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Subject: Your Submission

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Toxicology in Vitro

Dear Doctor Yu-Jen Chen,

I am pleased to confirm that your paper "Midostaurin (PKC412) modulates differentiation and maturation of human myeloid dendritic cells" has been accepted for publication in Toxicology in Vitro.

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Thank you for submitting your work to this journal.

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Toxicology in Vitro

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Abstract: Midostaurin, a tyrosine kinase inhibitor, has been shown efficacy against acute myeloid leukemia and various other malignancies in clinical trials. Prior studies indicate midostaurin affects the function of immune cells such as lymphocytes and macrophages. To understand the effect of midostaurin on human myeloid dendritic cells (DCs), we conducted an ex vivo study using immature DCs differentiated from CD14+ monocytes and further maturated using lipopolysaccharide. Addition of midostaurin to a culture of starting CD14+ monocytes markedly and dose-dependently reduced DC recovery. Mature DCs differentiating in the presence of midostaurin had fewer, shorter cell projections than those differentiating in the absence of midostaurin. Changes in morphological features characteristic of apoptotic cells were also evident. Moreover, midostaurin affected DC differentiation and maturation patterns; CD83 expression levels decreased, whereas CD14 and CD80 expressions increased. Additionally, DCs derived in the presence of midostaurin possessed a lower endocytotic capacity and less allostimulatory activity on naive CD4+CD45+RA+ T cell proliferation than those derived in its absence, suggesting that midostaurin redirects DC differentiation toward a less mature stage and that this effect is not solely due to its cytotoxicity. Whether this effect underlies immune suppression or tolerance to disease treatments with unwanted immune reactions needs further evaluation.

Midostaurin (PKC412) modulates differentiation and maturation of human myeloid dendritic cells

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Conflict of Interest Page

All authors have no conflict of interest.

ABSTRACT

Midostaurin, a tyrosine kinase inhibitor, has been shown efficacy against acute myeloid leukemia and various other malignancies in clinical trials. Prior studies indicate midostaurin affects the function of immune cells such as lymphocytes and macrophages. To understand the effect of midostaurin on human myeloid dendritic cells (DCs), we conducted an ex vivo study using immature DCs differentiated from $CD14^+$ monocytes and further maturated using lipopolysaccharide. Addition of midostaurin to a culture of starting $CD14^+$ monocytes markedly and dose-dependently reduced DC recovery. Mature DCs differentiating in the presence of midostaurin had fewer, shorter cell projections than those differentiating in the absence of midostaurin. Changes in morphological features characteristic of apoptotic cells were also evident. Moreover, midostaurin affected DC differentiation and maturation patterns; CD83 expression levels decreased, whereas CD14 and CD80 expressions increased. Additionally, DCs derived in the presence of midostaurin possessed a lower endocytotic capacity and less allostimulatory activity on naïve $CD4^+CD45^+RA^+$ T cell proliferation than those derived in its absence, suggesting that midostaurin redirects DC differentiation toward a less mature stage and that this effect is not solely due to its cytotoxicity. Whether this effect underlies immune suppression or tolerance to disease treatments with unwanted immune reactions needs further evaluation.

Keywords: dendritic cell; differentiation; maturation; midostaurin; PKC412

Abbreviations

CFSE	carboxyfluorescein succinimidyl ester
CTLA-4	cytotoxic T-lymphocyte antigen-4
DCs	dendritic cells
DMSO	dissolved in dimethyl sulfoxide
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
IL-1	interleukin-1
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
PBS	phosphate buffered saline
SD	standard deviation
PKC	protein kinase C
TNF-α	tumor necrosis factor-α

1. Introduction

Dendritic cells (DCs) are specialized leukocytes that present antigens to naïve T cells; they play a pivotal role in both cell-mediated and humoral immune responses *in vivo* (Banchereau and Steinman, 1998). The exceptional ability of DCs to stimulate T cells *in vitro* and *in vivo* has been attributed, at least in part, to their ability to capture antigens, migrate into lymphoid organs, and express high levels of immunostimulatory molecules such as major histocompatibility complex (MHC) class II, B7.1 (CD80), B7.2 (CD86), and IL-12 (Banchereau and Steinman, 1998). Upon exposure to various microbial and inflammatory products (e.g., lipopolysaccharide [LPS], interleukin-1 [IL-1], or tumor necrosis factor-α [TNF-α]), DCs mature and migrate into lymphoid tissues to interact with T and B cells (Jonuleit et al., 1997; Labeur et al., 1999; Cella et al., 1996; Kato et al., 1997).

Midostaurin (PKC412; N-benzoyl staurosporine), which is derived from the naturally occurring alkaloid staurosporine (Fabbro et al., 2000), is a small-molecule inhibitor of protein kinase C (PKC) isoforms α, β, γ, δ, ε, and η (Dekker and Parker, 1994; Goekjian and Jirousek, 2001). Midostaurin has also been shown to inhibit a variety of tyrosine kinases, including *fms*-like tyrosine kinase 3 (FLT3), platelet-derived growth factor-α and -β receptors, and c-kit (Furukawa et al., 2007; Levis and Small, 2005). Given that the kinases inhibited by midostaurin play key roles

in proliferation and differentiation of cells such as lymphocytes and hematopoietic stem cells, it is possible that they have roles in other cellular processes, such as immune cell response to stimuli. For example, midostaurin has been reported to inhibit proliferation of murine RAW 264.7 macrophages via induction of G2/M cell cycle arrest and apoptosis (Miyatake et al., 2007). Also, it significantly suppressed the LPS-induced release of TNF-α and nitric oxide, but enhanced IL-6 secretion (Piemonti et al., 1999). Furthermore, midostaurin has been reported as a FLT3 inhibitor. Previous reports showed that the administration of recombinant FLT3 ligand dramatically increased the number of DCs within the bone marrow and periphery in humans (Angelov et al., 2005; Diener et al., 2008; Shaw et al., 1998). On this basis, it was therefore reasonable to hypothesize that midostaurin may modulate the development of DCs.

Toward this end, we used human monocyte-derived DCs as an experimental model to examine the effect of midostaurin on the morphology, phenotype, and allostimulatory activity of mature DCs.

2. Materials and methods

2.1. Reagents

Midostaurin was provided by Novartis Pharma AG (Basel, Switzerland). It was dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C until further use.

2.2. Generation of human dendritic cells

Human peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation with Histopaque (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Erythrocytes were lysed by treating with 0.9% ammonium chloride for 3 min at 37°C. Subsequently, $CD14^+$ cells were purified by high-gradient magnetic sorting using the miniMACS system with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Bladbach, Germany). After incubating for 2 hours at 37°C, nonadherent cells were removed, and adherent cells were collected. The purity of isolated $CD14^+$ monocytes was over 90% on flow cytometric analysis. Immature DCs were generated from the $CD14^+$ monocytes by culturing in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 ng/ml GM-CSF (Schering-Plough, Munich, Germany), 50 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA), and midostaurin in concentrations of 0, 0.5, and 1.0 μM every 3 days for 6 days in a humidified 5% CO_2 incubator. The stability of midostaurin during 3 days is not

impaired noted in our previous report on its effect on megakaryocytic differentiation (Huang et al., 2009). To trigger DC maturation, immature DCs were incubated with LPS (Sigma, St Louis, MO, USA) for a further 24 hours. In some experiments, midostaurin was added to immature DCs (cells harvested on day 6 prior to LPS stimulation) to evaluate its sole effect on DC maturation.

2.3. Number of viable cells

DCs were harvested on day 7, and the numbers of viable cells were counted using the trypan blue dye exclusion test. The recovery rate of DCs was estimated by dividing the number of harvested DCs by the total number of sorted $CD14^+$ monocytes.

2.4. Flow cytometric analysis

Dual-color immunolabeling was performed using fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs). The mouse anti-human mAbs IgG1:FITC/mouse IgG1:PE, along with the appropriate isotype controls anti-CD14 (for IgG-FITC), anti-CD1a-PE, anti-CD80-PE, anti-CD86-PE, anti-CD83-PE, CD184-PE, CCR5-PE, CCR7-PE, anti-HLA-DR-PE, and anti-DC-sign-PE, were purchased from Serotec (Oxford, UK) and used for

characterization of DCs. After washing twice with phosphate buffered saline (PBS), 10^6 cells were processed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were collected and analyzed using the CellQuest Software (BD Biosciences).

2.5. Morphological observations

For morphological examination, DCs were cytocentrifuged onto a microscope slide using a Cytospin[4] (Shandon Southern Instrument Inc., Sewelicky, PA, USA), then stained with Liu's stain, and observed under an upright microscope (Olympus, Tokyo, Japan) at a magnification of 1000×.

2.6. Allogeneic naïve T cell proliferation and cytokine secretion

To purify $CD4^+CD45RA^+$ T cells, nonadherent cells from an isolated culture of mononuclear cells were used. Naïve T cells were enriched with a $CD4^+CD45RA^+$ T cell isolation kit (Miltenyi Biotec) using the MiniMACS system with magnetic Abs by a negative selection technique. Monocyte-derived DCs were harvested and irradiated (3,000 cGy) with 6 MeV X-rays generated by a linear accelerator (Clinac® 1800, Varian Associates, Inc., CA, USA) at a dose of 4.0 Gy/min in a single fraction. Full electron equilibrium was ensured for each fraction by using a parallel plate PR-60C

ionization chamber (CAPINTEL, Inc., Ramsey, NJ, USA). DCs irradiated at 30 Gy were incubated with 10^6 allogeneic naive T cells at ratios of 1:10 or 1:30 for 7 days; thereafter, 5-μM carboxyfluorescein succinimidyl ester (CFSE) was added to the T-cell cultures. These T cells were then collected, and the incorporated CFSE was detected using flow cytometry.

2.7. Endocytosis assay

For the uptake of FITC-dextran**,** DCs were incubated with 1 mg/ml of FITC-dextran in PBS supplemented with 10% fetal bovine serum (FBS) for 1 h at 37°C or at 4°C (as a control for background binding). Samples were analyzed by flow cytometry.

2.8. Statistical analysis

Results are presented as mean ± standard deviation (SD) and are from at least three independent experiments. Differences among multiple groups were examined for statistical significance using one-way ANOVA tests. *P* value for trend was analyzed using the General Linear Model procedure. Statistical analyses were performed using the SPSS software package, version 17.0 (SPSS Inc, Chicago, IL, USA); a *p* value less than 0.05 were considered significant.

3. Results

3.1. Effect of midostaurin on recovery rate of DCs

As shown in Figure 1, midostaurin added to the starting cells at the beginning of DCs differentiation reduced the recovery rates of LPS-triggered mature DCs in a dose-dependent manner (*p* value for trend = 0.018). To exclude the effect of LPS on DC viability, we also compared DC viability in culture with or without LPS at day 6. The viable cell counts were similar in the absence or presence of LPS at a midostaurin concentration of 1.0 μM (1.47×10^5 versus 1.41×10^5), indicating that LPS was unlikely to have a significant cytotoxic effect. Intriguingly, when midostaurin was added along with LPS at day 6, it did not affect the recovery rate of mature DCs (Fig. 1). These data suggest that midostaurin modulated the development of DCs at the differentiation stage, but not at the maturation stage. It also indicates that the target cells of midostaurin are likely the starting DC precursors rather than immature DCs.

3.2. Morphological changes

By observing Liu's staining under a light microscope, we observed the morphology of immature DCs collected on day 6 before the LPS trigger showed round contours without evident dendrites (data not shown). The LPS-triggered DCs observed on day 7 had morphological characteristics typical of mature DCs, including

loose adherence and multiple cytoplasmic projections with abundant cytoplasm (Fig. 2a and 2c). The majority of DCs derived from midostaurin-treated $CD14^+$ cells at the beginning of the DCs differentiation manifested fewer and shorter cell projections, indicating an inhibited DC differentiation (Fig. 2b). Moreover, cells with morphological features of apoptosis were also evident. However, no features typical of macrophages, such as pseudopods or abundant cytoplasmic vacuoles, could be noted. When midostaurin was added along with LPS at day 6, it did not affect the morphology of mature DCs (Fig. 2d). This suggests that, in terms of morphological changes, midostaurin inhibited differentiation of DCs, and did not induce dedifferentiation toward macrophages.

3.3. Modulation by midostaurin of DC surface marker expression

As shown in Table 1, the expression of CD83, a marker of mature DCs, was upregulated upon LPS treatment and was profoundly inhibited by treatment with midostaurin ($p < 0.05$). Midostaurin also increased the expression of CD14 ($p < 0.001$). The expression of CD80, a costimulatory molecule known as B7.1, was low in LPS-triggered mature DCs. In contrast, midostaurin treatment markedly increased the expression of CD80. Consistent with the recovery rate, when midostaurin was added along with LPS at day 6, it did not significantly affect expression of the above surface

markers. Thus, there was no difference in the expression of CD1a, CD86, HLA-DR, DC-sign, CD184, CCR5, and CCR7 with or without midostaurin treatment.

3.4. Effect of midostaurin on the capacity of DCs to stimulate allogeneic naive T cells

As demonstrated in Figure 3, midostaurin suppressed the allostimulatory activity of DCs by stimulating proliferation of naïve $CD4^+CD45^+RA^+$ T cells. Again, the impaired stimulation of allogeneic T cells by DCs could only be noted when midostaurin was added to the starting $CD14^+$ monocytes, but not when added to the immature DCs. Since midostaurin-treated cells possessed the allostimulatory activity, an important function of viable DCs, it suggests that the suppressed DC phenotype may not be solely due to cytotoxicity of midostaurin.

3.5. Effect of midostaurin on the endocytotic capacity of DCs

DCs lost their endocytotic capacity during maturation. Thus, immature DCs usually possess greater endocytotic capacity than do mature DCs. By assessing the uptake of FITC-dextran, we found that midostaurin diminished the endocytotic capacity of DCs (Fig. 4). This supports that the target of midostaurin is differentiating DCs at the stage before mature DCs.

4. Discussion

The findings of the present study suggest that midostaurin, an effective, multitarget, small-molecule therapeutic against acute myeloid leukemia and various malignancies, modulates the differentiation and maturation of human myeloid DCs. Treatment of $CD14^+$ monocytes with midostaurin suppressed the generation of DC and caused deviation of standard DC differentiation toward a state of suppressed phenotype maturation; this was accompanied by strikingly enhanced expression of CD80. Midostaurin treatment of naïve $CD4^+CD45^+RA^+$ T cells inhibited the endocytotic capacity and allostimulatory activity of DCs.

There is growing evidence that different stimuli skew the differentiation of monocytes into DCs with distinct phenotypes and functions. Dexamethasone at a low concentration (10^{-8} M) has been shown to direct the differentiation of human DCs to a less mature stage (Piemonti et al., 1999). DCs differentiated in the presence of platonin, an NF-kappa B inhibitor, were less mature in terms of CD83 expression and stimulatory effect on naïve T cells, but expressed more CD80 (Lee et al., 2006). This suggests that the changes in phenotype and function under modulation of DC differentiation by various treatments may not be parallel. In this study, midostaurin also had differential effects on the endocytotic ability and allostimulatory function of myeloid DCs.

The B7-1/B7-2–CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4) costimulatory pathway plays a crucial role in regulating T-cell differentiation, activation, and tolerance (Linsley et al., 1991b; Linsley et al., 1991a). CD28 and CTLA-4 are thought to have opposite functions in T-cell stimulation. It is known that CD28 can promote T-cell response, while conversely, CTLA-4 can inhibit T-cell response (Doyle et al., 2001; Linsley et al., 1991a). Notably, CD28 and CTLA-4 share two structurally homologous ligands, CD80 and CD86, which are expressed by antigen-presenting cells, including DCs. It has been suggested that CD80 might be the initial ligand responsible for maintaining immune tolerance through interaction with CTLA-4 (Fallarino et al., 1998). The inhibitory activity of CD80 could be overridden by the upregulation of CD86 on DCs as a result of inflammatory stimuli, leading to immune activation (Sansom et al., 2003). Our previous study demonstrated that platonin exhibited a similar effect to midostaurin (Lee et al., 2006). On this basis, platonin has been further testified as an effective immunosuppressant for preventing reject of skin allograft (Lee et al., 2006). In our study, midostaurin enhanced the expression of CD80, but not CD86, on mature DCs. Whether this increase in CD80 expression favors immune activation or tolerance remains to be determined.

Midostaurin has been demonstrated to be toxic to murine RAW 264.7 macrophages *in vitro* at an IC_{50} of 0.95–3.82 μM (Miyatake et al., 2007). In our study,

midostaurin at a relatively lower concentration of 0.25–1.0 μM inhibited the formation of DCs from human $CD14^+$ monocytes. However, the effect of midostaurin on DC differentiation does not seem to be attributable to cytotoxicity against the starting monocytes because the viability of $CD14^+$ monocytes was not affected (data not shown). DCs differentiated in the presence of midostaurin still possessed allostimulatory activity, supporting that midostaurin is not solely cytotoxic. Pharmacokinetic data obtained from 32 patients given between 12.5 and 300 mg of midostaurin per day for advanced cancer indicated that mean plasma concentrations on day 1 were in the range of 0.3–7.0 μmol/l (Propper et al., 2001). Therefore, the tested concentrations used in this *in vitro* study, 0–1.0 μM, might be relevant to the interpretation of this drug's clinical effects.

In conclusion, our data suggest that midostaurin modulates the differentiation, maturation, and function of DCs. This raises the interesting possibility that midostaurin may have novel pharmacological activities other than the current clinical indications in cancer. Thus, further investigation is warranted in order to understand the implications of DC modulation in the treatment of disorders with unwanted immune reactions, such as transplantation rejection or autoimmune diseases.

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References

Angelov, G.S., Tomkowiak, M., Marcais, A., Leverrier, Y., and Marvel, J., 2005. Flt3 ligand-generated murine plasmacytoid and conventional dendritic cells differ in their capacity to prime naive CD8 T cells and to generate memory cells in vivo. Journal of Immunology 175, 189-195.

Banchereau, J. and Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245-252.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G., 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. The Journal of Experimental Medicine 184, 747-752.

Dekker, L.V. and Parker, P.J., 1994. Protein kinase C--a question of specificity. Trends in Biochemical Sciences 19, 73-77.

Diener, K.R., Moldenhauer, L.M., Lyons, A.B., Brown, M.P., and Hayball, J.D., 2008. Human Flt-3-ligand-mobilized dendritic cells require additional activation to drive effective immune responses. Experimental Hematology 36, 51-60.

Doyle, A.M., Mullen, A.C., Villarino, A.V., Hutchins, A.S., High, F.A., Lee, H.W., Thompson, C.B., and Reiner, S.L., 2001. Induction of cytotoxic T lymphocyte antigen 4 (CTLA-4) restricts clonal expansion of helper T cells. The Journal of Experimental Medicine 194, 893-902.

Fabbro, D., Ruetz, S., Bodis, S., Pruschy, M., Csermak, K., Man, A., Campochiaro, P., Wood, J., O'Reilly, T., and Meyer, T., 2000. PKC412--a protein kinase inhibitor with a broad therapeutic potential. Anti-cancer Drug Design 15, 17-28.

Fallarino, F., Fields, P.E., and Gajewski, T.F., 1998. B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28. The Journal of Experimental Medicine 188, 205-210.

Furukawa, Y., Vu, H.A., Akutsu, M., Odgerel, T., Izumi, T., Tsunoda, S., Matsuo, Y., Kirito, K., Sato, Y., Mano, H., and Kano, Y., 2007. Divergent cytotoxic effects of PKC412 in combination with conventional antileukemic agents in FLT3 mutation-positive versus -negative leukemia cell lines. Leukemia 21, 1005-1014.

Goekjian, P.G. and Jirousek, M.R., 2001. Protein kinase C inhibitors as novel

anticancer drugs. Expert Opinion on Investigational Drugs 10, 2117-2140.

Huang, Y.C., Chao, D.K., Chao, K.S.C., and Chen, Y.J., 2009. Oral small-molecule tyrosine kinase inhibitor midostaurin (PKC412) inhibits growth and induces megakaryocytic differentiation in human leukemia cells. Toxicology in Vitro 23, 979-985.

Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J., and Enk, A.H., 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. European Journal of Immunology 27, 3135-3142.

Kato, T., Yamane, H., and Nariuchi, H., 1997. Differential effects of LPS and CD40 ligand stimulations on the induction of IL-12 production by dendritic cells and macrophages. Cellular Immunology 181, 59-67.

Labeur, M.S., Roters, B., Pers, B., Mehling, A., Luger, T.A., Schwarz, T., and Grabbe, S., 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. Journal of Immunology 162, 168-175.

Lee, J.J., Liao, H.F., Yang, Y.C., Liu, C.L., Chen, Y.Y., Lin, C.P., and Chen, Y.J., 2006. Platonin modulates differentiation and maturation of human monocyte-derived dendritic cells. International Immunopharmacology 6, 287-293.

Levis, M. and Small, D., 2005. FLT3 tyrosine kinase inhibitors. International Journal of Hematology 82, 100-107.

Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A., 1991a. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. Journal of Experimental Medicine 173, 721-730.

Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L.S., Damle, N.K., and Ledbetter, J.A., 1991b. CTLA-4 is a second receptor for the B cell activation antigen B7. Journal of Experimental Medicine 174, 561-569.

Miyatake, K., Inoue, H., Hashimoto, K., Takaku, H., Takata, Y., Nakano, S., Yasui, N., and Itakura, M., 2007. PKC412 (CGP41251) modulates the proliferation and lipopolysaccharide-induced inflammatory responses of RAW 264.7 macrophages. Biochemical and Biophysical Research Communications 360, 115-121.

Piemonti, L., Monti, P., Allavena, P., Sironi, M., Soldini, L., Leone, B.E., Socci, C.,

and Di Carlo, V., 1999. Glucocorticoids affect human dendritic cell differentiation and maturation. Journal of Immunology 162, 6473-6481.

Propper, D.J., McDonald, A.C., Man, A., Thavasu, P., Balkwill, F., Braybrooke, J.P., Caponigro, F., Graf, P., Dutreix, C., Blackie, R., Kaye, S.B., Ganesan, T.S., Talbot, D.C., Harris, A.L., and Twelves, C., 2001. Phase I and pharmacokinetic study of PKC412, an inhibitor of protein kinase C. Journal of Clinical Oncology 19, 1485-1492.

Sansom, D.M., Manzotti, C.N., and Zheng, Y., 2003. What's the difference between CD80 and CD86? Trends in Immunology 24, 314-319.

Shaw, S.G., Maung, A.A., Steptoe, R.J., Thomson, A.W., and Vujanovic, N.L., 1998. Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: implications for anti-cancer and anti-viral therapy. Journal of Immunology 161, 2817-2824.

Figure Legends

Fig. 1. Cell viability of DCs assessed by trypan blue exclusion assay. DCs were treated with DMSO and midostaurin at concentrations of 0.5 and 1.0 μM on day 0 or day 6 and collected after 7 days. Results expressed as the mean cell count ± SD (bars) of at least three independent experiments.

Fig. 2. Morphological observation of DCs on day 7. Cells were stained by Liu's method for morphological examination under light microscope (Magnification 1000×). DCs treated with (a) DMSO and (b) 1.0 μM midostaurin at the beginning of monocyte-derived DCs (day 0). DCs treated with (c) DMSO and (d) midostaurin at 1.0 μM concentration on day 6. All LPS-triggered DCs treated on day 6.

Fig. 3. Proliferation of allogeneic $CD4^+CD45RA^+$ naïve T cells stimulated by mature DCs generated at different dosages of midostaurin. DCs irradiated at 30 Gy were incubated with $1x10^6$ allogeneic naïve T cells at ratios of 1:10 or 1:30 for 7 days, after which 5 μM carboxyfluorescein succinimidyl ester (CFSE) was added to T cell cultures. The cells were then collected and the incorporated CFSE was detected using flow cytometry. Data from at least three independent experiments are expressed as mean ± SD.

Fig. 4. Endocytotic capacity of DCs. Uptake of FITC-dextran was used to assess the endocytotic capacity of DCs. DCs were incubated with 1 mg/ml of FITC-dextran in PBS supplemented with 10% FBS for 1 h at 4°C (as a control for background binding) or at 37°C. Samples were analyzed by flow cytometry. Data from at least three independent experiments are expressed as mean ± SD.

Table 1. Effect of midostaurin on expression of surface markers in dendritic cells (DCs)

Surface marker	midostaurin concentration (μM)	midostaurin added to starting $CD14^+$ cells		midostaurin added to immature DCs	
		Mean ± SD (%)	*p* value*	Mean ± SD (%)	*p* value*
CD14	0	21.6 ± 5.1	< ***0.001***	19.8 ± 4.7	0.848
	0.5	72.5 ± 8.1		19.1 ± 5.9	
	1.0	68.6 ±17.3		17.6 ± 6.4	
CD1a	0	9.9 ± 2.7	0.403	10.9 ± 1.9	0.564
	0.5	6.2 ± 2.4		9.8 ± 2.4	
	1.0	9.0 ± 5.7		11.3 ± 1.8	
CD83	0	48.1 ± 7.5	***0.044***	43.8 ± 12.9	0.653
	0.5	22.0 ± 21.7		48.8 ± 9.0	
	1.0	16.4 ± 15.3		50.7 ± 9.7	
CD80	0	55.3 ± 20.0	***0.034***	49.9 ± 18.1	0.985
	0.5	73.6 ± 2.6		52.0 ± 23.6	
	1.0	82.9 ± 8.1		52.2 ± 10.3	
CD86	0	95.0 ± 1.6	0.622	95.0 ± 3.8	0.538
	0.5	93.3 ± 2.9		96.5 ± 2.6	
	1.0	93.0 ± 4.2		97.2 ± 1.1	
HLA-DR	0	94.6 ± 2.6	0.067	95.1 ± 4.4	0.610
	0.5	97.2 ± 1.7		92.2 ± 5.9	
	1.0	98.2 ± 1.2		95.2 ± 3.3	
DC-sign	0	73.7 ± 18.8	0.480	82.9 ± 7.5	0.209
	0.5	89.6 ± 9.5		75.4 ± 23.0	
	1.0	82.7 ± 22.7		53.3 ± 30.7	
CD184	0	27.3 ± 18.2	0.682	26.9 ± 18.1	0.334
	0.5	38.1 ± 27.2		28.2 ± 9.0	
	1.0	42.6 ± 28.1		43.3 ± 19.8	
CCR5	0	5.0 ± 3.8	0.756	6.3 ± 4.9	0.850
	0.5	9.1 ± 10.6		5.4 ± 6.0	
	1.0	8.0 ± 7.7		4.1 ± 4.7	
CCR7	0	13.5 ± 19.1	0.909	12.9 ± 20.3	0.941
	0.5	19.4 ± 36.1		8.7 ± 13.0	
	1.0	24.3 ± 44.1		11.3 ± 17.5	

*ANOVA test

Figure 1

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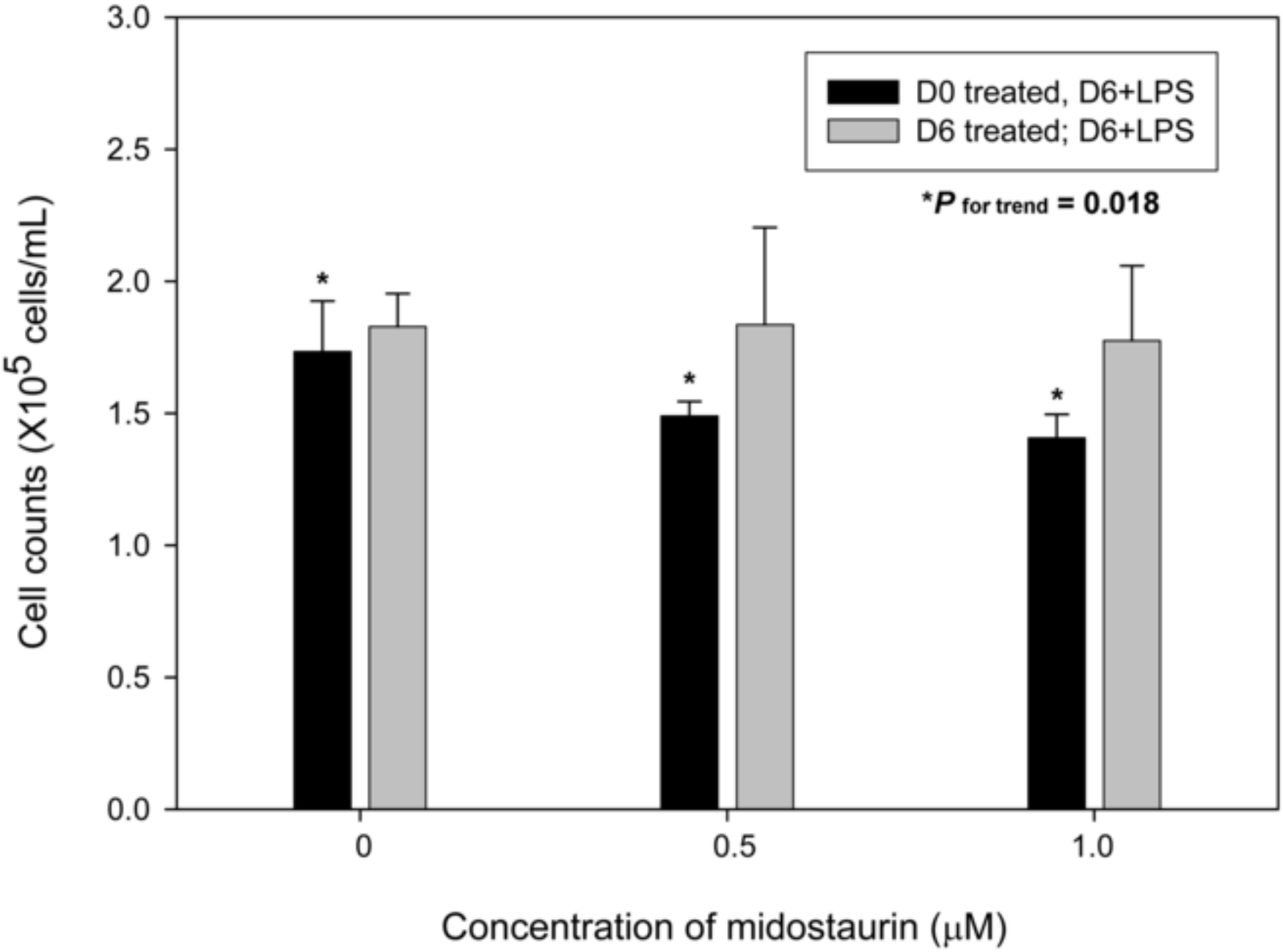

Figure 2

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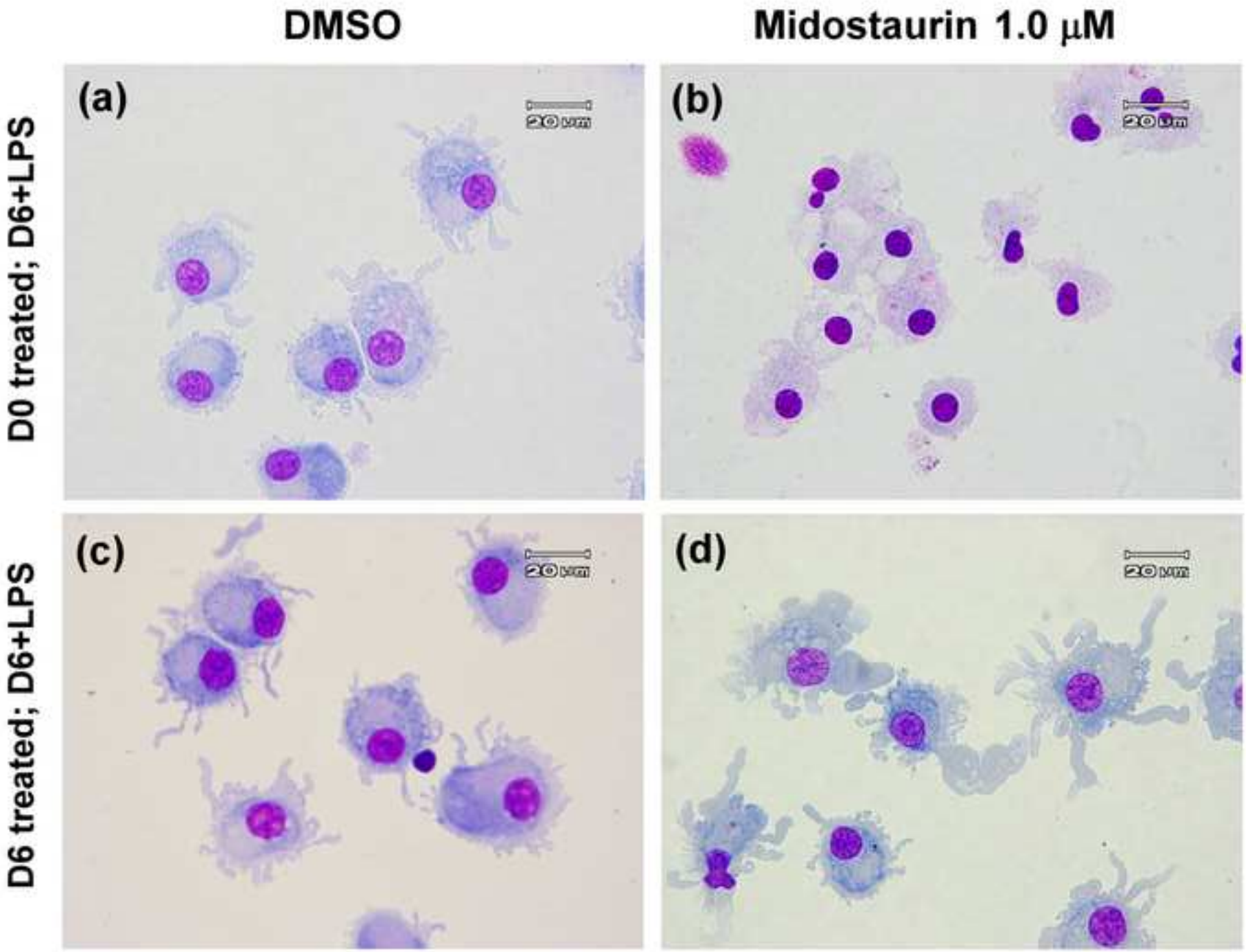

Figure 3

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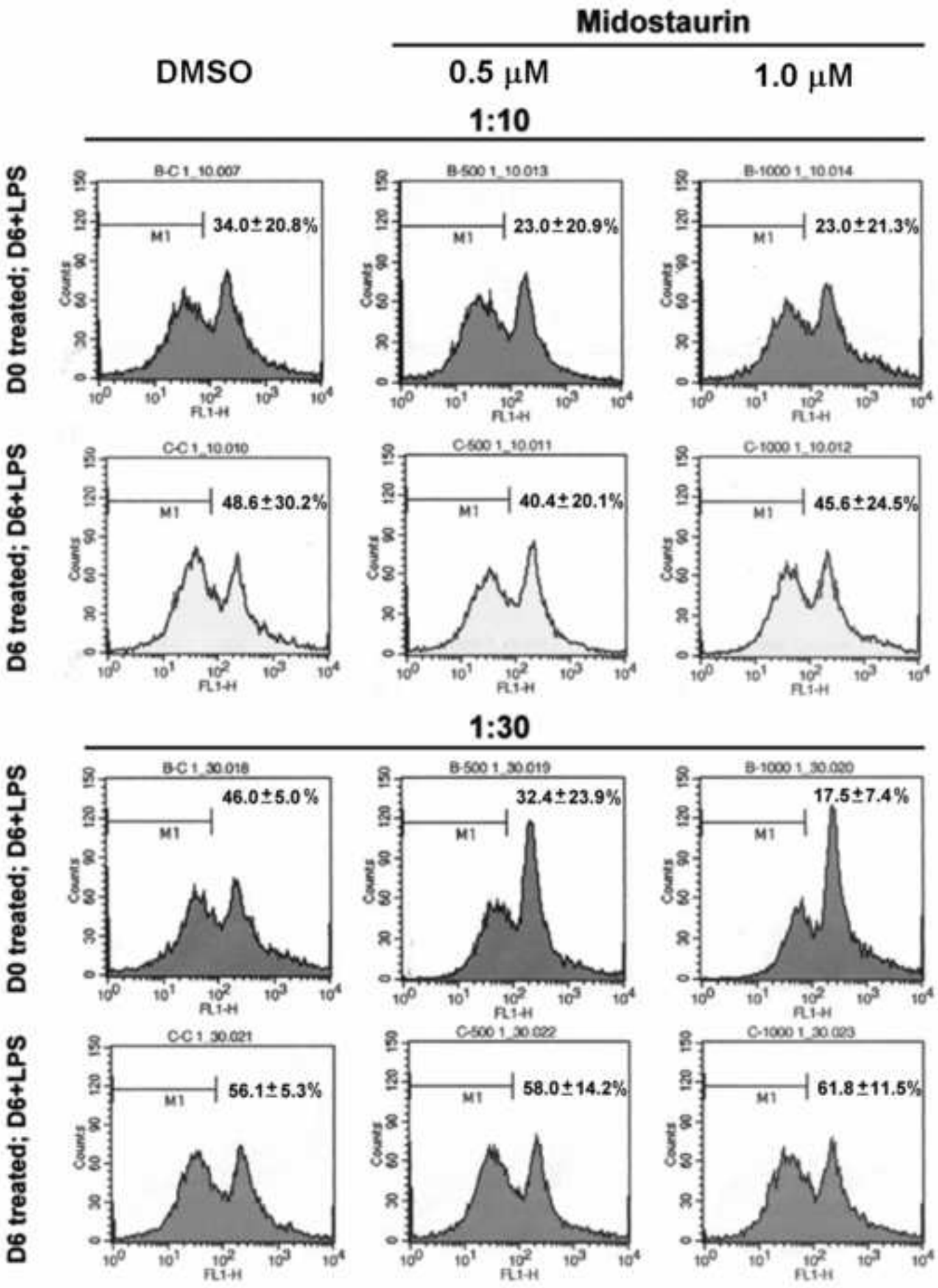

Figure 4

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