constructed as follows. The TE671/CD4 cells were inoculated with a CCR5 and puromycin-resistant gene-encoding murine leukemia virus (MLV) vector constructed as reported (Kubo et al., 2003), and were selected with puromycin. Puromycin-resistant cell pool was used in this study. TE671 and 293T cells expressing CD4 and a C-terminally VSV-G-tagged dominant negative mutant of ezrin (EZ-N) was constructed by inoculation of the TE671/CD4 and 293T/CD4 cells with an EZ-N-encoding MLV vector constructed as reported (Kubo et al., 2003), and designated as TE671/CD4/EZ-N and 293T/CD4/EZ-N. The VSV-G-tagged EZ-N plasmid was kindly provided from Dr. M. Arpin.

Transduction assay

To obtain HIV-1 vector particles, 293T cells (5×10^5) were plated onto a 10-cm dish and cultured for 2 days. The 293T cells were transfected with the R8.91, pTY-EFnLacZ, and one of HIV-1 Env expression plasmids. The transfected 293T cells were washed to remove the transfection complex 24 h after transfection, and continued to be cultured in fresh medium for additional 24 h. Culture supernatants of the transfected cells were diluted to make their titer about 60 blue-cell-forming units per a microscopic field in ezrin dominant negative mutant-free or siRNA-free cells, and were inoculated into target cells. Target cells (2×10^5) were plated onto a 6-cm dish and were inoculated 24 h after the plating. The inoculated cells were stained with 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside (X-Gal) (Wako) 2 days after inoculation. Numbers of blue cells were counted to estimate transduction titers.

Western immunoblotting

Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (BioRad), and were transferred onto a PVDF membrane (Millipore). The membrane was treated with an anti-VSV-G (Sigma), -ezrin, -radixin, -moesin, or -actin antibody (Santa Cruz), and then with a horseradish peroxidase-conjugated protein G (BioRad). Protein G-bound polypeptides were visualized by ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

FACS

To analyze cell surface expression of CXCR4 and CCR5, suspended cells were treated with a rat anti-CXCR4 or -CCR5 antibody (Tanaka et al., 2001). The cells were washed with PBS 3 times, and then treated with an FITC-conjugated anti-rat IgG antibody (Sigma). The cells were applied to a flow cytometer (Coulter). To analyze CD4 cell surface expression, cells were treated with an FITC-conjugated anti-CD4 antibody (Sigma).

Transfection of siRNA

Sequences of sense strands of two siRNAs against ezrin were GAAUCCUUAGCGAUGAGAUCU (siRNA-E1) and CCUGAUUCUCGCGAUUAUUCU (siRNA-E2). Sequences of sense strands of siRNAs against radixin and moesin were CGACAAGUUAACACCUAAAU (siRNA-R) and CUCCCAGACGGAUCUGUUGC (siRNA-M). An siRNA against green fluorescence protein (GFP) was used as control, and sequence of the sense strand was CUGGAGUUGUCCCAAUUCUUG. These siRNAs were synthesized by RNAi Co. LTD. Cells were transfected with one of these siRNAs (200 pmol) by the TransIT TKO reagent (10 l) (Mirus). To knockdown expression of all three ERM family proteins simultaneously, cells were co-transfected with three siRNAs (total 600 pmol) by the transfection reagent (30 l).

Semi-quantitative RT-PCR

Total RNA was isolated from siRNA-transfected cells. First strand cDNA was synthesized from the total RNA with ramdom hexamer by a reverse transcriptase (TAKARA). Semi-quantitative PCR was performed using the first strand cDNA as template to detect ezrin, radixin, moesin, and GAPDH mRNAs. Nucleotide sequences of the PCR primers for ezrin mRNA were GCACAAACTTACCAG and TGGTCCTGGCCTGGCTGTTA, for radixin mRNA were GGCAACACAAAGCTTTTGCAG and ATATATGCAAAATAACAGCTCTCA, for moesin mRNA were TGAGGCTGTGGAGTGGCAGCA and CTAGAGGCTGGGTGCCCATTA, and for GAPDH mRNA were AGGTCGGAGTCAACGGATTTGGT and GTGGGCCATGAGGTCCACCAC. These primers were synthesized by Genenet Co. Ltd.

Cell-fusion assay

Receptor protein-expressing cells (2×10^5) were plasted onto a 6-cm dish and cultured for 2 days. The cells were co-transfected with the siRNA and a -galactosidase expression plasmid, in which the -galactosidase gene is under the control of an HIV-1 long terminal repeat (LTR-LacZ), by the TransIT TKO transfection reagent (Mirus). 293T cells (2×10^5) were plated onto a 6-cm dish and cultured for 2 days. The cells were transfected with a Tat expression plasmid as control or the HIV-1 Env expression plasmid by the Lipofectamine transfection reagent (Invitrogen). The Env expression plmasids additionally encodes the tat gene. The transfected recipient cells (5×10^5) were added onto the HIV-1 Env-expressing 293T cells 24 h after transfection. If these cells fuse, the Tat protein activates the -galactosidase expression. -Galactosidase activity of cell lysates was measured 24 h after mixed culture by the high sensitive -galactosidase activity kit (Stratagene).

Statistical analysis

Differences between two groups of data were determined by the Student's t-test. Statistical significance was set at $P<0.05$ for all tests.

Acknowledgments

We thank Dr. U. Hazan for the NDK Env expression plasmid, Dr. Y. Yokomaku for the HXB2, JRFL, NH1, and NH2 Env expression plasmids, Dr. D. Trono for the R8.91 plasmid, Dr. L. Chang for the VSV-G expression plasmid and pTY-EFnLacZ,

and Dr. M. Arpin for the expression plasmids of the wild type and dominant negative forms of ezrin. The VSV-G, TAT, and LacZ-containing HIV-1 vector genome expression plasmids were onbatined through AIDS Research and Reference Reagent Program, NIAID, NIH, USA. This study was supported by a Health Science Research Grant from the Ministry of Health, Labour, and Welfare of Japan and by the 21st century Centers of Excellence (COE) program.

References

Algrain, M., Turunen, O., Vaheri, A., Louvard, D., Arpin, M., 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J. Cell Biol. 120 (1), 129–139.

Chang, L.J., Urlacher, V., Iwakuma, T., Cui, Y., Zucali, J., 1999. Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. Gene Ther. 6 (5), 715–728.

Das, V., Nal, B., Roumier, A., Meas-Yedid, V., Zimmer, C., Olivo-Marin, J.C., Roux, P., Ferrier, P., Dautry-Varsat, A., Alcover, A., 2002. Membrane-cytoskeleton interactions during the formation of the immunological synapse and subsequent T-cell activation. Immunol. Rev. 189, 123–135.

Doi, Y., Itoh, M., Yonemura, S., Ishihara, S., Takano, H., Noda, T., Tsukita, S., 1999. Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. J. Biol. Chem. 274 (4), 2315–2321.

Dumonceaux, J., Nisole, S., Chanel, C., Quivet, L., Amara, A., Baleux, F., Briand, P., Hazan, U., 1998. Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. J. Virol. 72 (1), 512–519.

Faure, S., Salazar-Fontana, L.I., Semichon, M., Tybulewicz, V.L., Bismuth, G., Trautmann, A., Germain, R.N., Delon, J., 2004. ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. Nat. Immunol. 5 (3), 272–279.

Fievet, B., Louvard, D., Arpin, M., 2006. ERM proteins in epithelial cell organization and functions. Biochim. Biophys. Acta.

Gupta, N., Wollscheid, B., Watts, J.D., Scheer, B., Aebersold, R., DeFranco, A.L., 2006. Quantitative proteomic analysis of B cell lipid rafts reveals that ezrin regulates antigen receptor-mediated lipid raft dynamics. Nat. Immunol. 7 (6), 625–633.

Iwakuma, T., Cui, Y., Chang, L.J., 1999. Self-inactivating lentiviral vectors with U3 and U5 modifications. Virology 261 (1), 120–132.

Iyengar, S., Hildreth, J.E., Schwartz, D.H., 1998. Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. J. Virol. 72 (6), 5251–5255.

Jimenez-Baranda, S., Gomez-Mouton, C., Rojas, A., Martinez-Prats, L., Mira, E., Ana Lacalle, R., Valencia, A., Dimitrov, D.S., Viola, A., Delgado, R., Martinez, A.C., Manes, S., 2007. Filamin-A regulates actin-dependent clustering of HIV receptors. Nat. Cell Biol. 9 (7), 838–846.

Jolly, C., Kashefi, K., Hollinshead, M., Sattentau, Q.J., 2004. HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. J. Exp. Med. 199 (2), 283–293.

Jolly, C., Sattentau, Q.J., 2005. Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity. J. Virol. 79 (18), 12088–12094.

Kameoka, M., Kitagawa, Y., Utachee, P., Jinnopat, P., Dhepakson, P., Isarangkura-na-ayuthaya, P., Tokunaga, K., Sato, H., Komano, J., Yamamoto, N., Oguchi, S., Natori, Y., Ikuta, K., 2007. Identification of the suppressive factors for human immunodeficiency virus type-1 replication using the siRNA mini-library directed against host cellular genes. Biochem. Biophys. Res. Commun. 359 (3), 729–734.

Kikuchi, S., Hata, M., Fukumoto, K., Yamane, Y., Matsui, T., Tamura, A., Yonemura, S., Yamagishi, H., Keppler, D., Tsukita, S., Tsukita, S., 2002. Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. Nat. Genet. 31 (3), 320–325.

Kizhatil, K., Albritton, L.M., 1997. Requirements for different components of the host cell cytoskeleton distinguish ecotropic murine leukemia virus entry via endocytosis from entry via surface fusion. J. Virol. 71 (10), 7145–7156.

Kozak, S.L., Heard, J.M., Kabat, D., 2002. Segregation of CD4 and CXCR4 into distinct lipid microdomains in T lymphocytes suggests a mechanism for membrane destabilization by human immunodeficiency virus. J. Virol. 76 (4), 1802–1815.

Kubo, Y., Ishimoto, A., Amanuma, H., 2003. N-Linked glycosylation is required for XC cell-specific syncytium formation by the R peptide-containing envelope protein of ecotropic murine leukemia viruses. J. Virol. 77 (13), 7510–7516.

Kusagawa, S., Sato, H., Tomita, Y., Tatsumi, M., Kato, K., Motomura, K., Yang, R., Takebe, Y., 2002. Isolation and characterization of replication-competent molecular DNA clones of HIV type 1 CRF01_AE with different coreceptor usages. AIDS Res. Hum. Retrovir. 18 (2), 115–122.

Lehmann, M.J., Sherer, N.M., Marks, C.B., Pypaert, M., Mothes, W., 2005. Actin- and myosin-driven movement of viruses along filopodia precedes their entry into cells. J. Cell Biol. 170 (2), 317–325.

Naghavi, M.H., Valente, S., Hatziioannou, T., de Los Santos, K., Wen, Y., Mott, C., Gundersen, G.G., Goff, S.P., 2007. Moesin regulates stable microtubule formation and limits retroviral infection in cultured cells. EMBO J. 26 (1), 41–52.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272 (5259), 263–267.

Nguyen, D.H., Giri, B., Collins, G., Taub, D.D., 2005. Dynamic reorganization of chemokine receptors, cholesterol, lipid rafts, and adhesion molecules to sites of CD4 engagement. Exp. Cell Res. 304 (2), 559–569.

Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., Kabat, D., 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. 72 (4), 2855–2864.

Pontow, S.E., Heyden, N.V., Wei, S., Ratner, L., 2004. Actin cytoskeletal reorganizations and coreceptor-mediated activation of rac during human immunodeficiency virus-induced cell fusion. J. Virol. 78 (13), 7138–7147.

Rana, T.M., 2007. Illuminating the silence: understanding the structure and function of small RNAs. Nat. Rev., Mol. Cell Biol. 8 (1), 23–36.

Roumier, A., Olivo-Marin, J.C., Arpin, M., Michel, F., Martin, M., Mangeat, P., Acuto, O., Dautry-Varsat, A., Alcover, A., 2001. The membrane-microfilament linker ezrin is involved in the formation of the immunological synapse and in T cell activation. Immunity 15 (5), 715–728.

Saotome, I., Curto, M., McClatchey, A.I., 2004. Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine. Dev. Cell 6 (6), 855–864.

Steffens, C.M., Hope, T.J., 2003. Localization of CD4 and CCR5 in living cells. J. Virol. 77 (8), 4985–4991.

Steffens, C.M., Hope, T.J., 2004. Mobility of the human immunodeficiency virus (HIV) receptor CD4 and coreceptor CCR5 in living cells: implications for HIV fusion and entry events. J. Virol. 78 (17), 9573–9578.

Takeuchi, K., Sato, N., Kasahara, H., Funayama, N., Nagafuchi, A., Yonemura, S., Tsukita, S., Tsukita, S., 1994. Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. J. Cell Biol. 125 (6), 1371–1384.

Tanaka, R., Yoshida, A., Murakami, T., Baba, E., Lichtenfeld, J., Omori, T., Kimura, T., Tsurutani, N., Fujii, N., Wang, Z.X., Peiper, S.C., Yamamoto, N., Tanaka, Y., 2001. Unique monoclonal antibody recognizing the third extracellular loop of CXCR4 induces lymphocyte agglutination and enhances human immunodeficiency virus type 1-mediated syncytium formation and productive infection. J. Virol. 75 (23), 11534–11543.

Tsukita, S., Yonemura, S., Tsukita, S., 1997. ERM proteins: head-to-tail regulation of actin–plasma membrane interaction. Trends Biochem. Sci. 22 (2), 53–58.

Viard, M., Parolini, I., Sargiacomo, M., Fecchi, K., Ramoni, C., Ablan, S., Ruscetti, F.W., Wang, J.M., Blumenthal, R., 2002. Role of cholesterol in human immunodeficiency virus type 1 envelope protein-mediated fusion with host cells. J. Virol. 76 (22), 11584–11595.

Yokomaku, Y., Miura, H., Tomiyama, H., Kawana-Tachikawa, A., Takiguchi, M., Kojima, A., Nagai, Y., Iwamoto, A., Matsuda, Z., Ariyoshi, K., 2004. Impaired processing and presentation of cytotoxic-T-lymphocyte (CTL) epitopes are major escape mechanisms from CTL immune pressure in human immunodeficiency virus type 1 infection. J. Virol. 78 (3), 1324–1332.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 24, Number 3, 2008

DOI: 10.1089/aid.2007.0092

Enhancement of OX40-Induced Apoptosis by TNF Coactivation in OX40-Expressing T Cell Lines *in Vitro* Leading to Decreased Targets for HIV Type 1 Production

YOSHIAKI TAKAHASHI,[1,*] REIKO TANAKA,[1] NAOKI YAMAMOTO,[2] and YUETSU TANAKA[1]

ABSTRACT

OX40, a member of the tumor necrosis factor receptor (TNF-R) superfamily, has been shown to play an important role in the survival of antigen-specific CD4$^+$ T cells. We have previously reported that stimulation of the OX40-expressing and HIV-1 chronically infected T cell line, ACH-2/OX40, with either OX40 ligand (OX40L)-expressing cells or with TNF resulted in the activation of HIV-1 followed by apoptotic cell death. In the present study we found that costimulation via OX40 and TNF-R in OX40-expressing HIV-1-infected T cell lines leads to a marked reduction of HIV-1 production associated with rapid cell death. Since HIV-1-negative OX40$^+$ T cell lines underwent rapid apoptotic cell death after OX40L and TNF stimulation, it was reasoned that the ACH-2/OX40 cell death was unlikely to be due to HIV-1 infection. Furthermore, we found that the OX40-mediated apoptosis of the CD4$^+$ T cell line, Molt-4/CCR5-OX40 (M/R5-OX40), required (1) signals mediated via the cytoplasmic tail of OX40, (2) activation of the caspase cascade, including caspase-8 and caspase-3, and (3) induction of endogenous TNF-α, but not of TNF-β, FasL, or TNF-related apoptosis-inducing ligand (TRAIL), suggesting that this apoptosis occurred indirectly via the TNF/TNF-R system. Finally, a fraction of primary activated CD4$^+$ T cells, expressing high levels of OX40, underwent apoptosis, as revealed by annexin V staining, after cocultivation with OX40L$^+$ cells. These results suggest a new biological role of the OX40L/OX40 system in controlling the fate of activated CD4$^+$ T cells and of controlling HIV-1 infection in inflammatory environments.

INTRODUCTION

OX40 (CD134) is a 50-kDa transmembrane protein that serves as a marker of activated T cells. It is a member of the tumor necrosis factor receptor (TNF-R) superfamily, a family that also includes TNF-R1, TNF-R2, CD30, CD40, CD27, CD95 (Fas), TNF-related apoptosis-inducing ligand receptor (TRAIL-R) 1, and TRAIL-R2.[1–3] Its ligand, in humans, OX40L (CD252), was originally identified on human T cell leukemia virus type-1 (HTLV-I)-infected T cell lines and was termed gp34.[4,5] OX40L belongs to the tumor necrosis factor (TNF) superfamily[6] and is expressed predominantly on normal activated dendritic cells (DCs),[7] B cells,[8,9] vein endothelial cells,[10] and stimulated monocytes.[11] OX40-mediated costimulation of CD4$^+$ T cells by OX40L induces nuclear factor-kappa B (NF-κB) activation through TNF-R-associated factor (TRAF)2 and TRAF5,[12] and is associated with a number of immune function activities. These include the enhanced synthesis of T helper (Th) 2 responses from naive CD4$^+$ T cells,[13–15] the production of both Th1 and Th2 cytokines,[16–18] the development and survival of memory CD4$^+$ T cells,[17] the prevention of peripheral CD4$^+$ T cell tolerance,[19] and the ability to block the inhibitory activity of CD4$^+$ CD25$^+$ T regulatory cells.[20] Ligation of OX40L on activated B cells and on immature DC, *in vitro*, results in enhanced immunoglobulin production[9] and maturation of DC,[7] respectively. In addition to these costimulatory functions, additional OX40/OX40L functions include not only the promotion of cell-to-cell adhesion between activated or HTLV-I$^+$ leukemic CD4$^+$ T cells and OX40L$^+$ vein endothelial cells,[21] but also the migration of CD4$^+$ T cells to B cell follicles in pe-

[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan.
[2]AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan.
*Present address: AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan.

ripheral lymph nodes.[22] The failure to properly control OX40/OX40L interaction has been suggested to cause immune abnormalities such as autoimmune diseases,[23–25] allergy,[26,27] or defective protection against pathogens.[15,28–30]

In addition to its ability to induce cell activation, the TNF-R superfamily is also associated with promoting cell death. One group of the TNF-R superfamily, consisting of Fas, TNF-R1, TRAIL-R1, and TRAIL-R2, mediates cell death through their intracytoplasmic death domain (DD). A second group, consisting of the DD-lacking receptors, TNF-R2, CD27, CD30, CD40, 4-1BB, and OX40, is capable of inducing cell death under certain conditions. Thus, for example, activation of TNF-R2 triggers apoptosis of a rhabdomyosarcoma cell line and of HeLa cells.[31] The activation of CD27 induces apoptosis of B cell lines[32] and the activation of CD30 by specific antibody mediates apoptosis of an anaplastic large cell lymphoma[33] and of a T cell hybridoma costimulated with anti-T cell receptor (TCR).[34] In addition, the activation of CD40 by antibody induces apoptosis in transformed cell lines and in normal activated $CD4^+$ T cells costimulated with anti-CD3 antibody.[31,35] The precise mechanisms of cell death induced by the DD-lacking TNF-R superfamily remain to be elucidated. Recently, it has been shown that TNF-R2 stimulation causes TNF-R1-dependent apoptosis by the depletion of the antiapoptotic proteins TRAF2 and IAP.[36,37] We have previously shown that OX40 stimulation activates human immunodeficiency virus type-1 (HIV-1) production in the chronically HIV-1-infected T cell line ACH-2/OX40 through the activation of NF-κB.[38] This mechanism is consistent with another member of the TNF-R superfamily, CD30.[39] Following stimulation with either OX40L or TNF, ACH-2/OX40 cells undergo not only HIV-1 activation but also apoptotic cell death within 48 h.[38]

In the present study, we examined the fate of cells following either OX40 stimulation alone or stimulation with OX40 in combination with TNF. Surprisingly, costimulation resulted in a marked decrease of HIV-1 production, as a consequence of rapid cell death of the T cell line. The cell death was reasoned not to be secondary to HIV-1 infection since OX40 stimulation alone and/or costimulation with TNF also induced cell death of HIV-1-negative T cell lines. Furthermore, the cell death in the T cell lines via OX40 stimulation was mediated indirectly by the TNF/TNF-R system. These observations suggest a new immunological role of OX40.

MATERIALS AND METHODS

Reagents

The medium used consisted of RPMI 1640 medium (Sigma, St. Louis, MO), supplemented with 10% fetal calf serum (FCS; Sigma), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (hereinafter called RPMI medium). Antihuman (h) CD3 (clone OKT-3) and anti-hCD4 (clone OKT-4) monoclonal antibodies (mAbs) were purchased from the American Type Culture Collection (Rockville, MD). Anti-hOX40 mAb (clone B-17D8; mouse IgG2b, κ) was newly generated by a previously described method.[38] Anti-hTNF-α, anti-hTNF-β, and anti-hTRAIL mAbs, for neutralization, were purchased from R&D (Minneapolis, MN). Anti-hFasL neutralizing mAb was purchased from BD Pharmingen (San Diego, CA). Anti-hCaspase-8 and anti-hCaspase-3 mAbs were purchased from Cell Signaling (Danvers, MA) and horseradish peroxidase (HRPO)-conjugated goat antimouse immunoglobulin G (IgG) Ab was purchased from Chemicon (Temecula, CA). Anti-hOX40L neutralizing mAb (clone 5A8)[40] and isotype control mouse IgG1 (mIgG1), anti-HTLV-I Tax mAb (clone TAXY-8),[41] have been described previously. Isotype control mIgG2a and mIgG2b were purchased from BD Pharmingen and ImmunoTools (Friesoythe, Germany), respectively. Recombinant human TNF-α (rhTNF-α), TNF-α (rhTNF-β), interleukin-4 (rhIL-4), and rhIL-12 were purchased from Peprotec (London, UK). rhIL-2 was purchased from Shionogi Pharmaceutical (Osaka, Japan). The broad-spectrum caspase inhibitor, z-VAD-fmk, was purchased from MBL (Nagoya, Japan), dissolved in dimethyl sulfoxide and diluted in medium prior to use. Apoptosis was assessed by the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kit (Sigma and R&D). The hTNF-α sandwich enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D. The advanced protein assay reagent was purchased from Cytoskeleton (Denver, CO).

Cells

The hOX40-transfected and the HIV-1 chronically infected human T cell line cells, ACH-2/OX40 (cell groups 4 and 10), and its vector control, ACH-2/control (mock), and the hOX40L-tranfected mouse SV-T2 cell line, SV-T2/OX40L (gp34), and its vector control, SV-T2/control (mock), have been previously described.[38] The cell lines utilized for transfections of OX40, OX40L, and control vector were the human T cell lines Molt-4/CCR5[42] [Molt-4/CCR5-OX40 (M/R5-OX40), -OX40L, and -control], CEM (CEM/OX40, /OX40L, and /control), Jurkat (Jurkat/OX40, /OX40L, and /control), and HIV-1 productively infected human T cell line Molt-4/IIIB [Molt-4/IIIB-OX40 (M/IIIB-OX40), -OX40L, and -control], the human promonocytic cell lines U937 (U937/OX40, /OX40L, and /control), THP-1 (THP-1/OX40, /OX40L, and /control), and HIV-1 chronically infected human promonocytic cell line U1 (U1/OX40, /OX40L, and /control), and the human B cell line BJAB (BJAB/OX40, /OX40L, and /control). Aliquots of each of these cell lines were transfected by electroporation of 10–15 μg of the individual plasmids, pCAGIPuro/OX40, pCAGIPuro/OX40L, and control pCAGIPuro, as previously described.[38] In addition, the Molt-4/CCR5 cell line was also transfected with an expression vector containing the OX40 cytoplasmic tail-deleted mutant, pCAGIPuro/OX40-del(6-725), constructed as previously described,[38] to generate Molt-4/CCR5-OX40del cells. For the selection of transfectants, 1 μg/ml puromycin (Wako, Osaka, Japan) was added to the culture media. Expression of OX40 or OX40L in selectively grown cells was determined by flow cytometric analysis, as previously described.[38]

Detection of cell death and apoptosis

SV-T2/OX40L and SV-T2/control stimulator cells were fixed with 4% paraformaldehyde (PFA) for 15 min and washed three times in phosphate-buffered saline (PBS) prior to use. The OX40-expressing responder cells, at 4×10^5 cells/ml, were cocultured with various ratios of PFA-fixed stimulator cells in RPMI medium in the presence or absence of rhTNF-α (2 ng/ml)

or in the presence or absence of rhTNF-β (10 ng/ml) in 48-well culture plates (0.5 ml/well). The cocultures were incubated overnight, or for the indicated periods, at 37°C in 5% CO_2 humidified atmosphere. For blocking of the OX40–OX40L interaction, anti-hOX40L mAb (5A8) was added at a final concentration of 10–40 μg/ml at 37°C for 1 h prior to coculture. In some cases, anti-hTNF-α, anti-hTNF-β, anti-hTRAIL, and anti-hFasL blocking mAbs were added at a final concentration of 30–100 μg/ml at 37°C for 1 h prior to coculture. For inhibition of the caspase-dependent pathways of apoptosis, z-VAD-fmk was included in the culture medium at concentrations of 100 μM. The viability of the responder cells was determined, in triplicate, using a hemocytometer after staining with 0.1% eosin-Y (Wako, Osaka, Japan). The eosin-Y-stained PFA-fixed stimulator SV-T2/OX40L or SV-T2/control cells could be easily distinguished from dead responder cells by their distinct morphology. Apoptotic and necrotic cells were detected by staining with annexin V-FITC and PI, according to the manufacturer's instructions, utilizing a FACSCalibur (Becton Dickinson, San Jose, CA). The data obtained were analyzed using CellQuest software (Becton Dickinson).

HIV-1 production assay

Production of HIV-1 was determined by the measurement of HIV-1 core p24 levels using a commercial ELISA kit (ZeptoMetrix Corporation, Buffalo, NY). Data were analyzed by the Student's *t*-test. To examine the effect of OX40L and/or TNF stimulation on HIV-1 acutely infected T cell lines, M/R5-OX40 and M/R5-control were infected with the HIV-1 molecular clone NL4-3[43] at a multiplicity of infection (MOI) of 0.01 in 0.1 ml for 3 h at 37°C, as previously described.[44] The infected cells were subsequently washed twice and cultured at 2×10^5 cells/ml for 24 h in 48-well culture plates (0.5 ml/well). The infected cells were cocultured with PFA-fixed SV-T2/OX40L or with PFA-fixed SV-T2/control cells at a cell-to-cell ratio of 2:1 in the presence or in the absence of rhTNF-α (2 ng/ml), for an additional 3 days. Cell-free supernatant fluid was collected and the levels of p24 were quantified.

Western blotting

Western blot analysis was performed as previously described.[38] Briefly, M/R5-OX40 cells (4×10^5 cells/well) were stimulated by PFA-fixed SV-T2/OX40L or by PFA-fixed SV-T2/control cells (2×10^5 cells/well) for 6 h in 12-well plates. Cell lysates were obtained by lysis of 2.5–4 $\times 10^7$ cells in 1 ml of a lysis buffer (10 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) on ice for 20 min, followed by centrifugation at $13,000 \times g$ for 10 min at 4°C. The cell lysates (6 μg protein/lane) were treated with an equal volume of 2× sample buffer [125 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1% bromophenol blue] without 2-mercaptoethanol, separated by SDS–polyacrylamide gel electrophoresis (PAGE), using a 12.5% gel, and then transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MA). The membrane was blocked with a blocking buffer [1% bovine serum albumin (BSA) in PBS] at 4°C overnight and incubated with the primary anti-hCaspase-8 and anti-hCaspase-3 mAbs (1:1000) according to the manufacturer's instructions. Mem-

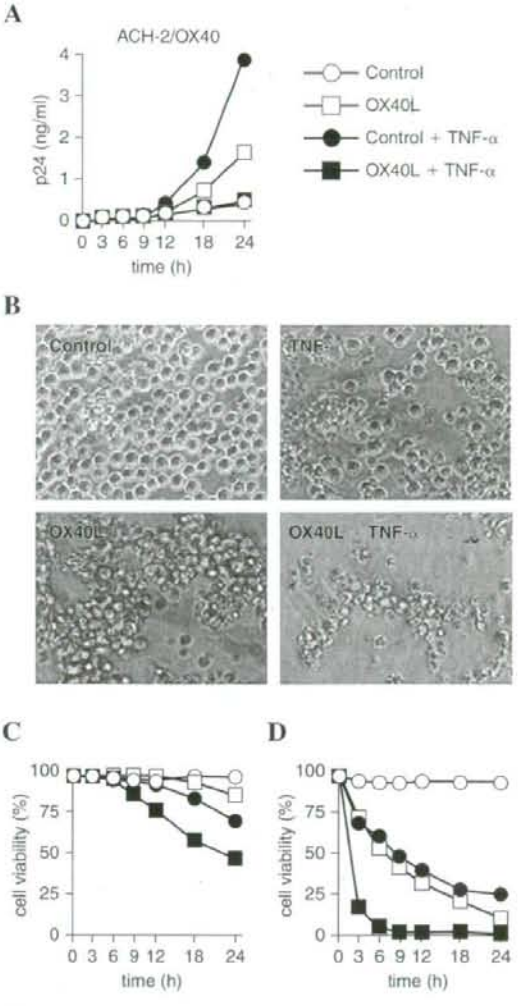


FIG. 1. Marked reduction of HIV-1 production and induction of rapid apoptosis by dual stimulation of ACH-2/OX40 cells with OX40L and TNF. ACH-2/OX40 cells (cell group 10) were cocultured with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells in the presence or in the absence of TNF-α at 2 ng/ml for 24 h. (**A**) The kinetics of HIV-1 production, as determined by the level of HIV-1 p24 in the culture supernatants, (**B**) microscopic observation of morphological changes, (**C**) the kinetics of cell death as determined by an eosin-Y dye exclusion assay, and (**D**) the kinetics of apoptosis as determined by annexin V/PI staining. Morphological changes were examined under an inverted microscope at 100× original magnification. The cell viability was shown as percentage of control. Representative results from three independent experiments are shown. The data presented are the mean values ± SD of triplicate determinations.

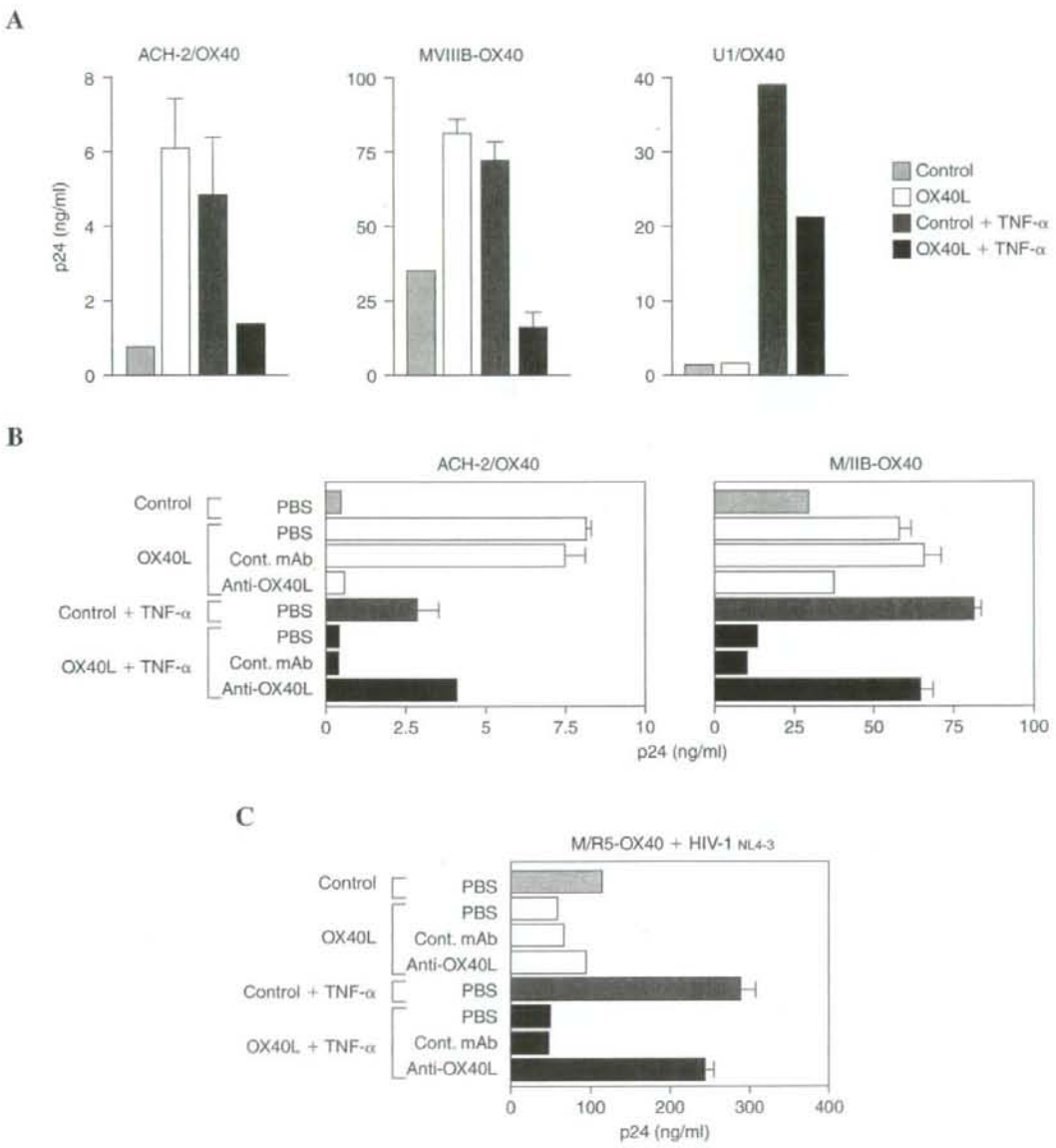


FIG. 2. Reduction of HIV-1 production in various HIV-1-infected cell lines. Several OX40-expressing cell lines were cocultured with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells in the presence or absence of TNF-α (2 ng/ml) for 24 h. The levels of HIV-1 p24 produced in the culture supernatants were determined by ELISA. (**A**) Representative data was obtained using the HIV-1 chronically infected ACH-2/OX40 cells (cell group 4), the HIV-1 productively infected Molt-4/IIIB-OX40 cells (M/IIIB-OX40), and the HIV-1 chronically infected U1/OX40 cells. (**B**) The blocking effect of the anti-OX40L mAb (Anti-OX40L, 10 μg/ml for ACH-2/OX40 and 40 μg/ml for M/IIIB-OX40), negative control mAb (Cont. mAb, the same conditions as above) or of PBS was determined using ACH-2/OX40 and M/IIIB-OX40 cells. (**C**) The Molt-4/CCR5-OX40 cells (M/R5-OX40) were acutely infected with HIV-1_{NL4-3} at an MOI of 0.01, were precultured for 24 h, and were stimulated with OX40L and/or with TNF for an additional 72 h. The blocking effect of equal volumes of anti-OX40L mAb (Anti-OX40L, 10 μg/ml), negative control mAb (Cont. mAb, 10 μg/ml), or of PBS was determined. The data presented are the mean values $\pm$ SD of triplicate determinations. Representative results from three independent experiments are shown.

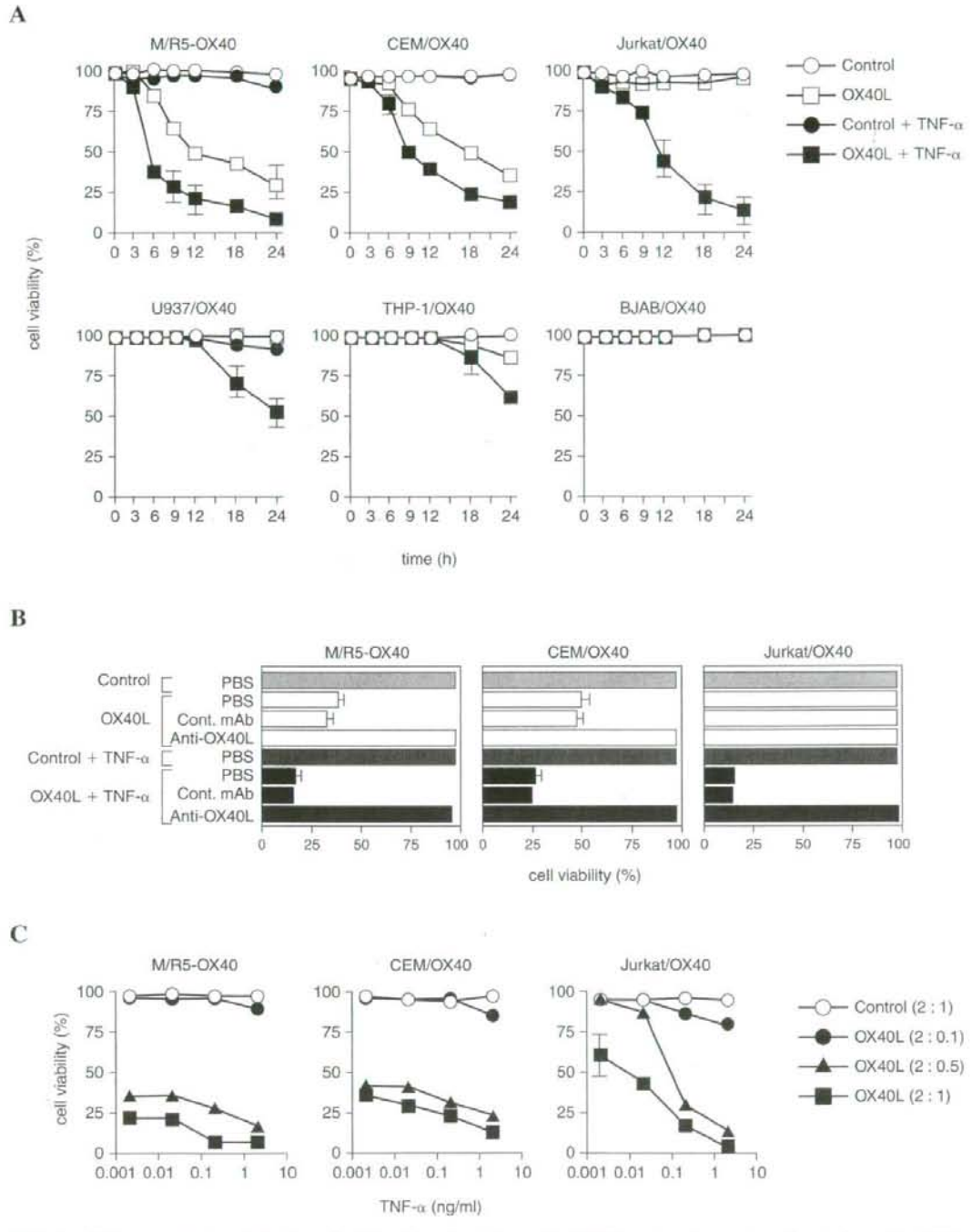
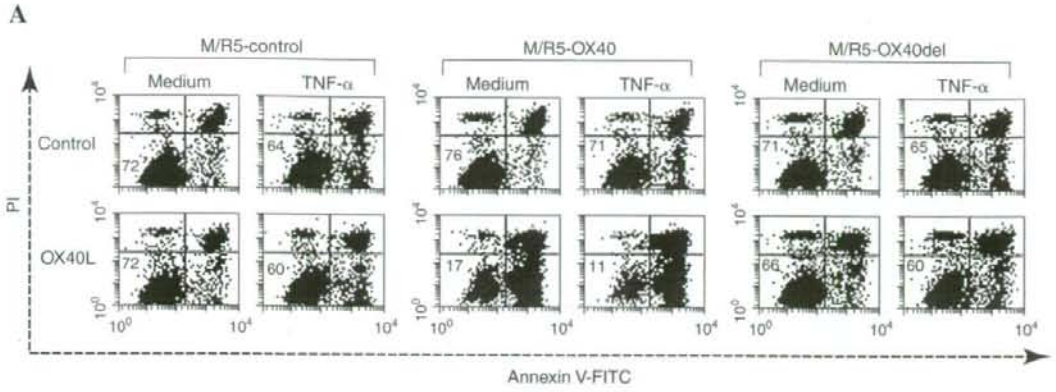
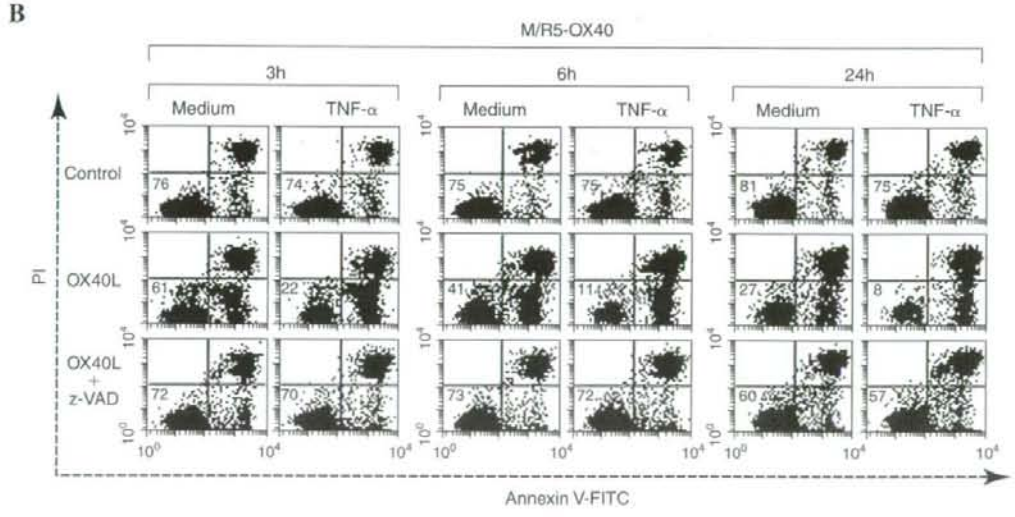
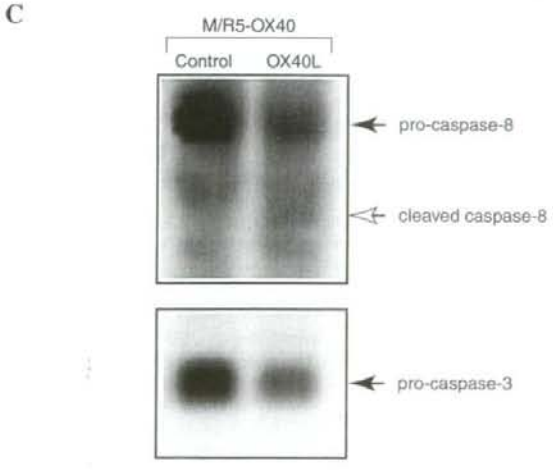


FIG. 3. Cell type-dependent induction of cell death by stimulation with OX40L alone or by combined activation with OX40L and TNF. Cell viability was determined by an eosin-Y dye exclusion assay, 24 h after stimulation by coculture with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells in the presence or absence of TNF-α (2 ng/ml or graded concentrations). (**A**) The three CD4$^+$ T cell lines, Molt-4/CCR5-OX40 (M/R5-OX40), CEM/OX40, and Jurkat/OX40, the two promonocytic cell lines U937/OX40 and THP-1/OX40, and the B cell lines BJAB/OX40 were examined. (**B**) The blocking effect of anti-hOX40L mAb (Anti-OX40L, 10 μg/ml) on cell death of OX40-expressing CD4$^+$ T cell lines was determined. (**C**) The cell death of OX40-expressing CD4$^+$ T cell lines was induced by the addition of various concentrations of TNF-α, up to 2 ng/ml, and by various ratios of stimulator to responder cell. Data presented are the mean values ± SD of triplicate determinations. Representative results from three independent experiments are shown.


A
M/R5-control
M/R5-OX40
M/R5-OX40del
Medium
TNF-α
Control
OX40L
PI
Annexin V-FITC


B
M/R5-OX40
3h
6h
24h
Medium
TNF-α
Control
OX40L
OX40L + z-VAD
PI
Annexin V-FITC


C
M/R5-OX40
Control
OX40L
pro-caspase-8
cleaved caspase-8
pro-caspase-3

branes were then incubated with HRPO-conjugated antimouse IgG Ab (1:2000). Signals were detected using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and the LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

TNF-α assay

As described above, cells (4×10^5 cells/well) were stimulated by PFA-fixed SV-T2/OX40L or by PFA-fixed SV-T2/control cells (2×10^5 cells/well), for the times indicated, in 12-well plates. For the determination of TNF-α production, culture supernatants and cell lysates ($5\text{-}6 \times 10^6$ cells/ml) were collected, and the concentrations of TNF-α were assayed using the Quantikine human TNF-α kit (R&D). The protein concentrations in cell lysates were determined using the advanced protein assay reagent.

Detections of apoptosis in primary T cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (5 U/ml) blood of normal healthy donors using standard density gradient centrifugation and a human lymphocyte separation medium (Sigma). The cells at the interface were collected and washed three times in PBS containing 2% FCS. PBMCs were resuspended at 1×10^6 cells/ml in RPMI medium, supplemented with 20 U/ml rhIL-2. Each well of 12-well plates was coated with 5 μg/ml anti-hCD3 mAb (clone OKT-3) for 1 h at 37°C and washed three times in PBS. Then, 1 ml of the cell suspension was dispensed into individual wells and cultured in the presence of either rhIL-12 or rhIL-4 at 20 ng/ml for 3 days at 37°C in a 5% CO_2 humidified incubator. The activated cells were harvested, adjusted to 2×10^5 cells/ml, and further stimulated using the same conditions at days 3 and 6. Activated PBMCs were harvested on day 9, in 20 U/ml rhIL-2-containing RPMI medium, and were then cocultivated for 24 h with either PFA-fixed SV-T2/OX40L or with PFA-fixed SV-T2/control cells at a cell to cell ratio of 1:1. Anti-hOX40L mAb (5A8) was added at 20 μg/ml to prevent OX40–OX40L interaction. The cells were then stained by Cy5-labeled anti-hCD4 mAb (OKT-4) followed by FITC-labeled annexin V to detect apoptosis. Apoptotic cells in the $CD4^+$ T cell gate were detected by FACSCalibur.

RESULTS

Combined stimulation of ACH-2/OX40 cell line cells with OX40L and TNF decreases HIV-1 production by inducing rapid apoptosis

We have previously reported that the chronically HIV-1-infected cell line ACH-2/OX40 produces large amounts of HIV-1 within 24 h, following stimulation by either coculture with OX40L-expressing cells or by the addition of TNF-α or -β. Such activation of HIV-1 replication is mediated primarily through activation of the NF-κB pathway.[38] In the present study, we examined the effect of dual stimulation by OX40L and TNF-α (or TNF-β, data not shown) on HIV-1 production. In contrast to the previously documented increase in HIV-1 production by activation via the ligation of either OX40 or TNF-R alone, dual stimulation of these two receptors resulted in a marked reduction of HIV-1 production in ACH-2/OX40 cells (Fig. 1A). The morphology of the ACH-2/OX40 cells was markedly altered following 24 h of dual stimulation (Fig. 1B). This morphological effect was associated with the induction of rapid apoptosis, as determined by apparent cell death (Fig. 1C) and by annexin V-FITC/PI staining (Fig. 1D). Of interest was the finding that all the dual receptor-stimulated ACH-2/OX40 cells became apoptotic as early as 9 h after stimulation, before HIV-1 production was detectable (Fig. 1A and D).

Dual receptor-induced activation also affects HIV-1 production in other HIV-1-infected cell line cells

To determine whether this HIV-1 reduction and this rapid cell death, induced by such dual receptor activation, were unique to the ACH-2/OX40 cells or whether they are general for all types of HIV-1-infected cells expressing these two receptors, a series of other HIV-1 productively or chronically in-

FIG. 4. Involvement of the OX40 cytoplasmic tail and of the caspase cascade in the induction of apoptosis of Molt-4/CCR5-OX40 cells. (**A**) Molt-4/CCR5-OX40 (M/R5-OX40), Molt-4/CCR5-OX40del (M/R5-OX40del), and Molt-4/CCR5-control (M/R5-control) cells were cocultured with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells, at a cell-to-cell ratio of 2:1 in the absence (Medium) or in the presence of 2 ng/ml TNF-α for 24 h. (**B**) The blocking of apoptosis of the M/R5-OX40 cells, induced by OX40L or by OX40L/TNF-α stimulation, by a caspase inhibitor, z-VAD-fmk. The caspase inhibitor z-VAD-fmk (z-VAD) was added, before stimulation, to M/R5-OX40 cells at a final concentration of 100 μM. The pretreated M/R5-OX40 cells were cocultured with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells, at a cell-to-cell ratio of 2:1 in the absence (Medium) or in the presence of 2 ng/ml TNF-α for 3, 6, and 24 h. Apoptotic and live cells were determined by a standard dual staining method using annexin V-FITC and PI. The cells were classified as undamaged cells, annexin V(−)/PI(−); early apoptotic cells, annexin V(+)/PI(−); and late apoptotic cells and necrotic cells, annexin V(+)/PI(+). The stimulating cells, which included the PFA-fixed SV-T2/OX40L or SV-T2/control cells, were included within the region of annexin V(+)/PI(+) cells (about 20% of the total cell number). The percentage of live cells is shown at the lower left quadrangle of each dot plot. (**C**) The cell lysates obtained from the M/R5-OX40 cells stimulated by PFA-fixed SV-T2/OX40L (OX40L) or by PFA-fixed SV-T2/control (Control) cells, at a cell-to-cell ratio of 2:1 for 6 h, were treated with an equal volume of 2× sample buffer without 2-mercaptoethanol, separated by SDS–PAGE, using a 12.5% gel, and then transferred to Immobilon-P Transfer Membrane. After blocking, the membranes were incubated with the primary anticaspase-8 and anti-caspase-3 mAbs (1:1000) followed by horseradish peroxidase-conjugated antimouse IgG Ab (1:2000). The reaction was detected using Super Signal West Femto Maximum Sensitivity Substrate and an image analyzer. Representative results from three independent experiments are shown.

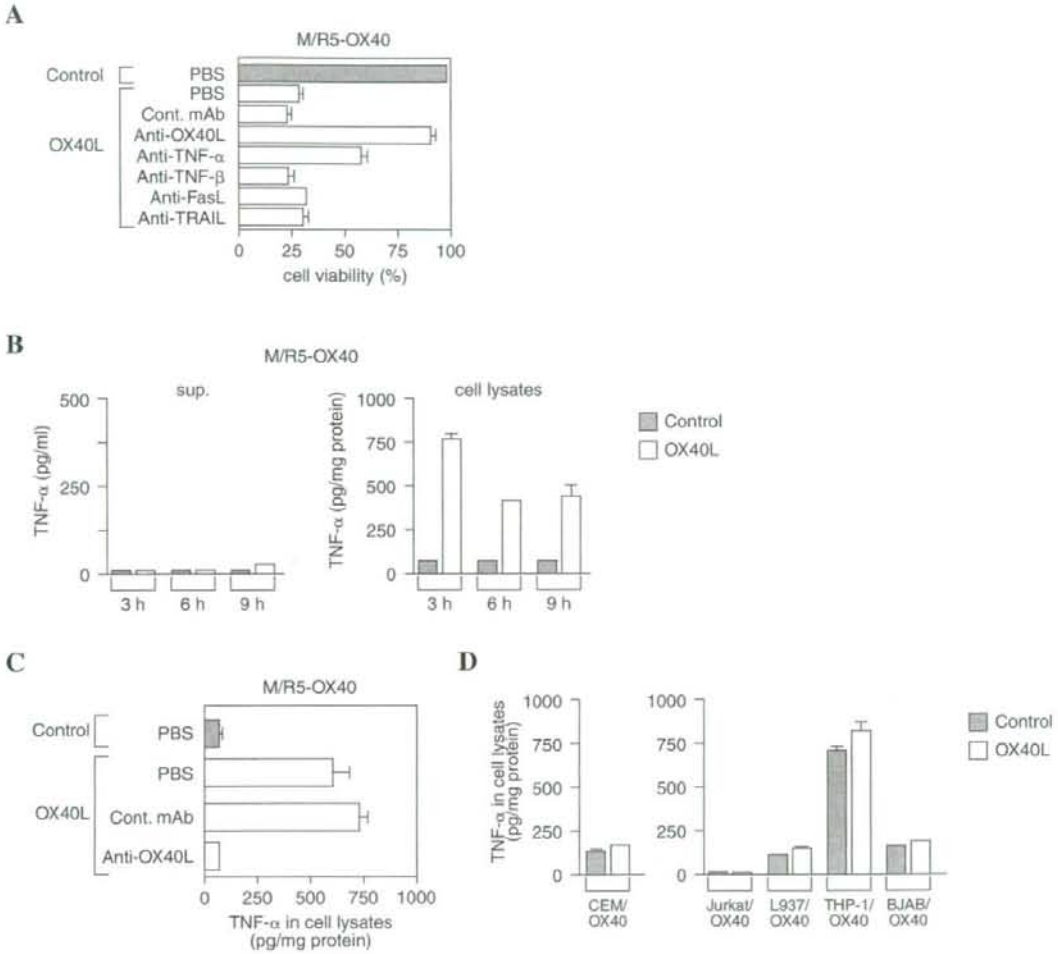


FIG. 5. OX40-induced apoptosis in Molt-4/CCR5-OX40 cells is mediated by endogenous TNF-α. (**A**) Molt-4/CCR5-OX40 (M/R5-OX40) cells were stimulated by PFA-fixed SV-T2/OX40L (OX40L) or by PFA-fixed SV-T2/control (Conrol) cells in the presence or in the absence of anti-hTNF-α (Anti-TNF-α, 30-100 μg/ml), anti-hTNF-β (Anti-TNF-β, 100 μg/ml), anti-hTRAIL (Anti-TRAIL, 100 μg/ml), anti-hFasL (Anti-FasL, 100 μg/ml), and anti-hOX40L (Anti-OX40L, 10 μg/ml) neutralizing mAb or with isotype control (Cont. mAb) for 24 h. Live cells were determined by annexin V-FITC/PI staining, followed by FACS analysis. (**B**) M/R5-OX40 cells were stimulated for 3, 6, and 9 h by PFA-fixed SV-T2/OX40L (OX40L) or by PFA-fixed SV-T2/control (Control) cells. TNF-α concentrations were determined in the culture supernatants (sup., left panel) and in the cell lysates (cell lysates, right panel) by an hTNF-α sandwich ELISA. (**C**) M/R5-OX40 cells were stimulated by PFA-fixed SV-T2/OX40L (OX40L) or by PFA-fixed SV-T2/control (Control) cells in the presence or in the absence of anti-hOX40L neutralizing mAb (Anti-OX40L, 10 μg/ml) or with isotype control (Cont. mAb) for 6 h. TNF-α concentrations were determined in the cell lysates. (**D**) The cells were stimulated by PFA-fixed SV-T2/OX40L (OX40L) or by PFA-fixed SV-T2/control (Control) cells for 6 h, in the case of CEM/OX40 cells, and for 24 h in all other cells. TNF-α concentrations were determined in the culture supernatants and in the cell lysates. The data presented are the mean values $\pm$ SD of triplicate determinations. Representative results from three independent experiments are shown.

fected cell lines transfected with OX40 were examined. These cell lines included the OX40-transfected T cell line, Molt-4/IIIB, Molt-4/IIIB-OX40 (M/IIIB-OX40), and the promonocytic cell line, U1, U1/OX40. The M/IIIB-OX40 cells produced relatively large amounts of HIV-1 upon stimulation with either OX40L or with TNF-α. The U1/OX40 cells produced significant levels of HIV-1 following stimulation with TNF-α, but produced little following ligation using OX40L. On the other hand, dual receptor activation with OX40L and TNF-α dramatically reduced HIV-1 production in M/IIIB-OX40 and in U1/OX40 cells, similar to our findings using ACH-2/OX40 cells (Fig. 2A). In M/IIIB-OX40 cells, moderate cell death was observed following OX40L stimulation, while dual stimulation, with OX40L and TNF-α, induced rapid cell death (data not

shown). In U1/OX40 cells, moderate cell death was induced only following dual stimulation (data not shown). The role of OX40/OX40L was underscored by the observation that antibody blockade of OX40L inhibited HIV-1 activation following OX40L stimulation. Antibody blockade also reversed HIV-1 reduction following dual stimulation with OX40L and TNF-α in ACH-2/OX40 and in M/IIIB-OX40 cells (Fig. 2B). Similarly, anti-OX40L blocking mAb partially or completely inhibited the cell deaths induced in ACH-2/OX40 and M/IIIB-OX40 cells following dual stimulation with OX40L and TNF-α (data not shown). The reduction of HIV-1 production resulting from dual receptor activation was also observed in the acutely HIV-1 NL4-3-infected Molt-4/CCR5-OX40 (M/R5-OX40) cells (Fig. 2C). This effect was also reversed by anti-OX40L blocking mAb. However, stimulation with OX40L alone did not enhance HIV-1 activation in acutely infected M/R5-OX40. The viability of the HIV-1 acutely infected M/R5-OX40 cells was rapidly reduced not only following dual stimulation with OX40L and TNF-α but also stimulation with OX40L alone as compared to ACH-2/OX40 and M/IIIB-OX40 cells (data not shown). This effect on cell viability was completely reversed with anti-OX40L blocking mAbs (data not shown). Therefore, these results support the view that OX40 stimulation by its natural ligand, OX40L, combined with stimulation by TNF-α, leads to a significant reduction of HIV-1 production, which is associated with rapid cell death, not only in an ACH-2 cell line, but also in Molt-4/IIIB, U1, and HIV-1 acutely infected Molt-4/CCR5 cell lines.

OX40 ligation-mediated cell death occurs independently of HIV-1

To determine whether the cell death of cells overexpressing OX40, induced by the stimulation with OX40L and/or TNF-α, was intrinsic to the cytopathic effects of HIV-1 infection, we established a series of HIV-1-negative/OX40-positive and control vector transfectants. These cells were stimulated by coculture with OX40L$^+$ cells in the presence or absence of TNF-α, followed by analysis of cell viability. As shown in Fig. 3A, cell death was seen in both the M/R5-OX40 and in the CEM/OX40 cells following stimulation by OX40L, and, furthermore, dual stimulation with OX40L and TNF-α synergistically accelerated the rate of cell death. In these two cell lines, TNF-α alone, at the doses utilized, had no effect on cell viability. On the other hand, in the case of another T cell line, Jurkat/OX40, and the two CD4$^+$ promonocytic cell lines, U937/OX40 and THP-1/OX40, dual receptor stimulation, but not OX40L stimulation alone, strongly induced cell death. However, the rate of cell death was more moderate in these cell lines than the rate observed with M/R5-OX40 and with CEM/OX40 cells. In contrast, the B cell line BJAB/OX40 was resistant to each of the stimulation protocols utilized above. As shown in Fig. 3B, death of OX40-expressing CD4$^+$ T cells, following stimulation with either OX40L or with OX40L and TNF-α, was completely inhibited by anti-OX40L blocking mAb. This shows that OX40 triggering by OX40L was necessary for the induction of cell death. Maximum cell death was observed under close cell-to-cell contact between OX40$^+$ responder and OX40L$^+$ stimulator cells and at higher TNF-α concentrations (more than 2 ng/ml) (Fig. 3C). Therefore, in the absence of HIV-1, OX40-mediated signaling is capable, on its own, of inducing cell death, and the dual stimulation with OX40L and TNF-α leads to an accelerated rate of cell death than stimulation with either ligand alone, which differs with respect to the cell line being studied.

OX40-mediated apoptosis in M/R5-OX40 cells is induced by signaling via the cytoplasmic tail of OX40 and by the caspase cascade

To confirm that the cell death, observed above, was dependent on signal transduction via OX40, we established a cell line expressing a deletion mutant of OX40 that lacked the cytoplasmic tail, Molt-4/CCR5-OX40del (M/R5-OX40del). M/R5-

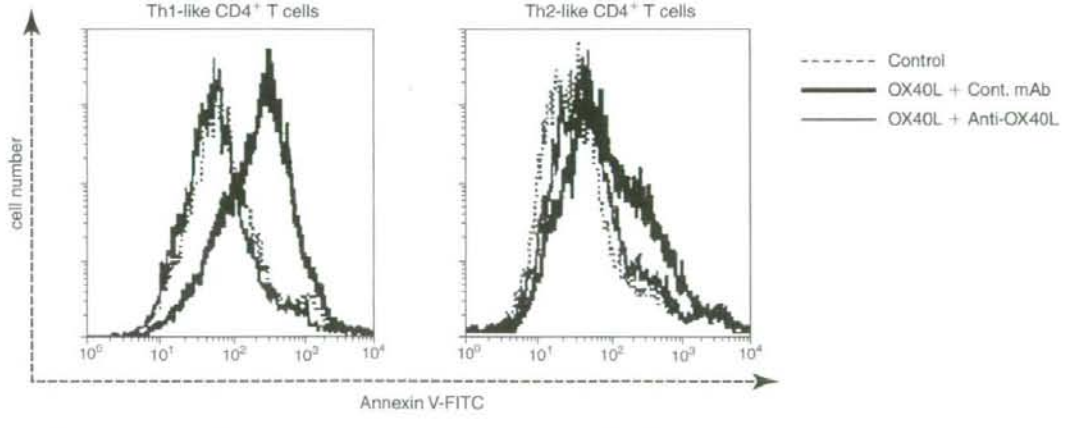


FIG. 6. Induction of apoptosis in primary activated CD4$^+$ T cells by cocultivation with OX40L-expressing cells. Two types of activated CD4$^+$ T cells from PBMCs of healthy donors, Th1-like and Th2-like, were activated in the presence of rhIL-12 and rhIL-4, respectively, on day 0, 3, and 6. After an additional 3 days, cells were cocultured with PFA-fixed SV-T2/OX40L (OX40L) or with PFA-fixed SV-T2/control (Control) cells in the presence of either anti-hOX40L blocking mAb (Anti-OX40L) or isotype control mAb (Cont. mAb). After 24 h, cells were stained by anti-hCD4-Cy5 and then stained by annexin V-FITC. Cells were analyzed with a FACSCalibur. Representative results from three independent experiments are shown.

OX40, M/R5-OX40del, and M/R5-control cells were stimulated with OX40L and/or TNF-α, followed by the determination of cell viability using annexin V-FITC/PI staining. At 24 h following stimulation, apoptosis was apparent in M/R5-OX40 cells; however, no detectable apoptosis was observed in M/R5-OX40del cells (Fig. 4A), suggesting a signal from OX40 is indeed required for apoptosis. On the other hand, M/R5-OX40 cells pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk and stimulated with OX40L, in the absence or presence of TNF-α, were prevented from undergoing apoptosis (Fig. 4B), suggesting that the apoptosis was caspase cascade dependent. In accordance with this, Western blot assays showed that in OX40L-stimulated M/R5-OX40 cells, the levels of pre-caspase-8 (57 kDa) and pro-caspase-3 (32 kDa) were markedly reduced, and cleaved caspase-8 (43 kDa) was increased after OX40L stimulation (Fig. 4C). Taken together, these results indicate that the OX40-mediated apoptosis of M/R5-OX40 cells is dependent on both signaling via the cytoplasmic tail of OX40 and on the caspase cascade.

OX40-induced apoptosis in M/R5-OX40 cells is mediated by endogenous TNF-α

To explore the mechanisms involved in OX40-mediated apoptosis, M/R5-OX40 cells were examined in more detail, since these cells undergo apoptosis by OX40L stimulation without exogenous TNF. First, we examined the effects of various mAbs on OX40L-triggered cell death. As shown in Fig. 5A, anti-hTNF-α blocking mAb, but none of anti-hTNF-β, anti-hTRAIL, and anti-hFasL blocking mAbs, inhibited the cell death, showing that TNF-α, but not TNF-β, TRAIL, and FasL, was involved in cell death after OX40L stimulation. Indeed, OX40L-stimulated M/R5-OX40 cells synthesized TNF-α, which was detected in cell lysates, but not in culture supernatants (Fig. 5B). The fact that TNF-α was not detected in culture supernatants suggests that endogenously synthesized TNF-α bound to its membrane receptors (TNF-RI and/or TNF-RII) on the surface of cells stimulated with OX40L. The production of endogenous TNF-α was completely inhibited by anti-OX40L neutralizing mAb, showing that this endogenous TNF-α production was mediated by the OX40L/OX40 interaction (Fig. 5C). These results suggest that endogenous TNF-α, induced by OX40 stimulation in the OX40$^+$ T cell line M/R5-OX40 mediates apoptosis. Furthermore, we also determined the induction of endogenous TNF-α in other OX40$^+$ cell lines. As shown in Fig. 5D, although endogenous TNF-α was induced in the T cell line CEM/OX40, it was not dependent on OX40L stimulation, unlike M/R5-OX40 cells. Interestingly, in another T cell line, Jurkat/OX40, endogenous TNF-α was not detected. On the other hand, two promonocytic cell lines, U937/OX40 and THP-1/OX40, strongly expressed endogenous TNF-α regardless of OX40 stimulation. The B cell line BJAB/OX40 also weakly expressed endogenous TNF-α regardless of OX40 stimulation. In all of these cell lines, soluble TNF-α was not detected in the culture supernatants after OX40L stimulation (data not shown).

Apoptosis of primary CD4$^+$ T cells by cocultivation with OX40L-expressing cells

Finally, we determined whether apoptosis can be induced in primary CD4$^+$ T cells by OX40-stimulation *in vitro*. We generated two types of activated CD4$^+$ T cells from PBMCs of healthy donors, Th1-like and Th2-like, which were activated in the presence of IL-12 and IL-4, respectively. The Th1-like CD4$^+$ T cells expressed higher levels of functional OX40 than the Th2-like CD4$^+$ T cells.[45] These two types of cells were cocultured for 24 h with PFA-fixed SV-T2/OX40L or with PFA-fixed SV-T2/control cells in presence of either anti-hOX40L blocking mAb or isotype control mAb. As shown in Fig. 6, OX40 stimulation increased the levels of annexin V binding, especially in the Th1-like CD4$^+$ T cells. Since anti-OX40L mAb inhibited annexin V staining, these results suggest that under Th1-like conditions some fractions of primary CD4$^+$ T cells undergo apoptosis following OX40 stimulation.

DISCUSSION

We show here, for the first time, that combined OX40L and TNF stimulation leads OX40$^+$ T cell lines and primary CD4$^+$ T cells to undergo apoptosis. Apoptosis-inducing effects have been described for a number of HIV-1 proteins, including Env,[46] Tat,[47] Nef,[48] Vpr,[49] and Vpu.[50] Recently, Lenardo *et al.* have shown that HIV-1 can induce necrosis of CD4$^+$ T cells.[51] However, the data presented herein, using the OX40 transfectants of the HIV-1-negative T cell line Molt-4/CCR5, clearly demonstrate that OX40-mediated cell death is independent of apoptosis- or of necrosis-inducing effects of HIV-1 proteins.

Stimulation of other DD-lacking members of the TNF-R superfamily, including TNF-R2, CD27, CD30, and CD40, induces the death of tumor cells and of normal cells under certain conditions. One known mechanism for TNF-R2-, CD30-, and CD40-induced cell death is that receptor stimulation activates cells to produce endogenous membrane TNF, which stimulates the DD-containing TNF-R1 to induce cell death.[31] A second mechanism suggested for CD40-mediated cell death is an amplification of the Fas-dependent apoptosis pathway.[52] In this study, we show that apoptosis in M/R5-OX40 cells, induced by OX40 stimulation, was efficiently blocked by the caspase inhibitor z-VAD-fmk, and was also blocked by anti-TNF-α neutralizing mAb. Moreover, the apoptosis of OX40-stimulated M/R5-OX40 cells was associated with the induction of endogenous TNF-α. These results suggest that the OX40-mediated apoptosis in M/R5-OX40 cells occurs indirectly, via a TNF/TNF-R system reminiscent of that mediated via TNF-R2, CD30, and CD40.

The response to coactivation by OX40L plus TNF-α in Jurkat/OX40 cells is also of great interest. In Jurkat/OX40 cells, apoptosis was not induced by soluble TNF-α or by OX40 stimulation and endogenous TNF-α was not induced by OX40 stimulation, unlike the results observed in M/R5-OX40 cells. However, rapid apoptosis was induced in Jurkat/OX40 cells by costimulation with OX40L plus TNF-α. These results suggest a new aspect of OX40 function in the induction of apoptosis in this cell line upon costimulation with OX40L plus TNF-α. The degree to which this mechanism contributes to OX40-induced apoptosis in other cell lines, such as M/R5-OX40, CEM/OX40, U937/OX40, and THP-1/OX40, is not currently known. On the other hand, U937/OX40 and THP-1/OX40 cells were relatively resistant, and BJAB/OX40 cells were completely resistant to the combined stimulation with OX40L and TNF-α (Fig. 3A). Since endogenous TNF-α is expressed in these cells irrespec-

tive of stimulation, these cells might have an endogenous mechanism to resist TNF-α-induced apoptosis. Furthermore, since normal B cells and monocytic cells do not express OX40, it might be possible that they lack the machinery for OX40-mediated intracellular signaling, or are equipped with an, as yet, undetermined anti-OX40 signal. Further studies are required to define the mechanisms of such resistance. In addition, since apoptosis of CEM/OX40 cells following OX40L stimulation could not be completely inhibited by the caspase inhibitor z-VAD-fmk at concentrations up to 100 μM (data not shown), it is possible that OX40-induced cell death may include additional apoptotic pathways, which may depend upon the cell line being studied.

It can be speculated that the survival or the apoptotic fate of $CD4^+$ T cells, after OX40 stimulation, is dependent on immunological environments. Kawamata *et al.* have shown that the cytoplasmic tail of OX40 binds TRAF2 and TRAF5, leading to NF-κB activation.[12] TRAF2 is required for the TNF-mediated activation of c-Jun N-terminal kinase (JNK) and of NF-κB, which leads to the generation of antiapoptotic signals.[3] It has been shown that TRAF2 can trigger cell death in the presence of the receptor-interacting protein (RIP), whereas in the absence of RIP, TRAF2 activates NF-κB.[53] RIP has also been implicated in caspase-8-independent necrosis.[54] Furthermore, Li *et al.* have shown that activation of TNF-R2 induces ubiquitination and proteasomal degradation of TRAF2, leading to the enhancement of TNF-induced apoptosis.[55] In addition, it has been demonstrated that stimulation of TNF-R2, CD30, or CD40 leads to selective enhancement of TNF-R1-mediated caspase-8-dependent cell death by depletion of both TRAF2 and the antiapoptotic IAP proteins.[37] Another suggested mechanism for CD40L-mediated $CD4^+$ T cell death is failure of the induction of the antiapoptotic proteins Bcl-2 and Bcl-X_L.[56] However, this mechanism cannot explain OX40-mediated apoptosis, since OX40 activation has been shown to induce these two antiapoptotic proteins.[57] Recently, Ma *et al.* have shown that in both OX40 and 4-1BB-expressing cells, combined stimulation by OX40 and 4-1BB induces reduced NF-κB activation, cell survival, and cell growth.[58] At present, it remains unclear whether OX40 activation directly mediates cell death. Taken together, it is interesting to speculate that OX40 stimulation induces cell death via an apoptotic pathway mediated by its cytoplasmic tail. Further studies are in progress to reveal the precise molecular mechanisms of OX40-induced apoptosis of T cells in various immunological environments.

In conclusion, the present study revealed a novel immunological function of OX40 in OX40-expressing $CD4^+$ T cells, with the control of cell death, in the presence of TNF, potentially resulting in a reduction of HIV-1 production.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan. We are grateful to Dr. A.A. Ansari (Emory University) for critical reading of this manuscript, Dr. H. Niwa (RIKEN) for the gift of the pCAGIPuro vector, and Dr. M. Baba (Kagoshima University) for the gift of the Molt-4/CCR5 cell line.

REFERENCES

1. Latza U, Durkop H, Schnittger S, *et al.*: The human OX40 homolog: cDNA structure, expression and chromosomal assignment of the ACT35 antigen. Eur J Immunol 1994;24:677–683.
2. Mallett S, Fossum S, and Barclay AN: Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes: A molecule related to nerve growth factor receptor. EMBO J 1990;9:1063–1068.
3. Baker SJ and Reddy EP: Modulation of life and death by the TNF receptor superfamily. Oncogene 1998;17:3261–3270.
4. Tanaka Y, Inoi T, Tozawa H, Yamamoto N, and Hinuma Y: A glycoprotein antigen detected with new monoclonal antibodies on the surface of human lymphocytes infected with human T-cell leukemia virus type-I (HTLV-I). Int J Cancer 1985;36:549–555.
5. Miura S, Ohtani K, Numata N, *et al.*: Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. Mol Cell Biol 1991;11:1313–1325.
6. Baum PR, Gayle RB 3rd, Ramsdell F, *et al.*: Molecular characterization of murine and human OX40/OX40 ligand systems: Identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. EMBO J 1994;13:3992–4001.
7. Ohshima Y, Tanaka Y, Tozawa H, Takahashi Y, Maliszewski C, and Delespesse G: Expression and function of OX40 ligand on human dendritic cells. J Immunol 1997;159:3838–3848.
8. Stuber E, Neurath M, Calderhead D, Fell HP, and Strober W: Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity 1995;2:507–521.
9. Morimoto S, Kanno Y, Tanaka Y, *et al.*: CD134L engagement enhances human B cell Ig production: CD154/CD40, CD70/CD27, and CD134/CD134L interactions coordinately regulate T cell-dependent B cell responses. J Immunol 2000;164:4097–4104.
10. Imura A, Hori T, Imada K, *et al.*: The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J Exp Med 1996;183:2185–2195.
11. Baba E, Takahashi Y, Lichtenfeld J, *et al.*: Functional CD4 T cells after intercellular molecular transfer of OX40 ligand. J Immunol 2001;167:875–883.
12. Kawamata S, Hori T, Imura A, Takaori-Kondo A, and Uchiyama T: Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF-kappa B activation. J Biol Chem 1998;273:5808–5814.
13. Flynn S, Toellner FM, Raykundalia C, Goodall M, and Lane P: CD4 T cell cytokine differentiation: The B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. J Exp Med 1998;188:297–304.
14. Ohshima Y, Yang LP, Uchiyama T, *et al.*: OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood 1998;92:3338–3345.
15. Akiba H, Miyahira Y, Atsuta M, *et al.*: Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. J Exp Med 2000;191:375–380.
16. Tanaka H, Demeure CE, Rubio M, Delespesse G, and Sarfati M: Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors. Role of stimulator/responder ratio. J Exp Med 2000;192:405–412.
17. Gramaglia I, Jember A, Pippig SD, Weinberg AD, Killeen N, and Croft M: The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J Immunol 2000;165:3043–3050.

18. De Smedt T, Smith J, Baum P, Fanslow W, Butz E, and Maliszewski C: OX40 costimulation enhances the development of T cell responses induced by dendritic cells in vivo. J Immunol 2002;168:661–670.
19. Bansal-Pakala P, Jember AJ, and Croft M: Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. Nat Med 2001;7:907–912.
20. Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, and Colombo MP: Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: A novel regulatory role for OX40 and its comparison with GITR. Blood 2005;105:2845–2851.
21. Imura A, Hori T, Imada K, *et al.*: OX40 expressed on fresh leukemic cells from adult T-cell leukemia patients mediates cell adhesion to vascular endothelial cells: Implication for the possible involvement of OX40 in leukemic cell infiltration. Blood 1997;89: 2951–2958.
22. Brocker T, Gulbranson-Judge A, Flynn S, Riedinger M, Raykundalia C, and Lane P: CD4 T cell traffic control: In vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. Eur J Immunol 1999;29:1610–1616.
23. Aten J, Roos A, Claessen N, Schilder-Tol EJM, Ten Berge IJM, and Weening JJ: Strong and selective glomerular localization of CD134 ligand and TNF receptor-1 in proliferative lupus nephritis. J Am Soc Nephrol 2000;11:1426–1438.
24. Ndhlovu LC, Ishii N, Murata K, Sato T, and Sugamura K: Involvement of OX40 ligand signals in the T cell priming events during experimental autoimmune encephalomyelitis. J Immunol 2001; 167:2991–2999.
25. Murata K, Nose M, Ndhlovu LC, Sato T, Sugamura K, and Ishii N: Constitutive OX40/OX40 ligand interaction induces autoimmune-like diseases. J Immunol 2002;169:4628–4636.
26. Jember AG-H, Zuberi R, Liu FT, and Croft M: Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. J Exp Med 2001;193:387–392.
27. Arestides RS, He H, Westlake RM, *et al.*: Costimulatory molecule OX40L is critical for both Th1 and Th2 responses in allergic inflammation. Eur J Immunol 2002;32:2874–2880.
28. Uchiyama T: Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu Rev Immunol 1997;15:15–37.
29. Kopf M, Ruedl C, Schmitz N, *et al.*: OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL responses after virus infection. Immunity 1999;11: 699–708.
30. Ekkens MJ, Liu Z, Liu Q, *et al.*: The role of OX40 ligand interactions in the development of the Th2 response to the gastrointestinal nematode parasite Heligmosomoides polygyrus. J Immunol 2003;170:384–393.
31. Grell M, Zimmermann G, Gottfried E, *et al.*: Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: A role for TNF-R1 activation by endogenous membrane-anchored TNF. EMBO J 1999;18:3034–3043.
32. Prasad KV, Ao Z, Yoon Y, *et al.*: CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. Proc Natl Acad Sci USA 1997;94:6346–6351.
33. Mir SS, Richter BW, and Duckett CS: Differential effects of CD30 activation in anaplastic large cell lymphoma and Hodgkin disease cells. Blood 2000;96:4307–4312.
34. Lee SY, Park CG, and Choi Y: T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. J Exp Med 1996;183:669–674.
35. Hess S and Engelmann H: A novel function of CD40: Induction of cell death in transformed cells. J Exp Med 1996;183:159–167.
36. Chan FK and Lenardo MJ: A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. Eur J Immunol 2000;30:652–660.
37. Fotin-Mleczek M, Henkler F, Samel D, *et al.*: Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. J Cell Sci 2002;115:2757–2770.
38. Takahashi Y, Tanaka Y, Yamashita A, Koyanagi Y, Nakamura M, and Yamamoto N: OX40 stimulation by gp34/OX40 ligand enhances productive human immunodeficiency virus type 1 infection. J Virol 2001;75:6748–6757.
39. Biswas P, Smith CA, Goletti D, Hardy EC, Jackson RW, and Fauci AS: Cross-linking of CD30 induces HIV expression in chronically infected T cells. Immunity 1995;2:587–596.
40. Tozawa H, Andoh S, Takayama Y, *et al.*: Species-dependent antigenicity of the 34-kDa glycoprotein found on the membrane of various primate lymphocytes transformed by human T-cell leukemia virus type-I (HTLV-I) and simian T-cell leukemia virus (STLV-I). Int J Cancer 1988;41:231–238.
41. Tanaka Y, Yoshida A, Tozawa H, Shida H, Nyunoya H, and Shimotohno K: Production of a recombinant human T-cell leukemia virus type-I trans-activator (tax1) antigen and its utilization for generation of monoclonal antibodies against various epitopes on the tax1 antigen. Int J Cancer 1991;48:623–630.
42. Baba M, Miyake H, Okamoto M, Iizawa Y, and Okonogi K: Establishment of a CCR5-expressing T-lymphoblastoid cell line highly susceptible to R5 HIV type 1. AIDS Res Hum Retroviruses 2000;16:935–941.
43. Adachi A, Gendelman HE, Koenig S, *et al.*: Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 1986;59:284–291.
44. Tanaka R, Yoshida A, Murakami T, *et al.*: Unique monoclonal antibody recognizing the third extracellular loop of CXCR4 induces lymphocyte agglutination and enhances human immunodeficiency virus type 1-mediated syncytium formation and productive infection. J Virol 2001;75:11534–11543.
45. Kondo K, Okuma K, Tanaka R, *et al.*: Requirements for the functional expression of OX40 ligand on human activated $CD4^+$ and $CD8^+$ T cells. Hum Immunol 2007;68:563–571.
46. Ohagen A, Ghosh S, He J, *et al.*: Apoptosis induced by infection of primary brain cultures with diverse human immunodeficiency virus type 1 isolates: Evidence for a role of the envelope. J Virol 1999;73:897–906.
47. Bartz SR and Emerman M: Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. J Virol 1999;73:1956–1963.
48. Xu X-N, Laffert B, Screaton GR, *et al.*: Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J Exp Med 1999;189:1489–1496.
49. Hrimech M, Yao XJ, Bachand F, Rougeau N, and Cohen EA: Human immunodeficiency virus type 1 (HIV-1) Vpr functions as an immediate-early protein during HIV-1 infection. J Virol 1999;73: 4101–4109.
50. Casella CR, Rapaport EL, and Finkel TH: Vpu increases susceptibility of human immunodeficiency virus type 1-infected cells to fas killing. J Virol 1999;73:92–100.
51. Lenardo MJ, Angleman SB, Bounkeua V, *et al.*: Cytopathic killing of peripheral blood CD4 (+) T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env. J Virol 2002;76:5082–5093.
52. Afford SC, Ahmed-Choudhury J, Randhawa S, *et al.*: CD40 activation-induced, Fas-dependent apoptosis and NF-kappaB/AP-1 signaling in human intrahepatic biliary epithelial cells. FASEB J 2001;15:2345–2354.

53. Pimentel-Muinos FX and Seed B: Regulated commitment of TNF receptor signaling: A molecular switch for death or activation. Immunity 1999;11:783–793.
54. Holler N, Zaru R, Micheau O, *et al.*: Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489–495.
55. Li X, Yang Y, and Ashwell JD: TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature 2002;416:345–347.
56. Blair PJ, Riley JL, Harlan DM, *et al.*: CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. J Exp Med 2000;191:651–660.
57. Rogers PR, Son J, Gramaglia I, Killeen N, and Croft M: OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 2001;15:445–455.
58. Ma BY, Mikolajczak SA, Danesh A, *et al.*: The expression and the regulatory role of OX40 and 4-1BB heterodimer in activated human T cells. Blood 2005;106:2002–2010.

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

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

Humanized Mice for Human Retrovirus Infection

Y. Koyanagi(✉), Y. Tanaka, M. Ito, and N. Yamamoto

Abstract Inbred mice with specific genetic defects have greatly facilitated the analysis of complex biological events. Several humanized mouse models using the C.B.-17 *scid/scid* mouse (referred to as the SCID mouse) have been created from two transplantation protocols, and these mice have been utilized for the investigation of human immunodeficiency virus type 1 (HIV-1) and human T-lymphotropic virus type I (HTLV-I) pathogenesis and the evaluation of antiviral compounds. To generate a more prominent small animal model for human retrovirus infection, especially for examination of the pathological process and the immune reaction, a novel immunodeficient mouse strain derived from the NOD SCID mouse was created by backcrossing with a common γ chain (γ_c)-knockout mouse. The NOD-SCID γ_c^{null} (NOG) mouse has neither functional T and B cells nor NK cells and has been used as a recipient in humanized mouse models

Y. Koyanagi
Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, 53 Shougoinkawara cho, Sakyou-ku, Kyoto 606-8507, Japan
ykoyanag@virus.kyoto-u.ac.jp

T. Nomura et al. (eds.), *Humanized Mice*.
Current Topics in Microbiology and Immunology 324.

for transplantation of human immune cells particularly including hematopoietic stem cells (HSC). From recent advances in development of humanized mice, we are now able to provide a new version of the animal model for human retrovirus infection and human immunity.

Abbreviations AIDS: acquired immunodeficiency syndrome; APC: antigen-presenting cell; ATL: adult T-cell leukemia; AZT: azidothymidine; CCR5: cc-chemokine receptor 5; DC: dendritic cell; ddI: dideoxyinosine; DN: double negative; γ_c: common gamma chain; GVHD: graft-versus-host disease; HAM: HTLV-I-associated myelopathy; HIV-1: human immunodeficiency virus type 1; HSC: hematopoietic stem cell; HTLV-I: human T-lymphotropic virus type I; IL: interleukin; MHC: major histocompatibility complex; PBMC: peripheral blood mononuclear cell; SCID: severe combined immunodeficiency; SP: single positive; TRAIL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand; TSP: tropical spastic paraparesis

1 Mouse/Human Chimeric Models for HIV-1 Infection Using SCID Mouse

The C.B.-17 *scid/scid* (SCID) mouse carries a spontaneously arising autosomal recessive mutation and was found to have severe combined immunodeficiency (SCID) by Bosma and colleagues (Bosma et al. 1983). This strain has a defect of DNA protein kinase and a lack of progenitors to T and B cells (Blunt et al. 1995; Boubnov and Weaver 1995; Kirchgessner et al. 1995; Miller et al. 1995; Peterson et al. 1995). Therefore, these mice are unable to reject the xenograft and they tolerate engraftment of human cells or tissues, followed by subsequent infection with human immunodeficiency virus type 1 (HIV-1). Two of these models are the SCID-hu thy/liv mouse developed by McCune and colleagues (McCune et al. 1988) and, the hu-PBL-SCID mouse developed by Mosier and colleagues (Mosier et al. 1988) (Fig. 1).

1.1 HIV-1 Infection in the SCID-hu thy/liv Mouse

The SCID-hu thy/liv mouse is generated by surgical coimplantation of a piece (1 mm) of human fetal thymus and liver under the murine kidney capsule. The implanted tissues produce a conjoint organ (thy/liv) that appears to reconstitute normal thymus for more than 1 year. The fetal liver provides hematopoietic precursors that are located in islets between the thymic lobes. Thymic epithelial cells are derived from the fetal thymus, whereas the hematopoietic cells including T cells and dendritic cells (DC) are derived from the liver (Vandekerckhove et al. 1992). The generated thymus is composed of more than 70% CD4CD8

Mouse strain	human tissue or cells	References
1) SCID-hu thy/liv mouse		
CB17 SCID mouse	human thy/liv tissue	McCune et al. 1988
2) Human-PBL-SCID mouse		
CB17 SCID mouse	human PBMC	Mosier et al.1991
NOD-SCID mouse	human PBMC	Koyanagi et al. 1997
NOG mouse	human PBMC	Nakata et al. 2005

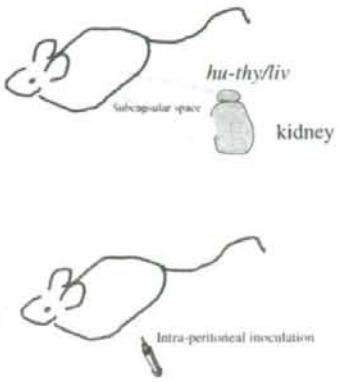


Fig. 1 Mouse/human chimeric models for HIV-1 infection. HIV-1-susceptible humanized mouse models have been reported from two transplantation protocols

double-positive (DP) cells, and the rest are CD4 or CD8 single-positive (SP) and double-negative (DN) T cells. Although these CD4 or CD8 SP cells migrate into the peripheral circulation and express a naive phenotype (>70% $CD45RA^+$), the numbers of these cells are very low (Jamieson and Zack 1999). In addition, no immune response to viral antigen has been found so far. Thus, the reconstitution of the human immune system is not complete in this mouse. Since some skill in the surgical operation to coimplant the thy/liv organ and systematic support on distribution of human fetal organs are required, the SCID-hu thy/liv mouse studies have been carried out in limited numbers of laboratories, mainly in the US.

The human thy/liv implant in SCID mice is highly susceptible to infection with HIV-1, including R5 and X4 HIV-1 (McCune et al. 1991; Namikawa et al. 1988). As the number of circulating human $CD4^+$ T cells is low as mentioned above, direct injection of virus into the implant is performed under anesthesia. The level of HIV-1 replication and potential with CD4 T cell depletion of X4 HIV-1 appears to be higher than that of R5 HIV-1 within a few weeks of infection by evaluation with PCR and flow cytometric analyses (Kaneshima et al. 1994). Immunohistological examination indicated that the infected cells initially appeared in the thymic cortical regions and subsequently spread through the entire organ after HIV-1 infection (Stanley et al. 1993). Flow cytometric analysis indicated that the CD4CD8 DP cells were almost completely eradicated and the ratio of $CD4^+$ and $CD8^+$ thymocytes was reversed after infection (Aldrovandi et al. 1993; Bonyhadi et al. 1993). Furthermore, SCID-hu thy/liv mouse have been also used to assess efficacy of several anti-HIV compounds administrated before infection, including azidothymidine (AZT) and 2′,3′-dideoxyinosine (ddI) (Kaneshima et al. 1991; McCune et al. 1990; Rabin et al. 1996).

1.2 HIV-1 Infection in the hu-PBL-SCID Mouse

The hu-PBL-SCID mouse is created by injection of peripheral blood mononuclear cells (PBMC) from healthy adults into the peritoneal cavity of SCID mice, and human $CD4^+$ and $CD8^+$ T cells circulate through the peritoneal cavity, peripheral blood, and organs such as spleen and liver for more than 1 month after PBMC injection (Mosier et al. 1988). The presence of human $CD4^+$ T cells makes this attractive as a model to study HIV-1 pathogenesis and evaluation of anti-HIV compounds (Mosier et al. 1991; Pastore et al. 2003; Hartley et al. 2004). Although it was initially reported as a model with little graft-versus-host disease (GVHD) in the hu-PBL-SCID mice, the high levels of T cell-reconstituted mice develop symptoms of GVHD within 2 months after injection of PBMC (Sandhu et al. 1995). Therefore, long-term observation may not be possible in this model. In addition, human $CD4^+$ and $CD8^+$ T cells in hu-PBL-SCID mice expressed the CD45RO antigen, a marker found in either activated or memory T cells (Tary-Lehmann and Saxon 1992). This is not similar to the ratio of normal adult PBMC, which contain approximately 50% $CD45RO^+$ and $CD45RA^+$ (naive) cells. Furthermore, human $CD4^+$ cells in the hu-PBL-SCID also express abundant levels of HIV-1 coreceptor CCR5, and accordingly, R5 but not X4 HIV-1 more actively replicates in this system (Mosier et al. 1993; Nakata et al. 2006). The relative ease with which this model can be generated and the high efficiency of R5 HIV-1 infection make this system very attractive to study HIV-1 pathogenesis for researchers who struggle in obtaining fetal organs for generating SCID-hu thy/liv mouse. Importantly, a significantly high level of HIV-1 replication correlates with severe depletion of human $CD4^+$ T cells within 2 weeks after infection, and the replication is dependent on Nef protein (Kawano et al. 1997). Thus, this model appears to be adequate for short-term investigation of HIV-1 replication and pathogenesis. As mentioned above, the reconstituted $CD4^+$ and $CD8^+$ T cells are strongly activated and have memory phenotypes, indicating that these cells are xenoreactive proliferated but anergic T cells (Tary-Lehmann and Saxon 1992; Tary-Lehmann at al. 1995). In this model, neither thymopoiesis nor hematopoiesis is generated (Koyanagi, unpublished observations). Thus, it is assumed that the pathological events of HIV-1 infection in this model include that in mature T cells in humans.

1.3 Human Acquired Immune Responses in the hu-PBL-SCID Mouse

Of interest, some immune responses are induced, including humoral and cellular immune responses in the hu-PBL-SCID mouse with administration of various antigens (Gorantla et al. 2005; Ifversen P and Borrebaeck 1996; Nonoyama et al. 1993; Sandhu et al. 1994). However, there are two major limitations to development of strong human immune responses in the hu-PBL-SCID mice. The first is the lack of

appropriate human antigen-presenting cells (APC) including DC. The second is the lack of a suitable microenvironment such as the presence of normal lymphoid organs and architecture that may facilitate induction and maintenance of immune effector cells (Tary-Lehmann et al. 1995). To overcome the lack of APC, Delhem et al. used autologous skin that contains tissue DC as a source of APC and succeeded in demonstrating the induction of primary MHC-restricted human T cell responses against HIV-1 envelope in the hu-PBL-SCID mice (Delhem et al. 1998). Santini et al. have reported that inactivated HIV-1-pulsed, monocyte-derived, and matured human DC can stimulate human anti-HIV-1 antibody production by B cells from HIV-1-negative donors in the SCID mouse system, and that this immune response is partially protective (Santini et al. 2000). However, there had been no attempts to overcome the lack of a suitable microenvironment in this hu-PBL-SCID mouse until our report (Yoshida et al. 2003).

To overcome the deficiency of a suitable microenvironment in the hu-PBL-SCID mouse system, we attempted to transfer PBMC together with inactivated HIV-1-pulsed autologous monocyte-derived DC directly into the mouse spleen (Yoshida et al. 2003). The intrasplenic inoculation of PBMC was found to reduce excessive GVHD compared to the intraperitoneal transfer method (Tanaka et al., unpublished observations). In addition, with this procedure we could obtain larger yields of human T cells than with the conventional intraperitoneal transfer. Therefore, we reasoned that the microenvironment in the mouse spleen should provide human T cells with optimum conditions for activation (Fig. 2). With this new immunizing protocol, we have succeeded in eliciting a protective $CD4^+$ T cell immunity against R5 HIV-1 infection. We were surprised to see that the immunized mice were totally resistant against challenge with live R5 HIV-1 (Yoshida et al. 2003). The protective immunity was induced equally with either R5 or X4 HIV-1 as an antigen. The sera from the immunized mice contained a soluble R5 HIV-1 suppressive factor that was mainly synthesized by human $CD4^+$ T cells in response to HIV-1 antigen, specific peptides of HIV-1 according to MHC class II haplotypes (Yoshida et al. 2005), but

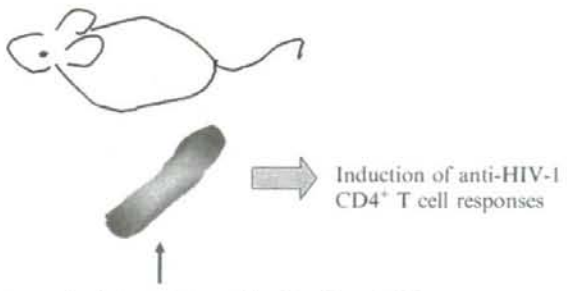


Fig. 2 Human acquired immune responses in the hu-PBL-SCID mouse. Protective $CD4^+$ T cell immunity against R5 HIV-1 infection is induced by PBMC transfer together with inactivated HIV-1-pulsed autologous DC directly into the mouse spleen