

Short communication

### S-NITROSOGLUTATHIONE MODULATES CXCR4 AND ICOS EXPRESSION

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**Abstract:** The expression of CXCR4, a membrane protein which is involved in the entry of HIV-1, is down-modulated from the cell surface by Phorbol 12-myristate 13-acetate (PMA) and the Ca<sup>+</sup> ionophore, Ionomycin. Inducible co-stimulator (ICOS), which contributes to lymphocyte proliferation, is up-regulated by PMA/Ionomycin. We examined the influence of S-nitrosoglutathione (SNG), an inhibitor of Vacuolar H<sup>+</sup>-ATPase (V-ATPase), on the expression of CXCR4 and ICOS in PMA/Ionomycin-treated peripheral mononuclear cells (PBMC), and of CXCR4 alone in lymphoid cell lines. In this report, we show that SNG interferes with both effects of PMA/Ionomycin, namely CXCR4 down-regulation and ICOS up-regulation. These studies imply opposing roles of V-ATPase in the regulation of CXCR4 and ICOS. The influence of SNG in modulating the susceptibility of T cells to HIV-1 and on their immune responses needs further investigation.

**Key words:** S-nitrosoglutathione (SNG), CXCR4, ICOS, HIV-1

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Abbreviations used: SNG – S-nitrosoglutathione; PMA – phorbol 12-myristate 13-acetate; V-ATPase – vacuolar H<sup>+</sup>-ATPase; ICOS – inducible co-stimulator; HIV – human immunodeficiency virus; CCP – clathrin-coated pit; PBMC – peripheral mononuclear cells; GRK – G-protein coupled receptor kinase.

## INTRODUCTION

Vacuolar H<sup>+</sup>-ATPase (V-ATPase), one of the membrane-associating proteins, is involved in the formation and maintenance of clathrin-coated pit (CCP) vesicles via the lowering of pH, contributing to the down-regulation of cell surface proteins and to intracellular protein transportation [1, 2]. In a previous study [3], S-nitrosoglutathione (SNG), which is a nitrogen superoxide donor reagent, was shown to inhibit the catalytic function of V-ATPase.

CXCR4 on the target cell's membrane are indispensable for the entry of T-cell tropic Human Immunodeficiency Virus 1 (HIV-1). CXCR4, whose natural ligand is SDF-1, functions as a co-receptor for T-cell tropic HIV-1 on the CD4<sup>+</sup> T lymphocyte [4]. On CD4<sup>+</sup> T lymphocytes, CXCR4 is down-modulated *in vitro* from the cell surface by stimulation with Phorbol 12-myristate 13-acetate (PMA) and the Ca<sup>+</sup> ionophore, Ionomycin [5]. To investigate the effect of V-ATPase on cell surface CXCR4 expression, we assessed CXCR4 expression in the presence of SNG in peripheral mononuclear cells (PBMC) and lymphoid cell lines under PMA/Ionomycin treatment. PMA/Ionomycin treatment up-regulates the cell surface expression of inducible co-stimulator (ICOS), which is a member of the CD28 family and is induced on activated T-lymphocytes; the stimulation of ICOS results in a strong enhancement of cell proliferation [6]. Though CD28 expression is down-regulated by the HIV-1 Nef protein-mediated CCP pathway in HIV-infected lymphocytes [7], the regulation of ICOS expression on HIV-infected cells is less understood. In this report, we show that SNG interferes with PMA/Ionomycin-induced CXCR4 down-regulation as well as with PMA/Ionomycin-induced up-regulation of ICOS.

## MATERIALS AND METHODS

### Cells

Lymphoid cell line, CEM.NKR-CCR5, and Jurkat Clone E6-1 were obtained from the NIH AIDS Research & Reference Reagent Program (Germantown, MD, USA). PBMC from healthy donors were isolated by centrifugation with Ficoll-Hy-paque (Pharmacia) and washed three times with Hank's balanced salt solution (HBSS). Cells were maintained in complete growth media of RPMI1640 with 10% FCS, 300 mg/ml L-glutamine, 100 mg/ml streptomycin and 100 units/ml penicillin.

### Cell stimulation, cell staining and flow cytometric analysis

In a 24-well polyethylene cell culture plate,  $0.5 \times 10^5$  to  $1 \times 10^6$  cells were cultured in 1 ml of complete growth media with 30 ng/ml PMA plus 300 ng/ml Ionomycin and 0.3, 1, and 3 mM SNG in 5% CO<sub>2</sub> and 37°C. Cells were harvested after 48 hrs culture, washed three times with HBSS and incubated with Mouse IgG isotype control antibody (BD Pharmingen), FITC conjugated hamster IgG isotype control antibody (e-Bioscience) or anti-ICOS-FITC (e-Bioscience), anti-CXCR4-PE (Clone 12G5) antibodies (BD Pharmingen) for

15 min at room temperature for cell staining. The cells were washed with HBSS and suspended in Cytotfix solution (BD Bioscience Pharmingen) and analyzed by FACS Calibur with Cell Quest software (Becton Dickinson).

## RESULTS AND DISCUSSION

CXCR4 is authentically expressed on the untreated CEM.NKR-CCR5 cell line (Fig. 1A) and is maximally internalized with PMA/Ionomycin treatment, as shown in Fig. 1A, column 3. In the presence of SNG, CXCR4 down-modulation by PMA/Ionomycin is inhibited. A reduction in the level of CXCR4 expression by PMA/Ionomycin was not found with 3 mM SNG (Fig. 1A, columns 4-6). SNG on CXCR4 by PMA/Ionomycin was also assessed to have an inhibitory effect in Jurkat Clone E6-1 cells. The baseline expression of CXCR4 was also authentically high on Jurkat Clone E6-1 (Fig. 1B). PMA/Ionomycin remarkably reduces CXCR4 expression from the Jurkat Clone E6-1 cell surface (Fig. 1B, column 3). However, even with 1 mM SNG, CXCR4 down-modulation was clearly inhibited. Inhibition of CXCR4 down-modulation was also confirmed with 0.3, 1 and 3 mM SNG (Fig. 1B, column 3 to 6).

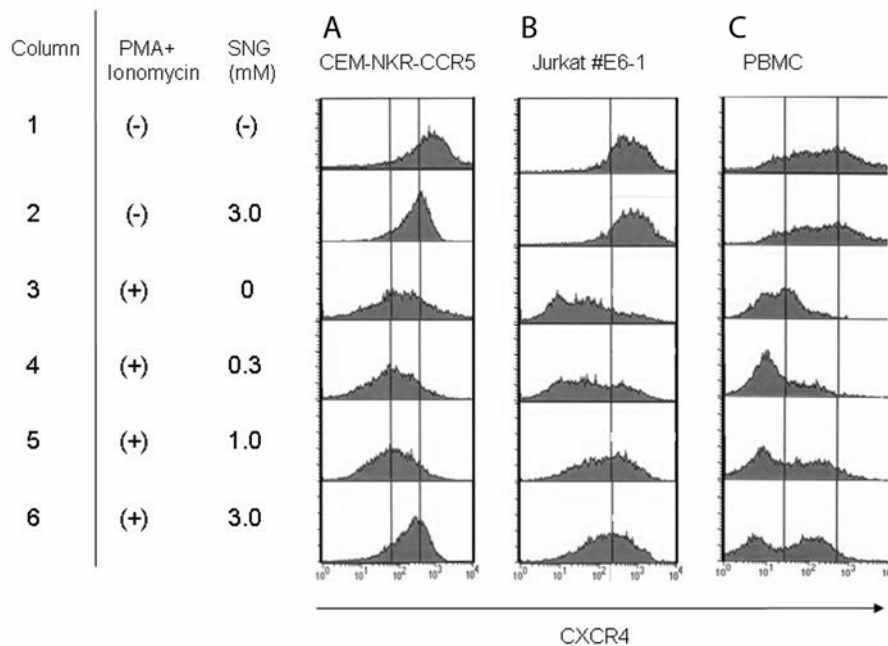


Fig. 1. Modulation of CXCR4 expression. CEM.NKR-CCR5 (A), Jurkat Clone E6-1 (B) and PBMC (C) were incubated with PMA/Ionomycin with 0.3, 1 or 3 mM S-nitrosoglutathione. Cells were harvested after 48 hrs incubation and stained with PE-conjugated CXCR4 antibody. Through the comparison of the Normal distribution curve fitted by Mean Fluorescence Intensity, the significance of SNG treatment was confirmed (data not shown). Experiments were performed more than two to three times independently and representative data are shown.

We examined whether this down-regulation of CXCR4 by PMA/Ionomycin can be inhibited by SNG in PBMC. CXCR4 down-modulation by PMA/Ionomycin was inhibited with 1 mM and 3 mM SNG (Fig. 1C). CXCR4 inhibition was more potent with 3 mM SNG than with 1 mM SNG.

Furthermore, we tested the inhibitory effect of SNG on CXCR4 expression in PBMCs treated with PMA/Ionomycin, PMA, and Ionomycin respectively. Comparing PBMC without SNG (Fig. 2A) and PBMC with SNG (Fig. 2B), CXCR4 down-modulation was interfered with by SNG in the treatment with PMA/Ionomycin and PMA alone (Fig. 2B, columns 2 and 3). PBMC treated with Ionomycin alone did not show strong CXCR4 down-modulation, so the interference with CXCR4 down-modulation by SNG was not clearly observed in the treatment with Ionomycin alone (Fig. 2B, column 4).

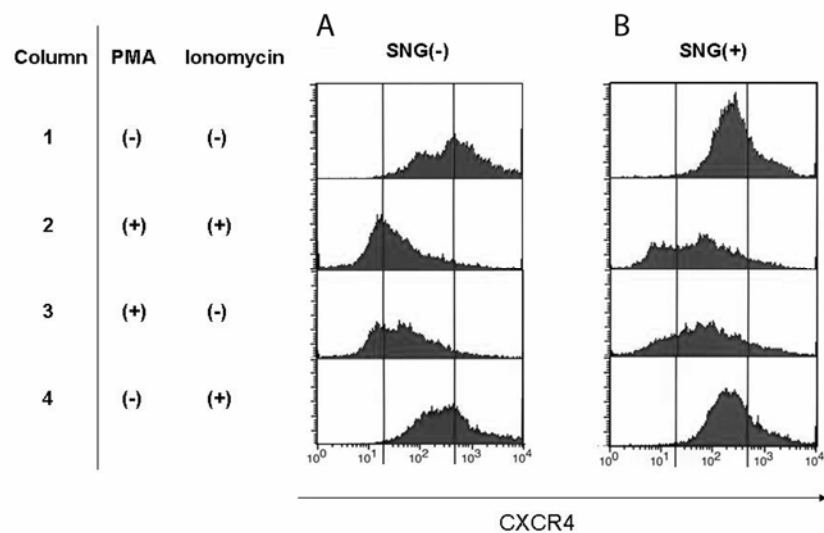


Fig. 2. Inhibitory effect of S-nitrosoglutathione on CXCR4 expression in PMA- and Ionomycin-treated PBMC. PBMCs were incubated for 48 hrs with PMA/Ionomycin, PMA or Ionomycin without (A) or with (B) 3 mM S-nitrosoglutathione. The cells were harvested and stained with PE-conjugated CXCR4 antibody. The experiments were performed twice independently and the representative data is shown.

ICOS is regularly induced with PMA/Ionomycin treatment in PBMC. However, we confirmed ICOS expression is not up-regulated with PMA/Ionomycin in the CEM.NKR-CCR5 or Jurkat E6.1 cell lines (data not shown). Therefore, we tested the inhibitory effect of SNG on PMA/Ionomycin treatment in PBMC. ICOS was obviously up-regulated in PBMC with PMA/Ionomycin as shown in Fig. 3, column 3. However, ICOS expression was inhibited in presence of SNG (Fig. 3, columns 4 to 6). This inhibition of ICOS expression was more clearly observed with 3 mM SNG (Fig. 3, column 6).

We also tested SNG for modulation of the expression of CCR5, which is the co-receptor for macrophage tropic HIV-1. Interestingly, CCR5 expression was not modulated by PMA/Ionomycin stimulation of CEM.NKR-CCR5 cells, and SNG did not show any further significant change in CCR5 expression (data not shown). Even though CCR5 is involved in membrane protein trafficking [8], SNG did not display a significant effect on CCR5 expression for PMA/Ionomycin-treated cells in our experiment.

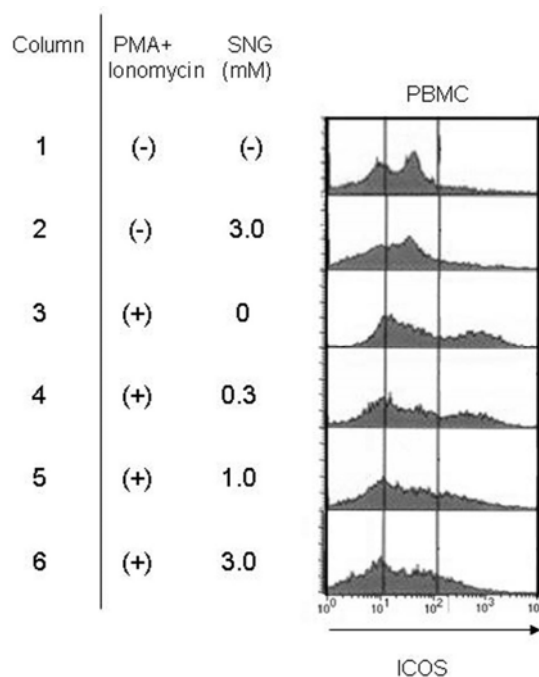


Fig. 3. Modulation of ICOS expression. PBMC were incubated with PMA/Ionomycin with 0.3, 1 or 3 mM S-nitrosoglutathione. Through the comparison of the Normal distribution curve fitted by Mean Fluorescence Intensity, the significance of SNG treatment was confirmed (data not shown). Experiments were performed independently more than three times and the representative data is shown.

V-ATPase plays an important role in intracellular membrane trafficking, protein processing, endocytosis and degradation, and coupled transport [1-3]. The V-ATPase of the CCP vesicle is inhibited by disulfide bond formation between two cysteine residues located at the catalytic site by SNG [3]. CXCR4 is one of the G-protein coupled receptors. Downstream of the protein kinase C signal pathway by PMA stimulation, G-protein coupled receptor kinases (GRKs) phosphorylate CXCR4. Then arrestin, which functions as an adaptor to AP-2 complex and Clathrin, binds to phosphorylated CXCR4. AP-2 complex, Clathrin and CXCR4 are involved in CCP vesicle formation and are internalized with the CCP pathway [5]. The lowering of pH by V-ATPase is essential for CCP vesicle formation and maintenance. Taken together, the interference function of SNG for

CXCR4 internalization is considered to be through V-ATPase inhibition by disulfide bond formation at the catalytic site of V-ATPase in the CCP vesicle.

ICOS is induced in the activated lymphocytes. ICOS expression is induced in both biological stimulation of the T-cell receptor (TCR) and biochemical activation with PMA/PHA [6]. ICOS is synthesized, modified and transported to be expressed on the cell surface. In our experiment, ICOS expression was interfered with by SNG. On the other hand, ICOS is expressed very slightly on unstimulated lymphocytes. However, ICOS does not increase in SNG treatment without PMA/Ionomycin treatment (Fig. 3, column 2). ICOS expression will increase if SNG inhibits ICOS down-regulation without PMA/Ionomycin treatment. Thus, our observation of the down-regulation of ICOS with SNG is considered to be through the inhibition of V-ATPase function in the up-regulation of ICOS.

In HIV-infected cells, V-ATPase has a binding site for HIV-1 Nef protein [9]. Nef also binds to the CD4 molecule in HIV-infected CD4+ T lymphocytes. The formation and involvement of the V-ATPase/Nef/CD4 complex in CCP is a critical step in CD4 down-regulation from the cell surface [10, 11]. Luo *et al.* showed that this CD4 down-modulation by HIV-1 Nef is inhibited by Ikarugamycin, an analogue of macrolides [12]. Ikarugamycin is an inhibitor of intracellular trafficking [13], thereby implying that CD4 down-modulation with HIV-1 Nef could also be blocked by a V-ATPase inhibitor, through the interference of pH control in the CCP. However, it has not been elucidated whether SNG is like Ikarugamycin in having an anti-Nef effect on CD4 expression.

Our results indicate that SNG plus PMA/Ionomycin treatment leads to high CXCR4 expression and low ICOS expression. When ICOS expression is down-modulated, the efficacy of co-stimulation by ICOS will be less and T-cell proliferation may be reduced. T-cell proliferation is low in HIV infection. Furthermore, as the CXCR4 tropic virus is dominant in the terminal stage of HIV/AIDS, the inhibition of PMA/Ionomycin-induced CXCR4 down-modulation by SNG is a good condition to monitor HIV infectivity. The influence of SNG in modulating the susceptibility of T cells to HIV and on their immune responses needs further investigation.

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