Targeting the Ataxia Telangiectasia Mutated-null phenotype in chronic lymphocytic leukemia with pro-oxidants

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ABSTRACT

Inactivation of the Ataxia Telangiectasia Mutated gene in chronic lymphocytic leukemia results in resistance to p53-dependent apoptosis and inferior responses to treatment with DNA damaging agents. Hence, p53-independent strategies are required to target Ataxia Telangiectasia Mutated-deficient chronic lymphocytic leukemia. As Ataxia Telangiectasia Mutated has been implicated in redox homeostasis, we investigated the effect of the Ataxia Telangiectasia Mutated-null chronic lymphocytic leukemia genotype on cellular responses to oxidative stress with a view to therapeutic targeting. We found that in comparison to Ataxia Telangiectasia Mutated-wild type chronic lymphocytic leukemia, pro-oxidant treatment of Ataxia Telangiectasia Mutated-null cells led to reduced binding of NF-E2 p45-related factor-2 to antioxidant response elements and thus decreased expression of target genes. Furthermore, Ataxia Telangiectasia Mutated-null chronic lymphocytic leukemia cells contained lower levels of antioxidants and elevated mitochondrial reactive oxygen species. Consequently, Ataxia Telangiectasia Mutated-null chronic lymphocytic leukemia, but not tumors with 11q deletion or *TP53* mutations, exhibited differentially increased sensitivity to pro-oxidants both *in vitro* and *in vivo*. We found that cell death was mediated by a p53- and caspase-independent mechanism associated with apoptosis inducing factor activity. Together, these data suggest that defective redox-homeostasis represents an attractive therapeutic target for Ataxia Telangiectasia Mutated-null chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) with defective DNA damage response (DDR) is refractory to conventional chemotherapeutics.[1] The loss of DDR occurs through inactivation of *ATM* and *TP53* genes.[2] *ATM* mutations appear in 13%-16% of CLLs and are distributed across a large coding region encompassing 64 exons.[3,4] The loss of ATM function typically occurs through the combined effect of 11q deletion (monoalleic *ATM* loss) and an *ATM* mutation, biallelic *ATM* mutations, or less frequently due to the presence of a single mutation, capable of exerting dominant-negative effect on the remaining *ATM* allele.[3,4] Compared to CLL tumors with 11q deletion only, those with inactivation of both ATM alleles exhibit abrogated DNA damage-induced apoptotic responses *in vitro* and rapid clonal expansion *in vivo*, leading to reduced overall and treatment-free survival.[1] Furthermore, in the phase III UK CLL4 trial that compared chlorambucil with fludarabine either alone or in combination with cyclophosphamide, CLL patients with biallelic *ATM* inactivation revealed progression-free survival inferior to tumors with a single mutation or 11q deletion, which was second only to tumors with loss/mutation of both *TP53* alleles.[5] Early cytogenetic studies showed that CLL progression is associated with the emergence of 11q deleted subclones.[6] More recent reports of the dynamic nature of clonal progression has led to the understanding that the selective pressure imparted by DNA damaging agents favors the expansion of pre-existing subclones with defective DDR and consequently successive rounds of treatment with these agents are less effective.[7,8] There is, therefore, a need for therapeutic strategies that act independently of the DDR pathway that can target ATM-null CLL cells.

ATM is a serine/threonine protein kinase activated by DNA double strand breaks (DSBs) that co-ordinates the activation of cell cycle checkpoints, DNA repair and p53-dependent apoptosis.[9] ATM phosphorylates numerous substrates and is involved in the regulation of a wide range of cellular processes. Consequently, deficiencies in any of these processes caused by the loss of ATM could be used as therapeutic targets.[10,11] Consistent with this notion, ATM-null CLL cells are defective in homologous recombination repair (HRR), a deficiency that can be exploited to induce tumor-specific killing via enhanced requirement for HRR upon PARP-inhibition.[11]

ATM is also implicated in redox homeostasis.[12] Deregulation of redox homeostasis results in oxidative stress and occurs either due to increased reactive oxygen species

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(ROS) production or reduced antioxidant capacity. A principle antioxidant pathway is regulated by the redox sensitive transcription factor, NF-E2 p45-related factor-2 (NRF2/NFE2L2). In unstressed cells, low levels of NRF2 are maintained through interaction with Kelch-like ECH-associated protein 1 (KEAP1), an adapter for the E3 ubiquitin ligase Cullin 3 (CUL3) that directs NRF2 for proteasomal degradation.[13] Modification of redox and electrophile sensitive cysteine residues inhibits the substrate adaptor activity of KEAP1 allowing NRF2 accumulation. The interaction of KEAP1 with NRF2 is further modulated by phosphorylation of NRF2 at serine 40 by protein kinase C (PKC).[14] Within the nucleus, NRF2 forms heterodimers with v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF) proteins (MAF-F, G and K) and binds to antioxidant response elements (AREs) in the promoters of target genes such as those required for glutathione synthesis and its own promoter.[15] This activity is regulated by the transcriptional repressor BTB and CNC homology 1 transcription factor (BACH1) which competes for the MAF binding partners and for binding to gene promoters.[16] In addition, genetic models suggest that binding of Nrf-2 and its activity are regulated by the ATM substrate, Brca1.[17]

The most compelling evidence for the role of ATM in redox homeostasis comes from studies of the human radiosensitivity syndrome, Ataxia Telangiectasia (A-T), where both *ATM* alleles are inactivated. Cells from these patients display increased oxidative stress as a consequence of elevated levels of ROS, increased oxidized/reduced glutathione (GSSG/GSH) ratio, diminished capacity to scavenge ROS and mitochondrial dysfunction.[12,18] Furthermore, ATM is directly activated by oxidative stress[19] and can exert antioxidant activity through regulation of the pentose-phosphate pathway.[20] Recent evidence suggests that ATM might regulate oxidative stress through NRF2. Namely, Nrf2 target gene expression was decreased in Atm-/- murine osteoblasts and this was rescued by the ectopic expression of PKC delta (PKCδ).[21]

In this study, we tested the hypothesis that ATM-null CLLs have an intrinsic defect in their antioxidant defenses that might be exploited to induce synthetic lethality of tumor cells by escalating oxidative stress. We show that compared to ATM-wild type (wt) CLL, ATM-null CLL tumors exhibited defective NRF2-dependent antioxidant transcriptional responses, decreased antioxidant capacity, elevated mitochondrial ROS, and increased sensitivity to pro-oxidants both *in vitro* and *in vivo*. Furthermore, we demonstrate that pro-oxidant treatment bypassed the need for a functional DRR and induced cell death via a p53- and caspase-independent mechanism involving apoptosis inducing factor (AIF).

Methods

Patients' samples and cell lines

Chronic lymphocytic leukemia samples were obtained from Birmingham and Bournemouth Hospitals (*Online Supplementary Table S1*). These were comprised of 3 CLLs with monoallelic *ATM* loss, 1 monoallelic ATM mutant, 3 biallelic *ATM* mutants, 5 with combined *ATM* mutation/deletion and 3 CLLs with *TP53* mutations. All patients' samples contained more than 90% tumor cells. South Birmingham Ethics Committee granted approval for the study. CII, HG3 and PGA isogenic CLL cell lines expressing short hairpin (sh)-RNA against GFP or ATM were generated as previously described.[11]

Chemicals

H_2O_2, tert-butylhydroquinone (tBHQ), *N*-acetyl-*p*-benzoquinone imine (NAPQI) and N-phenylmaleimide were purchased from Sigma-Aldrich (MO, USA), KU-55933 from Merck (KGaA, Darmstadt, Germany) and Z-VAD-FMK from EnzoLife Sciences (Exeter, UK). Parthenolide, dimethylamino parthenolide (DMAPT) and DMAPT-hydrochloride (DMAPT-HCl) were isolated and prepared as described (*Online Supplementary Appendix, Online Supplementary Table S2, and Online Supplementary Figures S1, S2 and S3*).

Quantitative real-time PCR

SYBR-Green quantitative real-time PCR (Q-PCR) (Life Technologies) was applied with primers against *NRF2, NQO1, GCLM, GSR* and *HMOX1* (*Online Supplementary Appendix*). Primers against β-ACTIN were used for normalization and quantification was achieved using the comparative Ct method.[22]

XChIP

XChIP was applied to primary CLL samples before and after treatment with 100 μM tBHQ for 6 h using anti-NRF2 and pre-immune antisera as previously described[23] (*Online Supplementary Appendix*).

Biochemical assays

GSH was quantified using a Glutathione Assay Kit (Sigma-Aldrich). To determine GSSG, GSH was first derivatized with N-ethylmaleimide (Sigma-Aldrich). Levels of NADPH and $NADP^+$ were quantified using an NADP/NADPH assay kit (Abcam, Cambridge, UK).

Mitochondrial ROS assay

Mitochondrial superoxide was detected using MitoSox Red (Life Technologies) in accordance with the manufacturer's instructions and quantified using an LSR II flow cytometer (BD Biosciences, Oxford, UK) (*Online Supplementary Appendix*).

RNA knockdown

Knockdown of gene expression in HaCat cells was achieved by transfection of siRNAs against ATM, KEAP1, BRCA1 or Scrambled siRNA with Oligofectamine (Life Technologies) in accordance with the manufacturer's instructions (*Online Supplementary Appendix and Online Supplementary Table S4*).

Immunoblotting

Immunoblotting was performed as previously described[24] (*Online Supplementary Appendix*).

Murine xenograft

Animals were treated in accordance with United Kingdom Home Office guidelines, Schedule 1. Subcutaneous tumors were initiated by injection of $5x10^6$ CII-isogenic cell lines with and without stable ATM-knockdown into 6-week old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. Tumors were grown for 15 days prior to treatment with 6 mg/kg parthenolide or vehicle via intra-peritoneal injection for 5 days.

Primary CLL tumor cells were engrafted as previously described.[25] Briefly, 6-week old NSG mice were sublethally irradiated (1.25 Gy) prior to intravenous co-injection of $50x10^6$ PBMC from an ATM-null CLL patient and $10x10^3$ $CD14^+$ mono-

cytes from a healthy donor. After three days, mice were randomized and treated with a daily dose of either 100 mg/kg DMAPT-HCl or vehicle by oral gavage for nine days. Splenic tumor burden was assessed by FACS analysis.

Statistical analysis

In vitro and *in vivo* data were analyzed using paired or unpaired 2-tailed Student's *t*-tests. Data are presented as ± standard error of the mean (SEM).

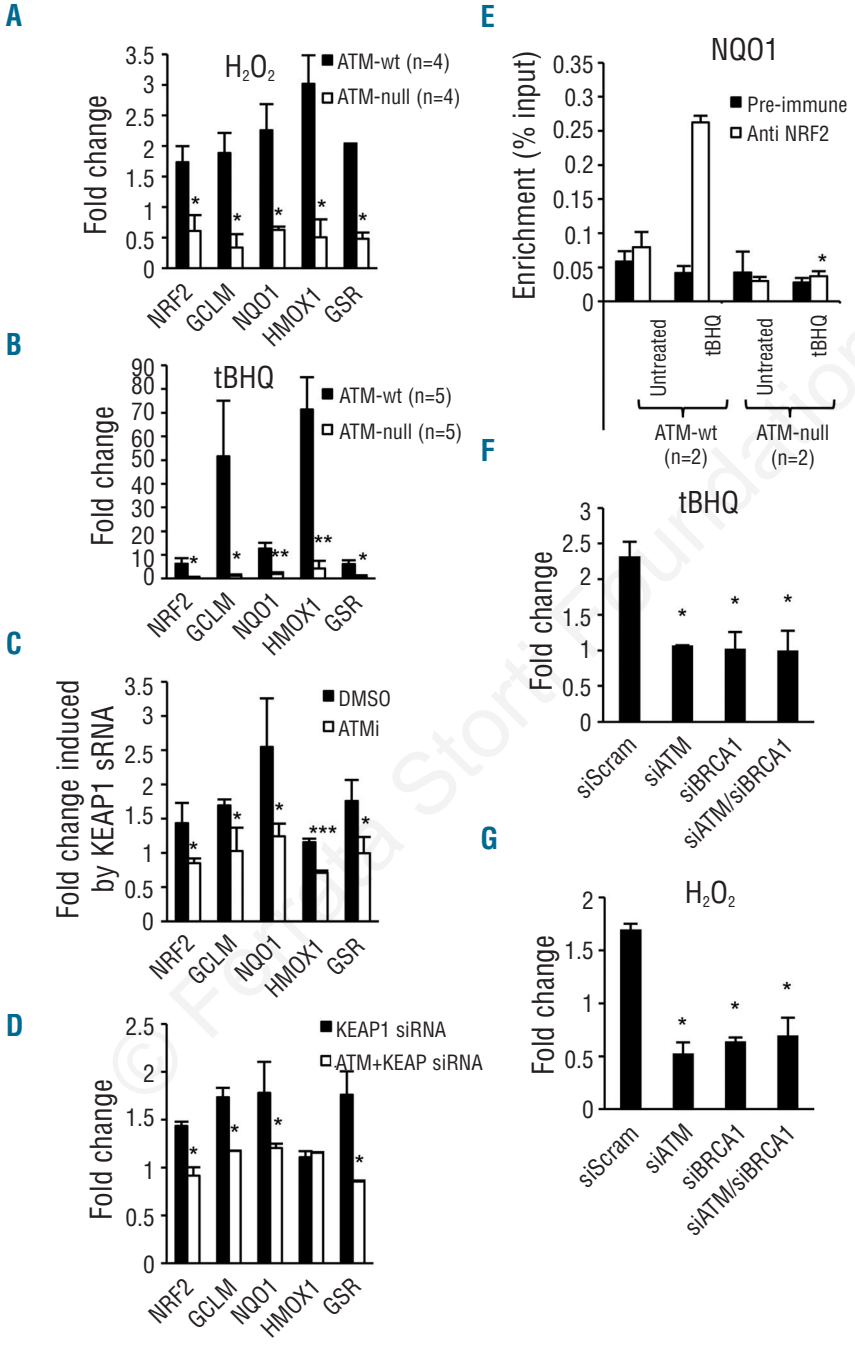

Figure 1. Induction of the NRF2 antioxidant response is defective in ATM-null CLL primary tumors. (**A**) Q-PCR showing differentially reduced induction of transcription of the NRF2-target genes (*NRF2, GCLM, NQO1, HMOX1* and *GSR*) in ATM-wt compared to ATM-null primary CLL samples following 6 h treatment with 100μM H_2O_2 and (**B**) 100μM tBHQ. (**C**) Q-PCR showing reduced induction of the NRF2 target genes in HaCaT cells following KEAP1-knockdown with and without ATM-knockdown (n=3) or (**D**) incubation of KEAP1- knockdown HaCaT cells with 10μM ATM kinase inhibitor KU-55933 (ATMi) (n=3). The inhibitor was added 48 h after transfection and incubated for 24 h. Data were normalized to β-ACTIN and expressed as fold-change relative to untreated cells using the comparative Ct method. (**E**) XChIP assay showing defective tBHQ-induced binding of NRF2 to ARE in the promoter of *NQO1* in ATM-null CLL cells compared to ATM-wt CLLs. XChIP was undertaken in accordance with the protocol described.[23] DNA was immunoprecipitated using anti-NRF2 antibody or pre-immune control. Enriched DNA was amplified using Q-PCR and data expressed as percentage of input. (**F**) Q-PCR showing the effect of ATM and BRCA1 knockdowns on tBHQ or (**G**) H_2O_2 induced expression of NRF2 target gene *NQO1* (n=3). Statistical significance was determined using Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant. Error bars represent SEM.

Results

Defective NRF2-regulated gene expression in ATM-null CLL cells

We have investigated the effect of ATM loss on NRF2 directed antioxidant responses by treating a panel of CLL tumors with H_2O_2 and measuring the induction of gene expression by Q-PCR. H_2O_2 induced a 1.7-3.0 fold increase in NRF2 target gene expression in ATM-wt tumors (n=4), whereas induction was significantly

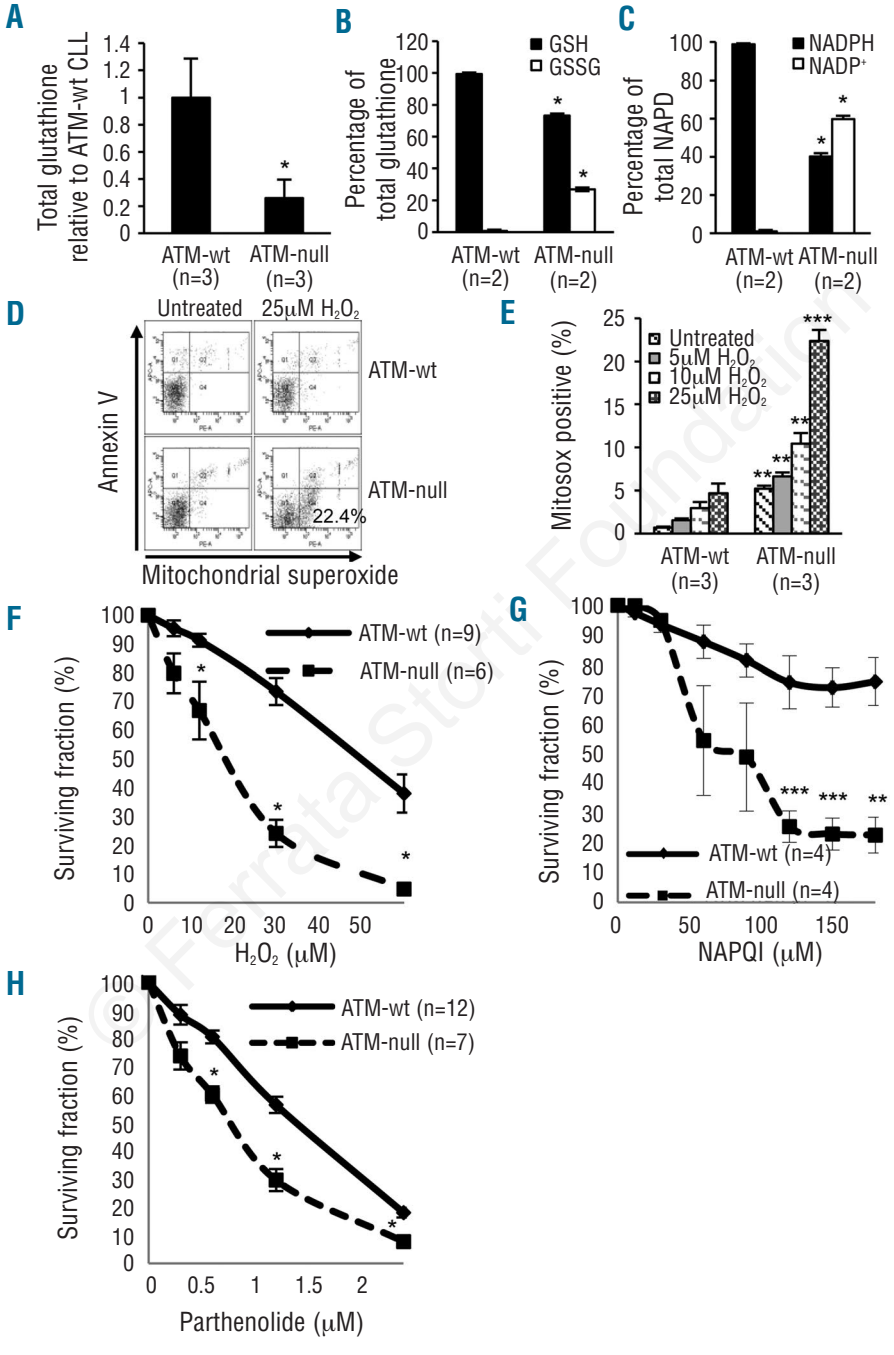

Figure 2. ATM-null CLL cells exhibit decreased antioxidant content, elevated levels of mitochondrial superoxide and differential sensitivity to pro-oxidant agents. (A) Total glutathione expressed relative to the levels in ATM wild-type cells are reduced in ATM-null primary CLL samples. (B) The ratios of GSSG:GSH and (C) NADP+:NADPH are differentially increased in ATM-null primary CLL tumors. (D) Mitochondrial superoxide measured by flow cytometry is differentially increased in ATM-null CLL tumors treated for 1 h with 0-25μM H_2O_2. Cells were dual stained with Annexin V-APC to permit exclusion of double-positive cells undergoing apoptosis. A representative dot plot is shown and (E) quantification is presented. ATM-null CLL primary tumors show differential sensitivity following treatment with (F) H_2O_2, (G) NAPQI or (H) parthenolide for 24 h. The surviving fraction was determined by flow cytometry (Beckman Coulter MCL-Epics flow cytometer) with Annexin V-FITC/propidium iodide labeling. The statistical significance was determined using Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 were considered significant. Error bars represent SEM.

impaired in ATM-null CLLs (n=4) (Figure 1A). Examination of previously generated expression data supported this observation.[26] Following exposure to IR, an insult that generates ROS, upregulation of 40 NRF2 regulated transcripts was significantly (P<0.05) impaired in ATM-null CLLs (n=6) compared to ATM-wt CLLs (n=5) (*Online Supplementary Figure S4A*).

To investigate a possible role for KEAP1 in the defective induction of NRF2 target genes in ATM-null CLL, KEAP1 activity was inhibited pharmacologically or by siRNA knockdown. Tumor cells were treated with tBHQ, the quinone radical of which is an electrophile that covalently modifies cysteine residues in KEAP1 preventing it from targeting NRF2 for degradation. In ATM-wt tumors (n=5), high-dose tBHQ (100 μM) induced a 5-71 fold upregulation of NRF2 target genes (Figure 1B). In comparison, significantly lower expression (0.5-4.3 fold) was induced in ATM-null tumors (n=5). A similar differential was observed with low-dose tBHQ (10 μM) indicating that the defective expression in ATM-null CLLs was not due to toxicity (*Online Supplementary Figure S4B*).

In accordance with a previous report, knockdown of KEAP1 also induced the expression of NRF2 target genes.[27] Inhibition of ATM function by siRNA-knockdown (n=3) (Figure 1C) or ATM-inhibitor (n=3) (Figure 1D) abolished induction of NRF2-target genes. These data indicated that the defective regulation of NRF2 transcripts in ATM deficient cells occurs downstream of KEAP1 function.

Consistent with an absence of a defect in the regulation of NRF2 by KEAP1 the expression of NRF2 mRNA and protein was comparable in ATM-wt and ATM-null tumors (*Online Supplementary Figure S4C and D*). Following treatment with tBHQ, no difference was observed between ATM-wt and ATM-null CLLs regarding the change in levels of NRF2 or other proteins involved in this antioxidant pathway: KEAP1, BACH1 and MAF protein (*Online Supplementary Figure S4D-F*).

A previous study suggested that ATM regulates NRF2 through PKCδ.[21] However, in keeping with the data from a proteomic study of ATM substrates,[10] we found no interaction between ATM and NRF2 in co-immunoprecipitation assay (*Online Supplementary Figure S5A*). Furthermore, we did not detect an interaction between ATM and PKCδ and loss of ATM did not alter the levels of PKCδ or the levels of phosphorylated-serine on NRF2 (*Online Supplementary Figure S5B and C*).

Brca1 was previously found to regulate Nrf-2 transcriptional activity.[17] Therefore, we next investigated the possibility that ATM co-operates with BRCA1 in the regulation of NRF2 activity. We found reduced induction of antioxidant genes in cells treated with either ATM or BRCA1-specific siRNAs (Figure 1F and G). This defect was not increased by the combined knockdown of both genes, suggesting ATM and BRCA1 may act in the same pathway that regulates NRF2 function.

These data suggest that in CLL cells, loss of ATM-function induces a defect in NRF2 regulated gene expression that is independent of PKCδ, KEAP1, BACH1 and MAF proteins and may involve co-operation with BRCA1.

ATM-loss reduces binding of NRF2 to antioxidant response elements

In the absence of any defect in the regulation of NRF2 protein levels, we considered whether the transcriptional deregulation in ATM-null CLL tumors arises at the point of NRF2 binding to AREs. We used XChIP and Q-PCR to measure tBHQ-induced binding of NRF2 to an ARE in the promoter region of the prototypic NRF2 target gene *NQO1*. Consistent with the observed difference in gene

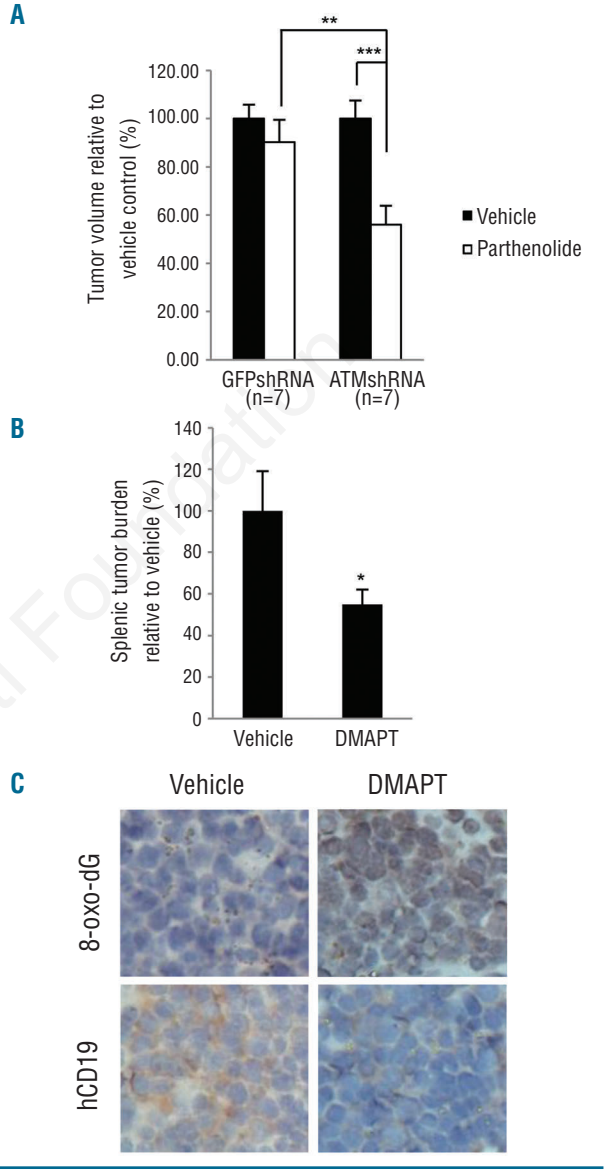

Figure 3. ATM-null cells are targeted by pro-oxidants *in vivo*. (A) Subcutaneous xenografts of ATMshRNA (n=7) and GFPshRNA (n=7) expressing CII-isogeneic cell lines were treated with 6 mg/kg parthenolide or vehicle by intraperitoneal injection for five days leading to a significant reduction in tumor volume. Tumor volume was calculated using the formula Vol = 0.5 x L x W^2 before and after treatment. (B) Xenografts of ATM-null primary chronic lymphocytic leukemia were established and treated by oral gavage with vehicle (n=5) or 100 mg/kg dimethylaminoparthenolide (DMAPT) (n=5) for nine days, leading to a significant reduced splenic tumor burden in DMAPT-treated animals, as determined by flow cytometry using antibodies against hCD19 (cl.HIB19), hCD3 (cl.SK7), hCD45 (cl.2D1) and mCD45 (cl.30-F11) (eBioscience Inc., San Diego, CA, USA) and CountBright absolute counting beads (Life Technologies). (C) Histological sections depicting differentially increased splenic 8-oxo-dG expression in DMAPT treated xenografts. Brown labeling indicates immobilization of anti-hCD19 (eBioscience) and anti-8-oxo-dG (Abcam) antibodies. Nuclei were counterstained blue with Hematocylin and images were captured at 200x magnification. Statistical significance was determined using Student's *t*-test. *P<0.05 was considered significant. Error bars represent SEM.

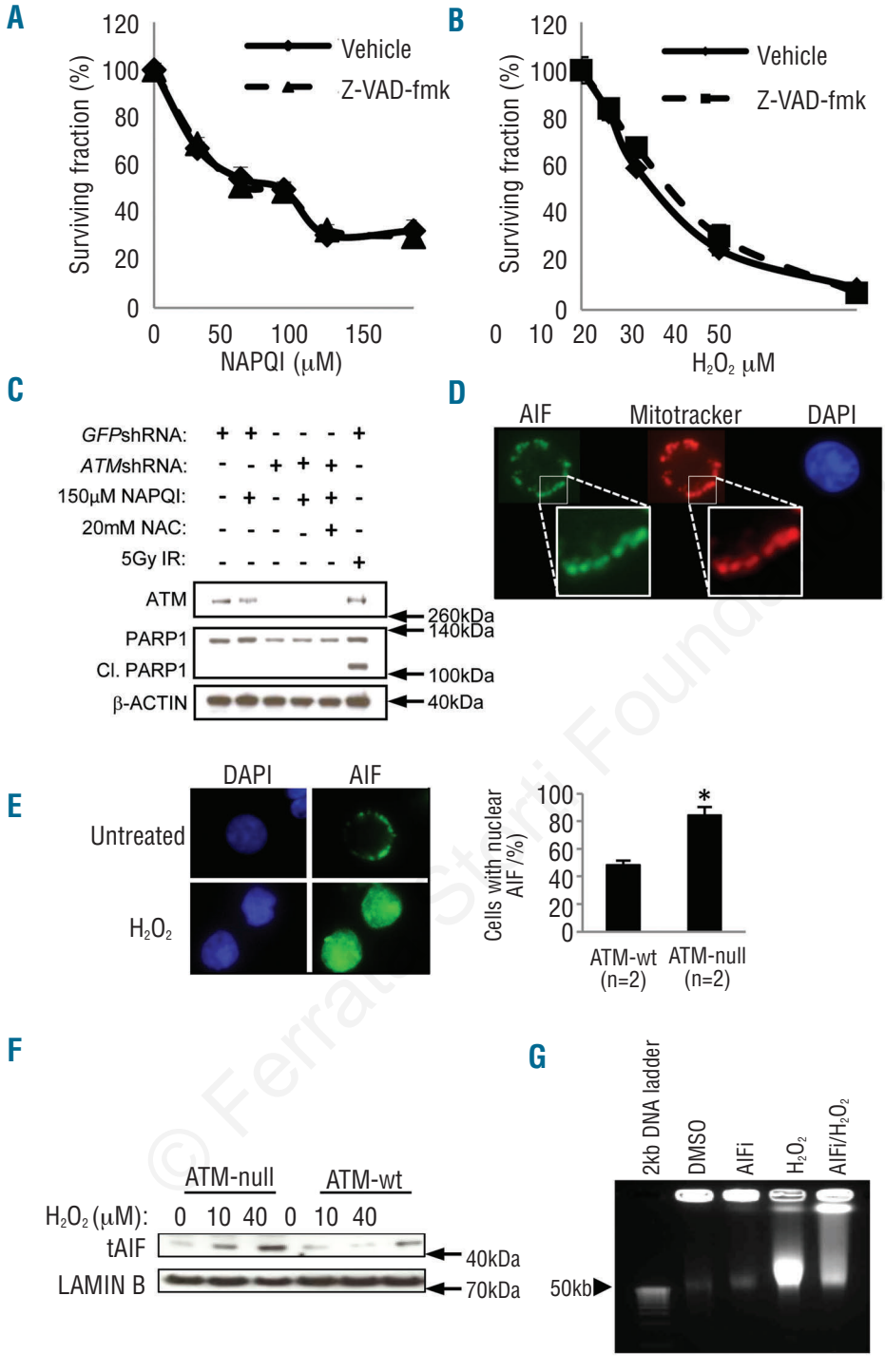

Figure 4. Pro-oxidant treatment induces caspase-independent, AIF-dependent apoptosis. Representative ATM-null primary CLL tumors were treated with (A) NAPQI or (B) H_2O_2 for 24 h in the presence or absence of 20 μM pan-caspase inhibitor (Z-VAD-FMK). The effect on apoptosis was analyzed using Annexin-V/PI labeling and flow cytometry. (C) Isogenic CII CLL cell lines were treated with NAPQI with and without antioxidant (NAC) for 24 h or irradiated (IR) and PARP cleavage induction visualized by immunoblotting. Anti-ATM (cl.11G12, Abcam), demonstrates ATM-knockdown and anti-ACTIN antibody (Sigma) was used as the loading control. (D) Immunofluorescence labeling shows colocalization of AIF (rabbit anti-AIF, Santa Cruz) (green) with mitochondria (MitotrackerRed, Life Technologies) (red). Nuclei were counterstained with DAPI (blue). (E) ATM-wt and ATM-null primary CLL cells were treated with 10 μM and 40 μM H_2O_2 for 6 h and labeled with anti-AIF antibody. Histogram shows quantification of cells with nuclear AIF. (F) Immunoblot of cellular fractions generated from cells treated with 10 μM or 40 μM H_2O_2 confirms increased H_2O_2-induced nuclear localization of AIF in ATM-null CLL. (G) The AIF-inhibitor, N-phenylmaleimide (50μM AIFi) reduced the generation of ~50kb DNA fragments in H_2O_2-treated primary CLL tumors. Agarose plugs containing cells treated as indicated were subjected to pulsed field gel electrophoresis as described[32] and the separated DNA was visualized with ethidium bromide. The statistical significance was determined using Student's *t*-test. *P<0.05 was considered significant. Error bars represent SEM.

induction, significantly less NRF2 bound to the promoter region of *NQO1* in ATM-null CLL compared to wild-type tumors (Figure 1E). This suggests that the reduced NRF2 dependent transcription in ATM-null primary CLL cells is caused by defective binding of NRF2 to AREs.

ATM-*null CLL cells exhibit reduced antioxidant capacity, elevated mitochondrial superoxide and increased sensitivity to pro-oxidants*

In agreement with the effect on antioxidant transcriptional responses, the level of total cellular glutathione was significantly lower in ATM-null compared to ATM-wt primary CLL (Figure 2A). This effect was recapitulated in three isogenic CLL cell lines with stable knockdown of ATM (*Online Supplementary Figure S6A*).

The regeneration of GSH from GSSG is catalyzed by glutathione reductase (GSR) utilizing NADPH as a co-factor. In ATM-wt tumors, virtually all glutathione was in the reduced form (GSH) whereas in ATM-null tumors only 70% was reduced and 30% was oxidized (GSSG), indicating increased oxidative stress in these cells (Figure 2B). Accordingly, the pool of $NADP^+$ was significantly elevated in ATM-null compared to ATM-wt CLLs (Figure 2C).

A-T cells display continuous oxidative stress due to intrinsic mitochondrial dysfunction and contain elevated superoxide levels.[28] To determine whether ATM-null CLL cells share this phenotype, the levels of mitochondrial ROS were examined using the mitochondrial superoxide sensitive dye, MitoSox Red. Consistent with observations in A-T cells, mitochondrial ROS levels were significantly higher in ATM-null (n=3) than wild-type CLLs (n=3) (P=0.007) (Figure 2D and E).

Increased mitochondrial ROS damages the organelle promoting further dysfunction in a positive-feedback loop.[28] To test whether this occurs in ATM-null CLLs, tumors were exposed to extracellular ROS. H_2O_2 induced significantly higher levels of mitochondrial superoxide in ATM-null CLLs (n=3) compared to wild-type CLLs (n=3) at all concentrations tested (Figure 2E). This suggests that the intrinsic mitochondrial dysfunction associated with ATM-deficiency can be further exacerbated using exogenous sources of ROS. Examination of mitochondrial DNA content showed that both ATM-null and ATM-wt CLL contained a comparable number of mitochondria but significantly more than PBMCs from normal donors (*Online Supplementary Figure S6B*) indicating that the increased mitochondrial ROS in ATM-null CLL is the result of mitochondrial dysfunction rather than mitochondrial number.

These data suggest that ATM-null CLL cells are under greater oxidative stress than their wild-type counterparts.

ATM-*null CLL cells show increased sensitivity to pro-oxidants* in vitro *and* in vivo

In view of the increased oxidative stress in ATM-null CLL cells, we determined if this translated to increased sensitivity to pro-oxidant treatment. ATM-null CLLs (n=6) were significantly more sensitive to H_2O_2 than ATM-wt CLLs (n=9) *in vitro* (Figure 2F). The effect of ATM-deficiency on H_2O_2-sensitivity was confirmed in a CII-isogenic cell line with stable knockdown of ATM (*Online Supplementary Figure S7A*).

Next, we addressed the sensitivity of ATM-null and wild-type primary CLLs to NAPQI and parthenolide, pro-oxidants that induce oxidative stress by depleting glutathione (GSH).[29,30] ATM-null tumors were significantly more sensitive than wild-type to both reagents (Figure 2G and 2H). The effects of H_2O_2, NAPQI and parthenolide were associated with oxidative damage, as treatment with N-acetyl cysteine (NAC), a glutathione precursor, protected cells (*Online Supplementary Figure S7B-D*). Importantly, the tumor-specific activity of both H_2O_2 and parthenolide was confirmed using PBMCs from normal donors (*Online Supplementary Figure S7E and F*).

To determine if the increased sensitivity to pro-oxidants was specific to the ATM-null status, we examined primary CLL cells with monoallelic 11q deletion or *TP53* mutations. The sensitivity of CLLs with either genotype did not significantly deviate from wild-type CLLs, suggesting that increased sensitivity to pro-oxidants is specifically associated with the ATM functional loss (*Online Supplementary Figure S7G and H*).

The *in vivo* efficacy of pro-oxidant therapy on ATM-deficient CLL was examined using two murine xenograft models. First, a subcutaneous xenograft model of CII-isogenic cell lines with and without stable ATM-knockdown was established. Parthenolide significantly inhibited the growth of ATM-knockdown tumors compared to either vehicle treated cells or to parthenolide treated wild-type tumors (44.0% *vs.* 9.7%) (Figure 3A).

Finally, an ATM-null primary CLL xenograft was established, and for systemic pro-oxidant treatment, dimethylamino parthenolide-hydrochloride (DMAPT-HCl), a water-soluble and orally bioavailable form of parthenolide,[31] was generated. Quantification of engrafted splenic CLL cells demonstrated that pro-oxidant therapy significantly reduced $hCD19^+$ tumor cell burden (P<0.05) (Figure 3B). Furthermore, engrafted cells displayed increased levels of 8-oxo-dG, indicating oxidative stress was induced following pro-oxidant treatment (Figure 3C).

These data show that pro-oxidant based therapies are effective against chemoresistant ATM-null CLL tumors *in vitro* and *in vivo*.

Pro-oxidant induced cell death in CLL is p53/caspase-independent and involves AIF

Since the DDR is defective in ATM-null CLL, a DDR-independent pathway must be operating to facilitate pro-oxidant induced cell death. Consistent with this, activation of the DDR was not detected in either ATM-null or wild-type CLL cells following treatment with therapeutically effective concentrations of H_2O_2 (*Online Supplementary Figure S8*), NAPQI or parthenolide (*data not shown*). To elucidate the mechanism of cell death induced by pro-oxidants, ATM-null primary CLL tumors were treated with NAPQI or H_2O_2 with and without pan-caspase inhibitor. Caspase inhibition did not significantly affect NAPQI or H_2O_2-induced cell death of ATM-null primary CLL tumors (Figure 4A and B). This was confirmed by immunoblotting in CII-isogenic cell lines which showed that, in contrast to ionising radiation, PARP1-cleavage was not induced by NAPQI (Figure 4C).

Next, we investigated the induction of cell death by an alternative mechanism involving the cellular redistribution of AIF. In response to stress, AIF is released from the mitochondria as a cleaved 57kDa pro-apoptotic protein (tAIF) that translocates to the nucleus where it co-operates with endonuclease G to cause large-scale DNA fragmentation and chromatin condensation.[33] In untreated cells, AIF staining co-localized with mitochondria (Figure

4D), whereas treatment with H_2O_2 led to nuclear translocation (Figure 4E and F). In agreement with the differential sensitivity of ATM-null CLL tumors to pro-oxidants, nuclear tAIF was induced with 10 μM H_2O_2, whereas in ATM-wt CLLs, 40 μM H_2O_2 was required to elicit the same effect (Figure 4F). Inhibition of AIF prevented H_2O_2-induced digestion of genomic DNA into ~50kb fragments, thus confirming the role of AIF in this process (Figure 4G).

These data demonstrate that pro-oxidants induce p53 and caspase-independent cell death in wild-type and ATM-null CLL associated with nuclear translocation of AIF.

Discussion

We show that the ATM-null phenotype in CLL can be targeted with pro-oxidant based therapies to induce selective killing. We demonstrate that a defect in the NRF2-directed antioxidant response is present in ATM-null primary CLLs. This correlated with reduced antioxidant capacity, increased mitochondrial ROS and increased sensitivity to pro-oxidants *in vitro* and *in vivo*. Previous reports have demonstrated increased sensitivity of CLL cells to pro-oxidant based therapies compared to non-tumor cells, but this is the first study to demonstrate that this approach specifically targets the ATM-null phenotype in CLL.[34,35]

Our data suggest that binding of NRF2 to target gene AREs is reduced in ATM-null CLL despite normal levels of NRF2, KEAP1, BACH1 and MAF proteins and normal levels of NRF2/MAF heterodimerization. Previous studies indicated that reduced PKCδ levels might contribute to defective Nrf-2 regulation in Atm-/- mice and that NRF2 stability is regulated by phosphorylation at ser40 by PKC.[14,21] We observed that PKCδ and serine-phosphorylated NRF2 levels are unaffected by the loss of ATM in CLL cells and that in agreement with previous proteomic screen,[10] both PKCδ and NRF2 are unlikely to be ATM substrates. In search for the mechanism of reduced NRF2 activity in ATM null cells, we confirmed the role of BRCA1 in the regulation of the NRF2 antioxidant response. Furthermore, by showing that the combined knockdown of ATM and BRCA1 does not cause further deregulation, we demonstrate that ATM and BRCA1 function in the same pathway to regulate NRF2-antioxidant responses. Previously, we have reported that ATM-null tumors are refractory to conventional DNA damaging therapies due to inactivation of the p53-dependent apoptosis pathway,[1,36] underscoring the need for a p53-independent strategy for the treatment of these tumors. In this study, we found that ATM-null cells are differentially sensitive to pro-oxidants as reflected by the appearance of tAIF in the nuclei of these cells at lower concentrations of H_2O_2 than in ATM-wt cells. The higher level of mitochondrial superoxide in ATM-null CLLs indicated mitochondrial dysfunction. Pro-oxidant treatment is likely to further damage the mitochondria, thus triggering the translocation of AIF and p53-independent cell death. Recent studies also suggest AIF translocation may occur due to an increase in inducible nitric oxide synthase (iNOS) activity following stimulation of JNK by ROS and the subsequent activation of ERK1/2.[37] In addition, ROS can induce the caspase-independent activation of the BH3 interacting domain death agonist (BID), thus leading to AIF translocation.[38]

Most importantly, using a pro-oxidant based strategy, we show that ATM-null CLL cells are significantly more sensitive than ATM-wt tumors and non-tumor cells to agents previously shown to stimulate NRF2-mediated adaptation to stress.[39,40] Thus both NAPQI and parthenolide represent novel clinically applicable approaches for the treatment of ATM-null CLL. Of note, acetaminophen, the NAPQI precursor, has previously been used in a phase I trial for the treatment of metastatic melanoma[41] as an approach to enhance the specificity of anti-cancer agents which deplete glutathione. Similarly, parthenolide and its water soluble derivative, DMAPT, have been shown to have activity against hematopoietic malignancies, including chemo-refractory CLL and acute myeloid leukemia.[42,43]

Targeting oxidative stress to bypass the defect in p53-dependent apoptosis is an attractive therapeutic strategy for several reasons. First, genetic variations present a challenging problem with respect to the treatment of CLL[8,44] and elevated oxidative stress can impact on disease progression by inducing oxidative DNA damage[45] and increasing the mutation rate that supports clonal diversity. Indeed, in CLL, oxidative stress is present early in the genesis of disease and is detectable at the stage of pre-malignant monoclonal B-lymphocytosis.[46] Thus to avoid the selection of adverse subclones with ATM-deficiency that occurs with conventional genotoxic agents,[7,8] it is preferable to target stress phenotypes common to all tumor cells and, as in this case, potential mechanisms of diversification.[47] Furthermore, elevated oxidative stress may provide a selective advantage for ATM-null CLL cells regarding their interactions with immune cells within lymphoid tissues. The activity of surrounding immune cells is suppressed by ROS and, therefore, loss of ATM and the associated increase in ROS production may facilitate immune evasion.[48] Finally, loss of ATM and the associated decrease in antioxidant levels may render the tumor cells more dependent on stromal cells for redox support,[49] thus providing a rationale for combined therapeutic approaches with pro-oxidants and inhibitors of tumor/microenvironment interactions.

Recently a number of p53-indendent treatments for CLL became available. These include use of immunomodulatory agents as well as inhibitors of B-cell receptor signaling that target either Bruton tyrosine kinase (ibrutininb) or PI3 kinase delta (idelalisib).[50] Pro-oxidant based therapies utilize oxidative stress as a cellular weakness of leukemic cells and represent an example of a synthetic lethality approach. It will be of interest to determine whether pro-oxidants could be used in combination with new targeted treatments to increase the benefit for patients with the ATM-null CLL phenotype.

Finally, it is important to note that the roles in DDR and oxidative stress responses denote two separate ATM functions. ATM is activated by oxidation of the cysteine residue at position 2991 in the FATC domain leading to formation of disulphide cross-linked dimers.[19] ATM mutated at C2991 is unresponsive to oxidative stress despite normal response to DNA damage. This raises the possibility that certain *ATM* mutations may not affect DDR and still render a phenotype amenable for targeting by pro-oxidants.

In summary, we show that the oxidative stress pheno-

type is a valid therapeutic target in the treatment of ATM-null CLL due to the intrinsic deficiencies in redox homeostasis. Significantly, this mode of therapy bypasses the DDR defect found in ATM-null CLL cells and therefore represents a feasible approach for treatment of patients that harbor these subclones.

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