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Dimethyl Fumarate Induces Apoptosis *via* Inhibiting NF-κB and STAT3 Signaling in Adult T-cell Leukemia/Lymphoma Cells

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Abstract. *Background/Aim: Adult T-cell leukemia (ATL) is a peripheral T lymphocytic malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) infection. Despite treatment that includes novel agents that have been developed, most of ATL patients relapse and acquire multidrug resistance. As a result, the creation of newer agents is critical. Dimethyl fumarate (DMF) has several effects in cancer cells, including cell signaling, proliferation and cell death. However, its antitumor effects on ATL cells remain unknown. In this study, we looked at DMF's antitumor effects on ATL cells. Materials and Methods: We examined the effects of DMF on proliferation and apoptosis using the trypan blue exclusion assay and annexin V/propidium iodide staining in HTLV-1-infected and transformed T-cell lines, MT-1 and MT-2 cells. We also evaluated the effects of DMF on the nuclear factor-kappa B (NF-κB) and signal transducers and activators of transcription 3 (STAT3) signaling pathways and anti-apoptotic proteins by immunoblotting. Results: DMF inhibited proliferation and induced apoptosis in MT-1 and MT-2 cells by activating poly ADP-ribose polymerase (PARP). Furthermore, DMF inhibited the constitutive activation of both canonical and non-canonical NF-κB pathways in MT-2 cells and the non-canonical NF-κB pathway in MT-1 cells. DMF also inhibited the constitutive tyrosine phosphorylation of STAT3 and the expression of anti-apoptotic proteins, c-IAP2 and survivin in both cells. Conclusion: These results indicate that DMF inhibits proliferation and induces apoptosis in HTLV-1-infected and transformed T-cells by suppressing NF-κB and STAT3 signaling pathways. DMF should be investigated further as a novel agent for ATL.*

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Adult T-cell leukemia (ATL) is a peripheral T lymphocytic malignancy, which is caused by the human T-cell leukemia virus type 1 (HTLV-1) (1-3). HTLV-1 is prevalent worldwide, with the highest known prevalence in Japan, Caribbean islands, South America, the African continent and Melanesia (4). The median age of diagnosis in Japan was 69 years, according to a Japanese nationwide epidemiological study of ATL diagnosed between 2012 and 2013 (5). Due to its resistance to conventional chemotherapy, aggressive ATL, including acute and lymphoma types and chronic types with poor prognostic factors, has a poor prognosis with a median survival duration of 13 months (6). Recently, newer agents have been developed. Mogamulizumab is a humanized anti-CC chemokine receptor type 4 (CCR4) antibody that has been approved in Japan for the treatment of newly diagnosed aggressive ATL in combination with a conventional dose-intensified multidrug regimen, namely, the modified LSG15 (mLSG15) regimen (VCAP-AMP-VECP: vincristine, cyclophosphamide, doxorubicin, and prednisolone; doxorubicin, ranimustine, and prednisolone; vindesine, etoposide, carboplatin, and prednisolone). In a randomized phase II study, the complete response rate as the primary endpoint was significantly higher in the mogamulizumab plus mLSG15 arm (52%) than in LSG15 alone (33%) (7). Despite the development of treatment incorporating such novel agents, most ATL patients relapse and require additional treatment. As a result, new therapeutic agents that effectively kill ATL cells need to be developed.

The proliferation of ATL cells is related to the constitutive activation of the nuclear factor-kappa B (NF-κB) pathway and Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins (8, 9). Furthermore, NF-κB is induced by a unique HTLV-1 gene, Tax, which has been linked to tumor growth in an *in vivo* model of ATL (9). Thus, the activation of various intracellular signaling molecules is critical for HTLV-1 oncogenesis. Many researchers have been working on developing drugs that target these signaling molecules. Dehydroxymethylepoxyquinomicin (DHMEQ), a novel NF-κB

inhibitor, inhibited NF-κB activation and induced apoptosis in primary ATL cells and cell lines derived from them (10). A proteasome inhibitor, bortezomib, also suppressed NF-κB activation *via* inhibiting the degradation of IκBα in ATL cells and suppressed tumor growth *in vivo* (11). Based on these results, a phase II study of bortezomib in relapsed or refractory aggressive ATL was conducted. Unfortunately, the study was terminated early because the single-agent activity did not appear to be very promising (12). We previously demonstrated that the naturally occurring rotenoid deguelin and the well-known polyphenol compound resveratrol suppressed cell growth and survival *via* inhibiting STAT3 activation in HTLV-1-infected and transformed cells (13, 14).

Dimethyl fumarate (DMF) is a drug approved by the US Food and Drug Administration and the European Medicines Agency and it is clinically used as first-line treatment of relapsing-remitting multiple sclerosis or as a systemic medication for moderate to severe psoriasis. It has been shown that DMF induces apoptosis in activated T cells (15). DMF is also known as an activator of nuclear factor-erythroid 2-related factor 2 (Nrf2) (16). DMF has shown to enhance the differentiation induced by vitamin D derivatives in acute myeloid leukemia cells and inhibit tumor growth *via* the vitamin D receptor and Nrf2 signaling (17). It inhibits the NF-κB pathway in breast cancer, cutaneous T-cell lymphoma (CTCL), and diffuse large B-cell lymphoma (DLBCL) cells (18-20). Furthermore, DMF has been shown to inhibit the STAT3 pathway in DLBCL and hepatocellular carcinoma cells, and the Wnt signaling pathway in chronic lymphocytic leukemia cells (18, 21, 22). However, DMF's antitumor effect and mechanism of action in ATL cells are unknown. In this study, we report that DMF induces cell death in HTLV-1-infected and transformed T cells by suppressing the NF-κB and STAT3 pathways.

Materials and Methods

Reagents and antibodies. Sigma-Aldrich supplied the DMF (Tokyo, Japan). Working solutions were prepared in RPMI 1640 medium from a stock solution prepared by dissolving in dimethyl sulfoxide (DMSO). Control cells were incubated with DMSO (maximum concentration) alone. Antibodies against phospho-STAT3 (Tyr705) (#9145), STAT3 (#4904), phosho-IκBα (Ser32) (#2859), IκBα (#9242), a cellular inhibitor of apoptosis protein-2 (c-IAP2) (#3130), cleaved poly ADP-ribose polymerase (PARP) (#9541), survivin (#28089), Bcl-extra-large (Bcl-xL) (#2764), NF-κB p65 (#6956), and β-actin (#4967) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against RelB (sc-48366), B-cell lymphoma-2 (Bcl-2) (sc-7382), histone deacetylase 1 (HDAC1) (sc-7872), and NF-κB p52 (sc-7386), and horseradish peroxidase (HRP) -conjugated secondary antibodies (sc-7074, sc-707692) were obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies against NF-κB p50 (616702) were obtained from Biolegend (San Diego, CA). Primary and secondary antibodies were diluted at 1:1000 and 1:5000, respectively.

Cell lines and culture. The Japanese Collection of Research Bioresources (JCRB) Cell Bank (Tokyo, Japan) provided the MT-1 (a leukemic T-cell line derived from ATL patients) and the MT-2 (a human HTLV-1-infected cell line) cell lines. Both cell lines were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 50 U/mL penicillin (Thermo Fisher Scientific, Tokyo, Japan), and 50 μg/mL streptomycin (Thermo Fisher Scientific) in a 5% CO_2 incubator at 37˚C.

Cell proliferation and cell death assay. Cell viability was determined using the trypan blue exclusion assay with a Countess II FL Automated Cell Counter after cells were cultured in RPMI1640 medium supplemented with 10% FBS in the presence or absence of DMF for 24 or 48 h, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The proliferation rate was defined as the percentage of surviving cells in each group compared to that in the untreated group. For apoptosis assay, cells (1×10^6/ml) were treated in the presence or absence of DMF for 24 or 48 h. Then, cells were stained with annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). The percentage of annexin V-positive cells was analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Immunoblotting. After being treated with RPMI 1640 supplemented with 10% FBS in the presence or absence of DMF, the MT-1 and MT-2 cells were washed twice with cold PBS. Then, we prepared cell lysates using ice-cold RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) with 5 mM NaF, 0.5 mM sodium orthovanadate, and 1% proteasome inhibitors, incubated for 30 min at 4˚C, and then centrifuged at 10,000 rpm for 10 min, while supernatants were retained. The cell lysates were eluted by incubating them for 5 min at 95˚C in sodium dodecyl sulfate sample buffer (Bio-Rad Laboratories, Hercules, CA, USa). The samples were loaded into 7-15% Tris-glycine gel (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK). Nuclear and cytoplasmic fractions from each extract were prepared using the Cytoplasmic and Nuclear Protein Extraction Kit (101Bio, Palo Alto, CA, USA) according to the manufacturer's instructions. Next, the immunoblotting assays were performed according to the instructions provided by the manufacturers of the antibodies. Blots were visualized by using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection reagent (GE Healthcare, Tokyo, Japan). Membranes were incubated in stripping buffer (Pierce, Rockford, IL, USA) according to the manufacturer's instructions to re-blotting with other antibodies.

Statistical analysis. JMP11 was used for the statistical analysis (SAS Institute, Cary, NC, USA). The data obtained were expressed as mean±standard deviation (SD). Statistical differences between test groups were analyzed using ANOVA with Bonferroni's multiple comparison test after verifying that the data met the assumptions of the statistical test employed. *p*-Values <0.05 were considered statistically significant.

Results

DMF suppresses cell proliferation and survival in MT-1 and MT-2 cells. We initially examined the effects of DMF treatment and discovered that it significantly inhibited the

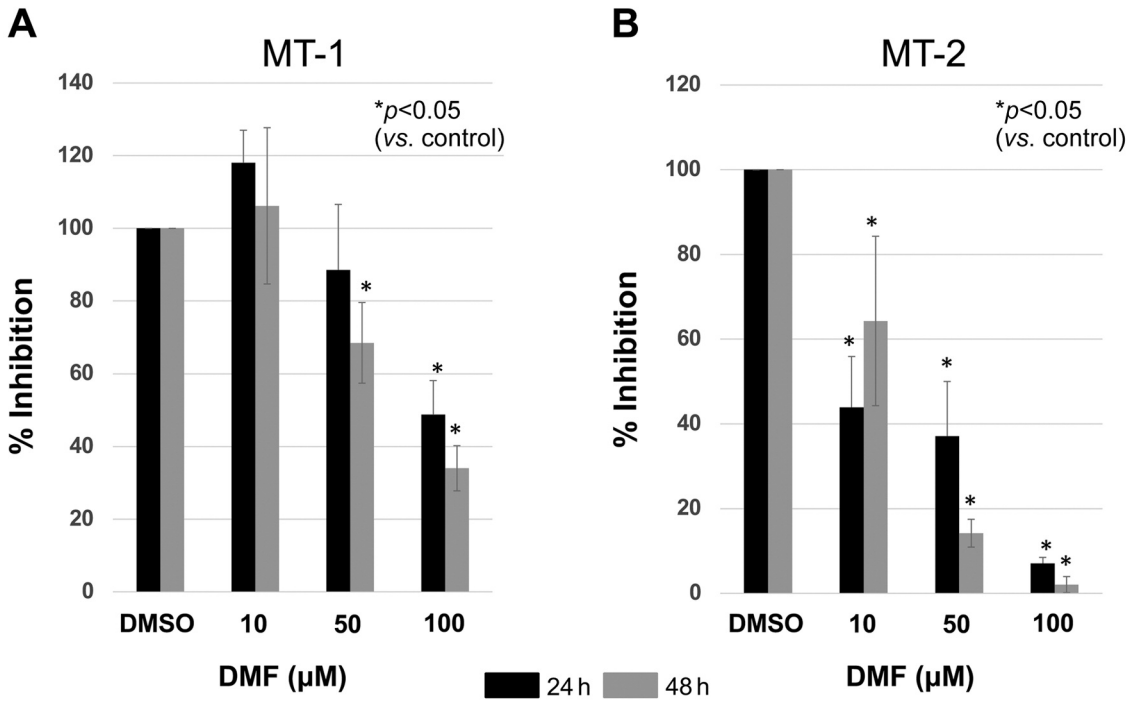

Figure 1. *Inhibitory effects of DMF on proliferation in MT-1 and MT-2 cells. (A) MT-1 and (B) MT-2 cells were cultured in 24-well tissue culture plates treated with DMSO as a control and DMF at the indicated concentrations for 24 and 48 h. Cell viability was measured using the trypan blue exclusion assay. Data are expressed as the mean percentage of the control cells representing the average of triplicate samples. Error bars represent one standard deviation (SD) from the mean of the triplicate wells. The results are representative of three independent experiments (DMF vs. control, *p-Value <0.05 using ANOVA with Bonferroni multiple comparison test).*

proliferation of MT-1 and MT-2 cells after 24 and 48 h in a dose-dependent manner as shown in Figure 1A and B. Next, we examined whether DMF induces cell death. However, the sensitivity of these cell lines varied, with MT-2 cells being more sensitive to DMF. Flow cytometric analysis with annexin V and PI staining of cells treated with 50 μM and 100 μM DMF for 24 and 48 h was used to determine the frequency of cell death. The frequency of apoptotic (annexin V-positive) cells was significantly higher in the presence of DMF than in its absence (Figure 2A-D). MT-2 cells were also more sensitive to DMF than MT-1 cells on induction of apoptosis. These results suggest that DMF suppressed cell proliferation and induced apoptosis in both MT-1 and MT-2 cells.

DMF inhibits phosphorylation of IκBα and induces PARP activation in MT-1 and MT-2 cells. To better understand the mechanism by which DMF suppresses the proliferation and induces apoptosis of MT-1 and MT-2 cells, we investigated whether DMF inhibits IkBα phosphorylation and induces PARP activation in both cells. IκBα normally binds to the complex of NF-κB p65 and p50, both of which are transcriptional regulators and are responsible for the suppression of nuclear translocation. As shown in Figure 3A, DMF treatment for 24 h inhibited IκBα phosphorylation in MT-1 and MT-2 cells. Furthermore, DMF induced PARP activation in a dose-dependent manner in both cells.

DMF inhibits nuclear translocation of NF-κB molecules in MT-1 and MT-2 cells. Next, we investigated if DMF suppresses the canonical and non-canonical NF-κB pathway in MT-1 and MT-2 cells. Since 100 μM DMF for 48 h caused a significant increase in apoptosis in MT-1 cells (Figure 2C), MT-1 cells were treated with 50 μM and 100 μM DMF for 48 h. As shown in Figure 3B, treatment with 100 μM DMF suppressed RelB expression in the nucleus of MT-1 cells. On the other hand, MT-2 cells were treated with 50 μM and 100 μM DMF for 24 h. Treatment with at least 50 μM DMF suppressed p52, RelB, and p65 expression in the nucleus of MT-2 cells.

DMF inhibits STAT3 phosphorylation, cIAP-2, and survivin expression in MT-1 and MT-2 cells. STAT3 was found to be phosphorylated all the time in MT-1 and MT-2 cells. DMF treatment for 24 h suppressed STAT3 phosphorylation in MT-1 and MT-2 cells, as shown in Figure 4. We then looked at how DMF affected anti-apoptotic proteins, including c-IAP2, survivin, Bcl-2, and Bcl-xL in both cells. DMF

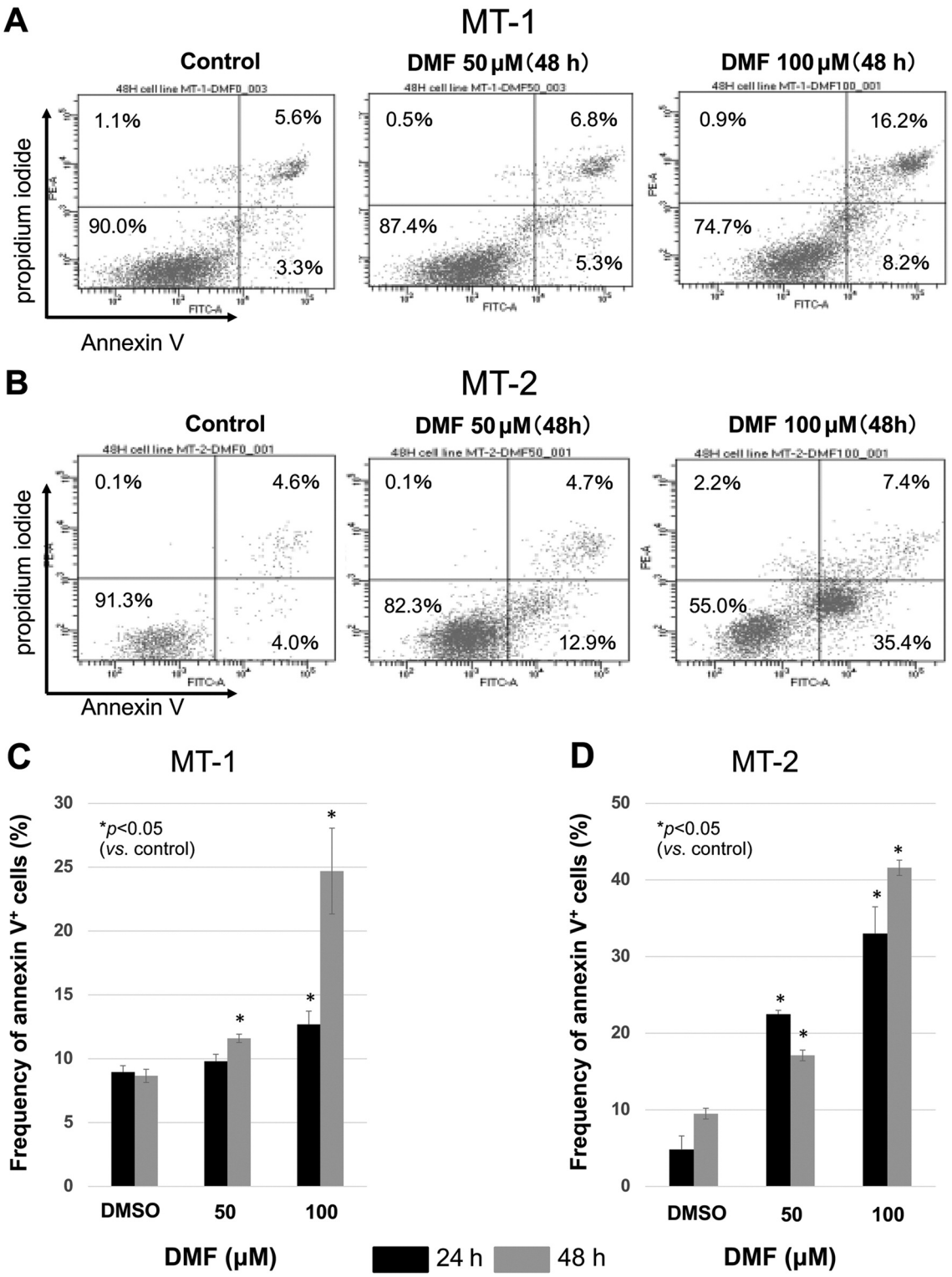

Figure 2. *DMF-mediated apoptosis in MT-1 and MT-2 cells. MT-1 and MT-2 cells were cultured in 24-well tissue culture plates treated with DMSO as a control and DMF at the indicated concentrations for 24 and 48 h. The degree of apoptosis was measured using flow cytometric analysis with annexin V and PI staining. The percentages of annexin V-positive cells are indicative of apoptotic cells. Representative dot plots of (A) MT-1 and (B) MT-2 cells are shown. Numbers in dot plots correspond to percentage of cells. Data representing the average of triplicate samples of (C) MT-1 and (D) MT-2 cells are shown. Error bars represent 1 SD from the mean for triplicate wells. Results are representative of two independent experiments (DMF vs. control, *p-Value <0.05 using ANOVA with Bonferroni multiple comparison test).*

inhibited cIAP-2 and survivin, both of which are known as downstream targets of NF-κB and STAT3, but not Bcl-2 and Bcl-xL in MT-1 and MT-2 cells (Figure 4).

Discussion

In this study, we demonstrated for the first time that DMF inhibited proliferation and survival of HTLV-1-infected and transformed cells. DMF is approved for the treatment of multiple sclerosis and psoriasis. It has been used in several clinical trials for its anti-inflammatory and anti-cancer properties (23). It has been shown that DMF induces cell death in various malignant cells including DLBCL, CTCL, breast cancer, hepatocellular carcinoma, melanoma, glioblastoma, lung cancer, and colorectal cancer (18-21, 24-27). Thus, DMF is a promising drug candidate for cancer therapy. In this study, we discovered that DMF significantly inhibits the proliferation and survival of MT-1 and MT-2 cells after 24 and 48 h (Figure 1 and Figure 2). MT-2 cells were more sensitive to DMF on apoptosis than MT-1 cells, similar to the antiproliferative effect of DMF on both cell lines. These findings could be attributed to differences in cell line characteristics; MT-1 cells are derived from ATL while MT-2 cells are derived from human cord leukocytes co-culturing with ATL cells (28, 29).

HTLV-1 Tax, the viral transcription activator protein, has been shown to activate various signaling pathways *via* direct interactions with signaling molecules (30). Despite the absence of Tax in primary ATL cells, these signaling pathways are activated, as well as HTLV-1-infected cells, in which Tax is present. These findings suggest that Tax-mediated signaling pathway activation is involved in the early stages of transformation. The NF-κB signaling pathway, among others, is constitutively activated in ATL cells (31). The canonical NF-κB pathway consists of p65 and p50 transcription factors, both of which are held in the cytoplasm by an inhibitor protein, IκBα. Phosphorylation of IκBα by upstream kinases leads to proteasomal degradation of IκBα. As a result, p65/p50 molecules can translocate to the nucleus, where they bind to DNA and induce gene transcription (32). The non-canonical NF-κB pathway consists of RelB and p52 transcription factors. This pathway is dependent on phosphorylation-induced p100 processing, which is activated by signaling from a subset of tumor necrosis factor receptors. The processing of p100 results in the production of p52 and the nuclear translocation of the RelB/p52 heterodimer (33, 34).

In this regard, DMF was tested to see if it inhibited the activation of the NF-κB pathway in MT-1 and MT-2 cells. DMF inhibited constitutive phosphorylation of IκB in MT-1 and MT-2 cells, as shown in Figure 3A. p65, p50, RelB, and p52 molecules were found in the nuclear fraction of MT-2 cells, indicating that both canonical and non-canonical pathways are active in MT-2 cells (Figure 3B). In addition, DMF suppressed p65, RelB, and p52 expression in the nucleus in a dose-dependent manner in MT-2 cells (Figure 3B). These results suggest that DMF inhibits both canonical and non-canonical NF-κB pathways in MT-2 cells. On the other hand, in MT-1 cells, we did not detect p65 molecule in the nucleus before treatment with DMF, suggesting that the canonical pathway is not activated in MT-1 cells (Figure 3B). Only RelB expression in the nucleus was suppressed by treatment with 100 μM DMF for 48 h in MT-1 cells (Figure 3B). DMF inhibits the non-canonical NF-κB pathway in MT-1 cells, according to these findings. However, DMF's inhibitory effect on NF-κB pathway molecules was significantly weaker in MT-1 cells than in MT-2 cells. The difference appeared to parallel the effect of DMF on cell proliferation and apoptosis levels in MT-1 and MT-2 cells. DMF has recently been shown to induce cell death by inhibiting the NF-κB pathway in CTCL and DLBCL (18, 20). DMF induced cell death in CTCL cells and inhibited CTCL tumor growth and metastasis *in vivo*, according to Nicolay *et al*. (20). They also demonstrated that DNA binding of p50, p65, and RelB proteins was inhibited after DMF treatment in CTCL cell lines. Schmitt *et al*. showed that DMF down-regulated several NF-κB gene signatures using gene set enrichment analysis in activated B-cell-like (ABC) DLBCL (18). Furthermore, they demonstrated that DMF treatment impaired the phosphorylation of IκBα at S32/36, indicating that DMF disrupted the chronic activation of the IκB kinase (IKK) complex. As expected, DMF treatment resulted in markedly decreased p65 translocation to the nucleus and in impaired binding of p65 and c-Rel to their consensus DNA sequence (18). These findings are consistent with our findings in MT-2 cells, which show that DMF strongly inhibits the NF-κB pathway in HTLV-1-infected T cells, MT-2. More research is needed to determine the precise mechanism by which DMF inhibits IκB phosphorylation in ATL cells.

Activation of the JAK/STAT signaling pathway is associated with cell proliferation in ATL (8). We found that DMF treatment simultaneously decreased the constitutive STAT3 phosphorylation in MT-1 and MT-2 cells (Figure 4), indicating a direct role of DMF in STAT3 inhibition. DMF inhibited STAT3 phosphorylation in ABC DLBCL, according to Schmitt *et al*. (18). DMF's direct modification and inhibition of JAK1 and TYK2 were also identified as another mechanism for its toxicity in ABC DLBCL. Future research should focus on determining the precise mechanism by which DMF inhibits STAT3 phosphorylation in ATL cells. We also discovered that DMF inhibited c-IAP2 and survivin protein expression in both cell lines (Figure 4). Both anti-apoptotic proteins have been previously reported as an NF-κB target and STAT3 (13, 14, 35). A recent study showed that NF-κB and IRF4 regulated several cancer genes such as MYC, CCR4, and c-IAP2 in ATL cells (36). These findings

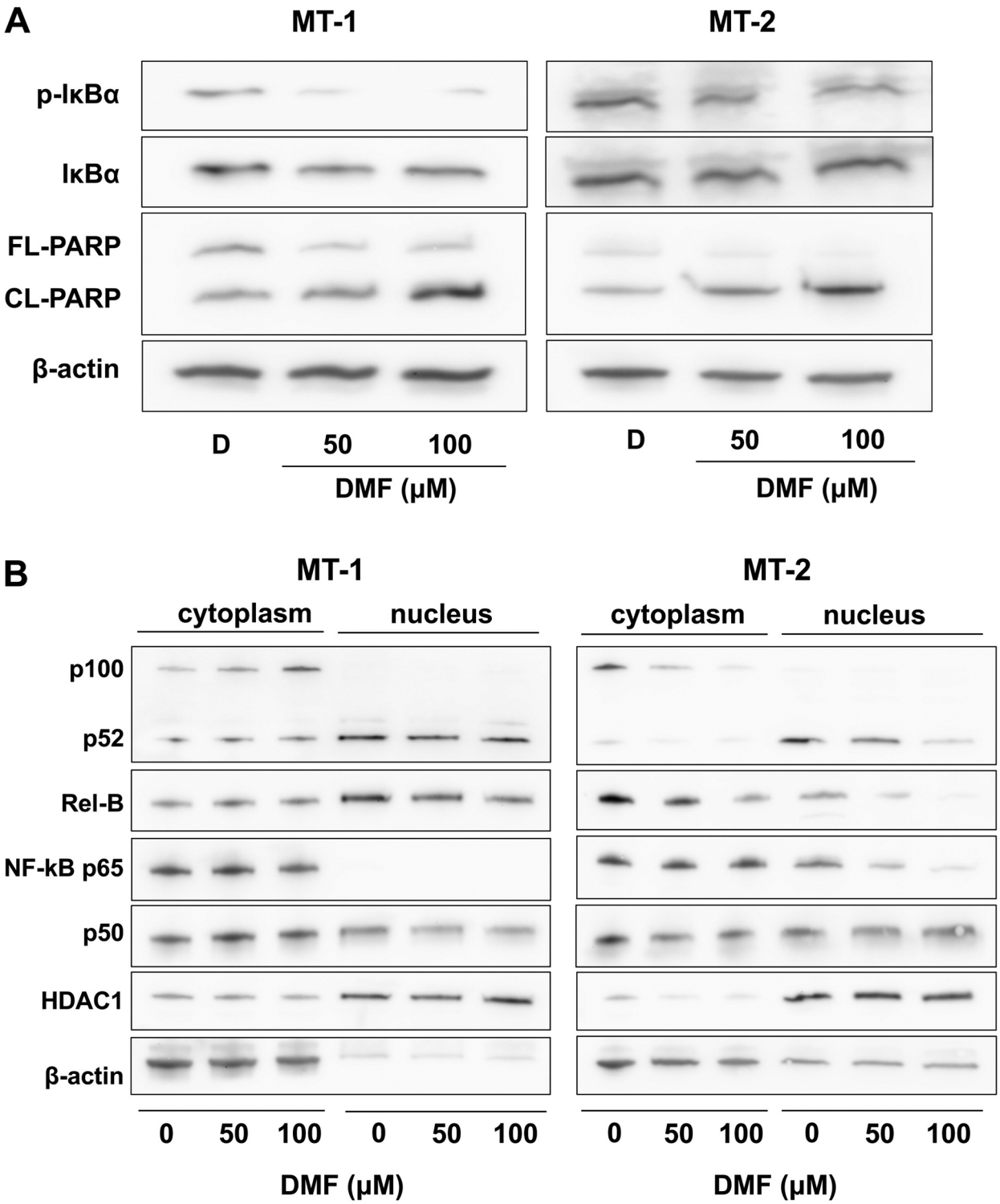

Figure 3. *DMF suppresses the NF-κB signaling pathways and activates PARP in MT-1 and MT-2 cells. (A) MT-1 and MT-2 cells were treated with DMSO and DMF at the indicated concentrations for 24 h. Immunoblotting was performed with specific antibodies against phospho-IκBα at Ser32, IκBα, cleaved PARP and β-actin. (B) MT-1 and MT-2 cells were treated with or without DMF at the indicated concentrations for 48 and 24 h, respectively. Nuclear and cytoplasmic fractions from each extract were prepared as described in Materials and methods. Immunoblotting was performed with specific antibodies against p52, RelB, p65, p50, HDAC1, and β-actin. All the immunoblots shown are representative of two independent experiments. FL-PARP, full-length PARP; CL-PARP, cleaved PARP; D, DMSO.*

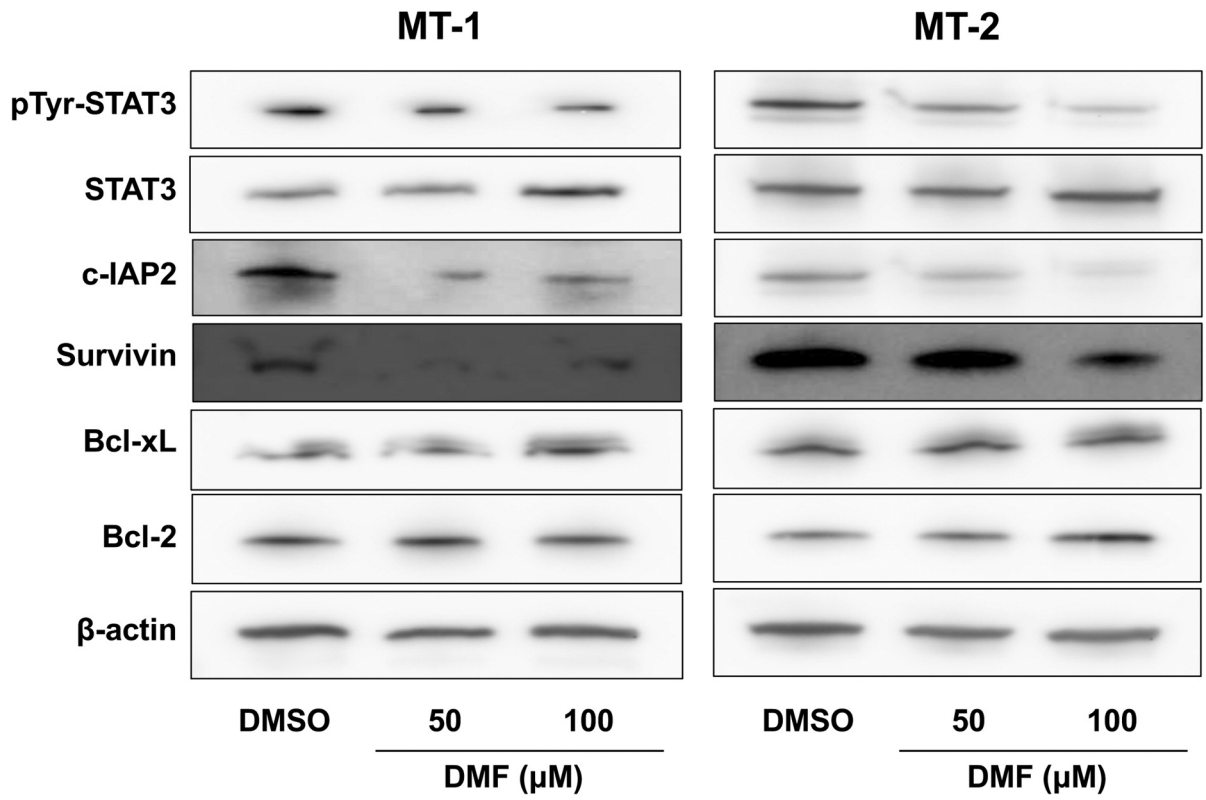

Figure 4. *DMF suppresses the constitutive phosphorylation of STAT3 at Tyr705 and c-IAP2 and survivin protein expression in MT-1 and MT-2 cells. MT-1 and MT-2 cells were treated with DMSO and DMF at the indicated concentrations for 24 h. Immunoblotting was performed with specific antibodies against phospho-STAT3 at Tyr705, STAT3, c-IAP2, survivin, Bcl-xL, Bcl-2 and β-actin. All the immunoblots shown are representative of two independent experiments.*

indicate that DMF induces apoptosis by inhibiting c-IAP2 and survivin expression.

Overall, by suppressing the NF-κB and STAT3 signaling pathways, DMF inhibits proliferation and induces apoptosis in HTLV-1-infected and transformed T cells. DMF should be investigated further as a novel ATL agent.

Conflicts of Interest

The Authors declare no competing financial interests.

Authors' Contributions

All Authors performed experiments. T.M. and S.I. designed the research, wrote the paper and discussed the manuscript.

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