minimum acceptable level of significance was $p \leq 0.05$ calculated through two-tailed unpaired Student's *t*-test and CHI Test.

3. Results

3.1. Lymphocytes from patients affected with A-T show accumulation of APs

To address the involvement of an abnormal autophagic process in the pathogenesis of A-T, we first analyzed, under TEM, the lymphocytes isolated from A-T patients or healthy controls maintained in normal culture conditions or in a serum-starved condition, which is a classical pro-autophagic stimulus. Ultrastructural analysis of healthy control lymphocytes by TEM showed the morphological aspects of a cell in unstarved culture conditions (Fig. 1A) and the accumulation of autophagic vesicles (AVs) at different maturation stages (*i.e.* APs and ALs) in a cell after serum starvation for 2 h (Fig. 1B). Ultrastructural analysis of lymphocytes from A-T patients at basal conditions revealed a general increase in AV size and number (Fig. 1C–F) not observed in healthy control cells.

Next we analyzed distinct types of AVs, such as APs and ALs. TEM revealed that A-T patients exhibit a higher number of APs and a decrease in ALs, as compared to healthy subjects (mean: 13.60 *vs* 2.30/100 μm^2; $p = 0.03$; mean: 2.10 *vs* 4.7/100 μm^2 respectively) (Fig. 1G). This suggests that fusion of APs to lysosomes and, thus, their conversion to autolysosomes, could be impaired in A-T. A further quantitative analysis of ALs revealed that, in the A-T patients, they were not detected in the majority of the fields analyzed, differently from the controls (83 *vs* 36% ALs negative fields, respectively; $p < 0.01$) (Fig. 1H), resulting in an APs/ALs ratio much higher in the

Table 1
Sequences of oligos used in Real-Time qPCR analysis.

Gene symbol	Gene name	Sequence (5′-3′)	
		Forward primer	Reverse primer
UVRAG	UV Radiation Resistance Associated	TGACAATTCGTTGCAGGCAGTTA	AGGCAACTTGACACCGCATACA
VPS11	Vacuolar Protein Sorting 11	CCACTTTGATGTGGAGACAGC	TGTATCGAAGGGCTTCCTGA
VPS 18	Vacuolar Protein Sorting 18	AGCGTCGCTACCTGGAGAG	GTACGTTCGGCTGGCTTC
CSTF	Cathepsin F	ACAGAGGAGGAGTTCCGCACTA	GCTTGCTTCATCTTGTTGCCA
CSTB	Cathepsin B	AGTGGAGAATGGCACACCCTA	AAGAAGCCATTGTCACCCCA
CSTD	Cathepsin D	AACTGCTGGACATCGCTTGCT	CATTCTTCACGTAGGTGCTGGA
CSTA	Cathepsin A	CAGGCTTTGGTCTTCTCTCCA	TCACGCATTCCAGGTCTTTG
CSTE	Cathepsin E	GGACATGATCCAGTTCACCGA	GGTTGGCACACTCCACAGCA
GBA	Glucosidase, beta, acid	TGGGTACCCGGATGATGTTA	AGATGCTGCTGCTCTCAACA
GUS	Glucoronidase	ATTGGAGGTGCAGCTGACTG	TCCTCATGCTTGTTGACACC
CD63	CD63 molecule	TCACGTTTGCCATCTTTCTG	TCGAAGCAGTGTGGTTGTTT
KIF1Bβ	kinesin family member 1B	GACCAAGCTCAGTGGTCTCTAAG	CCAGATGACCTGGTCGTATTGTGC
KIF2A	kinesin heavy chain member 2A	GCCTTTGATGACTCAGCTCC	TTCCTGAAAAGTCACCACCC
ARL8A	ADP-ribosylation factor-like 8A	AGTCCTGGGTAACAAGCGAGAC	GCAAGAGATGGAGTAGCAGCAG
ARL8B	ADP-ribosylation factor-like 8B	CACCTTCGTCAATGTCATCG	CCTATGTCCCAGATCTTTATTGTG
KIF3C	kinesin family member 3C	GAAGATGCTGGAGGACCTGCGG	GTAGGTGCCCCGGAGCTCCATAG
β-actin		GACAGGATGCAGAAGGAGAT	TTGCTGATCCACATCTGCTG

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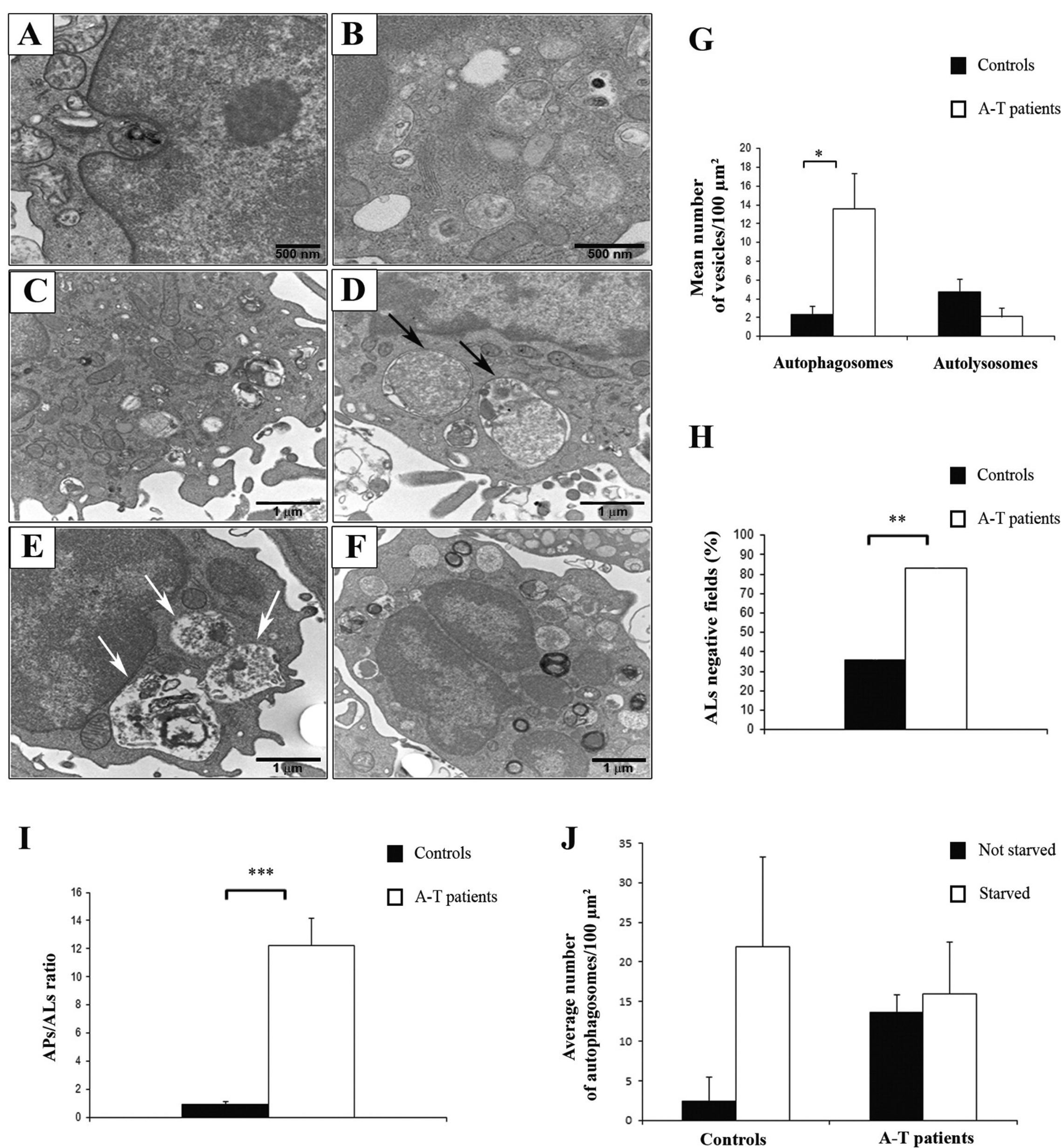

Fig. 1. Lymphocytes from patients affected with A-T show accumulation of autophagosomes. (A–B) Electron micrographs of freshly isolated lymphocytes from healthy controls in unstarved culture conditions or after serum starvation for 2 h showing the accumulation of autophagic vesicles (AVs). Scale bar: 500 nm. (C–F) Electron micrographs of lymphocytes isolated from A-T patients at basal conditions showing a general engulfment of AVs. (D) Autophagosomal vesicles (black arrows) with a double-membrane profile in the process of engulfment of targeted organelles. (E) Autolysosomes (ALs) (white arrows) containing degraded material. (F) Vacuolar structures in the late stage of the autophagolysosomal pathway. Scale bar: 1 μm. (G) Number of autophagic vesicles/100 μm^2 in freshly isolated lymphocytes from healthy controls and A-T patients. (H) Percentage of AL negative fields in fresh isolated lymphocytes from healthy controls and A-T patients. (I) APs/ALs ratio for freshly isolated lymphocytes from healthy controls and A-T patients. (J) Number of autophagosomes/100 μm^2 in freshly isolated lymphocytes from healthy controls and A-T patients at basal conditions and after serum starvation for 2 h. (Data expressed as mean ± S.D.; $^*p = 0.03$; $^{**}p < 0.01$; $^{***}p < 0.05$. Representative image, $n = 3$ subjects per group).

patients than in the controls (12.22 *vs* 0.93; $p = 0.03$) (Fig. 1I). Indeed, the APs number did not further increase under starvation in patient cells, indicating that in A-T an inhibition of autophagy flux rather than a stimulation of AP biogenesis occurs (Fig. 1J).

The formation of LC3 puncta is observed in cells during autophagic activation [19]. To further confirm that in A-T patients the autophagy was present at basal conditions, we tested the presence of LC3 puncta in lymphocytes from both A-T patients and controls. Immunofluorescence analysis documented the presence of LC3 puncta in A-T patients but not in the controls, who showed a diffuse staining pattern of LC3 (Fig. 2A). Moreover, as shown in Fig. 2A, in few lymphocytes from A-T patients the LC3 marker co-localized with LAMP2, in contrast to the controls, where no merge signal between LC3 and LAMP2 was documented. This result indicates that in the patients, even at basal conditions, autophagy was detectable, since the presence of the merge signal indicates that the fusion between APs and lysosomes occurred. A quantitative analysis of LC3 positive cells, revealed that in A-T patients the percentage of cells with a puncta pattern was much higher than in the controls (82 *vs* 12.25%) (Fig. 2B) and that in A-T patients about 4/5 of the total amount of LC3 molecules contained in the cells showed the puncta staining pattern (82% puncta *vs* 18% non-puncta; $^*p < 0.005$), thus resulting in a LC3 puncta cells/LC3 non-puncta cells ratio much higher in the patients than in the controls (6.56 *vs* 0.37) (Fig. 2B).

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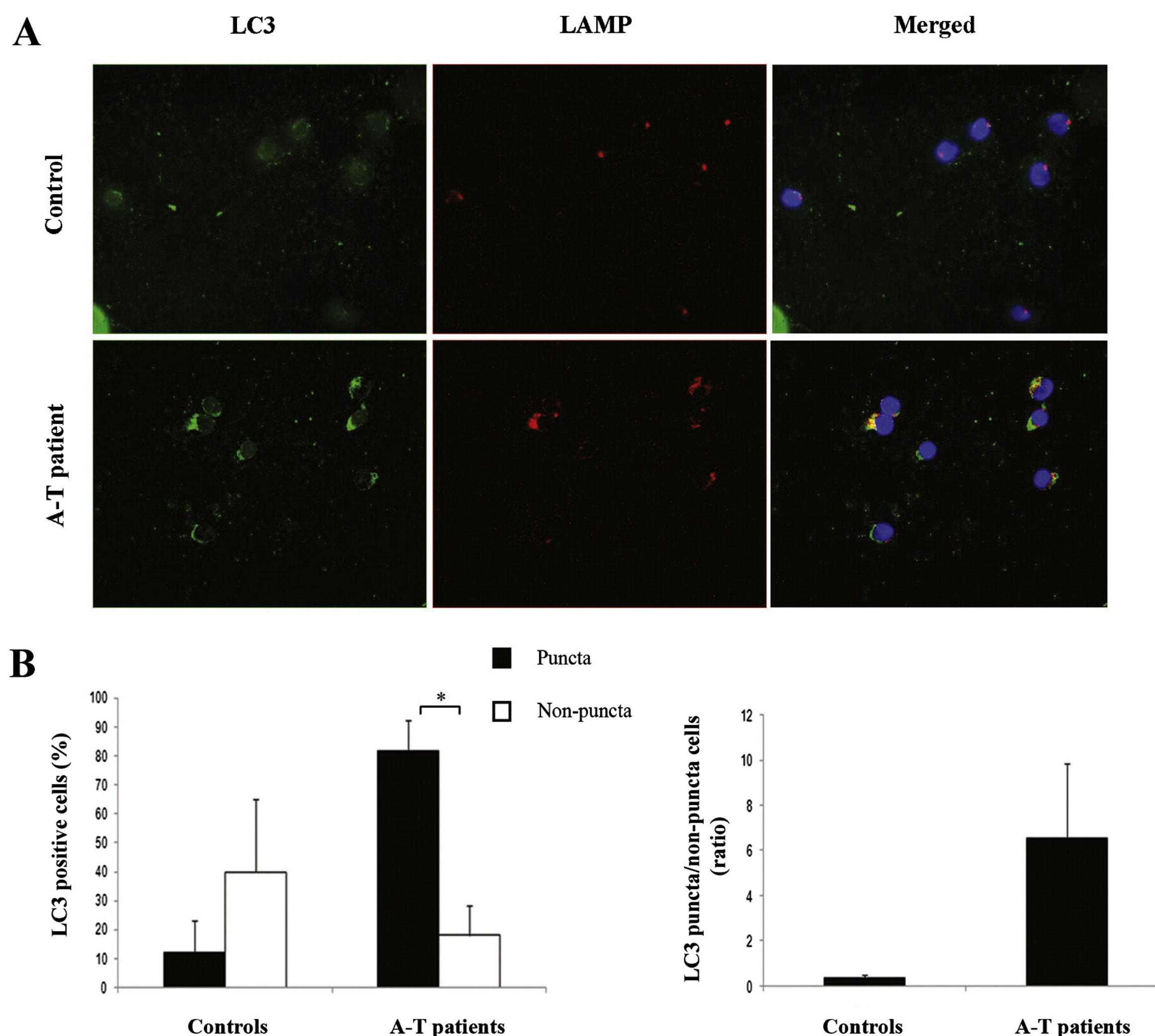

Fig. 2. Autophagy is active in lymphocytes from A-T patients at basal conditions. (A) Representative images of freshly isolated lymphocytes from healthy controls and A-T patients showing autophagosomes expressing LC3 marker (green punctate dots), lysosomes expressing LAMP2 marker (red dots) and autolysosomes (yellow dots). Cells were imaged by fluorescence microscopy. (B) Percentage of autophagic vesicles positive for LC3 marker and LC3 puncta cells/LC3 non-puncta cells ratio in freshly isolated lymphocytes from healthy controls and A-T patients (data expressed as mean ± S.E.; $^{*}p < 0.005$; representative image, $n = 4$ subjects per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. The accumulation of APs in lymphocytes from patients affected with A-T is associated to a reduced expression level of Vps18 mRNA

Autophagy consists of two consecutive stages: the early stage when the APs biogenesis occurs and the late stage characterized by the fusion between APs and endosomes-lysosomes, which leads to the formation of ALs, where the damaged proteins and the entire organelles are degraded by the lysosomal hydrolases and recycled to generate building blocks for anabolic processes. Of note, a normal biogenesis and function of lysosomes and their positioning in the cell, are essential for a proper autophagic process. Since several genes are activated during the process, playing different roles in the different steps, we analyzed through quantitative real-time PCR the relative expression levels of an array of genes differentially involved in the process.

In A-T patients at basal conditions, mRNA expression level of *UVRAG*, involved in the biogenesis of APs, was higher than controls (Fig. 3A), in keeping with the data obtained by TEM. The evaluation of the mRNA expression level of genes involved in the lysosome biogenesis and function (*CSTF*, *CSTB*, *CSTD*, *GBA*, *GUS*, *CD63*, *CSTA*, *CSTE*) revealed that, in A-T patients, at basal conditions, the expression of 6 out of the 8 genes analyzed was much higher than in the controls (Fig. 3B), while under starvation conditions the expression was comparable (data not shown), thus suggesting that in A-T, at basal conditions, the activation of the process was constitutively high. Taken together the results indicate that in A-T patients, the generation of both the APs and the lysosomes is very active. Moreover, the increased expression of 4 out of 5 cathepsins (*CSTB*, *CSTD*, *CSTA*, *CSTE*) and of the *GBA* hydrolase mRNAs in A-T at basal conditions indicates that lysosomal functionality is activated, as well (Fig. 3B).

Since we observed an inappropriate APs/ALs ratio at the morphological studies in A-T, we also evaluated the expression level of *Vps11* and *Vps18* mRNAs, whose products are involved in vesicular trafficking and fusion. As shown in Fig. 3C, at basal conditions, both *Vps11* and *Vps18* are less expressed in the patients than in the controls. The expression level of *Vps18* did not increase even after serum starvation, as compared to control expression.

To allow the encounter between APs and lysosomes, the vesicles have to be in the perinuclear zone. Since in the presence of nutrients, the lysosomes are linked to the plasmatic membrane through kinesins, we also evaluated the expression level of *KIF1Bβ*, *KIF2A*, *ARL8A*, *ARL8B* and *KIF3C*. As shown in Fig. 3D, at basal conditions, all the kinesins, and in particular the *KIF3C* molecule, were more expressed in A-T

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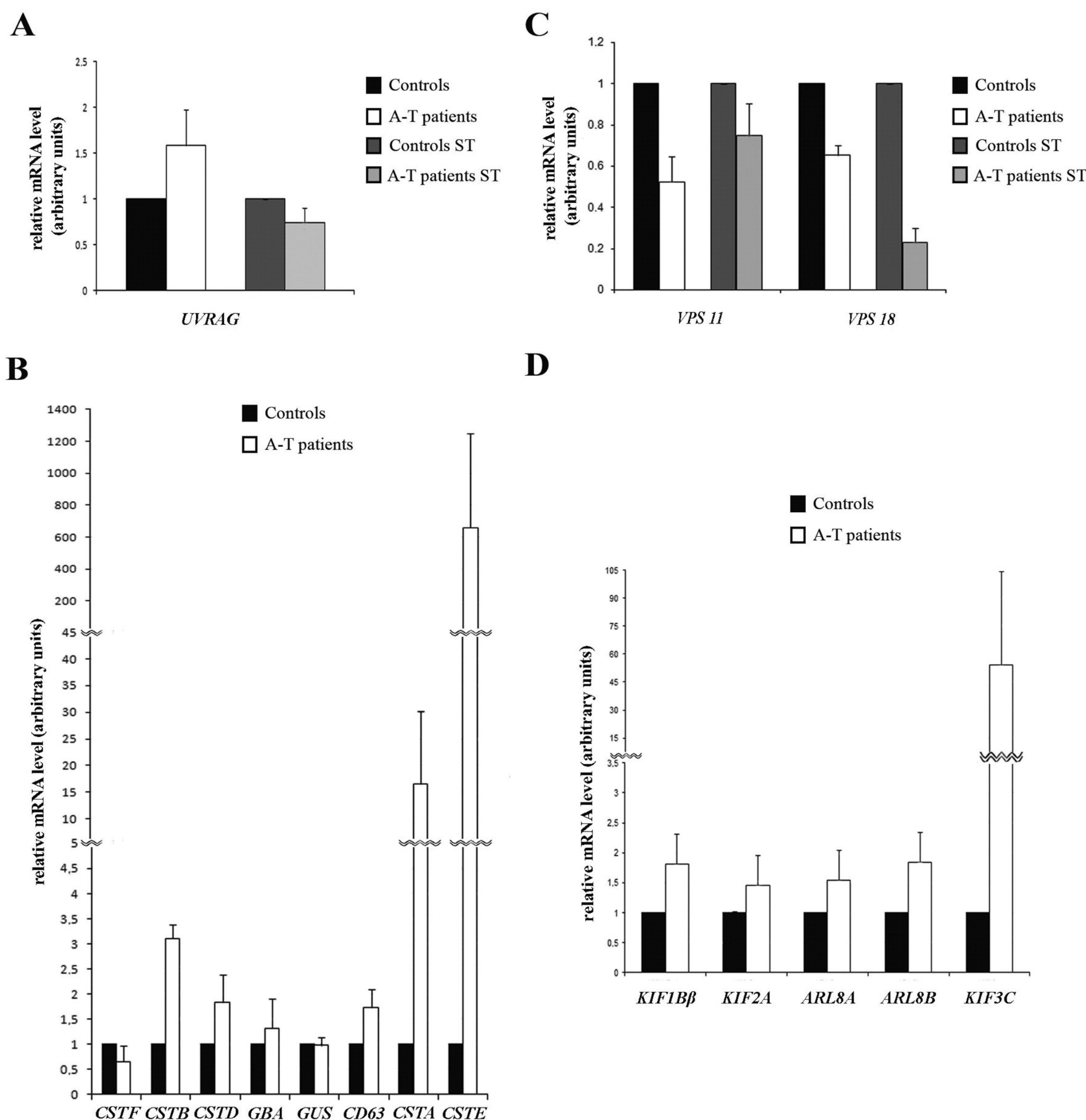

Fig. 3. The accumulation of autophagosomes in lymphocytes from A-T patients is associated to a reduced expression level of Vps18 mRNA. (A–B) Relative mRNA expression level of genes involved in the biogenesis of APs (*UVRAG*) and in lysosome biogenesis and function (*CSTF*, *CSTB*, *CSTD*, *GBA*, *GUS*, *CD63*, *CSTA*, *CSTE*), in freshly isolated lymphocytes from healthy controls and A-T patients, at basal conditions and after serum starvation for 2 h. (C–D) Relative mRNA expression level of genes involved in vesicular trafficking and fusion (*Vps11* and *Vps18*), at basal conditions and after serum starvation for 2 h, and in lysosomal positioning in the cell (*KIF1Bβ*, *KIF2A*, *ARL8A*, *ARL8B* and *KIF3C*) in freshly isolated lymphocytes from healthy controls and A-T patients, at basal conditions (data are expressed as mean ± S.E.; $n = 3$ subjects per group).

patients than in the controls, thus suggesting that the lysosomes are located in the peripheral zone of the cell, not favoring the fusion process between the vesicles.

3.3. Autophagy is increased in A-T patients despite an activated mTOR pathway

mTORC1 is an important sensor in regulating cell proliferation and inhibiting autophagy [28]. Several pathways regulate its activation, which eventually results in the phosphorylation of downstream targets S6K, S6 and 4EBP1, involved in protein synthesis and survival. By contrast, ATM inhibits mTORC1. We investigated the phosphorylation status of mTOR (Fig. 4A) and its downstream targets at basal conditions and after treatment with H_2O_2 at different time points (Fig. 4B). We found that in lymphoblastoid cell lines from A-T patients, the phosphorylation status of mTOR, S6K, S6 and 4EBP1 proteins, was increased, in particular after 30′ and 60′ of treatment with H_2O_2, indicating that in A-T patients mTORC1 is active, and that autophagy mechanism is inhibited. Collectively, these data suggest that in A-T patients there is a lower ATM-mediated inhibition on mTORC1 and of its downstream molecules, as compared to the control.

3.4. Betamethasone pre-treatment promotes autophagy inducing autophagosome formation and degradation

SQSTM1/p62 and LC3 are the canonical markers to evaluate the autophagic process. In particular, SQSTM1/p62 translocates to the autophagosome formation site, where it is incorporated and then degraded [29]. Its accumulation reveals an impaired autophagic flux [19]. LC3-II amount correlates with the number of autophagosomes, and is used as indicator of the autophagosome formation [30]. BAFA1, a specific inhibitor of vacuolar-type H^+ ATPase of the lysosome, increases LC3-II levels, mainly during H_2O_2 induced stress, indicating that a normal flux occurs. We showed that in lymphoblastoid cells from A-T patients, at basal conditions, SQSTM1 levels are accumulated as compared to controls (Fig. 5A). Such increase was more evident in

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Fig. 4. Autophagy is increased in A-T patients despite an activated mTOR pathway. Lymphoblastoid cells from the A-T patient and control were treated with 0.2 mM of H_2O_2 for different time points. (A) Fractioned lysates were loaded for detection of mTOR and p-mTOR. (B) Fractioned lysates were also loaded on another gel, for detection of p-S6K, p-S6, p-4EBPI and housekeeping protein. (C) p-mTOR/mTOR ratio in lymphoblastoid cells from the A-T patient and control.

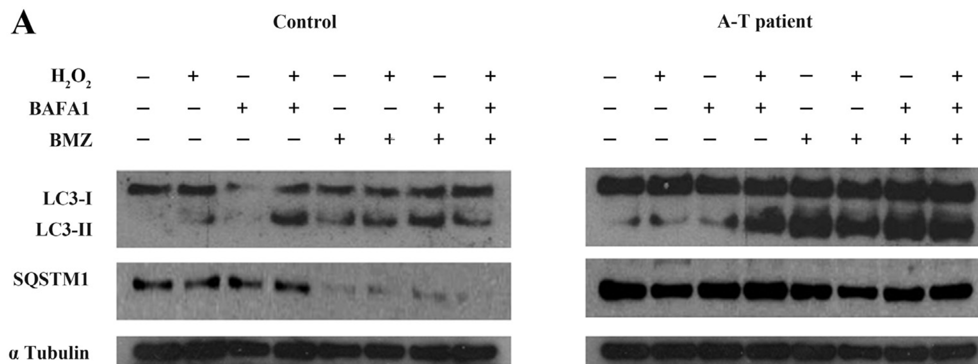

Fig. 5. Betamethasone pre-treatment promotes autophagy inducing autophagosome formation and degradation. (A) Lymphoblastoid cells from the A-T patient and healthy control were pre-treated or not with Betamethasone (BMZ) (80 nM) for 2 h, and H_2O_2 (0.2 mM) for 0.5 h in the presence or absence of 100 nM Bafilomycin (BAFA1) as indicated. Western blotting was performed to analyze the status of LC3, SQSTM1 and Tubulin.

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the presence of stress induced for 30′ by H_2O_2 (Fig. 5A). After treating for 1 h with BAFA1, we found that degradation of SQSTM1 was inhibited (Fig. 5A), as expected. Furthermore, a synergic effect of H_2O_2 and BAFA1 treatment resulted in a remarkable increase of SQSTM1 level (Fig. 5A). We also provided evidence that, in lymphoblasts from A-T patients at basal conditions, BMZ pre-treatment is able to promote the degradation of SQSTM1, as shown in Fig. 5A. However, BMZ is ineffective in counteracting the effect on the molecule of the H_2O_2 induced stress (Fig. 5A). By contrast, BMZ is able to counteract the inhibitory effect of the BAFA1 on SQSTM1 level (Fig. 5A). In the control, BMZ *per se* promotes the degradation of SQSTM1. As expected, BAFA1 increased LC3-II expression under the condition of H_2O_2 induced stress. This increase was even more evident after BMZ pre-treatment. In particular, the overexpression of LC3-II after BAFA1 treatment, suggests an enhancement of the autophagic flux and a BMZ positive effect on the modulation of the autophagic process.

Collectively, these results suggest that in A-T patients the process of APs maturation is active, while the fusion process between APs and ALs is inappropriate, the latter being the overall limiting step in the autophagic process. Moreover, we documented a positive effect of Betamethasone pre-treatment on molecules implicated in the cell clearance apparatus and promotion of autophagosome formation and subsequent degradation.

4. Discussion

In the present study we documented that lymphocytes isolated from A-T patients are characterized by an accumulation of APs, associated with a high expression of genes involved in the process of biogenesis and function of APs. This accumulation is not coupled to a parallel formation of autolysosomes, indicating the inappropriateness of the cell clearance apparatus, which ultimately results in the morphological appearance at the TEM of cytoplasmic engulfment of waste material.

Autophagy is markedly induced by stress conditions, such as starvation, and has two major purposes: to recycle essential macromolecules and energy to be reused in conditions of nutritional scarcity or to clear cells from altered intracellular components [31].

Autophagy is a dynamic flux including vesicle biogenesis, fusion between APs and lysosomes and, eventually, content degradation, and thus the increased number of APs herein observed may be due to an increased biogenesis or to a reduced degradation of the vesicles after the fusion with the lysosomes.

Moreover, it has recently been reported by Park et al. [32] that autophagy is upregulated in response to DNA damage and that impaired cell cycle progression mediated through the checkpoint kinase 1 (Chk1), that allows DNA repair, affects the functionality of autophagy.

In keeping with the data obtained by TEM, documenting an accumulation of APs, we found in A-T lymphocytes, an increased expression of the *UVRAG* mRNA, which is specifically involved in the initial step of formation of APs, thus confirming, also at molecular level, that the stage of biogenesis of APs properly occurs in A-T patients. Moreover, the evaluation of CD63, which is expressed on lysosomal membrane, revealed an increased mRNA expression in A-T lymphocytes, indicating a proper lysosomal biogenesis.

An accumulation of APs may be due to abnormalities of regulatory molecules [33]. Among the genes involved in lysosome functionality, cathepsins, lysosomal hydrolases belonging to the cysteine protease family A, B, D and F, and β-glucosidase and β-glucuronidase, help degrade unwanted intracellular or endocytosed proteins [21]. In brain neurons of CST $D^{-/-}$ or CST $B^{-/-}$/CST $L^{-/-}$ mice, the lack of CST is responsible for the absence of protein degradation, which results in the accumulation of vacuolar structures, a feature similar to that observed in A-T lymphocytes. CST E is an intracellular aspartic proteinase that is predominantly distributed in immune-related cells. The lack of CST E induces several abnormal membrane trafficking events resulting in the impairment of autophagic flux and the accumulation of toxic proteins and/or damaged organelles [34]. CST A is a serine protease, which regulates the lysosomal activity and stability of several lysosomal enzymes such as glycosidases, beta-D-galactosidase, as well as the transport of neuraminidase to mature lysosomes. CST A also triggers the degradation of LAMP 2A receptor involved in the autophagic process [35]. Similarly, in macrophages, the absence of CST S promotes an accumulation of APs [36], thus indicating a regulatory role for cathepsin family molecules. However, in A-T patients, even in unstimulated conditions, the mRNA expression levels of these proteases, *CSTB*, *CSTD*, *GBA*, *CSTA* and *CSTE*, are increased, thus indicating a constitutive hyperactivation of the lysosomal functionality. These data argue against the possibility that the accumulation of APs in A-T could be related to a reduced hydrolase activity.

The positioning of lysosomes plays an important role in that, only the lysosomes localized in the perinuclear area of the cell are able to fuse with APs and promote the cell clearance [24]. The subcellular redistribution of lysosomes mostly relies on their transport along microtubules mediated by the kinesins. The process ultimately leads to the formation of ALs where the degradation occurs. In this study, we found an overexpression in A-T cells of the kinesins KIF2A and KIF1B-β and the monomeric GTPase ARL8B and its close homolog ARL8A. A very impressive increase in KIF3C expression was also found. These results further support the inappropriateness in the process of lysosomal trafficking, which does not parallel AP formation, thus impairing the autophagic flux. By contrast, the A-T lymphocytes show a reduced expression of *Vps18* and *Vps11* mRNAs, whose protein products are involved in the vesicular trafficking and fusion between APs and lysosomes [25,37]. Both Vps11 and Vps18 are subunits of the Vps-C core complex also composed of Vps16 and Vps33 [38]. In eukaryotic organisms, the Vps-C complex acts as a tethering factor in endosome- and lysosome-related vesicle fusion process. In particular, the down-regulation of *Vps18*, by knocking-down or by using anti-Vps18 antibody, blocks AP-lysosome and early endosome fusion, leading to an accumulation of APs and late endosomes in yeast [38]. Additional data demonstrate that Vps18 plays a prominent role in the process [39]. However, the immunofluorescence staining experiments revealed that, at a certain extent, the fusion process between APs and lysosomes in A-T cells did take place. Thus, we would argue that the reduced expression of *Vps11* and *Vps18* represents the limiting factor of the process that does not allow an autophagic flux at a proper extent. Overall, the data herein reported indicate that the accumulation of APs may be due to the inefficacy of their degradation as a consequence of the reduced fusion process among APs and lysosomes in the perinuclear zone, determined by an altered regulatory control of the process. The increase in kinesins expression may be interpreted as an attempt to overcome the inappropriate AP-lysosome fusion process.

Taken together our data indicate that, in A-T lymphocytes, the fusion step between APs and lysosomes represents the limiting factor in the autophagy process.

Besides the nuclear activity of ATM, there is evidence that the molecule is also localized in the cytosol, within synaptosomal fractions [40], and, in particular, on the peroxisomes [41], where it induces pexophagy in response to ROS [42]. It is also well documented that ATM, in the cytosol, has a role in the autophagy pathway [12]. In fact, in the presence of elevated ROS levels, ATM activates the tumor suppressor TSC2, through the LKB1/AMPK pathway, to inhibit mTORC1 and, in turn, induces autophagy [43]. In this study, we documented in lymphoblasts from A-T patients, an increase of the phosphorylated S6K, S6 and 4EBP1 proteins, which are mTORC1 substrates, and, in particular, under stress conditions. This is in keeping with the inhibitory effect of ATM on mTORC1 signaling. In addition, the increased p-mTOR/mTOR ratio in A-T patients, under stress conditions, further supports the ATM-mediated inhibition of mTORC1. Since in this study we found an accumulation of APs, this would imply that autophagy is a complex process and that the phase of AP formation is dependent on different regulatory pathways, which are not dependent

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on the cytosolic form of ATM. Whether the dysregulation of the process, herein described, directly depends on the absence of ATM mediated control of the pathway upstream to mTORC1 or rather to the defect in DNA repair remains to be further clarified.

Several studies have focused on the relationship between glucocorticoids and autophagy. It is known that GCs are able to induce autophagy in different cell types [44,45], even though the explanation of the intimate molecular mechanism by which these drugs lead to this effect is not fully clear.

In the attempt to explain the beneficial effect of the steroids in A-T, we evaluated the effect of Betamethasone on molecular targets implicated in autophagy. We found that Betamethasone pre-treatment promoted the degradation of SQSTM1 protein, which was accumulated in lymphoblastoid cells obtained from A-T patients. The degradation of this protein is generally considered to parallel the progression of autophagy. We also found an accumulation of LC3-II protein after BMZ treatment, suggesting an enhancement of the autophagic flux and a Betamethasone-mediated positive effect on the process.

In conclusion, we provide evidence of the inadequacy of the cell clearance apparatus in the cells from A-T patients, characterized by an accumulation of un-degraded APs. The molecular studies led us to identify the fusion process between APs and lysosomes the limiting step in the overall process. To address the issue of whether the alterations observed in lymphocytes from A-T patients could also be responsible for neurodegeneration, further studies will be planned in $ATM^{-/-}$ neuronal cell lines.

Abbreviations

A-T	Ataxia Teleangiectasia
AP	Autophagosome
AL	Autolysosome
LC3	Microtubule-associated protein-light chain 3
SQSTM1	Sequestosome 1
BMZ	Betamethasone

Conflicts of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

This work was supported by Sparks Charity, A-T Society and Action for A-T (Grant number: 13FED01) and by “Associazione Immunodeficienze Primitive” (AIP). AF was partially supported by the Italian ONLUS Association “Gli Amici di Valentina”.

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