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ORIGINAL ARTICLE

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Control of *Atm*−/− thymic lymphoma cell proliferation in vitro and in vivo by dexamethasone

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Abstract *Aim*: Ataxia telangiectasia (A-T) is an autosomal recessive disease in humans caused by mutations in the *Atm* (A-T mutated) gene. The disease involves multiple organ systems, and is associated with a high incidence of leukemias and lymphomas that develop in childhood. We have reported previously that thymic lymphoma development in *Atm* knockout (*Atm*−/−) mice is associated with elevated spontaneous DNA synthesis in thymocytes, and that dexamethasone (Dex) attenuates the elevated DNA synthesis and prevents thymic lymphoma development. The primary objectives of the present study were (1) to investigate possible mechanisms underlying the tumor-suppressing effect of Dex on *Atm*−/− thymic lymphoma cells, and (2) to determine whether Dex is an effective tumor-suppressing treatment in mice bearing transplanted *Atm*−/− thymic tumors. *Methods*: Establishment of a number of *Atm*−/− thymic lymphoma (ATL) cell lines from *Atm*−/− mice, cell proliferation assays, cell cycle analyses, Western blotting and Hoechst nuclear staining were used to analyze the effects of Dex on *Atm*−/− thymic lymphoma cells. *Atm*−/− tumor cells were transplanted into the right flanks of *Atm*+/+ mice prior to the initiation of Dex treatment. *Results*: *Atm*−/− tumor cells were highly sensitive to Dex, both in culture and in vivo as ectopic tumors in mice. In cultured ATL-1 cells, Dex induced apoptosis, arrested the cell cycle at the G_1 phase and downregulated NF-κB and multiple cell cycle regulators, while upregulating the NF-κB inhibitor IκBα. In *Atm*+/+ mice transplanted subcutaneously with ATL-1 cells, tumor growth was either prevented completely or significantly suppressed by Dex treatment. *Conclusions*: Our findings identify potential mechanisms by which Dex affects the proliferation and survival of ATL-1 cells in culture, and provide evidence that Dex can suppress the proliferation of *Atm*−/− thymic lymphoma cells growing in the body. Together these results add to our earlier published data suggesting that the cellular pathways regulated by Dex may be promising therapeutic targets for prevention and treatment of thymic lymphomas in A-T individuals.

Keywords *Atm* gene knockout mice · Lymphoid malignancy · Dexamethasone · Apoptosis · Cell cycle

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Introduction

Ataxia telangiectasia, or A-T, occurs in humans who carry two mutant autosomal recessive alleles at the Ataxia Telangiectasia Mutated (*Atm*) gene locus. The resulting systemic syndrome is characterized by progressive neuronal degeneration, oculocutaneous telangiectasia, immunodeficiency, premature aging, infertility, and an increased risk of cancers [1, 2]. As many as one-third of A-T patients developed tumors in childhood [3], and most of these are lymphoreticular tumors of T-cell origin.

In several laboratories it has been shown recently that inactivation of the *Atm* gene is also associated with sporadic lymphoid tumors in non-A-T patients, and that loss of heterozygosity at 11q22-23 (the location of *Atm* gene) is a common event in lymphoid malignancy [4–8]. These observations suggest that inactivation of ATM kinase might be an important cause of lymphoid neoplasms in both A-T and non-A-T patients [5].

Atm−/− mice recapitulate some of the features of human A-T, especially with respect to their hypersensitivity to oxidative stress, and in the occurrence of lethal thymic lymphomas by 4–5 months of age [9, 10]. Lymphoma cells isolated from *Atm*−/− mice have abnormal chromosomes, and rearrangements at the T-cell receptor α/δ locus on chromosome 14, with an average of four

additional chromosomal rearrangements per tumor [11]. A-T patients and *Atm*−/− mice both show retarded thymic development [12], and we have found that spontaneous DNA synthesis is markedly elevated in thymocytes from *Atm*−/− mice as compared with thymocytes from *Atm*+/+ littermates [12, 13]. This behavior by *Atm*−/− thymocytes, which reflects a cellular dysfunction involved in cell cycle checkpoints, suggests that the ATM kinase is required during normal T-cell development.

In thymocyte progenitors and T cells, GC treatment induces cell cycle arrest at the G_1 phase, and triggers apoptosis [14]. We have shown previously that the increased spontaneous DNA synthesis in *Atm*−/− thymocytes is rapidly inhibited by Dex in vitro [12], and that administration of Dex to *Atm*−/− mice prevents thymic lymphoma development in these animals [13]. We show here that tumor cells cultured from thymic lymphomas of *Atm*−/− mice retain immature T cell characteristics, including (1) the requirement for IL-2 in culture, (2) a $CD4^+CD8^+$ (double positive, DP) surface phenotype in most cases, and (3) a high sensitivity to Dex treatment, both in culture and after transplantation into *Atm*+/+ recipient mice.

The cell cycle regulators cyclin D1, cyclin E, cdk2, cdk4, cdk6, E2F1 and retinoblastoma (Rb) play important roles during the G_1/S transition, but no prior studies have addressed the mechanisms by which Dex affects cell cycle regulators in *Atm*−/− tumors. We report here that amounts of the cell cycle components cyclin D1, cyclin E, cdk2, cdk4, cdk6, E2F1 and phosphorylated retinoblastoma (pRb) were markedly decreased in Dex treated *Atm*−/− thymic lymphoma cells relative to untreated cells. Dex treatment also dramatically increased apoptosis in these cells. In addition, NF-κB, which regulates cell growth, was downregulated simultaneously with upregulation of levels of NF-κB inhibitor IκBα. These results show for the first time that Dex directly affects cell cycling and initiates apoptosis in *Atm*−/− thymic lymphoma cells. These effects of Dex were confirmed by our finding that growth of solid tumors from these cells, when transplanted into animals, was either prevented completely or significantly suppressed by Dex treatment of the recipients. Together with our previous studies [13], this work provides critical evidence that Dex may be useful as a therapeutic agent for thymic lymphomas in A-T individuals.

Materials and methods

Cells and cell culture

Atm−/− thymic lymphoma cells were isolated from *Atm*−/− mice at 3–4 months of age using routine cell isolation methods. Cells from 26 individual thymic lymphomas were cultured as separate cell lines in RPMI-1640 medium (Life Technologies, Grand Island, N.Y.), supplemented with 10% FBS and antibiotics. To support the proliferation of these cells in vitro, 5 ng/ml of interleukin-2 (IL-2, Sigma) was added to the cell culture medium. After three to five passages, single-cell suspensions from two of these lines (called ATL-1 and ATL-2) were prepared for in vitro study or for transplantation into animals. Cells of the murine thymic lymphoma cell line, EL-4 [15] (purchased from ATCC), and the murine thymic leukemia cell line, ASL-1 [16] (gift from Dr. Jacqueline Dudley), were cultured in RPMI-1640 medium, as described above, but without IL-2.

Flow cytometry analysis of cultured tumor cell lines

Single-cell suspensions of the ATL-1 lines were prepared in HBSS buffer and stained with anti-CD4 antibody conjugated with phycoerythrin (PE) and anti-CD8 antibody conjugated with fluorescein isothiocyanate (FITC) antibodies (PharMingen, San Diego, Calif.). Analysis was performed with a Coulter EPICS Elite flow cytometer (Beckman Coulter).

Cell proliferation assays with cultured tumor cell lines

Cells were cultured in 96-well plastic tissue culture plates at 5×10^4 cells/well at 37°C for 24 h. For the last 4 h of culture, 0.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, N.J.) was added per well. The cells were then harvested and [^{3}H]thymidine incorporation into DNA measured as described previously [12]. Results were expressed as mean ± SD counts per minute of triplicate cultures.

Cell cycle analysis with cultured tumor cell lines

Dex-treated or control cells were washed with cold phosphate-buffered saline (PBS) and the pellets (1.5×10^6 cells) were resuspended in 0.2 ml cold PBS. The cells were then fixed with 75% (v/v) cold ethanol overnight at −20°C. After centrifugation, the cells were resuspended in staining solution (PBS containing 50 μg/ml propidium iodide and 100 μg/ml RNaseA) and incubated for 30 min at 37°C. DNA content was then analyzed by flow cytometry, using Multicycle software (Phoenix Flow Systems, San Diego, Calif.) to quantify cell numbers at different stages of the cell cycle. Gates were set to differentiate G_0/G_1, S and G_2-M phases, with apoptotic cells (sub-G_1) appearing to the left of the G_0/G_1 peak. Cells appearing in the sub-G_1 peak were not counted in the quantitation of cells in the G_0/G_1, S and G_2-M phases [17].

Western blot analysis of proteins in cultured cell lines

Total cellular extracts were prepared using RIPA buffer (50 m*M* Tris-HCl, pH 7.5, 1% NP-40, 0.5% sodium

deoxycholate, 0.1% SDS, 0.25 m*M* PMSF, 1 m*M* sodium orthovanadate, 1 m*M* sodium fluoride, and 1 μg/ml aprotinin, leupeptin and pepstatin). Nuclear extracts were prepared as described previously [18]. The whole cell lysates and the nuclear lysates were cleared by centrifugation at 13,000 *g* for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad Dc Protein Assay Reagent (Bio-Rad Laboratories, Hercules, Calif.).

Lysates (50 μg total protein per sample) were separated on SDS-PAGE gels, transferred to PVDF membranes (Millipore Corporation, Bedford, Mass.) and immunoblotted with primary antibodies. Anti-NF-κB p65, IκBα, cyclin D1, cdk4, cdk6, Rb, E2F1, cyclin E and cdk2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-phospho-Rb (pRb, Ser807/811) was from Cell Signaling Technology (Beverly, Mass.). Antibody to activated caspase-3 was purchased from BioVision (Mountain View, Calif.). Secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK). Immune complexes were detected on the membranes using enhanced chemiluminescence reagents (NEN Life Science Products, Boston, Mass.) according to the manufacturer's instructions. A monoclonal anti-β-actin antibody (Sigma) was used as a control for protein loading.

DNA staining of tumor cell lines with Hoechst 33342

ATL-1 and ASL-1 cells were cultured with or without Dex for 24 h as described above. The cells were then fixed in 3.7% paraformaldehyde for 15 min and stained with 0.5 μg/ml Hoechst 33342 (Sigma) for 30 min at room temperature. Stained nuclei were viewed with an Olympus System microscope model BX40 (Olympus America, N.Y.) equipped with an Olympus MagnaFire camera and matching software.

Transplantation of ATL-1 cells into animals, and Dex treatment of the recipients

Atm−/− thymic lymphoma cells were cultured as described above. For transplantation, 10^6 ATL-1 cells were injected subcutaneously into the left flanks of 8-week-old or 4-week-old *Atm*+/+ mice (bred from *Atm*+/− parents purchased from the Jackson Laboratory Bar Harbor, Me.), respectively, in two separate experiments [19]. Intraperitoneal Dex injections were started at 4 or 9 days after tumor cell injection. The diameters of tumors developing at the injection site were measured every other day with calipers. All of the mice (Dex-untreated and Dex-treated *Atm*−/− tumor cell recipients) were killed at 22 days after transplantation, and the tumors were excised and weighed.

Results

Growth of *Atm*−/− thymic lymphoma cells in vitro and in vivo

In a recent study, Liyanage et al. compared cultured thymic lymphoma cells taken from thymic lymphomas of eight different *Atm*−/− mice, and found that all were DP in their surface phenotype [11]. In this study, we cultured cells from thymic lymphomas of 26 individual *Atm*−/− mice, and found that 24 of the 26 cell lines established from these tumors, or 92%, were DP, while 2, or 8%, were $CD8^+$ only, or single positive (SP; data not shown). These results suggest that most thymic lymphomas from *Atm*−/− animals are arrested in, or arise from, thymocytes at the DP stage, with the remaining minority of tumors originating from $CD8^+$ SP cells. With regard to the $CD8^+$ SP cells, an interesting possibility suggested from our previous work [13] is that the $CD8^+$ SP tumor cell lines represent tumors arising in the small, unique population of intermediate SP cells [20–22], which in *Atm*−/− mice may resemble DP cells in their sensitivity to malignant transformation [22].

It has been reported previously that *Atm*−/− thymic lymphoma cells maintain their immature status after isolation and placement into culture, and that these cells require IL-2 supplementation in order to survive in this context [11]. Our results are consistent with this observation, in that all 26 thymic lymphoma cell lines we isolated, including the two SP lines, required supplementation of their culture medium with IL-2 (data not shown).

Before beginning the studies reported here, we transplanted cells of the independently derived ATL-1 and ATL-2 cell lines (both DP, grown from separate thymic lymphomas in two different mice) into *Atm*+/+, *Atm*+/− and *Atm*−/− mice at 4–8 weeks of age. The results disclosed that cells from either line had grown into palpable tumor masses by 1 week after injection in recipients of all three genotypes (data not shown). We therefore selected ATL-1 cells to represent the DP cell lines from all of our 26 established cultures, and we used *Atm*+/+ mice as recipients, to exclude the confounding effects of *Atm* gene deletion in *Atm*+/− and *Atm*−/− mice.

ATL-1 cells are sensitive to Dex

We have reported previously that Dex inhibits DNA synthesis in freshly isolated thymocytes from *Atm*−/− mice [12]. To determine whether this is also true for cultured *Atm*−/− thymic lymphoma cells, we treated all 26 of our *Atm*−/− thymic lymphoma cell lines with Dex, and found that cells of all 26 lines were sensitive to Dex in vitro. The top left and right panels of Fig. 1 show that in the two lines (ATL-1 and ATL-2) chosen randomly

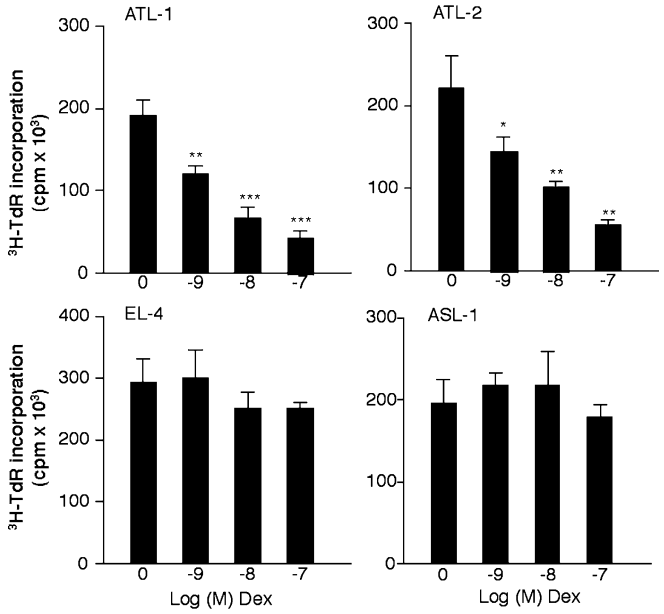

Fig. 1 Sensitivity to Dex in ATL-1, ATL-2, EL-4 and ASL-1 cells. Cells were cultured in 96-well plates at 5×10^4 cells/well in the presence (10^{-9}–10^{-7} *M*) or absence of Dex for 24 h. [^{3}H]Thymidine (0.5 μCi) was pulsed into each well at 20 h of culture. The results are expressed as mean ± SD counts per minute (cpm) for triplicate cultures. Cell proliferation values at different Dex doses in the ATL-1 and ATL-2 cells were significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

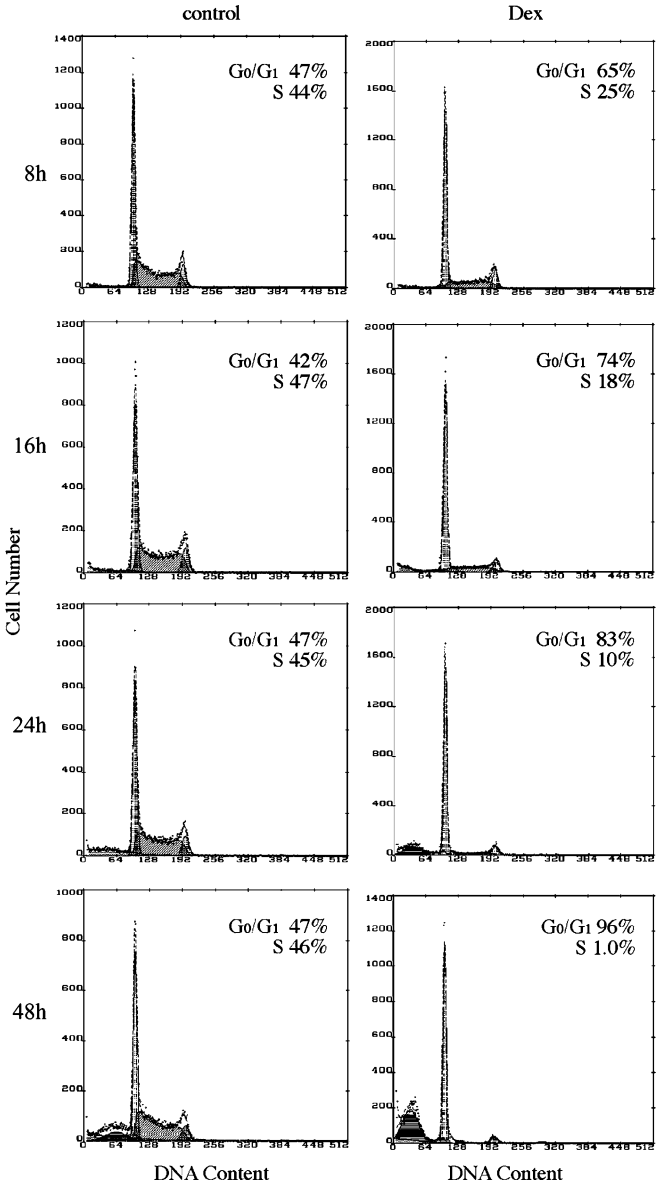

Fig. 2 Flow cytometric analyses of cell cycling in untreated versus Dex-treated ATL-1 cells. ATL-1 cells were either left untreated (*control*) or treated with 10^{-7} *M* Dex (*Dex*) for the indicated times after initiation of Dex treatment (8,16, 24 or 48 h). Percentages of cells in the G_0/G_1 and S phases of the cell cycle appear at the *top right* of each panel

from the 26 tumor cell lines we established (see above), DNA synthesis decreased in a dose-dependent fashion in cultures treated with 10^{-9}–10^{-7} *M* Dex. By contrast, DNA synthesis was unaffected by Dex treatment in two murine thymic tumor cell lines (EL-4 and ASL-1; bottom left and right panels of Fig. 1, respectively). These two control cell lines exhibited a $CD4^-CD8^-$ (double negative, DN) surface phenotype, and they displayed normal *Atm* gene function when ATM kinase activity was tested (data not shown).

Dex arrests ATL cell division at the G_1 phase

Dex is an anti-inflammatory model glucocorticoid (GC) that disrupts cytokine production and induces apoptosis of peripheral T cells and of T-cell progenitors in the thymus [23, 24]. It has been reported that Dex inhibits the proliferation of human acute lymphoblastic leukemia (ALL) cells by arresting cell cycling at the G_1 phase, and by activating apoptosis [25, 26]. To determine whether cell cycle arrest is involved in the antiproliferative effect of Dex on ATL cells, we used flow cytometry to compare DNA content in untreated versus Dex-treated ATL-1 cells at 8, 16, 24 and 48 h after the initiation of Dex treatment. Figure 2 shows that the percentages of cells in G_1 phase increased over time in Dex-treated ATL-1 cell cultures, while the proportions of cells in S phase decreased. This finding suggests that Dex inhibition of cell proliferation in ATL-1 cultures is due to cell cycle arrest at the G_1 phase. By contrast, Dex does not affect cell cycle events in ASL-1 cells (data not shown).

Dex inhibits NF-κB levels and translocation to the nucleus in ATL cells

The nuclear transcription factor NF-κB is involved in cell cycle regulation [27, 28]. The NF-κB inhibitor IκBα controls NF-κB activity by binding to it, making it unavailable for translocation into the nucleus. To determine whether and how Dex might downregulate

NF-κB expression and nuclear translocation in ATL-1 cells, we used Western blotting to compare levels of NF-κB (as the p65 subunit), and of its regulator IκBα, in Dex-treated versus untreated ATL-1 cells.

The results in Fig. 3a show that NF-κB levels were significantly lower in the Dex-treated versus untreated cells by 16 h and 24 h after the initiation of Dex treatment. Figure 3b shows that Dex treatment also significantly reduced translocation of NF-κB into the nuclear fraction (*nuc*) from the cytoplasm (*cyto*) during the culturing period. In the Dex-treated ATL-1 cultures, IκBα expression was increased at time points as early as 8 h after treatment (prior to the observation of decreased NF-κB at 16 h and 24 h; Fig. 3a). This suggests that Dex inhibition of NF-κB activation in ATL-1 cells is likely due to upregulation of IκBα levels.

G_1/S transition regulators are downregulated in Dex-treated ATL-1 cells

The cyclin D1/cdk4 and cyclin D1/cdk6 complexes control the G_1/S cell cycle transition by phosphorylating the retinoblastoma, or Rb, protein [29]. Upon phosphorylation of Rb, E2F1 is released from the inhibitory Rb/E2F1 complex, and this frees cyclin E to form the cyclin E/cdk2 complex, which is required for initiation of the G_1/S transition [30]. To determine how Dex checks cell cycling at the G_1 phase, we used Western blotting to compare the levels of cyclin D1, cdk4 and cdk6 in Dex-treated versus control untreated ATL-1 cell cultures. Figure 4a shows that Dex treatment progressively reduced levels of cdk4, cdk6 and cyclin D1 in these cells. In addition, levels of E2F1 and phosphorylated Rb (pRb), but not of Rb, were also decreased at 24 h and 48 h (Fig. 4b), as were levels of cdk2 and cyclin E (Fig. 4c). Together these results indicate that Dex arrests ATL-1 cells at the G_1 phase by affecting the levels of multiple cell cycle regulators.

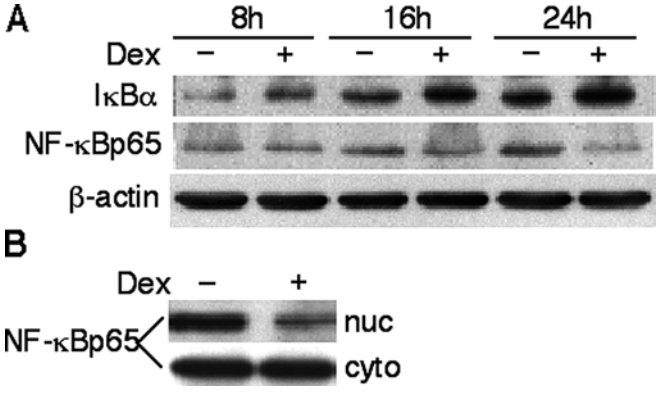

Fig. 3 Levels of IκBα and NF-κB p65 in Dex-treated versus untreated ATL-1 cells. **a** IκBα and NF-κB p65 levels were detected by Western blotting for ATL-1 cells grown in the presence (+) or absence (–) of Dex (10^{-7} *M*) at 8, 16 and 24 h. **b** Amounts of nuclear (*nuc*) versus cytoplasmic (*cyto*) NF-κB p65 in ATL-1 cells grown for 24 h in the presence (+) or absence (–) of Dex (10^{-7} *M*)

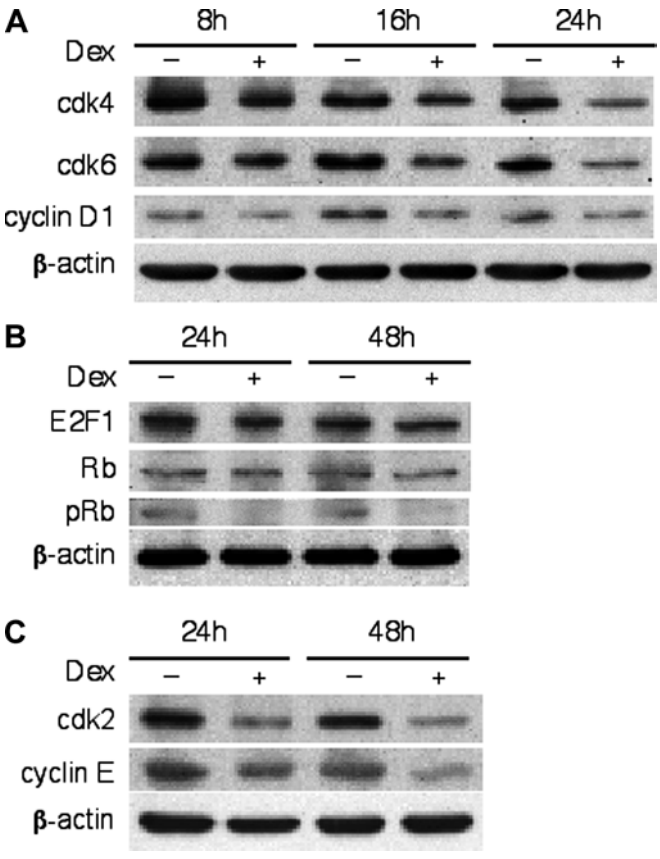

Fig. 4 G_1 phase-regulating molecules in Dex-treated versus untreated ATL-1 cells. **a** Levels of cdk4, cdk6 and cyclin D1 were compared by Western blotting for Dex-treated (+) and untreated (–) ATL-1 cells at 8, 16 and 24 h. **b** Levels of E2F1, Rb and pRb in Dex-treated (+) and untreated (–) ATL-1 cells at 24 h and 48 h. **c** cdk2 and cyclin E expression in Dex-treated (+) and untreated (–) ATL-1 cells at 24 h and 48 h

Dex induces apoptosis in ATL-1 cells

To further investigate Dex-induced cell growth inhibition in *Atm*–/– thymic lymphoma cells, ATL-1 cells were either left untreated or treated with Dex, after which they were stained with Hoechst 33342 (at 24 h). Figure 5a shows that Dex-treated ATL-1 cells (*Dex*) exhibited extensive nuclear condensation and fragmentation, both of which are changes typical of apoptotic cells [31]. Flow cytometry analysis of these cells confirmed that apoptosis was underway by showing that the percentage of cells in the sub-G_1 peak was increasingly elevated over the 48 h of culture in Dex-treated ATL-1 cells (Fig. 5b).

Dex can induce apoptosis either by activating specific death receptor pathways or by initiating the mitochondrial apoptotic pathway [31, 32]. In both cases, cell death requires downstream activation of the key effector enzyme caspase-3 [31, 33]. When activated caspase-3 levels (*act-caspase 3*) were compared by Western blotting for untreated versus Dex-treated ATL-1 cells, the amounts of activated caspase-3 were found to be significantly increased in Dex-treated ATL-1 cells (Fig. 5c).

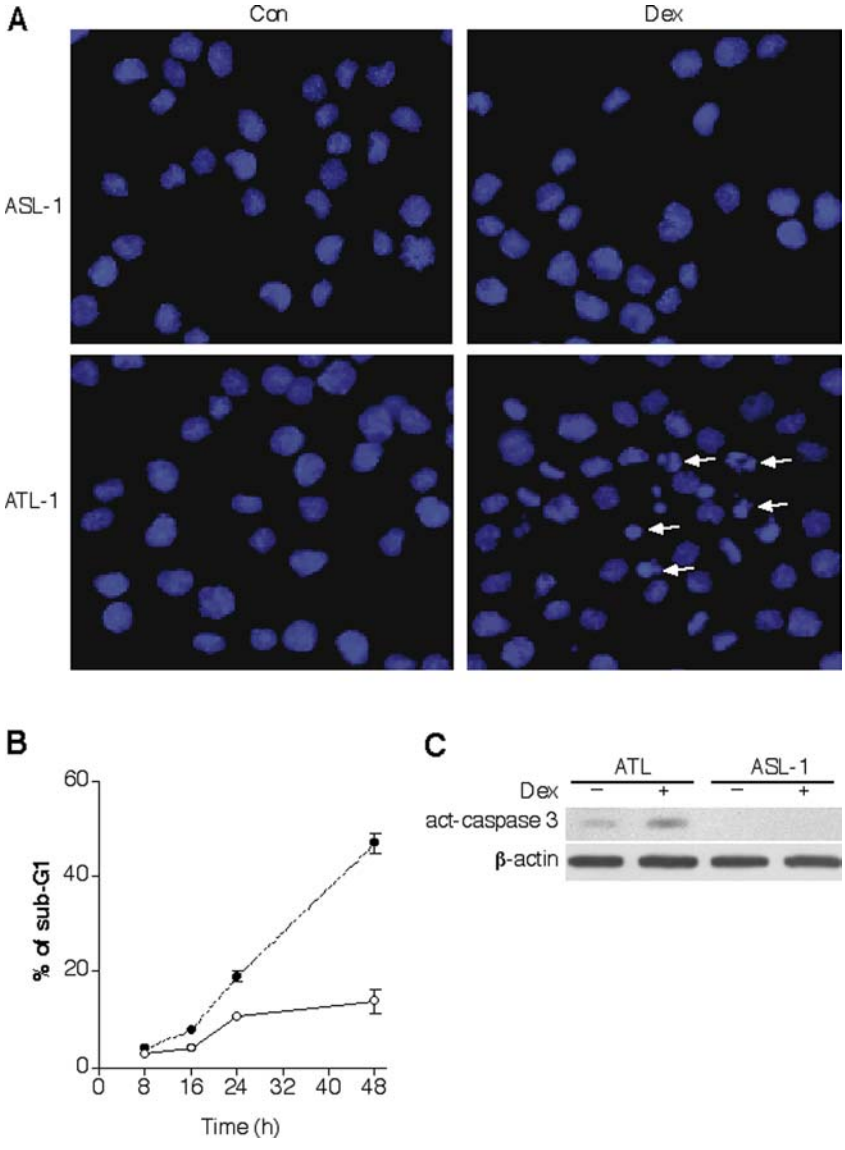

Fig. 5 Dex induces apoptosis in ATL-1 cells. **a** ASL-1 (*top panels*) or ATL-1 (*bottom panels*) cells were cultured without (*Con*) or with Dex (10^{-7} M; *Dex*) for 24 h, at which time the cells were fixed in 3.7% paraformaldehyde followed by staining with the DNA-binding dye Hoechst 33342. Apoptotic cells with pyknotic and condensed nuclei are indicated by *arrowheads* in the Dex-treated ATL-1 cells in the *bottom right panel* (original magnification ×400). **b** Summary of flow cytometry data from Dex-treated (*solid circles*, *dotted line*) versus control untreated ATL-1 cells (*open circles*, *solid line*), giving the percentages (means ± SD) of cells in the sub-G_1 peak at the indicated times after initiation of Dex treatment. **c** Western blots comparing levels of activated caspase-3 (*act-caspase 3*) in Dex-treated (+) versus untreated (−) ATL-1 and ASL-1 cells at 24 h after initiation of Dex treatment

Dex inhibits the in vivo growth of solid tumors in animals transplanted with *Atm*−/− thymic lymphoma cells

We have shown previously that Dex treatment in *Atm*−/− mice prevents in situ development of thymic lymphomas, which normally occurs in 100% of animals at about 4–5 months of age. The fact that Dex inhibits proliferation of *Atm*−/− ATL-1 thymic lymphoma cells in culture (Figs. 1, 2, 3, 4 and 5) suggested that it might inhibit the growth of solid ATL-1 cell tumors in recipient mice that have received transplanted ATL-1 cells.

In the first of two experiments, sixteen 8-week-old *Atm*+/+ mice were inoculated subcutaneously in the left flank with 1×10^6 ATL-1 tumor cells each. Beginning 4 days later, eight of the mice (the "treatment group") received 5 mg/kg of Dex daily for 12 days (intraperitoneally), while the remaining eight mice (the "control group") received the same volume of normal saline (NS, or *vehicle*) by the same route. Tumor development in the living animals was then observed every other day by palpation, and tumor diameters were assessed using calipers. At 22 days after tumor injection, the Dex-treated and control tumor recipient mice were killed, and the tumor masses were excised and weighed.

Figure 6a shows that large tumor masses had grown from the transplanted cells in all eight of the control untreated recipient mice, while the eight Dex-treated mice either had much smaller tumors (four mice) or no tumors at all (four mice) at the injection site. Figure 6b shows the mean tumor volumes for animals in both groups (Dex-treated vs vehicle-treated controls), measured from 12 to 22 days after they received ATL-1 cells, and Fig. 6c shows the mean tumor weights for the two groups at 22 days, at which time all the mice were killed.

This dramatic result suggests that low-dose Dex treatment (5 mg/kg) prevents ATL-1 cell proliferation in vivo, even at sites outside of the thymic microenvironment. To determine whether Dex can suppress the growth or reduce the sizes of established ATL-1 cell

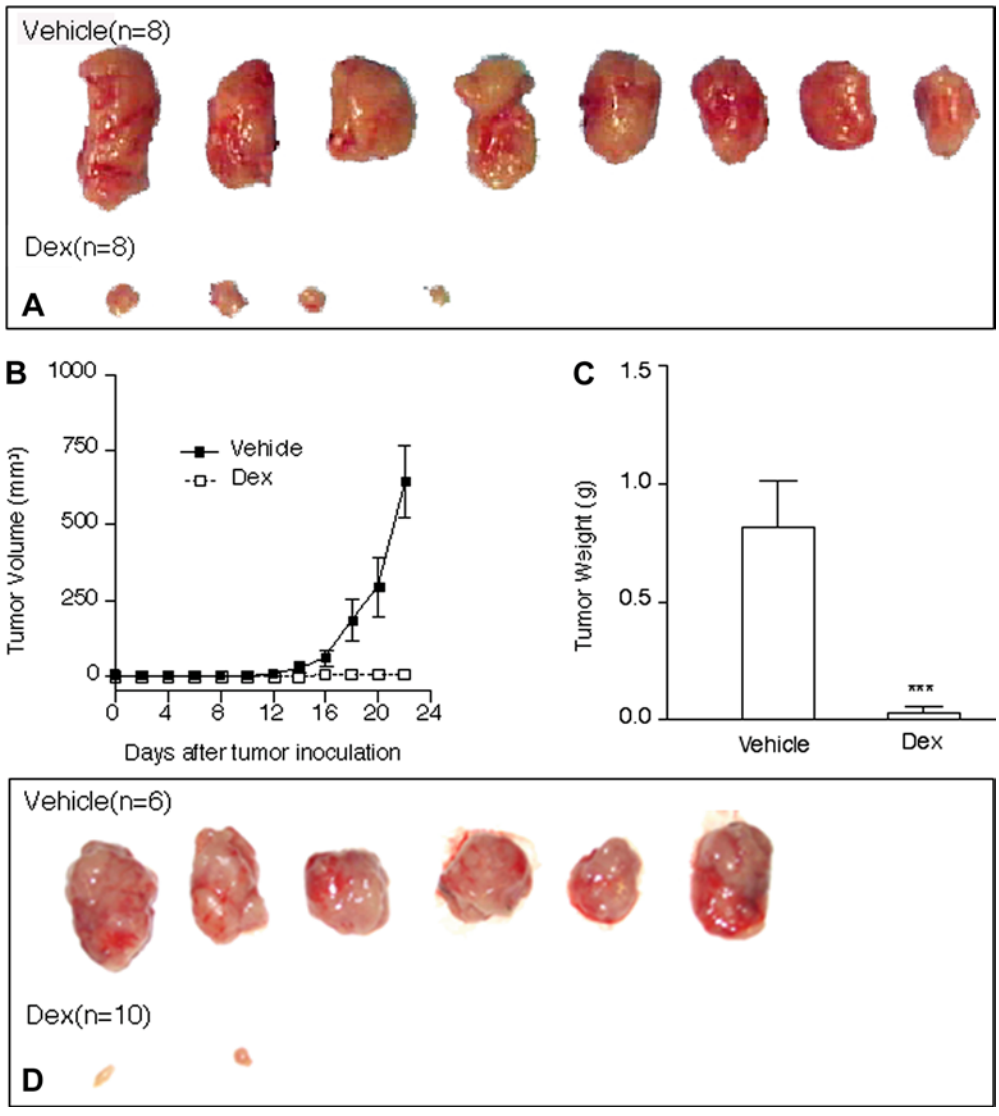

Fig. 6 Dex inhibits growth of solid tumors from ATL-1 cells transplanted into *Atm*+/+ mice. **a** Photographs of tumors taken from Dex-treated (*Dex*) versus saline-injected (*Vehicle*) mice that had received ATL-1 cell transplants. Dex treatment (5 mg/kg) was started 4 days after tumor cell injection. **b** Average volume of tumors growing from transplanted *Atm*−/− thymic lymphoma cells in living 8-week-old Dex-treated versus control recipient mice. **c** Weights of tumors from Dex-treated (*Dex*) versus control (*Vehicle*) tumor recipient mice (***$P < 0.001$). **d** Photographs of the tumors taken from Dex-treated (*Dex*) versus saline-injected (*Vehicle*) 4-week-old mice that received ATL-1 cell transplants, as in **a**, but with Dex treatment (10 mg/kg) started 9 days after tumor cell injection

solid tumors, a second set of experiments was performed, this time with sixteen 4-week-old *Atm*+/+ mice that had received ATL-1 cells, followed by Dex treatment in ten of the mice, at a higher dose (10 mg/kg) than that used previously (5 mg/kg), and with treatment started at 9 days (rather than 4 days) after the tumor cell injection, at which time the tumor mass was palpable and visible (about 2 mm in diameter). The photographs of the tumors in Fig. 6d show that while large tumors grew in the six untreated control mice, Dex arrested tumor development in two of the ten treated mice, and that it completely destroyed the tumors that had developed in the remaining eight mice.

Discussion

ATM is a pleiotropic protein kinase that protects cells from oxidative stress, and modulates many events in cell cycle progression and differentiation [34]. In A-T humans and in *Atm*−/− mice, T-cell development is retarded, and immature thymocytes fail to stop proliferating at developmental checkpoints that require temporary quiescence if the cells are to survive [12, 13]. We have shown previously that elevated spontaneous proliferation of primary cultured *Atm*−/− thymocytes can be attenuated by Dex [12], and that administration of Dex to *Atm*−/− mice prevents thymic lymphoma development [13]. We have proposed that this unchecked thymocyte proliferation in *Atm*−/− mice results both in apoptosis of the cells (most thymocytes) or in the development of thymic lymphomas from the few cells that escape apoptosis during this period [13].

In this study, we extended these prior findings by showing that Dex suppresses cell proliferation in *Atm*−/− thymic lymphoma cell cultures by affecting the cell cycle and by inducing apoptosis. In addition, we showed that Dex inhibits the growth of solid tumors in *Atm*+/+ mice that have received ATL-1 cell transplants.

In thymocytes, GCs cause G_1 cell cycle arrest, trigger secretion of glutathione (GSH), and induce apoptosis [35, 36]. GCs mediate their effects by binding to GC

receptors (GRs) in the cytoplasm, whereupon the bound GRs are translocated to the nucleus [37]. Once in the nucleus, the activated GRs bind to specific DNA sequences called GC responsive elements (GREs), enhancing or inhibiting transcription of various genes. Of these genes, NF-κB is a common target for GRs in the nucleus [38].

In the experiments reported here, Dex decreased intracellular NF-κB levels in ATL-1 cells, and prevented NF-κB nuclear translocation in *Atm*−/− thymic lymphoma cells by elevating IκBα. Dex also reduced the levels of cyclin D1, cdk4, cdk6 and pRb, together with levels of E2F1, cyclin E and cdk2, all of which are required in normal amounts for cell division. By contrast, the amounts of the cell cycling intermediate p27, which inhibits the formation of the cyclin E/cdk2 complex, were significantly increased in Dex-treated ATL-1 cells (data not shown). These results suggest that Dex downregulates cell cycling by (1) increasing production of IκBα, which binds to cytoplasmic NF-κB and blocks NF-κB nuclear translocation, and by (2) decreasing the amounts of the cell cycle regulators cyclin D1, cdk4, cdk6, E2F1, pRb, cyclin E and cdk2. In Dex-treated ATL-1 cell cultures, IκBα elevation was evident at 8 h, before levels of G_1/S phase related cell cycle molecules had decreased. This observation implies that upregulation of IκBα is critical for the inhibition of growth in ATL-1 cells by Dex.

Induction of apoptosis is another pathway by which ATM is known to control cell fate [39]. Thymocytes from *Atm*−/− mice have been shown to undergo spontaneous apoptosis [40], and to be more sensitive than normal thymocytes to GC-induced cell death [12]. In our hands, approximately 10% of ATL-1 cells underwent spontaneous apoptosis, but Dex treatment significantly increased the proportion of apoptotic cells in these cultures (Fig. 5b). Dex also increased the amount of activated caspase-3 in cultured *Atm*−/− tumor cells, which is the common executive effector for several different apoptotic cascades (Fig. 5c). By contrast, ASL-1 cells, which contain functional ATM, were completely resistant to Dex-induced apoptosis (Figs. 5a,c). These results suggest that cell cycle arrest is accompanied by increased apoptosis in Dex-treated ATL-1 cells, and that both effects contribute to the inhibition of their proliferation in vitro and in vivo.

At this point, it is not clear why *Atm*−/− thymic lymphoma cells are so sensitive to Dex. There are several possibilities. In most GC-sensitive cell types, GR levels are correlated with GC sensitivity [41, 42], and we have found that GR levels in ATL-1 cells are significantly higher than those of Dex-resistant ASL-1 cells (data not shown). This could therefore account for the high sensitivity of ATL-1 cells to Dex. Alternatively, several recent studies have shown that DP thymocytes in the normal thymus are more sensitive to Dex-induced apoptosis than are other T-cell progenitor subsets [43, 44]. Our data show that 92% of ATL-1 lines from 26 individual thymic lymphomas bearing *Atm*−/− mice are DP phenotype.

If the tendency of *Atm*−/− thymic lymphoma cells to be DP results from the inability of most thymocytes in *Atm*−/− mice to pass this checkpoint, the GC sensitivity of tumor cell lines derived from these cells may simply reflect a physiological property inherited from the tumor cells of origin, along with the DP surface phenotype. Finally, it is possible that Dex causes release of GSH from ATL-1 cells, thereby depleting the cells of their major antioxidant [35, 36, 45]. Since oxidative stress is already elevated in A-T [46–48], and elevated oxidative stress in their thymocytes makes them vulnerable to apoptosis [45], this effect of Dex is likely to exacerbate proapoptotic conditions in the A-T thymocytes, amplifying the tumor-preventing effects of Dex at low dosages.

Treatment of lymphoid malignancies in A-T patients is problematic, since A-T patients are highly sensitive to radiation [49]. Similarly, full-dose chemotherapy is also toxic to an unacceptable degree in A-T patients [50, 51]. The results reported here identify cellular pathways affected by GCs as potential therapeutic targets for prevention or treatment of lymphoreticular tumors in A-T. The data also suggest that Dex could be less toxic and more effective than irradiation or chemotherapy for suppression of tumor growth in these individuals. As noted above, A-T is a rare human autosomal recessive disease, but many lymphomas and leukemias occurring sporadically in non-A-T patients are associated with inactivation of the *Atm* gene [5]. Thus the study described here identifies Dex as an agent that may prove useful for the treatment of lymphoreticular malignancies in A-T and non-A-T patients as well.

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