

ATM kinase activity modulates Fas sensitivity through the regulation of FLIP in lymphoid cells

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Ataxia telangiectasia (A-T) is a rare cancer-predisposing genetic disease, caused by the lack of functional ATM kinase, a major actor of the double strand brakes (DSB) DNA-damage response. A-T patients show a broad and diverse phenotype, which includes an increased rate of lymphoma and leukemia development. Fas-induced apoptosis plays a fundamental role in the homeostasis of the immune system and its defects have been associated with autoimmunity and lymphoma development.

We therefore investigated the role of ATM kinase in Fas-induced apoptosis. Using A-T lymphoid cells, we could show that ATM deficiency causes resistance to Fas-induced apoptosis. A-T cells up-regulate FLIP protein levels, a well-known inhibitor of Fas-induced apoptosis. Reconstitution of ATM kinase activity was sufficient to decrease FLIP levels and to restore Fas sensitivity. Conversely, genetic and pharmacologic ATM kinase inactivation resulted in FLIP protein up-regulation and Fas resis-

tance. Both ATM and FLIP are aberrantly regulated in Hodgkin lymphoma. Importantly, we found that reconstitution of ATM kinase activity decreases FLIP protein levels and restores Fas sensitivity in Hodgkin lymphoma-derived cells. Overall, these data identify a novel molecular mechanism through which ATM kinase may regulate the immune system homeostasis and impair lymphoma development. (Blood. 2008;111:829-837)

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Introduction

Ataxia telangiectasia (A-T) is an autosomal recessive disorder characterized by cerebellar progressive neurodegeneration leading to ataxia, dilatation of blood vessels in the eye and facial area (telangiectasia), sensitivity to γ -irradiation, high incidence of tumorigenesis in the lymphoid system, and deficiency in immunoresponses. A-T pathology is characterized by the loss of functional ATM protein kinase. Following DNA damage, ATM is rapidly activated and (auto)phosphorylated,¹ and, in turn, it phosphorylates a number of substrates that all contribute to cell growth arrest or, alternatively, apoptosis (reviewed in Shiloh²). The higher cancer predisposition of A-T patients has been associated with the lack of DNA-damage response, which results in genomic instability.³ The immune system is the major target of tumor development in these patients, and lymphoma and leukemia are very frequent.^{4,5} This clinical feature is consistent with the central role of ATM in the management of the DNA DSBs generated during the immune system development and function in physiological conditions.⁶ Indeed most of the lymphomas developed in A-T patients are characterized by aberrant VDJ recombination.⁶ More interestingly, ATM expression is aberrantly low in several B- and T-cell lymphomas irrespective of A-T genotype.⁷⁻¹⁰

Fas (CD95/APO-1) is a transmembrane protein belonging to the tumor necrosis factor superfamily. Upon binding of Fas ligand or agonistic antibodies, the Fas receptor recruits several cytosolic

proteins to form the death-inducing signaling complex (DISC). This is necessary to catalyze dimerization and processing of procaspase-8 to generate the active caspase-8 tetramer, composed of 2 p18 and 2 p10 subunits, which initiates the caspase cascade.¹¹ Procaspase-8 activation is absolutely required to trigger receptor-activated apoptotic response,¹² and its catalytic activity has to be tightly regulated to avoid inappropriate activation and undesired cell death.¹³ FLIP protein is structurally similar to procaspase-8 and can therefore compete with procaspase-8 for binding to DISC, thus preventing caspase-8 activation and the following apoptotic cascade. Two isoforms of FLIP, arising from alternative splicing, are normally present in most of the cells. FLIP-long (FLIP-L), similarly to procaspase-8, has 2 DED domains that mediate the recruitment to the DISC, as well as a p18 and a p10 subunit, but it lacks the Cys residue in the active site and is therefore catalytically impaired. However, in some contexts FLIP-L can also dimerize and therefore promote caspase-8 activation.¹⁴ Conversely, FLIP-short (FLIP-S) contains only the DED domains and it behaves as a pure inhibitor of procaspase-8 activation and Fas-induced apoptosis.¹³

The death receptor system is essential for the regulation of the lymphoid system homeostasis.¹⁵ It is assumed that the negative selection process of B as well as T cells in the germinal center (GC) and thymus, respectively, depends on Fas system.^{16,17} Several lines of evidence indicate the importance of this system for the balance

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between B-cell proliferation and apoptosis.¹⁸ Indeed, mice lacking functional Fas expression suffer from autoimmunity and increased incidence of B-cell lymphomas.^{19,20} Patients with mutations that impair the function of proteins involved in Fas-dependent apoptosis develop the autoimmune lymphoproliferative syndrome (ALPS), which predisposes them to autoimmune disorders and to lymphoma development.^{21,22} Finally, Fas mutations were identified in lymphomas, in particular those deriving from GC B cells (reviewed in Muschen et al²³).

Classic Hodgkin lymphoma (cHL), a common human lymphoma, has been proposed to derive, most frequently, from GC cells.²⁴ Currently, the molecular pathogenesis of cHL remains unclear. Interestingly, Hodgkin/Reed Sternberg (HRS) cells, the malignant cells of cHL, resist to Fas-induced apoptosis,²⁵ and Fas resistance has been proposed to play an active role in the development of HRS cells. Indeed, these cells evade the control of the immune system and initiate the tumor growth. Recently, Fas resistance of HL-derived cell lines has been proposed to be caused by the aberrant up-regulation of FLIP proteins in these cells. Indeed, the specific down-regulation of FLIP expression by siRNA sensitizes these cells to Fas-induced apoptosis.^{26,27} Remarkably, immunohistochemistry studies have shown that most cases of Hodgkin disease are ATM negative,⁷ although ATM loss of heterozygosity is a rare event,²⁸ and therefore alternative mechanisms may account for ATM down-regulation.²⁹

Taking into account the linkage between Fas impairment and the development of those tumors that are more frequent in A-T patients, the question arises as to whether any relationship exists between Fas and ATM signaling pathways.

Here we show that ATM deficiency results in a significant resistance of lymphoid cells derived from A-T patients to Fas-induced apoptosis. Interestingly, loss of endogenous ATM kinase activity results in the aberrant up-regulation of FLIP protein levels. Consistently, ATM kinase activation down-regulates FLIP protein levels providing a novel mechanism to modulate Fas sensitivity. Furthermore Hodgkin lymphoma cells that are characterized by Fas resistance may be sensitized to Fas upon ATM kinase expression. These data point to ATM as a novel player in Fas-induced apoptosis and suggest a novel molecular mechanism for the increased lymphoma susceptibility of A-T patients and for the development of B-cell lymphoma.

Methods

DNA constructs

pcDNA3-Flag-ATM-wt and pcDNA3-Flag-ATM-Kin⁻ were kindly provided by M. Katan. shFLIP construct and its control were kindly provided by H. Walczak.³⁰

Antibodies and other reagents

The following antibodies and reagents were used: anti-phospho-Ser1981-ATM (Rockland, Philadelphia, PA), anti-ATM (MAT3; generously provided by Y. Shiloh), anti-phosphoSer15-p53 (Cell Signaling, Beverly, MA), anti-p53 (Pab240; Santa Cruz, Santa Cruz, CA), anti-phosphoThr68-Chk2 (Cell Signaling), anti-Chk2 (kindly provided by D. Delia), anti-pS139 H2A.X (UBI, Hauppauge, NY), anti-Fas IgM monoclonal antibody (CH11; UBI), anti-Flag (Sigma, St Louis, MO), anti-caspase-8 (clone 5F7; MBL, Watertown, MA), anti-FLIP (S and L) (H-202; Santa Cruz), anti-active caspase-3 (Cell Signaling), caspase-inhibitor zVAD (Biomol, Plymouth Meeting, PA), NCS (kindly provided by Y. Shiloh), and KU-55933 (kindly provided by KuDOS, Cambridge, United Kingdom).

Cell culture and transfections

C3ABR and L6 cells (kindly provided by M. Lavin and Y. Shiloh), as well as GM-03189 and GM-02782 cell lines, were cultured in RPMI 1640 medium with 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum. C3ABR and L6 cells were stably transfected by electroporation using 20 µg of the indicated constructs. Stably transfected cells were selected in the presence of 500 µg/mL G418. HL-derived cell line, L428, kindly provided by H. Kashkar and M. Kronke, was transfected by electroporation.

Analysis of apoptosis

C3ABR, L6, L6pCDNA, L6-Flag-ATM-wt, L6-Flag-ATM-Kin⁻, and L6-shFLIP cells lines were treated to undergo apoptosis with 250 to 500 ng/mL anti-Fas antibody. Where indicated in Western blot and immunofluorescence analysis, cells were also treated with NCS (100 ng/mL for 1 hour) or stimulated in the presence of 40 µM zVAD caspase-inhibitor, which was added 30 minutes before stimulation with Fas.

Apoptosis was quantified by propidium iodide (Sigma) nuclear staining or by the analysis of annexin V (Pharmingen, San Diego, CA) exposure using a FACScanto (Becton Dickinson, Lincoln Park, NJ). Specific apoptosis was determined as follows: (% of apoptotic cells with anti-Fas – % of apoptotic cells without anti-Fas)/(100 – % of apoptotic cells without anti-Fas).

Analysis of Fas receptor levels

To analyze the expression of Fas protein, cells were incubated for 30 minutes at room temperature with mouse antihuman Fas antibody (APO1; Transduction Laboratories, Lexington, KY). Next, cells were reacted with PE-conjugated goat anti-mouse IgG (H + L) (Pharmingen) for 30 minutes at room temperature. Cells were analyzed using a flow cytometer. For each cell line, incubation with PE-conjugated goat anti-mouse IgG alone served as negative controls. Mean fluorescence intensity of cells stained with anti-Fas was used to compare the level of Fas expression.

Flow cytometry of phospho-Ser1981-ATM in apoptotic cells

Our protocol is a variation of a recent method used to evaluate phosphopeptide status by flow cytometry.³¹ Cells (5×10^5) were fixed in 4% formaldehyde and incubated for 15 minutes at 4°C. They were then permeabilized by resuspending with vigorous vortexing in 1 mL ice-cold MeOH and incubated at –20°C overnight (O/N). Cells were washed and resuspended in PBS-Tween 0.5% containing 5% normal goat serum (NGS) containing antimouse phospho-Ser1981-ATM and rabbit active caspase-3 primary antibodies and incubated for 1 hour at room temperature. After washing and repeating the process with antimouse AlexaFluor488– and antirabbit AlexaFluor633–conjugated secondary antibodies, flow cytometry was evaluated in a FACScanto (Becton Dickinson).

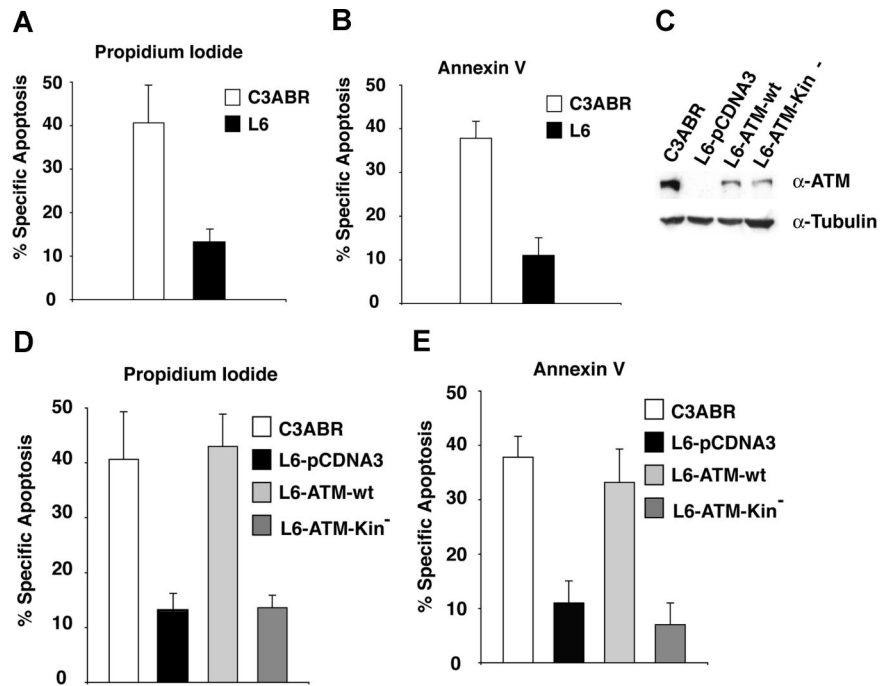
Immunofluorescence analysis

C3ABR, L6-pCDNA, L6-Flag-ATM-wt, and L6-Flag-ATM-Kin⁻ cells line were fixed and permeabilized, and immunofluorescence was carried out as previously described.³² Flag-ATM protein was visualized with monoclonal anti-Flag (Sigma) diluted 1:500 followed by fluorescein-conjugated antimouse antibody (Alexis, Bayport, MN) diluted 1:200 in blocking buffer. Phospho-S1981-ATM was labeled with anti-pS1981-ATM (Rockland) diluted 1:1000 followed by rhodamine-conjugated antirabbit diluted 1:600 (Alexis) or by fluorescein-conjugated antirabbit antibody diluted 1:200. Nuclei were visualized with Hoechst 33342 (Molecular Probes, Eugene, OR) diluted 1:20 000 in PBS-0.1% Triton X-100.

Immunoblotting

Cell extracts were prepared in immunoprecipitation (IP) buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 1 mM orthovanadate, 10 µg/mL TPCK, 5 µg/mL TLCK, 1 µg/mL leupeptin, 10 µg/mL soybean

Figure 1. ATM-deficient cells are resistant to Fas-induced apoptosis. ATM-proficient cells (C3ABR) and ATM-deficient cells (L6) were treated with 250 ng/mL anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation in (A) propidium iodide–stained cells (PI) or by the analysis of (B) annexin V binding, 24 hours after anti-Fas treatment. (C) ATM-deficient cells (L6) were stably transfected with ATM-wt, ATM-Kin⁻, or empty vector as control using 20 μ g of the indicated constructs. For immunoblotting, 80 to 100 μ g protein extract were separated by SDS-PAGE and transferred on nitrocellulose. ATM protein was revealed with anti-ATM (MAT3) antibodies. (D,E) Cells were treated to undergo apoptosis with 250 ng/mL anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation on PI nuclear staining (D) or by the analysis of annexin V exposure (E) at 24 hours after anti-Fas treatment.



trypsin inhibitor, 1 μ g/mL aprotinin).³³ For immunoblotting, 100 to 200 μ g protein extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membrane, and detected with specific antibodies. All immunoblots were revealed by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL).

Caspase-8 activity assay

To determine caspase-8 activity in C3ABR, L6pCDNA, L6-FlagATM-WT, and L6-FlagATM-KD cells lines, cells were induced to undergo apoptosis with 500 ng/mL anti-Fas mAb. Protein extracts were assayed for caspase-8 activity using IETD-AMC as a substrate and Ac-IETD-AMC at 37°C in 200 μ L assay buffer (20 mM Tris [pH 7.4], 0.1 M NaCl, 10% sucrose, 0.1% CHAPS, 10 mM DTT) containing 700 μ g protein extract. Reaction was started by the addition of 10 μ M Ac-IETD-AMC. Cleavage of the substrate as a function of time was monitored reading the absorbance at 460 nm upon excitation at 390 nm. The enzymatic activity was determined from the linear portion of the curve.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated using Trizol reagent (Invitrogen, Frederick, MD) and subjected to RT using oligo(dT) primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT reaction was then amplified by PCR using the primers described in Salon et al.³⁴ Amplification of actin was performed in the same PCR reaction as internal control. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical methods

All data were analyzed and presented as mean plus or minus SD ($n < 10$). The significance of differences between populations of data was assessed according to the Student 2-tailed *t* test with a level of significance of at least *P* less than .05 (alpha conventionally equal to .05). This analysis allows the estimation of the mean of a normally distributed population when the sample size is small.

Results

ATM-deficient cells are resistant to Fas-induced apoptosis

To investigate whether ATM could participate in Fas-mediated apoptosis, we compared the sensitivity to Fas of 2 lymphoblastoid cell lines widely used in studies on ATM activity, one established from an A-T patient (L6)³⁵ and the other one from a healthy control donor (C3ABR).³⁶ Fas was stimulated with agonistic anti-Fas antibodies that mimic the binding of Fas ligand and triggers the apoptotic response. Interestingly, L6 cells, which lack the expression of ATM protein, were significantly resistant to Fas-induced apoptosis (Figure 1A,B). Similar results were obtained also with other A-T lymphoblastoid cell lines, such as GM-03189 and GM-02782 (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). To address the question of whether ATM kinase activity is required for Fas sensitivity, we stably reconstituted ATM expression in L6 cells. For this purpose, L6 cells were stably transfected with constructs that allow the expression of either FLAG-ATM-wt protein (L6-ATM-wt) or the kinase-dead FLAG-ATM-Kin⁻ protein (L6-ATM-Kin⁻), or with the empty vector as control (L6-pCDNA). ATM expression was monitored by immunoblotting with specific antibodies. (Figure 1C). L6-ATM-wt and L6-ATM-Kin⁻ cells expressed the same levels of ATM protein. Interestingly, the reconstitution of the expression of ATM in the L6-ATM-wt cells dramatically sensitized these cells to Fas-induced apoptosis (Figure 1D,E; Figure S2). The expression of the ATM-kinase-defective mutant, FLAG-ATM-Kin⁻, completely failed to restore Fas sensitivity. Overall, these results suggest that ATM kinase activity enhances Fas-induced apoptosis.

Caspase activation is a prerequisite of Fas-dependent ATM activation

We therefore asked the question of whether Fas stimulation triggers ATM kinase activation and whether this may contribute to Fas

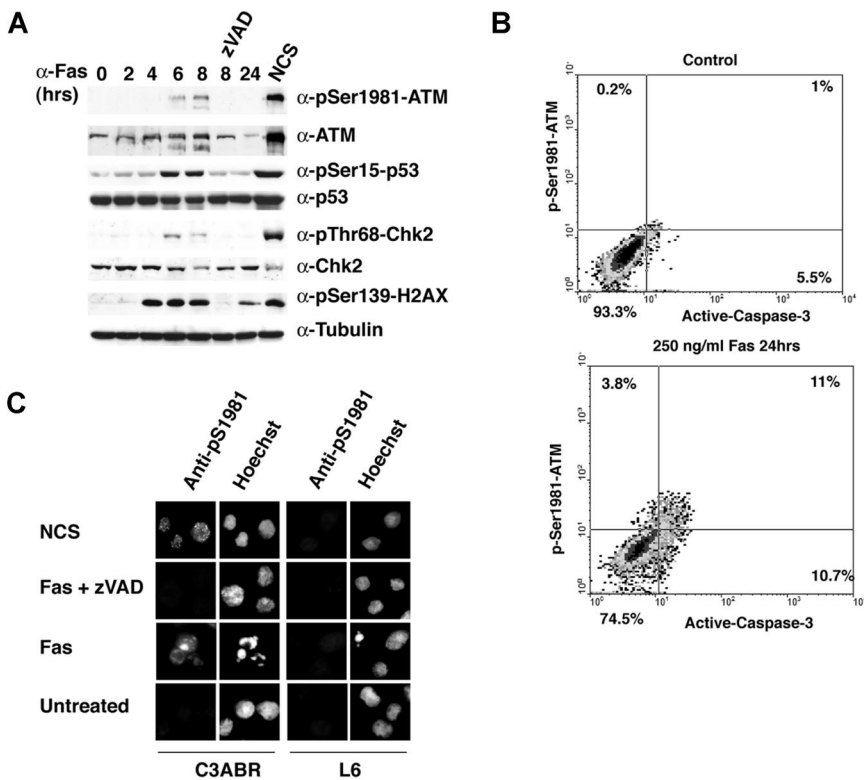


Figure 2. ATM kinase activation following Fas-induced apoptosis is a late passive event. (A) C3ABR cells were induced to undergo apoptosis with 250 ng/mL anti-Fas IgM monoclonal antibody. Untreated and NCS-treated cells that trigger DSB and classically induce ATM activation⁴² were used as controls. For immunoblotting, 80 to 100 μ g protein extract were separated by SDS-PAGE and transferred on nitrocellulose. The proteins of interest and their phosphorylation were revealed by immunoblotting with specific antibodies. (B) C3ABR cells were treated to undergo apoptosis with 250 ng/mL anti-Fas IgM monoclonal antibody (CH11; UBI). Untreated and treated cells were analyzed by flow cytometry for active caspase-3 and phospho-Ser1981-ATM. (C) C3ABR cells were treated to undergo apoptosis as in panel B. Untreated and NCS-treated cells were used as controls. Cells were fixed and permeabilized, and immunofluorescences were carried out as previously described.³² Nuclear condensation and fragmentation have been evaluated by Hoechst staining.

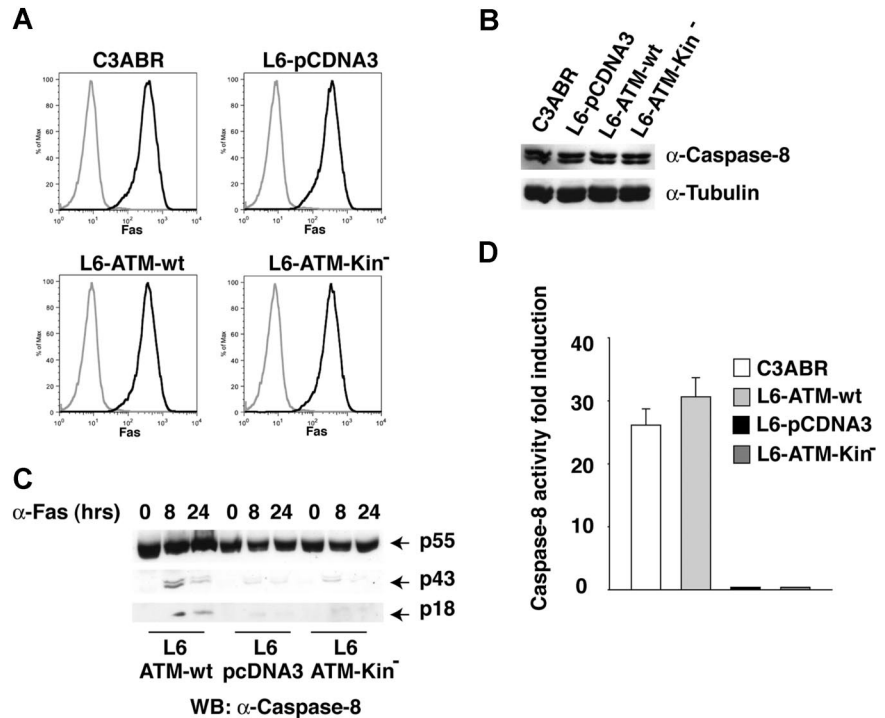
sensitivity. To evaluate the effect of Fas stimulation on ATM kinase activity, protein extracts at different times of stimulation were analyzed. Similarly to what has been described following DNA damage,³⁷⁻⁴¹ Fas stimulation resulted in the typical ATM-dependent phosphorylation cascade. In particular, ATM was phosphorylated on its autophosphorylating activating site (ie, Ser1981),¹ and p53, Chk2, and H2AX became phosphorylated at Ser15, Thr68, and Ser139, respectively (Figure 2A; Figure S3). These data indicate that Fas stimulation results in ATM activation. Importantly, ATM activation was completely prevented by preincubation with the general caspase-inhibitor z-VAD (Figure 2A), thus suggesting that, in the absence of caspase activation, no Fas-induced ATM activation occurs. To evaluate this possibility, we established a new flow cytometry-based assay that allowed us to analyze the levels of phospho-Ser1981-ATM versus caspase-3 activation, which accounts for apoptotic response. This analysis revealed that ATM activation occurs mainly in cells that activate caspase-3, supporting the hypothesis that Fas-dependent ATM activation is downstream of caspase-3 activation, and therefore most likely does not play a major role in Fas sensitivity (Figure 2B). Fas stimulation also resulted in the cleavage of ATM protein (Figure 2A), similarly to other apoptotic stimuli, which trigger ATM kinase cleavage most likely through caspase-3 activity.⁴³ Moreover the uncleavable mutant of ATM, ATM-D863A, previously characterized,⁴³ sensitized A-T cells to Fas-induced apoptosis to the same extent as ATM-wt, further confirming that the cleavage per se does not modulate Fas sensitivity (data not shown). Finally, Fas stimulation triggered ATM phosphorylation on Ser1981 only on those cells that showed apoptotic morphology characterized by nucleus condensation or fragmentation (Figure 2C). Overall, these findings strongly suggest that ATM activation upon Fas stimulation is a passive event subsequent to DNA fragmentation, and therefore most likely does not contribute significantly to cell fate.

ATM kinase activity down-regulates c-FLIP protein levels

To get more insight in the molecular mechanism by which ATM modulates Fas-induced apoptosis, we analyzed the expression profile of those proteins that are relevant for this signaling. Fluorescence-activated cell sorting (FACS) analysis showed that ATM expression and activity do not modulate the levels of Fas (Figure 3A). Immunoblotting analysis showed that all cell lines express comparable levels of caspase-8, independently on ATM activity (Figure 3B). Remarkably, despite the observation that caspase-8 is equally expressed in all cell lines, its activation following Fas cross-linking is significantly delayed in the ATM-deficient cells (L6-pCDNA) as well as in the ATM kinase activity-deficient cells (L6-ATM-Kin⁻) (Figure 3C,D). Full activation of caspase-8 upon Fas stimulation requires its processing, essential to get a stable active caspase-8 tetramer and to allow its release from the DISC and subsequent cleavage of cytoplasmic substrates, such as executioner caspases.¹¹ Immunoblotting experiments, using an anti-caspase-8 antibody raised against the p18 subunit, showed that the lack of ATM results in the delayed accumulation of the intermediate processing product p43 and of the p18 subunit (Figure 3C). Moreover, ATM deficiency delayed Fas-induced caspase-8 activation, measured as its ability to cleave its substrate peptide IETD (Figure 3D).

Because c-FLIP is a well-characterized inhibitor of Fas signaling, we wanted to investigate the possible relationships between ATM activity and c-FLIP expression. Importantly, the lack of ATM expression triggers the up-regulation of c-FLIP (Figure 4A), which may account for Fas resistance of A-T cells (Figure 1). Reconstitution of ATM kinase activity in L6-ATM-wt cells significantly decreased FLIP-L and FLIP-S expression levels, which may account for the recovery of Fas sensitivity (Figure 1). Again, the ATM-Kin⁻ mutant completely failed to down-regulate FLIP (Figure 4A). To test whether indeed ATM activity modulates Fas

Figure 3. ATM kinase activity promotes caspase-8 activation. (A) Fas receptor levels were detected by flow cytometry analysis. Cells were incubated with anti-Fas antibodies followed by PE-conjugated secondary antibodies (dark lines). For each cell line, an incubation with PE-conjugated alone served as negative controls (light lines). (B) Caspase-8 expression was revealed by immunoblotting on extracts obtained by the indicated cell lines. Protein extract (80-100 μ g) was separated by SDS-PAGE and transferred on nitrocellulose, and caspase-8 expression was revealed using specific antibodies. (C) Protein extracts from the indicated cell lines stimulated to undergo apoptosis with anti-Fas antibodies have been separated by SDS-PAGE, and caspase-8 was revealed by immunoblotting with specific antibodies. The arrows point to the entire protein, p55, as well as to the processing products p43 and p18. (D) Caspase-8 activity from the same extracts was measured by the hydrolysis of the caspase-8 substrate Ac-IETD-AMC.



sensitivity via the regulation of FLIP levels, we generated a stable A-T cell line, L6-shFLIP, where FLIP expression has been genetically reduced through specific shRNA that selectively targets FLIP-L and FLIP-S isoforms. These cells express low levels of FLIP proteins comparable with the endogenous level of ATM kinase-reconstituted cells (Figure 4B). Indeed, the reduction of FLIP sensitizes A-T cells to Fas-induced apoptosis (Figure 4C; Figure S2), indicating that the aberrant levels of FLIP proteins may be responsible for Fas resistance in A-T cells. Overall, these experiments show that ATM kinase sensitizes cells to Fas-induced apoptosis through the modulation of FLIP levels. This observation suggests that ATM kinase activity may down-regulate FLIP. Indeed, stimulation with neocarzinostatin (NCS), which classically triggers ATM kinase activation, resulted in a reduction of the levels of FLIP protein (Figure 5A). This effect is completely abrogated in cells that lack ATM protein or have been reconstituted with the ATM kinase-defective mutant (Figure 5A). According to these data, NCS treatment significantly sensitized cells to Fas-induced apoptosis (Figure 5B).

The observation that FLIP is aberrantly up-regulated in A-T cells as well as in A-T cells reconstituted with inactive ATM (Figure 4A) suggests that the endogenous basal activity of ATM is

sufficient to down-regulate FLIP protein levels. To unambiguously address this issue, ATM-proficient cells were incubated in the presence of the ATM kinase inhibitor KU-55933.⁴⁴ Indeed, this treatment triggered FLIP up-regulation (Figure 5C). Interestingly, preincubation with KU-55933 for 1 hour is not sufficient to increase FLIP protein levels (Figure 5C) and fails to protect cells from Fas-induced apoptosis (Figure 5D). Conversely, preincubation with KU-55933 for 8 hours, which is sufficient to trigger FLIP protein accumulation, dramatically impairs Fas-induced apoptosis to the same extent of A-T cells (Figure 5D). These data clearly show that ATM kinase activity is required to modulate Fas sensitivity through the control of FLIP protein levels.

ATM kinase activity modulates FLIP protein stability

To evaluate whether ATM modulates the mRNA levels of FLIP, we analyzed FLIP mRNA levels in ATM-proficient and ATM-deficient cell lines. RT-PCR experiments showed that the levels of FLIP transcripts are comparable in all cell lines independently on ATM expression and activity (Figure 6A), suggesting that FLIP regulation does not occur at transcriptional level. In agreement with this assumption, an exogenous FLAG-tagged FLIP-L driven by a

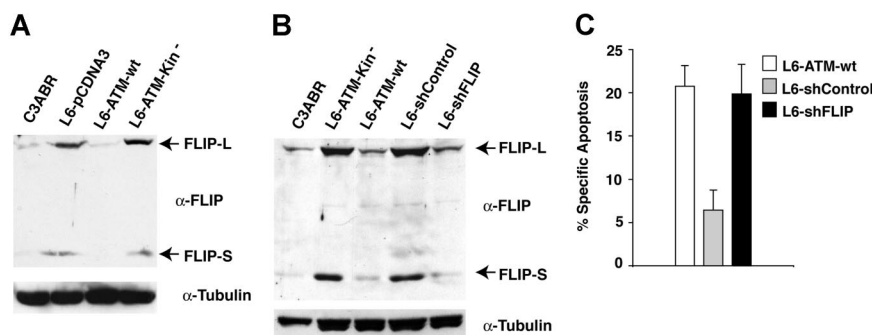


Figure 4. Basal ATM kinase activity regulates FLIP protein levels. (A) FLIP expression was revealed by immunoblotting on extracts obtained from the indicated cell lines. Protein extract (80-100 μ g) was separated by SDS-PAGE and transferred on nitrocellulose, and FLIP expression was revealed using specific antibodies. The arrows point to FLIP-L and FLIP-S isoforms. (B) ATM-deficient L6 cells were stably transfected with shFLIP or with a scrambled oligo as control. Protein extracts from the indicated cell lines were probed for FLIP expression by immunoblotting as described in panel A. The arrows point to endogenous FLIP-L and FLIP-S. (C) The indicated cell lines were stimulated to undergo apoptosis with 250 ng/mL anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation upon PI nuclear staining 24 hours after anti-Fas treatment.

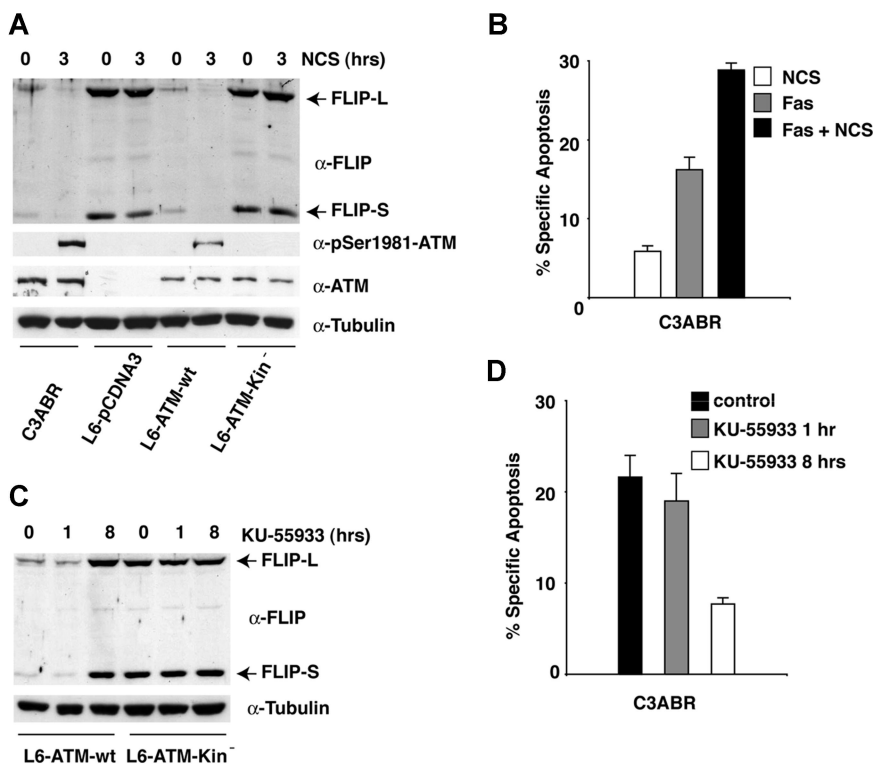


Figure 5. Modulation of ATM kinase activity results in the regulation of FLIP protein levels, which in turn determines Fas sensitivity. (A) Different cell lines were treated with NCS for 3 hours to trigger ATM kinase activation. Protein extract (80-100 μ g) was separated by SDS-PAGE and transferred on nitrocellulose, and FLIP expression was revealed using specific antibodies. (B) C3ABR cells were stimulated to undergo apoptosis with 250 ng/mL anti-Fas mAb in the presence or in the absence of NCS pretreatment for 3 hours. Apoptosis was determined by the analysis of DNA fragmentation in PI-stained cells 24 hours after anti-Fas treatment. (C) The indicated cell lines were incubated in the presence of the specific ATM kinase inhibitor KU-55933 (10 μ M) for 1 or 8 hours. Protein extract (80-100 μ g) was separated by SDS-PAGE and transferred on nitrocellulose, and FLIP expression was revealed using specific antibodies. (D) C3ABR cells were preincubated for 1 or 8 hours with the specific ATM kinase inhibitor KU-55933 (10 μ M) to allow endogenous ATM kinase inactivation and FLIP level up-regulation and then stimulated to undergo apoptosis with 250 ng/mL anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation in PI-stained cells 24 hours after anti-Fas treatment.

heterologous promoter was repressed similarly to the endogenous FLIP-L when stably transfected C3ABR-FLAG-FLIP-L cells were stimulated with NCS to trigger ATM kinase activity (Figure 6B). We therefore tested whether ATM kinase activity accelerates FLIP protein degradation by blocking nascent translation with cycloheximide (CHX). Cells were pretreated with the ATM kinase inhibitor KU-55933 for 8 hours to have the same initial levels of FLIP proteins and then, upon KU-55933 removal, they were incubated for different times with CHX. The degradation of both FLIP forms was significantly faster when L6 cells were reconstituted with ATM-wt than with its kinase-dead homologue (Figure 6C). This approach allowed us to conclude that FLIP protein degradation is significantly increased dependent on ATM kinase activation and that ATM kinase down-regulates FLIP protein stability.

ATM kinase activity sensitizes Hodgkin lymphoma cells to Fas-induced apoptosis

Resistance to death receptor-mediated apoptosis is supposed to be important for the deregulated growth of B-cell lymphoma. Hodgkin/Reed Sternberg (HRS) cells, the malignant cells of classical Hodgkin lymphoma (cHL), resist Fas-induced apoptosis. Fas resistance in this system is due to the aberrant up-regulation of FLIP proteins.^{26,27} Conversely, ATM expression and function are impaired in many HL cases⁷ and in several HL-derived cell lines.^{9,29} To test whether ATM loss of function may contribute to Fas resistance through FLIP protein up-regulation, we took advantage of a lymphoma cell line, L428, that has been previously characterized for the aberrant down-regulation of ATM activity²⁹ and for the aberrant up-regulation of FLIP protein levels.^{26,27} Transient transfection of ATM down-regulates FLIP levels (Figure 7A) and restores Fas sensitivity (Figure 7B), suggesting that targeting of ATM kinase activity significantly contributes to death receptor resistance of HL cell lines and most likely plays a functional role in this pathology.

Discussion

Important defects of the immune system, leading to a significant increase of lymphoma and leukemia development, are one of the major features of A-T syndrome.^{4,5} Since ATM kinase plays a central role in the DSB DNA-damage response and this response is required in some physiological contexts such as the immune system homeostasis, the lack of ATM activity has been proposed to be responsible for aberrant chromosomal translocations originated as a consequence of a failure of the DNA-damage response and indeed associated with several lymphomas and leukemias.⁶

Fas-dependent apoptosis plays a fundamental role in the regulation of the homeostasis of the lymphoid system.¹⁵ Failure in the Fas signaling causes, both in mice and in humans, autoimmunity as well as aberrant proliferation and lymphoma development.¹⁹⁻²²

We reasoned that since defects in Fas-induced apoptosis result in defects in the immune system that partially resemble some of the abnormalities characteristic of the immune system of A-T patients, ATM kinase may play a role in Fas-induced apoptosis. According to our hypothesis, the present article shows that cells that lack ATM kinase are significantly resistant to Fas-induced apoptosis (Figure 1). Reconstitution experiments showed that ATM catalytic activity is required to sensitize cells to Fas (Figure 1D,E). We could show that Fas stimulation triggers ATM kinase activation. However, our data strongly suggest that ATM activation upon Fas stimulation occurs when the apoptotic signaling is already irreversible, as a consequence of DNA condensation and fragmentation during the apoptotic response. Therefore ATM activation does not seem to play a major role in the sensitivity to Fas-induced apoptosis. This apparent paradox prompted us to investigate whether basal ATM kinase may modulate the level and/or the activity of any central player of Fas signaling. Fas sensitivity relies mainly on Fas

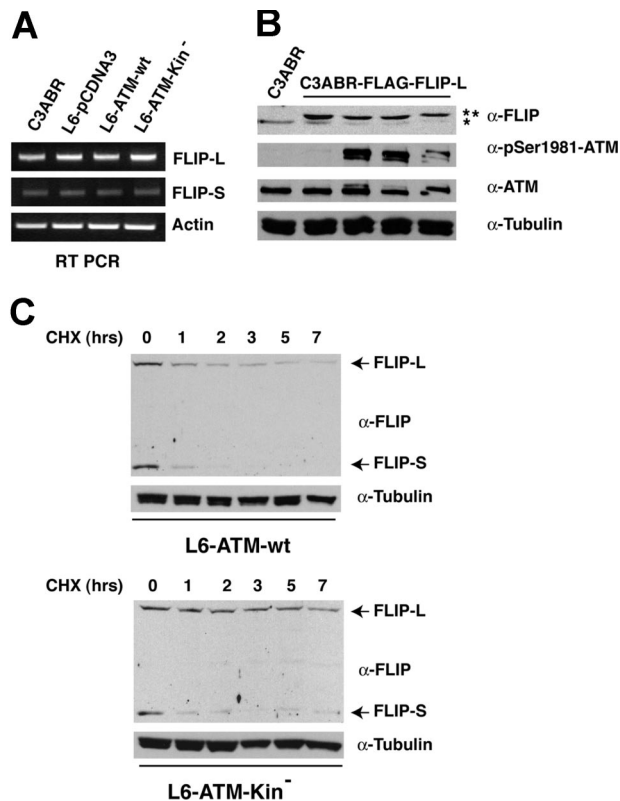


Figure 6. ATM kinase activity down-regulates FLIP protein stability. (A) RT-PCR analysis of FLIP-L and FLIP-S RNA expression levels was performed in the indicated cell lines. Amplified actin was used as an internal control. (B) C3ABR-FLAG-FLIP-L stably transfected cells were incubated with NCS for different times to trigger ATM kinase activation. Protein extract (80-100 μ g) was separated by SDS-PAGE and transferred on nitrocellulose, and endogenous (*) and transfected FLIP (**) expression was revealed using specific anti-FLIP antibodies. (C) Cells were pretreated with KU-55933 O/N, washed, and then incubated with CHX for the indicated times.

receptor expression on cell surface and on caspase-8 activity, which is absolutely required to drive the caspase cascade and execute the apoptotic program. Importantly, ATM protein does not modulate Fas receptor or caspase-8 protein levels (Figure 3A,B). However, A-T cells are impaired in caspase-8 activation consistent with their resistance to Fas (Figure 3C,D). It has been clearly established that FLIP proteins may modulate caspase-8 activation *in vitro* and *in vivo* (reviewed in Peter¹³). Moreover FLIP level is tightly regulated during T- and B-cell activation, and its decrease parallels the enhancement of Fas sensitivity (reviewed in Thome and Tschopp⁴⁵). Importantly, we could show that A-T cells significantly accumulate FLIP proteins (Figure 4A). Reconstitution of ATM kinase activity down-regulates FLIP proteins. Conversely, a catalytically inactive ATM fails to down-regulate FLIP. Importantly, there is a strict relationship between FLIP levels and the sensitivity of the different cell lines to Fas-induced apoptosis. To further test the hypothesis that ATM kinase sensitizes cells to Fas-induced apoptosis through the down-regulation of FLIP proteins, we interfered with FLIP expression in A-T cells, by specific shRNA constructs. Following this approach, it was possible to down-regulate FLIP to the same levels observed in ATM-proficient cells, which, in turn, resulted in the restoration of Fas sensitivity in A-T cells (Figure 4B,C). Therefore we concluded that ATM kinase activity modulates Fas sensitivity through the regulation of FLIP protein levels.

The observation that FLIP levels decrease in A-T cells upon reconstitution with kinase-active ATM but not with a kinase-defective mutant (Figure 4A) suggests that a basal ATM kinase

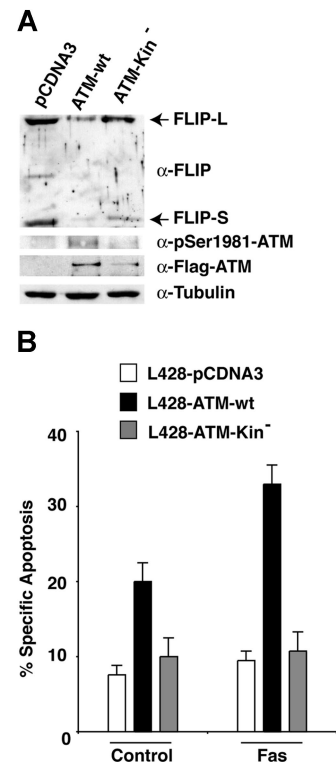


Figure 7. ATM kinase activity down-regulates FLIP protein levels and sensitizes Hodgkin lymphoma cell lines to Fas-induced apoptosis. (A) L428 HL cells were transiently transfected with the indicated constructs along with GFP. Twenty-four hours after transfection, GFP-positive cells were isolated by FACS sorting, and incubated for an additional 12 hours. For immunoblotting, 80 to 100 μ g protein extract were separated by SDS-PAGE and transferred on nitrocellulose. The proteins of interest and their phosphorylation were revealed by immunoblotting with specific antibodies. (B) L428 HL cells were transiently transfected with the indicated constructs along with GFP. Twenty-four hours after transfection, cells were stimulated to undergo apoptosis with 250 ng/mL anti-Fas mAb. Apoptosis was determined by the analysis of annexin V binding 24 hours after anti-Fas treatment, upon FACS sorting of GFP-positive cells.

activity may be sufficient to down-regulate FLIP levels. The presence of an endogenous basal ATM activity, which may be further induced upon DNA damage, has been already described.^{46,47} Consistent with the presence of such a basal activity, NCS treatment, which triggers ATM activation, down-regulates FLIP in the presence of a kinase-competent ATM protein (Figure 5A). Conversely, the treatment of ATM-proficient cells with the ATM kinase inhibitor KU-55933 triggers FLIP up-regulation (Figure 5C). These data allow us to propose that ATM kinase activity modulates FLIP protein levels. Consistently, while the decrease of FLIP levels following NCS treatment sensitizes cells to Fas-induced apoptosis (Figure 5B), the up-regulation of FLIP levels after 8 hours of preincubation with KU-55933 protects cells from Fas-induced apoptosis (Figure 5D). Overall, we provide evidence for A-T cell resistance to Fas-induced apoptosis and we demonstrate that ATM kinase activity may modulate Fas sensitivity through the regulation of FLIP proteins level. Moreover, we could show that ATM modulates FLIP protein stability (Figure 6). Further experiments will clarify the molecular mechanism beyond this regulation.

Overall, these findings point to the up-regulation of FLIP protein levels as a putative novel marker of A-T cell lines. We are currently investigating the levels of FLIP protein in heterozygous-derived A-T cell lines. This study along with further experiments on peripheral blood cells from A-T patients will address the

question whether FLIP up-regulation could be used as a novel A-T prognostic marker.

Importantly, A-T patients have an increased rate of lymphoma and leukemia development, with a frequent occurrence of B-cell lymphomas such as Hodgkin lymphomas.^{4,5} Interestingly, several independent studies on HL reported the aberrant down-regulation of ATM activity as a common event, thus suggesting that ATM loss may promote HL development.^{7,9} Furthermore it has been clearly shown that HLs are very resistant to Fas- and TRAIL-induced apoptosis and this correlates clearly with the aberrant up-regulation of FLIP levels. Indeed the down-regulation of FLIP is sufficient to sensitize back these cells to death receptor-induced apoptosis.^{26,27} It has been reported that NF κ B transcription factor up-regulates FLIP expression.⁴⁸ Interestingly, the transcription factors NF κ B and API are aberrantly activated in HL and have been proposed to be responsible for the modulation of the levels of most of the proteins aberrantly expressed in HL.⁴⁹⁻⁵¹ Indeed, repression of NF κ B activity triggers FLIP protein down-regulation in HSR cells.²⁷ We have shown that ATM kinase activity modulates FLIP protein levels. To test whether the lack of ATM kinase activity in HSR cells may contribute to FLIP down-regulation, we restored ATM activity in L428 cells, an HL-derived cell line previously characterized for aberrantly low ATM function²⁹ and for aberrantly high FLIP levels.^{26,27} Using this approach, we could show that ATM activity is sufficient to decrease FLIP levels and to sensitize L428 cells to Fas-induced apoptosis (Figure 7). This finding, along with the data in the literature on ATM deficiency in HL,^{7,9} allows us to speculate that A-T deficiency could also contribute to lymphoma development via the loss of control on FLIP levels that in turn triggers Fas resistance.

In summary, we identified a novel function for ATM kinase as a regulator of FLIP levels and of Fas sensitivity and suggested that this signaling may contribute to the homeostasis of the immune system. It is also tempting to speculate that failure of the ATM-dependent FLIP regulation might be at least partially responsible for the increased frequency of lymphomas associated with A-T, as well as for the development of lymphoma in those situations where ATM kinase activity is down-regulated through alternative mechanisms other than homozygous deletion. Furthermore, the induction of ATM activation may provide a novel tool to down-regulate FLIP protein levels and to sensitize those lymphomas where endogenous ATM is still functional, to death receptor-induced apoptosis. Importantly, treatment of tumor cells with DNA-damaging drugs such as 5-fluorouracil (5-FU) has been shown to down-regulate c-FLIP and thereby to sensitize cells to

death receptor-induced apoptosis.³⁰ We observed that ATM kinase activity is required for this effect (data not shown), suggesting that indeed this mechanism might be diagnostically and therapeutically relevant.

Finally, we provide novel evidence for a basal endogenous activity of ATM kinase independent of the exogenous DNA-damage induction, which probably accounts for differences in the level of expression of FLIP protein. This basal activity of ATM could be relevant also in other cellular processes and contribute, at least in part, to the complexity of A-T phenotype. Therefore, investigations comparing different structural and functional features of wt and A-T cells in the absence of DNA damage may strongly contribute to broadening current knowledge on ATM kinase function and A-T pathology.

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Authorship

Contribution: V.S. performed the research and analyzed the data; M.G.B. performed the research; S.C. generated the shFLIP cell lines; I.C. supervised the work with the ATM-reconstituted cell lines; M.T.C. assisted with the flow cytometry and apoptosis analysis; R.T. and Y.L. analyzed the data; E.C. wrote the paper; D.B. designed the research and wrote the paper.

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