The Thesis of Doctor of Medicine

Alloantigen-stimulated CD8$^+$ T cells suppress HIV-1 replication by inhibiting phosphorylated NF-κB p65 and Ets-1 nuclear translocation

(アロ抗原刺激 CD8 陽性 T 細胞はリン酸化 NF-κB p65 と Ets-1 核移行阻害により HIV-1 複製を抑制する)

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Abstract

[Objective] CD8$^+$ T cells from HIV-1-infected individuals possess HIV-1 suppressive activity in both MHC-I-restricted and -unrestricted manner. In addition, alloantigen-stimulated CD8$^+$ T cells can suppress HIV-1 replication by MHC-I-unrestricted and cell contact-dependent mechanism, but its mechanism is not fully understood. The aim of this study is to elucidate the suppressive mechanism for HIV-1 replication in CD4$^+$ T cells induced by alloantigen-stimulated CD8$^+$ T cells.

[Methods] Alloantigen-stimulated CD8$^+$ T cells (Raji-CD8$^+$ T cells) were cultured with autologous HIV-1-infected or -uninfected CD4$^+$ T cells. PHA-stimulated CD8$^+$ T cells were used as control. Nuclear and cytoplasmic extracts were prepared from the isolated CD4$^+$ T cells, and used for the analysis of transcription factors by EMSA and Western-blotting. I also analyzed surface molecules on Raji-CD8$^+$ T cells by flowcytometry to identify the HIV-1 suppressive molecule.

[Results] Raji-CD8$^+$ T cells suppressed HIV-1 replication, and inhibited NF-κB p65 and Ets-1 nuclear translocation in HIV-1-infected CD4$^+$ T cells in a cell contact-dependent manner. I found that NF-κB and Ets DNA-binding activity were reduced, and nuclear translocation of phospho-NF-κB p65 (Ser276) and Ets-1 was inhibited in CD4$^+$ T cells cultured with Raji-CD8$^+$ T cells. ICAM-1 expression was higher on Raji-CD8$^+$ T cells than on PHA-CD8$^+$ T cells. Neutralization of ICAM-1 on CD8$^+$ T cells or stimulation of LFA-1 on CD4$^+$ T cells did not affect the nuclear translocation of NF-κB p65, suggesting that ICAM-1 was not a primarily responsible molecule for the inhibition of HIV-1 replication.

[Conclusion] Inhibition of nuclear translocation of phospho-NF-κB p65 (Ser276) and Ets-1 plays important roles in suppression of HIV-1 replication in CD4$^+$ T cells co-cultured with alloantigen-stimulated CD8$^+$ T cells.

Abbreviations

AC: asymptomatic carriers

AIDS: acquired immune deficiency syndrome

AKIP-1: A-kinase interacting protein-1

APC: allophycocyanin

BTLA: B and T lymphocyte attenuator

CAF: $CD8^+$ cell antiviral factor

CTL: cytotoxic T lymphocytes

CTLA-4: cytotoxic T lymphocyte-associated protein-4

ELISA: enzyme-linked immunosorbent assay

EMSA: electrophoretic mobility shift assay

Ets: E26 transformation-specific

FITC: fluorescein isothiocyanate

HIV-1: human immunodeficiency virus type 1

HSP-90: heat shock protein-90

ICAM-1: intracellular adhesion molecules-1

IκB: inhibitory kappa B

IKK: inhibitory kappa B kinase

LFA-1: lymphocyte function-associated antigen-1

MACS: magnetic activating cell sorting

MAPK: mitogen-stress activated kinase

MHC: major histocompatibility complex

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor-kappa B

PBMC: peripheral blood mononuclear cells

PCR: polymerase chain reaction

PD-1: programmed death-1

PE: phycoerythrin

PerCP: peridinin chlorophyll protein

PHA: phytohaemagglutinin

PKAc: protein kinase A catalytic subunit

SIV: simian immunodeficiency virus

TCF: T cell factor

Tim-3: T cell immunoglobulin domain and mucin domain-3

VCAM-1: vascular cell adhesion molecule-1

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus responsible for the development of acquired immunodeficiency syndrome (AIDS) caused by its infection to $CD4^+$ T cells, macrophages, and dendritic cells. HIV-1 infection induces strong host immune responses, and $CD8^+$ T cells including HIV-1-specific cytotoxic T lymphocytes (CTL) play important roles in suppressing the HIV-1 replication in infected individuals [1-4]. Previous studies have shown that $CD8^+$ T cells of asymptomatic HIV-1 carriers (AC) suppress HIV-1 replication in autologous peripheral blood mononuclear cells (PBMC) [5-7]. The clinical significance of these $CD8^+$ T cells has been supported by the studies using a simian immunodeficiency virus (SIV)-infected monkey AIDS model system both *ex vivo* and *in vivo* [8-10]. It has been demonstrated that reduction of viremia in the acute phase of SIV infection is associated with the appearance of SIV-specific CTL, and that depletion of $CD8^+$ T cells from persistently SIV-infected monkeys causes an increase of viral load *in vivo* [9, 10]. These findings imply that $CD8^+$ T cells are the common effector cells contributing to both recovery from the acute phase and maintenance of an asymptomatic state in virus infections.

It has been reported that $CD8^+$ T cells of AC possess suppressive capacity in not only the major histocompatibility complex-I (MHC-I)-restricted but also the MHC-I-unrestricted mechanism [11]. The former is HIV-1-specific CTL activity, in which HIV-1-infected cells are led to apoptosis through perforins/granzymes pathway and Fas/Fas-ligand pathway [12-16]. The latter, on the other hand, is exerted by soluble factors and unknown membrane-bound factors without the killing of $CD4^+$ T cell. $CD8^+$

T cells produce various soluble factors that suppress HIV-1 replication *in vitro* such as macrophage inflammatory protein 1-α (MIP-1α), MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES), stromal cell-derived factor 1 (SDF-1), macrophage-derived chemokine (MDC), interleukin-16, interferons, and defensin [17-22]. However, neutralizing antibodies against these cytokines or chemokines only partially inhibit the suppressive activity of $CD8^+$ T cells of AC [5, 23, 24]. Mackewicz *et al*. has reported that $CD8^+$ T cells of AC produce an unknown soluble $CD8^+$ cell antiviral factor (CAF) that can suppress HIV-1 replication at a transcriptional level by non-cytolytic mechanism [25]. In addition to the CTL activity and soluble factors as the mechanism of $CD8^+$ T cell-mediated suppression, Liu *et al*. has reported that HIV-1-irrelevant alloantigen-stimulated $CD8^+$ T cells from HIV-1-uninfected healthy donors inhibit X4 and R5 HIV-1 replication in a cell contact-dependent manner [26]. Although this mechanism should have an important and unique role in HIV-1 suppression, its suppressive mechanism has not yet been elucidated.

In the present study, to clarify the unknown HIV-1-suppressive mechanism, I stimulated $CD8^+$ T cells by HIV-1-irrelevant antigen, alloantigen, to exclude the MHC-I-restricted CTL activity. PHA-stimulated $CD8^+$ T cells were used as control, which did not possess HIV-1-suppressive activity. These $CD8^+$ T cells were co-cultured with HIV-1-infected or -uninfected $CD4^+$ T cells, and nuclear and cytoplasmic extracts were prepared from the isolated $CD4^+$ T cells. Using these extracts, I analyzed transcriptional factors relating to HIV-1 replication and several molecules regulating these transcriptional factors in $CD4^+$ T cells. Moreover, to identify the candidate

molecules for unknown HIV-1-suppressive factor, I analyzed surface molecules on $CD8^+$ T cells.

2. Materials and Methods

2.1 Cell culture

2.1.1 Cell line

Raji cells (human B cell lymphoma cell line) were cultured in RPMI-1640 medium (Sigma-Aldrich, St.Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Inc., South Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.1.2 Isolation of peripheral blood mononuclear cells (PBMC)

This study was approved by the ethics committee of Kitasato University of Allied Health Sciences (No. 2009-011). After informed consent had been obtained from five healthy donors, PBMC were isolated by density gradient centrifugation with Ficoll-Hypaque (Lymphoprep™, AXIS-SHIELD PoC, Oslo, Norway), and stimulated with 1% Phytohemagglutinin-P (PHA-P, Sigma) for overnight. After washing, PBMC were cultured in RPMI-1640 medium (Sigma) containing 10 U/ml of recombinant human interleukin-2 (rhIL-2), 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.1.3 Induction of alloantigen-stimulated $CD8^+$ T cells

PBMC were stimulated with Raji cells treated with 0.05 mg/ml Mitomycin C (Sigma) in RPMI-1640 containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stimulation with Raji cells was performed periodically every week

for 4 weeks. The stimulated $CD8^+$ T cells were separated from the cell culture by magnetic activating cell sorting (MACS) separation, and used as alloantigen-stimulated $CD8^+$ T cells (Raji-$CD8^+$ T cells). The purity of isolated $CD8^+$ T cells was analyzed by staining with monoclonal antibodies against CD8 (fluorescein isothiocyanate, FITC) and CD3 (phycoerythrin, PE) (BioLegend, San Diego, CA) for flowcytometric analysis using MACSQuant flow cytometer (Milteny Biotec, Bergisch Gladbach, Germany).

2.1.4 Enrichment of $CD4^+$ T and $CD8^+$ T cells

$CD8^+$ T cells were enriched by MACS separation using CD8 microbeads from PHA-stimulated PBMCs originating from the same donor as that used for the Raji-$CD8^+$ T cell induction. The positively selected fraction was used as the PHA-stimulated $CD8^+$ T cells (PHA-$CD8^+$ T cells), and the negatively selected fraction was used as the $CD4^+$-enriched T cells. The $CD4^+$-enriched T cells were cultured with autologous PHA-$CD8^+$ T cells or Raji-$CD8^+$ T cells at a ratio of 1:3. After 1 or 4 days of culture, $CD8^+$ T cells were removed and $CD4^+$ T cells were enriched for the preparation of nuclear and cytoplasmic extracts.

2.1.5 Neutralization of interaction between ICAM-1 and LFA-1

PHA-$CD8^+$ T cells or Raji-$CD8^+$ T cells were treated with anti-ICAM-1 antibody or isotype control antibody (BioLegend) at 10 μg/ml for 2 h at 37°C with 5% CO_2. After washing with 10% FCS RPMI-1640, the cells were cultured with autologous

$CD4^+$-enriched T cells for 1 or 4 days. The $CD4^+$ T cells were isolated from the co-culture using Auto MACS, and used for preparation of nuclear and cytoplasmic extracts.

2.1.6 LFA-1 stimulation with recombinant ICAM-1

$CD4^+$-enriched T cells were isolated from PHA-stimulated PBMCs using Auto MACS, and cultured in a 96-well plates coated with 0.1, 1, 5, and 10 μg/ml recombinant human ICAM-1 IgG_1 Fc chimera (R&D Systems, Minneapolis, MN) or human IgG_1 isotype as control (Serotec, Oxford, OX5 1GE, UK). After 1 or 4 days of culture, $CD4^+$ T cells were collected and used for preparation of nuclear and cytoplasmic extracts.

2.2 HIV-1 infection and suppression assay

2.2.1 HIV-1 infection of $CD4^+$ T cells *in vitro*

HIV-1 used for *in vitro* infection was a filtered culture supernatant of MOLT4/LAI C-3 cells [27]. For HIV-1 infection, $CD4^+$ T cells isolated from HIV-1-unifected healthy donors were spinoculated with culture supernatants containing HIV-1 at a multiplicity of infection of 0.03 to 0.1 at room temperature for 1.5 h, extensively washed, and cultured in a medium containing recombinant human IL-2 at a concentration of 10^5 cells/200 μl in a 96-well round-bottom plate.

2.2.2 Measurement of HIV-1 p24 by ELISA

The concentration of HIV-1 p24 antigen in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA kits were kindly provided by Dr.Tanaka Y, the University of Ryukyu).

2.2.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA in the culture supernatants was extracted with the illustra RNAspin mini kit (GE Lifesciences, Little Chalfont, UK) in accordance with the manufacturer's instructions. The RNA was eluted in 40 μl of nuclease-free water. For the RT reaction, the eluted RNA sample was added to 2×Ampdirect Plus (Shimadzu, Kyoto, Japan), M-MLV (200 U/μl) (Invitrogen, Carlsbad, CA, USA), and HIV-1 LAI long terminal repeat (LTR) reverse primer (5'-agcactcaaggcaagcttta-3'). The RNA was reverse-transcribed at 37°C for 30 min followed by denaturation of the enzyme at 95°C for 5 min. The diluted cDNA was amplified in a reaction mixture containing HIV-1 LAI LTR forward primer (5'-ccctgattagcagaactacac-3') and BIOTAQ HS (5 U/μl) (Shimadzu) under conditions of 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min [28].

2.3 Preparation of nuclear and cytoplasmic extracts from $CD4^+$ T cells

Nuclear and cytoplasmic extracts were prepared from $CD4^+$ T cells using a Nuclear/Cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA). The protein concentration of the nuclear extracts was measured by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). The protein concentration in the cytoplasmic extracts

was measured using a 2D-Quant kit (GE Healthcare, Little Chalfont, UK).

2.4 Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear extracts from $CD4^+$ T cells; double-stranded DNA oligomers were labeled with [α-^{32}P]-dNTP with a Klenow Fragment and Random Primer DNA Labeling Kit ver.2.0 (Takara Bio Inc., Shiga, Japan). Table 1 shows the sequences of the double-stranded DNA oligomers used. For blocking of non-specific DNA-protein binding, one microgram of nuclear extract was incubated in reaction mixture including 20 mM HEPES-KOH (pH7.9), 50 mM KCl, 5% glycerol, 1 mM EDTA (pH 8.0), 10 mM DTT, BSA (10 mg/ml), and poly dI-dC (1 mg/ml) for 15 min on ice. After incubation, [α-^{32}P]-labeled DNA oligomers and/or cold-competitors were added to the reaction mixture for 20 min on ice. The reaction mixture was then loaded on a 5% polyacrylamide gel and electrophoresed in 0.5×TBE buffer containing 45 mM Tris, 45 mM boric acid, and 25 mM EDTA at 150V at 4 °C. The gel was dried, and DNA-protein complexes were detected by autoradiography.

2.5 Western-blotting

Nuclear and cytoplasmic extracts from $CD4^+$ T cells were fractionated by sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis (PAGE). Western analysis was performed using the following-primary antibodies: anti-PKA-C rabbit monoclonal antibody, anti-PKA-C phospho-Thr197 rabbit monoclonal antibody, anti-NF-κB p65 rabbit monoclonal antibody, anti-NF-κB p65 phospho-Ser276 rabbit polyclonal

antibody, anti-NF-κB p65 phospho-Ser536 rabbit monoclonal antibody, anti-Akt rabbit polyclonal antibody, anti-Akt phospho-Ser473 rabbit monoclonal antibody, anti-p38 MAPK rabbit polyclonal antibody, anti-p38 MAPK phospho-Thr180/Tyr182 rabbit monoclonal antibody, anti-Caspase-3 rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA), anti-Ets-1 mouse monoclonal antibody, anti-IκB-α rabbit polyclonal antibody, anti-HSP-90α/β mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-GAPDH mouse monoclonal antibody, and anti-beta actin mouse monoclonal antibody (Abcam, Cambridge, UK), anti-C11orf17 (AKIP-1) rabbit polyclonal antibody (Abnova, Taipei city, Taiwan), anti-histone H3 rabbit monoclonal antibody (BioLegend). Each protein was detected using the following appropriate-secondary antibodies: horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG polyclonal antibody and HRP-conjugated goat anti-mouse IgG (BioLegend), and visualized on X-ray film (FujiFilm Corporation, Tokyo, Japan) after incubation with a chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK).

2.6 Flowcytometric analysis

PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells were stained with the following fluorescent conjugated monoclonal antibodies: CD8 (FITC), VCAM-1 (PE), Fas-L (PE), BTLA (PE), 2B4 (PerCP/Cy5.5), PD-1 (PerCP/Cy5.5), Tim-3 (PE/Cy7), CTLA-4 (APC), and ICAM-1 (APC) (BioLegend,). The stained cells were analyzed by a MACSQuant flow cytometer (Miltenyi Biotec).

2.7 Statistics

Statistical significance was analyzed using the Student t test. Values were considered statistically significant when $P < 0.05$.

3. Results

3.1 Inhibition of NF-κB p65 and Ets-1 nuclear translocation in HIV-1-infected $CD4^+$ T cells co-cultured with Raji-$CD8^+$ T cells

$CD4^+$-enriched T cells from two donors were infected with HIV-1, and co-cultured with Raji-$CD8^+$ T cells which had been stimulated with alloantigen B cell line, Raji. $CD4^+$-enriched T cells were also co-cultured with autologous PHA-stimulated $CD8^+$ T cells (PHA-$CD8^+$ T cells) and used as control. First, I assessed the HIV-1-suppressive capacity by Raji-$CD8^+$ T cells by ELISA, Western blotting, and RT-PCR. It turned out that Raji-$CD8^+$ T cells remarkably suppressed HIV-1 replication in $CD4^+$ T cells compared with PHA-$CD8^+$ T cells at 4 days of incubation (Figure 1A-1C). From these results, I assumed that Raji-$CD8^+$ T cells might transduce some signals affecting the transcriptional factors in $CD4^+$ T cells leading to the suppression of HIV-1 replication. To investigate the mechanism of the suppression, I extracted the nuclear and cytoplasmic fraction from HIV-1-infected $CD4^+$ T cells cultured with either PHA-$CD8^+$ T cells or Raji-$CD8^+$ T cells, and analyzed transcriptional factors NF-κB p65 and Ets-1 by Western-blotting. I found that the amount of NF-κB p65 in nucleus was decreased in HIV-1-infected $CD4^+$ T cells co-cultured with Raji-$CD8^+$ T cells in comparison with those cultured with PHA-$CD8^+$ T cells (Figure 2A, 2B). In addition, Ets-1 was clearly decreased in the nuclei of HIV-1-infected $CD4^+$ T cells co-cultured with Raji-$CD8^+$ T cells (Figure 2A, 2C). These results suggested that inhibition of NF-κB p65 and Ets-1 nuclear translocation contributed to the suppression of HIV-1 replication by Raji-$CD8^+$ T cells.

3.2 Reduction of DNA-binding activity of transcriptional factors in CD4$^+$ T cells co-cultured with Raji-CD8$^+$ T cells

Next, to investigate further the mechanism for the suppression of HIV-1 replication, I examined DNA-binding activity of NF-κB and Ets in CD4$^+$ T cells cultured with PHA-CD8$^+$ T cells or Raji-CD8$^+$ T cells. For the biological safety and prevention of HIV-infection to the experimenter, all the following experiments were carried out using HIV-1-uninfected CD4$^+$ T cells. EMSA was performed for NF-κB and Ets as well as other transcriptional factors NFAT and TCF. I observed that DNA-binding activity of NF-κB and Ets was remarkably reduced in CD4$^+$ T cells cultured with Raji-CD8$^+$ T cells, whereas DNA-binding activity of neither NF-κB nor Ets was affected in CD4$^+$ T cells cultured with PHA-CD8$^+$ T cells (Figure 3A). In contrast, NFAT and TCF were not affected in CD4$^+$ T cells cultured with Raji-CD8$^+$ T cells. These results suggested that the reduction of DNA-binding activity of NF-κB and Ets in autologous CD4$^+$ T cells resulted in the suppression of HIV-1 replication in CD4$^+$ T cells. Western analysis revealed the reduced nuclear translocation of NF-κB p65 and Ets-1 in HIV-1-uninfected CD4$^+$ T cells, confirming the results of HIV-1-infected CD4$^+$ T cells (Figure 3B, 3C).

3.3 The effect of culture supernatants derived from CD8$^+$ T cells on NF-κB p65 and Ets-1 nuclear translocation

To investigate whether culture supernatants of CD8$^+$ T cells affect the NF-κB p65 and Ets-1 nuclear translocation, I extracted the nuclear and cytoplasmic fraction from CD4$^+$

T cells incubated with culture supernatants of PHA-CD8$^+$ T cells or Raji-CD8$^+$ T cells, and analyzed NF-κB p65 and Ets-1 by Western-blotting. I detected no evident reduction of nuclear localization of NF-κB p65 and Ets-1 in CD4$^+$ T cells incubated with culture supernatants (Figure 4A-4C). These results indicated that inhibiting of NF-κB p65 and Ets-1 nuclear translocation by Raji-CD8$^+$ T cells was exerted by cell contact-dependent manner, but not by soluble factors such as cytokines, chemokines, or CAF.

3.4 Cellular localization of phosphorylated NF-κB p65 (Ser276) in CD4$^+$ T cells

It has been known that phosphorylation of NF-κB p65 by a number of kinases affect the nuclear translocation of NF-κB p65 and its transcriptional activity [29-31]. To clarify the state of NF-κB p65 phosphorylation, I investigated whether NF-κB p65 in the cytoplasm and nucleus of CD4$^+$ T cells cultured with PHA-CD8$^+$ T cells or Raji-CD8$^+$ T cells was phosphorylated at serine residues 276 and 536. I observed less amount of phospho-NF-κB p65 (Ser276) in the nuclei of CD4$^+$ T cells cultured with Raji-CD8$^+$ T cells for one day than in those cultured with PHA-CD8$^+$ T cells (Figure 5A, 5B). After 4 days of culture, phospho-NF-κB p65 (Ser276) was not clearly detectable in the nuclei of CD4$^+$ T cells cultured with Raji-CD8$^+$ T cells and was retained only in the cytoplasm. In CD4$^+$ T cells cultured with PHA-CD8$^+$ T cells, on the other hand, phospho-NF-κB p65 (Ser276) was transported from the cytoplasm to the nucleus. The total phosphorylation rate at Ser276 in nucleus and cytoplasm of CD4$^+$ T cells did not differ, irrespective of culture with PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells (Figure 5C). In contrast to the Ser276, there was no difference in the amount of cytoplasmic

phospho-NF-κB p65 (Ser536) in $CD4^+$ T cells, irrespective of culture with PHA-$CD8^+$ T cells and Raji-$CD8^+$ T cells (Figure 5D, 5E). These results indicated that although NF-κB p65 was phosphorylated at Ser276 and Ser536 equally in $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and in those cultured with Raji-$CD8^+$ T cells, nuclear transport of phospho-NF-κB p65 (Ser276) was selectively inhibited by co-culture with Raji-$CD8^+$ T cells. It is thought that this mechanism plays a central role in the suppression of HIV-1 replication induced by cell contact with Raji-$CD8^+$ T cells.

3.5 Analysis of signal transducing molecules relating with phospho-NF-κB p65 (Ser276) nuclear translocation

Next, I analyzed the several signal transducing molecules relating with phospho-NF-κB p65 (Ser276) nuclear translocation. Akt, also known as protein kinase B (PKB) and p38 mitogen-activated protein kinase (MAPK) are known to regulate NF-κB signaling via activation of the IKK complex and IκB degradation [32-34]. To examine the possibility of participation of these molecules in the reduced nuclear translocation of NF-κB p65 induced by Raji-$CD8^+$ T cells, I analyzed the phosphorylation of Akt and p38 MAPK in $CD4^+$ T cells. I did not detect significant difference between $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and those cultured with Raji-$CD8^+$ T cells (Figure 6A-6C). Next, I analyzed the level of IκB-α degradation and expression of HSP-90, which interact with IKK and regulate NF-κB nuclear translocation [35, 36]. I found that the level of IκB-α degradation did not differ significantly between $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and those cultured

with Raji-$CD8^+$ T cells (Figure 6D, 6E). Similarly, the amount of HSP-90 showed no difference between the two cell types (Figure 6D, 6F).

Because protein kinase A (PKA) has been reported to phosphorylate NF-κB p65 at serine 276 [37, 38], I analyzed PKA catalytic subunits (PKAc) and phospho-PKAc (Thr197) in $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and those cultured with Raji-$CD8^+$ T cells. Neither PHA-$CD8^+$ T cells nor Raji-$CD8^+$ T cells affected the phosphorylation and the total amounts of PKAc in $CD4^+$ T cells (Figure 7A-7C). I also analyzed AKIP-1 because it binds to NF-κB p65 and regulates its phosphorylation at serine residue 276 by PKAc [39]. I found that AKIP-1 expression showed no difference between $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and those cultured with Raji-$CD8^+$ T cells (Figure 7D, 7E). These results indicated that Akt, p38 MAPK, IκB-α, HSP-90, PKAc, or AKIP-1 are probably not involved in the inhibition of nuclear translocation of phospho-NF-κB p65 (Ser276) in our experimental condition.

3.6 Flowcytometric analysis of surface molecules on Raji-$CD8^+$ T cells

To identify the molecules on Raji-$CD8^+$ T cells responsible for the suppression of HIV-1 replication in $CD4^+$ T cells, I examined the surface expression of co-stimulatory molecules, adhesion molecules, and negative regulatory molecules on Raji-$CD8^+$ T cells in comparison with PHA-$CD8^+$ T cells. It is noteworthy that expression of intercellular adhesion molecule-1 (ICAM-1) was significantly higher on Raji-$CD8^+$ T cells than PHA-$CD8^+$ T cells (Figure 8, Table 2). Expression of vascular cell adhesion molecule-1 (VCAM-1) was slightly higher on Raji-$CD8^+$ T cells than PHA-$CD8^+$ T cells.

Raji-CD8$^+$ T cells also showed higher expression of 2B4, programmed death-1 (PD-1), and T cell immunoglobulin domain and mucin domain-3 (Tim-3), suggesting that Raji-CD8$^+$ T cells might be more exhausted than PHA-CD8$^+$ T cells. Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and Fas-ligand (Fas-L) were not expressed on either PHA-CD8$^+$ T cells or Raji-CD8$^+$ T cells, whereas the expression of B and T lymphocyte attenuator (BTLA) was slightly higher on PHA-CD8$^+$ T cells than on Raji-CD8$^+$ T cells.

3.7 Influence of ICAM-1 neutralization on phospho-NF-κB p65 Ser276 nuclear translocation

As described above, expression of ICAM-1 on Raji-CD8$^+$ T cells was higher than on PHA-CD8$^+$ T cells. Interaction between ICAM-1 and LFA-1 is known to regulate cell adhesion and intracellular signaling [40-42]. Previous studies have shown that inhibition of this interaction suppresses HIV-1 replication by inducing soluble antiviral factors or by inhibiting viral transcription [43, 44]. Therefore, I speculated that ICAM-1 might be one of the candidate molecules for membrane-bound HIV-1-suppressive molecule. To test this hypothesis, I neutralized ICAM-1 on Raji-CD8$^+$ T cells with anti-ICAM-1 antibody, and analyzed its effect on phospho-NF-κB p65 (Ser276) nuclear translocation in HIV-1-uninfected autologous CD4$^+$ T cells from two donors. The result from one donor is shown in Figure 9. As expected from the above studies, the amount of phospho-NF-κB p65 (Ser276) and NF-κB p65 was remarkably reduced in the nucleus of CD4$^+$ T cells co-cultured with Raji-CD8$^+$ T cells compared with PHA-CD8$^+$ T cells,

both which had been treated with isotype control IgG. When Raji-$CD8^+$ T cells were pretreated with anti-ICAM1 antibody, the reduced amounts of phospho-NF-κB p65 (Ser276) in nucleus were not recovered (Figure 9A, 9B). Thus, nuclear translocation of phospho-NF-κB p65 (Ser276) was not affected by ICAM-1 neutralization.

3.8 Influence of LFA-1 signaling on NF-κB p65 nuclear translocation

Next, I stimulated $CD4^+$ T cells with increasing amount of recombinant ICAM-1 and investigated whether intracellular signaling derived from LFA-1 on $CD4^+$ T cells inhibited NF-κB p65 nuclear translocation. The result from one of two donors is shown in Figure 10. I could not detect significant difference in the amount of nucleus NF-κB p65 between control IgG and recombinant ICAM-1 (Figure 10A, 10B). These results indicated that ICAM-1 expressed on Raji-$CD8^+$ T cells was not a primary membrane-bound HIV-1-suppressive molecule.

4. Discussion

Previously, Liu *et al*. have reported that HIV-1-irrelevant antigen-stimulated $CD8^+$ CTL strongly suppress HIV-1 X4 and R5 virus in a cell contact-dependent manner [26], although its mechanism has been unclear. In the present study, to clarify the unknown HIV-1-suppressive mechanism, I used the $CD8^+$ T cells stimulated with HIV-1-irrelevant antigen, alloantigen, to exclude the HIV-1-specific CTL activity. I found that suppression of HIV-1 replication by alloantigen-stimulated $CD8^+$ T cells (Raji-$CD8^+$ T cells) was associated with the inhibition of nuclear translocation of NF-κB p65 and Ets-1 in HIV-1-infected $CD4^+$ T cells. The inhibition of nuclear translocation of NF-κB and Ets as well as their DNA-binding activity were also confirmed in HIV-1-uninfected $CD4^+$ T cells cultured with Raji-$CD8^+$ T cells. Culture supernatants of $CD8^+$ T cells did not affect the nuclear translocation of NF-κB p65 and Ets-1. I examined whether $CD4^+$ T cells were killed by PHA-$CD8^+$ T cells and Raji-$CD8^+$ T cells and found that neither cell death nor cleaved caspase-3 was evident in $CD4^+$ T cells cultured with both types of $CD8^+$ T cells (data not shown). These results suggested that Raji-$CD8^+$ T cells provided some signals to $CD4^+$ T cells leading to the inhibition of NF-κB p65 and Ets-1 nuclear translocation in cell contact-dependent manner, which may finally resulted in the suppression of HIV-1 replication.

To clarify the mechanisms of NF-κB p65 nuclear translocation, I analyzed Akt and p38 MAPK, which regulate NF-κB signaling via IKK activation [32-34], but these molecules were not related to inhibition of NF-κB p65 nuclear translocation. I did not find the evidence either showing that other possible regulatory molecules such as

IκB-α [35] and HSP-90 [36] are involved in the NF-κB p65 nuclear translocation in $CD4^+$ T cells, suggesting that the IKK-IκB pathway may not play a significant role in the present experimental system.

Next, I studied the phosphorylation of NF-κB p65 because it regulates nuclear translocation and transcriptional activity of NF-κB p65. Notably, nuclear translocation of phospho-NF-κB p65 (Ser276), but not phospho-NF-κB p65 (Ser536), was significantly inhibited in $CD4^+$ T cells co-cultured with Raji-$CD8^+$ T cells, indicating that inhibition of nuclear transport of phospho-NF-κB p65 (Ser276) was the responsible mechanism for the suppression of HIV-1 replication induced by Raji-$CD8^+$ T cells. No significant difference was observed between $CD4^+$ T cells cultured with Raji-$CD8^+$ T cells and those cultured with PHA-$CD8^+$ T cells in the expression level of PKAc that phosphorylates NF-κB p65 at Ser276 [37, 38] or AKIP-1 that regulates NF-κB p65 nuclear translocation via phosphorylation at Ser276 [39], although I cannot exclude the possibility that binding capacity of AKIP-1 for NF-κB p65 differs between them. Other molecules responsible for the inhibition of NF-κB p65 nuclear translocation, although not analyzed in this study, would be Ras-related associated diabetes (RRAD) that binds to the NF-κB p65 subunit and inhibits nuclear translocation [45] and programmed cell death 4 (PDCD4) that inhibits the nuclear localization of NF-κB p65 regardless of IKK activation and IκB degradation [46]. Moreover, nuclear/cytoplasm shuttling molecules, including importins or κB-Ras, could also affect the NF-κB p65 nuclear translocation [47, 48].

I found that ICAM-1 was highly expressed on Raji-$CD8^+$ T cells in comparison with

PHA-CD8$^+$ T cells. Thus, I examined whether ICAM-1 is a membrane-bound HIV-1-suppressive molecule by neutralizing it with anti-ICAM-1 antibody. ICAM-1 neutralization, however, did not affect the NF-κB p65 nuclear translocation. Stimulation of LFA-1 on CD4$^+$ T cells with recombinant ICAM-1 induced a slight but non-significant degree of NF-κB p65 nuclear translocation. These results suggested that interaction between ICAM-1 and LFA-1 might not directly relate to the inhibition of NF-κB p65 nuclear translocation induced by Raji-CD8$^+$ T cells. However, it would be possible that higher expression of ICAM-1 augments the cell contact between Raji-CD8$^+$ T cells and HIV-1-infected CD4$^+$ T cells, leading to the promoted suppression of HIV-1 replication. In addition to ICAM-1, 2B4 and Tim-3, known as the markers of exhausted CD8$^+$ T cells [49], tended to be highly expressed on Raji-CD8$^+$ T cells in comparison with PHA-CD8$^+$ T cells. Even when HIV-1-specific CTLs are exhausted and their CTL activity is attenuated by chronic viral infection *in vivo*, CTLs are known to keep the viral load at low level in asymptomatic state. Our results suggested that the exhausted Raji-CD8$^+$ T cells might also contribute to suppress the HIV-1 replication as in the case of HIV-1-specific CTL. In addition, 2B4 and Tim-3, another exhausted markers of CD8$^+$ T cells, have been reported to affect intracellular signaling and could be other possible candidates for membrane-bound HIV-1-suppressive molecules on Raji-CD8$^+$ T cells [50-52].

Our model for the mechanism of the suppression of HIV-1 replication induced by alloantigen-stimulated CD8$^+$ T cells is shown in Figure 11. In conclusion, the present study revealed that nuclear translocation of phospho-NF-κB p65 (Ser276) was inhibited

in $CD4^+$ T cells cultured with alloantigen-stimulated $CD8^+$ T cells and that DNA-binding activity of NF-κB p65 and Ets-1 was suppressed. This event will play an important role in the suppression of HIV-1 replication by alloantigen-stimulated $CD8^+$ T cells. In particular, the mechanism will have significant importance to suppress the replication of mutated HIV-1 escaping from CTL activity, to maintain the viral load in asymptomatic state at low level for a long-term. My findings would contribute to the development of therapeutic agents for AIDS by targeting the nuclear translocation of phospho-NF-κB p65 (Ser276).

5. Acknowledgements

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Table 1. Sequence of EMSA probes

NF-κB consensus	5'-AAAAGTTGATTTTACTTTCCCGGC-3'
NF-κB LTR	5'-ACAAGGGACTTTCCGCTGGGGACTTTCCA-3'
Ets consensus	5'-AAAGGGCTGCTTGAGGAAGTATAAGAAT-3'
NFAT LTR	5'-CAGAGAAGGTAGAAGAGGCCAATGAAGGAGAGAACAA-3'
TCF LTR	5'-GGAGTACTACAAGGACTGCT-3'

Table 2. Characterization of alloantigen-stimulated $CD8^+$ T cells.

	CTLA-4	BTLA	Fas-L	VCAM-1	ICAM-1*	2B4	PD-1	Tim-3
PHA8	0.05 ± 0.03	39.06 ± 16.06	1.03 ± 0.73	4.28 ± 2.79	21.05 ± 13.05	15.81 ± 8.34	11.32 ± 7.79	19.80 ± 11.75
Raji8	0.30 ± 0.24	28.82 ± 5.39	1.08 ± 1.04	12.16 ± 15.09	65.04 ± 14.54	31.61 ± 18.2	17.93 ± 10.46	41.58 ± 22.69

* $p < 0.01$ ICAM-1 expression on Raji8 significantly increased in comparison to PHA8

Figure 1

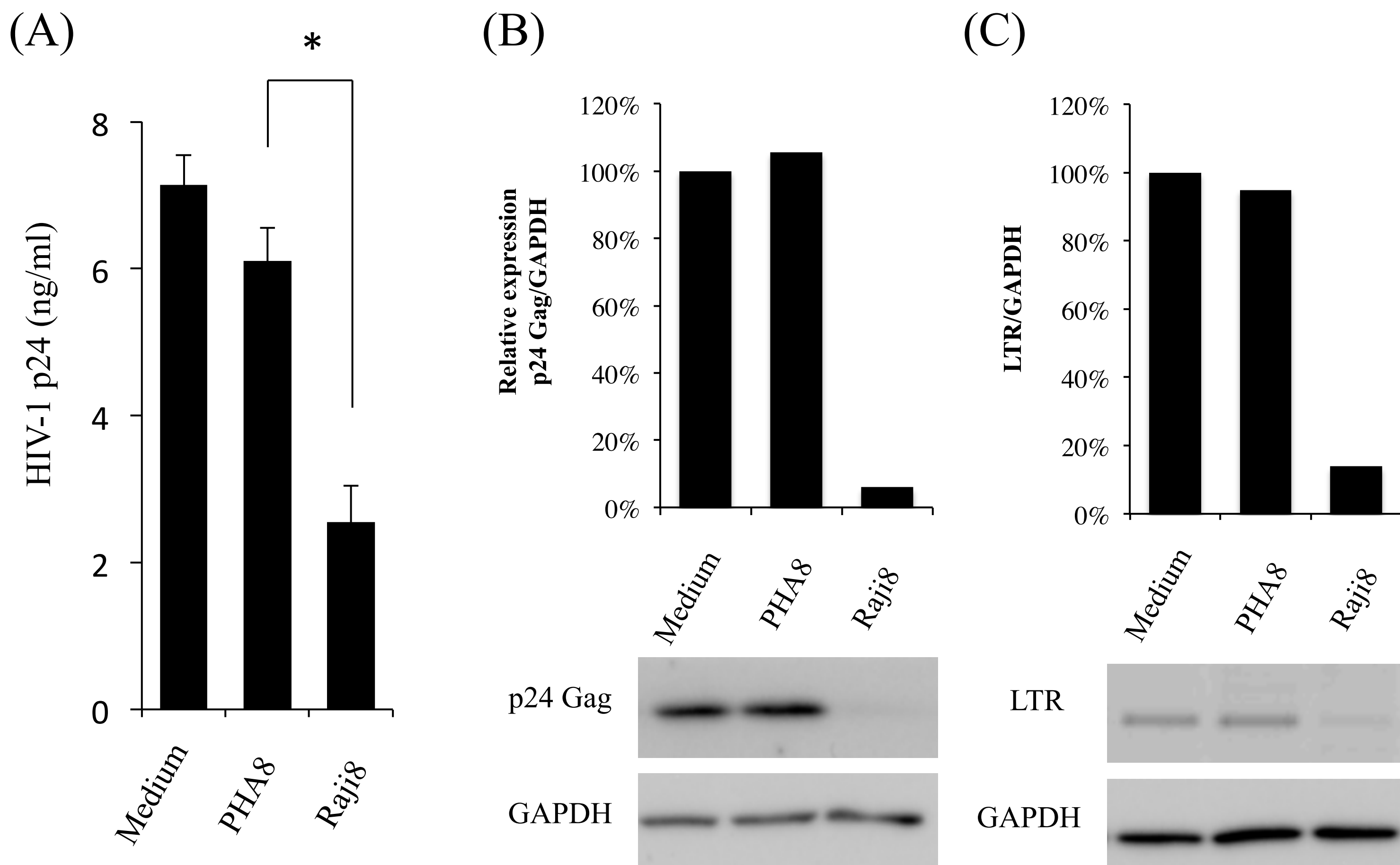

Figure 1. Suppression of HIV-1 replication in HIV-1-infected CD4$^+$ T cells co-cultured with alloantigen-stimulated CD8$^+$ T cells.

HIV-1-infected CD4$^+$ T cells were co-cultured with autologous PHA-stimulated CD8$^+$ T cells (PHA8) or alloantigen-stimulated CD8$^+$ T cells (Raji8) for 4 days, and nuclear and cytoplasmic extracts were prepared from the isolated CD4$^+$ T cells. HIV-1 suppression was evaluated by analysis of the amount of HIV-1 p24 Gag in the culture supernatants by ELISA (A), HIV-1 p24 Gag in CD4$^+$ T cells by Western-blotting (B), and HIV-1 LTR expression in the culture supernatants by RT-PCR (C). HIV-1 LTR expression in culture supernatants was normalized by GAPDH of HIV-1-infected CD4$^+$ T cells.

Figure 2

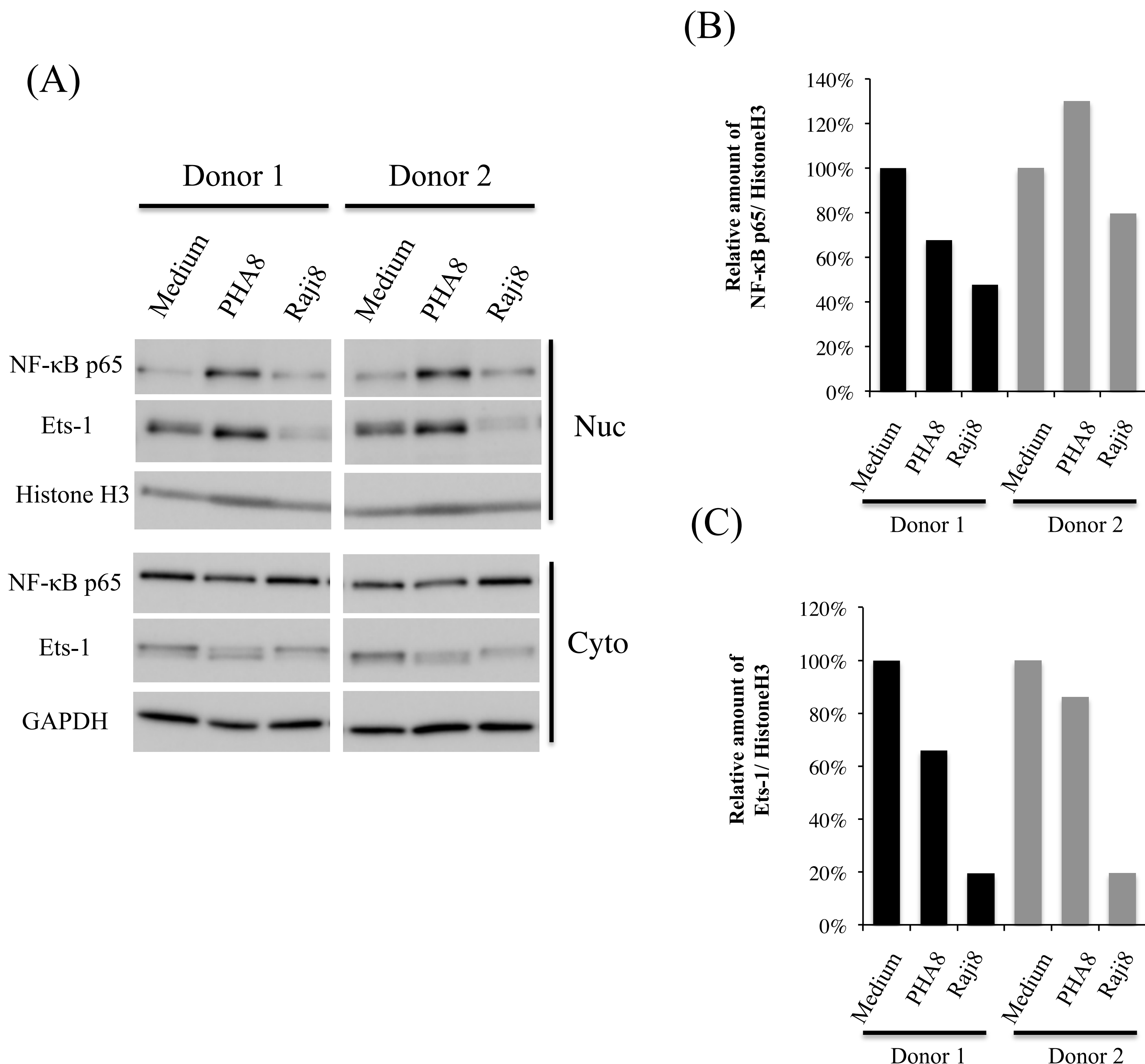

Figure 2. Localization of NF-κB p65 and Ets-1 in HIV-1-infected CD4$^+$ T cells.

HIV-1-infected CD4$^+$ T cells from two donors were cultured with PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells for 4 days, and nuclear and cytoplasmic extracts were prepared from the isolated CD4$^+$ T cells. (A) NF-κB p65 and Ets-1 expressions in CD4$^+$ T cells were analyzed by Western-blotting. (B) The relative amounts of nuclear NF-κB p65 in CD4$^+$ T cells were calculated considering the medium control as 100%. (C) The relative amounts of nuclear Ets-1 in CD4$^+$ T cells were calculated considering that those in the medium control as 100%. GAPDH was used as a cytoplasmic loading control and Histone H3 as a nuclear loading control, and representative data are shown.

Figure 3

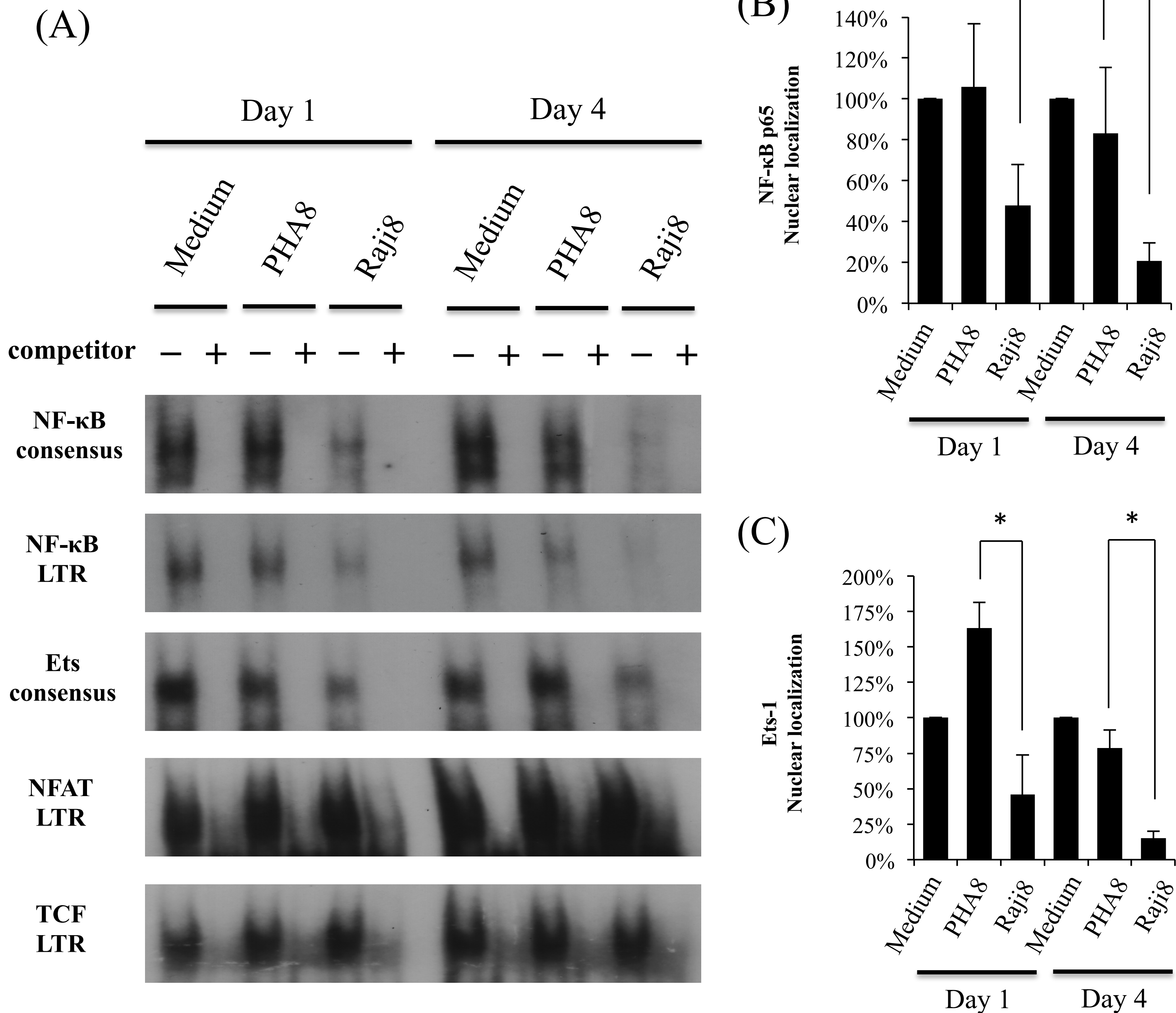

Figure 3. Suppression of transcriptional activity in CD4$^+$ T cells induced by alloantigen-stimulated CD8$^+$ T cells.

HIV-1-uninfected CD4$^+$ T cells were cultured with PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells for 1 or 4 days, and nuclear and cytoplasmic extracts isolated from autologous CD4$^+$ T cells were analyzed by EMSA and Western-blotting. (A) Nuclear extracts were incubated with ^{32}P-labeled DNA probes corresponding to the NF-κB consensus, NF-κB LTR, Ets consensus, NFAT LTR, and TCF LTR. The data for one of three independent experiments are shown. (B) Nuclear localization of NF-κB p65 in CD4$^+$ T cells was analyzed by Western-blotting, and calculated the relative amounts considering the medium control as 100%. (C) Nuclear localization of Ets-1 in CD4$^+$ T cells was analyzed by Western-blotting, and calculated the relative amounts considering the medium control as 100%. Western-blotting analysis was performed by three independent experiments. * $p < 0.05$ ** $p < 0.01$

Figure 4

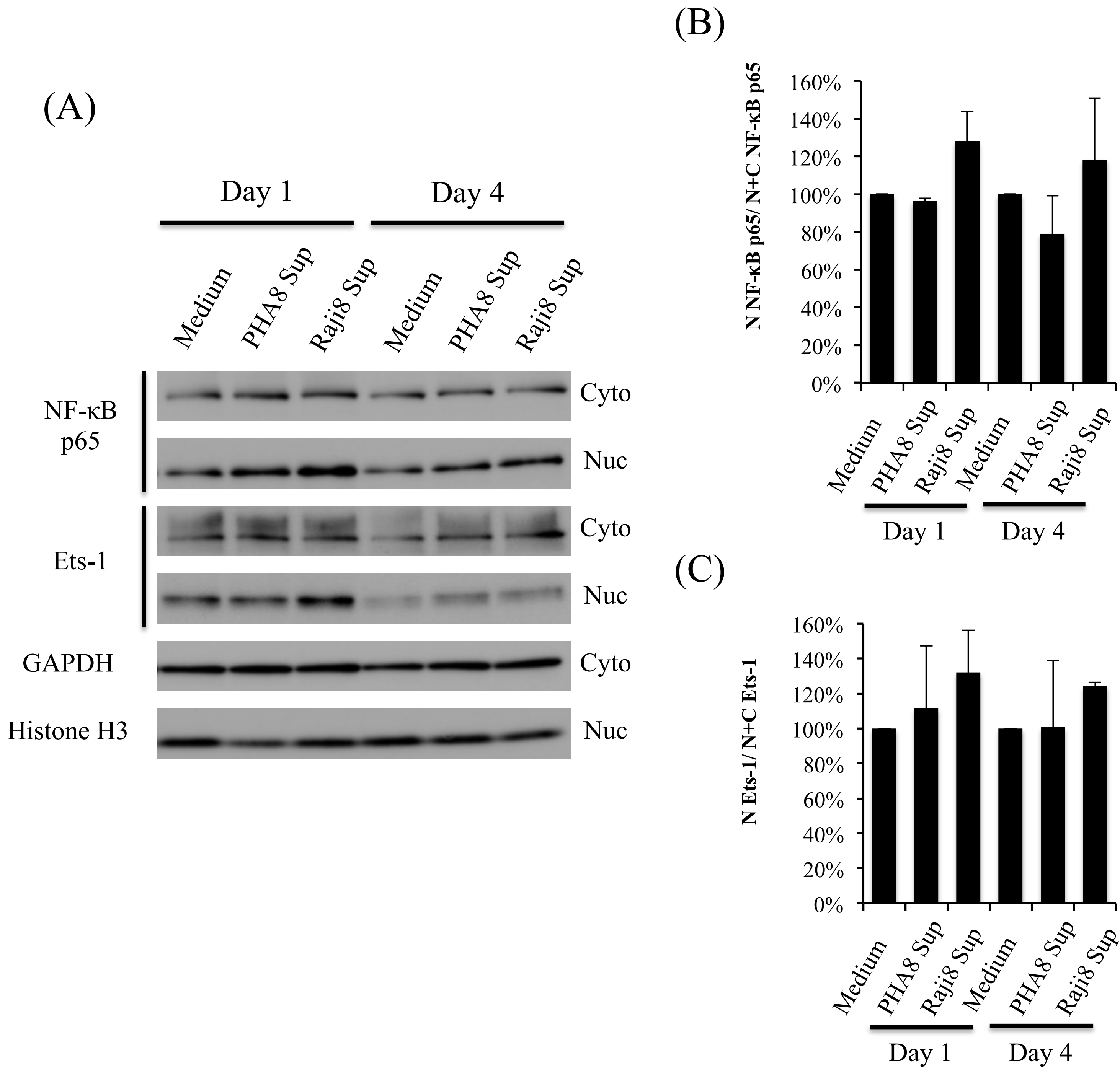

Figure 4. Analysis of NF-κB p65 and Ets-1 localization in CD4⁺ T cells incubated with culture supernatants.

HIV-1-uninfected $CD4^+$ T cells were incubated with culture supernatants of PHA-$CD8^+$ T cells and Raji-$CD8^+$ T cells (50% vol.) for 1 or 4 days, and nuclear and cytoplasmic extracts were prepared from the isolated $CD4^+$ T cells. (A) NF-κB p65 and Ets-1 localization in $CD4^+$ T cells incubated with culture supernatants were analyzed by Western-blotting. (B) The relative amounts of nuclear NF-κB p65 in $CD4^+$ T cells were calculated considering the medium control as 100%. (C) The relative amounts of nuclear Ets-1 in $CD4^+$ T cells were calculated considering the medium control as 100%. Western-blotting analysis was performed by two independent experiments.

Figure 5

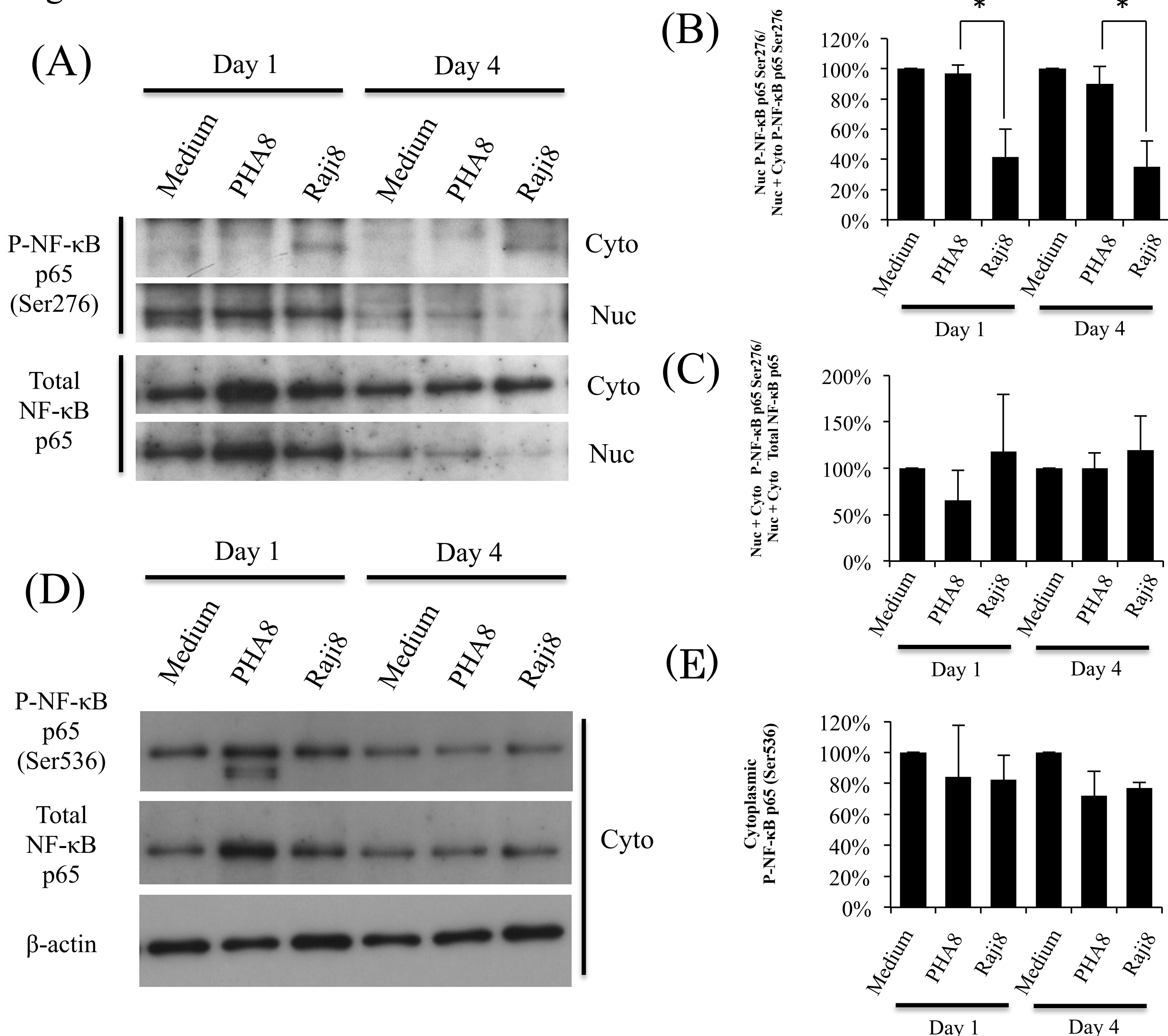

Figure 5. Analysis of phosphorylated NF-κB p65 in CD4⁺ T cells.

Nuclear and cytoplasmic extracts were isolated from HIV-1-uninfected $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and Raji-$CD8^+$ T cells. (A) Phospho-NF-κB p65 (Ser276) and total NF-κB p65 in $CD4^+$ T cells was analyzed by Western-blotting. (B) The amounts of nuclear phosphorylated NF-κB p65 (Ser276) were expressed as a ratio to its total amounts present in nuclei and cytoplasm. The percentage of nuclear phosphorylated NF-κB p65 (Ser276) was expressed relatively to those in the medium control (100%). (C) The relative amounts of nuclear and cytoplasmic phosphorylated NF-κB p65 (Ser276) were expressed as a ratio to the amounts of total nuclear and cytoplasmic NF-κB p65. The percentages of nuclear and cytoplasmic phosphorylated NF-κB p65 (Ser276) were expressed relatively to those in the medium control (100%). (D) Phospho-NF-κB p65 (Ser536) and total NF-κB p65 in $CD4^+$ T cells was analyzed by Western-blotting. (E) The percentages of cytoplasmic phosphorylated NF-κB p65 (Ser536) were expressed relatively to those in the medium control (100%). Western-blotting analysis was performed by three independent experiments. * $p < 0.05$

Figure 6

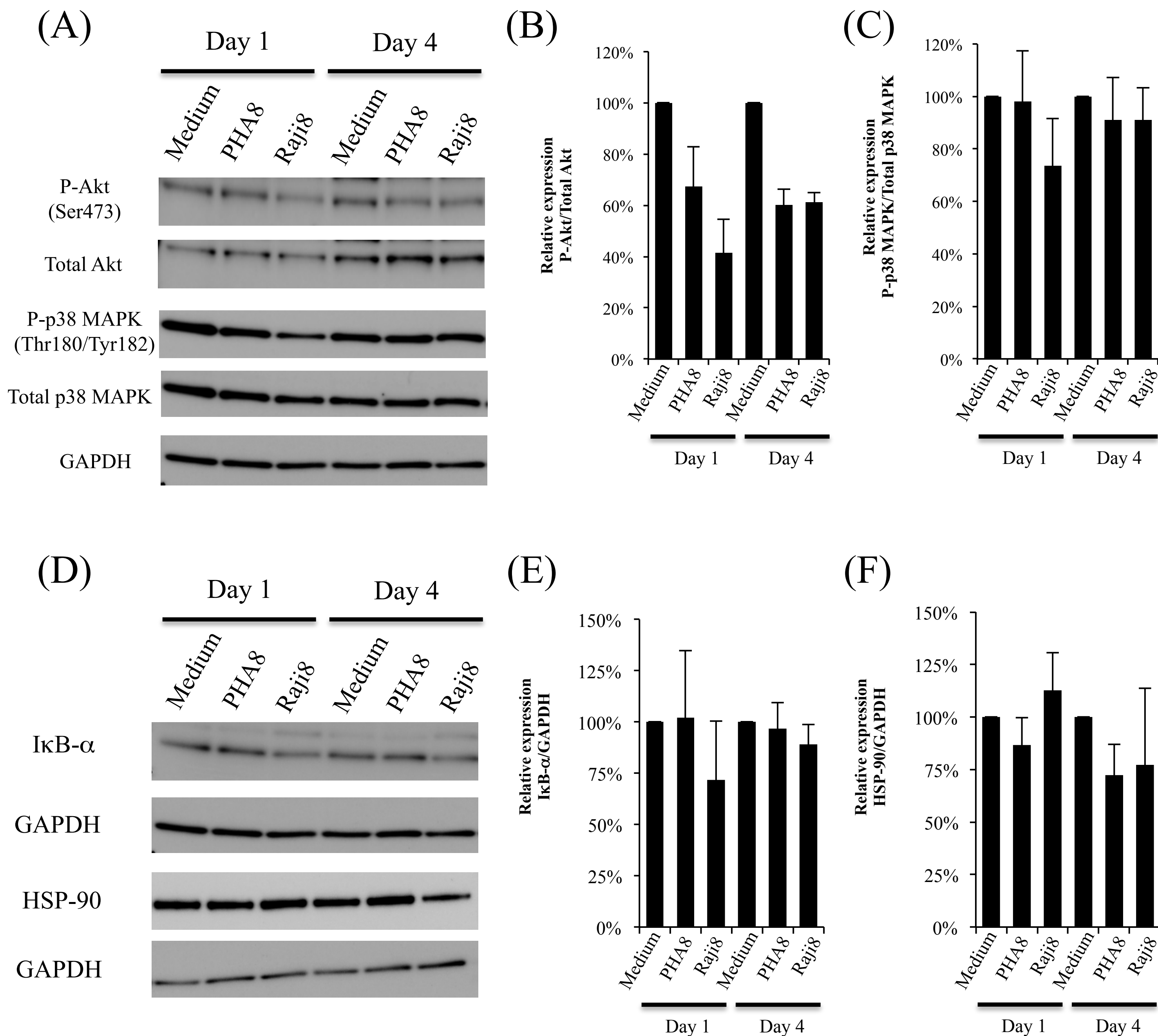

Figure 6. Analysis of Akt, p38 MAPK, IκB-α, and HSP-90 in the cytoplasm of CD4⁺ T cells

Cytoplasmic extracts were isolated from HIV-1-uninfected $CD4^+$ T cells cultured with PHA-$CD8^+$ T cells and Raji-$CD8^+$ T cells. (A) Akt and phospho-Akt (Ser473), p38 MAPK and phospho-p38 MAPK (Thr180/Tyr182) in $CD4^+$ T cells were analyzed by Western-blotting. (B) The relative amounts of phospho-Akt (Ser473) in $CD4^+$ T cells were calculated considering the medium control as 100%. (C) The relative amounts of phospho-p38 MAPK (Thr180/Tyr182) in $CD4^+$ T cells were calculated considering the medium control as 100%. (D) IκB-α and HSP-90 expression in $CD4^+$ T cells was analyzed by Western-blotting. (E) The relative amounts of IκB-α in $CD4^+$ T cells were calculated considering the medium control as 100%. (F) The relative amounts of HSP-90 in $CD4^+$ T cells were calculated considering the medium control as 100%. β-actin and GAPDH were used as a loading control. Western-blotting analysis was performed by four independent experiments.

Figure 7

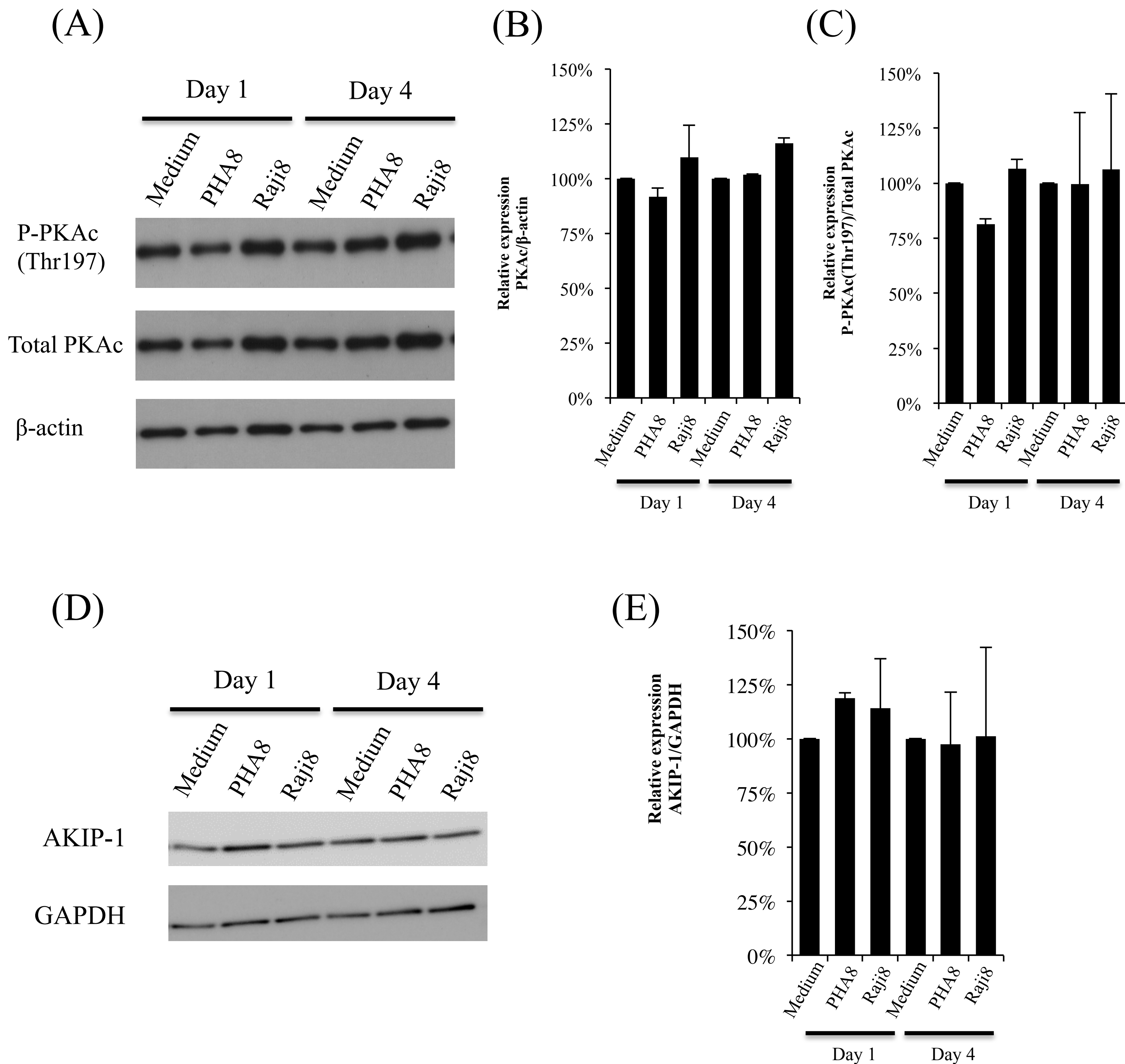

Figure 7. Analysis of PKAc and AKIP-1 in the cytoplasm of CD4$^+$ T cells.

HIV-1-uninfected CD4$^+$ T cells were co-cultured with PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells for 1 or 4 days, and nuclear or cytoplasmic extracts were prepared from the isolated CD4$^+$ T cells. (A) PKAc and phospho-PKAc (Thr197) in CD4$^+$ T cells were analyzed by Western-blotting. (B) The relative amounts of PKAc in CD4$^+$ T cells were calculated considering the medium control as 100%. (C) The relative amounts of phospho-PKAc (Thr197) in CD4$^+$ T cells were calculated considering the medium control as 100%. (D) AKIP-1 expression in CD4$^+$ T cells was analyzed by Western-blotting. (E) The relative amounts of AKIP-1 in CD4$^+$ T cells were calculated considering the medium control as 100%. β-actin and GAPDH was used as loading controls. Western-blotting analysis was performed by three independent experiments.

Figure 8

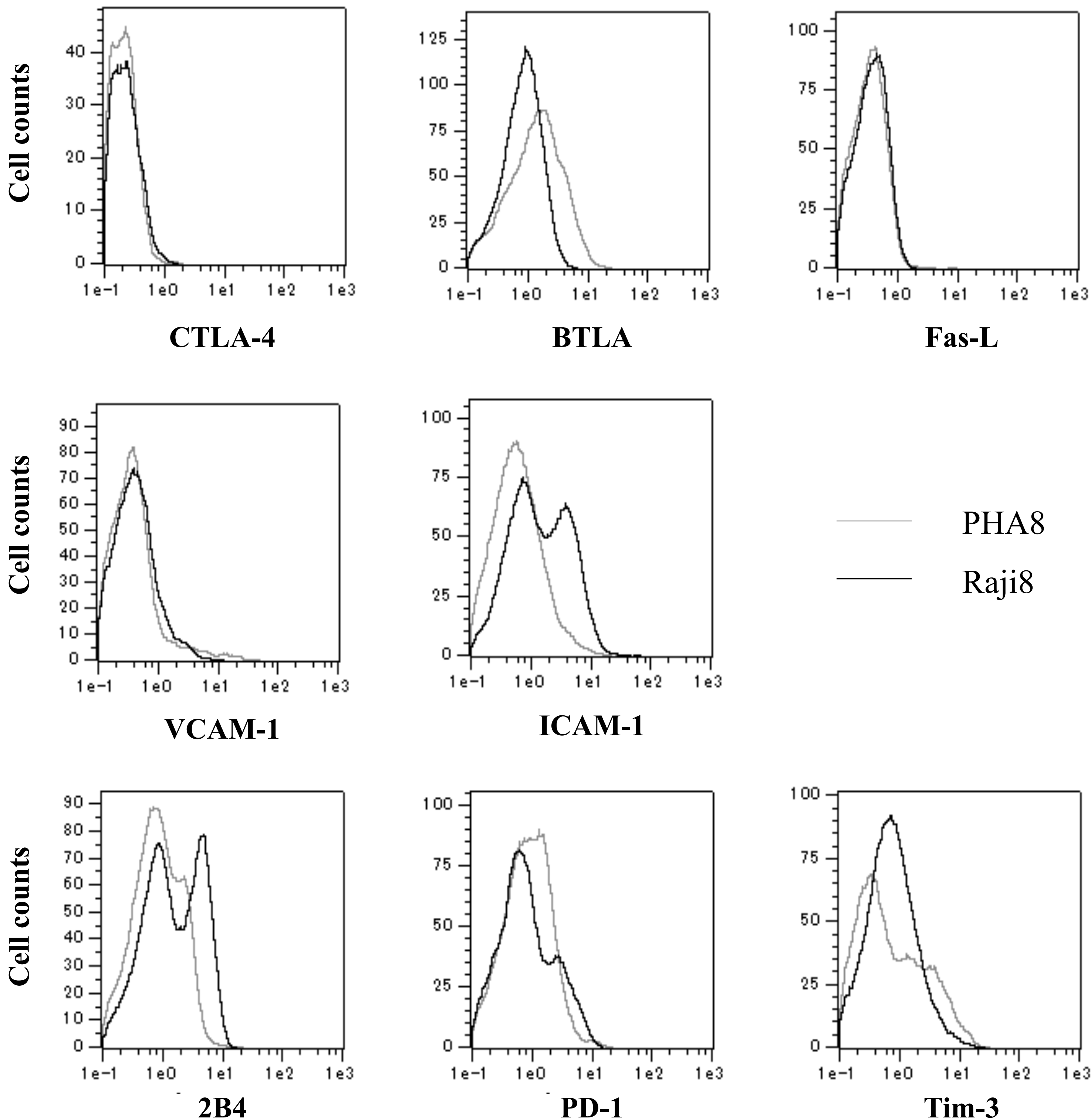

Figure 8. Characterization of surface molecules on alloantigen-stimulated CD8^{+} T cells.

Surface molecules of Raji-CD8^{+} T cells (black line) and PHA-stimulated CD8^{+} T cells (gray line) derived from three healthy donors were analyzed by flowcytometry using immunofluorescent antibodies against CD8, CTLA-4, BTLA, Fas-L, VCAM-1, ICAM-1, 2B4, PD-1, and Tim-3. The data for one of five independent experiments are shown.

Figure 9

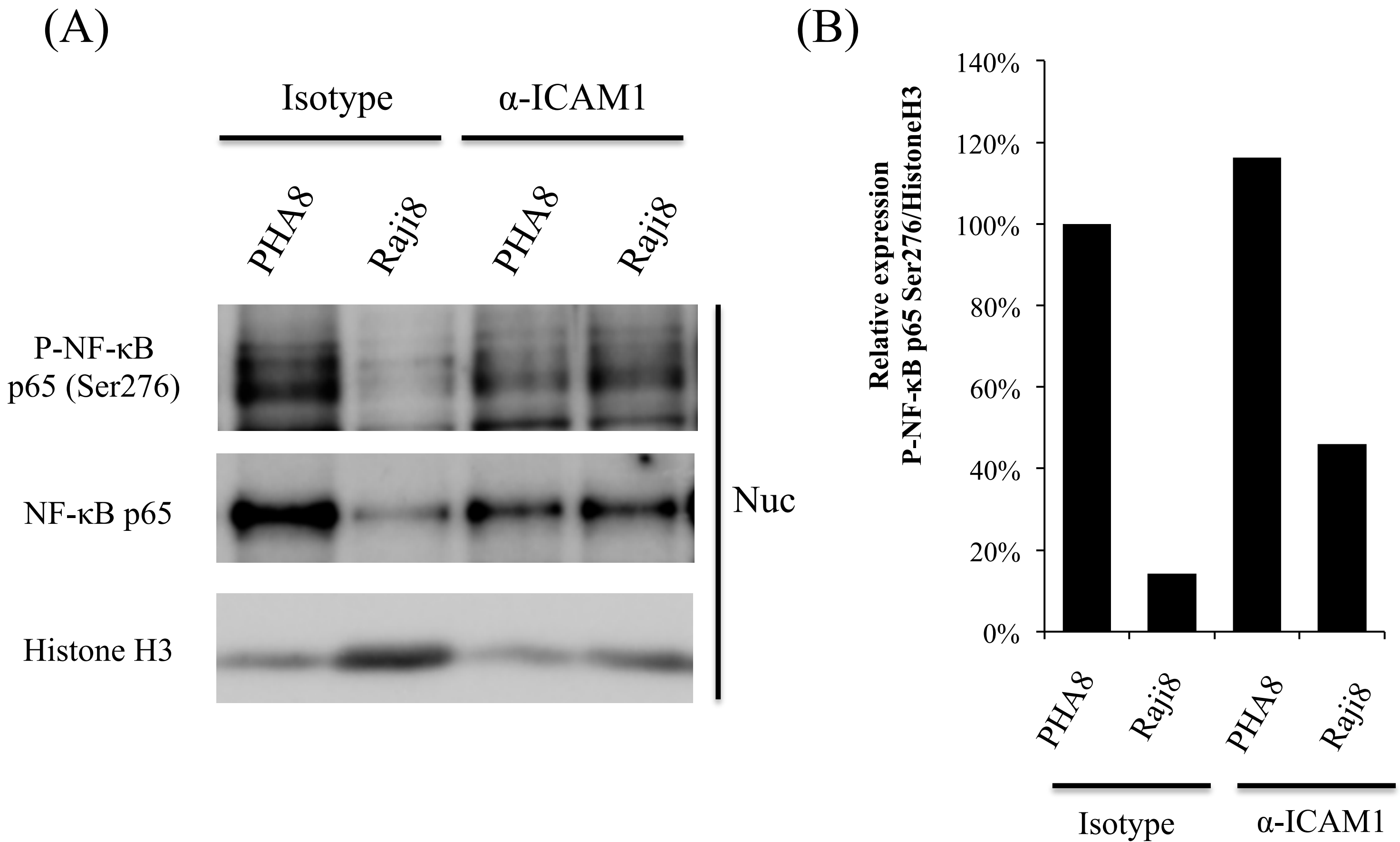

Figure 9. Effects of anti-ICAM1 neutralizing antibody on phospho-NF-κB p65 (Ser276) nuclear translocation.

HIV-1-uninfected CD4$^+$ T cells were co-cultured with PHA-CD8$^+$ T cells and Raji-CD8$^+$ T cells which had been pretreated with anti-ICAM-1 antibody or isotype control antibody. After 1 or 4 days of culture, CD8$^+$ T cells were removed and CD4$^+$ T cells were used for preparation of nuclear and cytoplasmic extracts. The data from one of two donors are shown. (A) Western-blotting analysis of nuclear phospho-NF-κB p65 (Ser276) and NF-κB p65 in CD4$^+$ T cells after 4 days of culture. (B) The relative amounts of phospho-NF-κB p65 (Ser276) in CD4$^+$ T cells were shown after normalization with Histone H3 used as a nuclear loading control, and calculated considering the PHA-CD8$^+$ T cells pretreated with isotype control as 100%.

Figure 10

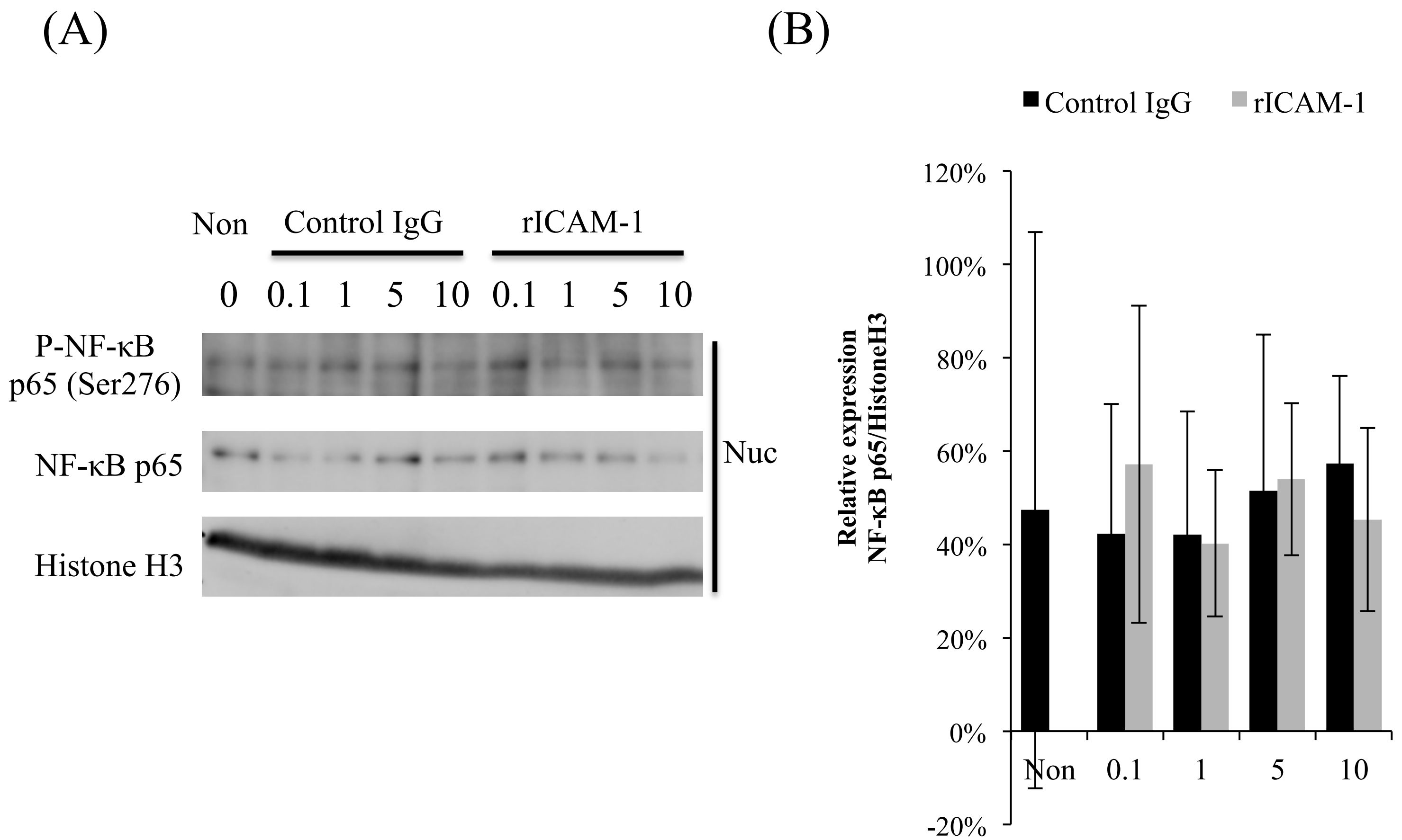

Figure 10. Analysis of NF-κB p65 localization in $CD4^+$ T cells stimulated by recombinant ICAM-1

HIV-1-uninfected $CD4^+$ T cells were isolated from PHA-stimulated PBMCs as described in Materials and Methods. $CD4^+$-enriched T cells were cultured in a 96-well plates coated with 0.1, 1, 5, and 10 μg/ml recombinant ICAM-1 IgG_1 Fc chimera or normal human IgG_1. After 1 or 4 days of culture, nuclear and cytoplasmic extracts were prepared from the CD4+ T cells. The data from one of two donors are shown. (A) Western-blotting analysis of nuclear phospho-NF-κB p65 (Ser276) and NF-κB p65 in $CD4^+$ T cells after 4 days of culture. (B) The relative amount of nuclear NF-κB p65 in $CD4^+$ T cells was shown after normalization with Histone H3 used as a nuclear loading control.

Figure 11

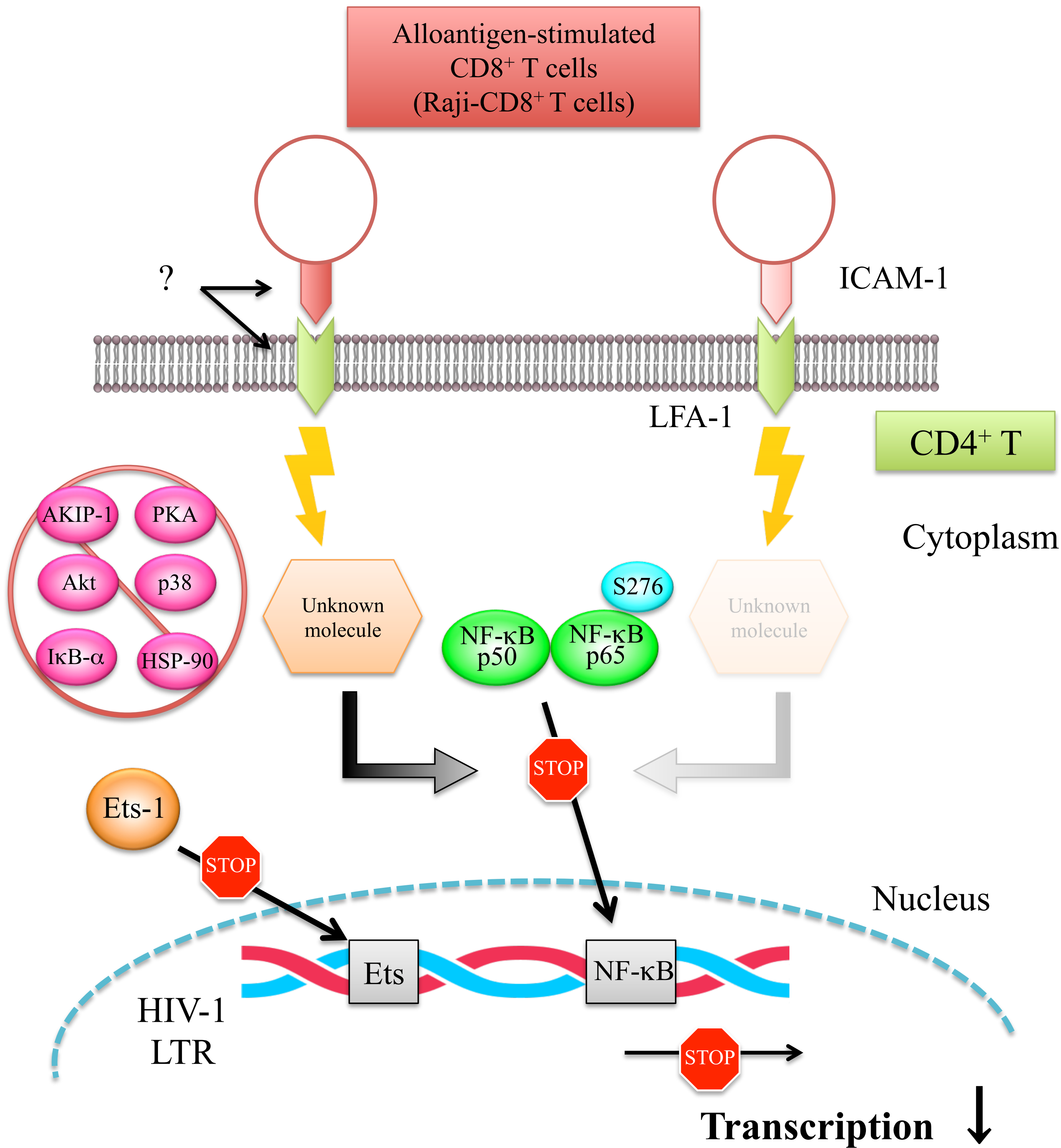

Figure 11. The model for the mechanism of suppression of HIV-1 replication induced by alloantigen-stimulated CD8⁺ T cells

Alloantigen-stimulated CD8⁺ T cells (Raji-CD8⁺ T cells) induce some signal that inhibits the nuclear translocation of phospho-NF-κB p65 (Ser276) and Ets-1 in autologous CD4⁺ T cells. Then, the reduced DNA-binding activity of NF-κB and Ets leads to the suppression of HIV-1 transcription/replication.