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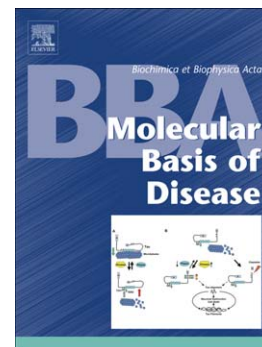
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***Regular papers*****HIV envelope protein gp120-triggered CD4<sup>+</sup> T-cell adhesion to vascular endothelium is regulated via CD4 and CXCR4 receptors**

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***Running title:*** gp120-induced T-cell adhesion to vascular endothelium

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## Summary

Activation of T-lymphocytes is an important component of inflammatory and infectious processes, including HIV infection. It is regulated via the actions of various cell-surface receptors, including CD4 and CXCR4. We examined the roles of CD4 and CXCR4 in the adhesive interaction of CD4+T-cells with the vascular endothelium. CD4+Jurkat cells were incubated in the presence or absence of anti-CD4 to stimulate CD4, or with SDF-1 alpha, a cognate ligand of CXCR4. Stimulation of CD4 or CXCR4 each significantly enhanced cell adhesion. We next stimulated the two receptors together, using gp120, a component of HIV. This enhanced cell adhesion was greater than stimulation of CD4 or CXCR4 individually. Western blotting revealed that stimulation of CXCR4 by SDF-1 alpha significantly increased the phosphorylation of ERK1/2 in Jurkat cells. Treatment with anti-CD4 also activated ERK1/2, although to a lesser extent. When the expression of CD4 was reduced by siRNA transfection, both CD4-dependent adhesion and MAPK activation were diminished. Furthermore, pre-treatment with fluvastatin, significantly attenuated observed Jurkat cell adhesion. These findings indicate novel mechanisms of CD4+T-cells recruitment to activated endothelium via CD4 and CXCR4, which are modulated by statin.

## 1. Introduction

Infection of target lymphocytes by human immunodeficiency virus type 1 (HIV-1) requires the expression of cofactors such as chemokines and their regulators on the lymphocyte surface, leading to a selective loss of CD4<sup>+</sup> T-cells and fatal immunodeficiency [1-3]. The process of HIV infection involves the binding of glycoprotein (gp) 120, a surface component of the viral envelope glycoprotein, to CD4 and CXCR4 receptors expressed on the target cell surface [4]. gp120 first binds to the CD4 receptor, resulting in exposure of novel binding sites in gp120 by which it binds to the co-receptor CXCR4 [5]. These interactions trigger the fusion and entry of the viral genome into the host lymphocyte [6]. CXCR4, which was first reported as a receptor for stromal cell-derived factor (SDF)-1 [7-10], is known to activate MAPK and divergent intracellular signal pathways [11]. Although the SDF-1 alpha-dependent CXCR4 signal transduction cascade has been studied in T-cells, the biological consequences of gp120-triggered CXCR4 signal transduction are not fully understood.

In the present study, we examined the effects of CD4 and CXCR4 on CD4<sup>+</sup> T-cell adhesion to vascular endothelium, and possible modulation by fluvastatin, a statin drug that inhibits 3-hydroxyl-3methylglutaryl co-enzyme A (HMG-CoA) reductase. Statins are used clinically to reduce serum levels of LDL cholesterol as a means to prevent cardiovascular disease [12]. In addition to their lipid-lowering effect, increasing evidence supports the anti-inflammatory potential of statins [13]. Our data indicate an anti-inflammatory role for statins in CD4<sup>+</sup> T-cells stimulated with gp120, suggesting a vascular protective role for these drugs in HIV-positive patients with atherosclerosis.

## 2. Material and Methods

### 2.1 Cell Culture.

A CD4<sup>+</sup> T-cell line (Jurkat cells) was obtained from ATCC (Manassas, VA) and was cultured in RPMI-1640 supplemented with 10% FCS (Life Technologies Oriental, Inc). Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical veins and cultured as described previously [14]. CD4<sup>+</sup> T-cells were prepared from human volunteers as follows. Freshly drawn human blood samples were separated by Histopaque (1.077 density) density-gradient centrifugation to obtain PBMC fractions. CD4<sup>+</sup> T-cells were purified from the PBMC fraction through negative selection using a magnetic cell separation system (Miltenybiotec, MACS CD4<sup>+</sup> T Cell Isolation Kit). The purity of CD4<sup>+</sup> T-cells was determined using a calibrated FACScan flow cytometer (Becton Dickinson, San Jose, CA). For use in a flow chamber apparatus, HUVECs were plated on 22-mm fibronectin-coated glass coverslips, as previously described [15].

### 2.2 Flow Cytometry.

Jurkat cells ( $1 \times 10^6$  cells/ml) were stained with 10  $\mu$ g/ml of anti-CXCR4 antibody (12G5) or anti-CD4 antibody (13B8.2)[16] for 45 minutes at 4°C, then washed and incubated for an additional 45 minutes at 4°C with 10  $\mu$ g/ml of anti-IgG FITC secondary antibody (Molecular Probes, Eugene, OR). The cells were washed and subsequently analyzed using a FACScan apparatus and Cell Quest software (Becton Dickinson, San Jose, CA).

### 2.3 Adhesion Assay under Laminar Flow.

The protocols of the adhesion assay under flow conditions have been previously described in detail [17, 18]. Briefly, endothelial monolayers on coverslips were stimulated with IL-1 $\beta$  (10 units/ml) and cultured for 4 hours at 37°C, then positioned in a flow chamber mounted under an inverted microscope. Jurkat cells or CD4 $^{+}$  T cells were then diluted in the perfusion medium to  $1 \times 10^5$  cells/ml. In some of the experiments, cells were treated with monomeric gp120 (HIV-1 B) (2.5  $\mu$ g/ml) (Immuno Diagnostics, Woburn, MA) and SDF-1  $\alpha$  (80 ng/ml) (PeproTech, Rocky Hill, NJ) at 37°C for 5 minutes, or with an anti-CD4 antibody (10  $\mu$ g/ml) (Beckman Coulter, Fullerton, CA) for 40 minutes at 4°C prior to the assay. PD98059 (Sigma, St. Louis, MO), a potent inhibitor of MEK (MAP kinase kinase), was used at 25  $\mu$ M for 4 hours at 4°C prior to the assay. The cells were drawn through the chamber at a controlled flow rate to generate a calculated wall shear stress of 1.0 dyne/cm $^2$  for 10 minutes. The entire period of perfusion was recorded on videotape using a digital video recorder equipped with a time generator. The captured images were transferred to a personal computer for image analysis to determine the numbers of adherent cells in 5 to 10 randomly selected x20 microscope fields from each experiment.

### 2.4 Western Blotting of MAPK.

Western blotting was conducted as previously described [19]. In brief, the cells were cultured in RPMI-1640 containing 0.1% FCS for 48 hours, and thoroughly washed with FCS-free RPMI-1640. Then, the cells were stimulated with the indicated amount of gp120 (2.5  $\mu$ g/ml) or SDF-1  $\alpha$  (80 ng/ml) for 5 minutes, or with anti-CD4 (10  $\mu$ g/ml) for 40 minutes, after which they were lysed with a lysis buffer (0.1 M Tris-HCl, 0.15 M NaCl, and 5 mM EDTA,

pH 7.4) containing 1% Triton X-100, 10 µg/ml of leupeptin, 60 U/ml of aprotinin, and 1 mM of PMSF. In some experiments, cells were pretreated with 10 µmol/L of fluvastatin for 48 hours prior to the assay. For Western blot with anti-ERK (K-23) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-ERK (E-4) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, 10 µg of each cell lysate was loaded into each lane. Immunoreactive bands were visualized using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system.

### 2.5 Translocation of RhoA.

To examine the translocation of RhoA from the cytosol to the membrane, membranes and total cell lysates of Jurkat cells ( $1 \times 10^6$ /mL) were prepared as described previously [18]. An equal amount of protein (10 µg) from each fraction was subjected to 12.5% SDS-PAGE, and Western blot analysis was performed using the anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive RhoA was detected with an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

### 2.6 Transfection of Jurkat Cells with siRNA.

The siRNA duplexes against human CD4 cDNA— 5'-GAU CAA GAG ACU CCU CAG UdGdA-3' (sense), 5'-ACU GAG GAG UCU CUU GAU CdTdG-3' (antisense) [20]—were synthesized, and scrambled siRNA (sciRNA) of CD4 and LacZ siRNA were used as controls with the following sequences: scrambled siRNA, 5'-AGU CAG AAG CAC UUC ACA GdAdG-3' (sense), 5'-CUG UGA AGU GCU UCU GAC UdGdT-3' (antisense); LacZ, 5'-



CGU ACG CGG AAU ACU UCG AdTdT-3' (sense), 5'-UCG AAG UAU UCC GCG UAC GdTdT-3' (antisense). Nucleofector (Amaxa, Cologne, Germany) was used for transfection of Jurkat cells following the manufacturer's instructions. In brief, the cells were resuspended ( $5 \times 10^6$  cells in 100  $\mu$ l) in an optimized transfection solution. Each sample was transfected with 5  $\mu$ M siRNA using electroporation with program C-16. After electroporation, cells were immediately transferred to 4.0 ml of complete medium.

### 2.7 Statistical Analysis.

Results are presented as means  $\pm$  SD. Data were analyzed using analysis of variance (ANOVA), with a value of  $p < 0.05$  considered significant.

### 3. Results

We first examined whether activation of CXCR4 and CD4 would induce Jurkat T-cell adhesion to vascular endothelium. Jurkat T-cells were incubated in the presence of SDF-1 alpha (80 ng/ml) at 37°C for 5 minutes, or anti-CD4 mAb (10 µg/ml) for 40 minutes, after which adhesion assays were carried out under flow conditions. As shown in **Figure 1A**, treatment with SDF-1 alpha and anti-CD4 each increased Jurkat T-cell adhesion to activated HUVECs (SDF-1 alpha,  $p < 0.05$  vs. control; anti-CD4,  $p < 0.05$  vs. control). Interestingly, treatment with gp120, expected to activate both CXCR4 and CD4, induced a much higher level of adhesion to HUVECs [gp120 (-),  $3.0 \pm 0.76$ ; gp120 (+),  $11.9 \pm 1.86$ ;  $p < 0.05$ ]. Under static conditions, Jurkat cell adhesion was maximal at 2.5 µg/ml of gp120 (data not shown). The surface expression of CXCR4 was not affected by SDF-1 alpha or gp120 in Jurkat cells (data not shown). To explore the responsible adhesion molecules on Jurkat T-cell adhesion, a fluorescent immunoassay was carried out using anti-LFA-1 and VLA-4 antibodies. The expression levels of LFA-1 and VLA-4 on their surface were not significantly changed before and after treatment with SDF-1 alpha, anti-CD4, and gp120 (data not shown). Adhesion assays were also carried out using freshly isolated CD4+ lymphocytes. As shown in **Figure 1B**, SDF-1 alpha, anti-CD4, and gp120 induced similar levels of CD4+ lymphocyte adhesion. To investigate intracellular machinery, we examined the phosphorylation of MAPK after CXCR4 or CD4 stimulation. We found that phosphorylation of ERK1/2 was enhanced by treatment with SDF-1 alpha and gp120, and to a lesser extent with anti-CD4 (**Figure 2A**). Pre-treatment with PD98059, a potent inhibitor of MEK, reduced observed phosphorylation of ERK1/2. PD98059 also inhibited SDF-1 alpha- [SDF-1(+),  $7.3 \pm 0.68$  cells/HPF; SDF-1(+)+PD98059(+),  $3.1 \pm 1.12$ ;  $p < 0.05$ ], anti-CD4- [anti-CD4(+),  $6.7 \pm 0.81$  cells/HPF; anti-CD4(+)+PD98059(+),  $4.1 \pm 0.67$ ;  $p < 0.01$ ] and gp120-induced [gp120(+),  $11.4 \pm 1.12$  cells/HPF; gp120(+)+PD98059(+),  $7.1 \pm 0.87$ ;  $p < 0.01$ ] Jurkat cell adhesion (**Figure 2B**). Further, the

involvement of RhoA, a member of the small GTP-binding protein family, was also examined because RhoA plays a role in cell motility and adhesion in various cell types [21-23]. As shown in Figure 2C, membrane translocation of RhoA was enhanced following treatment with SDF-1 alpha, anti-CD4, and gp120. In the presence of PD98059, these activations of RhoA were reduced.

To differentiate CD4-dependent signaling pathways from CXCR4-dependent pathways, siRNA against CD4 (siCD4) was transfected into Jurkat cells to inhibit CD4-dependent signaling. Transfection of siCD4 effectively reduced CD4 expression (**Figure 3A**) and both anti-CD4-induced [siLacZ(+),  $5.3 \pm 1.41$  cells/HPF; siCD4(+),  $4.0 \pm 1.15$ ;  $p < 0.05$ ] gp120-induced [siLacZ(+),  $9.2 \pm 1.4$  cells/HPF; siCD4(+),  $6.8 \pm 1.23$ ;  $p < 0.01$ ] cell adhesion were attenuated (**Figure 3B**)

It is reported by us and others, a pharmacological inhibitor of HMG-CoA reductase, statin reduces monocyte adhesion to HUVEC. We thus examined the effect of statin in Jurkat cell adhesion. Jurkat cells were incubated in the presence of 10  $\mu$ M of fluvastatin for 48 hours. In preliminary experiments, the viability of Jurkat cells in the presence of 10  $\mu$ M of fluvastatin was 90% (data was not shown). As shown in **Figure 4A–C**, pre-treatment with fluvastatin significantly inhibited adhesion induced by SDF-1 alpha, anti-CD4, or gp120, while treatment with fluvastatin did not change the baseline level of adhesion. We then determined the phosphorylation of ERK following incubation with fluvastatin. As demonstrated in Figure 4D, fluvastatin effectively inhibited ERK phosphorylation.

#### 4. Discussion

During the process of HIV infection, the interaction of gp120 with chemokine receptors, including CXCR4, is a critical step that permits fusion of the viral membrane with the cellular membrane and subsequent penetration of viral genetic material into the cytoplasm [24]. Chemokine receptors play key roles in leukocyte recruitment to the vascular endothelium. We therefore examined the effects of gp120 in CD4<sup>+</sup> Jurkat T cells, and found that it significantly induced an adhesive interaction with vascular endothelium (HUVEC cells). Stimulation of anti-CD4 or CXCR4 individually also induced CD4<sup>+</sup> Jurkat cell adhesion to vascular endothelium, although to a lesser extent than gp120. These findings may indicate an inflammatory potential of gp120 in T-cell activation.

The HIV envelope protein gp120 activates a variety of signaling pathways in lymphocytes, including the ERK pathway. As previously reported [25] [26], SDF-1 alpha induces the phosphorylation of MAP kinase via CXCR4 in both Jurkat T cells and CD4<sup>+</sup> T cells. Here, we have further demonstrated that ERK phosphorylation plays an important role in the enhanced adhesion of Jurkat T cells mediated by SDF-1 alpha, anti-CD4, and gp120. ERK1/2 phosphorylation is required for integrin activation in many cell types. For example, platelet activation factor (PAF) has been shown to induce CD11b/CD18 expression via ERK-dependent pathways in neutrophils. In another report, MCP-1-induced adhesion of monocytes was reduced by PD98059 treatment [27]. Therefore, we think that ERK1/2 activation could be one of primary mechanisms controlling cell adhesion.

A recent study by Brainard et al. using human T cells reported that CD4-dependent signal transduction was more significant than an SDF-1 alpha-dependent pathway [28]. In the present study, however, ERK1/2 activation upon CD4 stimulation was almost comparable to that upon CXCR4 stimulation (**Figure 2A**). This discrepancy may be explained, at least in

part, by the speculation that gp120 prepared from different HIV strains activates different signal pathways, or because different T-cell populations may possess different signal pathways. As attenuation of the CD4-dependent signal by siRNA did not affect SDF-1 alpha-dependent adhesion, we conclude that the expression of CD4 is not necessary for CXCR4 to induce ERK1/2 phosphorylation. Whether the CXCR4-dependent signaling pathway is identical to or distinct from that of CD4 should be clarified in future. Previous studies claimed that SDF-1 alpha induces internalization of CXCR4 in lymphocytes[29, 30], which was not observed in our condition. Considering the distinct sustained activation of CXCR4 by gp120 in neurons [31], different T-cell populations may possess different regulatory mechanisms of CXCR4 expression.

We also identified an involvement of RhoA GTPase following activation of ERK1/2 in SDF-1 alpha- and gp120-dependent Jurkat cell activation (**Figure 2C**). This suggests a sequential activation of ERK1/2 and RhoA by SDF-1 alpha and gp120 (**Figure 5**).

In the present study, we showed the potential activation of T-lymphocytes by gp120, which may therefore play a role in inflammation in HIV-positive subjects. Recent therapeutic advances in HIV infection have increased the number of HIV patients with chronic pathological conditions, such as atherosclerosis due to hyperlipidemia caused by anti-HIV medications. Our current study points to a unique molecular mechanism of inflammation induced by HIV envelope protein, gp120, and its modulation by statins. Though our data did not support their effects on transmigration of T cells, an anti-inflammatory role of statin could be demonstrated in HIV patients in future.

In summary, our findings indicate a role for gp120 in the induction of adhesion of Jurkat cells in a CXCR4- and CD4-dependent manner. We also found a protective role for fluvastatin in

the modulation of Jurkat cell adhesion to activated vascular endothelium induced by SDF-1 alpha and gp120.

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## Figure Legends

**Figure 1.** Treatment with SDF-1 alpha, anti-CD4, and gp120 enhances Jurkat T-cell and lymphocyte adhesion to activated HUVECs. A: Jurkat T cells were stimulated with gp120 (2.5 µg/ml) and SDF-1 alpha (80 ng/ml) at 37°C for 5 minutes, or anti-CD4 (10 µg/ml) for 40 minutes at 4°C, after which the adhesive interactions with HUVEC monolayers pre-treated with IL-1beta (10 units/ml) were analyzed using an *in vitro* flow chamber system at a shear stress of 1 dyne/cm<sup>2</sup>. The number of adhering cells was determined as described in Materials and Methods. The graph shown is representative of three experiments. \*  $p < 0.05$  vs. controls. B: CD4<sup>+</sup> lymphocytes were prepared from healthy donors as described in Materials and Methods, and adhesion assays were carried out after gp120, SDF-1 alpha, or anti-CD4 stimulation. #  $p < 0.05$  vs. control. The graph shown is a representative of three experiments.

**Figure 2.** Activation of ERK1/2 by SDF-1 alpha, anti-CD4, and gp120. A: Jurkat T cells were treated with SDF-1 alpha (80 ng/ml), anti-CD4 (10 µg/ml), or gp120 (2.5 µg/ml), then subjected to Western blotting using anti-ERK1/2 (bottom bars) and p-ERK (top) antibodies, as described in Materials and Methods. As pre-treatment with PD98059, 25µM for 4 hours, inhibited SDF-1 alpha-, anti-CD4 and gp120-induced Jurkat cell activation. The density of bands was quantified (bottom bar graph) using an LAS-1000 CCD camera with Image Gauge 3.4 software. B: Jurkat cells were treated (as described in Figure 1) in the presence or absence of PD98059, a potent inhibitor of MEK, prior to the adhesion assays. Quantification of p-ERK was normalized against total ERK. \*  $p < 0.05$  vs. controls. #  $p < 0.05$  vs SDF-1 alpha(+) PD98059(-). ##  $p < 0.01$  vs. anti-CD4(+) PD98059(-). ###  $p < 0.01$  vs. gp120(+) PD98059(-). Data are representative of results obtain in three independent experiments. C: RhoA activity in Jurkat T cells. RhoA activity in Jurkat T cells was determined by its translocation to the

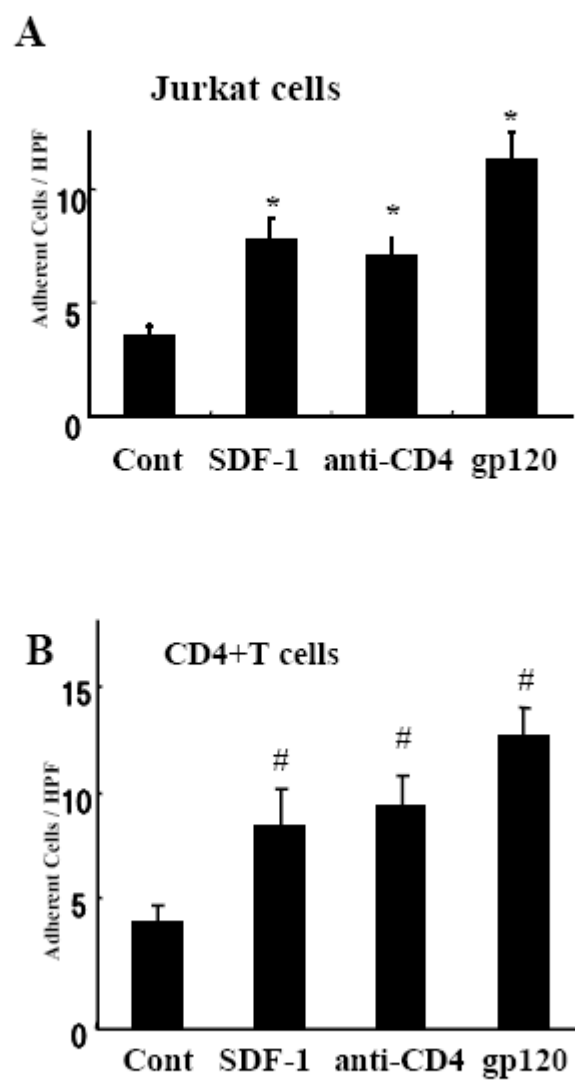
membrane fraction, using a RhoA antibody, as described in Materials and Methods. The cells were treated with gp120 (2.5  $\mu\text{g/ml}$ ) and SDF-1 alpha (80 ng/ml), or anti-CD4 (10  $\mu\text{g/ml}$ ) for 40 minutes at 4°C in the presence or absence of PD98059. The density of bands was quantified as described previously. Data are representative of results obtained in one of two independent experiments. Quantification of the RhoA in the membrane fraction was normalized against total cells. \* $P < 0.01$  vs anti-CD4(+) PD98059 (-), # $P < 0.01$  vs gp120 (+) PD98059 (-).

**Figure 3.** Effect of siCD4 to inhibit CD4-dependent signal in Jurkat T cells. A: FACS analysis of Jurkat cells transfected with siRNA against CD4 using an anti-CD4 mAb. The non-binding isotype control immunoglobulin G mAb (bold line) is indicated. The data shown are representative of two independent experiments. B: siCD4-transfected Jurkat T-cells (siCD4-Jurkat) and control siRNA-transfected Jurkat T-cells (siLacZ-Jurkat) were treated with SDF-1 alpha, anti-CD4, and gp120, as described in Materials and Methods, after which adhesion assays were carried out under flow conditions. Data shown are representative of three similar experiments. \* $p < 0.01$  vs. siLacZ-Jurkat anti-CD4. \*\*  $p < 0.05$  vs. siLacZ-Jurkat gp120.

**Figure 4.** Potential effects of fluvastatin in SDF-1 alpha- and gp120-induced Jurkat T-cell adhesion. A–C: Jurkat cells were incubated in the presence or absence of fluvastatin (10  $\mu\text{M}$ ) for 48 hours, followed by stimulation with SDF-1 alpha (80 ng/ml, A) and gp120 (2.5  $\mu\text{g/ml}$ , C) for 5 minutes or anti-CD4 (10  $\mu\text{g/ml}$ , B) for 40 minutes. Adhesion assays were carried out as described in Materials and Methods. Data shown are representative of three separate experiments. D: The phosphorylation of ERK1/2 after fluvastatin treatment was examined by Western blot, using the conditions described in Material and Methods. \* $P < 0.01$  vs SDF-1 alpha (+) Fluvastatin (-), \*\* $P < 0.01$  vs anti-CD4 (+) Fluvastatin (-), \*\*\* $P < 0.01$  vs gp120 (+)

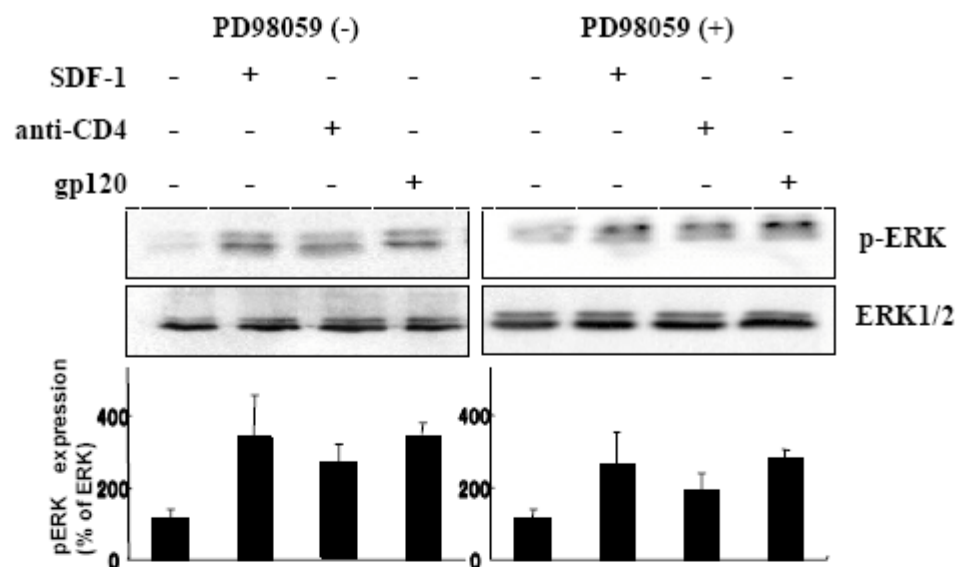
Fluvastatin (-).

**Figure 5.** Schematic representation of cell adhesion and ERK1/2 activation in CD4+ T cells induced by gp120 and SDF-1 alpha.

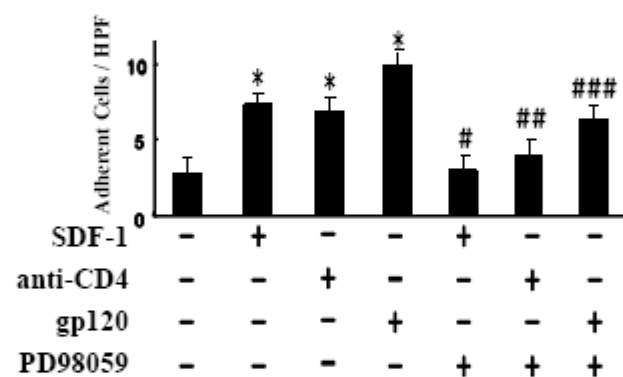
**Figure 1**

**Figure 2**

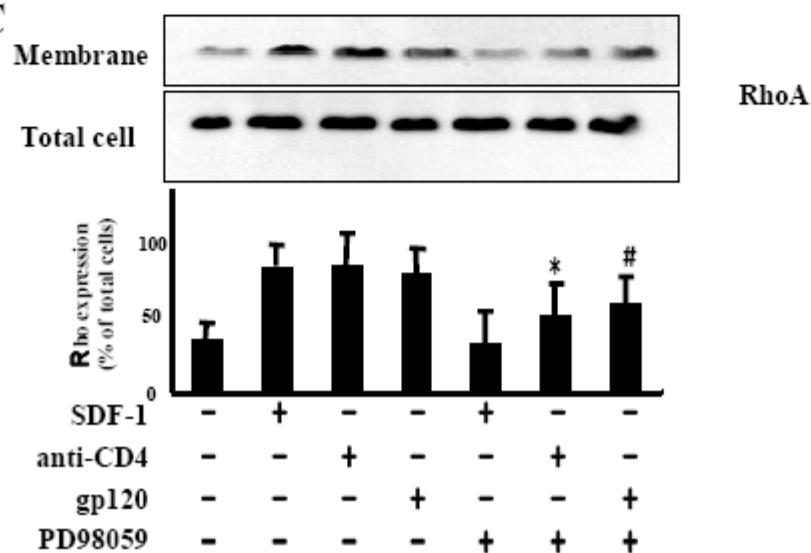
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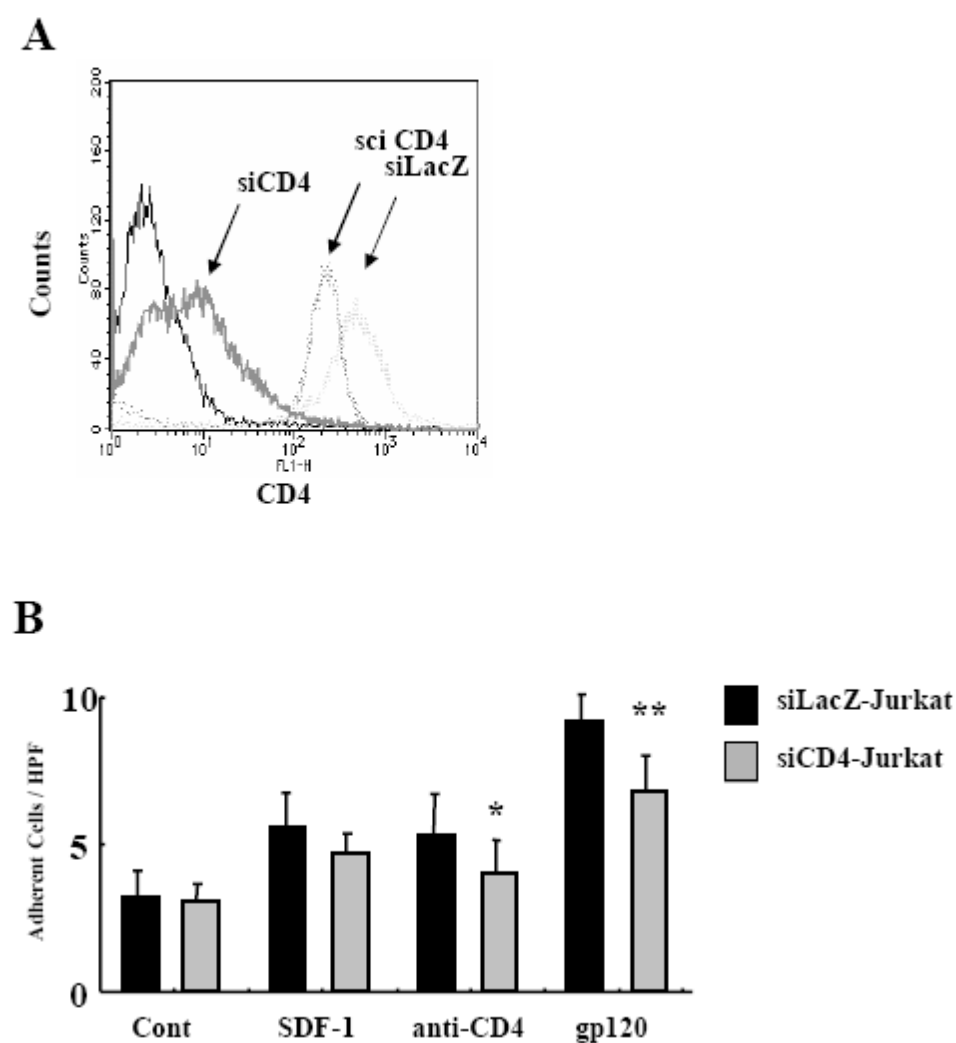
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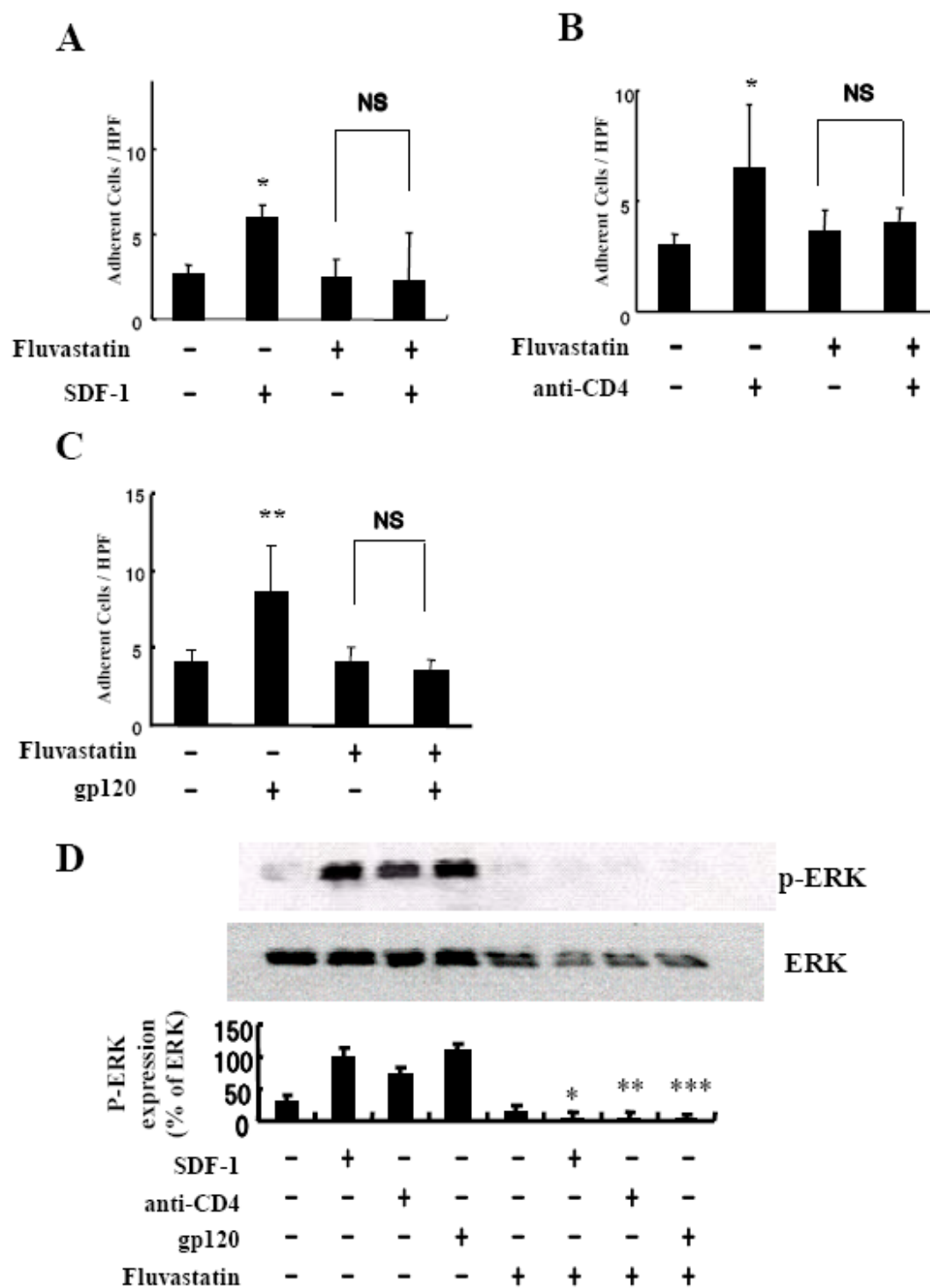
**C**





**Figure 3**

**Figure 4**



**Figure 5**