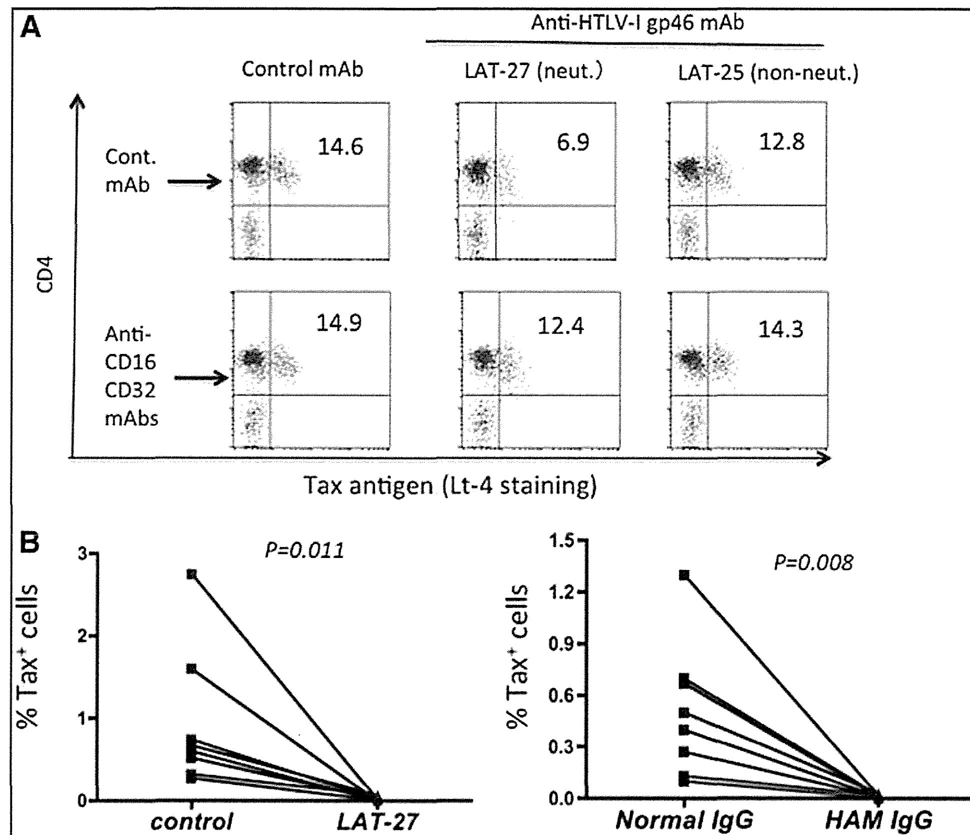

FIG. 4. Reduction of Tax-expressing cells in *in vitro* cultures of PBMCs from HAM patients in the presence of HTLV-1 neutralizing monoclonal antibody (mAb). (**A**) PBMCs from HAM patients were depleted of $CD8^+$ T cells and cultured *in vitro* for 24 h at 1×10^6 cells/ml in interleukin (IL)-2-containing medium in the presence or absence of 10 μg/ml antibodies indicated in the figure. The cells were then stained for cell surface CD4 and intracellular Tax antigen as described in the Materials and Methods section. The numbers in each dot-plot show the percentage of $CD4^+$ Tax^+ cells. The mixture of antibodies against human Fc receptors (FcR) (anti-CD16 and CD32) was added to block FcR function. Data shown are representative of three independent experiments using PBMCs from different donors. (**B**) PBMCs from HAM patients ($n = 8$) were depleted of $CD8^+$ T cells and cultured *in vitro* in IL-2-containing medium in the presence of (1) LAT-27 or an isotype control mAb at 10 μg/ml or (2) HAM-IgG or normal human IG at 100 μg/ml for 2 weeks. The cells were stained for Tax antigen and the total percentage of Tax^+ cells was calculated. The control used for LAT-27 was an isotype control (rat IgG2b anti-HCV mAb). The negative control mAb for anti-CD16 and CD32 was mouse IgG1 against HIV-1 (clone 2C2).

$CD4^+$ T cells but not normal PBMCs. However, no detectable reduction of Tax^+ cells was observed in the cultures treated with either LAT-27 or HAM-IgG cocultured in the presence of PBMCs (data not shown). Thus, these cells were washed and cocultured again for an additional 3 days with the same antibodies and fresh PBMCs.

As shown in Fig. 6A, although fresh PBMCs alone reduced the frequency of Tax^+ cells to some extent, a marked net reduction was seen in the presence of LAT-27 and HAM-IgG. In a similar fashion, the production of HTLV-1 p24 in the culture supernatants was markedly reduced by LAT-27 and HAM-IgG in the presence of autologous PBMCs. As shown in Fig. 6B, when these cultures were exposed one more time to the same antibodies and fresh PBMCs, LAT-27 IgG and HAM-IgG, but not $F(ab')_2$ of LAT-27 or normal IgG, further reduced the frequency of Tax^+ cells. These data suggest that the addition of LAT-27 as well as HAM-IgG eliminates the HTLV-1 gp46 antigen-expressing cells via an FcR-dependent manner while blocking the spread of HTLV-1 to new target cells including fresh PBMCs in the same cell cultures *in vitro*. The involvement of complement-dependent cytotoxicity was ruled out because the fetal calf serum used in the present study was heat inactivated prior to use.

ADCC against HTLV-1-infected cells by LAT-27

To examine whether LAT-27 could mediate ADCC in the present culture conditions, IL-2-dependent HTLV-1-infected T cells established from normal donors were labeled with ^{51}Cr and cocultured with fresh autologous PBMCs in the presence or absence of antibodies. Significant ADCC activity was induced by HAM-IgG, but not LAT-27, by 6 h (data not shown). However, after 24 h at a high effector-to-target cell ratio, LAT-27, but not the $F(ab')_2$ fragment of LAT-27, showed significant cytotoxicity ($p < 0.01$) (Fig. 7A). When the effector PBMCs were depleted of either $CD16^+$ or $CD56^+$ cells, but not $CD14^+$ or $CD19^+$ cells, the ADCC activity mediated by either LAT-27 or HAM-IgG was significantly reduced ($p < 0.01$) (Fig. 7B and C). These data suggest that the $CD16^+$ $CD56^+$ subpopulation of PBMCs [representing natural killer (NK) cells] were most likely the main effector cells involved in the cell lysis. These results

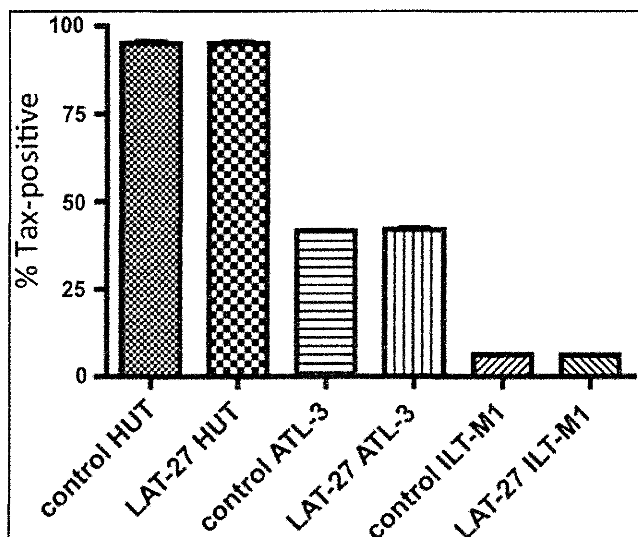

FIG. 5. LAT-27 alone does not affect long-term cultured HTLV-1-infected T cells. A standard HTLV-1-infected cell line HUT-102 (HUT), an IL-2-dependent CD4^{+} T cell line (ATL-3, generated from an ATL patient), and an IL-2-dependent CD8^{+} T cell line (ILT-M1) were cultured in the presence of 10 μg/ml of either LAT-27 or isotype control (control) for 4 days, and the frequencies of Tax^{+} cells were determined by flow cytometry ($n=3$).

demonstrate that the monoclonal LAT-27, similar to the polyclonal HAM-IgG, is able to induce ADCC against HTLV-1-infected cells by autologous NK cells while protecting the spread of new infection with HTLV-1.

Discussion

The present study demonstrates that the monoclonal anti-HTLV-1 gp46 antibody clone LAT-27 generated by our laboratory mediates both HTLV-1 neutralization and HTLV-1-specific ADCC, and such ADCC activity might be capable of eliminating HTLV-1-infected T cells *in vitro* in the presence of autologous fresh PBMCs. Although fresh PBMCs alone showed a partial but significant inhibitory activity against HTLV-1-infected cells during prolonged *in vitro* cultivation, the data obtained here suggest that the HTLV-1-specific ADCC activity is the direct mechanism for this eradication. Similar suppressive activities were demonstrated for human IgG from HAM patients. This mechanism may explain the previous findings reported by Tochikura *et al.*[28] on the HTLV-1 suppressing activity of human anti-HTLV-1 antibodies. Furthermore, this mechanism may also explain in part why HTLV-1 antigen-expressing cells are not found *in vivo* in anti-HTLV-1 antibody-positive individuals. Although it is not known where and when HTLV-1 is produced *in vivo* in the infected individual, the continued presence of CD8^{+} T cells and antibodies specific for HTLV-1 indicates that HTLV-1 should be expressed periodically. Based on the results presented in this article, it might be possible that HTLV-1 expression occurs upon T cell stimulation in the periphery, but as soon as the cells express HTLV-1 gp46 antigen they might be instantly killed by the combination of anti-HTLV-1 ADCC-inducing antibodies and activated NK cells.

We submit that the addition of fresh PBMCs to the autologous HTLV-1-producing T cell cultures may result in it becoming readily infected and immortalized by HTLV-1. Thus, it is clear that the presence of neutralizing antibody is essential for the prevention of new infection of PBMCs and since ADCC effector mechanisms are functional during this time period, their contribution to the control of infection deserves merit. Interestingly, the ADCC induced by LAT-27 progressed slowly and the elimination of Tax^{+} cells became evident only after two consecutive exposures every 3 days in the present cell culture conditions. Since there was heterogeneity of the intensity of gp46 expression among cells in a single HTLV-1-infected cell line (data not shown), the findings suggest that the lysis of such gp46^{low} cells by ADCC requires a prolonged incubation period. Alternatively, since the repeated exposure against PBMCs resulted in an accumulation of live PBMCs, it is possible that a large number of effector fresh PBMCs might be required for the complete eradication by LAT-27, possibly due to the relatively low affinity of LAT-27 for human FcR.

Cell depletion experiments in the present study showed that the effector cells involved in the HTLV-1-specific ADCC in fresh PBMCs were either CD16^{+} or CD56^{+} cells, representing the cytolytic human NK cell subset, although it remains to be confirmed with purified NK cells. Because there are abundant circulating NK cells in the periphery in healthy donors, these findings strongly suggest that the HTLV-1-specific ADCC responses in the presence of neutralizing antibodies might have a role in controlling HTLV-1 *in vivo* in concert with HTLV-1-sepecific CTL responses in healthy HTLV-1 carriers. This view is supported by the findings that the ADCC effector function of PBMCs is lower in both HAM/TSP and ATL patients than healthy HTLV-1 carriers or normal donors,[17,40] suggesting that defects in functional ADCC activities may contribute to the onset of HTLV-1-related diseases.

The level of ADCC of HTLV-1^{+} cells by LAT-27 was weaker than that induced by human polyclonal anti-HTLV-1 IgG. This might be due to the fact that LAT-27 is of rat origin and recognizes a single epitope on the gp46 (amino acids 191–196)[29] in contrast to the fact that HAM-IgG is of human origin and consists of high titers of polyclonal antibodies against multiple epitopes on gp46. In addition, it has been shown that mouse and rat IgG exhibit different ADCC activities with human NK cells depending on their subclasses, and that rat IgG2b (the subclass of LAT-27), but not IgG2a, triggers effective ADCC with human NK cells.[41] Along these lines, it is possible that a humanized form of LAT-27 utilizing the human IgG1- or IgG3-Fc portion as a backbone would be far more effective than even the rat IgG2b of LAT-27.

This hypothesis has been confirmed by preliminary experiments using humanized LAT-27 consisting of human IgG1, which was generated in collaboration with Dr. Shimizu of IBL Inc. (Tanaka *et al.*, unpublished observations). In addition, epitope specificity and/or the affinity of anti-gp46 antibodies may also be involved in determining the ADCC-inducing activities. For example, LAT-25, which belongs to the rat IgG2b subclass and recognizes a C-terminal region of the gp46, did not eradicate HTLV-1^{+} cells (Fig. 7). Similarly, Kuroki *et al.* showed that a human mAb recognizing gp46 amino acids 191–196 (similar to the epitope recognized by LAT-27) could induce ADCC, but another human mAb

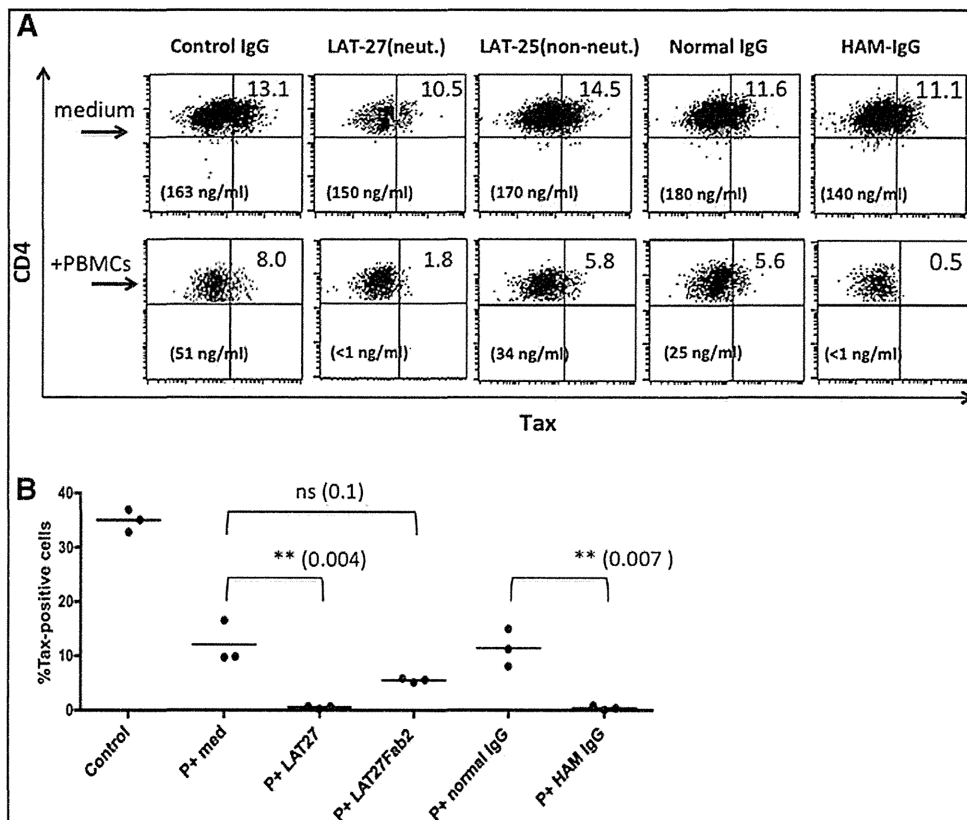

FIG. 6. Elimination of Tax$^+$ cells and reduction of HTLV-1 p24 production in IL-2-dependent HTLV-1-infected T cells cocultured with autologous PBMCs in the presence of LAT-27 or HAM-IgG. **(A)** IL-2-depnedent CD4$^+$ HTLV-1-infected T cells established from the PBMCs of normal donors were repeatedly exposed to autologous PBMCs (+PBMCs) in the presence of 10 μg/ml of LAT-27 or isotype control, or 100 μg/ml of HAM-IgG, or normal human IgG twice at 3 day intervals. Two days after the second exposure, the high forward and side scatter gated populations of cells that contained a majority of the HTLV-1$^+$ cells but not PBMCs were analyzed for the frequencies of CD4$^+$ Tax$^+$ cells. Percentages of CD4$^+$ Tax$^+$ cells are shown in the upper right quadrant. The numbers in parentheses show the levels of HTLV-1 p24 produced in the culture supernatants. Data shown are representative of three independent experiments using PBMCs from different donors. **(B)** As shown in **(A)**, IL-2-dependent CD4$^+$ HTLV-1-infected T cells were cultured *in vitro* either alone (control) or exposed to autologous PBMCs (P+) in the presence of 10 μg/ml of LAT-27 or F(ab′)$_2$ LAT-27, or 100 μg/ml of normal human or HAM-IgG in triplicate wells with three supplementations provided at 3 day intervals. Two days after the third exposure, the cells were examined for the frequencies of CD4$^+$ Tax$^+$ cells. Data shown are representative of three independent experiments using HTLV-1-infected cells and PBMCs from different donors.

recognizing the gp46 amino acids 187–193 could not, even though the two mAbs bind similarly to the cell surface of HTLV-1-infected cells and belong to the ADCC-inducing human IgG1.[22]

It remains to be determined whether there are clonal populations of human IgGs that can mediate both the neutralization and ADCC against HTLV-1. So far, it has been shown that the two activities could be operating separately by different epitope-specific human mAbs against gp46.[22] Recently, Kuo *et al.*[24] showed that both neutralizing and non-neutralizing mouse anti-gp46 mAbs can activate neutrophils and mediate its burst activity in the presence of an HTLV-1-infected MT-2 cell line, and concluded that HTLV-1-specific ADCC capacity is not coupled to the neutralizing capacity of the antibody. Thus, these articles highlight the finding of LAT-27 as a special antibody. Analyses of the conformational and antigenic structure of gp46 expressed on the cell surface will be necessary to address this issue further.

Another possible target for ADCC on HTLV-1-expressing cells is the envelope gp21; however, it has been unclear whether human anti-gp21 antibodies function in ADCC. In addition, the recent finding that the glycosylation of Fc-IgG plays an important role in anti-HIV-1 ADCC effector mechanisms[42] suggests that this issue needs to also be considered in the evaluation of anti-HTLV-1 gp46 antibodies and for vaccine formulations in general. Nevertheless, it is clear that the simultaneous operation of neutralization and ADCC by single or polyclonal antibodies is essential to recognize and eliminate HTLV-1$^+$ cells since not only T cells but also the NK cells are permissive to HTLV-1 infection.[43]

The present study also showed that fresh PBMCs had a partial and significant but not complete suppressive activity against autologous HTLV-1-infected cells in the absence of anti-HTLV-1 antibodies. Our preliminary experiments indicate that monocytes might be involved in this partial suppression because PBMCs depleted of CD14$^+$ cells, but not of NK cells, were no longer suppressive in the absence of LAT-27 (data not shown). Since HTLV-1-infected T cells are continuously activated due to the Tax antigen, one possible mechanism is a monocyte-dependent cell death (MDCD)

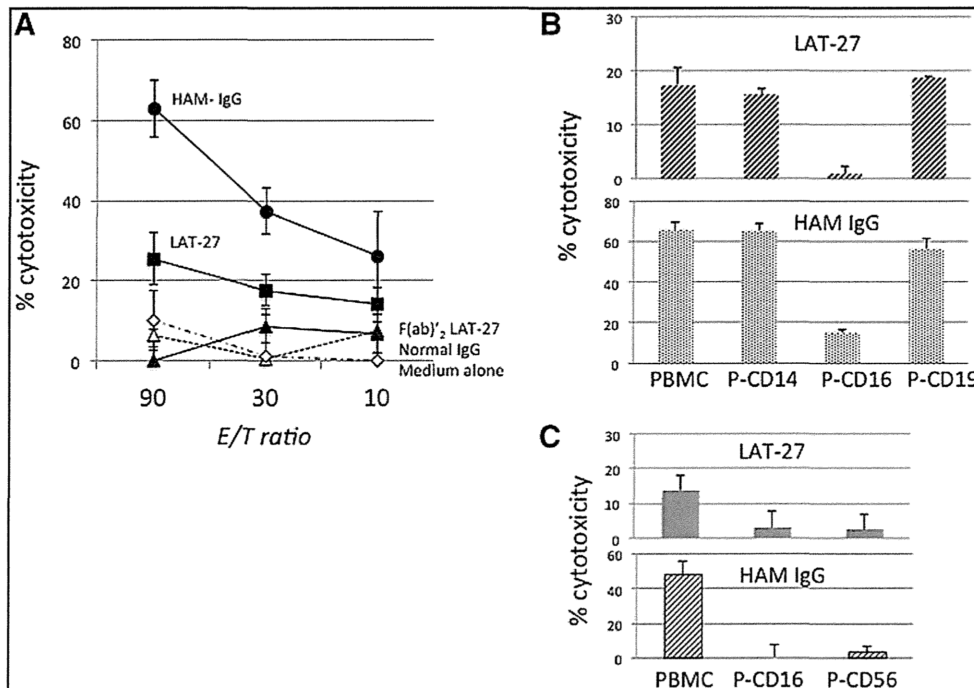

FIG. 7. The CD16$^+$ CD56$^+$ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. **(A)** ^{51}Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27 or F(ab′)$_2$ LAT-27, or 100 μg/ml of normal human or HAM-IgG for 24 h. Each coculture was performed in triplicate, and the amount of radioactivity in the culture supernatants was determined. Data shown are representative of three independent experiments. **(B, C)** Effector PBMCs before or after depletion of CD14$^+$, CD16$^+$, CD19$^+$, or CD56$^+$ cells were assayed for ADCC activity against autologous HTLV-1-infected cells in the presence of LAT-27 (10 μg/ml) or HAM-IgG (100 μg/ml) in triplicate wells in the 24 h ^{51}Cr-release assay. Data shown are representative of two independent experiments.

against activated autologous T cells.[44] Further studies are in progress to address this mechanism.

Based on the data presented herein, it is suggested that humanized LAT-27 mAb might have potential as a passive vaccine against HTLV-1 infection for HTLV-1-uninfected individuals at high risk of HTLV-1 infection, including babies born to HTLV-1 carriers and drug abusers who are also at high risk of HIV infection, and for HTLV-1 carriers whose anti-HTLV-1 neutralizing and ADCC-inducing antibody titers are low. One concern is the potential interference of LAT-27 activity by other nonneutralizing or non-ADCC-inducing antibodies that may interfere with the binding of LAT-27 to gp46. We have performed some experiments and obtained data showing that LAT-12, which blocked the binding of LAT-27 to HTLV-1-infected cells, did not interfere with either LAT-27-mediated syncytium blocking[29] and/or the eradication of HTLV-1-infected cells with autologous PBMCs (Supplementary Fig. S4). It seems likely that the binding affinities of neutralizing antibodies to gp46 expressed on actively living cells are higher than those of nonneutralizing antibodies. Thus, validation of humanized LAT-27 in animal models is currently one of our objectives.

Acknowledgments

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Y.Tak. and A.H. carried out the ADCC assays. R.T. and A.K. produced, purified, labeled monoclonal antibodies, confirmed their specificities, and made in-house EILSA for p24. M.S. participated in the determination of proviral loads and performed the statistical analysis. M.K. established HTLV-1-infected cells from patients and participated in the design of the study. A.A.A. participated in the design of the study and helped to draft the manuscript. Y.T. conceived the study, participated in its design and coordination, carried out the coculture assays, and drafted the manuscript. All authors read and approved the final manuscript.

Author Disclosure Statement

No competing financial interests exist.

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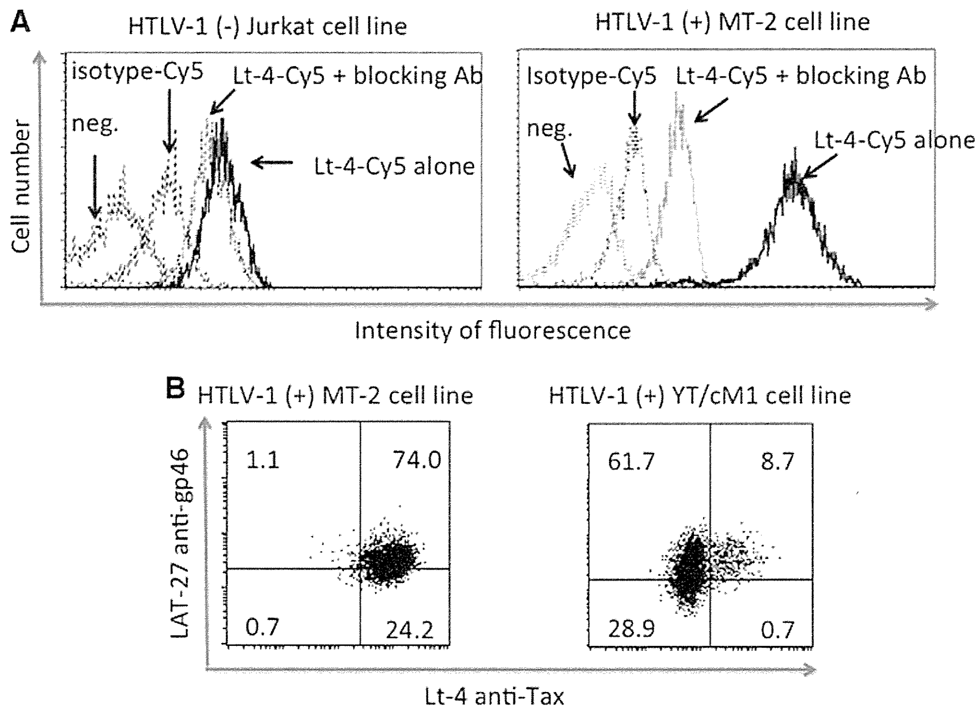
Supplementary Data

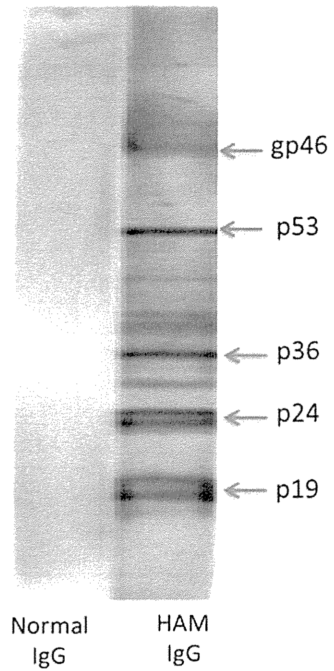


SUPPLEMENTARY FIG. S1. Flow cytometry of Tax and HTLV-I gp46 antigens. **(A)** Tax-specific and nonspecific staining by Cy5-labeled Lt-4. HTLV-I-negative Jurkat cells and HTLV-I-positive MT-2 cells were stained with either Cy5-labeled Lt-4 or Cy5-labeled mouse isotype control (IgG3) in the presence or absence of a 500 times excess of nonlabeled Lt-4 (blocking Ab). **(B)** Typical dual staining of MT-2 and another HTLV-I-immortalized T cell line (YT/cM1) with FITC-LAT-27 and Cy5-Lt-4. Negative controls for the two mAbs were obtained from cells stained in the presence of a 500 times excess of nonlabeled homologous blocking mAbs as explained above.

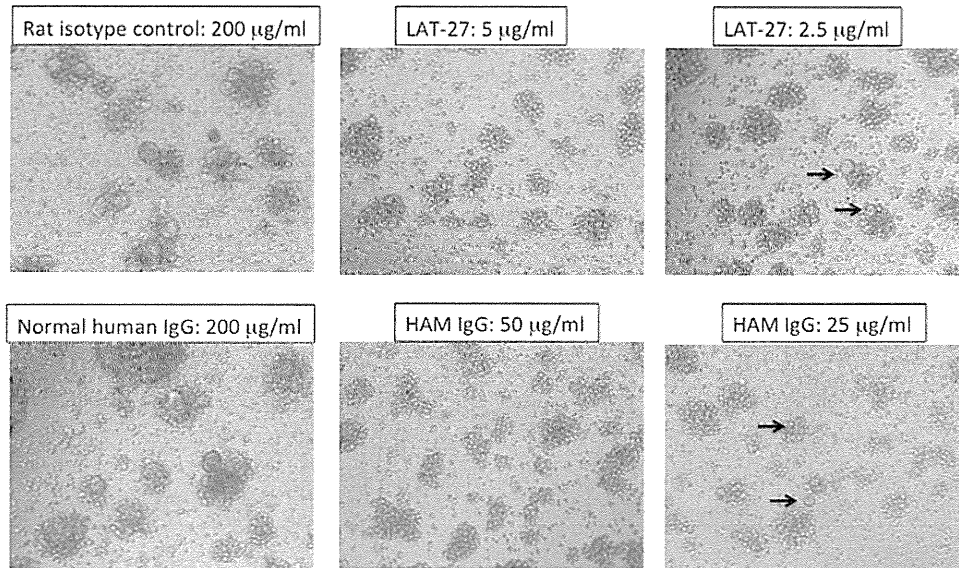
A HTLV-I-coated gelatin particle agglutination assay (SERODIA®HTLV-I)

Sample (1 mg/ml)	Titer
Normal-IgG	< 8 x
HAM-IgG	4,096 x

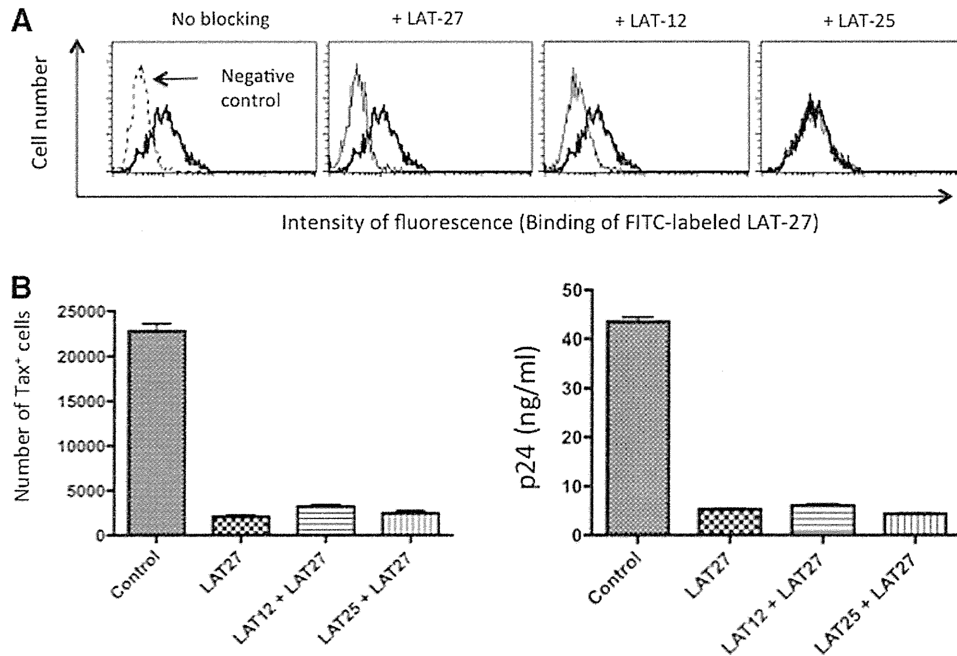
B Western blot analysis (PROBLOT HTLV-I)



SUPPLEMENTARY FIG. S2. Characterization of anti-HTLV-I antibody profile of HAM-IgG. (**A**) Purified HAM-IgG at 1 mg/ml was serially diluted and subjected to a commercial anti-HTLV-I agglutination assay (SERODIA®HTLV-I, Fujirebio Inc.). Titers were expressed as the reciprocal dilution that showed a positive reaction. (**B**) Using a commercial anti-HTLV-I western blot assay, IgG (10 μg/ml) from pooled plasma of normal donors and HAM patients was examined for HTLV-I antibodies.



SUPPLEMENTARY FIG. S3. Titration of HTLV-I-neutralizing antibody titers of LAT-27 and HAM-IgG. Two-fold diluted IgG samples were added to the coculture of ILT-M1 and Jurkat cells, and the minimum IgG concentration required for complete blockade of syncytium formation was determined. Note that the control rat isotype (rat IgG anti-HCV) and control IgG from pooled normal human plasma did not neutralize even a 200 μg/ml (final concentration). Arrows indicate small syncytia escaped from neutralization.



SUPPLEMENTARY FIG. S4. Lack of interference by nonneutralizing anti-gp46 mAb in LAT-27 mediated HTLV-1 suppression in the presence of autologous PBMCs. **(A)** Binding of FITC-labeled LAT-27 to ILT-M1 cells in the presence of a 10 times higher concentration of competing mAb was analyzed by flow cytometry (FCM). Dotted line, binding of FITC-isotype control; thick and thin lines, bindings of FITC-LAT-27 in the absence and presence of competitors, respectively. **(B)** As shown in Fig. 6, the IL-2-dependent HTLV-1-infected $CD4^+$ T cells were exposed to autologous PBMCs with 10 μg/ml of isotype control (control) or LAT-27 in the presence or absence of 100 μg/ml of LAT-12 or LAT-25 twice at 3 day intervals. Two days after the second exposure, the absolute Tax^+ cell number/culture and HTLV-1 p24 levels produced in the culture supernatants were quantitated by FCM and ELISA, respectively. In the absence of PBMCs, the numbers of Tax^+ cells were 47,200 + 5,200, which was not affected by the addition of only LAT-12, LAT-25, or LAT-27 (data not shown) ($n = 4$).

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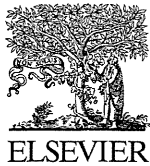

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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Protective Roles of Epithelial Cells in the Survival of Adult T-Cell Leukemia/Lymphoma Cells

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Adult T-cell leukemia/lymphoma (ATL) is a highly invasive and intractable T-cell malignancy caused by human T-cell leukemia virus-1 infection. We demonstrate herein that normal tissue–derived epithelial cells (NECs) exert protective effects on the survival of leukemic cells, which may partially account for high resistance to antileukemic therapies in patients with ATL. Viral gene–silenced, ATL-derived cell lines (ATL cells) dramatically escaped from histone deacetylase inhibitor–induced apoptosis by direct co-culture with NECs. Adhesions to NECs suppressed $p21^{Cip1}$ expression and increased a proportion of resting G0/G1 phase cells in trichostatin A (TSA)–treated ATL cells. ATL cells adhering to NECs down-regulated CD25 expression and enhanced vimentin expression, suggesting that most ATL cells acquired a quiescent state by cell-cell interactions with NECs. ATL cells adhering to NECs displayed highly elevated expression of the cancer stem cell marker CD44. Blockade of CD44 signaling diminished the NEC-conferred resistance of ATL cells to TSA-induced apoptosis. Co-culture with NECs also suppressed the expression of NKG2D ligands on TSA-treated ATL cells, resulting in decreased natural killer cell–mediated cytotoxicity. Combined evidence suggests that interactions with normal epithelial cells augment the resistance of ATL cells to TSA-induced apoptosis and facilitate immune evasion by ATL cells. *(Am J Pathol 2013, 182: 1832–1842; http://dx.doi.org/10.1016/j.ajpath.2013.01.015)*

Adult T-cell leukemia/lymphoma (ATL) is an intractable and fatal T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1).[1] After more than two decades of long-term latency, approximately 4% of HTLV-1 carriers develop ATL.[2,3] A striking feature of ATL is aggressive invasion of leukemia cells into the skin and epithelial linings of the gastrointestinal tract and lung.[4,5] Leukemia cells that have invaded the tissues are resistant to chemotherapy, presenting a major obstacle to the effective treatment of ATL.[6] Therefore, understanding the mechanisms by which tissue-infiltrating leukemia cells acquire resistance to chemotherapy is key to developing new promising treatments for patients with ATL.

HTLV-1 proteins are generally undetectable in HTLV-1–infected cells isolated from HTLV-1 carriers because of viral gene silencing. Such silencing is observed not only in asymptomatic carriers but also in patients with ATL, indicating that it allows ATL cells to evade the host immune response *in vivo*.[7,8] However, the mechanisms leading to viral gene silencing are poorly understood. Freshly isolated HTLV-1–infected cells begin to express viral genes after overnight culture *in vitro*, implying that an unknown mechanism exists to suppress viral genes *in vivo*.[9] Recently, primary ATL cells were shown to be well maintained *in vitro* by co-culture in direct contact with stromal cells.[10] Furthermore, type I interferon–induced HTLV-1 *gag* expression was suppressed in an ATL cell line when it was co-cultured with stromal cells.[11] These observations suggest that viral gene silencing occurs in ATL cells by interactions with the host microenvironment.

Epigenetic regulations, such as histone acetylation, are also assumed to be involved in viral gene silencing in ATL

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cells.[12] Thus, the *trans*-activity of the HTLV-1 long terminal repeats (LTRs) is induced by histone deacetylase (HDAC) inhibitors in HTLV-1–infected MT4 cells.[13] Also, HTLV-1 Tax expression was doubled when primary ATL cells were treated with HDAC inhibitors.[14] These observations indicate that in ATL cells cultured *in vitro*, viral gene expression is suppressed by epigenetic regulation and that HDAC inhibitors can reactivate silenced viral genes. Taken together, available evidence indicates that viral gene expression is suppressed in ATL cells by the dual action of the microenvironment of the host-tumor interface and epigenetic regulation in host cells.

Stromal fibroblasts constituting the bone marrow microenvironment prevent chemotherapy-induced apoptosis in acute myeloid leukemia and chronic lymphocytic leukemia cells.[15,16] Recently, stromal cell–derived factor-1α and its cognate receptor CXCR4 have emerged as critical mediators of leukemic/stromal cell interactions.[17] Blockade of the stromal cell–derived factor-1α–CXCR4 axis by the CXCR4 antagonist AMD3100 suppresses the migration of cultured ATL cells,[18] suggesting that this axis may also be involved in formation of the stromal niche environment in ATL.

The present study was undertaken to determine whether epithelial cells, another major component constituting the microenvironment of the host-tumor interface, affect the survival and phenotype of ATL cells. We show that co-culture with normal tissue–derived epithelial cells (NECs) increases a proportion of ATL cells in G0/G1 phase and rescues ATL cells from HDAC inhibitor–induced apoptosis. Adhesions to NECs induced prominent surface expression of CD44 on ATL cells; they also induced internalization of CD44 and nuclear translocation of cyclin D1 in a fraction of HDAC inhibitor–treated ATL cells, thus enabling such ATL cells to resume cell-cycle progression and leading to the ultimate survival of ATL cells. ATL cells co-cultured with NECs down-regulated the expression of NKG2D ligands, suggesting that this interaction also facilitates immune evasion by tumor cells.

Materials and Methods

Cells

The HTLV-1–positive ATL cell lines ATL-CR and ATL-TH were obtained from the Reference Center for HTLV Infection (Rio de Janeiro, Brazil). These cell lines were established from Brazilian patients with ATL after they provided informed consent. Briefly, peripheral blood mononuclear cells (PBMCs) isolated from patients with ATL were cultured in the presence of recombinant IL-2. After long-term culture, they acquired IL-2 independence, resulting in establishment of the ATL-CR and ATL-TH cell lines. Jurkat and HUT78, a T-cell lymphoma line derived from a patient with Sezary syndrome,[19] were used as HTLV-1–negative T-cell lymphoma cells. ATL-CR, ATL-TH, Jurkat, and HUT78 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. HEK293 and HEK293T, human embryo kidney epithelial-like cell lines, and primary normal human dermal fibroblasts were maintained in Dulbecco's minimal essential medium supplemented with 10% FBS and penicillin/streptomycin. 1C3IKEI, a primary normal human embryonic pancreas–derived epithelial-like cell line, was purchased from the RIKEN BioResource Center (Tsukuba, Japan) and maintained in Dulbecco's minimal essential medium supplemented with 15% FBS. KHYG-1, an NKG2D$^+$ natural killer (NK) cell line,[20] was purchased from the Health Science Research Resources Bank (Osaka, Japan) and was maintained in Dulbecco's minimal essential medium supplemented with 100 U of recombinant human IL-2 (Shionogi, Osaka, Japan), 10% FBS, and penicillin/streptomycin. Fresh ATL cells were obtained from patients with chronic or acute ATL after informed consent was provided. Briefly, PBMCs isolated from heparinized peripheral blood by Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) were resuspended in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin and were subjected to experiments immediately.

Antibodies, Plasmids, and Reagents

Rat anti-human/mouse CD44 antibody (clone IM7; eBioscience Inc., San Diego, CA) was used for flow cytometry (FCM) and immunofluorescence staining. Mouse anti-human MICA/B antibody (clone 6D4; eBioscience) was used for FCM. Mouse anti-human CD25 (clone BC96; eBioscience), polyclonal rabbit anti-human fibronectin (Dako, Glostrup, Denmark), mouse anti-human vimentin (clone V9; Dako), polyclonal rabbit anti-human p21^{Cip1} (eBioscience), and mouse anti-human Ki-67 (clone MIB-1; Dako) antibodies were used for immunofluorescence staining. HTLV-1 LTR luciferase plasmids have been described previously.[21] HDAC inhibitors, such as trichostatin A (TSA), valproic acid sodium salt (VPA), and sodium butyrate (NaB), were purchased from Sigma-Aldrich (St. Louis, MO).

Co-Culture

ATL cells were co-cultured with NECs using a direct co-culture system that allowed for cell-cell contact. NECs were labeled with 1 μmol/L of 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 1×10^6 cells/mL in PBS for 20 minutes at 37°C and then washed three times in PBS. CFSE-labeled NECs were used immediately for each experiment. ATL cells, 5×10^5, were directly co-cultured with CFSE-labeled NECs growing as monolayers in 24-well plates. Cell culture inserts (pore size, 0.4 μm; Invitrogen, Camarillo, CA) were used for an indirect co-culture system. ATL cells were co-cultured with CFSE-labeled NECs using the indicated concentrations of TSA. After the indicated co-culture time, whole cells in co-culture were harvested for subsequent experiments. In some experiments, nonadhering ATL cells in the supernatant were collected separately from the ATL cells adhering to the

monolayer of labeled NECs, and then the adhering ATL cells with labeled NECs were collected after rinsing twice gently with RPMI 1640 medium to remove nonadhering ATL cells. For the proliferation assay, ATL-CR cells were labeled with CFSE and then co-cultured with unlabeled HEK293T cells for 72 hours.[22]

FCM Analysis

Cell-cycle distribution of ATL cells was analyzed by DNA content using the propidium iodide (PI) staining method. Co-cultured cells were harvested after the indicated durations of TSA treatment. After fixation in 70% ethanol at −20°C overnight, cells were incubated in a PI/RNase A staining buffer (50 μg/mL of PI and 20 μg/mL of RNase A in PBS). Cells were then analyzed by FCM using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). CFSE and PI were detected in the FL-1 and FL-2 channels, respectively. Co-cultured ATL cells gated on CFSE-negative cells were acquired in 10,000 events. Expression of cell-surface molecules was analyzed using FCM. Analysis was performed using CellQuest Pro software version 6.0 (Becton Dickinson Immunocytometry Systems, San Jose, CA). Sigmoid-like dose-response curves were drawn using the logistic curve-fitting software ImageJ, version 1.46 (NIH, Bethesda, MD).[23]

Immunofluorescence Staining

Cells cultured on chamber slides were fixed with 4% paraformaldehyde for 15 minutes. For intracellular staining, cells were treated with PBS containing 0.1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) for 4 minutes and then fixed with ice-cold 70% methanol for 4 minutes. Nonspecific binding was blocked with 0.05% Tween 20 in PBS containing 0.1% goat serum for 10 minutes. After incubation with primary antibodies for each targeted protein, Alexa Fluor–conjugated goat polyclonal antibody was used as a secondary antibody. Images were acquired using an Olympus DP70 camera with its own Olympus DP controller software version 1.2.1.108 (Olympus, Tokyo, Japan). Three-dimensional analyses were reconstructed by images acquired by confocal microscopy using a laser scanning confocal microscope (FV-300; Olympus, Tokyo, Japan).

Functional Assays

Reporter genes were introduced into ATL cells directly using FuGENE transfection reagents (Roche Diagnostics, Branchburg, NJ) according to the manufacturer's instructions. Briefly, 5×10^5 ATL-CR cells were cultured in 60-mm dishes and cotransfected with 1 μg of HTLV-1 LTR firefly luciferase reporter plasmid together with 50 ng of the *Renilla* luciferase reporter pRL-TK. Eighteen hours after transfection, ATL-CR cells were washed five times in PBS and then were co-cultured with HEK293T cells in the presence of TSA in triplicate wells of 24-well plates (2×10^5 cells per well). Reporter activities were measured using the Dual-Luciferase reporter assay system (Promega, San Luis Obispo, CA). Briefly, cells were lysed in 1× passive lysis buffer, and firefly and *Renilla* luciferase activities were measured using a Turner 20/20 luminometer (Promega). Reporter activities were normalized using *Renilla* luciferase values.

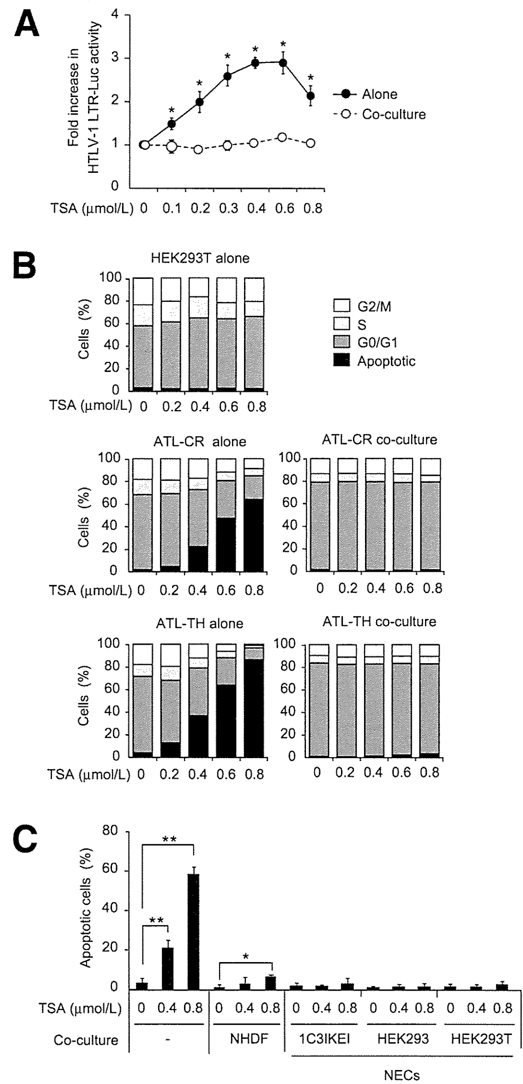

Figure 1 Direct co-culture with NECs rescued ATL cells from TSA-induced apoptosis. **A:** Transcriptional activity of HTLV-1 LTRs in TSA-treated ATL-CR cells. ATL-CR cells were cotransfected with 1 μg of HTLV-1 LTR firefly luciferase reporter plasmid together with 50 ng of pRL-TK 1 day before co-culture with HEK293T cells. After 18 hours of co-culture with the indicated concentrations of TSA, reporter activities were measured and normalized to *Renilla* luciferase values. The values indicate the means ± SD fold increases obtained by three independent experiments normalized to the untreated control sample. **B:** Cell-cycle analysis in ATL-CR and ATL-TH cells. ATL cells, 5×10^5 per well, were cultured alone or with HEK293T cells in the presence of the indicated concentrations of TSA for 48 hours. HEK293T cells were labeled with CFSE 1 day before co-culture. After the whole co-cultured cells were harvested, cell-cycle analysis was performed by FCM. **C:** Percentage of apoptotic ATL-CR cells co-cultured with normal human dermal fibroblasts (NHDFs), 1C3IKEI cells, HEK293 cells, and HEK293T cells. The data are presented as the means ± SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

Cytotoxicity Assays

Cytotoxicity assays used a combination of two dyes: CFSE was used to label effector cells (KHYG-1 cells) and HEK293T cells and PI was used to stain target cells (ATL-CR cells). CFSE-labeled effector cells were incubated with nonlabeled target cells cultured alone or co-cultured with CFSE-labeled HEK293T cells at effector to target cell ratios of 3:1, 6:1, or 9:1 in triplicate wells of 24-well plates for 18 hours. Apoptotic ATL-CR cells were evaluated by the percentage of sub G0/G1 phase cells in CFSE-negative cells as determined by FCM. Mean values were calculated from three independent experiments.

Statistical Analysis

Data were analyzed using either the Student's *t*-test or repeated-measures analysis of variance. A $P < 0.05$ was considered statistically significant.

Results

Direct Co-Culture with NECs Rescues ATL Cells from TSA-Induced Apoptosis

HTLV-1 gene expression is hardly detectable in ATL cells because of gene silencing. With the aim of eliminating viral gene silencing, we treated the IL-2—independent ATL-derived cell line ATL-CR with TSA. This treatment increased the amount of HTLV-1 p19 gag protein in the supernatants and lysates prepared from cultured ATL-CR cells (data not shown) and enhanced the transcriptional activity of HTLV-1 LTRs in a dose-dependent manner (Figure 1A), indicating that TSA effectively eliminates viral gene silencing. However, when ATL-CR cells were co-cultured with HEK293T cells (used as NECs), treatment with TSA did not increase the transcriptional activity of HTLV-1 LTRs, indicating that co-culture with NECs blocked TSA-induced viral gene reactivation (Figure 1A).

Next, we performed cell-cycle analysis to determine whether HEK293T cells affect the fate of TSA-treated ATL-CR and ATL-TH cells (Figure 1B). When ATL-CR cells were cultured alone in the presence of TSA, they displayed a dose-dependent increase in the percentage of apoptotic cells and a decrease in the percentage of G0/G1 phase cells; similar changes were observed in TSA-treated ATL-TH cells but not in TSA-treated HEK293T cells. ATL-CR and ATL-TH cells co-cultured with HEK293T cells showed no increase in the percentage of apoptotic cells; instead, the percentage of G0/G1 phase cells was increased. The ability of HEK293T cells to prevent apoptosis was evident even at an ATL-CR to HEK293T cell ratio of 25:1 (data not shown). Similar apoptosis-protective effects were also observed when ATL-CR cells were co-cultured with primary epithelial cells 1C3IKEI or primary dermal fibroblasts (Figure 1C). These results indicate that co-culture with NECs generally prevents ATL cells from TSA-induced apoptosis.

Cell-Cell Contact—Dependent Interactions with NECs Are Required to Rescue ATL Cells from HDAC Inhibitor—Induced Apoptosis

To examine whether cell-cell contact with NECs is required to rescue ATL cells from TSA-induced apoptosis, we co-cultured ATL-CR cells in direct or indirect contact with HEK293T cells in the presence of TSA. When ATL-CR cells were separated by 0.4-μm membranes from HEK293T cells, the ratio of apoptotic ATL-CR cells was lower than when they were cultured alone but showed a marked increase compared with when ATL-CR cells were cultured in direct

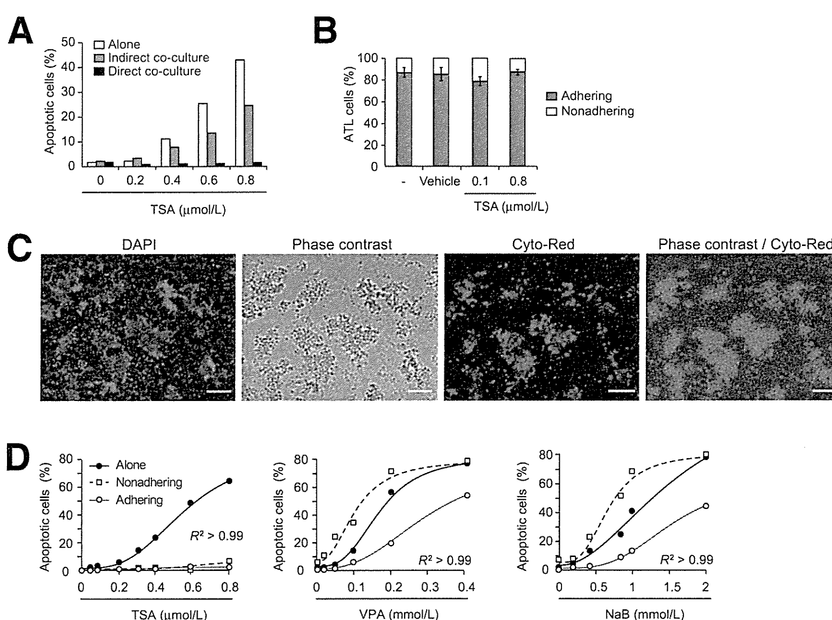

Figure 2 Cell-cell contact—dependent interactions with NECs are required to rescue ATL cells from TSA-induced apoptosis. **A:** Percentage of apoptotic ATL-CR cells. ATL-CR cells were directly co-cultured with HEK293T cells or indirectly co-cultured with HEK293T cells using cell culture inserts (pore size, 0.4 μm) in the presence of the indicated concentrations of TSA for 48 hours. **B:** Means ± SD percentage of ATL-CR cells adhering to HEK293T cells after co-culture for 24 hours. **C:** Most of the ATL-CR cells (red) showed adhesion-dependent growth on a monolayer of HEK293T cells after 24 hours of direct co-culture. Scale bars: 50 μm. **D:** Percentage of apoptotic ATL-CR cells. ATL-CR cells were co-cultured with HEK293T cells in the presence of the indicated concentrations of TSA, VPA, and NaB. After co-culture, nonadhering ATL-CR cells in the supernatant were collected separately from the ATL-CR cells adhering to the monolayer of CFSE-labeled HEK293T cells. The data are presented as the means ± SD of three independent experiments.

contact with HEK293T cells (Figure 2A), indicating that efficient suppression of apoptosis requires cell-cell contact. Approximately 84% of ATL-CR cells adhered to a monolayer of HEK293T cells by 24 hours after co-culture with HEK293T cells (Figure 2, B and C). There was no correlation between the dose of TSA and the adhesiveness of ATL-CR cells (Figure 2B), suggesting that TSA treatment did not augment the adhesive activity of ATL cells. To examine whether the extracellular matrix can substitute for NECs, ATL-CR cells were cultured in an extracellular matrix (BD Matrigel Matrix; Becton Dickinson)–coated dish in the presence of TSA. These experiments showed that extracellular matrix alone was completely ineffective in reducing TSA-induced apoptosis in ATL cells (data not shown).

We next examined whether ATL-CR cells co-cultured with HEK293T cells acquire apoptosis resistance to treatment with other HDAC inhibitors, such as VPA and NaB (Figure 2D). TSA-treated ATL-CR cells directly co-cultured with HEK293T cells acquired dramatic apoptosis resistance regardless of whether they adhered to HEK293T cells. In contrast, apoptosis resistance to VPA or NaB was acquired only in adhering ATL-CR cells, and the extent of acquired resistance was diminished. These results indicate that cell-cell contact–dependent interactions with NECs make ATL-CR cells more or less resistant to apoptosis induced by HDAC inhibitors.

Co-Culture with NECs Induces a Quiescent State in TSA-Treated ATL Cells

To examine the cellular changes that follow co-culture with NECs more closely, we analyzed cell-cycle distribution in ATL cells cultured alone, co-cultured with NECs, or co-cultured with NECs in the presence of TSA (Figure 3A). Co-culture with NECs decreased the proportion of S and G2/M phase cells and increased the proportion of G0/G1 phase cells. Co-culture in the presence of 0.8 μmol/L TSA further strengthened these changes. In contrast, TSA-treated ATL-CR cells cultured alone did not show such cell-cycle changes and underwent apoptosis (Figure 3B). Furthermore, when CFSE-labeled ATL-CR cells were cultured alone or with HEK293T cells for 72 hours, co-cultured ATL-CR cells showed less proliferation than those cultured alone, in the presence and absence of 0.8 μmol/L TSA (Figure 3C). This is consistent with the observation that co-culture with NECs induces G0/G1 accumulation in ATL-CR cells and allows them to escape from TSA-induced apoptosis (Figure 3A).

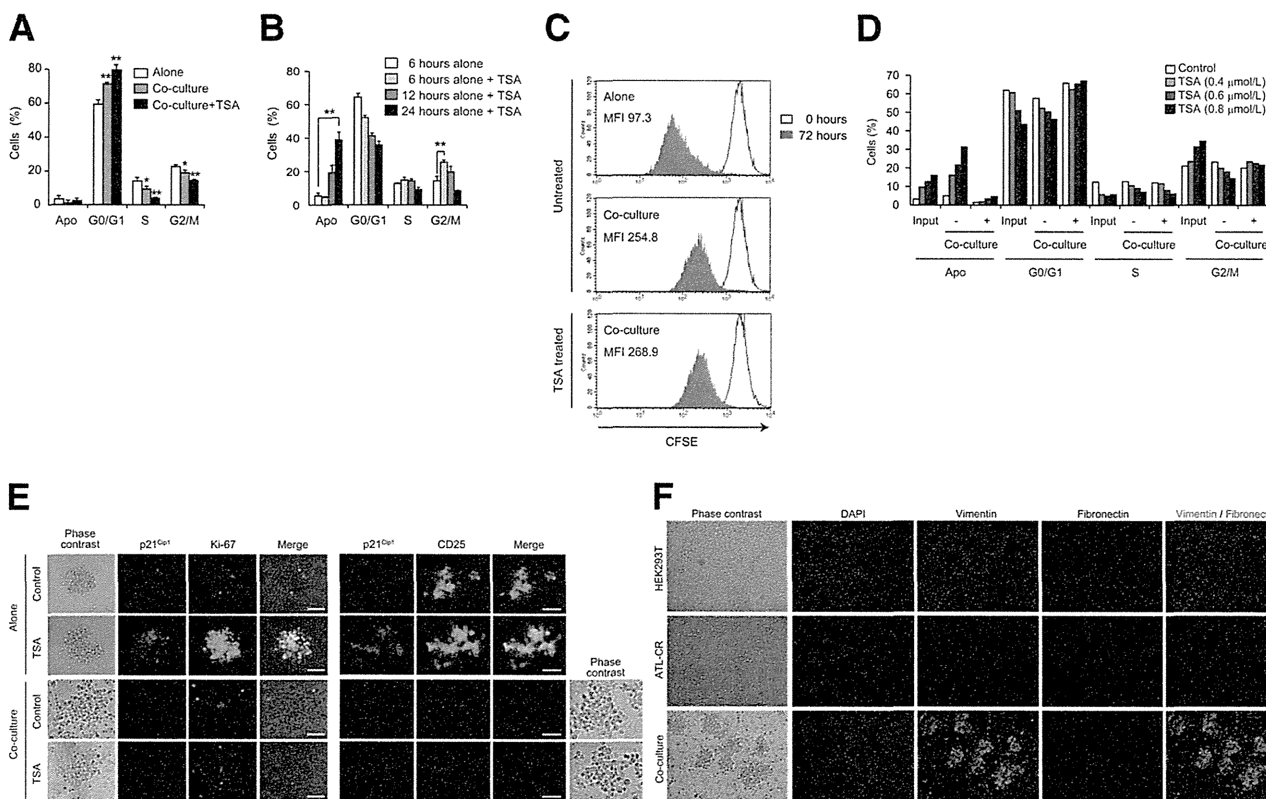

Figure 3 Co-culture with NECs induced a quiescent state in TSA-treated ATL-CR cells. **A:** Cell-cycle analysis in ATL-CR cells. ATL-CR cells co-cultured with HEK293T cells were treated with 0.8 μmol/L TSA for 24 hours. **B:** Changes in cell-cycle distribution were followed for 24 hours in ATL cells cultured alone in the presence or absence of TSA. **C:** Proliferation analysis in ATL-CR cells. CFSE-labeled ATL-CR cells were cultured alone or with HEK293T cells in the presence or absence of TSA for 72 hours. CFSE intensity was analyzed after gating on live cells. **D:** Recovery of TSA-pretreated ATL-CR cells by direct co-culture with NECs. ATL-CR cells were pretreated with the indicated concentration of TSA for 18 hours and then were cultured alone or with HEK293T cells in fresh RPMI 1640 medium for 24 hours. ATL-CR alone (input) indicates TSA-pretreated ATL-CR cells cultured alone. **E:** $p21^{Cip1}$, Ki-67, and CD25 expression in ATL-CR cells. ATL-CR cells were cultured alone or with HEK293T cells in the presence of 0.8 μmol/L TSA. **F:** Expression of vimentin and fibronectin in ATL-CR cells co-cultured with HEK293T cells. Apo, apoptotic cells. The data are presented as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$. Scale bars: 50 μm (**E** and **F**).

Next, to examine whether NECs have a protective role on ATL-CR cells pretreated with TSA, ATL-CR cells were incubated with TSA for 18 hours and then were transferred to the fresh medium free of TSA. Pretreatment of ATL-CR cells with TSA increased the proportion of G2/M phase cells in a dose-dependent manner (Figure 3D). These TSA-pretreated cells (shown as input cells) were transferred to the fresh TSA-free medium and were cultured for an additional 24 hours in the presence or absence of HEK293T cells. In the absence of HEK293T cells, the proportion of cells in the G0/G1, S, and G2/M phases continued to decline, and ATL-CR cells continued to undergo apoptosis. In contrast, the ratio of apoptotic cells did not increase appreciably in the presence of HEK293T cells. These results suggest that co-culture with HEK293T cells induced ATL-CR cells to enter the G0/G1 resting phase.

Ki-67 antigen is present during all active phases of the cell cycle (G1, S, G2, and M) but is absent from cells in resting G0 phase. When ATL-CR cells were stained with anti—Ki-67 antibody, most cells were not stained; however, when they were treated with TSA, 44.2% of the cells became positive with Ki-67 and entered the cell cycle (data not shown). TSA increases $p21^{Cip1}$ levels in cancer cells, leading to cell-cycle arrest.[24] When ATL-CR cells cultured alone were treated with TSA, they exhibited markedly induced expression of $p21^{Cip1}$ and Ki-67 and retained high expression of CD25 (Figure 3E). In contrast, when ATL-CR cells were treated with TSA and co-cultured with NECs, expression of $p21^{Cip1}$ and Ki-67 remained low, and expression of CD25 was strongly diminished (Figure 3E).

Vimentin is a mesenchymal intermediate filament supporting the structural integrity of quiescent cells while participating in adhesion, survival, growth regulation, and cell signaling processes.[25] It is a growth-regulated protein whose expression is induced in quiescent cells ≤2 hours after mitogenic stimulation, even when protein synthesis is inhibited.[26] We, therefore, examined the expression of vimentin and fibronectin, another mesenchymal marker, in ATL cells adhering to NECs. Expression of vimentin, but not fibronectin, was markedly elevated in ATL-CR cells co-cultured with HEK293T cells (Figure 3F). These results are consistent with the idea that adhesion to NECs induced cellular quiescence and resistance to TSA-induced apoptosis in ATL cells through vimentin expression.

ATL Cells Adhering to NECs Augment CD44 Expression

Cancer stem cell (CSC)—like cells often exhibit markers of epithelial-mesenchymal transition, such as vimentin.[25] Recent evidence indicates that CD44 directly reprograms stem cell properties in colon cancer cells.[27] We, therefore, examined whether the cellular quiescence achieved by co-culture with NECs was accompanied by elevated expression of CD44, a hyaluronan receptor known to be involved in cellular quiescence and now recognized as a marker of CSC-like cells (Figure 4A). When we compared cell surface expression of CD44 on ATL cells co-cultured with NECs and cultured alone in the presence or absence of TSA, CD44 expression was markedly increased in ATL-CR cells co-cultured with HEK293T cells compared with ATL cells cultured alone regardless of the presence or absence of TSA. Staining for CD44 was more pronounced in ATL-CR cells located at the upper and central regions of colonies, whereas vimentin tended to be evenly expressed on all ATL-CR cells (Figure 4, B and C). These observations suggest the involvement of CD44-mediated interactions in the induction of cellular quiescence in co-cultured ATL cells.

ATL Cells that Form Colonies on the Monolayer of NECs Show Intracellular Expression of CD44

ATL cells that formed colonies on the monolayer of NECs were more strongly positive for CD44 than were those that did not form colonies and floated in the medium (Figure 5A). To

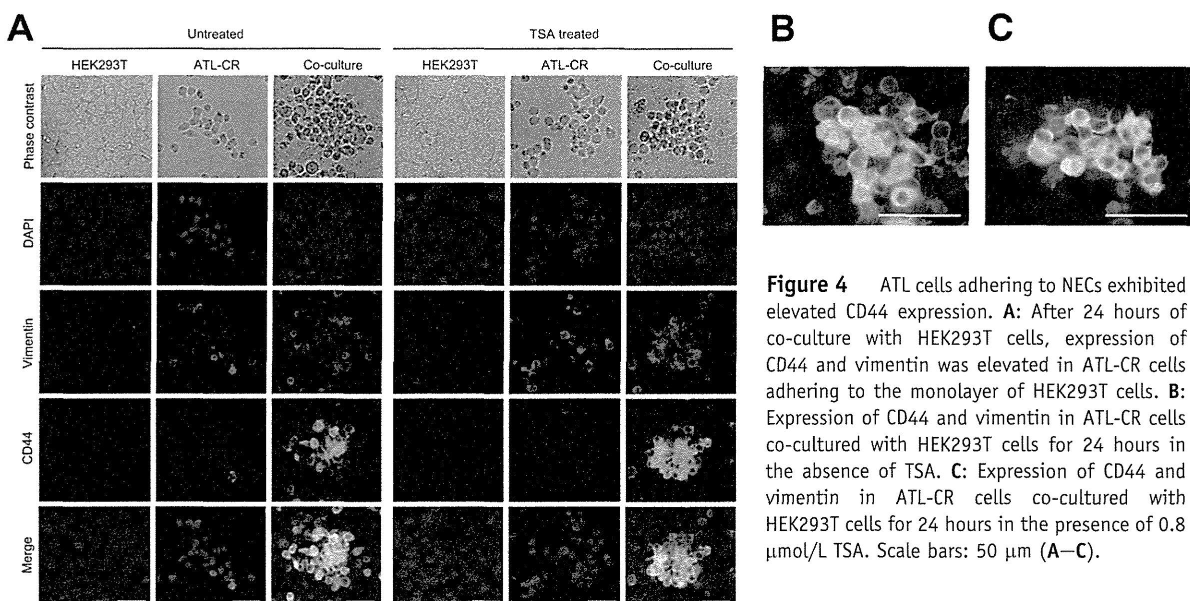

Figure 4 ATL cells adhering to NECs exhibited elevated CD44 expression. **A:** After 24 hours of co-culture with HEK293T cells, expression of CD44 and vimentin was elevated in ATL-CR cells adhering to the monolayer of HEK293T cells. **B:** Expression of CD44 and vimentin in ATL-CR cells co-cultured with HEK293T cells for 24 hours in the absence of TSA. **C:** Expression of CD44 and vimentin in ATL-CR cells co-cultured with HEK293T cells for 24 hours in the presence of 0.8 μmol/L TSA. Scale bars: 50 μm (**A—C**).

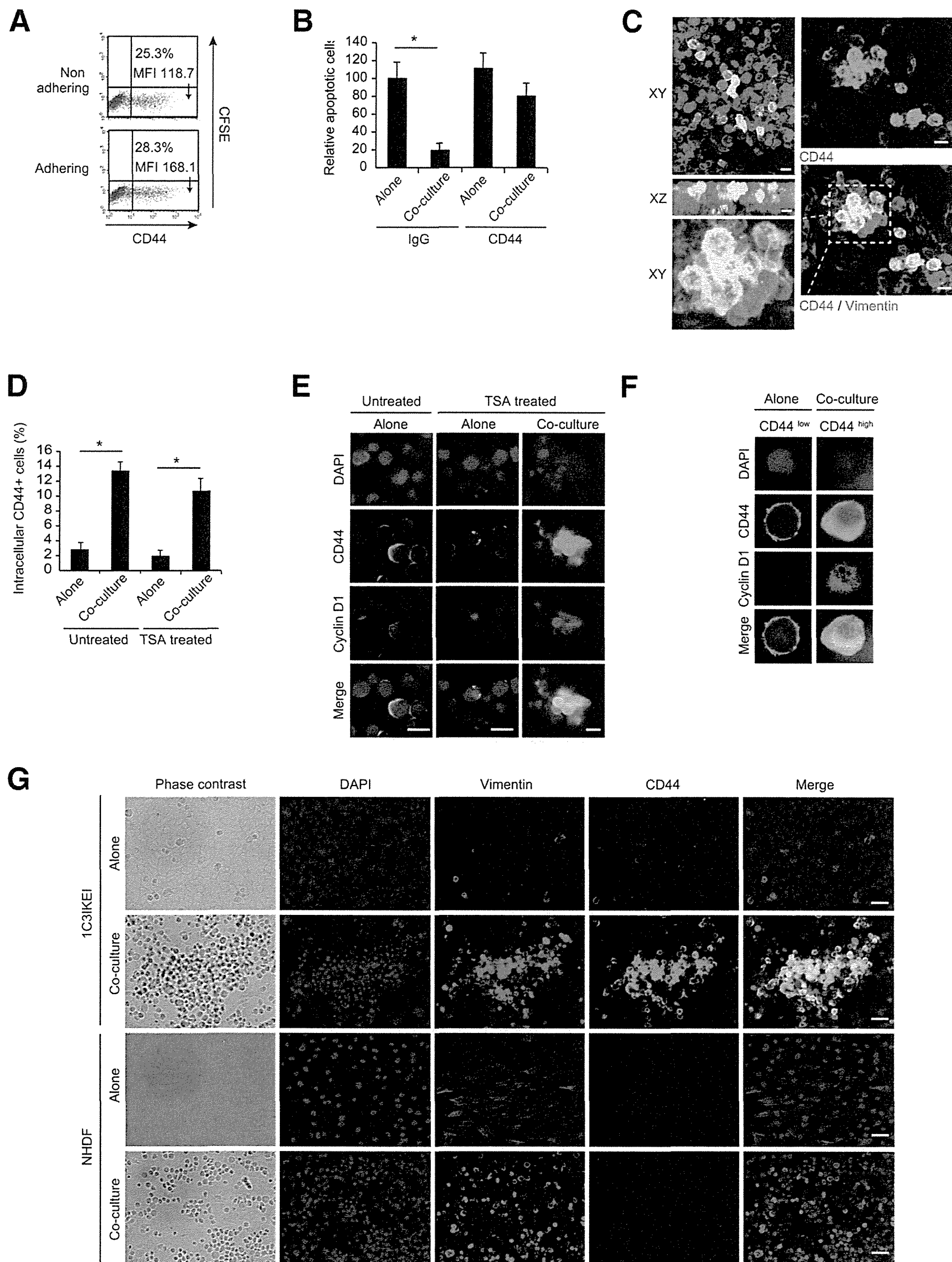

Figure 5 A fraction of ATL cells forming colonies on the monolayer of NECs showed intracellular expression of CD44. **A:** Cell surface expression of CD44 on adhering and nonadhering ATL-CR cells. ATL-CR cells were co-cultured with HEK293T cells for 24 hours. **B:** Blocking antibody for CD44 inhibited the apoptosis-protective effects conferred by co-culture with HEK293T cells. ATL-CR cells cultured alone were pretreated with anti-CD44 antibody or control IgG for 1 hour and then were cultured for an additional 24 hours with or without HEK293T cells in the presence of 0.8 μmol/L TSA. **C:** Three-dimensional analysis by confocal microscopy. Expression of CD44 was observed on ATL-CR cells that formed colonies on the monolayer of HEK293T cells. Expression was particularly pronounced on ATL-CR cells located at the upper and central regions of the colonies. **D:** Percentage of intracellular $CD44^+$ cells. **E:** CD44 and cyclin D1 expression. The TSA concentration used in **D** and **E** was 0.8 μmol/L. **F:** Nuclear localization of cyclin D1 in $CD44^+$ high cells. ATL-CR cells were cultured in the absence of TSA. **G:** CD44 and vimentin expression on ATL-CR cells co-cultured with 1C3IKEI and normal human dermal fibroblast (NHDF) cells. The data are presented as the means ± SD of three independent experiments. $^*P < 0.01$. Scale bars: 10 μm (**C** and **E**); 50 μm (**G**).

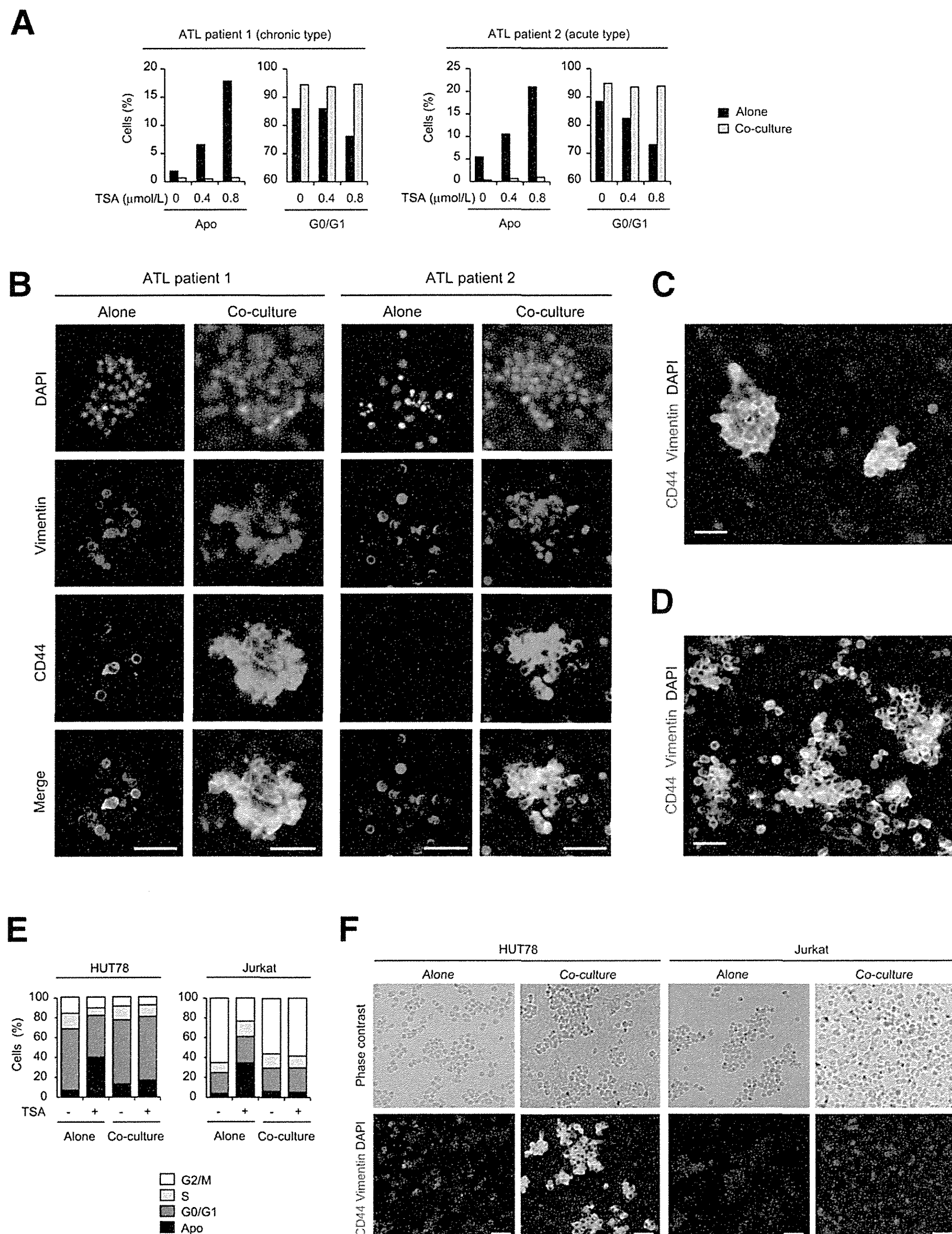

Figure 6 Fresh ATL cells adhering to NECs formed colonies, escaped from TSA-induced apoptosis, and augmented CD44 expression. **A–D:** PBMCs derived from patients with ATL with chronic type (patient 1) and acute type (patient 2) were cultured alone or with HEK293T cells with or without 0.8 μmol/L TSA, and then cell-cycle analysis and immunofluorescence staining for CD44 and vimentin were performed. **A:** Percentage of apoptotic cells (Apo) and G0/G1 phase cells. **B:** Expression of CD44 and vimentin in TSA-treated ATL cells. **C** and **D:** Expression of CD44 and vimentin in TSA-untreated ATL cells derived from patient 1 (**C**) and patient 2 (**D**). **E:** Cell-cycle analysis in HTLV-1–negative T-cell lymphoma cells. HUT78 and Jurkat cells co-cultured with HEK293T cells were treated with 0.8 μmol/L TSA for 48 hours. **F:** Expression of CD44 and vimentin was examined in HTLV-1–negative T-cell lymphoma cells co-cultured with HEK293T cells for 24 hours in the presence of 0.8 μmol/L TSA. Scale bars: 50 μm (**B–D** and **F**).

examine whether CD44 is involved in apoptosis resistance, we pretreated ATL-CR cells cultured alone with 40 μg/mL of anti-CD44 antibody or control IgG for 1 hour and then cultured for an additional 24 hours in the presence or absence of HEK293T cells (Figure 5B). Treatment with anti-CD44 antibody significantly reduced apoptosis resistance in ATL-CR cells cultured alone and co-cultured with NECs. These results indicate that elevated CD44 expression is involved in conferring apoptosis resistance on TSA-treated ATL-CR cells. Three-dimensional analysis by confocal microscopy revealed a population of ATL cells strongly positive for intracellular CD44; such cells were located on top of colony-forming, vimentin-positive ATL cells (Figure 5C). The proportion of cells showing strong intracellular staining for CD44 was significantly higher in

ATL cells co-cultured with NECs than in ATL cells cultured alone (means ± SD: 10.68% ± 1.65% for TSA-treated co-cultured ATL cells and 1.91% ± 0.47% for TSA-treated ATL cells cultured alone; $P < 0.001$) (Figure 5D).

Recently, it was reported that nuclear translocation of CD44 promotes cell proliferation through direct binding to the promoter region of the cyclin D1 gene.[28] We, therefore, examined whether cells positive for intracellular CD44 express cyclin D1. In TSA-untreated ATL cells, CD44 expression was restricted to the cell surface, with no evidence of nuclear translocation of cyclin D1 (Figure 5E). In TSA-treated ATL cells cultured alone, CD44 surface expression was low, with little detectable nuclear translocation of cyclin D1. In contrast, a fraction of ATL cells co-cultured with NECs was strongly positive for intracellular CD44 and cyclin D1 regardless of whether they were treated with TSA. In ATL cells co-cultured with NECs, nuclear translocation of cyclin D1 was induced after nuclear translocation of CD44 (Figure 5F). These results suggest that nuclear translocation of cyclin D1 enables a fraction of ATL cells to enter the cell cycle from the resting G0/G1 phase, thus accounting for the slower, yet demonstrable cell proliferation observed in Figure 3C.

We confirmed that as in co-culture with HEK293T cells, co-culture with primary epithelial 1C3IKEI cells induced expression of CD44 on a fraction of colony-forming ATL cells (Figure 5G). However, when ATL-CR cells were co-cultured with normal human dermal fibroblasts, they neither formed colonies nor increased CD44 expression (Figure 5G). These results suggest that NECs and fibroblasts confer apoptosis resistance on ATL cells through distinct mechanisms.

Freshly Isolated ATL Cells Form Colonies when Co-Cultured with NECs, Escape TSA-Induced Apoptosis, and Augment CD44 Expression

To assess the clinical relevance of cell-cell interaction between ATL cells and NECs, we isolated PBMCs from two patients with ATL. Patient 1, with chronic-type ATL, had a white blood cell count of 6100/μL, of which 37.3% were lymphocytes. Patient 2, with acute-type ATL, had a white blood cell count of 29,520/μL, of which 88% were lymphocytes. Patient-derived PBMCs were cultured alone or with HEK293T cells with or without 0.8 μmol/L TSA, and then cycle analysis and immunofluorescence staining for CD44 and vimentin were performed. ATL cells co-cultured with HEK293T cells showed no increase in the percentage of apoptotic cells when treated with TSA for 48 hours, indicating that direct co-culture with NECs rescued ATL cells from TSA-induced apoptosis (Figure 6A). When co-cultured with HEK293T cells, a sizable proportion of ATL cells from both patients formed colonies on the monolayer of HEK293T cells and markedly increased CD44 and vimentin expression in the presence (Figure 6B) and absence (Figure 6, C and D) of TSA. Therefore, co-culture with NECs induced similar phenotypic changes in both ATL cell lines and freshly isolated ATL cells.

Next, we examined whether NEC-induced protection occurs in leukemic cells other than ATL cells. When the HTLV-1−negative T-cell lymphoma cells Jurkat and HUT78 were cultured alone in the presence of TSA, they both displayed an increase in the percentage of apoptotic cells, and this increase was abrogated by co-culture with HEK293T cells (Figure 6E). Furthermore, similar to ATL cell lines and freshly isolated ATL cells, HUT78, an epidermotropic HTLV-1−negative T-cell lymphoma cell line established from a patient with Sezary syndrome,[19] formed colonies on the monolayer of HEK293T cells and markedly increased CD44 and vimentin expression (Figure 6F). In contrast, Jurkat cells co-cultured with HEK293T cells neither formed colonies nor increased CD44 expression, suggesting that they presumably escaped from TSA-induced apoptosis by mechanisms distinct from those operating in ATL and HUT78 cells (Figure 6F).

Co-Culture with NECs Reduces the Expression of NKG2D Ligands on ATL-CR Cells

MICA and MICB (MHC class I chain-related proteins A and B) are stress-inducible NKG2D ligands that interact with the activating NKG2D receptor expressed on NK

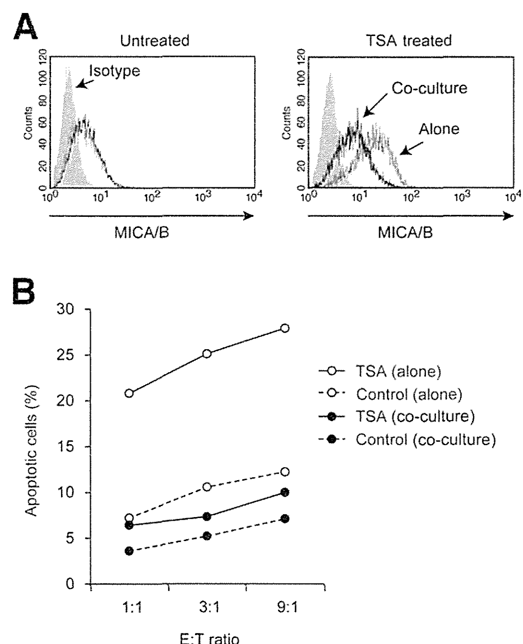

Figure 7 Direct co-culture with NECs reduced the expression of NKG2D ligands on ATL-CR cells. **A:** Induction of MICA/B expression on ATL-CR cells treated with TSA for 18 hours. ATL-CR cells were cultured alone or with HEK293T cells in the presence of 0.2 μmol/L TSA or vehicle. The cell surface expression of MICA/B on ATL-CR cells was analyzed by FCM using an anti-human MICA/B antibody. **B:** Susceptibility of TSA- or vehicle-treated ATL-CR cells to NK cell−mediated cytotoxicity. ATL-CR cells were treated with 0.2 μmol/L TSA or vehicle for 18 hours. CFSE-labeled KHYG-1 cells were used as effector cells and ATL-CR cells as target cells in cytotoxicity assays. Effector cells were incubated with nonlabeled target cells cultured alone or with CFSE-labeled HEK-293T cells at the indicated effector to target cell (E:T) ratios in triplicate wells of 24-well plates for 18 hours. Apoptotic ATL-CR cells were evaluated by FCM for the percentage of sub G0/G1 phase cells in the CFSE-negative cells. The data are presented as the means ± SD of three independent experiments.

cells.[29–31] This interaction activates NK cells, thereby facilitating the elimination of tumor cells. To examine whether co-culture with NECs affects immunologic properties of ATL cells, we cultured ATL-CR cells in the presence or absence of HEK293T cells in a medium containing 0.2 μmol/L TSA and examined MICA/MICB expression on ATL-CR cells. ATL-CR cells co-cultured with HEK293T cells expressed decreased levels of MICA/MICB (Figure 7A). Consistent with this, they became less susceptible to NK cell–mediated cytotoxicity (Figure 7B). These results suggest that interactions with NECs help ATL cells evade NKG2D-mediated immune attack.

Discussion

We demonstrated that in ATL cells cultured alone, TSA treatment induces p21[Cip1] accumulation, leading to cell-cycle arrest and apoptosis, whereas co-culture with NECs induces G0/G1 accumulation in TSA-treated ATL cells, enabling them to stay in a quiescent state and to acquire apoptosis resistance (Figures 1, 2, and 3). This resistance was acquired even when ATL cells cultured alone were pretreated with TSA and then co-cultured with NECs in fresh TSA-free medium, indicating that the contact with NECs subsequent to exposure to TSA enabled ATL-CR cells that otherwise were destined to undergo apoptosis to recover and resume growth (Figure 3). HDAC inhibitors, such as TSA, VPA, and NaB, induce not only transcriptional activation of viral and host genes but also genomic instability by a variety of mechanisms.[32–34] Thus, the present data suggest that NECs have a key role in guarding ATL cells from genomic instability, including reactivation of viral genes.

Co-culture with NECs induced cellular quiescence in ATL cells as assessed by cell-cycle analysis and staining with Ki-67 (Figure 3). ATL-CR cells that adhered to NECs displayed enhanced expression of CD44 and vimentin (Figures 3, 4, and 5). In this regard, treatment of CSCs with high concentrations of hyaluronan induces cellular quiescence, epithelial-mesenchymal transition, and a multidrug-resistant phenotype.[35] Expression of vimentin and CD44 might be induced by hyaluronan fragments in the extracellular matrix produced by NECs. Accumulating evidence indicates a close link between epithelial-mesenchymal transition and cancer stemness.[25] It is, therefore, possible that direct contact with NECs induces CSC-like phenotypes in ATL cells.

Co-culture with NECs induced expression of CD44 and vimentin in not only ATL cell lines but also ATL cells freshly isolated from patients (Figure 6, A–C), strongly suggesting the potential clinical importance of our observation. Indeed, recent work has shown that CD44 is expressed on skin-infiltrating tumor cells in patients with ATL.[36] Taken together, the present study suggests that interactions with epithelial cells induce CSC-like phenotypes in ATL cells and make them highly resistant to chemotherapies. Co-culture with NECs exerted antiapoptotic effects on two HTLV-1–negative T-cell lymphoma cells, Jurkat and HUT78 (Figure 6E), and induced the expression of CD44 and vimentin in HUT78 cells (Figure 6F). These observations suggest that NECs may play a protective role in lymphomas other than ATL.

Co-culture with NECs reduced expression of the stress-inducible NKG2D ligands MICA and MICB in TSA-treated ATL cells (Figure 7A), presumably because it reduced cellular stresses incurred by epigenetic changes. Reduced MICA and MICB expression made ATL cells less susceptible to cytotoxicity mediated by $NKG2D^+$ NK cells (Figure 7B). These results suggest that NECs not only induce CSC-like phenotypes in tissue-infiltrating ATL cells but also facilitate immune evasion by tumor cells.

HDAC inhibitors have emerged as a new class of promising chemotherapeutic agents against cancer.[32] However, monotherapeutic clinical trials with HDAC inhibitors have met with only limited success in most types of cancers.[37,38] The therapeutic efficacy of HDAC inhibitors in patients with ATL is still a controversial issue.[39,40] Furthermore, the safety of this treatment has not been established because it could activate viral genes.[14] The present work suggests that the therapeutic effectiveness of HDAC inhibitors will be reduced against leukemia cells that have invaded epithelial tissues and, thereby, acquired resistance to the inhibitors.

In conclusion, the results of the present study suggest that a propensity of leukemia cells to infiltrate epithelial tissues might produce at least two pathologic outcomes in patients with ATL: survival of leukemia cells through the acquisition of CSC-like phenotypes and evasion of the host immune response through reduced expression of NKG2D ligands.

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