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DHMEQ, a novel nuclear factor-κB inhibitor, induces selective depletion of alloreactive or PHA-stimulated peripheral blood mononuclear cells, decreases production of Th1 cytokines, and blocks maturation of dendritic cells

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Key Words: DHMEQ, Th1 cytokine, NF-κB, dendritic cell, CD40

Running title: DHMEQ against inflammatory diseases

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The abbreviations are: DHMEQ, Dehydroxymethylepoxyquinomicin; PBMCs, peripheral blood mononuclear cells; MTT, 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; GVHD, graft-versus-host disease; DCs, dendritic cells; APCs, antigen-presenting cells; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-2, interleukin 2; PHA, phytohemagglutinin.

Summary

Dehydroxymethylepoxyquinomicin (DHMEQ), a novel nuclear factor κB (NF-κB) inhibitor, has been shown to be active against variety types of solid tumors as well as hematological malignant cells. This study explored the anti-inflammatory effects of DHMEQ in vitro. DHMEQ inhibited the proliferation of PHA-stimulated or alloreactive peripheral blood mononuclear cells (PBMCs) in mixed lymphocyte cultures as measured by 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. On the other hand, DHMEQ did not affect viability of resting PBMCs. In addition, real-time PCR showed that DHMEQ decreased PHA-stimulated expression of Th-1 cytokines including IL-2, IFN-γ, and TNF-α in PBMCs as well as Jurkat T-lymphoblastic leukemia cells in association with decreased levels of p65 isoforms of NF-κB in nucleus. Furthermore, we found that DHMEQ inhibited endocytic capacity of dendritic cells in conjunction with down-regulation of expression of cell surface antigen CD40, suggesting that DHMEQ blocked maturation as well as function of dendritic cells. Taken together, DHMEQ may be useful for treatment of inflammatory diseases including graft-versus-host disease after allogenic hematopoietic stem cell transplantation.

Introduction

GVHD is the major complication after allogeneic stem cell transplantation (HST) and is a manifestation of the alloreactive response to host histocompatibility differences mediated by mature donor T cells [1-3]. GVHD is induced by a three-step process where the innate and adaptive immune systems interact. (i) The conditioning regimen with irradiation and/or chemotherapy lead to damage of host tissues throughout the body and activate the secretion of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1) [4]. These cytokines enhance donor T-cell recognition of host alloantigens by increasing expression of major histocompatibility complex (MHC) antigens and other molecules on host antigen-presenting cells (APSs), such as dendritic cells (DCs). (ii) Host APCs present alloantigen in the form of a peptide human leukocyte antigen (HLA) complex to the resting T cells. The interaction of APCs and donor T cells triggers costimulatroy signals, which further activates these immune cells to produce cytokines, including IL-2 and IFN-γ, which primes mononuclear phagocytes to produce TNF-α and IL-1 [5]. (iii)Effector functions of mononuclear phagocytes and neutrophils are triggered through a secondary signal provided by mediators, such as lipopolysaccharide (LPS), that leaks through the damaged intestinal mucosa caused by irradiation and/or chemotherapy. Released inflammatory chemokines

thus recruits effector cells into target organs, resulting in tissue destruction and, in some cases, death. Thus,inappropriate production of c ytokines is intimately involved in pathogenesis of acute GVHD [6,7].

NF-κB is a generic term for a dimeric transcription factor formed by the hetero- or homodimerization of a number of the rel family members [8]. To date, five rel proteins have been identified: RelA (p65), RelB and cRel, each having transactivation domains, and p50 and p52, which are expressed as the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), respectively. These precursors require post-translational processing and do not contain transactivation domains. The most abundant and active forms of NF-κB are dimeric complexes of p50/RelA (p50/p65). NF-κB is considered to play a pivotal role in immune and inflammatory responses through the regulation of genes encoding proinflammatory cytokines. These proinflammatory cytokines are supposed to be critical mediators of graft-versus-host disease (GVHD) [6-7, 9-11]. Therefore, a rationale target for either prevention or treatment of GVHD may be NF-κB.

Dehydroxymethylepoxyquinomicin (DHMEQ), a specific inhibitor of nuclear factor κB (NF-κB) nuclear translocation, has been shown to be active against variety types of solid tumors as well as hematological malignant cells [12-16].

We have previously shown that DHMEQ blocked TNF-α-induced nuclear

translocation of NF-κB in Jurkat T-lymphoblastic leukemia cells [17]. These observations raised the possibility that DHMEQ might inhibit exaggerated cytokine production in inflammatory disease such as GVHD.

Materials and Methods.

Reagents. DHMEQ was synthesized in our laboratory [18]. It was dissolved in DMSO to prepare a 10 µg/ml solution and subsequently diluted in culture medium to a final DMSO concentration of <0.1%.

Cells. The acute lymphoblastic T-cell leukemia Jurkat cells were cultured in standard RPMI 1640 medium (Sigma, St. Louis, Missouri) supplemented with 10% FBS. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers after obtaining informed consent.

MTT assays. PBMCs ($4x10^6$/ml) from healthy volunteers were cultured in the following conditions with or without various concentrations of DHMEQ (0.5-3 µg/ml) for 3 days in 96-well plates (Flow Laboratories, Irvine, CA). (i) Culture medium alone (control); (ii) culture medium plus phytohemagglutinin (PHA, 5 µg/ml); (iii) culture medium plus irradiated (3 Gy) allogenic PBMCs ($4x10^6$/ml). After culture, cell number and viability were evaluated 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. All experiments were done in triplicate and repeated at least three times.

Assessment of apoptosis. PBMCs were plated at a density of $2x10^6$/ml and incubated

with PHA (5 μg/ml) or various concentrations of DHMEQ (0.5-3 μg/ml) either alone or in combination in 12-well plates (Flow Laboratories, Irvine, CA). The ability of DHMEQ to induce apoptosis of PHA-stimulated PBMCs was measured by annexin V-FITC apoptosis detection kit according to the manufacturer's instruction (Pharmingen, Inc., San Diego, CA).

Generation of monocyte-derived dendritic cells. DCs were generated by differentiation of PBMCs in the presence of 50 ng/ml GM-CSF (Sigma, Saint Louis, MO, USA) and 10 ng/ml IL-4 (Sigma). The medium was replenished with cytokines every other day. Maturation of differentiated DC was accomplished by treating with TNF-α (10 ng/ml, 48 hrs)(Sigma) for another two days. On day 5 of culture, 0.5 μg/ml DHMEQ was added to examine whether DHMEQ blocked maturation of DCs. Cells were harvested for further experiments at day 7 of culture.

Flow cytometry analysis. To measure whether DHMEQ blocks maturation of DCs, levels of CD40 antigen on cell surface of DCs were measured byusing flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). The phycoerythrin (PE)-cojugated anti-human CD40 monoclonal antibody (mAb) and PE Mouse IgG1 K Isotype Control was purchased from eBioscience (San Diego, CA, USA). Live cells ($1x10^4$) were gated and analyze.

Analysis of endocytic capacity. For the analysis of endocytic activity, $1x10^5$ cells were incubated with the fluorescein (FITC)-dextran (40,000 MW, molecular probes; Invitrogen, Karlsruhe, Germany) for 1 h at 37°C. The isotype control used FITC Mouse IgG1 (eBioscience, San Diego, CA, USA). The cells were washed 4 times and immediately analyzed on a FACSCalibur cytometer.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). RNA isolation and cDNA preparation were performed as described previously [19]. Real-time PCR was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) as described previously [19]. Primers for PCR are shown in Table 1. PCR conditions for all genes were as follows: a 95°C initial activation for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds, and fluorescence determination at the melting temperature of the product for 20 seconds on an ABI PRISM 7000 (Applied Biosystems).

Western blot analysis. Western blot analysis was performed as described previously [19]. Cells were suspended in ice-cold extraction buffer containing 20 mmol/L HEPES (pH 7.9), 20% glycerol, 10 mmol/L NaCl, 0.2 mol/LEDTA (pH 8.0), 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 1 mmol/L DTT, 100 μg/mL phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 10 μg/mL leupeptin. After 10 minutes of

incubation on ice, nuclei were collected by a short spin in a microcentrifuge. The supernatant was saved as a cytoplasmic fraction, and the nuclei were resuspended in ice-cold extraction buffer containing 300 mmol/L NaCl. After 30 minutes of incubation, the supernatant was collected by centrifugation at 13,500 rpm for 10 minutes at 4°C. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 10% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies. Anti-IκBα (Imgenex, San Diego, CA), anti-phospho- IκBα (Cell Signaling Technology, Beverly, MA), anti- p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA), anti- HistoneH1 (Santa Cruz Biotechnology) and anti- α-tubulin (Santa Cruz Biotechnology) antibodies were used.

Statistical analysis. Statistical analyses were carried out by paired *t*-test using the SPSS software (SPSS Japan, Tokyo, Japan). To assess the difference between two groups under multiple conditions, one-way ANOVA followed by Boneferroni's multiple comparison tests was performed by using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA). The results were considered to be significant when the P-value was < 0.05, and when the P-value was < 0.01, highly significant.

Results.

DHMEQ inhibits proliferation of PHA-stimulated or alloreactive PBMCs. To examine whether DHMEQ affects proliferation or survival of PBMCs, MTT assays were performed on resting, PHA-stimulated, or alloreactive PBMCs (Figs 1A,B). Exposure of PBMCs to PHA (5 μg/ml, 3 days) increased their proliferation by approximately 2.7-fold (Fig 1A). DHMEQ (0.5-3 μg/ml, 3 days) inhibited PHA-stimulated proliferation of PBMCs in a dose-dependent manner (Fig 1A). Similarly, DHMEQ (0.5-3 μg/ml, 3 days) significantly decreased alloreactive proliferation of PBMCs in a dose-dependent manner, although, inhibition was less potent compared to that induced in PHA-stimulated PBMCs (Fig 1B). Of note, DHMEQ did not affect the viability of resting PBMCs under the identical culture condition (Figs 1 A,B).

DHMEQ induces apoptosis of PHA-stimulated PBMCs. To investigate the mechanism by which DHMEQ inhibits the proliferation of activated PBMCs, we utilized annexin V staining. Approximately 9 % of cells became annexin V positive after incubation with DHMEQ (3 μg/ml, 24 hrs) (Fig 2). When cells were exposed to PHA (5 μg/ml, 24 hrs) in combination with DHMEQ (3 μg/ml, 24 hrs), annexin V positive population increased by 30 % (Fig 2), suggesting that DHMEQ induced apoptosis of

PHA-stimulated PBMCs.

Effect of DHMEQ on PHA-stimulated expression of cytokine genes. Increased levels of cytokines, including IFN-γ, IL-2 and TNF-α, are associated with pathogenesis of acute GVHD [6-7, 9-11]. In order to analyze the effect of DHMEQ on PHA-stimulated cytokine production, PBMCs ($4x10^6$/ml) from healthy donors were cultured with or without DHMEQ (1μg/ml) for 3 hrs and then exposed to PHA (5 μg/ml, 3 hrs) or not. Exposure of PBMCs to PHA profoundly stimulated expression of *IFN-γ, IL-2* and *TNF-α* (Fig 3A). Pre-incubation of these cells with DHMEQ (1 μg/ml, 3 hrs) prominently blocked PHA-stimulated expression of these cytokine genes (Fig 3A). Exposure of only DHMEQ did not change expression levels of *IFN-γ, IL-2* and *TNF-α* compared with control (Fig 3A). Similarly, PHA increased expression of *IL-2* and *IFN-γ* in Jurkat cells and pre-incubation of these cells with DHMEQ (1μg/ml) decreased these levels by approximately half (Fig 3B).

Effect of DHMEQ on PHA-stimulated NF-κB activity in Jurkat cells. Expression of cytokine genes is regulated by NF-κB. Activation of NF-κB involves two important steps: (*i*) phosphorylation and subsequent degradation of IκBα caused by IκBα kinase resulting in release of NF-κB, and (*ii*) the nuclear translocation of the activated NF-κB. To elucidate the effect of DHMEQ on these steps, we measured the levels of NF-κB

proteins in nucleus and the levels of IκBα proteins in cytoplasm in Jurkat cells after exposure to PHA alone or in combination with DHMEQ. Jurkat cells ($5x10^6$/ml) were cultured with either DHMEQ (1 μg/ml) or control diluent. After 3 hrs, cells were washed twice with PBS and exposed to PHA (5 μg/ml) for 3 or 24 hrs. Exposure of Jurkat cells to PHA increased levels of NF-κB in nucleus. When PHA was combined with DHMEQ PHA-stimulated up-regulation of NF-κB was blocked. Concurrently, DHMEQ inhibited PHA-stimulated phosphorylation of IκBα and down-regulation of IκBα in cytoplasm (Fig 4), suggesting that DHMEQ blocked PHA-induced nuclear translocation of NF-κB in Jurkat cells via inhibition of degradation of IκBα. These observations are reminiscent of our previous studies showing that DHMEQ blocked TNF-α-induced nuclear translocation of NF-κB in Jurkat cells [17].

Effect of DHEMQ on maturation of DCs. NF-κB regulatesthe differ entiation and activation of DCs [20,21], which plays a pivotal role in initiation of GVHD. We therefore explored whether DHMEQ affected maturation of DCs. For this purpose, we measured levels of CD40 antigen on cell surface of DCs. Increased expression of CD40 associates with maturation of DCs [20,21]. As we expected, DHMEQ decreased levels of TNF-α-stimulated expression of CD40 in monocyte- derived DCs (Fig 5A).

Effect of DHMEQ on endocytic capacity of DCs. Furthermore, we explored whether

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DHMEQ affected function of DCs. we monitored DC capacity to internalize FITC-labeled dextran as an assay of DC endocytic capacity (Fig 5B). To this end, we decided to use immature DCs because these normally show pronounced internalization capacity as compared with mature DCs [20,21]. Exposure of DCs to DHMEQ (0.5 or 1 μg/ml) reduced their endocytic ability (Fig 5B), suggestingthat DHMEQ may affect DC function by impeding antigen uptake for further processing and presentation.

Discussion.

This study found that DHMEQ, a novel NF-κB inhibitor, inhibited proliferation of both PHA-stimulated and alloreactive PBMCs (Fig 1). In addition, DHMEQ inhibited expression of Th1 cytokines in these cells (Fig 3). Furthermore, DHMEQ blocked maturation and endocytic capacity of DCs (Figs 5A,B). Mature dendritic cells express high levels of CD40 and act as APCs [20,21]. Antigen recognition by T cells induces the expression of CD40 ligand (CD40L). CD40L engages CD40 on APCs and stimulates the secretion of cytokines which further activate T cells [22,23]. The interaction of CD40L on T cells with CD40 on DCs thus initiates uncontrolled immunoreaction involved in GVHD. Hence, blockade of maturation of DCs by DHMEQ is probably useful to prevent and/or treat GVHD.

Previously, we showed that DHMEQ down-regulated LPS-stimulated expression of inflammatory cytokines including IL-6, IL-12, IL-1β, and TNF-α in association with blockade of nuclear translocation of NF-κB in murine macrophage RAW264.7 cells [24]. The study also showed that DHMEQ blunted the function of RAW264.7 cells; DHMEQ inhibited phagocytosis of Escherichia coli in these cells [24]. In addition, we have demonstrated that DHMEQ decreased severity of collagen-induced arthritis in murine model [25]. More recently, we have shown that DHMEQ prevented allograft

rejection and prolonged allograft survival in association with inhibition of mixed lymphocyte reaction and decreased in production of IFN-γ in murine cardiac transplantation model [26]. These observations augment the evidence that DHMEQ possesses anti-inflammatory activity.

Recent in vitro studies performed by other investigators demonstrated that bortezomib (PS-341; Velcade, Millenium Pharmaceuticals, Cambridge, MA), the proteasome inhibitor, possessed anti-inflammatory activity; bortezomib inhibited the proliferation of alloreactive T lymphocytes and decreased the production of Th1 cytokines [27]. The other group found that bortezomib inhibited the cytokine production and endocytic capacity of DCs [28]. Moreover, bortezomib protected murine from lethal GVHD in association with reduced serum levels of pro-inflammatory cytokines [29,30]. Of note, bortezomib did not compromise donor engraftment [29,30]. Bortezomib is a well known NF-κB inhibitor and induce apoptosis of variety types of cancers in which NF-κB is activated [31]. More selective NF-κB inhibitor PS1145, an inhibitor of IKK, also succeeded to manage GVHD in murine model [29]. Together with our observations, NF-κB may be a critical mediator of GVHD and this nuclear transcription factor can be a promising molecular target to prevent or treat GVHD.

Taken together, DHMEQ, a novel NF-κB inhibitor may be useful for prevention or

treatment of GVHD. Further studies are warranted to clarify the mode of action of this agent in inflammatory diseases.

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References

1. Hakim FT, Mackall CL. The immune system: effector and target of graft-versus-host disease. In: JLM Ferrara, HJ Deeg, SJ Burakoff (eds). Graft-vs-host Disease, 2nd Edn. Marcel Dekker: New York, 1997.

2. Ferrara JL, Levy R, Chao NJ. Pathophysiologic mechanisms of acute graft-vs-host disease. Biol Blood Marrow Transplant 1999;5:347-356.

3. Korngold R. Biology of graft-vs.-host disease. Am J Ped Hematol/Oncol 1993;15:18-27.

4. Xuu CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophoshamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of actute and chronic graft-versus-host disease in H-2-incompativle taransplanted SCID mice. Blood 1994;83:2360-7.

5. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-57.

6. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, Ferrara JL . Acute graft-versus-host disease does not require alloantigen expression on host epithelium.

Nat Med 2002;8:575-81.

7. Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-versus-host disease. Blood 1992;80:2964-8.

8. Karin M and Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol 2000;18:621-63.

9. Hill G R, Teshima T, Rebel VI, Krijanovski OI, Cooke KR, Brinson YS, Ferrara JL. The p55 TNF-alpha receptor plays a critical role in T cell alloreactivity. J. Immunol 2000;164:656–63.

10. Reddy P, Teshima T, Kukuruga M, Ordemann R, Liu C, Lowler K, Ferrara JL. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. J. Exp. Med 2001;194:1433–40.

11. Ferrara JL, Abhyankar S, Gilliland DG.Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1. Transplant Proc. 1993;25:1216-7.

12. Umezawa K. Inhibition of tumor growth by NF-kappaB inhibitors. Cancer Sci 2006;97:990-5.

13. Kikuchi E, Horiguchi Y, Nakashima J, et al. Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. Cancer Res. 2003;63:107-10.

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14. Matsumoto G, Namekawa J, Muta M, Nakamura T, Bando H, Tohyama K, Toi M, Umezawa K. Targeting of nuclear factor kappaB Pathways by dehydroxymethylepoxyquinomicin, a novel inhibitor of breast carcinomas: antitumor and antiangiogenic potential in vivo. Clin Cancer Res 2005;11:1287-93.

15. Watanabe M, Ohsugi T, Shoda M, et al. Dual targeting of transformed and untransformed HTLV-1-infected T cells by DHMEQ, a potent and selective inhibitor of NF-kappaB, as a strategy for chemoprevention and therapy of adult T-cell leukemia. Blood 2005;106:2462-71.

16. Horie R, Watanabe M, Okamura T, et al. DHMEQ, a new NF-kappaB inhibitor, induces apoptosis and enhances fludarabine effects on chronic lymphocytic leukemia cells. Leukemia 2006;20:800-6.

17. Ariga A, Namekawa J, Matsumoto N, Inoue J, Umezawa K. Inhibition of tumor necrosis factor-alpha -induced nuclear translocation and activation of NF-kappa B by dehydroxymethylepoxyquinomicin. J Biol Chem 2002;277:24625-30.

18. Suzuki Y, Sugiyama C, Ohno O, Umezawa K. Preparation and biological activities of optically active dehydroxymethylepoxyquinomicin, a novel NF-kB inhibitor. Tetrahedron 2004;60:7061-6.

19. 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;64:1521-9.

20. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;19:245-52.

21. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.

22. Carvalho-Pinto CE, Garcia MI, Mellado M, Rodriguez-Frade JM, Martin-Caballero J, Flores J, Martinez A C, Balomenos D. Autocrine production of IFN- gamma by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/lpr mice. J Immunol 2002;169:1058-67.

23. Lalor PF, Shields P, Grant A, Adams DH. Recruitment of lymphocytes to the human liver. Immunol Cell Biol 2002;80:52-64.

24. Suzuki E, Umezawa K. Inhibition of macrophage activation and phagocytosis by a novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin. Biomed Pharmacother 2006;60:578-86.

25. Wakamatsu K, Nanki T, Miyasaka N, Umezawa K, Kubota T. Effect of a small molecule inhibitor of nuclear factor-kappaB nuclear translocation in a murine

model of arthritis and cultured human synovial cells. Arthritis Res Ther 2005;7:1348-59.

26. Ueki S, Yamashita K, Aoyagi T, et al. Control of allograft rejection by applying a novel nuclear factor-kappaB inhibitor, dehydroxymethylepoxyquinomicin. Transplantation 2006;82:1720-7.

27. Blanco B, Perez-Simon JA, Sanchez-Abarca LI, et al. Bortezomib induces selective depletion of alloreactive T lymphocytes and decreases the production of Th1 cytokines. Blood 2006;107:3575-83.

28. Nencioni A, Schwarzenberg K, Brauer KM, Schmidt SM, Ballestrero A, Grunebach F, Brossart P. Proteasome inhibitor bortezomib modulates TLR4-induced dendritic cell activation. Blood 2006;108:551-8.

29. Vodanovic-Jankovic S, Hari P, Jacobs P, Komorowski R, Drobyski WR. NF-{kappa}B as a target for the prevention of graft versus hos disease: comparative efficacy of bortezomib and PS-1145. Blood 2006;107:827-34.

30. Sun K, Welniak LA, Panoskaltsis-Mortari A, et al. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. Proc.Natl. Acad. Sci. USA 2004;101:8120-5.

31. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome

inhibition as an effective anticancer therapy. Annu Rev Med 2006;57:33-47.

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Figure legends.

Fig.1 DHMEQ induces proliferation of alloreactive (A) or PHA-stimulated in PBMCs. MTT assay. (A), PBMCs from healthy volunteers (n=4) were cultured with PHA (5 μg/ml) or various concentrations of DHMEQ (0.5-3 μg/ml) either alone or in combination. Their viability was assed by MTT assay on day 3 of culture. **(B),** PBMCs were obtained from healthy volunteers (n=5) and were cultured with irradiated (3 Gy) allogeneic PBMCs. These cells were exposed to various concentrations of DHMEQ (0.5-3 μg/ml). Their viability was assed by MTT assay on day 3 of culture. Results represent the mean ± SD of 4 **(A)** or 5 **(B)** experiments performed in triplicate plates. The statistical significance was assessed by paired *t*-test.

Fig 2. DHMEQ induces apoptosis of PHA-stimulated PBMCs. Annexin V staining. PBMCs from healthy volunteers were cultured with PHA (5 μg/ml) or various concentrations of DHMEQ (0.5-3 μg/ml) either alone or in combination. After 24hrs, cells were stained with annexin-V/ propidium iodide, and analyzed by flow cytometry. The result represents one of the three experiments performed independently.

Fig 3. Effect of DHMEQ on PHA-stimulated expression of inflammatory cytokine genes in PBMCs. PBMCs form healthy volunteers **(A)** (n=3)or Jurkat cells **(B)** were pre-treated with either DHMEQ (1 μg/ml) or control diluent for 3 hrs and then exposed

to PHA (5 μg/ml) or without for 3 hrs. Cells were harvested and RNA was extracted. cDNAs were synthesized and subjected to real-time PCR using SYBRGreen nucleic acid gel staining solution to measure the levels of IFN-γ, IL-2 and TNF-α in these cells. Results represent the mean ± SD of three experiments with triplicate dishes per experimental point. The statistical significance was determined by one-way ANOVA followed by Boneferroni's multiple comparison tests.

Fig 4. Effect of DHMEQ on NF-κB. Western blot analysis. Jurkat cells were pretreated with either DHMEQ (1 μg/ml) or ontrol diluent for 3 hrs and then exposed to PHA (5 μg/ml) for the indicated time periods. The cytoplasmic and nuclear extracts of these cells were prepared and subjected to Western blot analysis to measure the level of IκBα, p–IκBα and p65 of NF-κB, respectively. α-tubulin or HistoneH1 expression was used as a loading control.

Fig 5. (A), Cell surface expression of CD40 on monocyte-derived DCs. Monocyte-derived DCs were generated from PBMCs from healthy volunteers (n=2) by culture in the presence of GM-CSF (50 ng/ml)and IL -4 (10 ng/ml)for 5 days. These cells were then exposed to TNF-α (10 ng/ml) either alone or in combination with DHMEQ (0.5 μg/ml). After 2 days, DCs were harvested, stained with anti-CD40 antibody and analyzed by flow cytometry. The figure represents one of the three

experiments performed independently. **(B), Exposure of DCs to DHMEQ blunts endocytic capacity.** Monocyte-derived DCs were generated from PBMCs by culture in the presence of GM-CSF and IL-4 for 5 days. These cells were then exposed to DHMEQ (0.5 or 1 μg/ml). After 2 days, cells were cultured with FITC-dextran for 1h at 37°C, washed 4 times, and analyzed by flow cytometry. The figure represents one of the three experiments performed independently.

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’
TNF-α	Forward	5’-CCTCCTCTCTGCCATCAAGA-3’
	Reverse	5’-GGAAGACCCCTCCCAGATAG-3’
18S	Forward	5’-AAACGGCTACCACATCCAAG-3’
	Reverse	5’-CCTCCAATGGATCCTCGTTA-3’

Fig.1

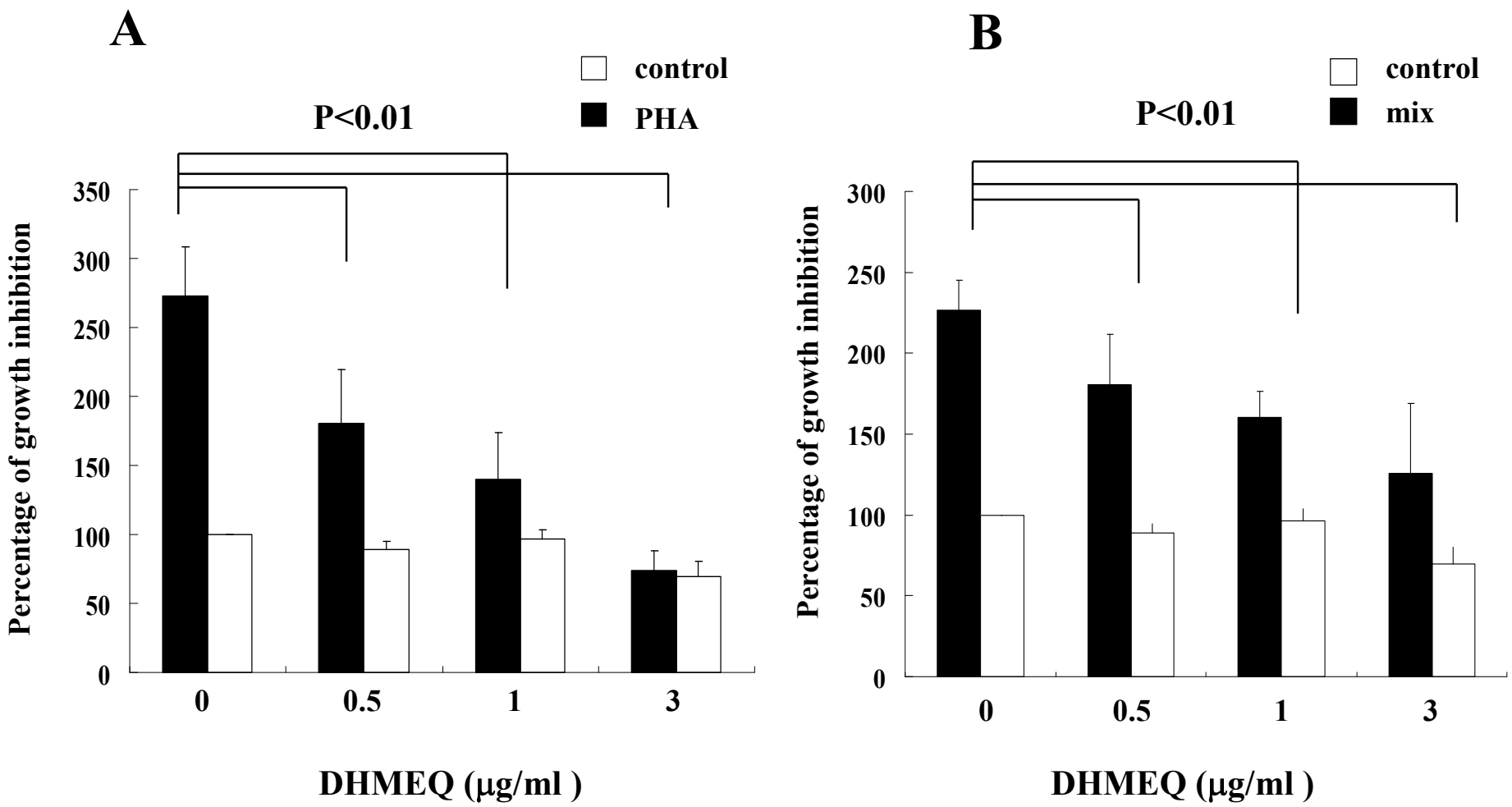
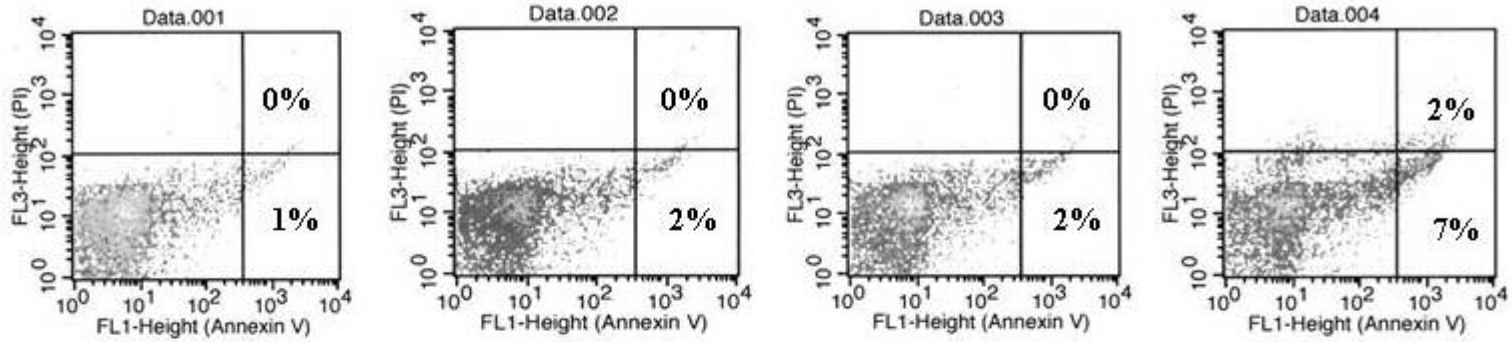
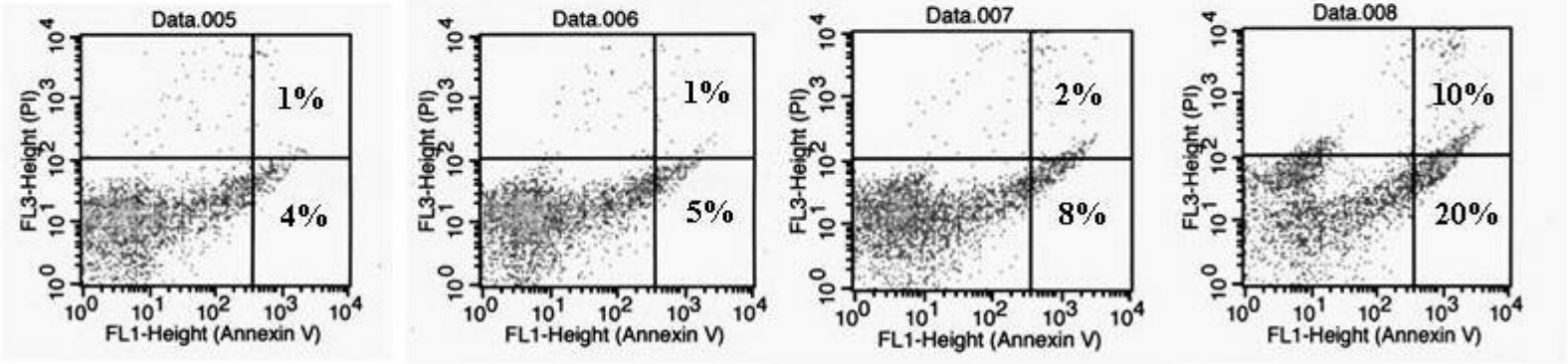

A
control
PHA
P<0.01
Percentage of growth inhibition
350
300
250
200
150
100
50
0
0
0.5
1
3
DHMEQ (μg/ml)
B
control
mix
P<0.01
Percentage of growth inhibition
300
250
200
150
100
50
0
0
0.5
1
3
DHMEQ (μg/ml)

Fig.2

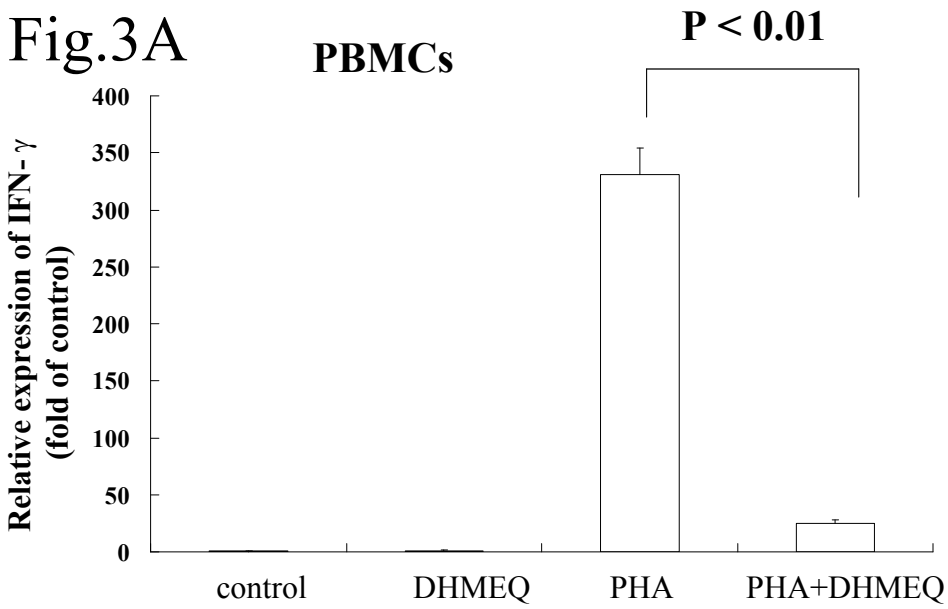


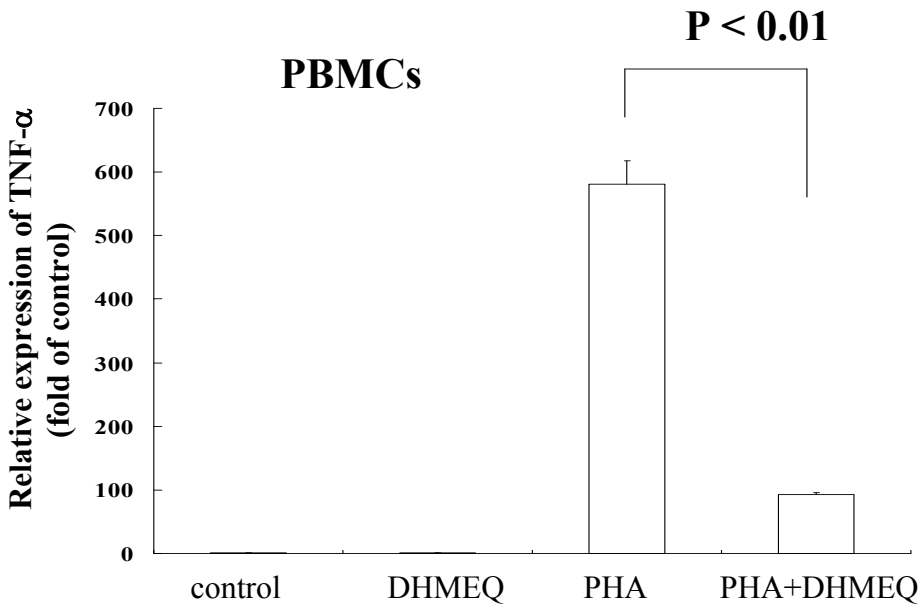
PHA 5 (μg/ml)	-	-	-	-
DHMEQ 1 (μg/ml)	-	0.5	1	3



PHA 5 (μg/ml)	+	+	+	+
DHMEQ 1 (μg/ml)	-	0.5	1	3

Fig.3A


PBMCs
P < 0.01
Relative expression of IFN-γ (fold of control)
400
350
300
250
200
150
100
50
0
control
DHMEQ
PHA
PHA+DHMEQ


PBMCs
P < 0.01
Relative expression of TNF-α (fold of control)
700
600
500
400
300
200
100
0
control
DHMEQ
PHA
PHA+DHMEQ

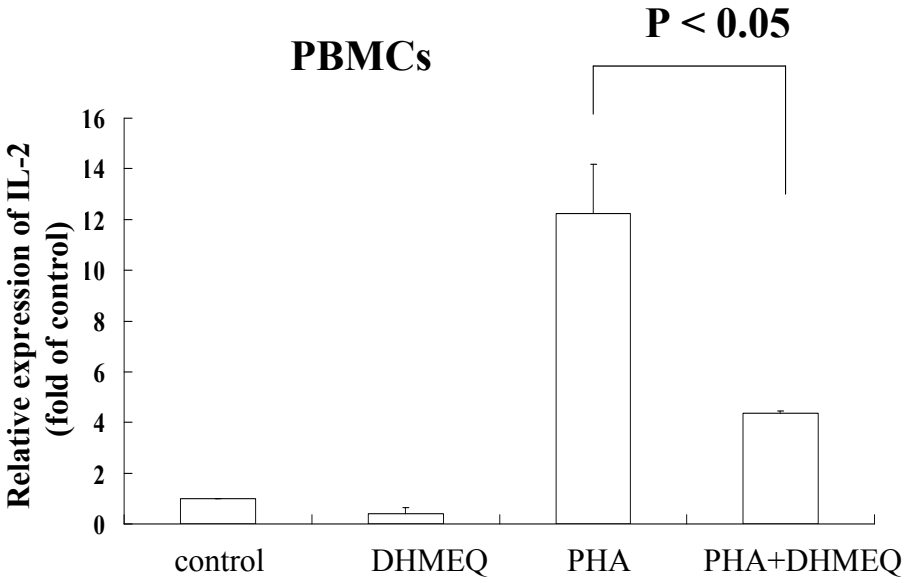
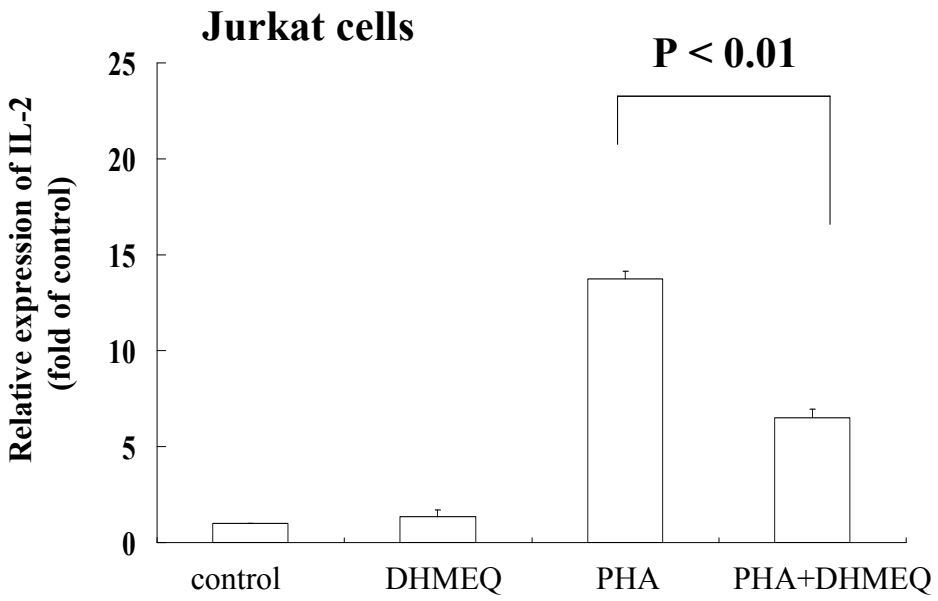

PBMCs
P < 0.05
Relative expression of IL-2 (fold of control)
16
14
12
10
8
6
4
2
0
control
DHMEQ
PHA
PHA+DHMEQ

Fig.3B


Jurkat cells
P < 0.01
Relative expression of IL-2 (fold of control)
25
20
15
10
5
0
control
DHMEQ
PHA
PHA+DHMEQ

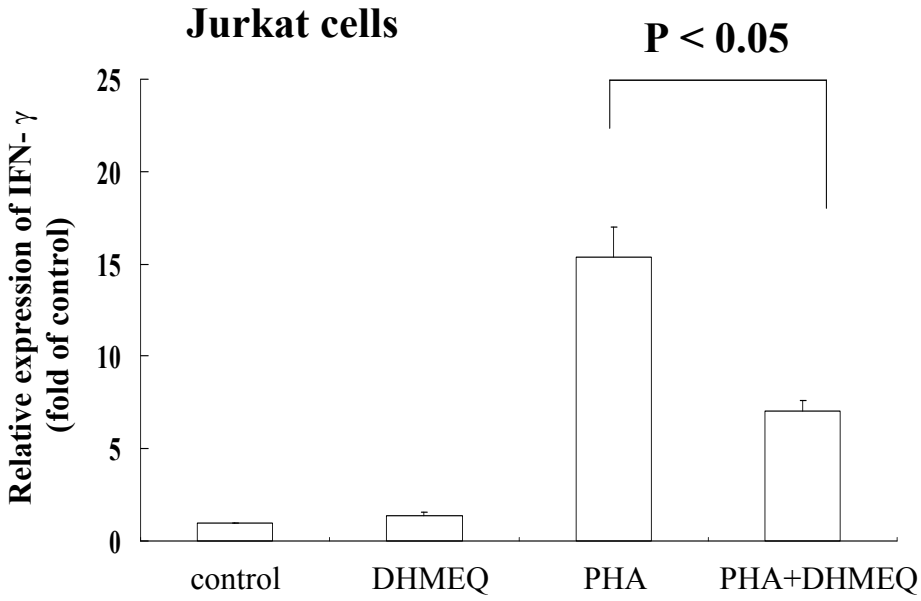

Jurkat cells
P < 0.05
Relative expression of IFN- γ (fold of control)
25
20
15
10
5
0
control
DHMEQ
PHA
PHA+DHMEQ

Fig.4

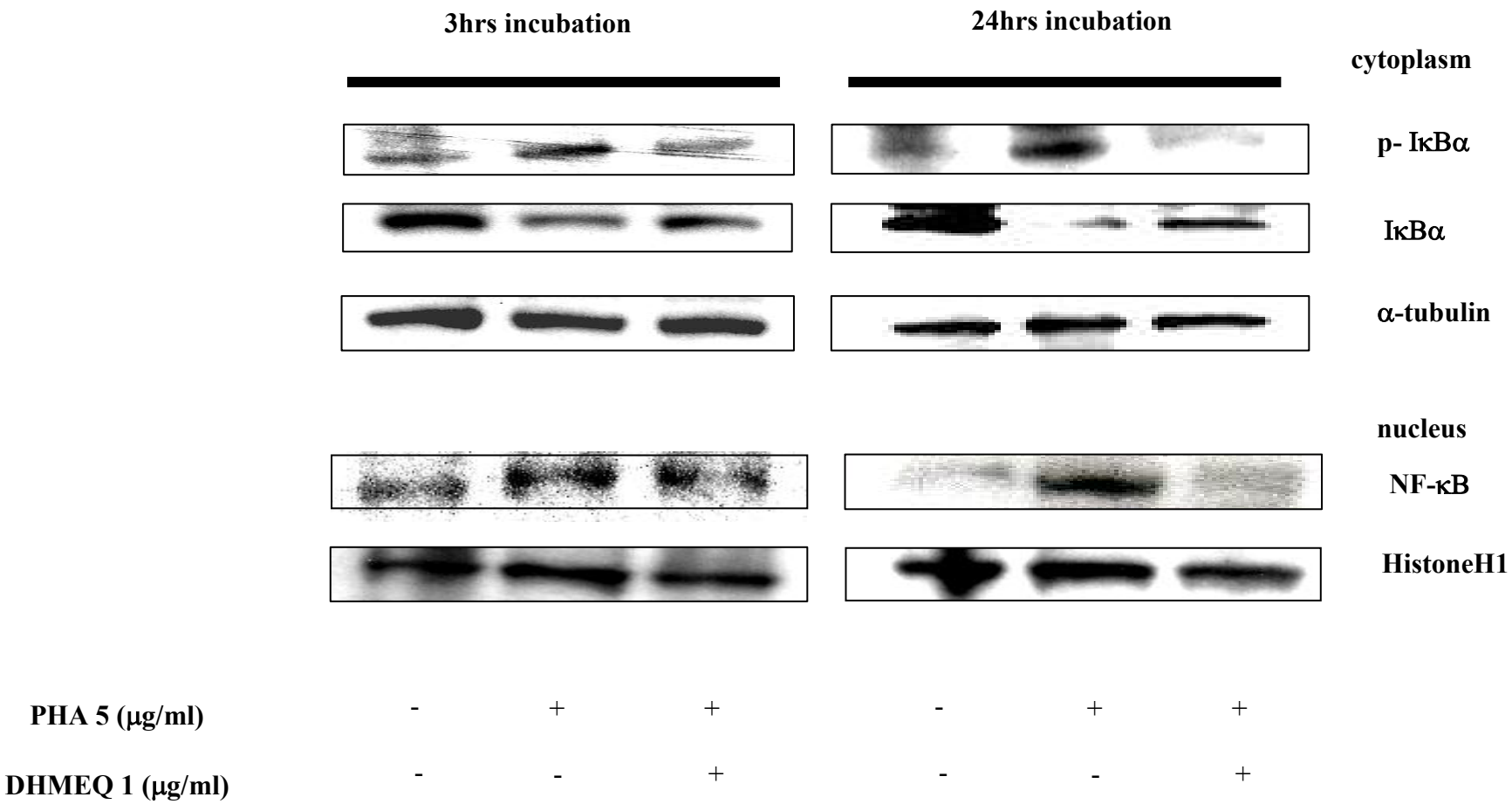
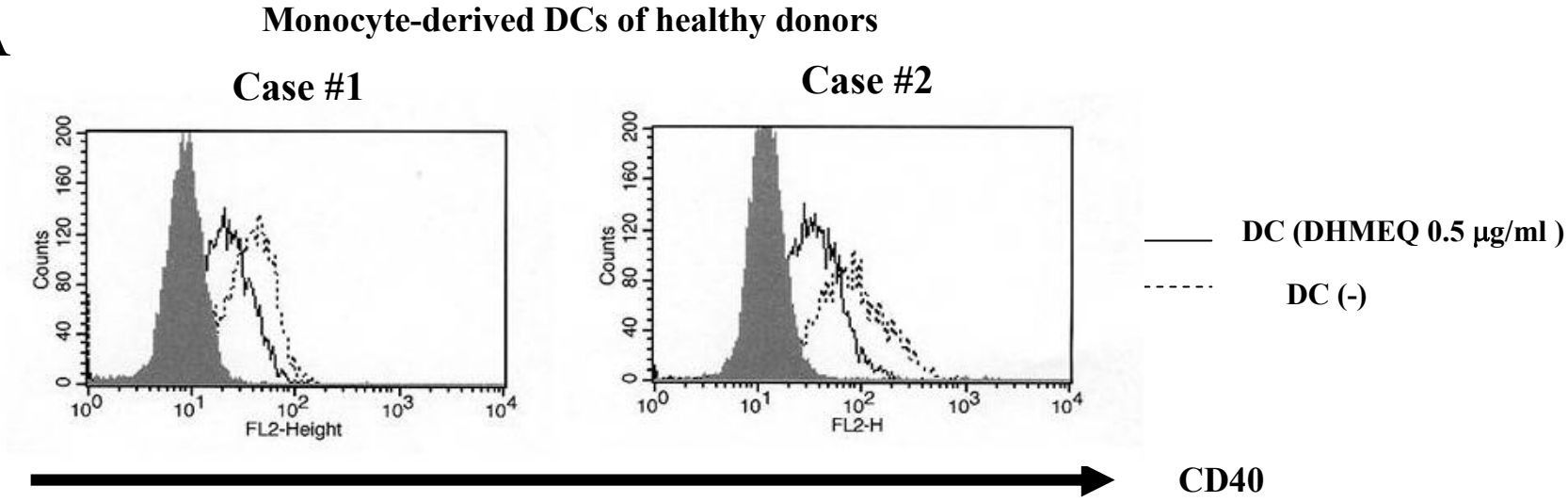
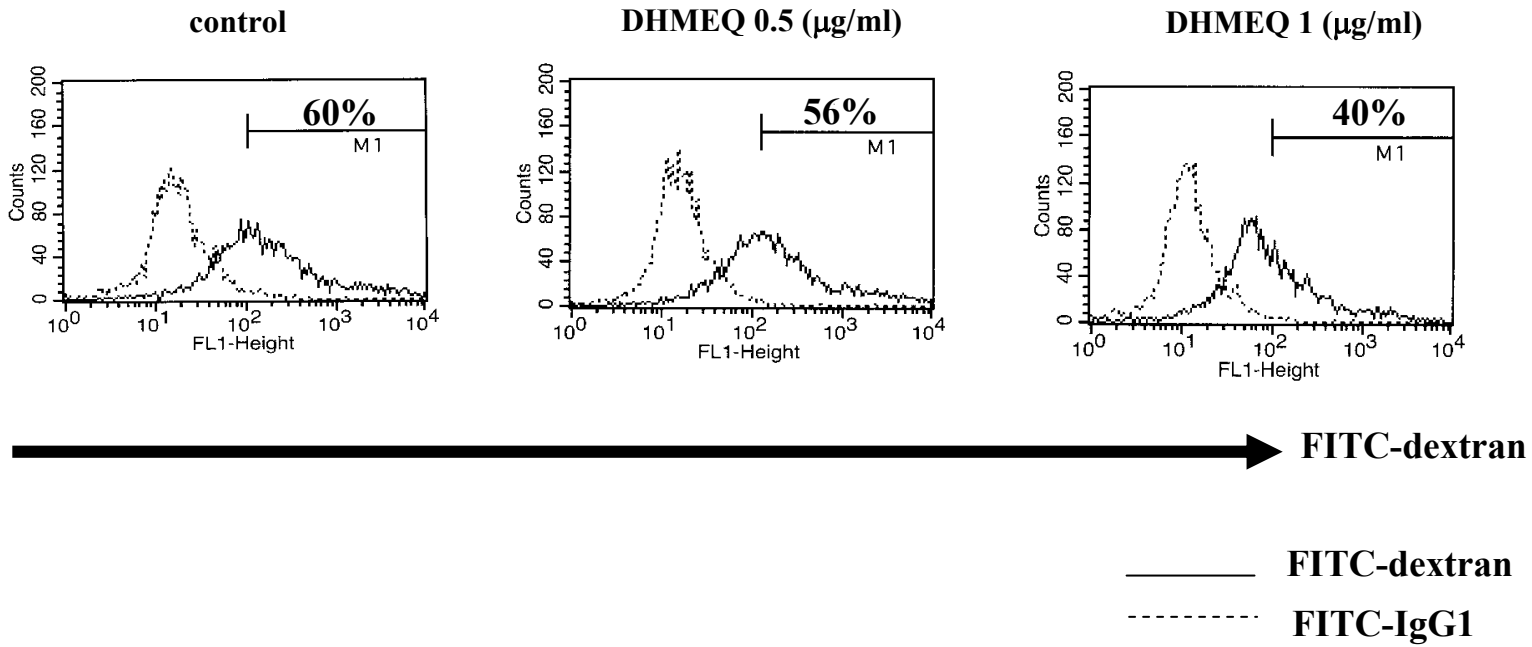

3hrs incubation
24hrs incubation
cytoplasm
p- IκBα
IκBα
α-tubulin
nucleus
NF-κB
HistoneH1
PHA 5 (μg/ml)
- + + - + +
DHMEQ 1 (μg/ml)
- - + - - +

Fig.5A


Monocyte-derived DCs of healthy donors
Case #1
Case #2
Counts
FL2-Height
FL2-H
DC (DHMEQ 0.5 μg/ml)
DC (-)
CD40

B


control
DHMEQ 0.5 (μg/ml)
DHMEQ 1 (μg/ml)
60%
56%
40%
M1
Counts
FL1-Height
FITC-dextran
FITC-dextran
FITC-IgG1