Sunitinib, an orally available receptor tyrosine kinase inhibitor, induces monocytic differentiation of acute myeogenouse leukemia cells that is enhanced by 1,25-dihydroxyviatmin D_3.

To the Editor: Sunitinib, an orally available multitargeted receptor tyrosine kinases (RTK) inhibitor, effectively induced growth arrest and apoptosis of acute myelogenous leukemia (AML) cells with gain of function mutation in fms-like tyrosine kinase 3 (*FLT3*) (*FLT3-ITD*) *in vitro* and *in vivo*.[1] This study fount that sunitinib induced monocytic differentiation of AML cells irrespective of *FLT3* gene status. For example, exposure of HL60 cells with the wild-type *FLT3* as well as MOLM13 cells expressing *FLT3-ITD* to sunitinib increased levels of CD11b antigen, a marker of myeloid differentiation, on their cell surface (Figs 1A). Sunitinib also increased levels of CD14 cell surface antigens and stimulated their endocytic capacity, as measured by internalization of FITC-labeled dextran (data not shown). Moreover, sunitinib stimulated production of inflammatory cytokines, such as interferon-γ **(**IFN-γ) and tumor necrosis factor-α (TNF-α) in AML cells (Figs 1B,C), indicating monocytic differentiation of AML cells. Sunitinib-mediated monocytic differentiation was further confirmed by morphological change (D). Interestingly, sunitinib-induced differentiation was significantly potentiated in the presence of $1,25(OH)_2D_3$, a known inducer of monocytic differentiation in leukemia cells (Figs 1A-C).[2] Furthermore, $1,25(OH)_2D_3$

potentiated the ability of sunitinib to inhibit the clonogenic growth of AML cells (Figs 1E).

CCAAT/enhancer binding protein α (C/EBPα) belongs to a family of nuclear transcription factor and is required for myeloid differentiation.[3] Sunitinib increased levels of C/EBPα in association with downregulation of c-Myc, a negative regulator of differentiation, in MOLM13 and HL60 cells (Fig 2). Sunitinib might induce monocytic differentiation of AML cells via modulation of these transcription factors.

We next examine whether sunitinib affected activity of normal hematopoietic cells *in vivo*. Treatment of C57BL/6 mice (n=5) with sunitinib (20 mg/kg, 5 days) significantly increased population of $CD14^+$ mononuclear cells and $CD8^+$ T cells in their spleen as well as bone marrow (Table 1). Also, sunitinib stimulated production of IFN-γ and TNF-α in their spleen mononuclear cells (Fig 3). These observations suggested that sunitinib activated the inflammatory cells *in vivo*.

Finally, we attempted to verify the molecular mechanisms by which sunitinib induced monocytic differentiation of hematopoietic cells. Notable, when AML cells were pre-incubated with anti-human TNF-α monoclonal antibody infliximab (100 ng/ml), sunitinib-induced expression of CD11b was almost completely blocked (Table 2), suggesting the involvement of TNF-α-mediated signaling in sunitinib-inducing monocytic differentiation of AML cells. This study utilized the very low concentrations

of sunitinib (2.5-20 nM), which was not able to dephosphorylate FLT3 and its downstream signal pathways in MOLM13 and HL60 cells (data not shown). Sunitinib also induced the differentiation of U937 cells which did not express FLT3 on their cell surface (data not shown). Thus, sunitinib-induced differentiation was probably independent of FLT3. Recently, erlotinib as well as gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR), induced growth arrest and differentiation of AML cells *in vitro* and *in vivo*, which was independent of EGFR signaling.[4,5] Erlotinib and gefitinib could produce TNF-α, resulting in differentiation of AML cells.

Taken together, sunitinib induced monocytic differentiation of AML cells irrespective of the *FLT3* gene status. Clinical studies with this class of agents should include the individuals with AML expressing the wild-type *FLT3*.

Acknowledgements

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Figure Legend

Fig 1. Effects of 1,25(OH)$_2$D$_3$ on sunitinib-induced differentiation and growth arrest of AML cells. (A) MOLM13 and HL60 cells were cultured with either sunitinib (2.5-20 nM) and/or 1,25(OH)$_2$D$_3$ (0.005-0.5 μM). After 2 days, CD11b expressing population was measured by FACS. Results represent the mean ± SD of three experiments performed twice in duplicate plate. The statistical significance of difference was determined by one-way ANOVA followed by Boneferroni's multiple comparison tests. *, $p< 0.01$, with respect to control cells treated with either sunitinib or 1,25(OH)$_2$D$_3$ alone. VitD, 1,25(OH)$_2$D$_3$. **Effects of 1,25(OH)$_2$D$_3$ and/or sunitinib on macrophage-related cytokines. (B, C)** MOLM13 and HL60 cells were cultured with either sunitinib (2.5-20 nM) and/or 1,25(OH)$_2$D$_3$ (0.01-0.5μM). After 2 days, RNA was extracted and cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of **(B)** *IFN-*$\gamma$and **(C)** *TNF-*α. Results represent mean ± SD of triplicate cultures. The statistical significance of difference between cytokine production induced by either 1,25(OH)$_2$D$_3$ and/or sunitinib was determined by one-way ANOVA followed by Boneferroni's multiple comparison tests. *, $p<0.01$. VitD, 1,25(OH)$_2$D$_3$. **(D) May-Giemsa stain.** MOLM13 cells were culture by replacing culture media containing sunitinib (2.5 μM) or control diluent (DMSO) every another day. After 8 days, cells were harvested and cytospin preparations were stained with May-Giemsa. Their

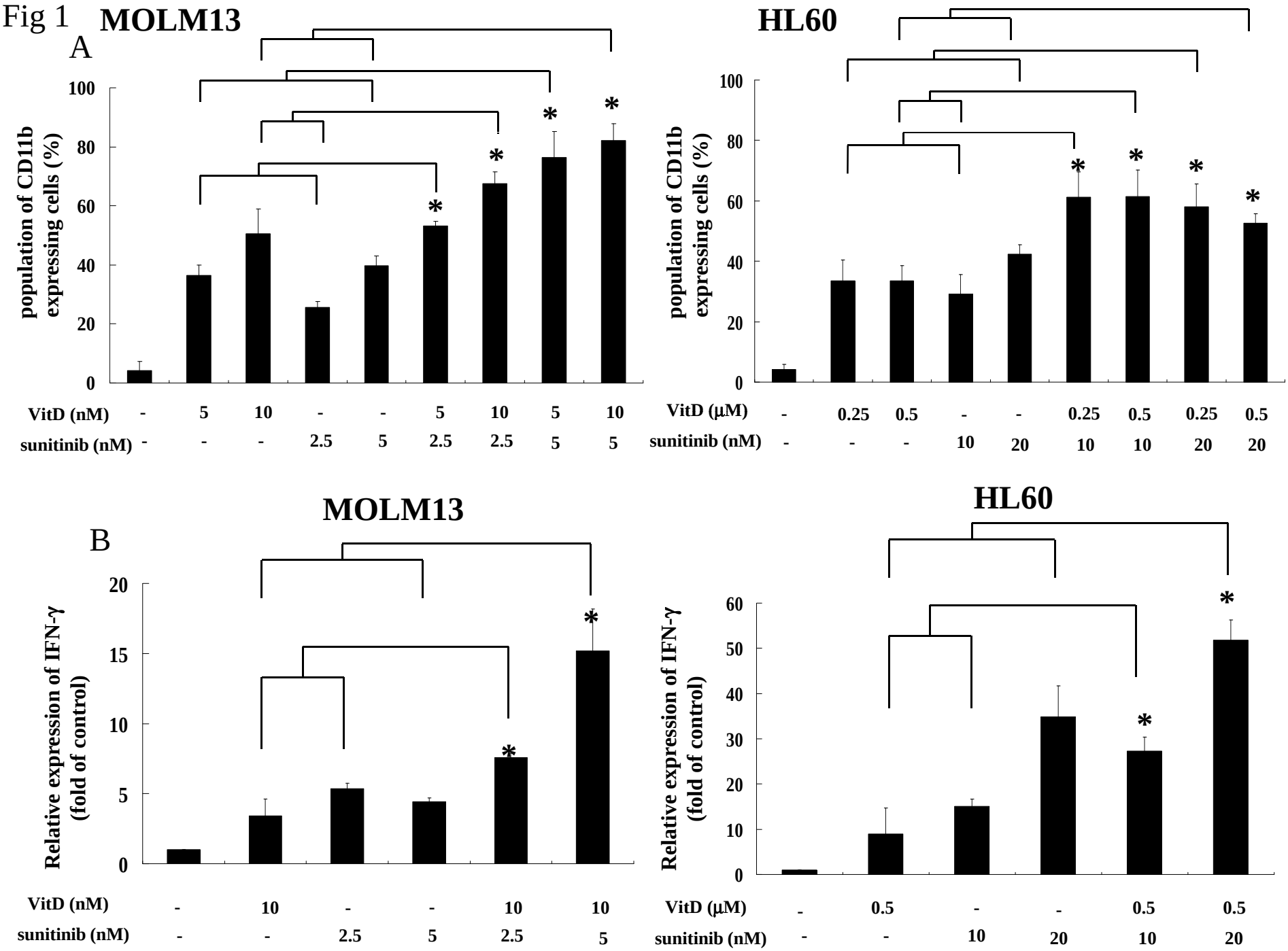
morphology was assessed under a light microscope. Original magnifications were x 400. **1,25(OH)$_2$D$_3$ potentiated anti-proliferative effect of sunitinib. (E) Colony forming assay.** MOLM13 and HL60 cells were cloned in soft agar and cultured with sunitinib (2.5-20 nM) or 1,25(OH)$_2$D$_3$ (1.25-50 nM) either alone or in combination. After 10 days, colonies were counted. Results are expressed as a mean percentage of control plates containing diluent. The statistical significance of difference between growth inhibition produced by either sunitinib or 1,25(OH)$_2$D$_3$ alone and those resulting from the combination of both was determined by one-way ANOVA followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate. *, p<0.01. VitD, 1,25(OH)$_2$D$_3$.

Fig 2. Effects of sunitinib on differentiation-related molecules. Western blot analysis. MOLM13 and HL60 cells were cultured with sunitinib (5-20 nM). After 2 days, cells were harvested and subjected to Western blot analysis to monitor the levels of C/EBPα and c-Myc. Each lane was loaded with 30 μg of nuclear proteins. Levels of GAPDH were measured as a loading control.

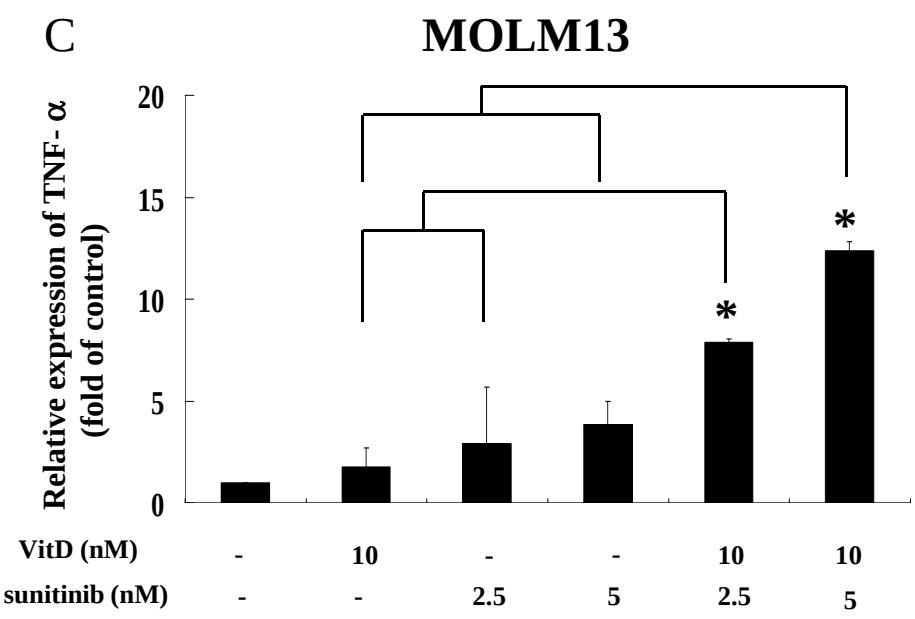
Fig 3. Effects of sunibinit on macrophage-related cytokines *in vivo*. Either sunitinib (n=5, 20 mg/kg) or control diluent (n=5) was administered to C57BL/6 mice by gavage for 5 days. At the end of the experiments, spleen was removed and RNA was extracted. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of

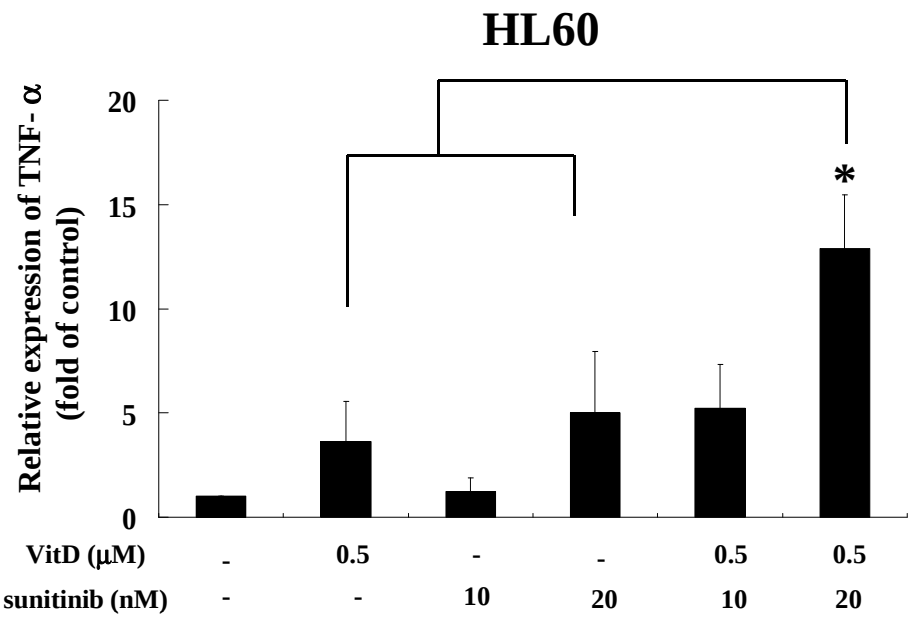
IFN-γ and *TNF-α*. Results represent mean ± SD of triplicate cultures. *, $p<0.05$; **, $p<0.01$.

Fig 1

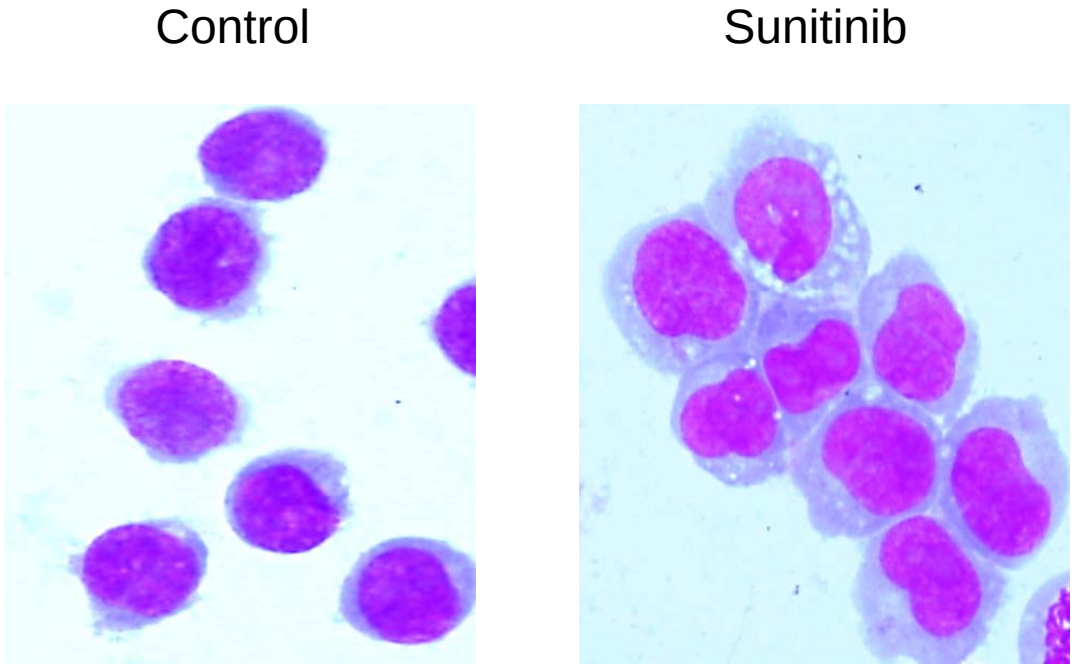

A
MOLM13
population of CD11b expressing cells (%)
0
20
40
60
80
100
VitD (nM) - 5 10 - - 5 10 5 10
sunitinib (nM) - - - 2.5 5 2.5 2.5 5 5
HL60
population of CD11b expressing cells (%)
VitD (μM) - 0.25 0.5 - - 0.25 0.5 0.25 0.5
sunitinib (nM) - - - 10 20 10 10 20 20
B
MOLM13
Relative expression of IFN-γ (fold of control)
0
5
10
15
20
VitD (nM) - 10 - - 10 10
sunitinib (nM) - - 2.5 5 2.5 5
HL60
Relative expression of IFN-γ (fold of control)
0
10
20
30
40
50
60
VitD (μM) - 0.5 - - 0.5 0.5
sunitinib (nM) - - 10 20 10 20

C


MOLM13
Relative expression of TNF- α (fold of control)
20
15
10
5
0
*
*
VitD (nM) - 10 - - 10 10
sunitinib (nM) - - 2.5 5 2.5 5


HL60
Relative expression of TNF- α (fold of control)
20
15
10
5
0
*
VitD (μM) - 0.5 - - 0.5 0.5
sunitinib (nM) - - 10 20 10 20

C


Control
Sunitinib

E

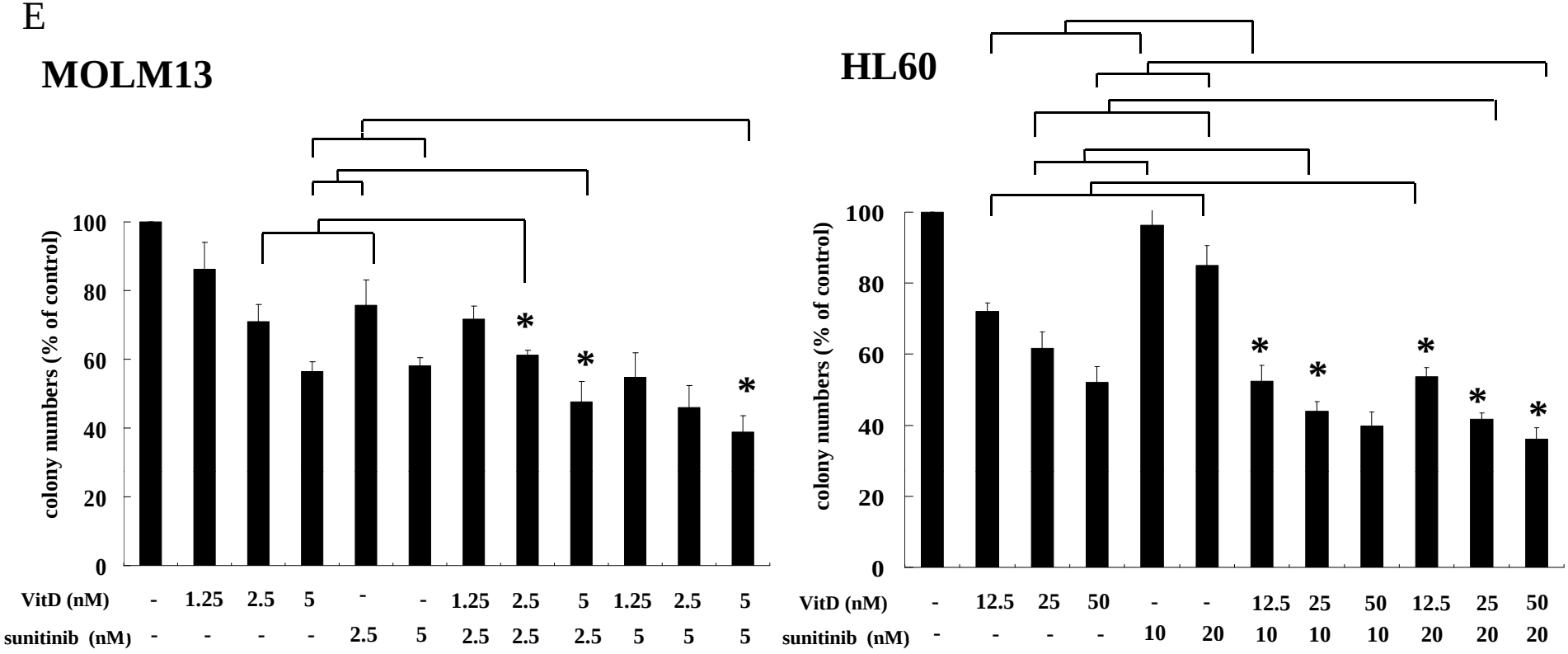

MOLM13
colony numbers (% of control)
100
80
60
40
20
0
VitD (nM) - 1.25 2.5 5 - - 1.25 2.5 5 1.25 2.5 5
sunitinib (nM) - - - - 2.5 5 2.5 2.5 2.5 5 5 5
*
HL60
colony numbers (% of control)
100
80
60
40
20
0
VitD (nM) - 12.5 25 50 - - 12.5 25 50 12.5 25 50
sunitinib (nM) - - - - 10 20 10 10 10 20 20 20
*

Fig 2

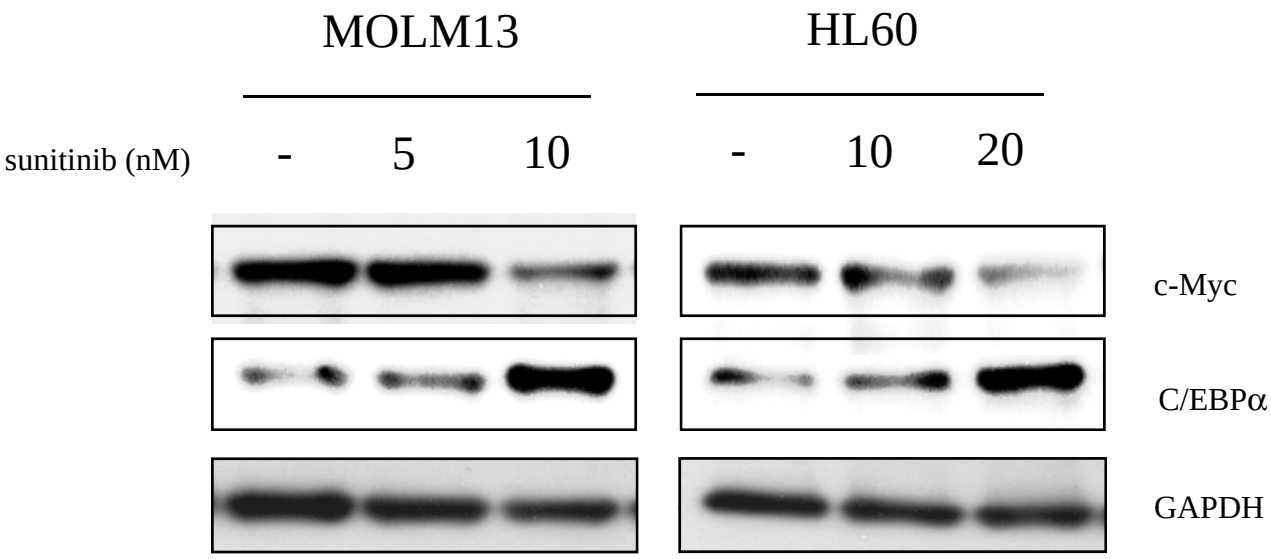

MOLM13
HL60
sunitinib (nM)
-
5
10
-
10
20
c-Myc
C/EBPα
GAPDH

Fig 3

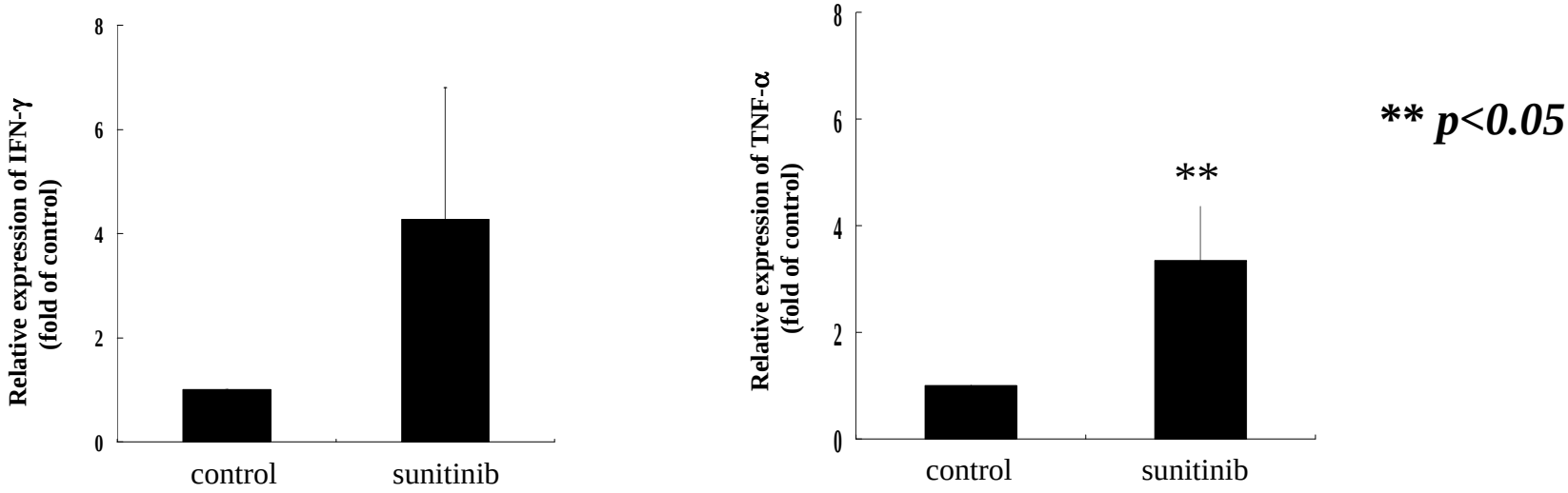

Relative expression of IFN-γ (fold of control)
0
2
4
6
8
control
sunitinib
Relative expression of TNF-α (fold of control)
0
2
4
6
8
**
control
sunitinib
** p<0.05

Table 1. CD14 and CD11b expression.

	CD14	CD8
control spleen	15.5±1.9%	35.5±2.9%
sunitinib spleen	22.3±6.1%**	39.6±2.6%**
control BM	42.3±1.1%	10.6±3.8%
sunitinib BM	47.0±3.6%**	22.4±2.3%*

Mice were treated with either sunitinib (20 mg/kg/day, for 5 days) or control diluent by gavage. At the end of experiments, mice were sacrificed, and spleen and BM were removed. Population of CD14- or CD8-expressing cells was quantified by FACS.
* $p<0.01$, ** $p<0.05$. BM; bone marrow

Table 2. Expression of CD11b.

	control	infliximab 100 ng/ml
MOLM13		
control	4.3 ± 1.9%	3.4 ± 0.5%
sunitinib 2.5nM	25.6 ± 2.0%	3.5 ± 0.3%
sunitinib 5 nM	39.8 ± 3.3%	4.5 ± 0.6%
HL60		
control	4.1 ± 1.7 %	0.8 ± 0.04%
sunitinib 10 nM	29.2 ± 6.5 %	3.4 ± 0.3%
sunitinib 20 nM	42.3 ± 3.2 %	5.8 ± 0.2%

AML cells were pre-incubated with anti-human TNF-α monoclonal antibody infliximab (100 ng/ml) for 1 hr, and then exposed to various concentrations of sunitinib (2.5-20 nM). After 2 days, cells were harvested, and CD11b expressing population was analyzed by flow cytometry.