Fludarabine induces growth arrest and apoptosis of cytokine- or alloantigen-stimulated peripheral blood mononuclear cells, and decreases production of Th1 cytokines via inhibition of nuclear factor κB.

Chie Nishioka,[1, 2] Takayuki Ikezoe,[1] Kazuto Togitani,[1] and Akihito Yokoyama [1]

[1]Department of Hematology and Respiratory Medicine, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan, [2]Research Fellow of the Japanese Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo 102-8472, Japan.

Category: Original Article

Subject category: Experimental Therapeutics and Clinical Medicine

Key Words: Fludarabine, NF-κB, Th1 cytokines

Running title: Fludarabine and GVHD

* Address correspondence: Takayuki Ikezoe, MD
Department of Hematology and Respiratory Medicine, Kochi University,
Nankoku, Kochi 783-8505, Japan.
Tel: +81-88-880-2345, Fax: +81-88-880-2348, e-mail: ikezoet@kochi-u.ac.jp

The abbreviations are: PBMCs, peripheral blood mononuclear cells; MTT,

3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; GVHD, graft-versus-host disease; IFNγ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; PHA, phytohemagglutinin.

Summary

Fludarabine is a purine analogue that has demonstrated significant activity in B-cell malignancies, including chronic lymphocytic leukemia. Fludrabine also possesses an immunosuppressive effect and is being used to prevent graft versus host disease (GVHD) in the hematopoietc stem cell transplantation. However, the molecular mechanism by which fludarabine inhibits immunoreaction remains to be fully elucidated. This study found that fludarabine inhibited tumor necrosis factor α (TNF-α)-stimulated degradation of IκBα, resulting in blockade of nuclear translocation of nuclear factor κB (NF-κB) in Jurkat T-cells, as measured by Western blot analysis, as well as immunocytochemistry. The ability of fludarabine to inhibit NF-κB was further confirmed by electrophoretic mobility shift assay. We also found that fludarabine induced growth arrest and apoptosis of alloreactive as well as TNF-α-stimulated peripheral blood mononuclear cells (PBMCs). In addition, fludarabine inhibited TNF-α-stimulated production of IL-2 and IFN-γ, which play important roles in onset of GVHD, in Jurkat cells. Taken together, fludarabine is useful for management of immune disease including GVHD via inactivation of NF-κB.

Introduction

Fludarabine is a purine analogue that has demonstrated significant activity in B-cell malignancies, including chronic lymphocytic leukemia and indolent non-Hodgkin's lymphoma.[1] Fludarabine is converted intracellularly into its active metabolite F-ara-ATP, which inhibits DNA as well as RNA synthesis, resulting in induction of growth arrest and apoptosis of malignant B-cells.[2] Also, fludarabine has been included in conditioning regimens for stem cell transplantation because of its immnunosuppressive effect,[3] although the mechanisms by which fludarabine inhibits T-cell function remain to be fully elucidated.

Nuclear factor κB (NF-κB) is a transcription factor that regulates expression of various genes involved in cell cycle regulation and inhibition of apoptosis, as well as, genes involved in inflammatory and immune responses.[4-6] We previously showed that inactivation of NF-κB by eight herbal mixture PC-SPES successfully rescued mice from LPS-induced septic shock in association with decreased production of inflammatory cytokines.[7]

GVHD is initiated by tissue damage caused by conditioning regimen which consists of chemotherapy and radiotherapy, resulting in release of proinflammatory cytokines including IL-1β and TNF-α.[8-10] Donor T-cell recognition of host alloantigens

activates T-cells and further stimulates cytokine production.[11-13] Released cytokines recruits secondary effectors such as macrophages and NK cells to the target organs, especially to gastrointestinal tract, which damage tissues, resulting in the leak of endotoxin across damaged mucosal barriers, further exacerbating the cytokine cascade. These events destruct tissues and causes, in some cases, death.

This study explored the medicinal action of fludarabine as an immunosuppressive agent and found that it possessed anti-NF-κB activity.

Materials and Methods.

Cells. PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-Conray. PBMCs were suspended in standard RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated in six-well culture plates (Costar, NY, USA) for 2 h at 37 °C with 5% CO_2. The acute lymphoblastic T-cell leukemia Jurkat cells were cultured in standard RPMI 1640 medium (Sigma) supplemented with 10% FBS.

Chemical. Fludarabine phosphate or cytarabine was provided by Schering AG (Berlin, Germany) or Nihon Shinyaku Co. Ltd (Tokyo, Japan), respectively. TNF-α was purchased from Sigma Chemical, Co. (St Louis, MO, USA).

Western blot analysis. Western blot analysis was performed as described previously (7) Anti- IκBα (Imgenex, San Diego, CA), anti-phospho- IκBα, anti-cleaved PARP (Cell Signaling, Beverly, MA), anti-p65 subunit of NF- κB (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-tubulin (Santa Cruz Biotechnology) antibodies were used.

Immunocytochemistry. Jurkat cells were cultured either with or without fludarabine (2.5 μM) for 3 hrs and then exposed to TNF-α (20 ng/ml). After 15 min, cells were harvested and cytocentrifuge slides were prepared. Anti-p65 (Santa Cruz Biotechnology) and anti-mouse IgG antibodies (Amersham Corp., CA, USA) were used

for immunocytochemistry. Immune complexes were visualized using the LSAB2 system (Dako, Corp., Carpinteria, CA). Sections were counterstained with hematoxylin and mounted.

MTT assays. The effect of fludarabine on proliferation of PBMCs was assessed by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium salt (MTT) (Sigma), to a colored formazan product. PBMCs (4 x 10^6/ml) were placed in 96-well plates (Flow Laboratories, Irvine, CA) and cultured in the following conditions in the presence or absence of various concentrations of fludarabine (0.5-5 μM) for 3 days. (1) Culture medium alone (control); (2) culture medium plus phytohemagglutinin (5 μg/ml) or TNF-α (20 ng/ml); (3) culture medium plus irradiated (3 Gy) allogenic PBMCs (4 x 10^6/ml). All experiments were done in triplicate and repeated at least three times.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay was done as previously described.[7] Briefly, 4 μg unclear extracts were incubated with 16 fmol ^{32}P-end labeled, NF-κB binding probe. The DNA-protein complex was separated from the free oligonucleotide on a 5 % polyacrylamide gel. Gels were dried and exposed to Kodak XAR film (Eastman Kodak, New Haven, CT).

Assessment of apoptosis. TNF-α (20 ng/ml)-stimulated PBMCs (1 x 10^6 cells/ml) were

place in 12-well plates and cultured with/without fludarabine (0.5-5 μM). After 24 hrs, apoptosis was evaluated by annexin V staining.

RNA isolation and reverse transcription-polymerase chain reaction. RNA isolation and cDNA preparation were performed as described previously.[14] We measured expression of *18S* for normalization as previously described.[14] Real-time PCR was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) as described previously. [14] Primers for PCR are shown in Table 1. PCR conditions for all genes were as follows: 95°C initial activation for 10 minutes followed by 45 cycles of 95°C for 15 s and 60°C for 30 s, and fluorescence determination at the melting temperature of the product for 20 seconds on an ABI PRISM 7000 (Applied Biosystems).

Statistical analysis. To assess the difference between two groups under multiple conditions, one-way ANOVA followed by Boneferroni's multiple comparison tests were performed using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results

Effect of fludarabine on TNF-α- induced phosphorylation and degradation of IκBα and nuclear translocation of NF-κB (p65) in Jurkat cells. In resting T cells, NF-κB proteins are sequestered in the cytoplasm as latent precursors by IκBα.[15] Upon phosphorylation at Ser32 and Ser36 residues, IκBα undergoes degradation by the 26S proteasome, resulting in release of NF-κB, leading to nuclear translocation and binding to a specific consensus sequence in the DNA. To investigate whether fludarabine inhibits TNF-α-stimulated NF-κB activity in jurkat cells, control and fludarabin-treated Jurkat cells were exposed to TNF-α (20 ng/ml) for various durations and the kinetics of IκBα phosphorylation and degradation were studied by Western blot analysis using cytoplasmic extracts (Figure 1a). As shown in Figure 1a, no significant difference in pattern of IκBα phosphorylation was observed, but the levels of IκBα were higher in fludarabin-treated Jurkat cells than those in control cells, suggesting that fludarabin prevented TNF-α-stimulated degradation of IκBα in Jurkat cells. We next examined the levels of NF-κB in nucleus in these cells. TNF-α (20 ng/ml, 0-60 min) stimulated accumulation of NF-κB in nucleus in both of control and fludarabine-treated cells; however, levels of NF-κB were significantly lower in fludarabin-treated cells (Figure 1b). The ability of fludarabine to inhibit TNF-α-stimulated nuclear translocation of

NF-κB was further comfirmed by immunocytochemistry (Figure 1c). One the other hand, another nucleoside analogue cytarabine did not affect TNF-α-stumulated nuclear translocation of NF-kB in Jurkat cells (Figure 1c).

Effect of fludarabine on NF-kB activity in Jurkat cells. We next examined whether fludarabine affected NF-κB activity in Jurkat cells by utilizing electrophoretic mobility shift assay (Figure 2). Exposure of Jurkat cells to TNF-α stimulated NF-kB/DNA binding (Figure 2). Pre-incubation of Jurkat cells with fludarabine (2.5 mM, 60 min) disrupted formation of TNF-α-stimulated NF-kB/DNA binding complex (Figure 2). The NF-kB/DNA complex was competed with 100 times molar excess of unlabeled oligonucleotides but not with the same molar excess of mutated oligonucleotides, confirming the specificity of NF-κB binding (Figure 2).

Fludarabine inhibits proliferation of TNF-α-, PHA-, and alloantigen- activated PBMCs cells. NF-κB is activated by extracellular signals including TNF-α [16, 17] and PHA, [18] and regulates proliferation of activated T lymphocytes.[18] We therefore examined whether fludarabine affected proliferation of T lymphocytes. Either TNF-α (20 ng/ml) or PHA (5 μg/ml) stimulated proliferation of PBMCs from healthy volunteers (n=3) by 1.25- and 2.5-fold, respectively, on the third day of culture, as measured by MTT assay. (Figures 3a, b). Exposure of these cells to fludarabine (0.5-5

μM) significantly inhibited their proliferation. Likewise, irradiated (3 Gy) allogeneic PBMCs stimulated proliferation of normal PBMCs by 2-fold, and fludarabine effectively blocked alloreactive expansion of PBMCs (Figure 3c). On the other hand, fludarabine did not affect viability of the resting PBMCs (Figures 3a-c). Control studies found that cytarabine also inhibited proliferation of TNF-α or PHA-stimulated, but not resting PBMCs (Figures 3d, e).

Fludarabine induces apoptosis of TNF-α-activated T cells. To investigate the mechanism by which fludarabine inhibits the proliferation of activated PBMCs, we utilized annexin V staining. PBMCs from healthy volunteers were cultured in the presence of TNF-α (20 ng/ml) either with or without fludarabine. After 24 h, apoptosis was assessed by Annexin V binding and propidium iodide (PI) staining using FACSCan. As shown in Figure 4, fludarabine (0.5-5 μM, 24 h) induced approximately 26 % of TNF-α-activated PBMCs to become Annexin +/PI -, a feature characteristic of apoptosis (Figure 4a). On the other hand, approximately 14 % of control cells became apoptotic after 24 h (Figure 4a). In addition, Western blot analysis showed that exposure of PBMCs to fludarabine (2.5 or 5 μM, 24 h) induced cleavage of PARP, which is thought to be a late event in apoptosis, in TNF-α-stimulated PBMCs, confirming the ability of fludarabine to induce apoptosis (Figure 4b).

Effect of fludarabine on TNF-α-stimulated cytokine production. Furthermore, we studied whether fludarabine affected TNF-α-stimulated production of proinflammatory cytokines in Jurkat cells and PBMCs. Exposure of Jurkat cells to TNF-α (20 ng/ml, 1h) profoundly induced expression of IFN-γ and IL-2, and pre-incubation of these cells with fludarabine (2.5 μM, 3 hrs) decreased TNF-α-stimulated expression of either IFN-γ and IL-2 by approximately 90 % and 80 %, respectively, as measured by real-time PCR (Figure 5a, b). Likewise, fludarabine decreased cytokine-stimulated production of TNF-α in PBMCs (Figure 5c, d).

Discussion

Fludarabine has been used for treatment of B-cell malignancies such as chronic lymphocytic leukemia. Fludarabine induced apoptosis of malignant B-cells.[19] We have recently shown that fludarabine was also active against T-cell malignancies; fludarabine induced growth arrest and apoptosis of adult T-cell leukemia (ATL) cells. We found that NF-□B abberantly expressed in nucleus in ATL cells. Exposure of ATL cells to fludarabine inhibited nuclear accumulation of NF-κB and downregulated levels of NF-κB-target molecule XIAP, which is a member of inhibitor of apoptosis proteins, in these cells. [20]

Similarly, this study demonstrated that fludarabine blocked TNF-α-induced nuclear translocation of NF-κB in Jurkat T-cells, resulting in inactivation of this prosurvival nuclear transcription factor. As expected, fludarabine inhibited cytokine-, as well as alloantigen-stimulated proliferation of PBMCs in conjunction with decreased in production of Th1 cytokines such as IL-2 and IFN-γ. IL-2 is secreted by donor $CD4^+$ T cells in the first several days after on-set of GVHD induction, [21] and induces cytotoxic T-lymphocyte (CTL) response. Monoclonal antibodies against IL-2 or its receptor was able to prevent GVHD when administered shortly after the infusion of T cells. [22, 23] In addition, both of cyclosporine and FK506, an immunosuppressive agent, dramatically

reduced IL-2 production and effectively prevent GVHD. [24] IFN-γ is also crucial cytokine in the second step of the pathophysiology of acute GVHD, which primes mononuclear phagocytes to produce TNF-α and IL-1. High IFN-γ levels were associated with acute GVHD, [25-27] and a large proportion of T-cell clones isolated from GVHD patients produced IFN-γ. [28] Thus, inhibition of production of IL-2 and IFN-γ may alleviate severity of acute GVHD.

Another nucleoside analogue cytarabine also inhibited the proliferation of TNF-α or PHA-stimulated proliferation of PBMCs (Figures 3d, e); however, cytarabine did not affect TNF-α induced nuclear translocation of NF-κB in Jurkat cells (Figure 1c). These observations suggested that inhibition of NF-κB was not universal among nucleoside analogues.

Taken together, fludarabine acts as an immunosuppressive agent via inhibition of NF-κB. Further studies are warranted to evaluate the effect of fludarabine in inflammatory diseases including GVHD.

Acknowledgements

This work was supported in part by Grant-in-Aid from the Ministry of Education, Culture Sports, Science, and Technology of Japan and the Fund for Academic Research from Kochi University.

References

1. Keating MJ, Kantarjian H, Talpaz M, Redman J, Koller C, Barlogie B, *et al.* Fludarabine: a new agent with major activity against chronic lymphocytic leukemia. *Blood* 1989; **74**: 9-25.

2. Noker PE, Duncan GF, El Dareer SM, Hill DL.Disposition of 9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-phosphate in mice and dogs. *Cancer Treat Rep* 1983; **67**: 445-456.

3. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat Med* 1999; **5**: 444-447.

4. Wang Y, Wu TR, Cai S, Welte T, Chin YE. Stat1 as a component of tumor necrosis factor alpha receptor 1-TRADD signaling complex to inhibit NF-kappaB activation. *Mol Cell Bio.* 2000; **20**: 4505-4512.

5. Aggarwal BB. Nuclear factor-kappaB: the enemy within. *Cancer Cell* 2004; **6**: 203-208.

6. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; **431**: 461-466.

7. Ikezoe T, Yang Y, Heber D, Taguchi H, Koeffler HP. PC-SPES: potent inhibitor of

nuclear factor-kappa B rescues mice from lipopolysaccharide-induced septic shock. *Mol Pharmacol* 2003; **64**:1521-1529.

8. Xun CQ, Thompson JS, Jennings CD, Brown SA,Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft versus host disease in H-2 incompatible transplanted SCID mice. *Blood* 1994; **83**: 2360-2367.
9. Gonzalez M, Quezada SA, Blazar BR, Panoskaltsis-Mortari A, Rudensky AY, Noelle RJ. The balance between donor T cell anergy and suppression versus lethal graft-versus-host disease is determined by host conditioning. *J Immuno.* 2002; **169**: 5581-5589.
10. Reddy P. Pathophysiology of acute graft-versushost disease. *Hematol Oncol* 2003; **21**: 149-161.
11. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, *et al*. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 1999; **285**: 412-415.
12. Zhang Y, Shlomchik WD, Joe G, Louboutin JP, Zhu J, Rivera A, *et al.* APCs in the liver and spleen recruit activated allogeneic CD8+ T cells to elicit hepatic

graft-versus-host disease. *J Immunol* 2002; **169**:7111-7118.

13. Schmaltz C, Alpdogan O, Muriglan SJ, Kappel BJ, Rotolo JA, Ricchetti ET, *et al.* Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* 2003; **101**: 2440-2445.
14. Ikezoe T, Tanosaki S, Krug U, Liu B, Cohen P, Taguchi H, Koeffler HP. Insulin-like growth factor binding protein-3 antagonizes the effects of retinoids in myeloid leukemia cells. *Blood* 2004; **104**: 237-242.
15. Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649-683.
16. Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U S A* 1989; **86**: 2336-2340.
17. Israel A, Le Bail O, Hatat D, Piette J, Kieran M, Logeat F, *et al.* TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors. *EMBO J* 1989; **8**: 3793-3800.
18. Blanco B, Perez-Simon JA, Sanchez-Abarca LI, Carvajal-Vergara X, Mateos J, Vidriales B, *et al.* Bortezomib induces selective depletion of alloreactive T

lymphocytes and decreases the production of Th1 cytokines. *Blood* 2006; **107**: 3575-3583.

19. Sanhes L, Tang R, Delmer A, DeCaprio JA, Ajchenbaum-Cymbalista F. Fludarabine-induced apoptosis of B chronic lymphocytic leukemia cells includes early cleavage of p27kip1 by caspases. *Leukemia* 2003; **17**:1104-1111.
20. Nishioka C, Ikezoe T, Yang J, Koeffler HP, Taguchi H. Fludarabine induces apoptosis of human T-cell leukemia virus type 1-infected T cells via inhibition of the nuclear factor-kappaB signal pathway. *Leukemia* 2007; **21**: 1044-1049.
21. Via CS, Finkelman FD. Critical role of interleukin-2 in the development of acute graft-versus-host disease. *Int Immunol* 1993; **5**: 565-572.
22. Ferrara JL, Marion A, McIntyre JF, Murphy GF, Burakoff SJ. Amelioration of acute graft vs host disease due to minor histocompatibility antigens by in vivo administration of anti-interleukin 2 receptor antibody. *J Immunol* 1986; **137**: 1874-1877.
23. Herve P, Wijdenes J, Bergerat JP, Bordigoni P, Milpied N, Cahn JY, ***et al.*** Treatment of corticosteroid resistant acute graft-versus-host disease by in vivo administration of anti-interleukin-2 receptor monoclonal antibody (B-B10). *Blood* 1990; **76**: 2639-2640.

24. Li XC, Demirci G, Ferrari-Lacraz S, Groves C, Coyle A, Malek TR, *et al.* IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat Me.* 2001; **7**: 114-118

25. Szebeni J, Wang MG, Pearson DA, Szot GL, Sykes M. IL-2 inhibits early increases in serum ã-interferon levels associated with graft-versus-host disease. *Transplantatin* 1994; **58**: 1385-1393.

26. Wang MG, Szebeni J, Pearson DA, Szot GL, Sykes M. Inhibition of graft-versus-host diseaseby interleukin-2 treatment is associated with altered cytokine production by expanded graft-versus-host-reactive $CD4^+$ helper cells. *Transplantation* 1995; **60**: 481-490.

27. Troutt AB, Maraskovsky E, Rogers LA, Pech MH, Kelso A. Quantitative analysis of lymphokine expression in vivo and in vitro. Immunol Cell Biol 1992; **70**: 51-57.

28. Velardi A, Varese P, Terenzi A, Dembech C, Albi N, Grossi CE, *et al.* Lymphokine production by T-cell clones after human bone marrow transplantation. *Blood* 1989; **74**: 1665-1672.

Figure legends

Figure 1. Effect of fludarabine on TNF-α induced phosphorylation and degradation of IκB (a) and nuclear translocation of NF-κB (b), (c) in Jurkat cells. Jurkat cells were pretreated with either fludarabine (2.5 μM) or diluent for 3hrs. At the end of the treatment, cells were washed twice with PBS and exposed to TNF-α (20 ng/ml) for the indicated time periods. The cytoplasmic **(a)** and nuclear extracts **(b)** of these cells were prepared and subjected to Western blot analysis to measure the level of IκBα and phospho-IκBα, and p65 of NF-κB, respectively. **(c) Immunocytochemistry.** Jurkat cells were cultured either with or without fludarabine (2.5 μM) or cytarabine (2.5 μM) for 3 hrs. At the end of the treatment, cells were exposed to TNF-α (20 ng/ml) for 15 min. Cells were harvested and cytocentrifuge slides were prepared. Anti-NF-κB and anti-mouse IgG antibodies were used and immune complexes were visualized using the LSAB2 system. Sections were counterstained with hematoxylin.

Figure 2. Effect of fludarabine on TNF-a-stimulated NF-kB/DNA binding formation in Jurkat cells. Jurkat cells were pretreated with either fludarabine (2.5 mM) or diluent for 3h. At the end of the treatment, cells were washed twice with phosphate-buffered saline and exposed to TNF-α (20 ng/ml) for 60 min. Nuclear extracts were prepared and electrophoretic mobility shift assay was performed as

described in Materials and methods. The arrow indicates the gel location of NF-κB bound to DNA. WT, wildtype oligonucleotide; mt, mutant oligonucleotide; TNF-α, tumor necrosis factor a; NF-κB, nuclear factor κB.

Figure 3. Fludarabine inhibits the proliferation of TNF-α- (a, d), PHA- (b, e), or alloantigen- (c) stimulated PBMCs. PBMCs from healthy volunteers were cultured with/without TNF-α (20 ng/ml) **(a, d)** or PHA (5 μg/ml) **(b, e)** in the presence or absence of various concentrations of fludarabine (0.5-5 μM) **(a, b)** or cytarabine **(d, e)**. **(c)** PBMCs were cocultured with irradiated (3 Gy) allogeneic PBMCs and exposed to various concentrations of fludarabine (0.5-5 μM). Cell viability was assed by MTT assay on day 3 of culture. Results represent the mean ± SD of three experiments done in triplicate. Statistical analysis was performed by one-way ANOVA followed by Boneferroni's multiple comparison tests.

Figure 4. Fludarabine induces apoptosis of activated T cells. (a) TNF-α-stimulated PBMCs (1 x 10^6 /ml) were cultured with/without fludarabine. The ability of fludarabine to induce apoptosis was assessed by propidium idodide (PI) and annexin V staining. Lower left quadrants; viable cells (Annexin V- / PI-). Lower right quadrants; early apoptotic cells (Annexin V+/ PI-). Upper left quadrants; necrotic cells (Annexin V- / PI+). Upper right quadrants; late apoptotic cells (Annexin V+ / PI+). The result shows

one of the representative experiments performed three times independently. Flu, Fludarabine. **(b) Western blot analysis of activated caspase (cleaved PARP) in PBMCs.** TNF-α-stimulated PBMCs (1 x 10^6 /ml) were cultured with/without fludarabine (2.5 or 5 μM). After 24hrs, cells were harvested, and proteins were extracted and subjected to Western blot analysis. The membranes were sequentially proved with anti-PARP and anti-α-tubulin antibodies.

Figure 5. Effect of fludarabine on TNF-α-stimulated cytokine expression in Jurkat cells. (a, b) Jurkat cells and **(c, d)** PBMCs were pretreated with either fludarabine (2.5 μM) or diluent for 3 hrs. At the end of the treatment, cells were washed twice with PBS and treated with TNF-α (20 ng/ml) for 1h. Cells were harvested and RNA was extracted. cDNAs were synthesized and subjected to real-time PCR by using SYBY Green I nucleic acid gel staining solution. Results represent the mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Boneferroni's multiple comparison test. Flu, Fludarabine.

Table 1. PCR primers.

Protein	Direction	Primer
IL-2	Forward	5’-TGCAACTCCTGTCTTGCATT-3’
	Reverse	5’-TCCAGCAGTAAATGCTCCAG-3’
IFN-γ	Forward	5’-TCATCCAAGTGATGGCTGAA-3’
	Reverse	5’-CTTCGACCTCGAAACAGCAT-3’
18S	Forward	5’-AAACGGCTACCACATCCAAG-3’
	Reverse	5’-CCTCCAATGGATCCTCGTTA-3’

Figure 1a

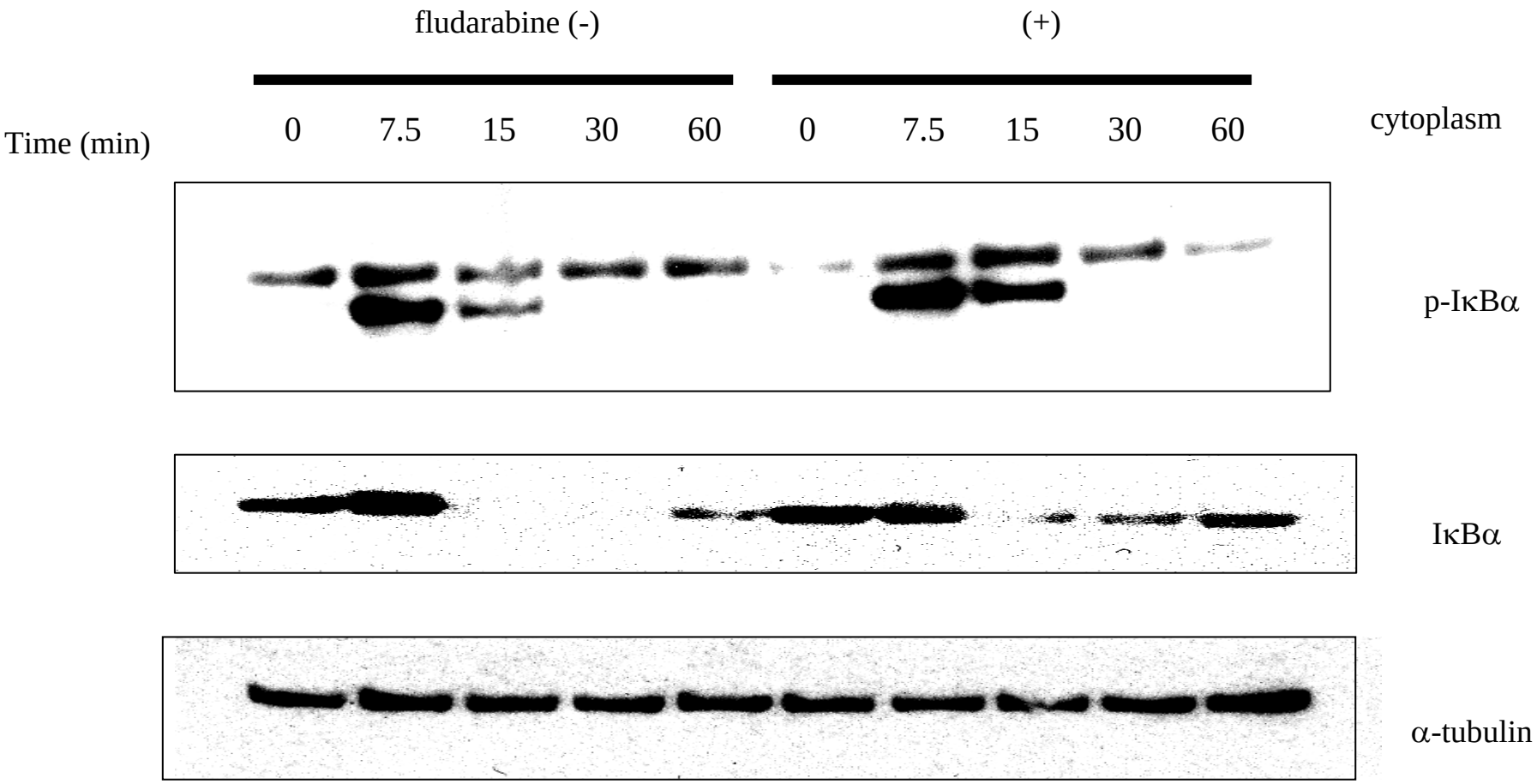

fludarabine (-)
(+)
Time (min)
0
7.5
15
30
60
0
7.5
15
30
60
cytoplasm
p-IκBα
IκBα
α-tubulin

Figure 1b

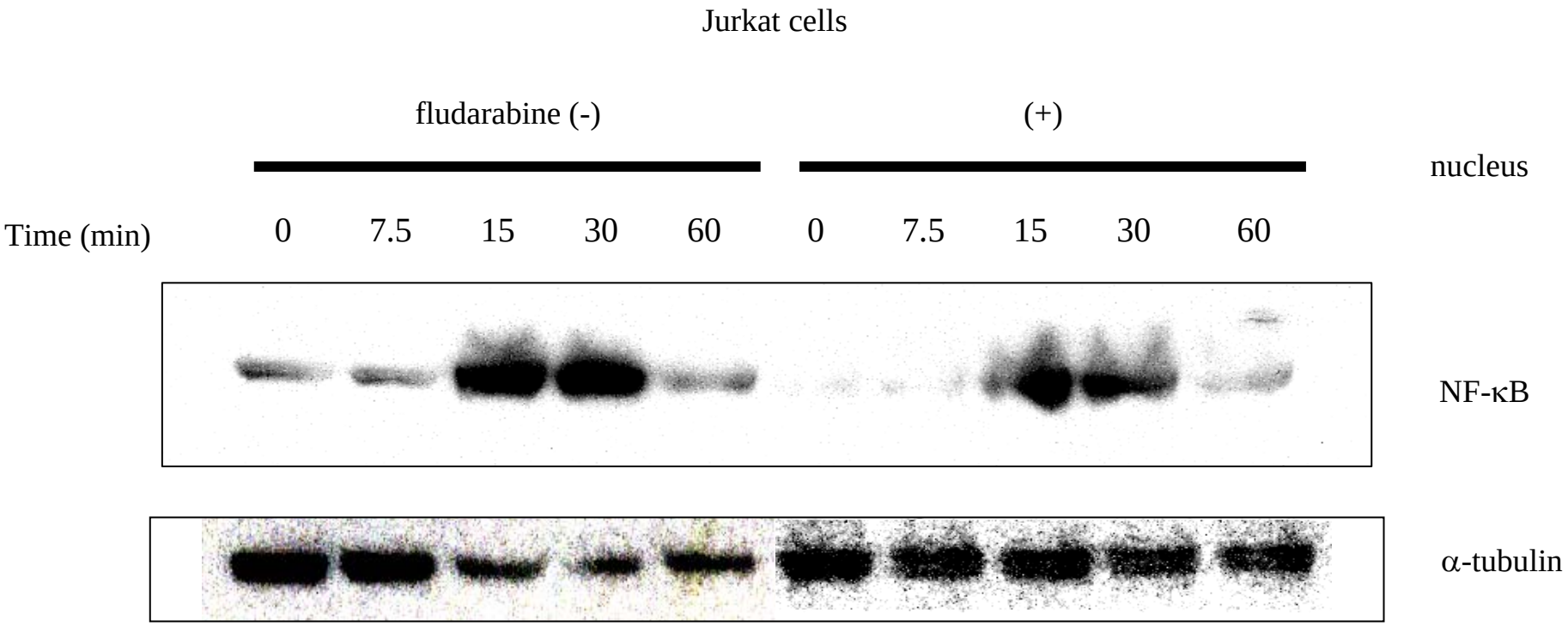
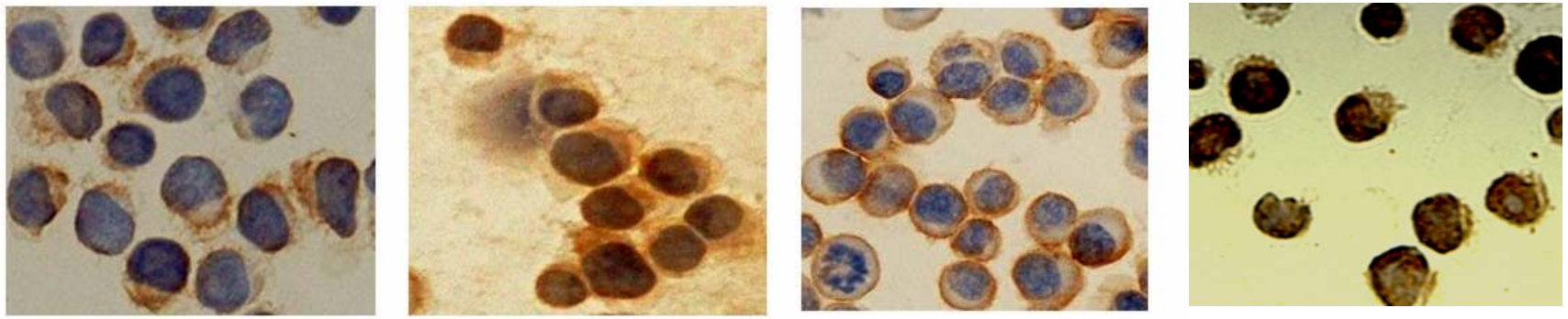

Jurkat cells
fludarabine (-)
(+)
nucleus
Time (min)
0
7.5
15
30
60
0
7.5
15
30
60
NF-κB
α-tubulin

Figure 1c

TNF-α	-	+	+	+
Fludarabine	-	-	+	-
cytarabine	-	-	-	+

NF-κB



IgG

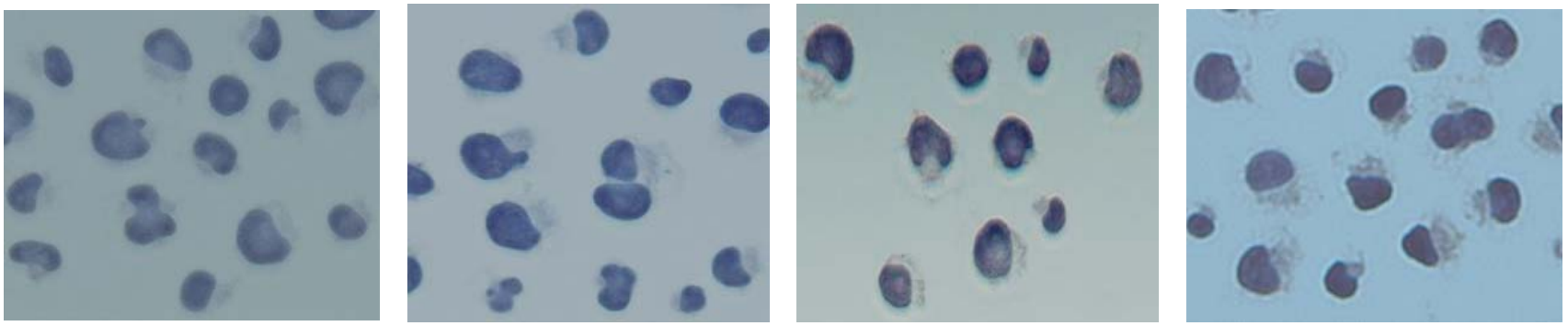


Figure 2

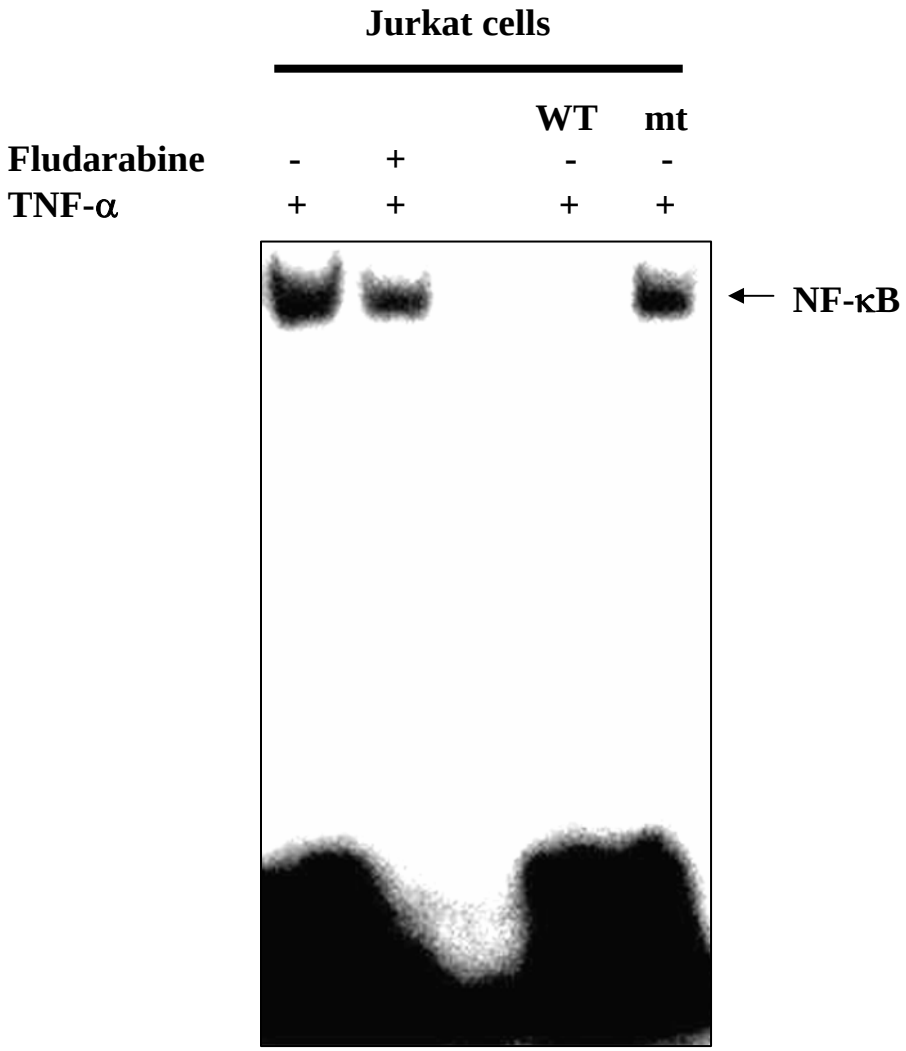
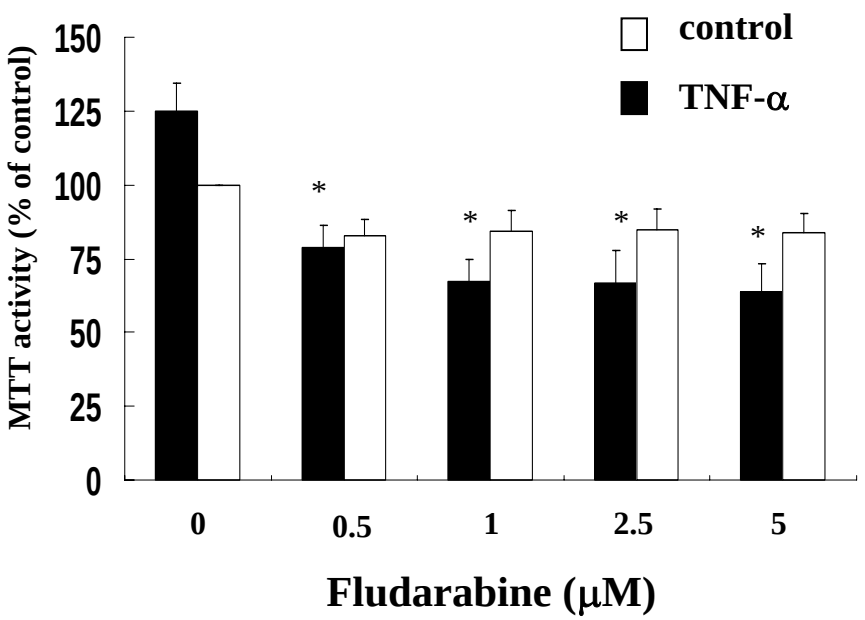
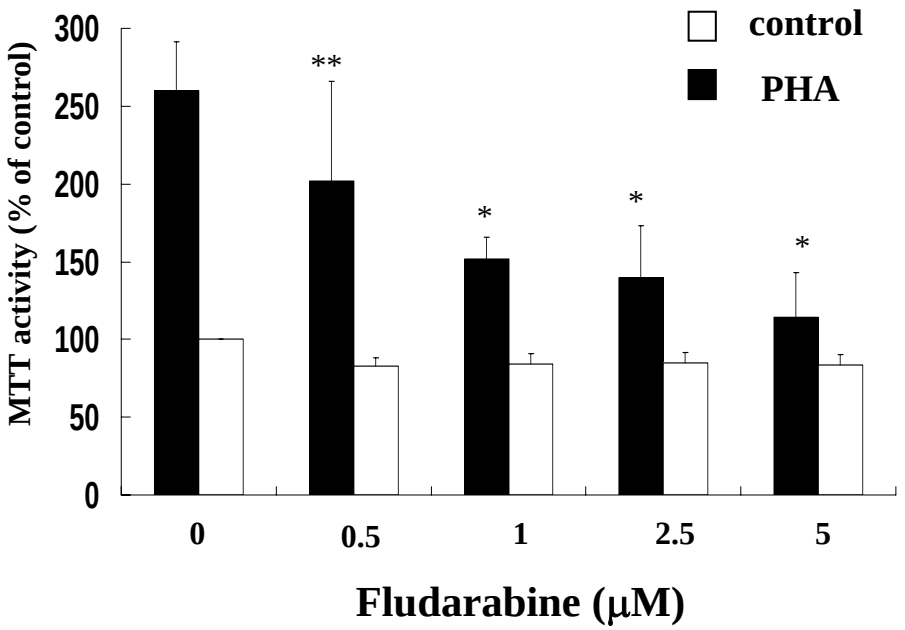


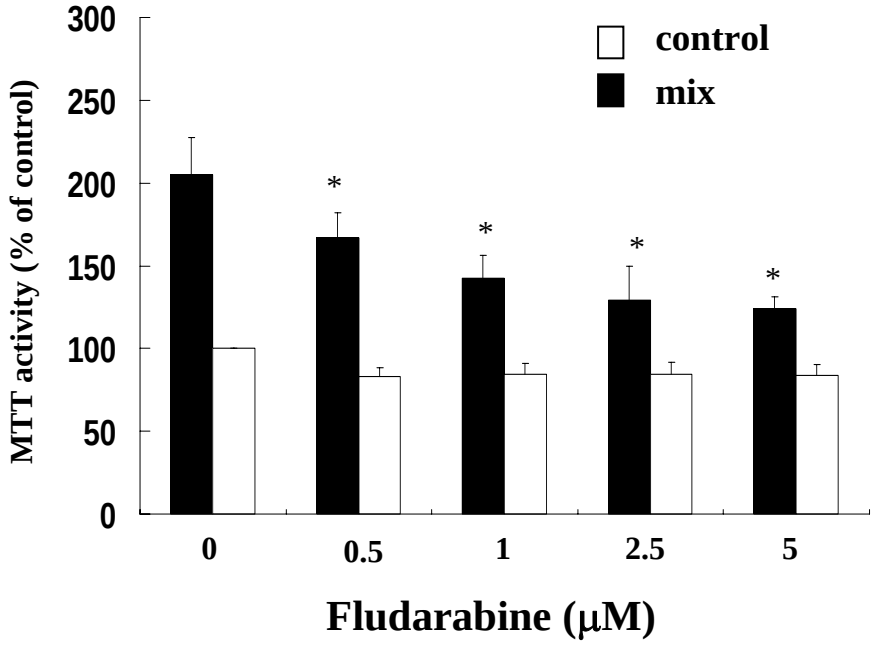
Figure 3 a



b

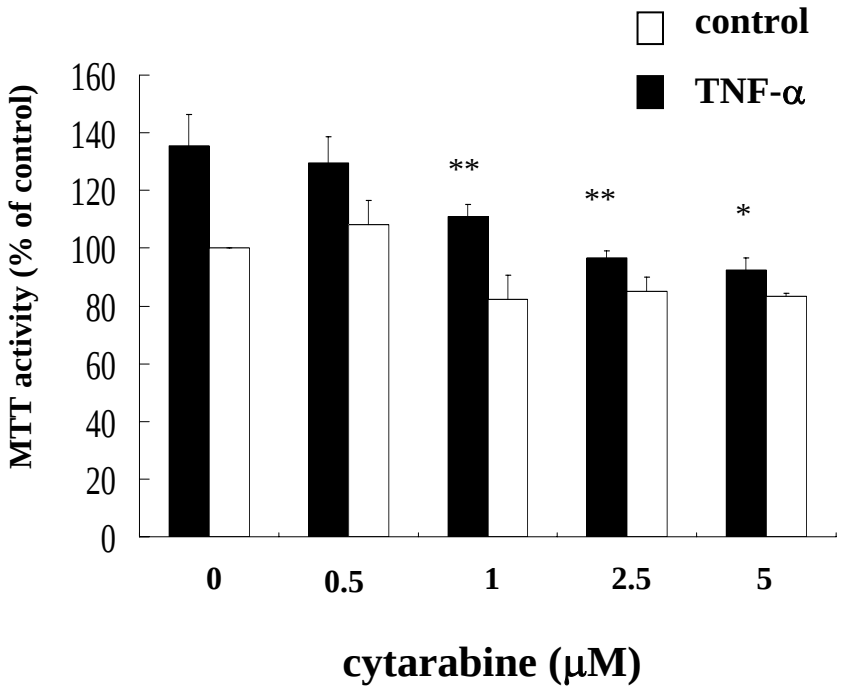


c

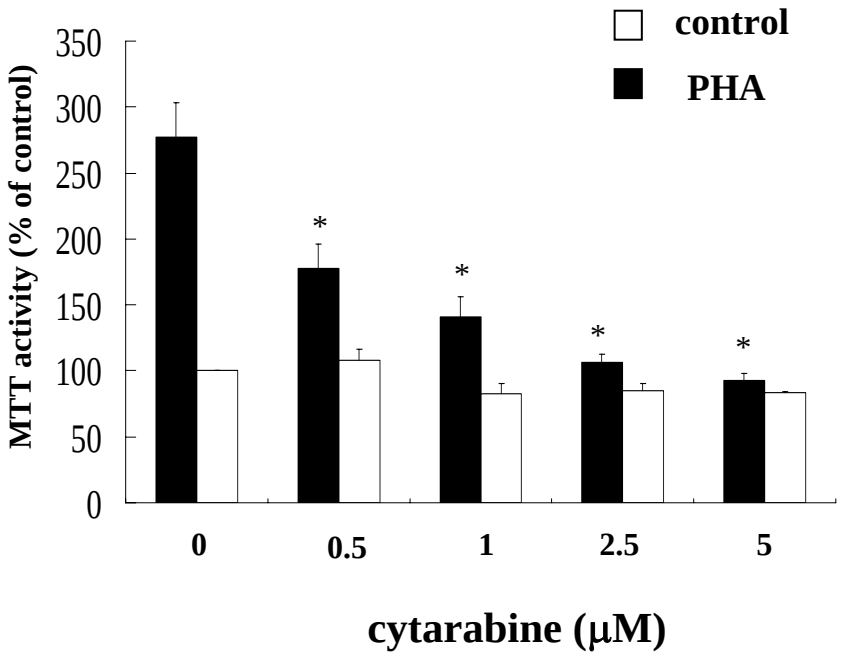


* $p<0.01$
** $p<0.05$

Figure 3 d



e

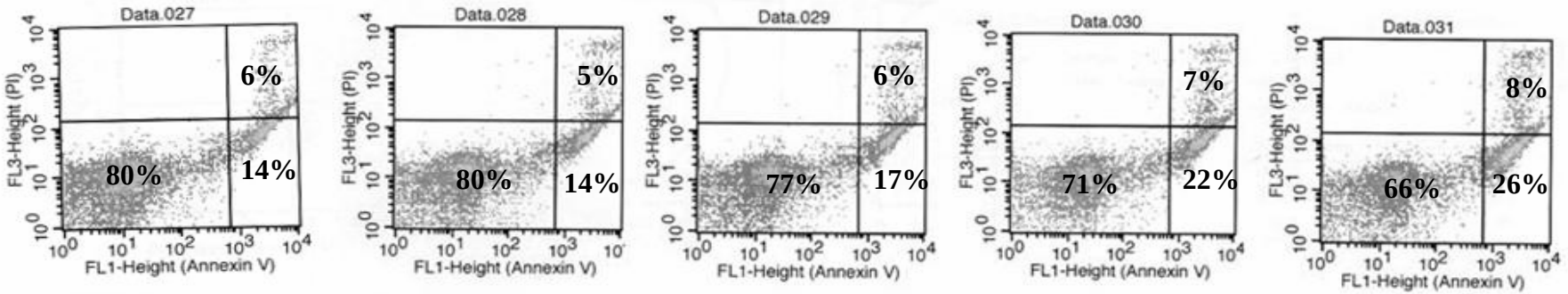


* $p<0.01$

** $p<0.05$

Figure 4

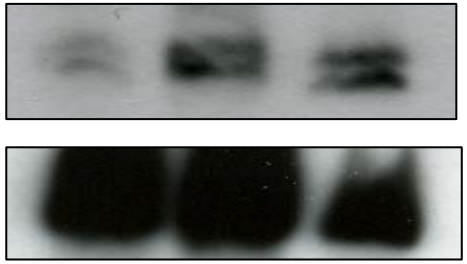
a



TNF-α 20 (ng/ml)	+	+	+	+	+
Flu (μM)	−	**0.5**	**1**	**2.5**	**5**

b

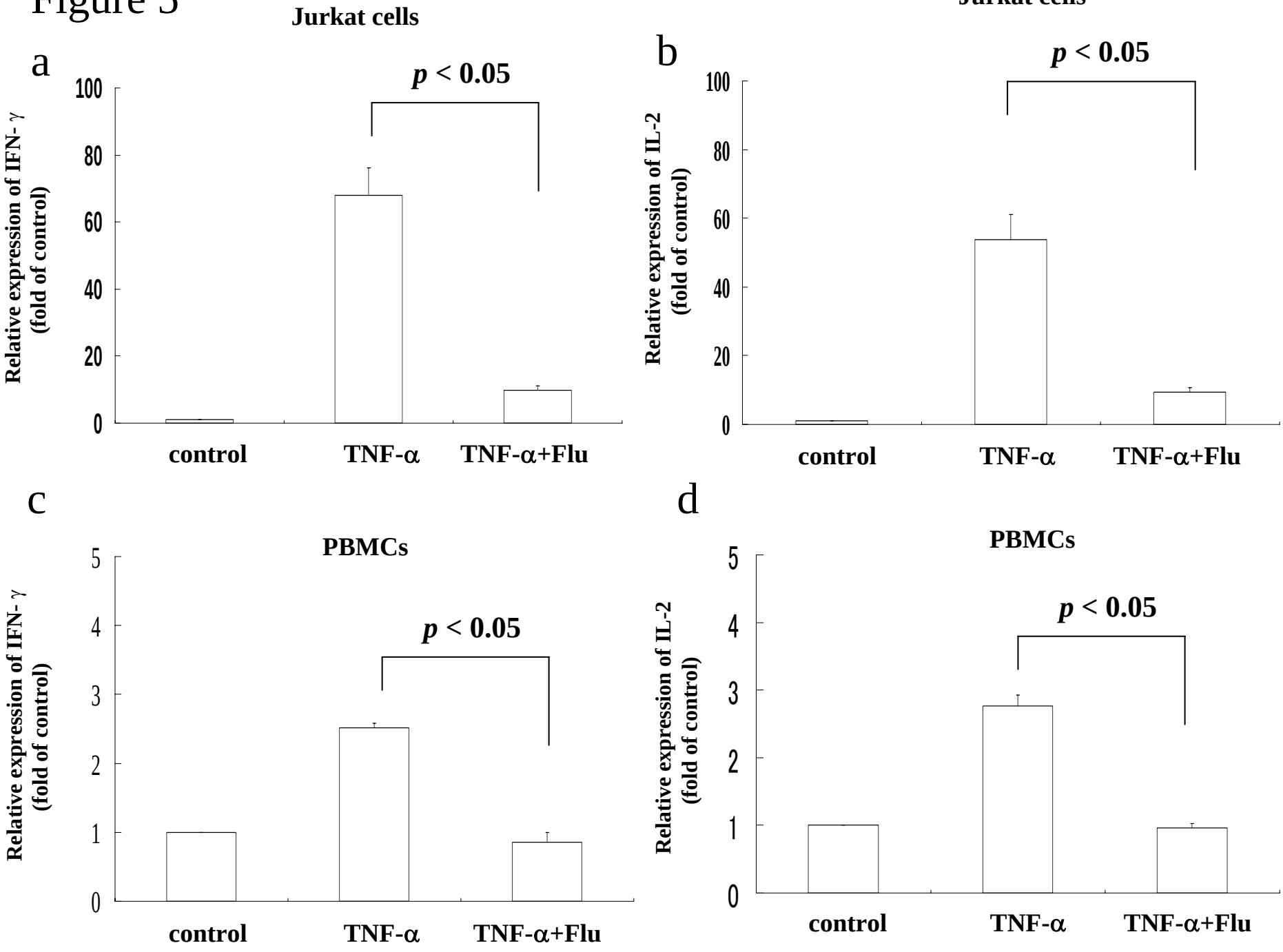
TNF-α 20 (ng/ml)	+	+	+
Flu (μM)	−	**2.5**	**5**



cleaved-PARP

α-tubulin

Figure 5


a
Jurkat cells
p < 0.05
Relative expression of IFN- γ (fold of control)
100
80
60
40
20
0
control
TNF-α
TNF-α+Flu
b
Jurkat cells
p < 0.05
Relative expression of IL-2 (fold of control)
100
80
60
40
20
0
control
TNF-α
TNF-α+Flu
c
PBMCs
p < 0.05
Relative expression of IFN- γ (fold of control)
5
4
3
2
1
0
control
TNF-α
TNF-α+Flu
d
PBMCs
p < 0.05
Relative expression of IL-2 (fold of control)
5
4
3
2
1
0
control
TNF-α
TNF-α+Flu