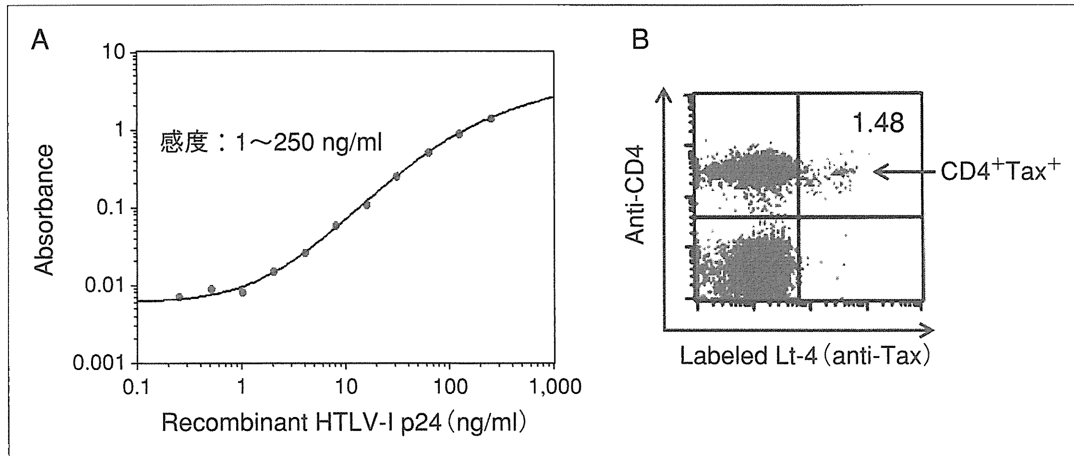

図2　簡便なHTLV-I抗原の定量(ELISA/FCM)
A：Gag p24抗原のサンドイッチELISA(直接法)．直接ELISAなので，反応は1ステップで終了し，発色も含めて1時間弱で終了する．細胞培養上清でも，細胞可溶化溶液でも測定が可能である．
B：Tax抗原のフローサイトメトリー(FCM)．HTLV-I感染者の新鮮PBMCを15～24時間培養し，生細胞を30分anti-CD4で染色後，PFA固定/サポニン膜透過液で処理してanti-Tax Lt-4で30分の最終染色を行う．その後，常法に従ってFCMを行い，解析する．

に結合し，引き続きエンベロープgp21を使って細胞に侵入すると考えられている[10]．しかし，他のウイルスと比較してHTLV-I粒子の大部分が感染粒子として不完全でありその感染性が非常に低い．また易熱性で冷蔵してもすぐに失活する[11]．したがって，*in vitro*でも生体内でも感染成立にはウイルス粒子単独でなく，HTLV-I産生細胞と標的細胞が直接コンタクトすることが必要と考えられている．このようにHTLV-Iがcell to cell感染で伝染する環境では，エイズやインフルエンザウイルスの場合とは異なり，中和抗体がウイルスに直接ウイルス粒子に干渉することはない．このことは，種々のHTLV-I野生株においてエピトープの変異がほとんどないこと[12]と一致し，HTLV-Iの標準株を標的として感染防御ワクチンを開発する妥当性を強く後押しする．

HTLV-IがヒトT細胞のみならず，調べたすべての脊椎動物細胞株やヒトのリンパ球以外の種々のヒトの血液細胞に感染するため，受容体は長い間不明のままであったが，エンベロープgp46の機能的領域が徐々に解明され，これまで受容体機能を持つ3分子が報告されている．すなわち，heparan sulfate proteoglycans (HSPG), neuropilin1 (NRP-1)およびglucose transporter type 1 (GLUT1)であるが，これら3分子が複合してHTLV-I感染に関与することが推測されている[13)14]．HSPGへの結合はgp46のC末領域(CTD)が，NRP-1とGLUT1への結合にはアミノ酸88-120の受容体結合領域(RBD)が関与するとされている[14]．しかし，ヒト生体内の感染にこのような受容体候補が実際にどのように働いているのか，特にCD4陽性T細胞への感染においてはどうなのかは今後の研究課題である．また，HTLV-I感染予防において受容体を標的とする戦略も考えられるが，今のところ実現性に乏しい．

感染中和エピトープ

ヒト血清抗体や免疫動物のHTLV-I中和抗体の認識エピトープを調べると，興味あることに，上記のウイルス受容体との結合関連領域RBDに含まれない領域〔特に中央部のプロリンリッチ領域(PRD)内のアミノ酸190番付近や，gp46の高次構造〕にも中和エピトープが存在する[9)15)16]．gp46は感染においてその高次構造の動的変化が重要なことが示唆される．したがって，HTLV-Iの初感染あるいは体内での再感染を防ぐ機能を持つ能動ワクチンは，このようにHTLV-Iエンベロープの中和エピトープを認識する抗体を効率よく誘導する機能が求められる．HTLV-Iキャリア由来のB細胞をEBV感染でトランスフォームして得られたヒト型HTLV-I中和単クロン抗体の研究がいくつか報告され，ヒトにおいてもPRD領域

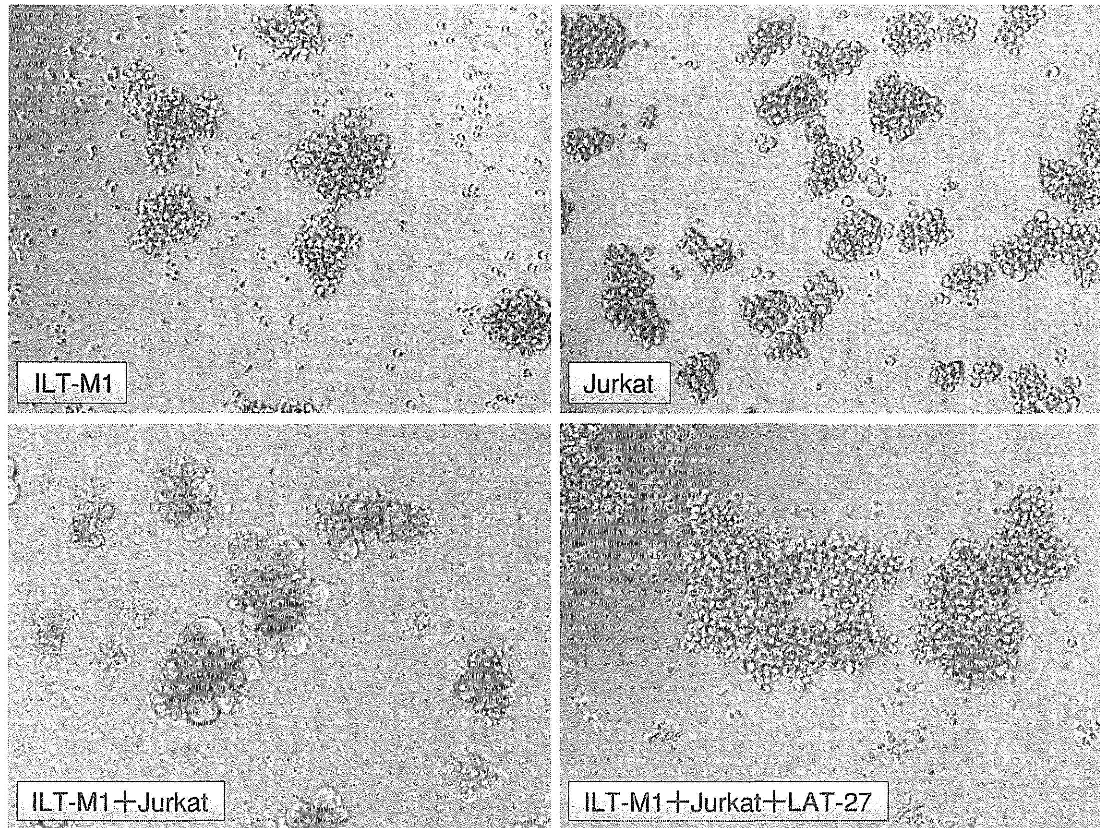

図3　HTLV-I感染の中和能：合胞体形成の阻止

が中和エピトープの集中する領域として認識されることが証明されている[9)17)]．これらの事実から，能動ワクチンの標的候補としてgp46 PRD領域のエピトープがワクチン候補として注目される．

感染防御ワクチン開発の研究基盤の確立

われわれが2011年から再開したHTLV-Iのワクチン開発へ向けた基礎研究では以下に示すように，まずHTLV-I感染防御能を評価する実験系の確立から行った[18)19)]．

(1)HTLV-I感染を定量するアッセイ系の確立：これまで作製した単クロン抗体ライブラリーを駆使し，HTLV-I抗原のp24やgp46を定量するELISA法，Tax抗原発現細胞のフローサイトメトリー法を確立した(図2)．

(2)*In vitro*感染系の確立：合胞体形成を指標とする感染実験において，HTLV-IはT細胞以外のヒト細胞株や異種動物の細胞株にも感染するが，あえて標的をヒトT細胞株にこだわり，種々のHTLV-I産生細胞株と種々のヒトT細胞株の組み合わせで最も判定しやすい合胞体を形成する組み合わせをスクリーニングした．ILT-M1とJurkat細胞が選択された．図3に示すようにこの系では，細胞と細胞を1：1で混合し一晩培養するだけで巨大な合胞体が形成される．5 μg/ml以上のLAT-27を添加すると合胞体形成が完全に阻止される．他のアッセイ系ではできた合胞体数を測定して％中和効率を算定しているが，この系では合胞体形成の完全阻止を中和のエンドポイントとしてヒト血清の中和抗体価の定量に用いている．100％防御でなければ，感染が成立するからである．また，ILT-M1細胞は混合培養により効率よく活性化PBMCを不死化する能力を持ち合わせるため，2～3週間後にTax陽性細胞のフローサイトメトリーを行えば，正常T細胞への感染が確認できるので，この系も中和抗体の機能確認に利用できる．抗体の不死化阻止効率と合胞体阻止効率はほぼ相関した(未発表)．

(3)動物感染実験系の確立：これまでHTLV-I感染感受性動物としてウサギが使用されていたが，近交系ラットも感受性が低いながらもHTLV-Iに感染することに注目した．このラットの系では，

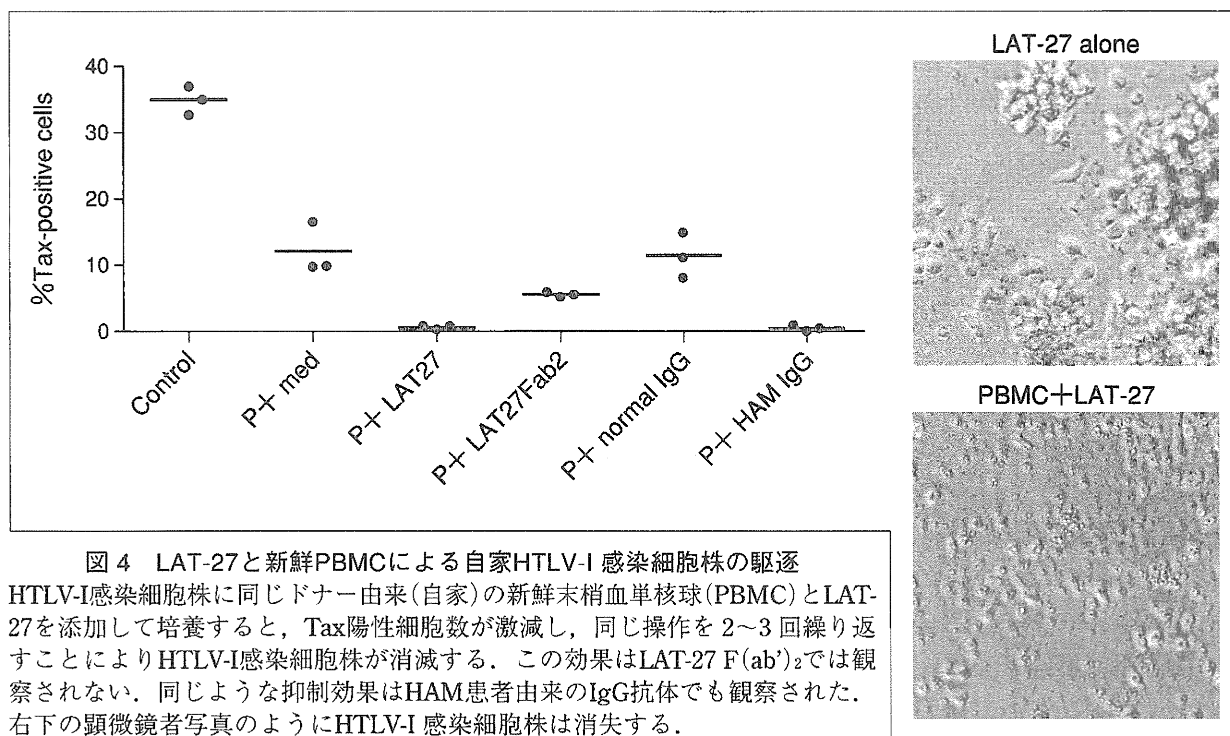

図4　LAT-27と新鮮PBMCによる自家HTLV-I感染細胞株の駆逐
HTLV-I感染細胞株に同じドナー由来（自家）の新鮮末梢血単核球（PBMC）とLAT-27を添加して培養すると，Tax陽性細胞数が激減し，同じ操作を2〜3回繰り返すことによりHTLV-I感染細胞株が消滅する．この効果はLAT-27 F(ab')$_2$では観察されない．同じような抑制効果はHAM患者由来のIgG抗体でも観察された．右下の顕微鏡者写真のようにHTLV-I感染細胞株は消失する．

経口を含めた種々の感染ルートからのHTLV-I感染を中和抗体が防御できるかを検討している[19]．また，HTLV-I陰性成人のPBMCを免疫不全マウスに移植したヒト化マウスにおける中和抗体の感染防御実験系についてもほぼ確立した．

(4)ワクチン候補は主に合成ペプチドを基本としてデザインしており，ラットやマウスに免疫し，種々の候補の中和抗体誘導能を比較している．これまでのところ，gp46抗原のPRD内のアミノ酸配列を持つペプチドが中和抗体誘導能に優れていた（未発表）．

HTLV-I感染自家細胞株の駆逐

上記の基盤を使って，実際に中和活性を持つLAT-27や他の抗体のウイルス産生抑制およびTax陽性細胞の駆逐能を比較検討した．そして，中和単クロン抗体LAT-27がHTLV-I感染者PBMC培養においてTax陽性細胞の出現頻度を下げ，さらに培養を続けるとT細胞の不死化をも顕著に抑制することを見出した[18]．このような効果は非中和anti-gp46抗体では観察できなかった．そのメカニズムをさらに探るため，健常人のT細胞をHTLV-Iで不死化した細胞株を樹立し，自家新鮮PBMCとLAT-27存在下で混合培養したところ，HTLV-I感染細胞株が駆逐された．LAT-27のFc部を消化したF(ab')$_2$フラグメントは感染中和活性を持つものの駆逐効果は観察できなかった[20]．つまり，駆逐にはIgG-Fc部が関与しているのである．同じような駆逐効果はHTLV-Iに対して高力価の中和能を示すHAM患者血清由来のIgGでも観察された（図4）．駆逐のメカニズムの一つにはナチュラルキラー細胞（NK）をエフェクターとするADCCの関与が示唆された．ヒトHTLV-I抗体を介するADCCによるHTLV-I感染細胞株の障害性については過去に多くの研究発表がなされているが[9)21)22)]，このような*in vitro*の実験結果からも，HTLV-I感染防御を目的とするワクチン標的とすべき第一の候補はHTLV-Iエンベロープgp46であること，抗体を使った受動免疫ワクチン候補としてヒト化したLAT-27があげられることが導き出される．

結　論

いまだHTLV-I能動ワクチンの開発には成功していないが，これまで私どもが開発した*in vitro*と*in vivo*のワクチン評価基盤を用いて安全性に優れた合成ペプチドワクチンを中心にスクリーニングを進めている．

これまで抗体の受動免疫によるHTLV-I母乳初期感染の防御についてはウサギでの感染実験で

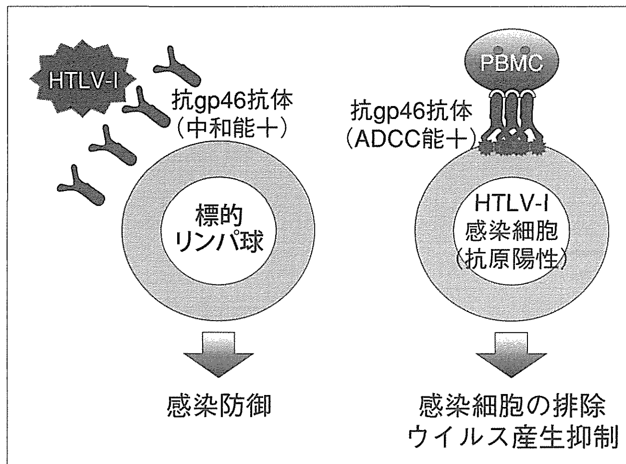

図5 HTLV-I Env gp46抗体を介するHTLV-I感染制御
ワクチン作製への礎

明らかにされ[23]，ヒトanti-HTLV-I抗体がサルにおいて受動免疫効果を持つことも報告されているが[24]，HTLV-Iの制御において抗体がどれだけ重要なのかを実証する研究はなかった 本稿では，LAT-27の中和効果および新鮮自家PBMCとの共同によるADCC様のウイルス感染細胞駆除効果を紹介した 同じ効果はHAM患者由来ポリクローナルIgGでも観察されたことから，1つの抗体がこのような2つの機能を持たなくとも，中和機能を持つ抗体とADCC機能を持つ別の抗体がいっしょに十分にあれば*in vivo*におけるHTLV-I感染防御と感染細胞の監視は十分にできることと推察できる(図5)

現在，LAT-27をヒト型化する作業を進行している 近い将来，ヒト化LAT-27は中和抗体活性の低いHTLV-Iキャリア 特に妊婦の方々を対象とした受動免疫抗体薬として活用できることを期待しており，現在，ラットでの垂直感染阻止実験を行っている LAT-27のようなHTLV-I感染細胞上のウイルス抗原を認識する抗体医薬は，現在のanti-CCR4[25]やanti-CD25抗体[20]と併用することによりさらに高いウイルス制御活性を発揮すると期待される

雑 談

ATLウイルスは，米国で1980年に米国Gallo博士らが報告した"みなしごウイルス"ヒトT細胞白血病ウイルス(human T-cell leukemia virus type-I, HTLV-I)と同一であることがわかり，2つの名前はHTLV-Iに統一された その後，HTLV-Iがエイズの原因ウイルス(HTLV-III)の仲間であるとされ，HTLV-Iは "human T lymphocyte-tropic virus type-I" と名称が勝手に変更された また最近の論文ではHTLV-1と記載されることが多く見られるが正しくはHTLV-Iであり，それはhuman T-cell leukemia virus type-Iの略である (ウラシマタロウ

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Elimination of Human T Cell Leukemia Virus Type-1-Infected Cells by Neutralizing and Antibody-Dependent Cellular Cytotoxicity-Inducing Antibodies Against Human T Cell Leukemia Virus Type-1 Envelope gp46

Yuetsu Tanaka,[1] Yoshiaki Takahashi,[1] Reiko Tanaka,[1] Akira Kodama,[1] Hideki Fujii,[1] Atsuhiko Hasegawa,[2] Mari Kannagi,[2] Aftab A. Ansari,[3] and Mineki Saito[4]

Abstract

Human T cell leukemia virus type-1 (HTLV-1) is prevalent worldwide with foci of high prevalence. However, to date no effective vaccine or drug against HTLV-1 infection has been developed. In efforts to define the role of antibodies in the control of HTLV-1 infection, we capitalized on the use of our previously defined anti-gp46 neutralizing monoclonal antibody (mAb) (clone LAT-27) and high titers of human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). LAT-27 and HAM-IgG completely blocked syncytium formation and T cell immortalization mediated by HTLV-1 *in vitro*. The addition of these antibodies to cultures of $CD8^+$ T cell-depleted peripheral blood mononuclear cells (PBMCs) from HAM/TSP patients at the initiation of culture not only decreased the numbers of Tax-expressing cells and the production of HTLV-1 p24 but also inhibited the spontaneous immortalization of T cells. Coculture of *in vitro*-HTLV-1-immortalized T cell lines with autologous PBMCs in the presence of LAT-27 or HAM-IgG, but not an $F(ab')_2$ fragment of LAT-27 or non-neutralizing anti-gp46 mAbs, resulted in depletion of HTLV-1-infected cells. A 24-h ^{51}Cr release assay showed the presence of significant antibody-dependent cellular cytotoxicity (ADCC) activity in LAT-27 and HAM-IgG, but not $F(ab')_2$ of LAT-27, resulting in the depletion of HTLV-1-infected T cells by autologous PBMCs. The depletion of natural killer (NK) cells from the effector PBMCs reduced this ADCC activity. Altogether, the present data demonstrate that the neutralizing and ADCC-inducing activities of anti-HTLV-1 antibodies are capable of reducing infection and eliminating HTLV-1-infected cells in the presence of autologous PBMCs.

Introduction

Human T cell leukemia virus type-1 (HTLV-1) is the first human retrovirus that was etiologically associated with adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] HTLV-1 is prevalent worldwide with foci of high prevalence in southwest Japan, the Caribbean islands, South America, and a part of Central Africa. The total number of HTLV-1 carriers is currently estimated to be 10–20 million.[5] The majority of HTLV-1 carriers remain asymptomatic throughout their lives, and approximately 5% of HTLV-1-infected individuals will develop either ATL or HAM/TSP after prolonged latency periods.

HTLV-1 is transmitted through contact with bodily fluids containing infected cells most often from mother to child through breast milk or via blood transfusion. It has been previously established that HTLV-1 efficiently spreads from cell to cell via the formation of virological synapses.[6] More recently, however, the formation of extracellular HTLV-1 viral particles similar to the formation of bacterial films has also been shown to be effective in viral transmission.[7] HTLV-1-antigen-expressing cells are difficult to detect at least in fresh peripheral blood mononuclear cells (PBMCs) from HTLV-1-infected individuals.[8] However, when these PBMCs are isolated from the blood and cultured *in vitro*, some T cells begin to produce HTLV-1 antigen[9,10] followed by spontaneous immortalization of the cells in media containing interleukin-2 (IL-2).[11]

[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan.
[2]Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan.
[3]Department of Pathology, Emory University School of Medicine, Atlanta, Georgia.
[4]Department of Microbiology, Kawasaki Medical School, Okayama, Japan.

Although it has been suggested that HTLV-1 can stay dormant in infected cells and become resistant to immune effector mechanisms by ratcheting down its antigen production,[12] the continued presence of strong $CD8^+$ cytotoxic T lymphocyte (CTL) responses[13] and readily detectable levels of antibodies specific for HTLV-1 antigens in HTLV-1-infected people[14] indicates that persistent production of HTLV-1 must occur *in vivo* to maintain such effector mechanisms. Escape from immune effector mechanisms by spontaneous mutation of key residues is unlikely, due to the high degree of genomic stability that is characteristic of the HTLV-1 genome.[15] It has been suggested that HTLV-1-infected cells expressing HTLV-1 antigens occur at a low enough frequency that they are constantly being eliminated by HTLV-1-specific CTL *in vivo*[10] without leading to immune exhaustion. Besides CTL and virus neutralizing antibodies, there has been renewed interest in the potential role of antibody-dependent cellular cytotoxicity (ADCC) as an effector mechanism against a number of viral infections. This view has been highlighted by the recent demonstration of the potential role of ADCC in the only known partially successful human RV144 trial of a vaccine against human immunodeficiency virus type-1 (HIV-1).[16] The ADCC activity against HTLV-1 was first reported by Miyakoshi *et al.* in 1984[17] followed by a number of other reports.[18–21]

So far, several lines of evidence show that the HTLV-1 envelope gp46 antigen serves as a major target of ADCC.[22–24] Antibodies against gp46 antigen are commonly detected in the sera of HTLV-1-infected individuals.[25–27] However, the precise role of ADCC effector mechanism(s) in controlling HTLV-1 infection has been lacking. A possible involvement of anti-HTLV-1 antibodies in the suppression of spontaneous HTLV-1 antigen expression by HTLV-1-infected cells was first reported by Tochikura *et al.*[28] These investigators showed that serum IgG from HTLV-1-infected donors interfered with HTLV-1 antigen expression by *in vitro*-cultured PBMCs from both ATL patients and healthy HTLV-1 carriers. However, the precise mechanism by which this was mediated remained unclear.

In efforts to define the role of antibodies with neutralizing and ADCC-inducing activities in the control of HTLV-1 infection, we capitalized on the use of our previously defined rat anti-gp46 neutralizing monoclonal antibody (mAb) (LAT-27)[29] and pooled human anti-HTLV-1 IgG purified from HAM/TSP patients (HAM-IgG). Studies were conducted to evaluate the potential of these antibodies to block HTLV-1 infection and eliminate HTLV-1-infected cells from autologous T cell cultures that had previously been infected with HTLV-1 *in vivo* or *in vitro*. Results of these studies show that monoclonal LAT-27 and the polyclonal HAM-IgG are not only capable of mediating neutralization and ADCC, but are also highly effective in the elimination of HTLV-1-infected cells in the presence of fresh autologous PBMCs while preventing *de novo* infection with HTLV-1.

Materials and Methods

Reagents

The medium used throughout was RPMI 1640 medium (Sigma-Aldrich. Inc., St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (hereinafter called RPMI medium). Anti-human CD3 (clone OKT-3) and anti-CD28 (clone 28.2) mAbs were purchased from the American Type Culture Collection (Rockville, MD) and Biolegend (San Diego, CA), respectively.

The rat and mouse mAbs utilized in the studies reported herein were produced and characterized by our laboratory previously.[29–34] These antibodies were rat IgG2b anti-gp46 (clones LAT-27 and LAT-25), rat IgG2a anti-gp46 (clone LAT-12), rat IgG2b anti-HCV (clone Mo-8), rat IgG2a anti HTLV-1 p24 (clone WAG-24), mouse IgG1 anti-HTLV-1 gp46 (clone MET-3), mouse IgG3 anti-HTLV-1 Tax (clone Lt-4), mouse IgG1 anti-p24 (clone NOR-1), and mouse IgG1 anti-HIV-1 p24 (clone 2C2). These in-house mAbs were purified from the ascites fluids of groups of CB.17-SCID mice carrying the appropriate hybridoma cell line. The ascites fluid was subjected to ammonium sulfate precipitation followed by gel filtration using Superdex G-200 (GE Healthcare, Tokyo, Japan). Aliquots of these mAbs were labeled with either fluorescein isothiocyanate (FITC), Alexafluor 488, Alexafluor 647, HRP (Dojindo, Kumamoto, Japan), or Cy-5 (GE Healthcare) according to the manufacturer's instructions. The FITC- or phycoerythrin (PE)-labeled mouse mAbs against human CD3, CD4, CD8, CD14, CD16, CD19, or CD56 and unlabeled mouse anti-CD16 and anti-CD32 mAbs were purchased from Abcam.

For cell depletion, magnetic beads labeled with anti-CD4, CD8, CD14, CD16, CD19, and antimouse IgG (Dynal) and those labeled with anti-CD56 mAb (LifeTec) were used according to the manufacturer's recommendations. Mitomycin-C (MMC) was commercially purchased from Kyowa Kirin (Tokyo, Japan) and used at 50 μg/ml in RPMI medium. A purified $F(ab')_2$ fragment of LAT-27 IgG generated by enzymatic digestion of LAT-27 IgG was purchased from IBL Inc. (Gunma, Japan). Human IgG was purified from pooled plasma from three normal donors (normal IgG) and three HAM patients (HAM-IgG) using protein-G affinity purification kits (GE Healthcare).

The protocols for the use of human PBMCs and animals were approved by the Human IRB and the Institutional Animal Care and Use Committee (IACUC) on clinical and animal research of the University of the Ryukyus prior to initiation of the present study.

Cell cultures

PBMCs were isolated from heparinized blood by standard density gradient centrifugation using Lympholyte (Cedarlane, Burlington, Canada). Some PBMCs were cryopreserved using a cell freezing media (Cell reservoir, Nakarai Tesque Inc., Kyoto, Japan). The method to activate PBMCs with anti-CD3 and CD28 mAbs has been described previously.[34] The HTLV-1-producing T cell lines utilized included MT-2, HUT102, IL-2-dependent $CD4^-CD8^+$ ILT-M1 cells derived from an HAM/TSP patient, $CD4^+CD8^-$ ILT-H2 cells, ATL-3 cells derived from ATL patients, and a number of other T cell lines derived from normal PBMCs following *in vitro* immortalization by cocultivation with MMC-treated ILT-M1 cells. These cell lines were maintained in culture using RPMI medium containing 20 U/ml IL-2.

The syncytium inhibition assay was performed using an assay that involved the coculture of ILT-M1 and Jurkat cells.[35] A suspension of ILT-M1 cells in a volume of 25 μl containing 5×10^4 cells in 20 U/ml IL-2 media was mixed with 50 μl of serially diluted antibody to be tested in a flat-bottom 96-well microtiter plate for 5 min followed by the addition of 5×10^4 Jurkat cells in a volume of 25 μl of medium. After coculture for 18$\sim$24 h at 37°C in a 5% CO_2 humidified incubator, syncytium formation was microscopically observed using an inverted microscope and the minimum concentration of antibody that showed complete blocking of syncytium formation was determined. In some experiments, gp46 antigen that had been affinity purified from the culture supernatants of MT-2 cells using our anti-gp46 mAb (MET-3) antibody-coupled Sepharose 4B column (GE Healthcare) was used as a target antigen to serve as a specificity control to block the syncytia neutralization of antibodies.[36]

The HTLV-1-immortalization inhibition assay was performed according to the method described previously with a slight modification.[29] Briefly, PBMCs from HTLV-1-negative healthy donors were activated with immobilized OKT-3 together with soluble anti-CD28 mAb overnight, and these cells (5×10^4 cells) were cocultured with an equal number of MMC-treated ILT-M1 cells in wells of round-bottom 96-well microtiter plates (BD) in 0.2 ml media containing 20 U/ml IL-2 at 37°C in a humidified 5% CO_2 incubator in the presence or absence of the test antibodies. The medium was replaced with fresh IL-2-containing media with or without antibody every 3–5 days. Aliquots of the cocultured cells were monitored every week for intracellular expression of Tax antigen, and the culture supernatants were monitored for the production of p24.

The assay for inhibition of spontaneous HTLV-1 antigen expression in PBMCs from HAM/TSP patients was performed as follows. PBMCs from HAM/TSP patients after depletion of CD8^{+} cells were cultured *in vitro* at 1×10^6 cells/ml in 20 U/ml IL-2-containing RPMI medium at 37°C in a 24-well plate (BD) in the presence of various anti-HTLV-1 mAbs, HAM-IgG, or controls. After 24 h, cells were harvested and an aliquot stained with anti-CD3, CD4, or CD8 mAb, followed by fixation and subsequent intracellular Tax staining. The frequency and absolute cell numbers of Tax-positive cells were analyzed by flow cytometry (FCM) using the Flowcount (Coulter). The remaining cells were further cultured for 2$\sim$6 weeks with a change of media with or without antibody every 3$\sim$4 days. If necessary, cultures were split into 1:2 or 1:4.

The elimination of HTLV-1 antigen-expressing cells was tested as follows. The IL-2-dependent HTLV-1-infected T cell lines established from PBMCs of normal donors (2×10^5 cells/ml) were cocultured with autologous fresh PBMCs (2×10^6 cells/ml) in 20 U/ml IL-2-containing RPMI medium in triplicate in a round-bottom 96-well microtiter plate (BD) in the presence or absence of various antibodies. After initial coculture for 3 days, these cultures were split, and one was cultured in the presence and the other in the absence of fresh PBMCs and antibodies for 3 days. If necessary, these cells were further treated with antibodies and fresh PBMCs every 3 days. These cell cultures were periodically monitored for changes in the levels of Tax-expressing cells and levels of p24 production.

Flow cytometry (FCM) and enzyme-linked immunosorbent assay (ELISA)

For the detection of HTLV-1 antigen-expressing cells, sample cells were analyzed using polychromatic FCM. Briefly, live cells were Fc receptor-blocked with 2 mg/ml pooled normal human IgG in FACS buffer [phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide] for 10 min on ice, and prestained with fluorescent dye-labeled mAbs for 30 min. After washing with FACS buffer, the cells were fixed in 4% paraformaldehyde (PFA) in PBS for 5 min at room temperature followed by permeablization and washing in 0.5% saponin + 1% BSA (Sigma) containing FACS buffer. The cells were incubated with 0.1 μg/ml of Cy5-labeled anti-Tax antibody (clone Lt-4) for 30 min. Negative control cells were stained with Cy5-Lt-4 in the presence of 50 μg/ml of unlabeled Lt-4. These cells were analyzed using a FACSCalibur (BD) and the data obtained were analyzed using the Cell Quest software (BD). Typical staining of HTLV-1-infected T cell lines with Lt-4 and LAT-27 is also shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/aid).

Production of HTLV-1 was determined by the measurement of the HTLV-1 core p24 antigen levels in the culture supernatants using our in-house formulated and standardized ELISA kit using a pair of anti-HTLV-1 p24 mAbs. The sensitivity of this assay was determined to be 0.5 ng/ml of p24 (data not shown).

ADCC assay

HTLV-1-immortalized target cells from healthy donors were labeled with ^{51}Cr for 60 min as described previously[37] and mixed with varying ratios of fresh PBMCs (varying effector-to-target cell ratios) in the presence or absence of various antibodies for the indicated period of time in 20 U/ml IL-2-containing medium. Appropriate controls were included with each assay including target cells cultured in media alone (spontaneous release) and in 0.5 N HCl (100% release). After brief centrifugation, supernatants were harvested and ^{51}Cr activity in each sample was determined using a gamma counter. The net percentage ^{51}Cr release was calculated using standard methods as follows (cpm in experiment – cpm in medium)/(cpm in 0.5 N HCl – cpm in medium) $\times$ 100. In some experiments, PBMCs were depleted of CD4^{+}, CD8^{+}, CD14^{+}, CD16^{+}, CD19^{+}, or CD56^{+} cells using appropriately conjugated immunomagnetic beads and tested for their effector activity.

Statistical analysis

Data were tested for statistical significance by the Student's *t* test using Prism software (GraphPad Software).

Results

HTLV-1 neutralizing activities of LAT-27 and human anti-HTLV-1-IgG in vitro

The syncytium inhibition assay has been generally used to evaluate HTLV-1 neutralization titers of anti-HTLV-1

antibodies. To optimize the syncytium inhibition assay, we screened various coculture combinations of HTLV-1-producing cells with a variety of HTLV-1-negative target cells, and selected the HTLV-1-producing T cell line ILT-M1 and the HTLV-1-negative T cell line Jurkat. Overnight coculture of the ILT-M1 and Jurkat cells resulted in the generation of numerous large syncytia (Fig. 1). Using this assay system, we titrated the syncytia-blocking activity of monoclonal LAT-27 and polyclonal IgG purified from pooled plasma from HAM patients (HAM-IgG). HAM-IgG was used as a positive anti-HTLV-1 antibody control because it contained high titers of antibodies against HTLV-1 antigens (Supplementary Fig. S2). The minimum concentrations required for the "complete" inhibition of syncytia formation by LAT-27 and HAM-IgG antibodies were calculated to be 5 μg/ml and 50 μg/ml, respectively (Supplementary Fig. S3). To adjust for decay in antibody activities during cultivation at 37°C, we used LAT-27 and HAM-IgG at concentrations of 10 and 100 μg/ml, respectively, in all subsequent experiments.

To confirm the gp46 specificity of LAT-27 and HAM-IgG in this syncytium inhibition assay, an affinity-column-purified gp46 antigen[36] was added to an aliquot of either LAT-27 or HAM-IgG solution prior to cocultivation. Controls consisted of incubating an aliquot of the cocultures in media alone (shaded bars denoted by 0) or media containing 10 μg/ml of gp46 (dark bars also denoted by 0). As shown in Fig. 2, HAM-IgG incubated in media alone clearly inhibited syncytia formation in a dose-dependent manner (at 12.5 ~ 100 μg/ml). However, preincubation of the HAM-IgG at 12.5 ~ 100 μg/ml with 10 μg/ml of affinity-purified gp46 resulted in significant reversal of inhibition, suggesting that gp46 was the main target for the neutralization activity present in the human anti-HTLV-1 antibodies. Similar results were obtained when LAT-27 instead of HAM-IgG was preincubated with gp46 (data not shown).

LAT-27 as reported previously[29] and HAM-IgG completely inhibited HTLV-1-mediated T cell immortalization of normal activated T cells *in vitro* at concentrations of 10 μg/ml and 100 μg/ml, respectively (Fig. 3).

Effect of antibodies on spontaneous HTLV-1 antigen expression in vitro

To evaluate the role of anti-gp46 neutralizing antibodies against T cells carrying endogenous HTLV-1 from naturally infected donors, we cultured freshly isolated PBMCs from HAM/TSP patients in the presence or absence of various antibodies for 24 h. To exclude any potential effects of $CD8^+$ CTL that are present within the PBMCs from the HAM/TSP patients,[10] PBMCs were depleted of $CD8^+$ T cells prior to the assay. For quantitation of the frequencies of HTLV-1 antigen-expressing cells, we stained an aliquot of the cells for the expression of intracellular Tax antigen utilizing our standardized anti-Tax mAb, which has generally been used to detect HTLV-1-infected cells.[10,38] At a concentration of 10 μg/ml, LAT-27 reduced the frequency of Tax^+ cells (Fig. 4A). This reduction was antigen and epitope specific since neither the isotype control rat IgG2b mAb (anti-HCV envelope) nor the anti-gp46 nonneutralizing mAb (LAT-25) and the other anti-gp46 nonneutralizing mAbs (clones LAT-12 and MET-3) that compete with LAT-27 in an antibody binding assay showed any detectable inhibitory effect (data not shown). The reduction in the frequency of Tax^+ cells by LAT-27 was partially reversed by a mixture of anti-CD16 and anti-CD32 mAbs when added at the initiation of the assay, suggesting an involvement of Fc receptors in this reduction assay.

As shown in Fig. 4B, after prolonged culture (2 weeks) the suppressive effect of LAT-27 became more evident since there remained few if any Tax^+ cells in the LAT-27-treated cultures of PBMCs from each of the HAM patients tested. A similar suppressive effect was observed for HAM-IgG but not normal human IgG (Fig. 4B). It should be noted that in the present culture conditions, similar to what has been generally observed for the PBMC cultures from HTLV-1-infected

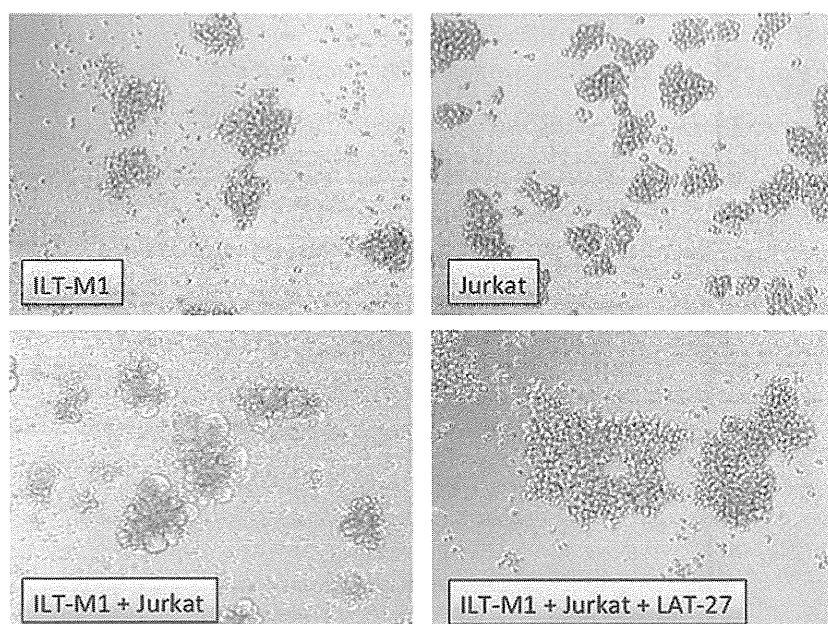

FIG. 1. Human T cell leukemia virus type-1 (HTLV-1)-mediated syncytia formation. $HTLV\text{-}1^+$ ILT-M1 and $HTLV\text{-}1^-$ Jurkat cells were either cultured alone or cocultured at a cell-to-cell ratio of 1:1 in the presence or absence of 10 μg/ml LAT-27 for 18 h. Syncytia were microscopically observed using an inverted microscope at a magnification of 100×. Representative data from three independent experiments are shown.

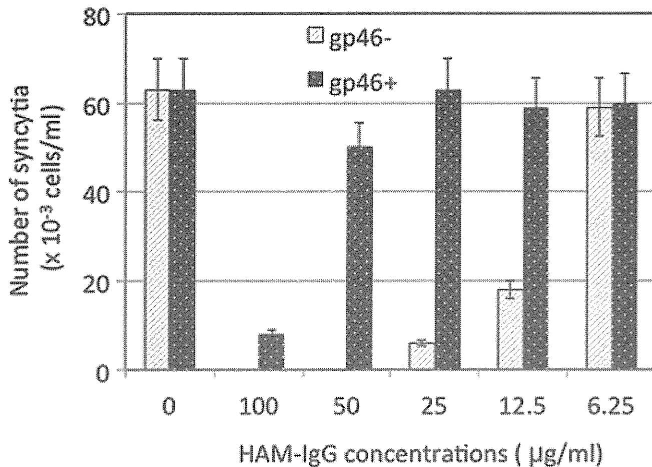

FIG. 2. Anti-gp46 antibodies are major HTLV-1 neutralizing antibodies in HAM-IgG. HAM-IgG at graded concentrations ($0 \sim 100\,\mu$g/ml) was preincubated with either affinity-purified gp46 antigen (black bars) at $10\,\mu$g/ml for 10 min or incubated with medium alone (gray shaded bars, labeled as "gp46-") and tested for syncytia inhibition activity. The numbers of syncytia were manually counted using a "Burker-Turk" hemocytometer. Representative data from three independent experiments are shown.

donors, the frequency of Tax$^+$ cells gradually decreased during 2 weeks in culture even in IL-2 medium alone and thus it was not likely due to an effect of the addition of the control rat isotype IgG or normal IgG. Spontaneous immortalization of T cells by HTLV-1 was observed in the PBMC cultures from two-thirds of the HAM patients treated with medium alone, isotype control, or normal IgG, but not in those treated with LAT-27 or HAM-IgG, as judged 6 weeks after culture (data not shown).

Importantly, neither LAT-27 nor HAM-IgG (data not shown) showed any detectable blocking effects on HTLV-1 Tax expression (Fig. 5) and p24 antigen production (data not shown) in long-term cultured HTLV-1-infected cell lines. It is known that the levels of Tax-positive cells vary depending on the cell lines being utilized due to DNA methylation, hypoacetylation of histones, or epigenetic changes of provirus.[39] Thus, we reasoned that the addition of the neutralizing antibodies blocks *de novo* expansion of HTLV-1 infection and/or eliminates the HTLV-1 gp46 expressing T cells via an FcR-dependent pathway in combination with effector cells contained within the PBMCs.

FcR-dependent elimination of HTLV-1-infected cells by antibodies

Due to the limitations on the availability of PBMC samples from HAM/TSP patients, we established a number of IL-2-dependent HTLV-1-infected CD4$^+$ T cell lines from PBMCs of normal donors to determine whether ADCC was involved in the suppression of HTLV-1-infected cells. These HTLV-1$^+$CD4$^+$ T cell lines were cocultured with autologous fresh PBMCs in the presence or absence of various antibodies including F(ab′)$_2$ of LAT-27, which showed HTLV-1 neutralization at a minimum concentration of $2.5\,\mu$g/ml (data not shown). HAM-IgG was included as an ADCC-positive control. After 3 days in culture, these cells were stained for cell surface CD4 and intracellular Tax antigen, and analyzed on a gated population of cells that displayed high forward and side scatters, which included a majority of the HTLV-1-infected

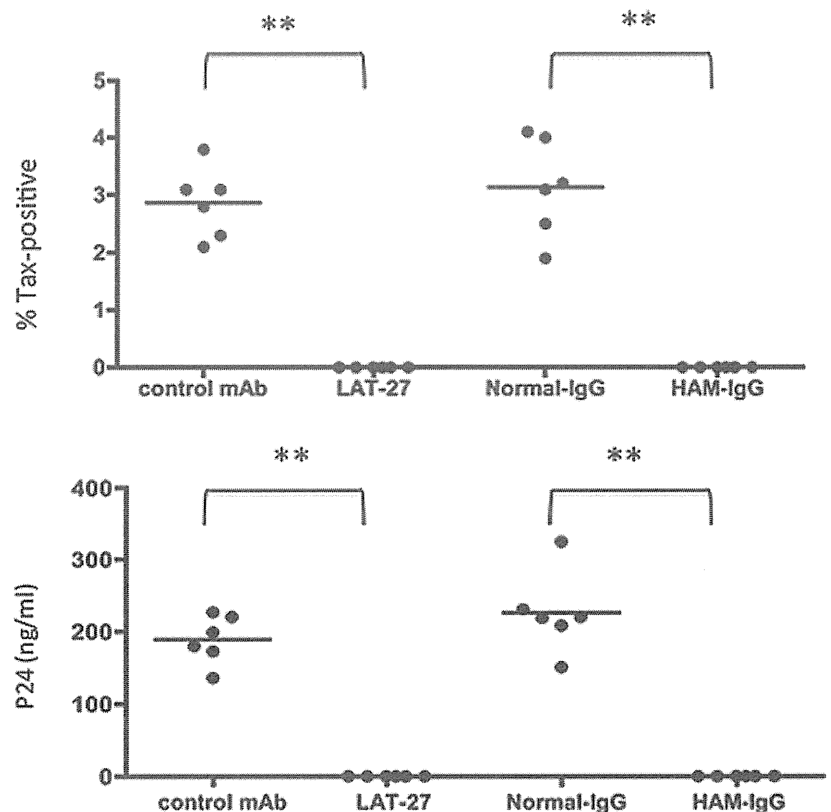

FIG. 3. LAT-27 and HAM-IgG completely block HTLV-1-mediated T cell immortalization *in vitro.* Activated peripheral blood mononuclear cells (PBMCs) from normal donors were seeded into six wells of 96-well U-bottom plates (1×10^5 cells/0.1 ml/well) and cocultured with an equal number of mitomycin C-treated ILT-M1 cells in the presence or absence of $10\,\mu$g/ml of LAT-27 or rat isotype control, $100\,\mu$g/ml of normal human IgG, or HAM-IgG. Half of the medium was replaced every $3 \sim 5$ days with new similar fresh media, and if necessary, cultures were split into 1:2. Each data point reflects the frequency of Tax$^+$ cells or the levels of p24 in the culture supernatants of each well 6 weeks after culture. Data shown are representative of three independent experiments. The differences between the controls and the experimental data were highly significant, denoted as $^{**}p < 0.01$. The negative control used for LAT-27 was an isotype control (rat IgG2b anti-HCV).

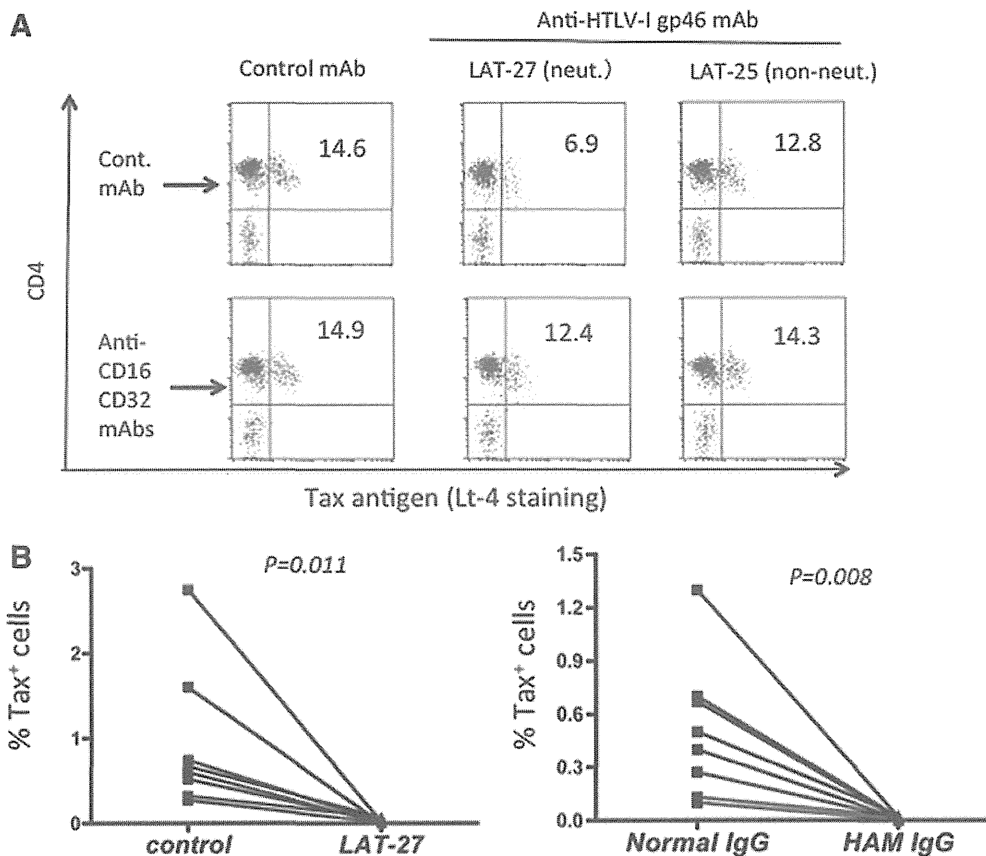

FIG. 4. Reduction of Tax-expressing cells in *in vitro* cultures of PBMCs from HAM patients in the presence of HTLV-1 neutralizing monoclonal antibody (mAb). **(A)** PBMCs from HAM patients were depleted of $CD8^+$ T cells and cultured *in vitro* for 24 h at 1×10^6 cells/ml in interleukin (IL)-2-containing 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

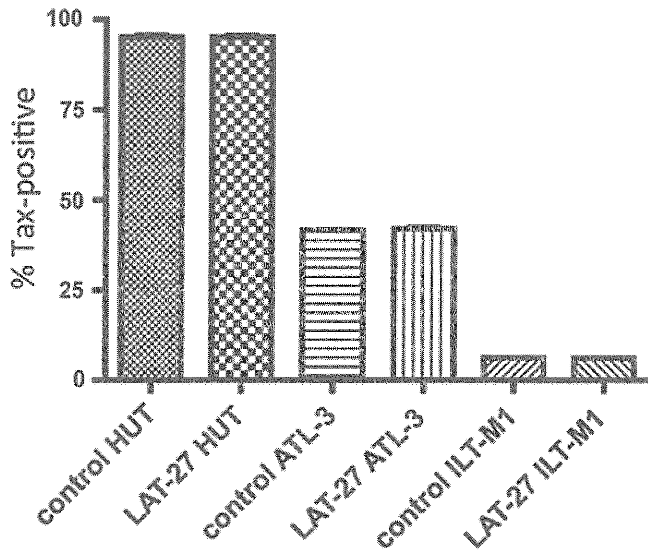

FIG. 5. LAT-27 alone does not affect long-term cultured HTLV-1-infected T cells. A standard HTLV-1-infected cell line HUT-102 (HUT), an IL-2-dependent CD4^{+} T cell line (ATL-3, generated from an ATL patient), and an IL-2-dependent CD8^{+} T cell line (ILT-M1) were cultured in the presence of 10 μg/ml 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

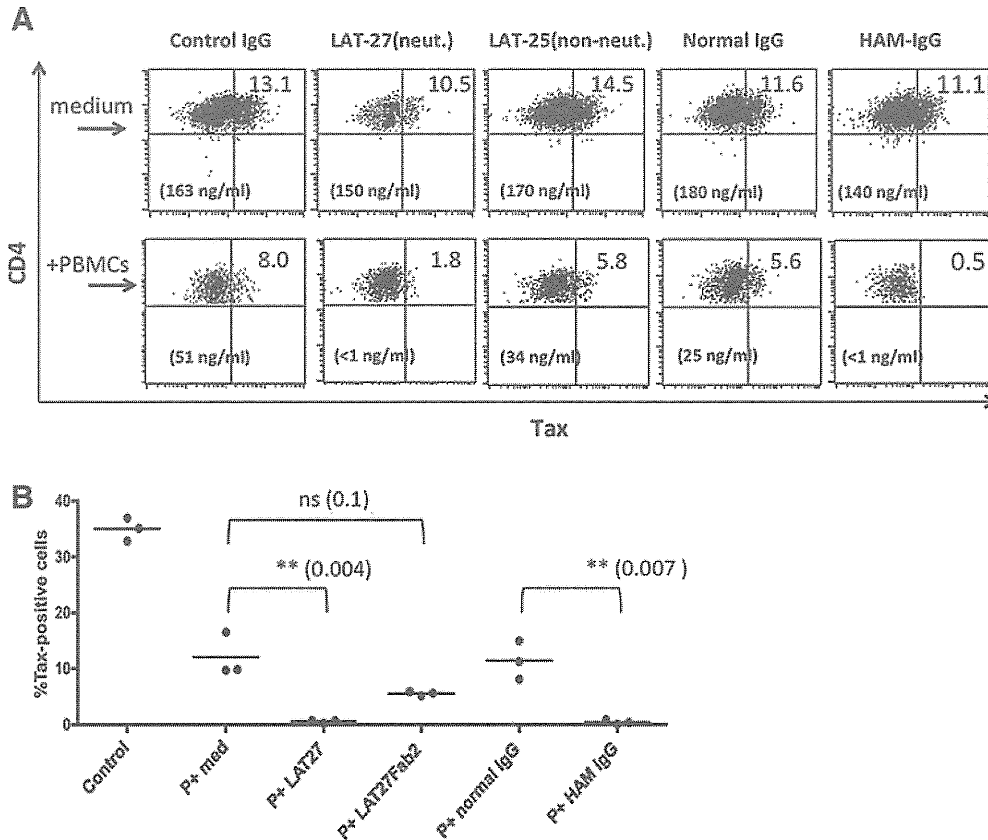

FIG. 6. Elimination of Tax$^+$ cells and reduction of HTLV-1 p24 production in IL-2-dependent HTLV-1-infected T cells cocultured with autologous PBMCs in the presence of LAT-27 or HAM-IgG. **(A)** IL-2-depnedent CD4$^+$ HTLV-1-infected T cells established from the PBMCs of normal donors were repeatedly 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)

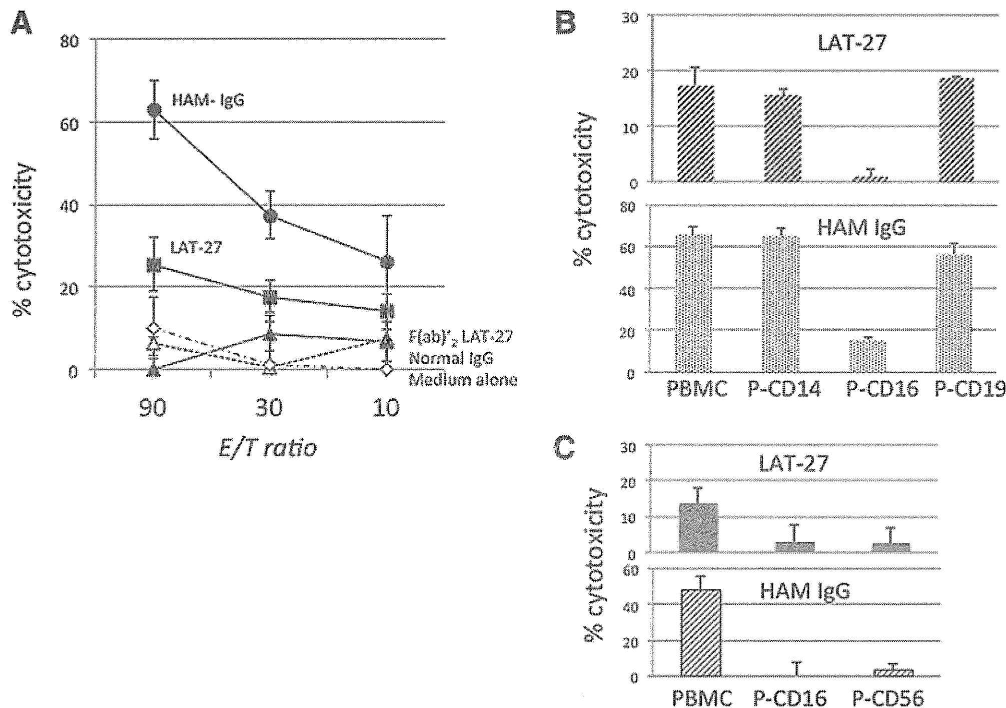

FIG. 7. The CD16+ CD56+ PBMCs mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of LAT-27 or HAM-IgG. **(A)** ^{51}Cr-labeled HTLV-1-infected cells were cocultured *in vitro* with autologous fresh PBMCs at various E/T ratios in the presence or absence of 10 μg/ml of LAT-27 or F(ab′)$_2$ 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.

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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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Address correspondence to:
Yuetsu Tanaka
Department of Immunology
Graduate School of Medicine
University of the Ryukyus
207 Uehara, Nishihara-cho
Okinawa 903-0215
Japan

E-mail: yuetsu@s4.dion.ne.jp

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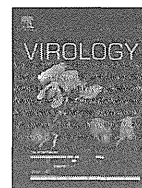

HTLV-1 infects human mesenchymal stromal cell *in vitro* and modifies their phenotypic characteristics ☆

Evandra Strazza Rodrigues [a,b], Mayra Dorigan de Macedo [a,b], Mariana Tomazini Pinto [a,b], Maristela Delgado Orellana [a,c], Maurício Cristiano Rocha Junior [a,b], Danielle Aparecida Rosa de Magalhães [a], Yuetsu Tanaka [d], Osvaldo Massaiti Takayanagui [c], Dimas Tadeu Covas [a,c], Simone Kashima [a,b,*]

[a] *Regional Blood Center of Ribeirão Preto, University of São Paulo, Brazil*
[b] *School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil*
[c] *School of Medicine of Ribeirão Preto, University of São Paulo, Brazil*
[d] *University of the Ryukyus, Okinawa, Japan*

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ABSTRACT

The typical characteristics of mesenchymal stem cells (MSCs) can be affected by inflammatory microenvironment; however, the exact contribution of HTLV-1 to MSC dysfunction remains to be elucidated. In this study, we demonstrated that MSC cell surface molecules VCAM-1 and ICAM-1 are upregulated by contact with HTLV-1, and HLA-DR was most highly expressed in MSCs co-cultured with MT2 cells. The expression levels of VCAM-1 and HLA-DR were increased in MSCs cultured in the presence of PBMCs isolated from HTLV-1-infected symptomatic individuals compared with those cultured with cells from asymptomatic infected individuals or healthy subjects. HTLV-1 does not impair the MSC differentiation process into osteocytes and adipocytes. In addition, MSCs were efficiently infected with HTLV-1 *in vitro* through direct contact with HTLV-1-infected cells; however, cell-free virus particles were not capable of causing infection. In summary, HTLV-1 can alter MSC function, and this mechanism may contribute to the pathogenesis of this viral infection.

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Background

Human T lymphotropic virus type 1 (HTLV-1) is a human retrovirus that is associated with two major diseases known as adult T-cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Only a small fraction (0.25% to 5%) of HTLV-1-infected individuals develops HAM/TSP, whereas 2% to 5% develop ATLL (Osame et al., 1986; Uchiyama, 1997), but other inflammatory disorders, including myositis, peripheral neuropathy, uveitis, arthritis, Sjögren syndrome, and alveolitis, have also been related to HTLV-1 infection (Oh and Jacobson, 2008). HAM/TSP is characterized as a chronic progressive inflammatory disease that initiates as an inflammatory disorder and progresses to a chronic long-term degenerative disease (Araújo et al., 1995). This inflammatory response can be characterized by the presence of HTLV-1-specific antibodies and the infiltration of T lymphocytes into the peripheral blood and cerebrospinal fluid (CSF). In response to the presence of HTLV-1, CNS infiltrating lymphocytes and resident cell populations trigger the release of cytotoxins, particularly pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), to cause an inflammatory response (Umehara et al., 1993, 1994; Jacobson et al., 1992).

It is still unclear why only a small subset of HTLV-1-infected individuals develops clinical diseases whereas the majority remains HTLV-1 asymptomatic carriers (HACs). Previous studies

Abbreviations: MSC, mesenchymal stromal cells; BM, bone marrow; PBMC, peripheral blood mononuclear cells; HAM/TSP, HTLV-associated myelopathy/tropical spastic paraparesis; HAC, HTLV asymptomatic carriers; ATLL, adult T-cell leukemia/lymphoma; CMV, cytomegalovirus; HHV, human herpesvirus, HHV8; PPARγ, peroxisome proliferator-activated receptors gamma; RUNX-2, runt-related transcription factor-2; CD73, NT5E, 5-nucleotidase, ecto; HLA-class I, human major histocompatibility complex, class I; CD45, leukocyte common; CD90, THY1, Thy-1 cell surface antigen; CD105, ENG, endoglin; CD106, VCAM1, vascular cell adhesion molecule 1; CD13, aminopeptidase-N; HLA-ABC, human major histocompatibility complex (MHC) class I, HLA-A, B, C; CD271, NGFR, nerve growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, beta-actin; NRP1, neuropilins 1; HSPG2, heparan sulfate proteoglycan 2; GLUT1, solute carrier family 2 (facilitated glucose transporter), member 1; URE, expression relative units

☆Institution where processing and analysis were performed: Molecular Biology Laboratory, Regional Blood Center of Ribeirão Preto, Rua Tenente Catão Roxo 2501, 14051-140 Ribeirão Preto, São Paulo, Brazil.

* Corresponding author at: Regional Blood Center of Ribeirão Preto, Avenida Tenente Catão Roxo 2501, Ribeirão Preto, São Paulo, Brazil. Fax: +55 16 2101 9309.
E-mail address: skashima@hemocentro.fmrp.usp.br (S. Kashima).

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have suggested that this result is due to a genetic susceptibility associated with deregulated immune responses and an increased viral or proviral load (Nagai et al., 1998).

Mesenchymal stromal cells (MSCs) are multipotent precursor cells with numerous immunomodulatory properties that have been isolated from multiple tissues, such as the bone marrow (BM), adipose tissue, umbilical cord blood, dental pulp, and the connective tissue of almost all organs (Friedenstein et al., 1976; da Silva Meirelles et al., 2006). The minimum criteria for MSC definition include plastic adherence, the surface expression of CD105 (endoglin, SH2), CD73 (ecto-5′-nucleotidase), and CD90 (Thy1), the absence of hematopoietic markers, such as CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR, and their *in vitro* differentiation into chondrogenic, adipogenic, and osteogenic cells (Dominici et al., 2006). These stromal cells have remarkable immunomodulatory and regenerative properties due to the secretion of anti-inflammatory proteins and suppress the activity of various immune cells, such as alloantigen-activated T and B lymphocytes. In contrast, it has been reported that the functionality and ability of MSCs to inhibit immune cells can be modulated by inflammatory mediators released from activated immune cells, such as IFN-γ, IL1β, and TNF-α. MSCs may provide a balance of immune responses by acquiring a pro-inflammatory or an anti-inflammatory capacity depending on the stimulus and the physiological conditions of the organism (Waterman et al., 2010; Auletta et al., 2012).

Due to the biological importance of MSCs, studies have investigated the interaction between MSCs and viruses and have demonstrated that MSCs can be cell targets for several infections (Marandin et al., 1995; Scadden et al., 1990; Smirnov et al., 2007; Choudhary et al., 2011). For example, human immunodeficiency virus (HIV-1) is able to infect bone marrow-derived MSCs, and this infection affects MSC biology, impairing their clonogenic potential, cell differentiation, and phenotype (Wang et al., 2002; Cotter et al., 2008, 2011, 2007). Human vessel wall-derived MSCs are also susceptible to HIV, and this susceptibility is connected with a dysregulation of their survival and differentiation potential (Gibellini et al., 2011). Human cytomegalovirus (CMV) infects MSCs, thereby changing their structure and function, and this process alters their morphological character and impairs their process of differentiation. Therefore, MSCs may be considered a site of CMV latency and reactivation (Smirnov et al., 2007; Wei et al., 2011). Other studies have shown that MSCs are susceptible to herpes simplex virus type-1 (HSV-1) and that this infection induces a cytopathic effect with viral productive replication (Choudhary et al., 2011).

Although MSC are considered a target of several viral infections, there are no reports in the literature on the susceptibility of MSCs to HTLV infection, and the biological effects of HTLV-1 on MSCs are unknown. Therefore, in this study, we demonstrate that HTLV-1 is able to infect human bone-marrow MSCs *in vitro* and induce alterations to the MSC phenotype. The interaction between HTLV-1 and MSC contributes to the current understanding of HTLV-1 dissemination and persistence and provides new information on HTLV-1 tropism and associated diseases.

Results

HTLV-1 exposition modifies MSC morphology

Morphology and ultrastructural analyses were performed to identify alterations in MSCs induced by HTLV-1. For this purpose, three distinct culture conditions were evaluated: (1) MSCs in the presence of 100 ng of cell-free virus, (2) MSCs co-cultured with MT2 cells in a transwell system, and (3) MSCs co-cultured with MT2 cells subjected to irradiation (60,000 Gy). As the control culture, MSCs were grown in the same medium in the absence of virus. The light microscope analyses showed that the MSC morphology was not affected by exposure to HTLV-1. The cells maintained their fibroblastoid-like, spindle-shaped morphology, similar to the control MSCs (Fig. 1A and B). However, the ultrastructural analysis of MSCs cultured in the presence of HTLV-1 showed morphological changes, including an increase in the number of intracellular vesicle structures, compared with the control MSC (Fig. 1C and D). In addition,

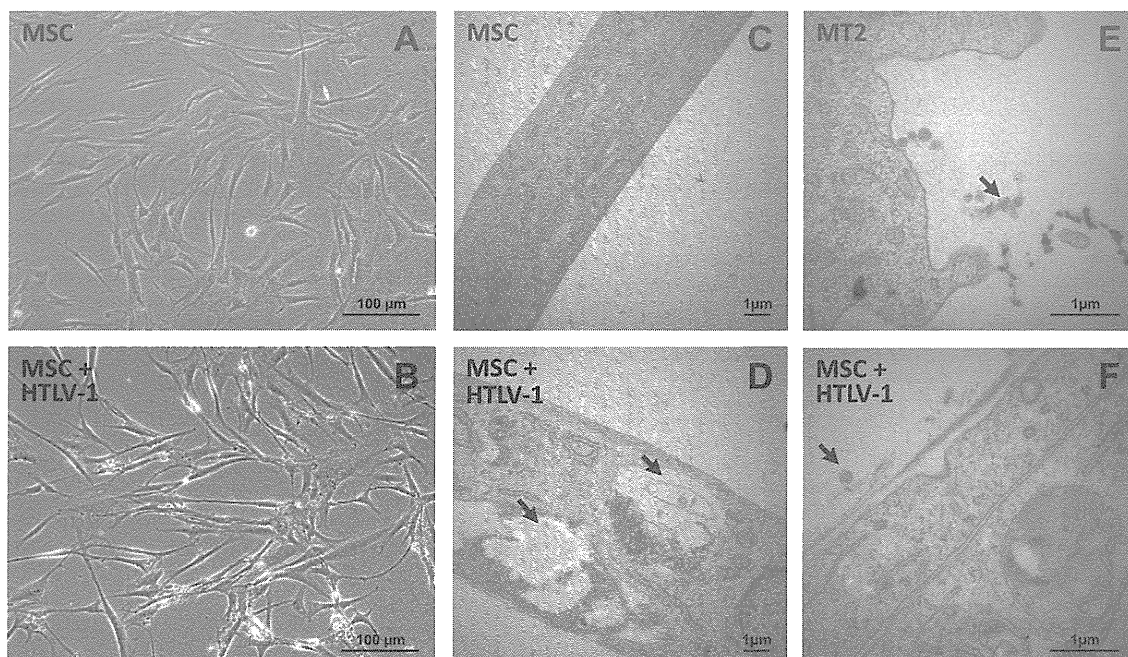

Fig. 1. Characterization of MSCs exposed to HTLV-1. (A and B) Morphological analysis by light microscopy of (A) control bone marrow (BM)-mesenchymal stromal cells (MSCs) and (B) MSCs exposed to HTLV. The scale bars represent 100 μm. (C–F) Ultrastructural analysis by transmission electron microscopy of (C) control MSCs and (D) MSCs exposed to HTLV with numerous intracellular vesicles (indicated by arrows); (E) HTLV-1 particles from MT2 cells are observed in the extracellular space (arrows), and (F) HTLV-1 virus particles are found in close association with MSCs (arrows). Scale bars = 1 μm.

we observed the release of HTLV-1 virions by MT2 cells (Fig. 1E) co-cultured in close proximity to MSCs (Fig. 1F).

MSCs exposed to HTLV-1 showed alterations in the immunophenotypic properties

To investigate the effect of HTLV-1 exposure on the physiological features of MSCs, we analyzed the expression level of MSC surface molecules by flow cytometry. The analysis revealed that the expression of adhesion molecules, such as VCAM-1 (CD106) and ICAM-1 (CD54), were significantly increased in MSCs exposed to HTLV-1 ($p < 0.01$) compared with the controls. The higher expression levels of CD106 and CD54 observed in MSCs cultured with HTLV-1-irradiated cells and in MSCs cultured with cell-free HTLV-1 were similar (Fig. 2A and B). We also observed that the expression of aminopeptidase N (CD13) was 1.3-fold lower in MSCs cultured with irradiated MT2 cells ($p = 0.007$; Fig. 2C). The expression of the MHC class II cell surface receptor (HLA-DR) protein was 7.7-fold higher in the same cell population ($p = 0.0006$). It is important to note that this difference was observed only between MSCs that had direct contact with HTLV-1-infected-cells and MSCs cultured in the absence of HTLV-1 (Fig. 2C and D). Significant differences were also observed in the expression of the NGF receptor p75NTR protein (CD271), which was reduced (2.5-fold) in MSCs cultured with cell-free concentrated HTLV-1 ($p = 0.0247$) compared with MSC control cultures (Fig. 2E). The expression levels of HLA-ABC, CD73, endoglin (CD105), and CD45 were similar ($p > 0.05$) in MSC control cultures and MSCs exposed to HTLV-1 (Supplementary material 1 A–D).

The variations observed in the expression of the surface molecules CD106, CD54, and CD271 were also evaluated by qPCR. The transwell culture resulted in an increase of 5.3- to 2.3-fold in the mRNA expression levels of CD106 and CD54 on MSCs (Supplementary material 2A and 2B). The most significant increase was obtained in the presence of cell-free HTLV-1 and through the direct contact of infected cells and MSCs (6.8- to 18-fold). We also observed that the CD271 gene expression was downregulated (4.5-fold) in MSCs cultured in the presence of HTLV-1 compared with MSCs cultured without HTLV-1 (Supplementary material 2C).

MSC immunophenotypic characteristics are altered by PBMCs from HTLV individuals

Because the group of HTLV-1-infected individuals is composed of both asymptomatic and symptomatic individuals, we examined

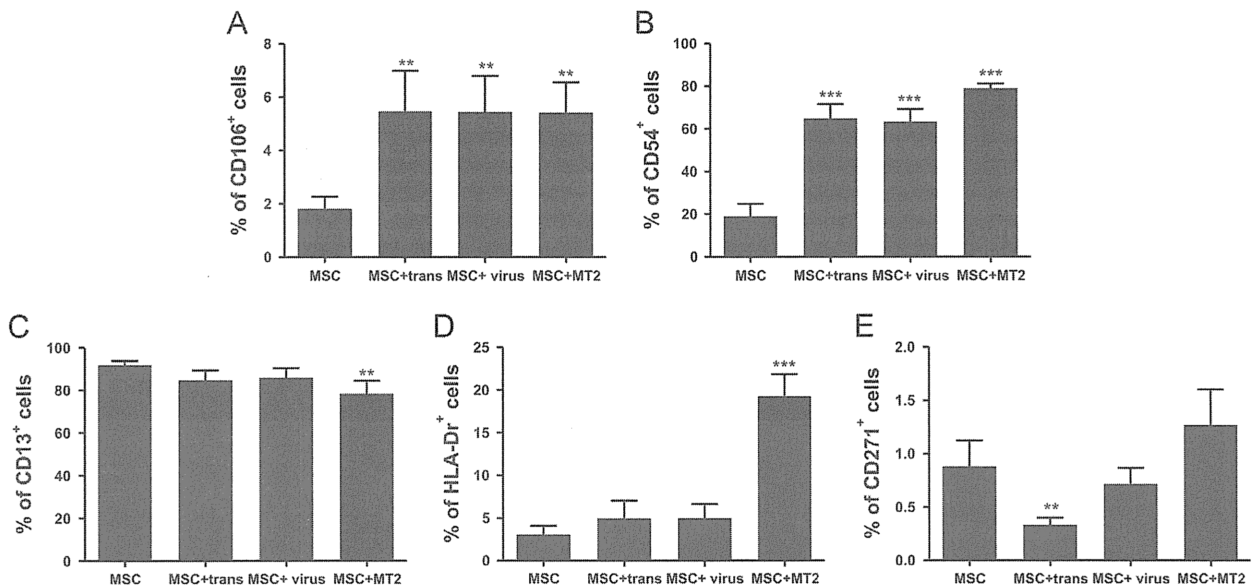

Fig. 2. Effect of HTLV-1 on MSC surface molecule expression. MSCs were cultured for 18 h under different growing conditions: control bone marrow (BM)-mesenchymal stromal cells (MSCs), MSCs cultured with MT2 cells using a transwell system (MSC+trans), MSCs cultured with cell-free HTLV-1 particles (MSC+virus), and MSCs cultured in the presence of irradiated MT2 cells (MSC+MT2). The data are presented as the means ± SEM of the percentage of cells stained with (A) anti-CD106, (B) anti-CD54, (C) anti-CD13, (D) anti-HLA-DR, and (E) anti-CD271, as evaluated by flow cytometry. The results were obtained from at least three independent experiments and evaluated using a one-tail Mann–Whitney t test. The asterisks denote significant differences compared with the MSC control culture (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

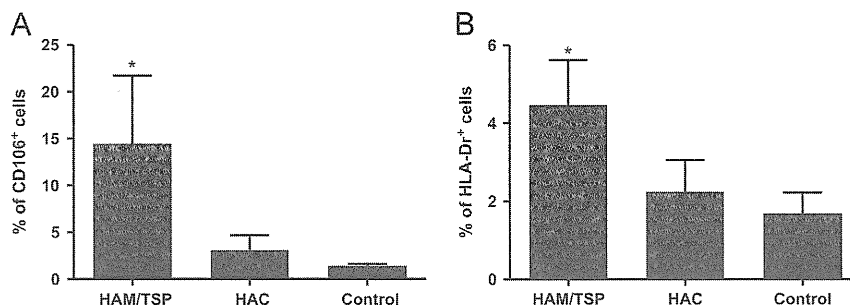

Fig. 3. Immunophenotypic profile of MSCs co-cultured with PBMCs from HTLV-1-infected individuals. MSCs were cultivated with PBMCs isolated from HTLV-1-infected symptomatic individuals (human T lymphotropic virus type 1 (HTLV-1) associated myelopathy/tropical spastic paraparesis; HAM/TSP), asymptomatic HTLV-1 carriers (HAC), and healthy subjects (control) using a transwell system. The data are presented as the means ± SEM of the percentage of cells stained with (A) anti-CD106, (B) anti-CD54, (C) anti-CD271, and (D) anti-HLA-DR antibodies, as evaluated by flow cytometry. The results were obtained from three independent experiments. We used the Mann–Whitney one-tail t test. *$p < 0.05$ compared with healthy subjects (control).

whether the PBMCs isolated from these individuals can modulate the expression of adhesion molecules (CD106 and CD54), HLA-DR, and CD271 in MSCs. All of the individuals included in this assay exhibited a $CD4^+/CD8^+$ ratio within the normal limits. As shown in Fig. 3, CD106 was overexpressed (10-fold) in the MSCs cultured with PBMCs from the HTLV-1 symptomatic group compared with MSCs cultured with cells obtained from healthy subjects or asymptomatic infected individuals ($p=0.0189$; Fig. 3A). The expression of HLA-DR was 2.6-fold higher in MSCs cultured with PBMCs isolated from HTLV-1 symptomatic individuals compared with that obtained in MSCs cultured with cells from healthy subjects or asymptomatic infected individuals ($p=0.0175$; Fig. 3B). The CD54 and CD271 expression levels showed no differences between MSCs cultured with PBMCs from HTLV-1-infected individuals or from healthy subjects (Data not shown).

HTLV-1 exposure does not alter the MSC differentiation potential

We also evaluated the effect of HTLV-1 on the MSC ability to differentiate into adipocytes and osteocytes. For this purpose, MSCs were cultivated with virus particles (using a transwell system or cell-free concentrated HTLV-1) or directly co-cultured with irradiated MT2 cells for a period of 18 h. After this period, the MSCs were differentiated into adipocytes and osteocytes for 22 days using the appropriate induction culture medium. In response to adipogenic differentiation, we observed changes in the cell morphology and the formation of lipid vacuoles, as visualized through Sudan II/Scarlet staining, regardless of the presence or absence of HTLV-1 (Fig. 4C, E, G, and I). The analysis of the peroxisome proliferator-activated receptor gamma (*PPAR-γ*) gene expression showed a 70-fold increase in MSCs subjected to

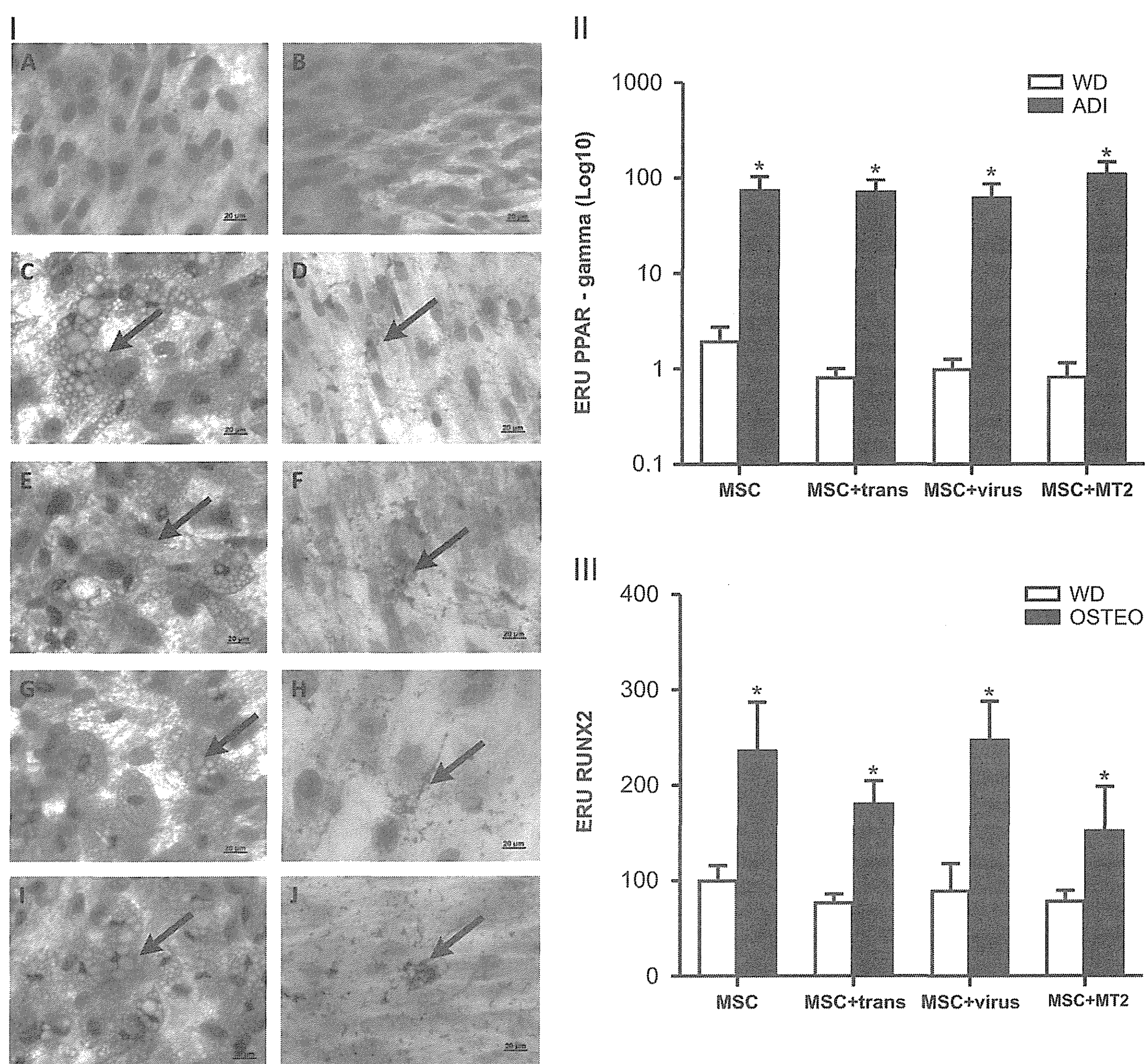

Fig. 4. Adipogenic and osteogenic differentiation of MSCs exposed to HTLV-1. (I) Morphologic analyses of bone marrow (BM)-mesenchymal stromal cells (MSCs) cultured for 18 h in the presence or absence of HTLV-1. MSCs were stained by Sudan II/Scarlet (C, E, G, and I) and the von Kossa method (D, F, H, and J) to evaluate the adipogenic and osteogenic differentiation potential, respectively. (A and B) Control MSCs cultured on standard medium; (C and D) control MSCs; (E and F) MSCs cultured with MT2 cells using a transwell system; (G and H) MSCs cultured with cell-free HTLV-1 particles; and (I and J) MSCs cultured in the presence of irradiated MT2 cells. The arrows indicate lipid vacuoles (C, E, G, and I) and mineralization nodules (D, F, H, and J). The scale bars represent 20 μm. (II) PPARγ gene expression in MSCs during adipogenic differentiation, as determined by qRT-PCR. (III) Level of Runx2 gene expression during osteoblastic differentiation, as determined by qRT-PCR. Control mesenchymal stromal cells (MSCs), MSCs cultured with MT2 cells in a transwell system (MSC+trans), MSCs cultured with cell-free HTLV-1 particles (MSC+virus), and MSCs cultured in the presence of irradiated MT2 cells (MSC+MT2). ADI: adipogenic induction; WD: without differentiation induction; OSTEO: osteogenic induction. The data are reported using expression relative units (ERU), and the results are represented as the means ± SEM ($n=4$ per group). The asterisks denote significant differences relative to the undifferentiated control ($^*p \leq 0.05$).