Efficient induction of HIV-1 replication in latently infected cells through contact with CD4+ T cells: Involvement of NF-κB activation

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Abstract

Reservoir cells latently infected with HIV-1 pose one of the major obstacles that hamper ultimate eradication of HIV-1 from infected patients. In this report, we showed that direct contact with MOLT-4 T cells induced HIV-1 replication in J22-HL-60 latently infected cells without any additional stimulus. Neutralization experiments revealed that pro-inflammatory cytokines, whose production was increased following cell–cell contact, were unlikely to be primarily involved in the induced HIV-1 replication. Cell–cell contact, but not soluble components in the culture supernatant, caused a rapid phosphorylation and degradation of IκBα, which led to elevated NF-κB DNA binding activity in J22-HL-60 cells. Furthermore, forced expression of a super-repressor form of IκBα or pretreatment with ritonavir efficiently blocked the activation of NF-κB and HIV-1 replication in J22-HL-60 cells co-cultured with MOLT-4 T cells. Moreover, either resting or PHA stimulated primary CD4+ T cells induced HIV-1 replication in J22-HL-60 cells in a similar way with that of MOLT-4 cells. These results indicated that direct contact with CD4+ T cells induced HIV-1 replication in latently infected cells and provide insight into the molecular mechanism of virus release from myeloid progenitor cells latently infected with HIV-1.

Keywords: HIV-1; Reservoir; Cell–cell contact; Latency; Co-culture

Introduction

The use of highly active antiretroviral therapy (HAART) in HIV-1-infected individuals has dramatically improved the clinical outcome in a majority of infected patients (reviewed in Pomerantz and Horn, 2003). However, the presence of cellular reservoirs that contain latent provirus capable of producing infectious particles after cellular activation leads to a rebound of the viral load after interruption of HAART (Pierson et al., 2000). The persistence of these latently infected viral reservoirs, despite prolonged HAART treatments, represents a major obstacle to the eradication of HIV-1 in infected patients (Finzi et al., 1997; Wong et al., 1997). CD4+ T cells are thought to be the major reservoirs of both actively replicating and latent HIV-1 throughout the course of the disease (Bukrinsky et al., 1991; Siliciano et al., 2003). In addition to CD4+ T cells, cells of the monocytic lineage are infected by HIV-1 and accumulating evidence suggests that purified CD34 positive populations of myeloid progenitor cells are susceptible to HIV-1 infection as well (Louache et al., 1994). A greater understanding of the molecular mechanisms of viral latency and its reactivation is essential for eradicating HIV-1. Previous studies demonstrated that cell–cell contact was essential for
induction of HIV-1 replication in latently infected cell lines (Devadas et al., 2004; Poli et al., 1994; Duh et al., 1989). However, the mechanism of HIV-1 replication induced by cell–cell contact in latently infected myeloid progenitor cells has remained unknown. In the present study, the J22-HL-60 cell line, established from HL-60 promyelocytic/myeloblastic cell line after infection with monocytotropic HIV-1 JR-FL strain, was used as a standard model.

Induction of HIV-1 replication by various extracellular stimuli is often mediated by NF-κB, which is known to bind to the κB sites within the HIV-1 long terminal repeat (LTR) enhancer region (Fauci, 1996). NF-κB is a dimeric transcription factor composed of p50, p52, RelA, RelB or c-Rel. In most unstimulated cells, NF-κB is retained in the cytoplasm through binding to IκB proteins, which mask the nuclear localization signal of NF-κB. Cellular stimulation triggers phosphorylation of specific serine residues of IκB proteins followed by its ubiquitination and rapid proteasome-mediated degradation. Released NF-κB then translocates to the nucleus and enhances transcription of target genes by binding to specific consensus sequences in their promoter region. Protease inhibitors (PIs) such as ritonavir have been successfully used in the clinical treatment of human immunodeficiency virus 1 (HIV-1) infection, resulting in a marked decrease in HIV-1 viral load and subsequent increase in CD4+ T-cell counts in patients (Collier et al., 1996a, 1996b). Other investigators showed that ritonavir suppressed proliferation of Kaposi’s sarcoma and prostate cancer cells via inhibition of NF-κB activity; ritonavir treatment of the human dermal microvessel endothelial cells (HMEC) blocked TNF-α-induced NF-κB activation; PIs inhibited Toll-like receptor 2 (TLR2)- and TLR4-induced NF-κB activation (Pati et al., 2002; Equils et al., 2004). Ritonavir was also shown to inhibit the 20S proteasome function (Andre et al., 1998). In this study, we demonstrate a marked increase in virus release from J22-HL-60 cells co-cultured with T cells, which is mediated by NF-κB and completely ablated by ritonavir.

Results

Co-culture induces HIV-1 replication in J22-HL-60 cells

To investigate effects of cell–cell interaction on the induction of HIV-1 replication in chronically HIV-1-infected J22-HL-60 cells, we co-cultured J22-HL-60 cells with various B and T-cell lines such as L428, KM-H2, MOLT-4, Jurkat, MT-2 and MT-4 at a ratio of 1:1, and the viral p24 antigen released in culture supernatants was quantified at 24 and 48 h after co-cultivation. Although the JR-FL strain is originally infectious, HIV-1 released from J22-HL-60 cells has proved not to be infectious (Qi et al., unpublished observation). Thus, p24 determined in this study is derived solely from J22-HL-60 cells. As shown in Fig. 1, co-culture strongly enhanced HIV-1 p24 release from J22-HL-60 cells. Notably, co-culture with T-cell lines induced p24 release from J22-HL-60 cells more efficiently than that with B-cell lines. Since MOLT-4 cells are broadly used and also free of known viruses, we chose this cell line for further studies.

Live cell contact is required for the co-culture-induced HIV-1 replication

We compared the kinetics of virus production induced by co-culture or TNF-α, one of the well-studied cytokines inducing HIV-1 replication in J22-HL-60 cells. As shown in Fig. 2, significant increase in p24 release from J22-HL-60 cells was first noticed at 36 h after co-cultivation, which continued throughout the experiment and finally exceeded the levels achieved by TNF-α stimulation. In contrast, TNF-α-induced p24 production was evident as early as 24 h after stimulation and reached a peak around 60 h (Fig. 2).

To determine whether cell–cell contact is essential for the induced HIV-1 expression in J22-HL-60 cells, J22-HL-60 and MOLT-4 cells were grown in separate trans-well compartments which allow exchange of soluble factors between the two cell lines, but not a physical contact between J22-HL-60 and MOLT-4 cells. As shown in Fig. 3A, p24 production was much reduced when cells were cultured separately in trans-wells or when J22-HL-60 cells were treated only with the supernatant of MOLT-4 cells. These results indicate that cell–cell contact is required for the maximal induction of HIV-1 replication in J22-HL-60 cells after co-culture with MOLT-4 cells and that contribution of soluble factors in supernatants is quite limited.

We next examined whether live MOLT-4 cells are necessary for the induction of HIV-1 replication in J22-HL-60 cells after co-culture with MOLT-4 cells. For this purpose, we used live, heat-inactivated, or 3% paraformaldehyde-fixed MOLT-4 cells for co-culture with J22-HL-60 cells (Fig. 3B). Neither heat-inactivated nor paraformaldehyde-fixed MOLT-4 cells were able to induce HIV-1 replication in J22-HL-60 cells, indicating that this induction requires the presence of live MOLT-4 cells. The results also suggest that the induction of HIV-1 replication is not simply a result of allo-reactive immunological response.
Co-culture-induced p24 release does not depend on TNF-α

Because some cytokines are known to induce HIV-1 replication, we investigated by RT-PCR how co-culture of J22-HL-60 and MOLT-4 cells influences mRNA expression of cytokines. Total RNA was isolated 24 and 48 h after co-culture and subjected to quantitative RT-PCR or semi-quantitative RT-PCR analysis. The results of quantitative RT-PCR clearly demonstrated that expression of mRNAs for IL-1β, TNF-α or M-CSF was significantly up-regulated in the mixture of the two types of cells at 24 and 48 h, when compared to MOLT-4 or J22-HL-60 cells cultured independently (Fig. 4A). Semi-quantitative PCR studies revealed that levels of mRNAs for other cytokines including IL-2, IL-4, IL-8, IL-10, IFN-γ, and GM-CSF remained unchanged after co-culture (data not shown). Based on these results, we next tested recombinant cytokines TNF-α, IL-1β and M-CSF for induction of HIV-1 replication in J22-HL-60 cells. Among these, only TNF-α was able to induce release of p24 in J22-HL-60 cells. To determine if TNF-α plays a role in the co-culture-induced HIV-1 replication, J22-HL-60 and MOLT-4 cells were treated with either anti-TNF-α antibody or control mouse IgG for 3 h before and during co-culture. As expected, anti-TNF-α antibody markedly suppressed TNF-α-mediated induction of p24 release from J22-HL-60 cells. However, the co-culture-induced p24 release was virtually unaffected in the presence of anti-TNF-α antibody (Fig. 4B).

NF-κB mediates co-culture-induced p24 release

To investigate if NF-κB is involved in the co-culture-induced p24 release, we first analyzed the phosphorylation status of IκBα by Western blotting because phosphorylation of IκBα usually precedes NF-κB activation induced by extracellular stimuli such as TNF-α and LPS. Phosphorylation of IκBα at the Serine 32 residue was induced in a relatively slow kinetics with a peak at 60 to 120 min after starting co-culture of J22-HL-60 and MOLT-4 cells (Fig. 5A, right panel), whereas TNF-α stimulation led to a rapid phosphorylation of IκBα as early as 5 min after stimulation. This phosphorylation decreased at 60 min and then increased again (Fig. 5A, left panel). Consistently, TNF-α stimulation resulted in a nearly complete disappearance of IκBα within 30 min, while co-culture induced a moderate decrease in IκBα 1 to 2 h later (Fig. 5A).

To further characterize the status of NF-κB activation, we examined NF-κB DNA binding activity, using a 32P-labeled...
A synthetic oligonucleotide containing the HIV-1 κB tandem motif. The results showed that the NF-κB DNA binding activity reached a peak around 2 h after starting co-culture (Fig. 5B). To know if NF-κB plays a key role in co-culture-mediated HIV-1 replication, J22-HL-60 cells were infected with retrovirus capable of expressing a super-repressor form of IκBα (SR-IκBα) that can specifically suppress NF-κB-dependent transcription (Brockman et al., 1995). The viability of J22-HL-60
cells was not altered by introduction of SR-IκBα (date not shown). Infected J22-HL-60 cells were then stimulated with TNF-α or co-cultured with MOLT-4 cells for the indicated time periods were separated on a 10% polyacrylamide gel and subjected to Western blotting with anti-phospho-IκBα, anti-IκBα or anti-GAPDH antibodies. (B) Nuclear extracts prepared from J22-HL-60 cells, J22-HL-60 cells stimulated with 1 ng/ml of TNF-α for 30 min, MOLT-4 cells or co-cultured J22-HL-60 and MOLT-4 cells were subjected to electrophoretic mobility shift assay with a 32P-labeled κB DNA probe. Oct-1 binding was studied in parallel to verify equivalent loading. (C) J22-HL-60 cells stably expressing a super-repressor-IκBα (SR-IκBα) or empty vector were stimulated with TNF-α (1 ng/ml) for 30 min or co-cultured with MOLT-4 cells for 2 h. Nuclear extracts were subjected to EMSA as described in panel B. (D) HIV-1 replication was monitored in J22-HL-60 cells stably expressing or not SR-IκBα and either stimulated with TNF-α or co-cultured with MOLT-4 cells. Release of p24 was analyzed at 24 and 48 h later.

Fig. 5. NF-κB mediates co-culture-induced p24 release. (A) Whole cell lysates (30 μg/lane) prepared from J22-HL-60 cells stimulated with TNF-α or co-cultured with MOLT-4 cells were separated on a 10% polyacrylamide gel and subjected to Western blotting with anti-phospho-IκBα, anti-IκBα or anti-GAPDH antibodies. (B) Nuclear extracts prepared from J22-HL-60 cells, J22-HL-60 cells stimulated with 1 ng/ml of TNF-α for 30 min, MOLT-4 cells or co-cultured J22-HL-60 and MOLT-4 cells were subjected to electrophoretic mobility shift assay with a 32P-labeled κB DNA probe. Oct-1 binding was studied in parallel to verify equivalent loading. (C) J22-HL-60 cells stably expressing a super-repressor-IκBα (SR-IκBα) or empty vector were stimulated with TNF-α (1 ng/ml) for 30 min or co-cultured with MOLT-4 cells for 2 h. Nuclear extracts were subjected to EMSA as described in panel B. (D) HIV-1 replication was monitored in J22-HL-60 cells stably expressing or not SR-IκBα and either stimulated with TNF-α or co-cultured with MOLT-4 cells. Release of p24 was analyzed at 24 and 48 h later.

Ritonavir blocks co-culture-induced p24 release through inhibition of NF-κB activation

A previous study suggested that protease inhibitor (PI) pretreatment blocks activation of NF-κB induced by TNF-α or Toll-like receptor ligands (Equils et al., 2004). We examined if ritonavir blocks NF-κB activation induced by cell–cell contact. Treatment with ritonavir for 2 h before and during co-culture efficiently suppressed activation of NF-κB in a dose-dependent manner (Fig. 6A). HIV-1 replication induced by co-culture was almost completely inhibited by 1 μM of ritonavir (Fig. 6B). Based on the SR-IκB results, it is reasonable to assume that pretreatment with ritonavir ablated HIV-1 replication in co-cultured J22-HL-60 cells through NF-κB inhibition.

CD4+ T cells stimulates HIV-1 replication in J22-HL-60 cells

Finally, we asked if primary CD4+ T cells were able to stimulate HIV-1 replication in J22-HL-60 cells. Co-culture with primary CD4+ T cells indeed induced p24 release from J22-HL-60 cells, which was greatly enhanced when primary CD4+ T cells had been stimulated with PHA before co-culture. Again, ritonavir treatment ablated the induced replication of HIV-1 (Fig. 7A). In parallel, we monitored the evolution of expression of
activation markers in primary CD4+ T cells after co-culture with J22-HL-60 cells pretreated or not with ritonavir. Co-culture with J22-HL-60 cells increased cell surface expression of activation markers CD25 and CD69 in a time-dependent manner, and this activation was not affected by ritonavir (Fig. 7B).

Discussion

We have demonstrated in this study that cell–cell contact is essential for the co-culture-induced HIV-1 replication in J22-HL-60 cells. A variety of membrane proteins including T-cell receptor (TCR) and major histocompatibility complex (MHC) molecules are involved in multiple immune responses. It has been shown that they complete their specific function accompanied with dynamic lateral and vertical movements on the cell membrane during the period of cell-to-cell interaction (Baba et al., 2001). Previous studies have shown that two distinct signals are required for the induction of cell proliferation and cytokine production in resting T cells (Chambers and Allison, 1999; Linsley and Ledbetter, 1993). The first signal is mediated through an interaction between peptide-loaded MHC-II molecules located on APC and the TCR/CD3 complex located on CD4+ T lymphocytes and the second signal is initiated through an interaction between either CD80 or CD86 on the APC and CD28 on CD4+ T cells. In order to investigate whether these two signals are required for the induction of HIV-1 replication in J22-HL-60 cells by cell–cell contact, we analyzed the expression of MHC-II molecules on J22-HL-60 cells (for first signal), CD80 or CD86 on J22-HL-60 cells and CD28 on MOLT-4 cells (for second signal) by FACS analysis. Our results indicated that J22-HL-60 cells express CD80 and CD86 but not MHC-II molecules, and MOLT-4 cells does not express CD28 (unpublished data). Therefore, these two kinds of signal may not be involved in the case of co-cultivation of J22-HL-60 cells and MOLT-4 cells.

Stimulation of a member of the tumor necrosis factor receptor (TNFR) superfamily, including TNFRI (CD120a), TNFRII (CD120b), Fas (CD95), CD40, CD30, CD27, 4-1BB (CD137) and OX40 (CD134), by their ligand molecule has been shown to trigger a variety of cellular activities, such as cell growth, differentiation, immunological responses and programmed cell death (Gruss and Dower, 1995; Chougnet et al., 2001; Herbein, 1997). In particular, TNF superfamily proteins have been reported to enhance HIV-1 replication in cells latently infected with HIV-1; TNF-α has been shown to enhance HIV-1 replication.
replication in mononuclear phagocytes and chronically infected cell lines including J22-HL-60 cells (Matsuyama et al., 1991; Folks et al., 1989; Matsuyama et al., 1989; Osborn et al., 1989); gp34–OX40 interaction through cell–cell contact also induced HIV-1 replication in latently infected T cells (Takahashi et al., 2001). Earlier reports described that up-regulation of cytokine expression by cell–cell contact contributes to replication of HIV-1 in latently infected cells (Devadas et al., 2004; Borghi et al., 2000). In our experiments, although expression of mRNA for cytokines M-CSF, TNF-α and IL-1β was enhanced by cell–cell contact, M-CSF and IL-1β showed no appreciable effect on virus production in J22-HL-60 cells. Moreover, neutralizing antibody against TNF-α was unable to inhibit co-culture-induced HIV-1 replication. Nevertheless, we cannot exclude
possible involvement of cytokines that were not examined in this study, and it is formally possible that such cytokines may be secreted from activated T cells after cell–cell contact and can stimulate J22-HL-60 cells. Paraformaldehyde-fixed MOLT-4 cells failed to induce HIV-1 replication in J22-HL-60 cells. It is thus possible that J22-HL-60 cells somehow stimulated MOLT-4 cells initially, which in turn gave back signals to J22-HL-60 cells and induced HIV-1 replication. This “outside-in” signaling model is consistent with the observed delay in phosphorylation of IκBα, degradation of IκBα and release of p24 induced by cell–cell contact as compared to those induced by TNF-α. The hypothesis was further supported by the evidence that primary CD4+ T cells (both resting and PHA simulated) exhibited elevated levels of surface activation markers CD25 and CD69 compared to resting CD4+ T cells (Fig. 7).

The replication of HIV-1 is closely associated with the activation status of its host cells and is dependent on a number of host factors. The HIV-1 long terminal repeat (LTR) contains activation status of its host cells and is dependent on a number of host factors. The HIV-1 long terminal repeat (LTR) contains

### Materials and methods

#### Cell culture

J22-HL-60 is a monocytic cell line that contains one copy of monocyte-tropic JR-FL HIV-1 strain (Kitano et al., 1990), L428 and KM-H2 are B lymphocytic cell lines. MOLT-4, Jurkat, MT-2 and MT-4 are T-cell lines. All the cell lines used in this study were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., Canada), 100 μg/ml of penicillin and 100 μg/ml of streptomycin. J22-HL-60 cells (2×10^5/ml) and MOLT-4 cells (2×10^5/ml) were co-cultured in 1.5 ml of the regular medium at ratio of 1:1. For trans-well experiments, J22-HL-60 cells were plated on the plastic substrate of 24-well plates, and MOLT-4 cells were plated in the trans-well insert in the same condition as for co-culture. Supernatants were collected at 24 and 48 h after starting co-culture. Alternatively, J22-HL-60 cells were treated with the supernatant of MOLT-4 cells (2×10^5/ml), which had been filtrated with a 0.45 μm filter and centrifuged at 53,000 rpm for 30 min. CD4+ T cells were isolated from PBMC by negative selection with magnetic beads coated with antibodies that remove non-CD4+ T cells. CD4+ T cells (1×10^6/ml) were washed once with RPMI 1640 medium and co-cultured with J22-HL-60 cells (2×10^5/ml). For activation of CD4+ T cells, CD4+ T cells were pretreated with PHA (5 μg/ml) 1 day before co-culture and washed with RPMI 1640 for 3 times to remove PHA then subjected to co-culture. Supernatants were collected at 24 and 48 h after starting co-culture. Plat-E packaging cells were described previously (Morita et al., 2000) and were maintained in DMEM supplemented with 10% fetal calf serum, blasticidin S (10 μg/ml), puromycin (1 μg/ml), penicillin G (100 U/ml) and streptomycin (100 μg/ml). Plat-E cells were transfected with pMRX-SR-IκBα-puro (pMRX-SR-IκBα-puro was constructed by inserting an EcoRI fragment encoding SR-IκBα into the EcoRI site of pMRX-puro) or pMRX-puro (Nonaka et al., 2005) using fugene 6 transfection reagent (Roche, Indianapolis, IN, USA). Culture supernatants of Plat-E cells were collected 72 h after transfection and used for infection of J22-HL-60 cells in the presence of 10 μg/ml of polybrene.

#### Quantification of HIV-1 replication

Culture supernatants were assayed for HIV-1 p24 antigen using an ELISA kit Lumipulse according to the manufacturer’s instructions. Assays were performed in triplicate.

#### Fixation or heat treatment of MOLT-4 cells

MOLT-4 cells were washed three times in 1× PBS (pH 7.2) and incubated either at room temperature for 30 min with 3% paraformaldehyde in 1× PBS or at 60 °C for 10 min. Cells were then washed three times with 1× PBS and twice with RPMI 1640 medium before placing them in co-culture.
Quantitative RT-PCR

Total RNA was extracted using Isogen (Nippon Gene Co., Toyama, Japan), treated with DNase I (GIBCO BRL) and subjected to quantitative RT-PCR. First-strand cDNA synthesis was conducted with 1 μg of total RNA using the SuperScript First-Strand Synthesis System for RT-PCR with random hexamers (Invitrogen Life Technologies, Carlsbad, CA). The amount of target transcripts was subsequently quantified by real-time PCR using ABI 7700 sequence detector system and SYBR green core reagent kit (Applied Biosystems) according to the manufacturer's protocol. The PCR cycling was done with a denaturation step at 95 °C for 10 min and 45 cycles of denaturation (95 °C for 15 s), annealing and extension (60 °C for 3 min). The primer sequences were as follows: for TNF-α, forward: 5′-ACAACGCTTAGCCCATGTT-3′ and reverse: 5′-AAAGTACACCTGCCCAACACT-3′; for IL-1β, forward: 5′-GGATATGAGCAACAAGTG-3′ and reverse: 5′-ATGGATTGTGGAAGGACTG-3′; for M-CSF, forward: 5′-ATGGATCAGCGGGGCGCGC-3′; and reverse: 5′-CTTACCATGGGATCACC-3′; for 18S ribosomal RNA, forward: 5′-GTAACCGTGTAAACCCATT-3′ and reverse: 5′-CCATCCGAGTAGTACC-3′. The copy number of 18S ribosomal RNA normalized the relative copy numbers of target transcripts.

Neutralization with anti-TNF-α antibody

J22-HL-60 cells (2 × 10⁵/ml) and MOLT-4 cells (2 × 10⁵/ml) were treated for 2 h with 1500 μg/ml anti-TNF-α antibody (SIGMA USA) or 1500 μg/ml goat anti-mouse IgG (American Qualex USA) and then placed in co-culture. Supernatants were collected at 24 and 48 h after starting co-culture. Culture supernatants were assayed for HIV p24 antigen production with the ECL detection reagent (Amersham) on Hyperfilm-ECL (Amersham).

Preparation of nuclear extracts

Cells were suspended in hypotonic buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM MgCl₂, 1 mM DTT and 0.1 mM EDTA) supplemented with protease and phosphatase inhibitors, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM Na₂VO₄ and 20 mM β-glycerophosphate. After 10 min incubation at 4 °C, Nonidet P-40 was added to 1%. Soluble and insoluble fractions were separated by centrifugation. The supernatant was recovered as a cytoplasmic extract and the nuclear pellet was washed with hypotonic buffer and resuspended in extraction buffer (50 mM HEPES [pH 7.8], 50 mM KCl, 350 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 2.5% glycerol). After 30 min incubation at 4 °C, with occasional agitation, DNA pellets were eliminated by centrifugation. The supernatant was transferred as a nuclear extract.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μg) were incubated in 20 μl of binding buffer (10 mM HEPES [pH 7.8], 100 mM NaCl, 1 mM EDTA, 2.5% glycerol containing 1 μg of poly[d(Ⅰ–C)] and 0.5 ng 32P-labeled κB probe derived from the H-2Kb promoter (Kieran et al., 1990) or 32P-labeled Oct-1 probe (Mori et al., 2000)) and incubated for 30 min at room temperature. Samples were run on a 5% polyacrylamide gel containing 2.5% glycerol in 0.5× TBE and retarded bands were revealed by autoradiography.

FACS analysis

Activation status of CD4⁺ T cells was examined with FITC-conjugated anti-CD25 or PE-conjugated anti-CD69 antibodies (DakoCytomation, Carpinteria, CA, USA). The population of CD4⁺ T cells were gated out by forward and side scatter signals (FSC/SSC) and 20,000 events were processed for each sample at a rate of 300–500 events/s. Dot-plot statistics were calculated by the instrument software (Cell Quest).

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