

Figure legends

Figure 1. Amino acid sequence of a protein and peptides used in this study. Amino acid sequence of the RNA binding domain of NS1 protein is shown with the position of the three α -helices indicated by underlines (A). Residues involved in direct RNA binding, Arg38 and Lys41, are indicated by arrowheads (26). The mutant peptides were derived from the helices-2 and -3 of the RNA-binding domain and three hydrophobic residues are replaced by alanine, indicated by italic font, for each peptide (B).

Figure 2. Secondary structures of human U6 snRNA (A) and model RNAs, U6-45 (B), and U6-34 (C). The NS1 binding site of human U6 snRNA is indicated by broken lines according to Qin *et al.* (10). U6-45 was designed to include the NS1 binding site. The two strands were connected with the stable UUCG loop, and the terminal base pairs were changed to GC pairs to increase the production yield for *in vitro* transcription by using the T7 RNA polymerase. A model RNA, U6-34, includes the NS1 protein binding site (C). Two strands were also connected with the stable UUCG loop, and the GU base pair closing the UUCG loop was changed to a GC pair. These modifications are indicated by italic font. The residue number is defined for U6-45, which does not correspond to that of U6 snRNA for convenience. The secondary structure of U6-34 reflected the result of the present NMR analysis.

Figure 3. Results of RNase digestion analysis summarized on the secondary structures. Filled and open arrows indicate the sites digested by RNase T₁ and T₂, respectively. Open characters indicate the sites digested by RNase V₁. For all cases, a larger arrow or character indicates stronger digestion. The mutation sites are indicated by arrows.

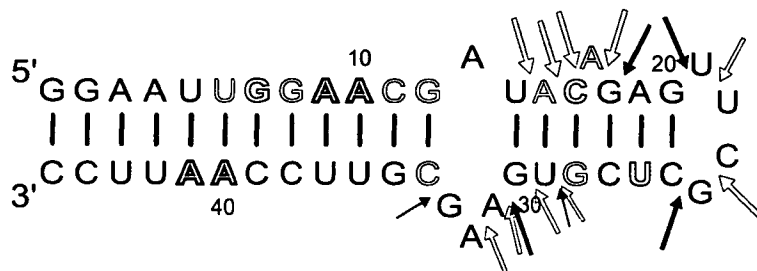
Figure 4. Competitive gel shift assay. Each lane contains 0.05 μM of U6-45. Lanes 2-7 each contain 0.12 μM of NS1 protein. Lanes 3-7 contain 0.05, 0.25, 0.5, 0.75 and 1.0 μM of competitor RNA, respectively. The competitor RNAs are indicated for each panel.

Figure 5. Gel shift assay for the RNA-binding ability of the peptides. RNA samples were analyzed by non-denaturing PAGE. The concentration of RNA was 0.05 mM for each lane in 5 μL of the loading buffer. Lane 1 is RNA alone. Lanes 2-4 (5-7) contain 0.25, 0.5 and 1.0 mM of NS1-2 (NS1-3).

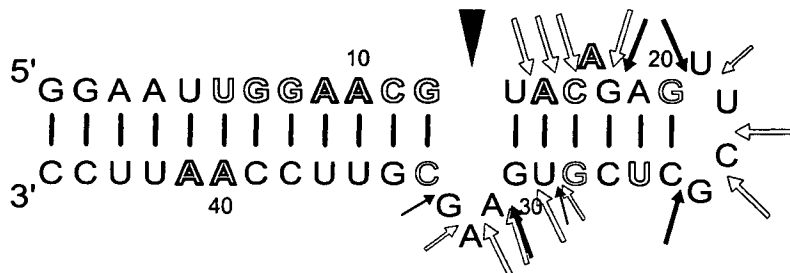
Figure 6. Peptide titration of imino proton spectra of U6-34. The concentration of U6-34 was 0.1 mM and the molar ratios of U6-34 and NS1-2 (A) or NS1-3 (B) were indicated for each row. The signal assignments are shown in the bottom row of (A). 600 MHz ^1H -NMR spectra were measured at a probe temperature of 22 $^{\circ}\text{C}$. For each experiment, 400 of scans were accumulated and 3.0 Hz of line broadening was applied prior to the Fourier transformation.

Figure 7. Chemical shift perturbation of U6-34 upon binding of NS1-2. For the complex, the concentrations of U6-34 and NS1-2 were 0.1 and 1.8 mM, respectively. (A) 2D NOESY spectra of U6-34 in the absence (left) and presence (right) of NS1-2. The NOE connectivity is depicted by lines. The residue number labels indicate intraresidue H6/H8-H1' crosspeaks. (B) Mapping of the chemical shift change on the secondary structure. Medium characters indicated the residues whose chemical shifts were changed by 0.05 - 0.1 ppm. Large characters indicated the residues with chemical shift changes of 0.1 ppm or more. Opened large characters indicated the residues with large shifts or disappearances. Italic fonts indicate the residues that could not be assigned in the complex.

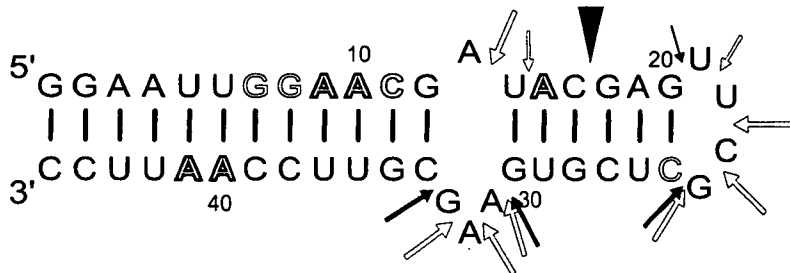
U6-45



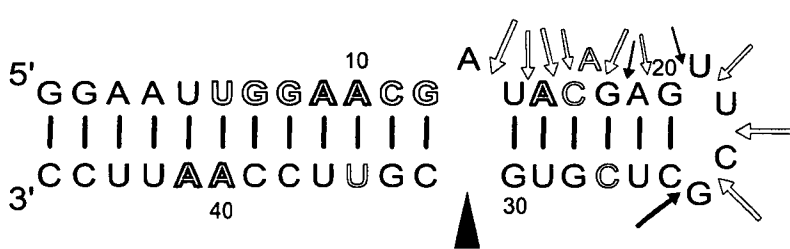
M1



M2



M3



M4

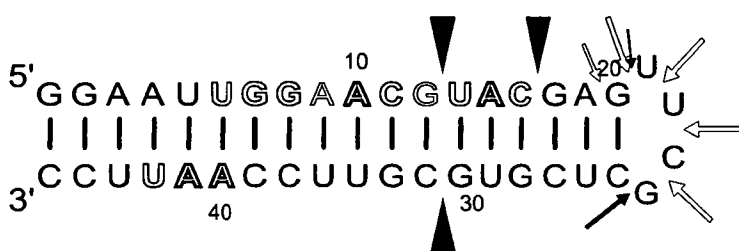


Fig. 3

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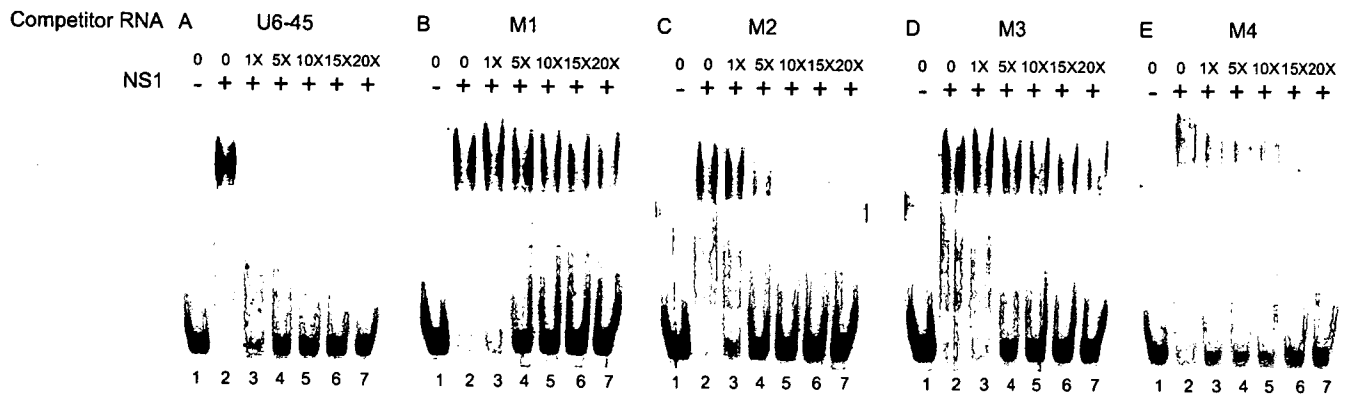


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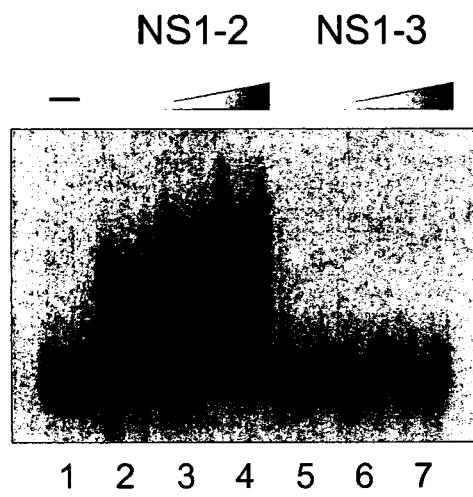


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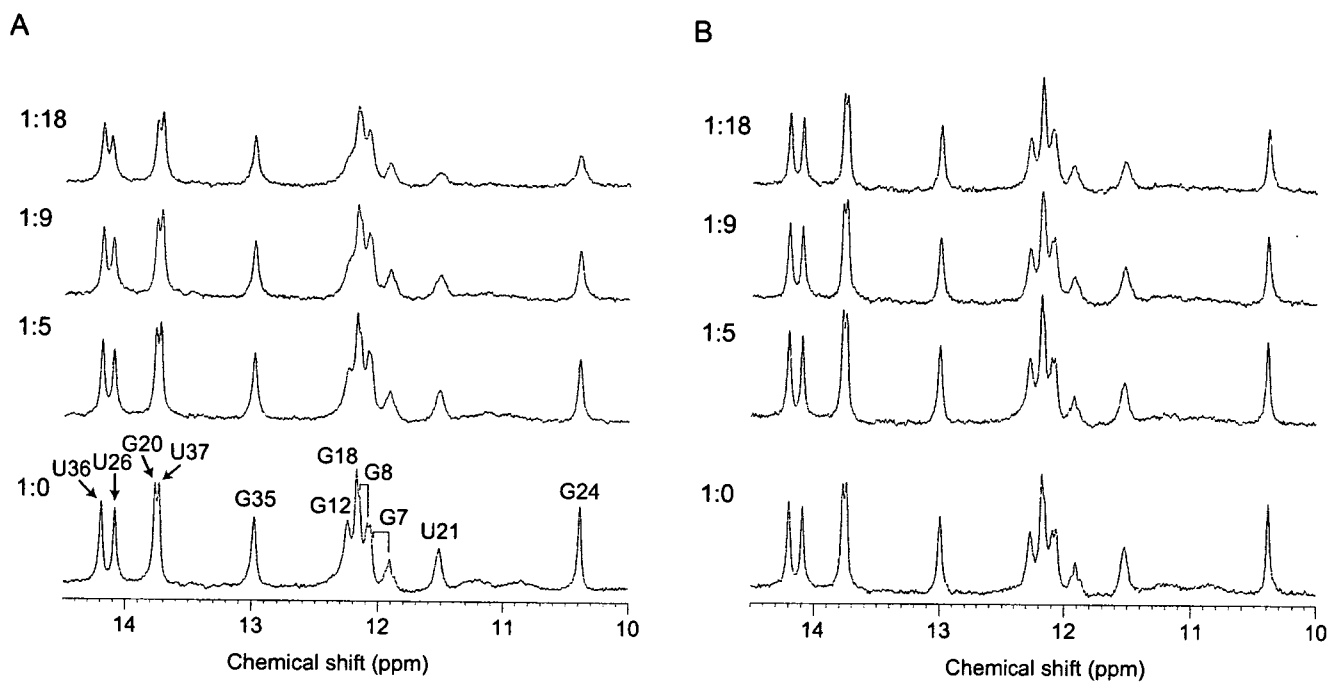


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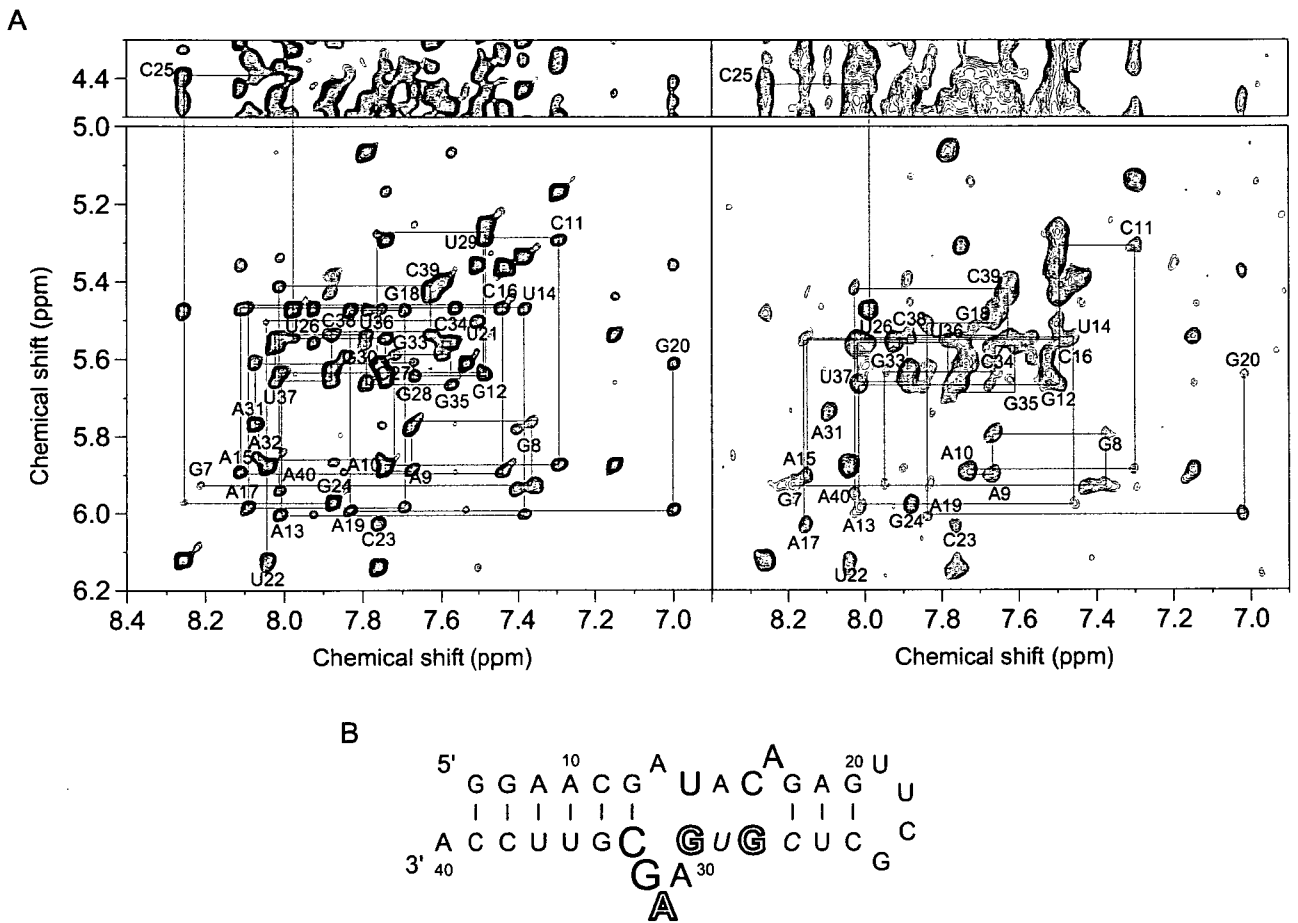


Fig. 7
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The Tax protein of HTLV-1 promotes oncogenesis in not only immature T cells but also mature T cells

To the editor:

Adult T-cell leukemia/lymphoma (ATLL) is a fatal T-cell neoplasm caused by infection with human T-cell leukemia virus type 1 (HTLV-1). Hasegawa *et al.*¹ have described transgenic mice that express HTLV-1 Tax viral regulatory protein/oncoprotein in thymocytes. These mice developed pre-T-cell (CD4⁺CD8⁻) lymphoma and leukemia. This is the first report that Tax alone is sufficient to promote oncogenesis in T cells. Tax is encoded by the *pX* region in HTLV-1, and is thought to play a central role in the leukemogenesis of ATLL (ref. 2). Transgenic mice expressing Tax develop tumors, but until the report of Hasegawa *et al.*¹, none of these mice had been reported to develop ATLL-like

T-cell lymphoma or leukemia³. The lymphoma cells in the transgenic mice developed by Hasegawa *et al.*¹, however, were immature T cells, whereas oncogenic cells in ATLL are generally mature lymphocytes⁴.

We have developed a transgenic mouse model of HTLV-1 by using a mouse lymphocyte-specific protein-tyrosine kinase (*Lck*) distal promoter⁵ to express the *Tax* gene in mature thymocytes and peripheral T lymphocytes. These mice were maintained under pathogen-free conditions, and, by the age of 21 months, 22% (14 of 63) had developed T-cell leukemia/lymphoma. The mice developed marked splenomegaly, hepatomegaly and lymphadenopathy (Fig. 1a). Total white blood cell count in trans-

genic mice with lymphoma was markedly elevated compared with that in nontransgenic mice. Large and abnormal leukemic cells comprised >80% of the peripheral blood mononuclear cells in a blood smear (Fig. 1b, left panel). We also observed a 'flower cell' morphology, albeit at low incidence (<5%) (Fig. 1b, right panel). Histological examination showed diffuse, large-cell lymphomas in the spleen, lymph nodes, liver, thymus, bone marrow, kidney, lung, meninges and skin (Fig. 1c,d).

On the basis of our pathological and histopathological findings, the disease presented by our transgenic mice corresponds to pre-T-cell leukemia/lymphoma, as defined in the transgenic mice reported by Hasegawa

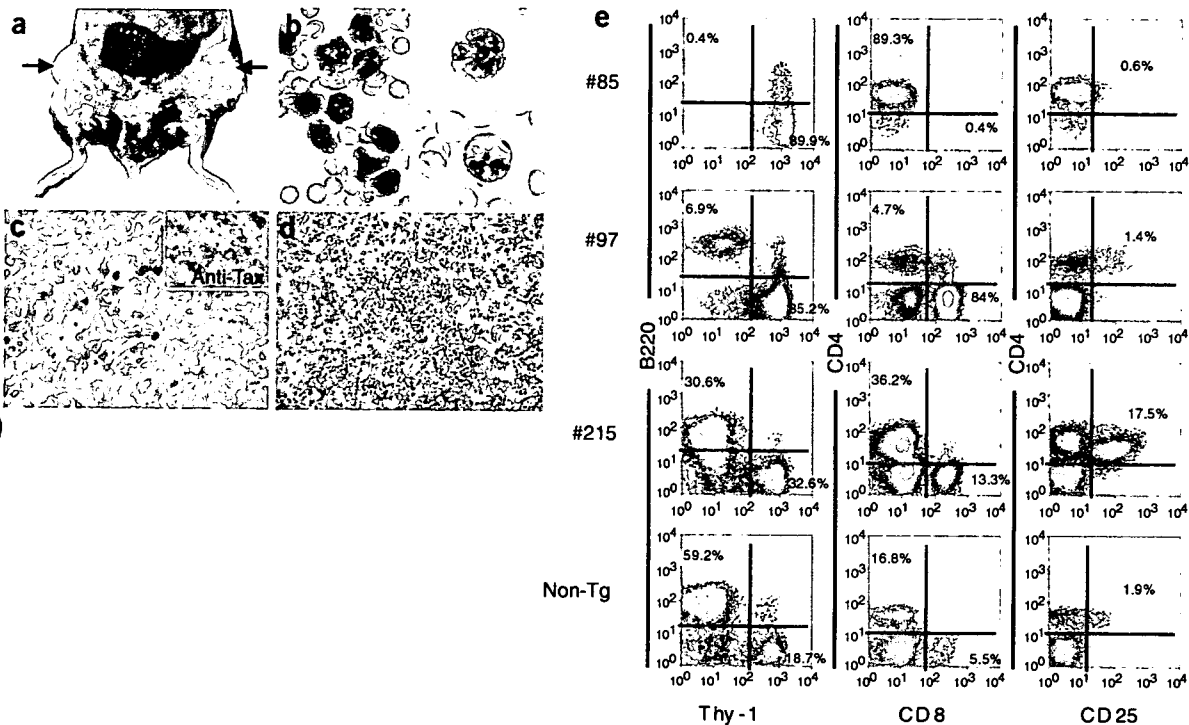


Figure 1 Pathological findings and flow cytometric analysis of lymphoma cells in Tax transgenic mice. (a) Marked splenomegaly, hepatomegaly and lymphadenopathy. (b) Left, peripheral blood smears from transgenic (Tg) mouse #85 with a high white blood cell count (178,000 mm³). Right, leukemic cells with multilobulated nuclei, the 'flower cell' morphology. (c) H&E staining shows diffuse large-cell lymphoma in an inguinal lymph node. Inset, immunohistochemical staining with antibodies to Tax. The anti-Tax/HTLV-1 Tax hybridoma 168A51-42 (Tab176) was obtained from B.C. Langton (through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, US National Institutes of Health). (d) Histology (H&E staining) shows infiltration of leukemic cells in the liver. (e) Flow cytometry reveals the phenotype of leukemic cells using the indicated fluorescence marker-conjugated antibodies. All data were gated for viable cells. Percentages represent proportions of viable cells in each region or quadrant. Phenotype of lymph node cells from mice #85 and #97 were CD4⁺ and CD8⁺, respectively. Both leukemic cells from #85 and #97 were negative for CD25. In mouse #215, expression of Thy-1 in cells, which confirmed their identity as CD4⁺CD25⁺ T cells, was elevated compared with that in control mice (17.5% versus 1.9%). Spleen cell suspensions from a littermate (non-Tg) of mouse #85 were used as a control. Both #85 and the littermate control mouse were 9 months old. Mice #97 and #215 were 8 and 20 months old, respectively. All procedures involving animals and their care were approved by the animal care committee of Kumamoto University in accordance with Institution and Japanese government guidelines (Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan, and the Law for the Humane Treatment and Management of Animals).

CORRESPONDENCE

*et al.*¹. Thus, we further characterized the leukemic cells in our transgenic mice. Flow cytometry revealed that the major phenotype of the leukemic cells was CD4⁺ or CD8⁺ T cells and that these T cells were mature (Fig. 1e). Thus, one major difference between our model and that reported by Hasegawa *et al.*¹ is the maturation stage of T cells in the leukemic cells. Our results provide additional support for the hypothesis that HTLV-I Tax promotes oncogenesis not only in immature T cells, as reported by Hasegawa and his colleagues¹, but also in mature T cells.

Tax has been shown *in vitro* to induce the expression of a variety of host genes, including those encoding interleukin (IL)-2 and the α -chain of the IL-2 receptor (IL-2R/CD25)^{6,7}. Although it is generally agreed that activation of the IL-2/IL-2R (CD25) loop contributes to T-cell activation and proliferation—with subsequent events leading to malignant transformation in ATLL—there is little *in vivo* evidence for the expression of IL-2 by HTLV-I-infected T cells. In our mouse model, the clonal pheno-

type of malignant cells was either CD4⁺ or CD8⁺, but CD25⁻, whereas ATLL usually exhibits a CD4⁺CD25⁺ T-cell phenotype⁴. It is unclear why the transgenic leukemia cells did not express CD25, although the expression of Tax in these cells was very high (Fig. 1c, inset). To date, we have observed CD4⁺CD25⁺ T-cell proliferation during polyclonal expansion of leukemia cells in only one of 63 mice tested up to the age of 21 months (Fig. 1e, #215). It has been reported that the expression of IL-2R (CD25) in HTLV-I-infected T cells seems to be stimulated by IL-2, which is induced by parasite antigen in human carriers dually infected with *Strongyloides stercoralis* and HTLV-I (ref. 8). This report and our results for transgenic mice reared under pathogen-free conditions suggest that the expression of CD25 in proliferating CD4⁺CD25⁺ HTLV-I-infected T cells might require cofactors, such as other infectious agents, that produce IL-2.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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M-CSF-Mediated Macrophage Differentiation but not Proliferation Is Correlated with Increased and Prolonged ERK Activation

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M-CSF is a cytokine essential for both the proliferation and differentiation of monocytes/macrophages. In this study, we established a new M-CSF-mediated differentiation-inducing system, and examined how the level and duration of the activation of ERK preceded M-CSF-mediated differentiation. TF-1-fms human leukemia cells rapidly proliferated in response to M-CSF. However, in the presence of a phorbol ester, TPA, TF-1-fms cells definitely switched their responsiveness to M-CSF from proliferation to differentiation, as evidenced by a more drastic morphological change and the appearance of cells with a higher level of phagocytic activity. In TF-1-fms cells expressing HIV-1 Nef protein in a conditionally active-manner, both M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation were inhibited by the activation of Nef. The Nef-active cells showed perturbed patterns of ERK activation. Under the proliferation-inducing conditions (TPA-free), parental or Nef-inactive cells showed modest ERK activation following M-CSF stimulation, whereas Nef-active cells showed an earlier and transient ERK activation, despite a decrease in their proliferation rate. Under the differentiation-inducing conditions, parental or Nef-inactive cells showed increased and prolonged ERK activation following M-CSF stimulation, whereas Nef-active cells showed transient ERK activation. These results supported the idea that the increased and prolonged ERK activation led to M-CSF-mediated macrophage differentiation but not to proliferation.

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Macrophage colony-stimulating factor (M-CSF) is a cytokine that supports both proliferation and differentiation of the cells of monocytic lineage (Roth and Stanley, 1992). The biological effects of M-CSF are mediated by a receptor tyrosine kinase, Fms (Sherr et al., 1985). The binding of M-CSF leads to the autophosphorylation of tyrosine residues in the cytoplasmic domain of Fms and subsequent interactions of the phosphorylated residues with other proteins, resulting in the initiation of multiple pathways (Bourette and Rohrschneider, 2000). Thus, to investigate which pathway leads to the proliferation or differentiation, mutant Fms proteins in which the tyrosine residues are substituted with phenylalanine have been generated and expressed in various cell types, such as NIH3T3 fibroblasts (Roussel et al., 1990), Rat-2 fibroblasts (van der Geer and Hunter, 1993), FDC-PI myeloid progenitor cells (Bourette et al., 1995) and M1 myeloid cells (Marks et al., 1999). However, because the pathway that is predominantly utilized and whether cells proliferate or differentiate in response to M-CSF depend on the cell type, the exact differences in the signaling events between these distinct cellular responses to M-CSF are still unclear. Culture systems in which the same cells distinctly respond to different stimuli (proliferation versus differentiation) are useful for clarifying this issue. For example, rat neuronal PC12 cells proliferate in response to EGF, whereas stimulation with NGF causes neuronal differentiation (Marshall, 1995). Of importance, studies with this culture system have revealed that the increased and prolonged activation of extracellular signal-regulated kinase (ERK) is critical for the neuronal differentiation of PC12 cells, but not for proliferation (Marshall, 1995). It has been also shown that the increased and prolonged activation of ERK is critical for the differentiation of

megakaryocytes (Melemed et al., 1997; Racke et al., 1997) and muscle cells (Gredinger et al., 1998). Furthermore, the functional maturation of macrophages induced by lipopolysaccharide seemed to require increased and prolonged ERK activation (Valledor et al., 2000). However, the role of ERK activation in M-CSF-mediated macrophage differentiation is not fully understood.

In this study, we first attempted to establish a new M-CSF-mediated differentiation-inducing system, using human leukemia TF-1-fms cells which essentially showed a proliferative response to M-CSF (Suzu et al., 1997). We assessed whether 12-O-tetradecanoylphorbol 13-acetate (TPA) triggered their differentiation and M-CSF accelerated the process. TPA has been characterized by its ability to induce the differentiation of several leukemia cell lines (Kitamura et al., 1989; Racke et al., 1997; He et al., 1999). We next attempted to clarify whether

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the increased and prolonged activation of ERK preceded M-CSF-mediated differentiation, by utilizing TF-I-fms cells expressing a conditionally active HIV-1 Nef protein (Suzu et al., 2005). Previously, we showed that Nef activation inhibited the M-CSF-mediated proliferation of TF-I-fms cells but enhanced the activation of ERK following M-CSF treatment (Suzu et al., 2005). Nef is a major determinant of the pathogenicity of HIV-1 (Fackler and Baur, 2002; Peterlin and Trono, 2003; Qiao et al., 2006), and has been shown to bind to and activate Hck, a Src kinase (Saksela et al., 1995; Moarefi et al., 1997). The activation of Hck was one possible molecular mechanism by which Nef caused the inhibition of M-CSF-mediated cell proliferation (Suzu et al., 2005) and the enhancement of ERK activation (Schrager et al., 2002; He et al., 2004). Based on these findings, we carefully examined how Nef affected the M-CSF/TPA-mediated differentiation of TF-I-fms cells and the level/duration of ERK activation in the differentiation-inducing conditions.

Materials and Methods

Cell culture and reagents

TF-I cells (Kitamura et al., 1989) were routinely cultured with RPMI 1640 medium (Sigma, St. Louis, MO) — 10% fetal calf serum (FCS) in the presence of recombinant human granulocyte/macrophage-CSF (GM-CSF) (2 ng/ml; PeproTech, London, UK). TF-I-fms cells (Suzu et al., 1997) were maintained with RPMI 1640 — 10% FCS in the presence of M-CSF (100 ng/ml; a gift from Morinaga Milk Industry, Kanagawa, Japan) and G418 (200 µg/ml; Life Technologies, Grand Island, NY). TF-I-fms cells expressing the HIV-1 Nef-murine estrogen receptor hormone-binding domain (Nef-ER) fusion protein (TF-I-fms-Nef-ER) (Walk et al., 2001; Suzu et al., 2005) were maintained with RPMI 1640 — 10% FCS containing M-CSF, G418, and puromycin (1.5 µg/ml; Sigma). In this system, Nef was basally inactive but its function was inducibly activated by the estrogen analogue, 4-hydroxytamoxifen (4-HT; Sigma) (Suzu et al., 2005). In this study, we also established TF-I cells expressing the Nef-ER fusion protein with the plasmid pEBB-Nef-ER-IRES-puro (Walk et al., 2005). TF-I-Nef-ER cells were maintained with RPMI 1640 — 10% FCS containing GM-CSF and puromycin. To activate Nef, 4-HT was added to the culture at a final concentration of 1 µM. TPA (Sigma) was added to the culture at a final concentration of 100 ng/ml. PD98059 (ERK kinase inhibitor) and PP2 (Src kinase inhibitor) were purchased from Sigma. U0126 was purchased from Calbiochem (San Diego, CA).

Cell count and viability analysis

The viable cell counts were obtained by enumerating the cells that excluded trypan blue dye on a hemocytometer. The adherent cells were harvested by trypsinization. The viability of cells was also examined with the propidium iodide (PI) exclusion method (Okada et al., 1998). Cells were suspended in phosphate-buffered saline (PBS) containing 0.1% Na₂S₂O₈, 3% FCS and 2 µg/ml PI. The uptake of PI in each cell was analyzed with a FACSCalibur using Cell Quest Software (Becton Dickinson, Mountain View, CA).

Analyses of expression of Fms and CD204, and phagocytic activity

The cell surface expression of Fms was analyzed by flow cytometry using Flag-tagged M-CSF (Suzu et al., 2005). In brief, cells were incubated with the Flag-tagged M-CSF followed by biotin-labeled anti-Flag M2 antibody (Sigma) and phycoerythrin (PE)-labeled streptavidin (PharMingen, San Jose, CA). The analyses were performed with a FACSCalibur. The cell surface expression of CD204 was determined using anti-CD204-FITC (clone E-5) (Tomokiyo et al., 2002). The phagocytic activity was determined by measuring the uptake of fluorescent microspheres (Fluoresbrite Carboxylate Microspheres, 0.7 µm in diameter, Polysciences, Warrington, PA). Cells cultured on a 6-well tissue culture plate were incubated with the fluorescent microspheres for 5 h and washed with PBS. The cells showing phagocytized particles were analyzed by flow cytometry.

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed essentially as described previously (Suzu et al., 2000, 2005). Cells were

depleted of M-CSF for 14 h in RPMI 1640 — 10% FCS with or without TPA, and then stimulated with M-CSF for the indicated periods. In selected experiments, 4-HT was added to the culture at the initiation of M-CSF deprivation/TPA pre-treatment. Then, the cells were solubilized with the Nonidet P-40 lysis buffer. The immunoprecipitation was performed with anti-phosphotyrosine mouse IgG conjugated to agarose (PY99; Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies (purchased from Santa Cruz) used for Western blotting were as follows: anti-Fms (C-20), anti-phosphotyrosine (PY99), anti-ERK (K-23), and anti-phosphorylated ERK (E-4). The rabbit antiserum to Nef was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD). The detection was performed using the ECL system (Amersham, Buckinghamshire, UK). The relative intensity of bands on scanned gel images was quantified by using the NIH image software.

Results

Proliferative- and differentiative properties of TF-I-fms cells

TF-I-fms cells were derived from TF-I cells by introducing the wild-type *c-fms* gene into the parental cells (Fig. 1A). The proliferation of TF-I and TF-I-fms was entirely dependent on GM-CSF and M-CSF, respectively (Fig. 1B) (Kitamura et al., 1989; Suzu et al., 1997). TF-I-fms cells lost their responsiveness to GM-CSF, due to a loss of the expression of the GM-CSF receptor α chain (data not shown). In the presence of M-CSF, TF-I-fms cells neither adhered to dishes (Fig. 1C) nor showed a differentiated morphology (Fig. 1D, top part). On the other hand, when cultured with media containing TPA alone, the cells tended to adhere to dishes and show a flattened morphology (Figs. 1C and D, middle part). Of note, M-CSF accelerated the differentiation-like process. Most TF-I-fms cells cultured in the presence of both M-CSF and TPA adhered to dishes and showed a mature macrophage-like morphology (Figs. 1C and D, bottom part), which closely resembled the primary macrophages obtained by culturing human peripheral blood monocytes with M-CSF (Hashimoto et al., 1999). In parallel with the morphological change, these cells showed more of an increase in granularity than cells cultured with TPA alone (Fig. 1E). More importantly, these cells showed higher phagocytic activity: the percentage of cells phagocytizing the microbeads and their mean fluorescence intensity (MFI) were higher in the culture containing M-CSF and TPA than in the culture containing TPA alone (Fig. 1F, left part). In addition, these cells showed a higher level of CD204 (class A macrophage scavenger receptor) (Fig. 1F, right part). These results indicated that the cells obtained by culturing TF-I-fms cells with both M-CSF and TPA were functionally mature macrophages. As mentioned above and shown in the upper panel of Figure 2A, the culture of TF-I-fms cells with TPA resulted in the appearance of adherent cells and the addition of M-CSF further increased the number of adherent cells. However, there was no significant difference in total number of viable cells between the two treatments (TPA alone versus M-CSF + TPA) (Fig. 2A, lower part). Moreover, there was no significant difference in the percentage of PI-positive dead cells between the two treatments (Fig. 2B), excluding the possibility that the acceleration of TF-I-fms cell differentiation by M-CSF and TPA reflected a survival-enhancing or anti-apoptotic function of M-CSF. It was therefore likely that TF-I-fms cells definitely switched their responsiveness to M-CSF from proliferation to macrophage differentiation in the presence of TPA.

Requirement of ERK activation for M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation of TF-I-fms cells

We next examined whether the activation of ERK was required for M-CSF-mediated proliferation and M-CSF/TPA-mediated

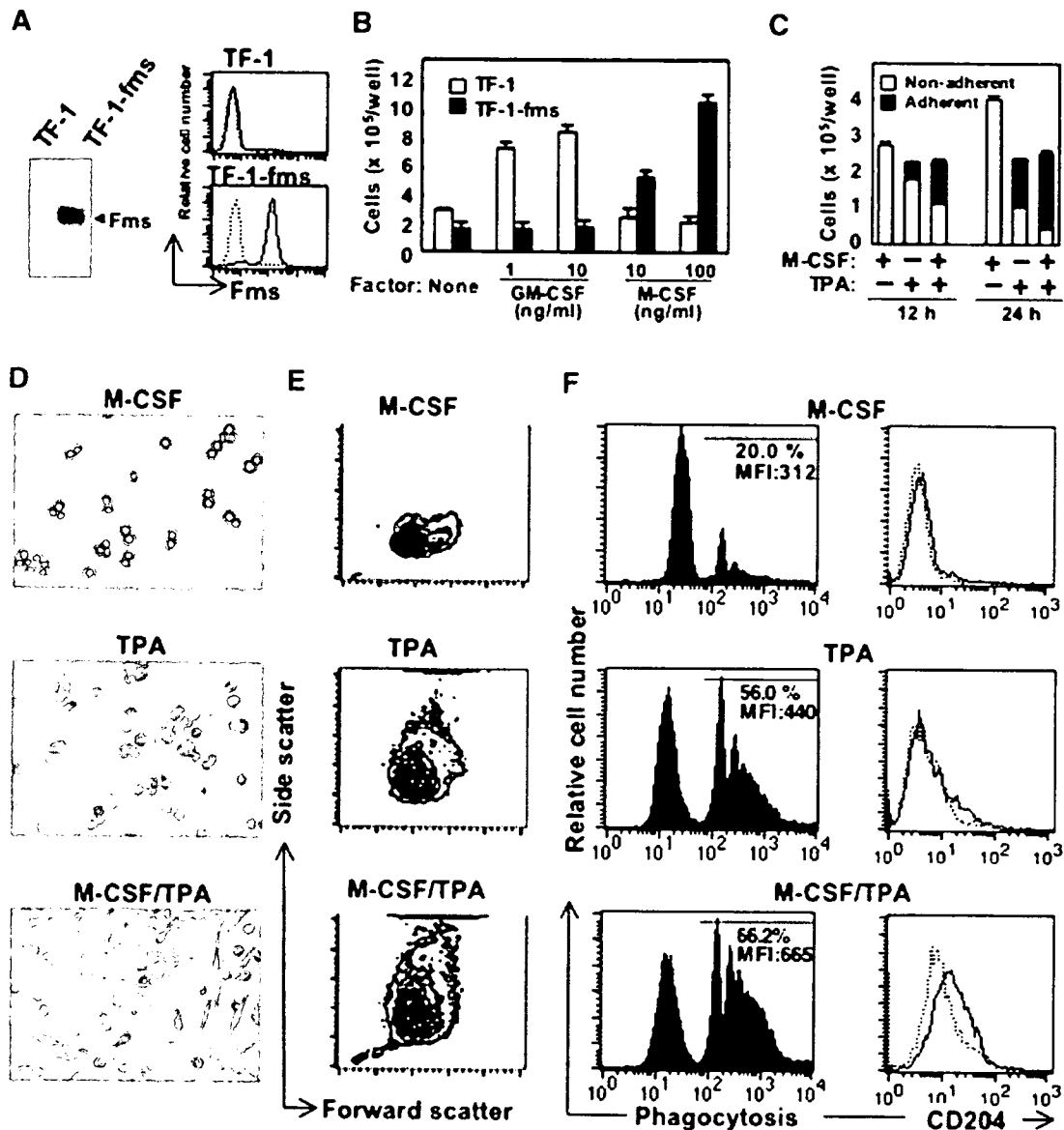


Fig. 1. The expression of Fms in and proliferative and differentiative properties of TF-1-fms cells. **A:** The total cell lysates from TF-1 or TF-1-fms cells were analyzed for the expression of Fms by Western blotting. Alternatively, the level of cell surface Fms expression was analyzed by flow cytometry with Flag-tagged M-CSF (solid lines). The profiles of cells incubated with a Flag-tagged protein, which is unrelated to M-CSF, are also shown as a control (broken lines). **B:** TF-1 or TF-1-fms cells were seeded into 6-well culture plates at a density of 5×10^4 cells/ml in the absence or presence of the indicated concentrations of cytokines. TF-1 and TF-1-fms cells were cultured for 3 and 2 days, respectively. After the cultures, viable cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. **C:** TF-1-fms cells were seeded at a density of 1×10^5 cells/ml, and cultured in the presence of M-CSF, TPA, or both. After culturing for 12 or 24 h, cells adhering to the dishes and non-adherent cells were enumerated. **D–F:** TF-1-fms cells were seeded at a density of 1×10^5 cells/ml, and cultured for 2 days in the presence of M-CSF (M-CSF), TPA (TPA), or both (M-CSF/TPA). **D:** The morphology of cells after culturing is shown. The cells cultured with M-CSF alone were photographed after a 5-fold dilution with media. **E:** The results of flow cytometric analyses of cells for forward and side scatters are shown. An equal number of cells were analyzed and the results are presented as counter plots. **F:** The phagocytic activity of cells was determined by the procedures described in the Materials and Methods (left panels). The percentage and mean fluorescence intensity (MFI) of cells in the region indicated by solid lines are shown. Alternatively, the cells were analyzed for the expression of CD204 by flow cytometry (right parts).

differentiation of TF-1-fms cells, using pharmacological inhibitors. As shown, PP2 (the inhibitor specific for Src kinases), PD98059 (the inhibitor specific for ERK kinase, MEK), and U0126 (another MEK inhibitor) significantly reduced the rate of M-CSF-mediated proliferation (Fig. 3A). The reduced

proliferation rate correlated well with the increase in the percentage of PI-positive dead cells (Fig. 3A). Thus, we could not exclude the possibility that the inhibitory effect of these inhibitors on M-CSF-mediated proliferation reflected their cytotoxicity. Of importance, however, these inhibitors, in

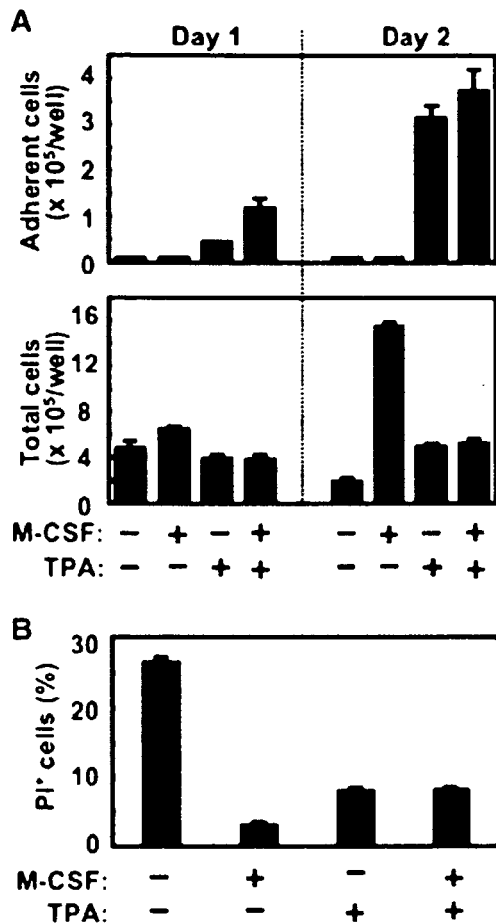


Fig. 2. A comparison of cell viability/death between the culture with TPA alone and that with M-CSF plus TPA. **A** and **B**: TF-I-fms cells were seeded into 6-well culture plates at a density of 1×10^5 cells/ml. Then, the cells were cultured in the absence of additives, or the presence of M-CSF, TPA, or both. **A**: After culturing for 1 or 2 days, cells adhering to the dishes (upper part) and all viable cells (lower part) were enumerated. **B**: After culturing for 2 days, the percentage of PI-positive dead cells in the wells was determined by flow cytometry. Error bars from triplicate assays are shown. These results are representative of two independent experiments.

particular U0126, significantly inhibited the differentiation of TF-I-fms cells induced by M-CSF and TPA without increasing the percentage of PI-positive dead cells (Fig. 3B). These results indicated that the differentiation of TF-I-fms cells was dependent on the activation of ERK.

The level and duration of ERK activation in parental TF-I-fms cells

We next examined whether the increased and/or prolonged activation of ERK preceded the differentiation. We pretreated TF-I-fms cells with TPA or left them untreated, stimulated then with M-CSF or left them un-stimulated, and analyzed the activation of ERK by using an antibody specific for phosphorylated ERK (Fig. 4). Consistent with an earlier report (He et al., 1999), the treatment of TF-I-fms cells with TPA led to the ERK activation (Figs. 4A and B). Following M-CSF stimulation, a number of molecules were shown to be rapidly tyrosine-phosphorylated in both TPA-pretreated cells and

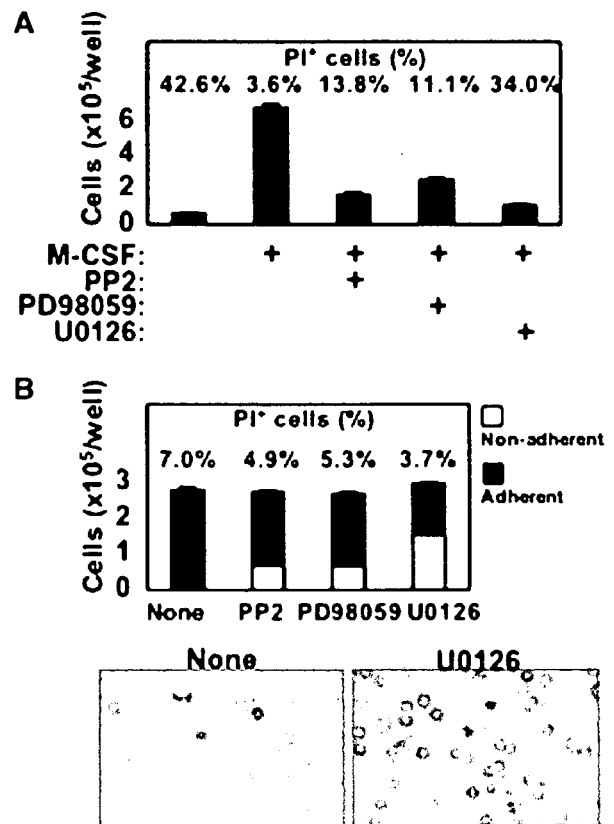


Fig. 3. Effects of pharmacological inhibitors on the proliferation and differentiation of TF-I-fms cells. **A**: TF-I-fms cells were seeded into 6-well culture plates at a density of 5×10^4 cells/ml in the absence or presence of the indicated inhibitors. M-CSF was added at a final concentration of 100 ng/ml. Both PP2 and U0126 were added at a final concentration of 10 μ M. PD98059 was used at a final concentration of 50 μ M. After culturing for 2 days, viable cells were enumerated. Error bars from triplicate assays are shown. Simultaneously, the percentage of PI-positive dead cells was determined by flow cytometry. These results are representative of two independent experiments. **B**: TF-I-fms cells were suspended at 1×10^5 cells/ml in medium containing M-CSF and TPA, and then cultured for 1 day in the absence or presence of the indicated inhibitors. The cells adhering to the dishes and non-adherent cells were enumerated. Error bars from triplicate assays are shown. Simultaneously, the percentage of PI-positive dead cells was determined by flow cytometry. These results are representative of two independent experiments. The morphology of cells after culturing is shown.

untreated cells (Fig. 4A). The most prominent band at 150–160 kD, the tyrosine-phosphorylation level of which was reduced in the pretreated cells, seemed to be Fms (Suzu et al., 2005). The reduction of Fms phosphorylation in the pretreated cells was likely to be due to the reduced cell surface expression of Fms, because the pretreatment caused an increase in the intracellular form of Fms (gp130) and concomitant decrease in the cell surface form of Fms (gp150) (Fig. 4A), as seen in TPA-treated p388D1 macrophages (Wilhelmsen and van der Geer, 2004). Despite the reduced Fms phosphorylation, the level of ERK activation following M-CSF stimulation in TPA-pretreated cells was higher than that in untreated cells (Figs. 4A and B). Although the higher level of ERK activation following M-CSF stimulation seemed to be due to the high baseline level (Fig. 4A, the bar graph), the extent of the activation apparently correlated with the differentiation of TF-I-fms cells.

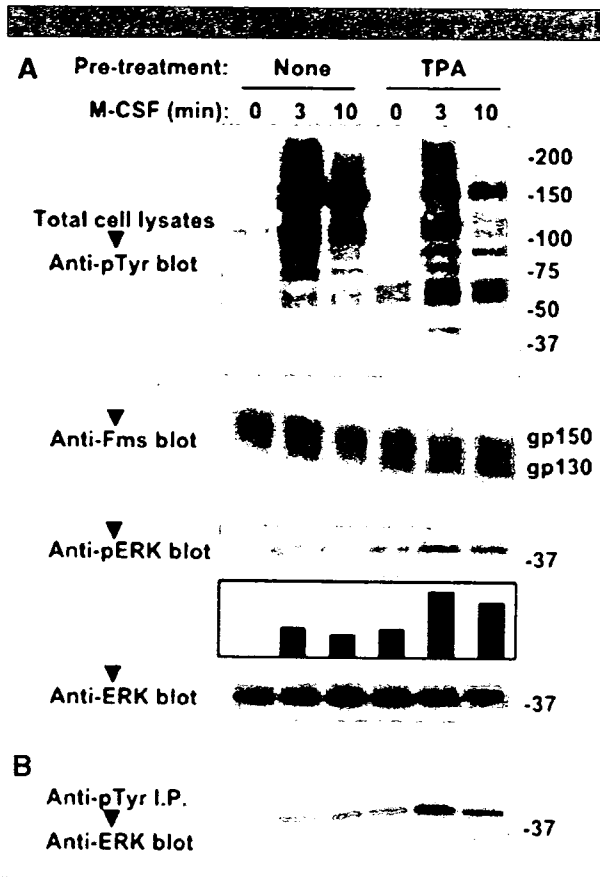


Fig. 4. The level of phosphorylation of ERK in TF-1-fms cells after pre-treatment with TPA and re-stimulation with M-CSF. TF-1-fms cells were deprived of M-CSF with or without TPA for 14 h and then re-stimulated with M-CSF for the indicated periods. **A:** Total cell lysates were analyzed with Western blotting using an antibody specific for phosphotyrosine (pTyr), Fms, phosphorylated ERK (pERK), or total ERK. The bottom blot was to show that comparable amounts of proteins were loaded in the upper blots. The relative intensities of bands on scanned gel images were quantified using the NIH Image software and the level of phosphorylated ERK normalized to the total amount of ERK is shown in the bar graph. **B:** The immunoprecipitates with anti-pTyr antibody were analyzed using anti-ERK antibody.

The proliferative/differentiative properties and the level/duration of ERK activation in TF-1-fms cells expressing conditionally active HIV-1 Nef

As mentioned above, we previously established TF-1-fms cells expressing the conditionally active HIV-1 Nef protein (the Nef-ER fusion protein) and showed that the activation of Nef by 4-HT caused the inhibition of the M-CSF-mediated proliferation of TF-1-fms cells (Figs. 5A and B; Suzu et al., 2005). By using the newly established TF-1 cells expressing the Nef-ER (Fig. 5A), we excluded the possibility that the inhibitory effect of Nef was due to non-specific cytotoxic activity of the viral protein: the activation of Nef did not affect GM-CSF-mediated proliferation of TF-1 cells (Fig. 5B). In this study, we found that Nef activation also caused the inhibition of the M-CSF/TPA-mediated differentiation of TF-1-fms cells (Fig. 5C). Consistent with its inhibitory effect on both M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation, Nef perturbed the patterns of ERK activation following the stimulation of TF-1-fms-Nef-ER cells with M-CSF (Figs. 5D and E). The untreated control cells

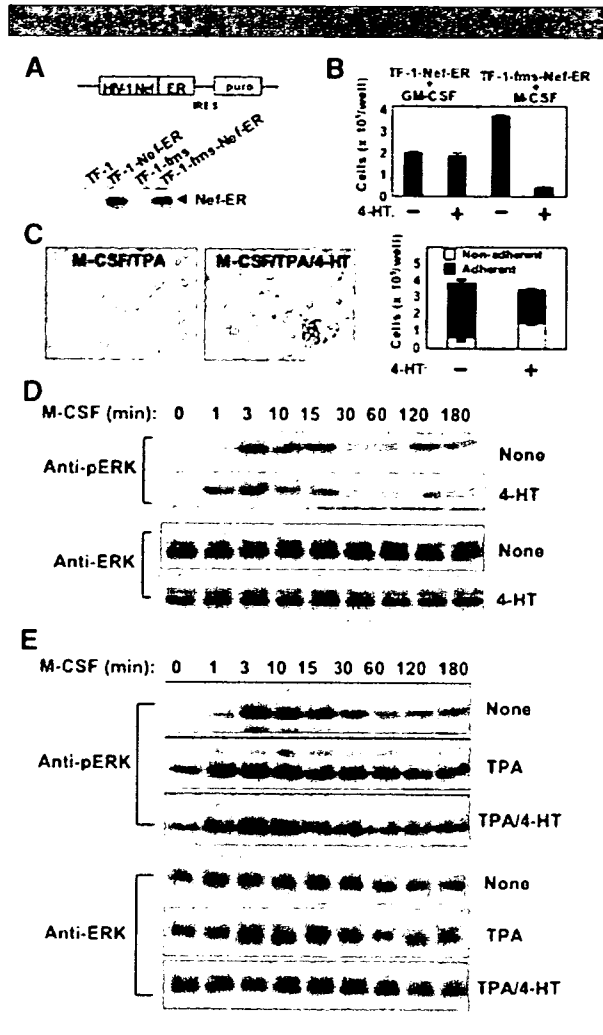


Fig. 5. Establishment of cells expressing the conditionally active Nef, proliferative/differentiative properties, and ERK activation. **A:** Schematic diagram of the Nef-ER-IRES-puro construct. ER, estrogen receptor hormone-binding domain; IRES, internal ribosomal entry sequence; puro, puromycin resistance gene. Total cell lysates from parental cells (TF-1 and TF-1-fms) or cells stably expressing Nef-ER (TF-1-Nef-ER and TF-1-fms-Nef-ER) were analyzed for the expression of Nef-ER by Western blotting with anti-Nef antibody. **B:** TF-1-Nef-ER cells were seeded at a density of 2×10^4 cells/ml and cultured in the presence of GM-CSF alone or GM-CSF plus 4-HT for 4 days. Similarly, TF-1-fms-Nef-ER cells were seeded at a density of 1×10^4 cells/ml and cultured in the presence of M-CSF alone or M-CSF plus 4-HT for 4 days. After the cultures, viable cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. **C:** TF-1-fms-Nef-ER cells were suspended in medium containing M-CSF and TPA, and then cultured for 2 days in the absence (M-CSF/TPA) or the presence of 4-HT (M-CSF/TPA/4-HT). The cells adhering to the dishes and non-adherent cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. **D:** TF-1-fms-Nef-ER cells were deprived of M-CSF for 14 h and then re-stimulated with M-CSF for the indicated periods. 4-HT was added to the culture at the beginning of the period of deprivation. Total cell lysates were prepared and analyzed by Western blotting using the antibody specific for phosphorylated ERK (upper part) or anti-total ERK antibody (lower part). **E:** As in D, TF-1-fms-Nef-ER cells were deprived of M-CSF for 14 h and then re-stimulated with M-CSF for the indicated periods. TPA or 4-HT was added to the culture at the beginning of the period of deprivation, as indicated. Total cell lysates were analyzed by Western blotting using the antibody specific for phosphorylated ERK (upper part) or anti-total ERK antibody (lower part). The result is representative of two independent experiments.

showed a biphasic ERK activation following M-CSF treatment. The early phase occurred between 1 and 3 min whereas the late phase started after 120 min (Fig. 5D). In contrast, the Nef-active cells whose proliferation rate was low showed an earlier but transient ERK activation following M-CSF stimulation (Fig. 5D). On the other hand, TPA-pretreated cells showed an increased and prolonged activation following M-CSF treatment when compared to the untreated control cells (Fig. 5E). Of importance, however, the Nef-active TPA-pretreated cells showed a transient ERK activation (Fig. 5E). These results suggested that the increased and prolonged activation of ERK correlated well with M-CSF/TPA-mediated differentiation, but not with M-CSF-mediated proliferation, of TF-1-fms cells.

Discussion

We established a new macrophage differentiation-inducing system that was dependent on M-CSF activity. TF-1-fms cells definitely switched their responsiveness to M-CSF from proliferation to differentiation in the presence of TPA (Fig. 1). Although the treatment with TPA alone triggered the macrophage differentiation of the cells, the presence of M-CSF enhanced the process: (1) the combination of both M-CSF and TPA caused more drastic morphological changes (Figs. 1C and D); (2) the culture in the presence of both M-CSF and TPA contained more adherent cells than that in the presence of TPA alone (Fig. 2A, upper part); (3) the phagocytic activity and the expression of CD204 were significantly higher in cells treated with M-CSF and TPA than those treated with TPA alone (Fig. 1E). The macrophage differentiation by M-CSF and TPA did not reflect the survival-enhancing/anti-apoptotic function of M-CSF (Fig. 2A, lower part and B).

The experiments with the pharmacological inhibitor U0126 showed that M-CSF/TPA-mediated differentiation of TF-1-fms was dependent on ERK activation (Fig. 3B). That the Src inhibitor PP2 was also a potent inhibitor of the responses (Fig. 3B) might reflect the finding that the activation of ERK by M-CSF was in part dependent on the activity of Src kinases (Cheng et al., 1999; McMahon et al., 2001). However, the involvement of the increased and/or prolonged ERK activation in M-CSF-mediated differentiation is somewhat controversial. Unlike murine M1 cells expressing wild-type Fms, the cells expressing Y559F mutant Fms showed an impaired differentiative response to M-CSF and a reduced level of ERK activation (McMahon et al., 2001). The enforced expression of a scaffolding protein, Gab2, in wild-type Fms-expressing FDC-PI cells resulted in an acceleration of the differentiation process and increased ERK activation (Liu et al., 2001). In contrast, the enforced expression of an adapter protein, Mona, in wild-type Fms-expressing FDC-PI cells resulted in increased and prolonged ERK activation, but not an acceleration of M-CSF-mediated differentiation (Bourgin et al., 2000). Moreover, 32D myeloid cells expressing the Y559F mutant Fms showed a "hyper-proliferative" response to M-CSF and prolonged ERK activation (Rohde et al., 2004). Our studies with parental and Nef-expressing TF-1-fms cells (Figs. 4 and 5) supported the former idea that increased and prolonged ERK activation led to M-CSF-mediated macrophage differentiation, but not to cell proliferation. The TPA-pretreated parental TF-1-fms cells (differentiative) showed increased and prolonged ERK activation following M-CSF stimulation (Fig. 4). In contrast, the Nef-active TPA-pretreated TF-1-fms (un-differentiative) showed transient ERK activation (Figs. 5C and E). The pharmacological agent GF109203X, a potent inhibitor of the expression of MAPK phosphatase-1 (Vallador et al., 1999), caused TF-1-fms cells to differentiate in the presence of M-CSF and the cells pretreated with GF109203X showed a sustained ERK activation following M-CSF treatment (data not shown), further supporting the idea.

The molecular mechanisms whereby Nef inhibited the M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation of TF-1-fms cells remained to be elucidated. In TF-1-fms cells, Nef activation induced the activation of Hck and its constitutive association with Fms (Suzu et al., 2005). The unphysiological behavior of Hck might explain the inhibitory effect of Nef on the responsiveness to M-CSF. Yet, under the proliferation-inducing conditions (TPA-free), the activation of Nef resulted in an earlier ERK activation (Fig. 5D). This might be explained by the finding that Nef induced ERK activation in CD4⁺ T cells (Schrager et al., 2002) and podocytes (He et al., 2004) in a Src-dependent manner. Further experiments are required to understand the molecular mechanisms whereby Nef rapidly terminated ERK activation in the presence of TPA (Fig. 5E). In summary, we showed that M-CSF-mediated macrophage differentiation, but not proliferation was correlated with increased and prolonged ERK activation, by using a newly established macrophage-inducing system. The culture system with Nef-expressing TF-1-fms cells provides a useful tool for determining the temporal regulatory mechanism of ERK activation and its contribution to M-CSF-mediated proliferation/differentiation.

Acknowledgments

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Immunity to infection

Proliferative activation up-regulates expression of CD4 and HIV-1 co-receptors on NK cells and induces their infection with HIV-1

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NK cells play important roles in immune surveillance against malignancy and virus-infected cells. NK cell functions are affected in patients infected with HIV-1; however, whether there is direct interaction between NK cells and HIV-1 remains controversial. In this study the expression of CD4, an important receptor for HIV-1, was up-regulated on NK cells co-cultured with an NK cell-selective stimulating cell line, HFWT, and rIL-2. Although the level of CD4 was lower on NK cells than on CD4⁺ T cells, expression of the HIV-1 co-receptor CCR5 was clearly up-regulated on CD4⁺ NK cells. CD4⁺ NK cells expressed higher levels of HLA-DR and CD25 than CD4⁻ NK cells, suggesting that they were highly activated. Cell-free HIV-1 could not infect the NK cells, but NK cells were infected when co-cultured with HIV-1-infected T cells. Using this co-culture system, we can better understand how HIV-1 infects NK cells and how NK cell functions are affected in AIDS.

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Introduction

NK cells play critical roles in immune surveillance without deliberate prior sensitization or restriction by MHC. Human NK cells can be divided into CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ populations: the former are highly cytotoxic, while the latter produce large amounts of cytokines. The cytotoxicity of NK cells is regulated by various activating and inhibitory receptors at the cell surface [1]. In AIDS patients, numbers and function of NK cells are altered. For example, in viremic HIV-1 patients, expression levels of

inhibitory receptors are increased. Expression levels of activation receptors, antibody-dependent cellular cytotoxicity (ADCC), production of anti-HIV-1 CC-chemokines, and maturation of DC by NK cells are all affected by HIV infection [2]. However, because CD4, a key receptor for HIV-1 infection, is generally not found on peripheral blood (PB) NK cells, it is difficult to clarify the direct interaction between HIV-1 and NK cells. At present, HIV-1 infection of NK cells remains a controversial issue. Valentin *et al.* demonstrated that HIV-1-infected NK cells constitute a long-living viral reservoir in AIDS patients [3]. In contrast, Mavilio *et al.* reported that HIV-1 proviral DNA was not detected in freshly isolated blood NK cells from viremic patients, although the phenotype and function of the NK cells were dramatically changed [4]. Therefore, it is thought that one approach to revealing NK cell abnormality in AIDS is to find evidence of HIV-1 infection of NK cells.

CD4 is generally expressed on helper T cells and monocytes/macrophages as well as on subsets of leukocytes such as dendritic cells [5], $\gamma\delta$ T cells [6],

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Abbreviations: APC: allophycocyanin · CB: cord blood ·

CBMC: cord blood mononuclear cell · PB: peripheral blood