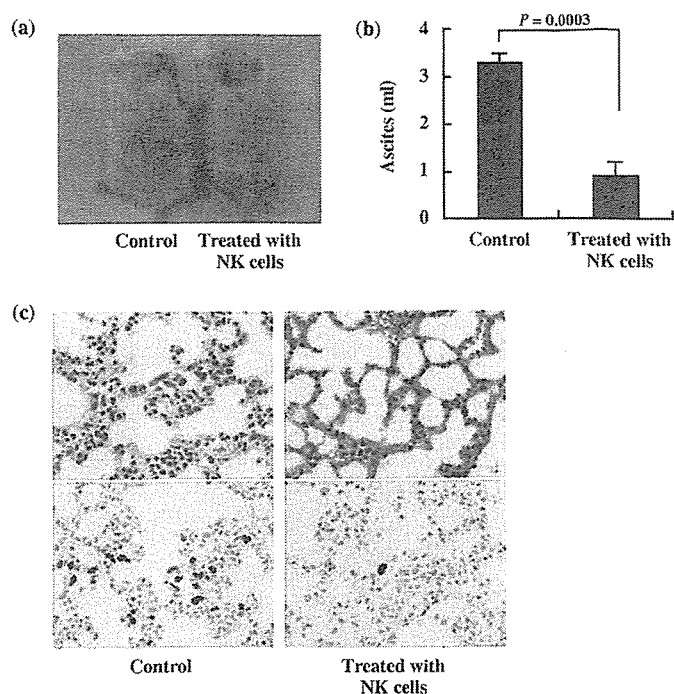


**Fig. 4.** Cytotoxic activity of activated natural killer (NK) *in vitro* culture cells. (a) Spontaneous cytotoxic activity of freshly isolated peripheral blood mononuclear cells (KT-1 and KN-2 represent samples from two donors) and NK cell line KHYG-1 against K-562 cells at different effector-to-target (E/T) ratios. (b) Cells cultured for 2 weeks against K-562 cells at different E/T ratios. (c) Cytotoxic activity of activated NK cells (NK-1 and NK-2 represent samples from two donors) against primary effusion lymphoma cells *in vitro* at various E/T ratios.

animal model without changes in its histomorphology or tumor marker expression would be useful to understand and investigate the mechanism of PEL cell growth and infiltration.

In patients with cancer and viral infection, NK-cell function has been shown to be impaired, as determined by the reduced proliferation, response to interferon (IFN), and cytotoxicity of the cells of patients *ex vivo*.<sup>(51,52)</sup> In the present study, we inoculated activated NK cells to treat tumor-bearing mice to further clarify the role of NK cells in tumor growth and infiltration. Transfer of activated NK cells in T, B and NK knock-out NOG mice showed significant inhibition of tumor and ascites formation as well as infiltration. T, B and NK knock-out NOG mice treated with activated NK cells rejected the tumor cells to a similar extent as T and B knock-out NOD/SCID mice.

Natural killer cells kill target cells by various mechanisms. One way is by the release of cytoplasmic granules – complex



**Fig. 5.** Inhibition of primary effusion lymphoma (PEL) cell growth and infiltration in NOG mice. T, B and natural killer (NK) knock-out NOG mice were injected with PEL cells ( $2 \times 10^6$ ) intraperitoneally in the abdominal region. Mice were administered either RPMI-1640 or activated NK cells ( $1 \times 10^7$ ) intraperitoneally on days 4, 10 and 17 followed by observation for up to 3 weeks. (a) Photograph of ascites-bearing control PEL mice and activated NK-treated PEL mice. (b) Volume of ascites in control PEL mice and activated NK-treated PEL mice. Volume of ascites in mice inoculated with BCBL-1 cells, shown as the mean  $\pm$  s.e.m. from six mice ( $P = 0.0003$ ). (c) Hematoxylin-eosin (HE) and immunohistochemical staining of the lung of NOG mice 3 weeks after inoculation of PEL cells using anti-LANA. Upper and lower panels show HE and immunohistochemical staining, respectively. Left and right panels represent the data from control mice and mice treated with activated NK cells, respectively. Magnification,  $\times 40$ . The data represent six mice in each group and three healthy donors (two mice for each donor).

organelles that combine specialized storage and secretory functions with the generic degradative functions of lysosomes. These granules contain a number of proteins, such as perforins and granzymes, which lyse target cells. Increased perforin and granzyme expression in activated NK cells was significantly correlated with inhibition of tumor growth and metastasis in the NOG mouse model. Perforin- and granzyme-mediated apoptosis is the principal pathway used by NK cells to eliminate tumor and virus-infected cells.<sup>(53)</sup> Studies in perforin-deficient mice have revealed that this protein is required for most NK-cell cytotoxicity.<sup>(54)</sup>

In summary, NK knock-out NOG mice were very efficient in the formation of primary tumors and organ infiltration. These results indicate that activated human NK cells prevent tumor growth and infiltration in NOG mice. Finally, our results suggest that NK cells play a critical role in tumor growth and infiltration, and that activated NK cells could be a promising immunotherapeutic strategy against cancer or viral infection either alone or in combination with conventional therapy. The reproducible growth behavior and preservation of characteristic features of PEL cells also suggest that the NOG mouse model system described in the present study may provide a novel opportunity to understand and investigate the mechanism of pathogenesis and malignant cell growth of PEL.

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## References

- Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. *World Health Organization Classification of Tumors, Pathology and Genetics of Tumors of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press, 2001.
- Gaidano G, Carbone A. Primary effusion lymphoma: a liquid phase lymphoma of fluid-filled body cavities. *Adv Cancer Res* 2001; **80**: 115–46.
- Cesarman E, Knowles DM. The role of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in lymphoproliferative diseases. *Semin Cancer Biol* 1999; **9**: 165–74.
- Klein U, Ghoghini A, Gaidano G *et al*. Gene expression profile analysis of AIDS-related primary effusion lymphoma (PEL) suggests a plasmablastic derivation and identifies PEL-specific transcripts. *Blood* 2003; **102**: 4115–21.
- Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N Engl J Med* 1995; **332**: 1186–91.
- Horenstein MG, Nador RG, Chadburn A *et al*. Epstein-Barr virus latent gene expression in primary effusion lymphomas containing Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. *Blood* 1997; **90**: 1186–91.
- Nador RG, Cesarman E, Chadburn A *et al*. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood* 1996; **88**: 645–56.
- Schmidt M, Deschner EE, Thaler HT, Clemmets L, Good RA. Gastrointestinal cancer studies in the human to nude mouse heterotransplant system. *Gastroenterology* 1977; **72**: 829–37.
- Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature* 1983; **301**: 527–30.
- Schuler W, Bosma MJ. Nature of the scid defect: a defective VDJ recombinase system. *Curr Top Microbiol Immunol* 1989; **152**: 55–62.
- Kamel-Reid S, Letarte M, Sirard C *et al*. A model of human acute lymphoblastic leukemia in immune-deficient SCID mice. *Science* 1989; **246**: 1597–600.
- Mosier DE, Gulizia RJ, Baird SM, Wilson DB. Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* 1988; **335**: 256–9.
- Ito M, Hiramatsu H, Kobayashi K *et al*. NOD/SCID/ $\gamma$ c<sup>null</sup> mouse: An excellent recipient mouse model for engraftment of human cells. *Blood* 2002; **100**: 3175–82.
- Dorshkind K, Pollack SB, Bosma MJ, Phillips RA. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (scid). *J Immunol* 1985; **134**: 3798–801.
- Feuer G, Stewart SA, Baird SM, Lee F, Feuer R, Chen ISY. Potential role of natural killer cells in controlling tumorigenesis by human T-cell leukemia viruses. *J Virol* 1994; **69**: 1328–33.
- Welsh RM. Regulation of virus infections by natural killer cells: a review. *Nat Immun Cell Growth Regul* 1986; **5**: 169–99.
- Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989; **47**: 187–376.
- Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat Rev Immunol* 2002; **2**: 957–64.
- Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat Immunol* 2004; **5**: 996–1002.
- Karre K, Ljungger HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defense strategy. *Nature* 1986; **319**: 675–8.
- Pena J, Alonso C, Solana R, Serrano R, Carracedo J, Ramirez R. Natural killer susceptibility is independent of HLA class I antigen expression on the cell lines obtained from human solid tumors. *Eur J Immunol* 1990; **20**: 2445–9.
- Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. *J Exp Med* 1993; **178**: 1321–36.
- Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 2000; **356**: 1795–9.
- Ullum H, Gotzsche PC, Victor J, Dickmeiss E, Skinhoj P, Pedersen BK. Defective natural immunity: an early manifestation of human immunodeficiency virus infection. *J Exp Med* 1995; **182**: 789–99.
- Ahmad R, Menezes J. Defective killing activity against gp120/41-expressing human erythroleukaemic K562 cell line by monocytes and natural killer cells from HIV-infected individuals. *AIDS* 1996; **10**: 143–9.
- Scott-Algara D, Paul P. NK cells and HIV infection: lessons from other viruses. *Curr Mol Med* 2002; **2**: 757–68.
- Bonaparte MI, Barker E. Inability of natural killer cells to destroy autologous HIV-infected T lymphocytes. *AIDS* 2003; **17**: 487–94.
- Sirianni MC, Libi F, Campagna M *et al*. Downregulation of the major histocompatibility complex class I molecules by human herpesvirus type 8 and impaired natural killer cell activity in primary effusion lymphoma development. *Br J Haematol* 2005; **130**: 92–5.
- Sirianni MC, Vincenzi L, Topino S *et al*. NK cell activity controls human herpesvirus 8 latent infection and is restored upon highly active antiretroviral therapy in AIDS patients with regressing Kaposi's sarcoma. *Eur J Immunol* 2002; **32**: 2711–20.
- Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998; **16**: 356–93.
- Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? *Nat Immunol* 2002; **3**: 6–8.
- Koo GC, Dumont FJ, Tutt M, Hackett J Jr, Kumar V. The NK-1.1(–) mouse: a model to study differentiation of murine NK cells. *J Immunol* 1986; **137**: 3742–7.
- Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* 2001; **13**: 459–63.
- Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nature* 2001; **2**: 293–9.
- Dewan MZ, Terashima K, Taruishi M *et al*. Rapid tumor formation of human T-cell leukemia virus type 1-infected cell lines in novel NOD-SCID/ $\gamma$ c<sup>null</sup> mice: suppression by an inhibitor against NF- $\kappa$ B. *J Virol* 2003; **77**: 5286–94.
- Dewan MZ, Uchihara JN, Terashima K *et al*. Efficient intervention of growth and infiltration of primary adult T-cell leukemia cells by an HIV protease inhibitor, ritonavir. *Blood* 2006; **107**: 716–24.
- Renne R, Zhong W, Herndier B *et al*. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat Med* 1996; **2**: 342–6.
- Katano H, Hoshino Y, Morishita Y *et al*. Establishing and characterizing a CD30-positive cell line harboring HHV-8 from a primary effusion lymphoma. *J Med Virol* 1999; **58**: 394–401.
- Katano H, Sato Y, Kurata T, Mori S, Sata T. High expression of HHV-8-encoded ORF73 protein in spindle-shaped cells of Kaposi's sarcoma. *Am J Pathol* 1999; **155**: 47–52.
- Dorshkind K, Pollack SB, Bosma MJ, Phillips RA. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (scid). *J Immunol* 1985; **134**: 3798–801.
- Visonneau S, Cesano A, Torosian MH, Miller EJ, Santoli D. Growth characteristics and metastatic properties of human breast cancer xenografts in immunodeficient mice. *Am J Pathol* 1998; **152**: 1299–311.
- Cavacini LA, Giles-Komar J, Kennel M, Quinn A. Effect of immunosuppressive therapy on cytolytic activity of immunodeficient mice: implications for xenogeneic transplantation. *Cell Immunol* 1992; **144**: 296–310.
- Hochman PS, Cudkovic G, Dausset J. Decline of natural killer cell activity in sublethally irradiated mice. *J Natl Cancer Inst* 1978; **61**: 265–8.
- Huang YW, Richardson JA, Tong AW, Zhang BQ, Stone MJ, Vietta ES. Disseminated growth of a human multiple myeloma cell line in mice with severe combined immunodeficiency disease. *Cancer Res* 1993; **53**: 1392–6.
- Lapidot T, Sirard C, Vormoor J *et al*. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645–8.
- Taghian A, Budach W, Zietman A, Freeman J, Gioioso D, Suit HD. Quantitative comparison between the transplantability of human and murine tumors into the brain of NCr/Sed-nu/nu nude and severe combined immunodeficient mice. *Cancer Res* 1993; **53**: 5018–21.
- Tanaka T, Tsudo M, Karasuyama H *et al*. Novel monoclonal antibody against murine IL-2 receptor beta-chain: Characterization of receptor expression in normal lymphoid cells and EL-4 cells. *J Immunol* 1991; **147**: 2222–8.
- Noguchi M, Yi H, Rosenblatt HM *et al*. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 1993; **73**: 147–57.
- Puck JM, Deschenes SM, Porter JC *et al*. The interleukin-2 receptor gamma chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum Mol Genet* 1993; **2**: 1099–104.
- Welsh RM. Regulation of virus infections by natural killer cells: a review. *Nat Immun Cell Growth Regul* 1986; **5**: 169–99.
- Whiteside TL, Herberman RB. Role of human natural killer cells in health and disease. *Clin Diagn Laboratory Immunol* 1994; **1**: 125–33.
- Whiteside TL, Vujanovic NL, Herberman RB. Natural killer cells and tumor therapy. *Curr Top Microbiol Immunol* 1998; **230**: 221–44.
- Trapani JA, Davis J, Sutton VR, Smyth MJ. Proapoptotic functions of cytotoxic lymphocyte granule constituents *in vitro* and *in vivo*. *Curr Opin Immunol* 2000; **12**: 323–9.

# Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment

Yukari Muguruma, Takashi Yahata, Hiroko Miyatake, Tadayuki Sato, Tomoko Uno, Jobu Itoh, Shunichi Kato, Mamoru Ito, Tomomitsu Hotta, and Kiyoshi Ando

Hematopoiesis is maintained by specific interactions between both hematopoietic and nonhematopoietic cells. Whereas hematopoietic stem cells (HSCs) have been extensively studied both *in vitro* and *in vivo*, little is known about the *in vivo* characteristics of stem cells of the nonhematopoietic component, known as mesenchymal stem cells (MSCs). Here we have visualized and characterized human MSCs *in vivo* following intramedullary transplantation of enhanced green fluorescent pro-

tein-marked human MSCs (eGFP-MSCs) into the bone marrow (BM) of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Between 4 to 10 weeks after transplantation, eGFP-MSCs that engrafted in murine BM integrated into the hematopoietic microenvironment (HME) of the host mouse. They differentiated into pericytes, myofibroblasts, BM stromal cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells, which constituted the functional compo-

nents of the BM HME. The presence of human MSCs in murine BM resulted in an increase in functionally and phenotypically primitive human hematopoietic cells. Human MSC-derived cells that reconstituted the HME appeared to contribute to the maintenance of human hematopoiesis by actively interacting with primitive human hematopoietic cells. (Blood. 2006; 107:1878-1887)

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## Introduction

Mesenchymal stem cells (MSCs) present in bone marrow (BM) are thought to give rise to cells that constitute the hematopoietic microenvironment (HME).<sup>1</sup> MSCs have been isolated from BM and various tissues from humans and many other species, expanded in culture, and shown to differentiate into osteocytes, chondrocytes, adipocytes, and myoblasts under defined conditions *in vitro*.<sup>2</sup> In culture, MSCs produce a number of cytokines and extracellular matrix proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis.<sup>3,4</sup> They also support the development of hematopoietic colonies *in vitro*.<sup>4</sup> However, in contrast to hematopoietic stem cells (HSCs) that have been prospectively isolated and extensively studied at the single-cell level both *in vitro* and *in vivo*, MSCs have only been defined and isolated by physical and functional properties *in vitro*. Consequently, little is known about their phenotypic and functional characteristics *in vivo*.

Systemic administration of MSCs for facilitation of bone marrow transplantation has been proposed based on the *in vitro* characteristics of MSCs.<sup>5</sup> In recent studies, cotransplantation of human MSCs and HSCs resulted in increased chimerism or accelerated hematopoietic recovery (or both) in animal models and in humans,<sup>6-9</sup> suggesting a role for MSCs in the engraftment and repopulation of HSCs. Although the existence of donor MSCs has been documented in the BM of recipient animals following MSC

infusion,<sup>9,10</sup> the methods used to detect engraftment, such as polymerase chain reaction (PCR) or staining of cytospin samples, could not unambiguously distinguish engraftment from cell survival or nonspecific lodgment on the vascular bed. In addition, Awaya et al examined stromal cells of patients who received BM transplants and confirmed that all donor signals were, in fact, derived from macrophages.<sup>11</sup> To our knowledge, there is no physical evidence that transplanted human MSCs have indeed engrafted in the BM of adult animals and directly participated in the enhanced engraftment of HSCs.

To assess the engraftment, spatial distribution, and lineage commitment of MSCs as well as their roles in hematopoiesis *in vivo*, we transplanted enhanced green fluorescent protein (eGFP)-marked human MSCs into the tibiae of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice by intra-bone marrow transplantation (IBMT), a method previously shown to improve the engraftment of both hematopoietic and nonhematopoietic cells in mice.<sup>12-14</sup> We used a dual-color genetic marking strategy<sup>15</sup> along with immunofluorescent staining to distinguish and investigate transplanted cells *in situ*. We show that transplanted human MSCs integrated into the functional components of the HME and that these MSC-derived cells appeared to be actively involved in the maintenance of human hematopoiesis in murine BM.

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## Materials and methods

### Isolation of human cord blood CD34<sup>+</sup> cells

Human umbilical cord blood (CB) samples were obtained from full-term deliveries with informed consent of the mother and used in accordance with the institutional guidelines approved by the Tokai University Committee on Clinical Investigation. CD34<sup>+</sup> cells were selected using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's instructions as described previously.<sup>12</sup> The purity of selected cord blood CD34<sup>+</sup> (CBCD34) cells was always greater than 95%, and they were cryopreserved in liquid nitrogen until use. In some experiments, CBCD34 cells were further fractionated into CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> populations at the day of transplantation.

### Human MSCs

Human MSCs were purchased from Cambrex BioScience Walkersville (Walkersville, MD) and cultured according to the directions supplied by the company. The ability to differentiate into adipocytes, chondrocytes, and osteoblasts was confirmed *in vitro* before they were used for the experiments.<sup>2</sup>

### Antibodies

The following antibodies were used for tissue immunostaining: anti-CD15 (80H5, 1:75; Coulter/Immunotech, Marseille, France), anti-CD31 (1:100; TECNE, Minneapolis, MN), anti-CD34 (My10, 1:20; BD Biosciences, San Jose, CA), anti-CD45 (2D1, 1:75; BD Biosciences), anti-glycophorin A (JC159, 1:400; Dako, Glostrup, Denmark), anti-N-cadherin (1:20; IBL, Gunma, Japan), antiosteocalcin (1:25; Biogenesis, Poole, United Kingdom), anti-smooth muscle (SM) actin (1A4, 1:800; Sigma-Aldrich, St Louis, MO), anti-alkaline phosphatase (B4-78, 1:30; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), antivimentin (1:400; Progen Biotechnik, Heidelberg, Germany), anti-GFP (1:500; MBL, Nagoya, Japan), antiosteopontin (10A16, 1:100; IBL), and anti-SDF-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). The following monoclonal antibodies (mAbs) were used for flow cytometry: fluorescein isothiocyanate-conjugated anti-CD19 (SJ25C1; BD Biosciences), phycoerythrin-conjugated anti-CD33 (WM53) and anti-CD34 (581), and allophycocyanin-conjugated anti-CD45 (J.33; all from Coulter/Immunotech).

### Experimental animals, lentiviral gene transduction, and cell transplantation

Eight- to 10-week old male NOD/Shi-*scid* (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan) and housed in sterile microisolation cages in the animal facility of Tokai University School of Medicine. Mice were given autoclaved food and water. Twenty-four hours before transplantation, mice were irradiated with 300 cGy from an x-ray irradiator (HW-300, Hitex, Osaka, Japan) and thereafter fed acidified water. All procedures were approved by the Animal Care Committee of Tokai University. Prior to transplantation into mice, MSCs and CBCD34 were genetically marked with eGFP or its yellow variant enhanced yellow fluorescent protein (eYFP). Transduction of CBCD34 and MSCs was carried out as described previously.<sup>15,16</sup> For *in situ* examination of transplanted cells,  $1 \times 10^6$  eGFP-marked MSCs and  $2 \times 10^5$  eYFP-marked CBCD34,  $2$  to  $4 \times 10^5$  CD34<sup>+</sup>CD38<sup>+</sup> cells, or  $1.5$  to  $4 \times 10^4$  CD34<sup>+</sup>CD38<sup>-</sup> cells were suspended in 10  $\mu$ L PBS and transplanted directly into the right tibia of NOD/SCID mice using a Hamilton syringe equipped with a 31-gauge needle.<sup>12</sup> In some experiments, gene-marked MSCs or CBCD34 cells were separately transplanted by IBMT or by the intravenous route.

### Analysis of human cell homing

Staining of CBCD34 cells with PKH26 dye (Sigma-Aldrich) and analysis of cells that homed into BM were conducted as described previously.<sup>12</sup> Each mouse received  $1 \times 10^6$  MSCs into the right tibia and 10  $\mu$ L PBS into the

left tibia by IBMT, followed by administration of  $1 \times 10^6$  PKH26-labeled CBCD34 cells into the retro-orbital plexus. Twenty hours after transplantation, mice were humanely killed, and BM cells were collected separately from the tibiae that had been injected with MSC and the PBS.

### Analysis of human cell engraftment

A total of  $1 \times 10^6$  MSCs was injected into the right tibiae of irradiated NOD/SCID mice, and then  $5 \times 10^4$  CBCD34 cells were injected into the retro-orbital plexus of the mice. Control groups received the same amount of PBS in the right tibia. At 6 weeks after transplantation, mice were humanely killed, and BM cells were collected separately from each tibia. Aliquots of cells were used to examine the percentages of CD45<sup>-</sup>, CD19<sup>-</sup>, CD33<sup>-</sup>, and CD34-expressing cells in the respective tibia. Two-color flow cytometric analysis was conducted using FACSCalibur. Quadrants were set to include at least 97% of the isotype-negative cells. The proportion of each lineage was calculated from 20 000 events acquired using CellQuest software (Becton Dickinson, San Jose, CA). Remaining cells were saved for the clonogenic cell assay and secondary transplantation.

### Clonogenic cell assay

Human hematopoietic cells were isolated from BM cells of mice given transplants using CD45 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. The purity of selected cells was 36% to 96% (mean, 73%). CD45-enriched populations containing 10 000 CD45<sup>+</sup> cells were plated in MethoCult GFH4434V (StemCell Technologies, Vancouver, BC, Canada). The number and morphology of colonies formed during the 14-day culture period were determined under inverted microscope. Morphologic designation of colony type by light microscopy was confirmed by Wright-Giemsa staining of cytospin preparations. The specificity of the assay was confirmed by PCR on individual colonies using primers specific for the human chromosome 17- $\alpha$  satellite sequence<sup>12</sup> and the expression of CD45 by flow cytometric analysis.

### Secondary transplantation

BM cells obtained separately from each tibia of primary mice were intravenously transplanted into irradiated secondary recipient mice. Because the number of cells recovered from one tibia was small ( $2.4$ - $3.5 \times 10^6$ /tibia), we used NOD/SCID IL-2R $\gamma^{\text{null}}$  mice, which have been shown to be a better recipient of human cells than NOD/SCID mice,<sup>17,18</sup> as secondary recipients. Six weeks after transplantation, BM cells were obtained from tibiae and femurs of each secondary recipient, and the presence of human cells was analyzed by flow cytometry.

### Tissue processing and immunofluorescent staining

Anesthetized mice were perfused with 4% paraformaldehyde in PBS. The tibiae were excised, decalcified, infiltrated with sucrose, embedded in OCT compound (Sakura, Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections of decalcified bone were obtained using a cryostat microtome (CM3050, Leica, Germany) and stored at  $-80^\circ\text{C}$  until staining. Immunofluorescent staining and enzyme immunohistochemistry were performed as described previously.<sup>16</sup> For immunofluorescent analysis, slides were examined and images were captured using an LSM510 META confocal microscope and a 63  $\times$ /1.2 numeric aperture (NA) c-Apochromat objective lens (Carl Zeiss, Jena, Germany). Images were transferred to Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

### Quantitative microscopic examination

**Spatial distribution of human hematopoietic cells.** The location of cells was designated as either endosteal (within 12 cells of the endosteum) or central ( $>12$  cells of the endosteum) as described previously.<sup>19</sup> To accurately assess the proportion of endosteal area, the diameter of the diaphyseal shaft was evaluated by counting the number of cells from one side of the endosteum to the other along the line perpendicular to the longitudinal axis of the bone. CD34-, CD15-, and glycophorin A-reactive

human hematopoietic cells present in the diaphyseal shaft were counted under light microscopy with assistance of a Zeiss KS400.

**Interaction between human MSCs and human HSCs.** Entire fields of longitudinal sections cut through the center of bones were examined for cell counting. All eGFP-expressing cells and immunophenotyped human hematopoietic cells present in BM were counted under fluorescent microscopy. When cells were in physical contact, they were categorized as interacting.

### Statistical analysis

All values are presented as the mean plus or minus standard deviation (SD). The 2-sided *P* value was determined, testing the null hypothesis that the 2 population medians are equal. *P* below .05 was considered significant.

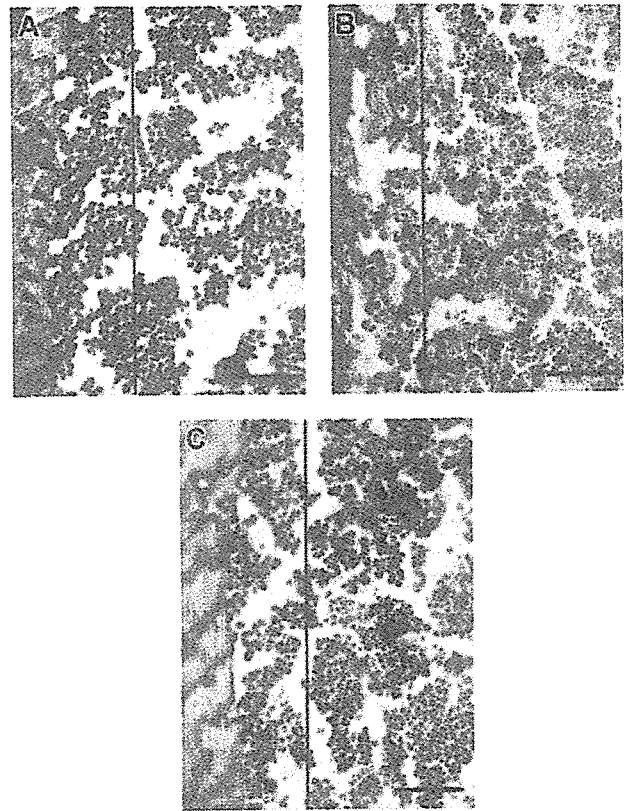
## Results

### Spatial distribution of human hematopoietic cells in the murine BM compartment

The potential of human HSCs *in vivo* can be assessed by using the SCID-repopulating cell (SRC) assay based on the ability to reconstitute hematopoiesis in the host following transplantation into NOD/SCID mice.<sup>20</sup> However, analysis of SRCs by flow cytometry does not allow identification of individual SRCs *in situ*. Consequently, the behavior of transplanted SRCs during repopulation has not been elucidated. To address this issue, we prepared mice that were highly chimeric with human hematopoietic cells by directly injecting CBCD34 cells into the tibia<sup>12</sup> and investigated the spatial organization of individual engrafted cells in murine BM at 10 weeks after transplantation. Close examination of the bone specimens revealed that cells stained positive for human CD34, a marker of human HSCs and progenitor cells, were specifically localized to an area near the endosteum of the bone (the endosteal region) (Figure 1A). In contrast, cells positively stained for human CD15, a marker of committed myelocytes, or glycoprotein A (GlyA), a marker of the erythrocyte lineage, were distributed distant to the endosteum (Figure 1B-C). These results reflect previous morphologic studies of BM that found that hematopoietic stem and progenitor cells are concentrated in the endosteal region rather than the central marrow area, which mostly is composed of mature cells.<sup>19,21,22</sup> To quantitatively confirm these observations, the numbers of CD34<sup>+</sup>, CD15<sup>+</sup>, and GlyA<sup>+</sup> cells present in the endosteal region or in the central BM cavity of the diaphyseal shaft were counted. Consistent with the microscopic observations, a surprisingly higher proportion of CD34<sup>+</sup> cells was located in the endosteal region compared to the lineage-committed cells (Table 1). Because the endosteal region consisted of only 21.4% ± 3.8% of the BM cavity examined for this assay, there were approximately 10-fold more CD34<sup>+</sup> cells in the endosteal area. By visualizing the fate of transplanted SRCs *in situ* and confirming their physiologic organization in BM, these results validate the SRC assay for human cell analysis. Hence, we used this xenogeneic transplantation system for the phenotypic and functional characterization of human MSCs *in vivo*.

### Visualization of human MSCs and human CBCD34 engraftment in the murine BM compartment

The route of administration is an important factor for effective delivery of transplanted cells into the target tissue. We compared the degree of cell engraftment in BM at 4 and 10 weeks after IBMT or intravenous transplantation of eYFP-marked CBCD34 cells



**Figure 1. Spatial distribution of human hematopoietic cells in the murine BM compartment.** Representative pictures of bone specimens from NOD/SCID mice at 10 weeks after IBMT of  $2 \times 10^5$  CBCD34 cells are shown. Slides were stained with either anti-CD34, anti-CD15, or anti-glycoprotein A antibody, and cells expressing the respective antigens were distinguished by brown reactive products of DAB on immunohistochemistry. CD34<sup>+</sup> cells are localized to the endosteal region (A; black arrowheads), whereas CD15<sup>+</sup> (B) and GlyA<sup>+</sup> cells (C) are distributed away from the endosteum. Vertical lines demarcate 12 cells of the endosteum. Bars represent 100  $\mu$ m. Images were obtained using an Olympus AX80 microscope and a 20  $\times$  0.7 NA UPlanApo objective lens. Images were captured using a DP50 digital camera fitted with Viewfinder Lite (all from Olympus, Tokyo, Japan).

(eYFP-CBCD34; Figure 2A) or eGFP-marked MSCs (eGFP-MSCs; Figure 2B). When excess numbers of eYFP-CBCD34 cells ( $2 \times 10^5$ ) were transplanted intravenously or by IBMT, similar amounts of eYFP<sup>+</sup> human hematopoietic cells (eYFP-cells) that expressed human CD45, a pan-leukocyte marker, were observed in BM at both time points examined (Figure 2C-D and data not shown). In marked contrast to the sufficient engraftment of human hematopoietic cells, intravenous administration of  $1 \times 10^6$  eGFP-MSCs resulted in virtually no visible MSCs in BM at either 4 (Figure 2F) or 10 weeks after transplantation. At 10 weeks after transplantation, analysis of 250 bone sections prepared from 5 mice yielded a total of only 10 eGFP-MSCs in BM. However, when the same number of MSCs was administered by IBMT, eGFP-MSCs were clearly identified in BM at the both time points. At 4 weeks after transplantation, eGFP-MSCs were broadly distributed in BM (Figure 2G-H). At 10 weeks after transplantation, eGFP-MSCs were detected in all sections examined (> 1000 sections), although the number of eGFP-MSCs per section varied considerably (range, 1-57; median, 13; mean, 14.8; from 91 sections) depending on the plane of sectioning. Interestingly, unlike eYFP-cells, which migrated into the contralateral tibia,<sup>12</sup> no eGFP-MSCs were detected in the BM of the tibia that had not received injections (data not shown), suggesting no or minimal migration of transplanted MSCs in this system.

**Table 1. Spatial distribution of human hematopoietic cells in the murine BM compartment at 10 weeks after IBMT of CBCD34**

	Total no. cells counted	Total no. cells in the endosteal region	Cells in the endosteal region/slide, %
CD34	1469	1086	71.4 ± 12.5*
CD15	2940	451	14.9 ± 6.2
GlyA	1514	171	10.7 ± 2.2

Fourteen slides from at least 8 different mice were examined to count each cell type. Slides containing  $115 \pm 19$  cells in the diaphyseal shaft were chosen for this analysis. Because the endosteal region was arbitrarily decided within 12 cells of both endosteum, the endosteal area comprised approximately  $21.4\% \pm 3.8\%$  of the BM cavity in this study. The proportion of cells located in the endosteal area was calculated for each slide and expressed as the means  $\pm$  SD.

\* $P < .001$  relative to the CD15 and GlyA groups.

### Integration of human MSCs into the murine microenvironment

Having confirmed the effectiveness of delivering MSCs into BM, IBMT was used for all subsequent experiments. The phenotypes of transplanted MSCs and their progeny at 10 weeks after transplantation were investigated in detail. In contrast to the bone specimens prepared at 4 weeks after transplantation, in which eGFP-MSCs were located throughout the marrow cavity (Figure 2G-H), eGFP-MSCs at 10 weeks after transplantation were preferentially localized to the endosteal region ( $73.8\% \pm 18.4\%$ ,  $n = 251$  eGFP-MSCs), frequently within 5 cells from the surface of the bone. When human MSC-derived eGFP-expressing cells (eGFP-cells) were found away from the endosteum, they were often associated with the vasculature (Figure 3A,D). Those vasculature-associated eGFP-cells expressed  $\alpha$ -SM actin (Figure 3B-C,E-F), the expression of which has been documented in pericytes, SM cells of the vascular wall as well as myofibroblasts in BM.<sup>23,24</sup> A total of  $59.9\% \pm 21.6\%$  of eGFP-cells ( $n = 251$ ) found in BM were positive for  $\alpha$ -SM actin.

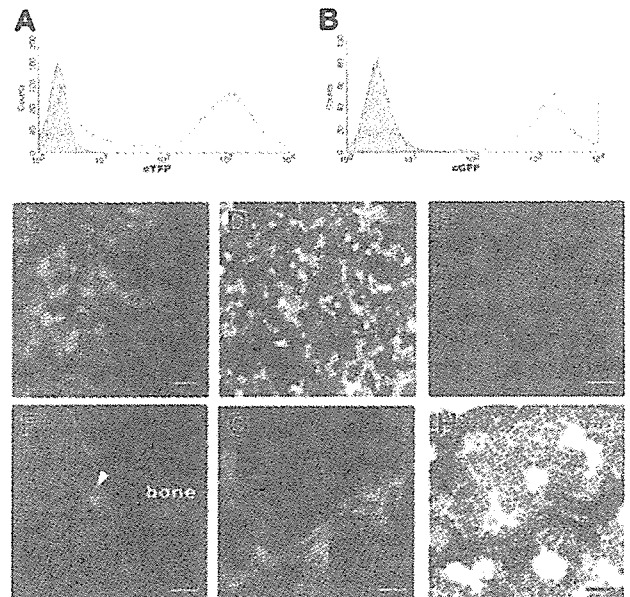
Two other types of eGFP-cells that were negative for  $\alpha$ -SM actin were also present in BM: flattened cells located in the hematopoietic cords but not specifically associated with the vasculature (Figure 3G) and cells characterized by long cytoplasmic extensions, so-called reticular cells (Figure 3H) that are considered to be the predominant cells of the HME.<sup>25,26</sup> BM was often interspersed with a fine network of cell processes that expressed alkaline phosphatase (ALP; Figure 3I), an enzyme that distinguishes reticular cells from the stromal component of acid phosphatase- or nonspecific esterase-expressing macrophages.<sup>11,25</sup> A total of  $28.2\% \pm 11.2\%$  of eGFP-cells ( $n = 242$ ) in BM were ALP<sup>+</sup>.

In addition, eGFP-cells were found within or on the surface of the bone (Figure 3J-O). Cells in the bone stained positive for osteocalcin, a specific marker of mature osteoblasts and osteocytes (Figure 3K-L), indicating an active participation in skeletal remodeling.<sup>27,28</sup> Interestingly, eGFP-cells on the bone surface resembled spindle-shaped osteoblasts (Figure 3M; see also Figure 7), a key component of the stem cell niche in murine hematopoiesis.<sup>29-32</sup> These cells expressed osteopontin (data not shown) and N-cadherin (Figure 3M-O), both of which are involved in regulating murine HSCs.<sup>29,33,34</sup> In a small number of mice examined (4 of 39), eGFP-cells associated with the vasculature stained positive for CD31 (Figure 3P-R) and CD34 (Figure 3S-U), markers of endothelial cells. MSCs expanded *ex vivo* prior to transplantation did not react with any of these markers either by the immunohistochemical or flow cytometric method, but they did stain positive for vimentin, a marker of fibroblasts (data not shown). These results indicate that

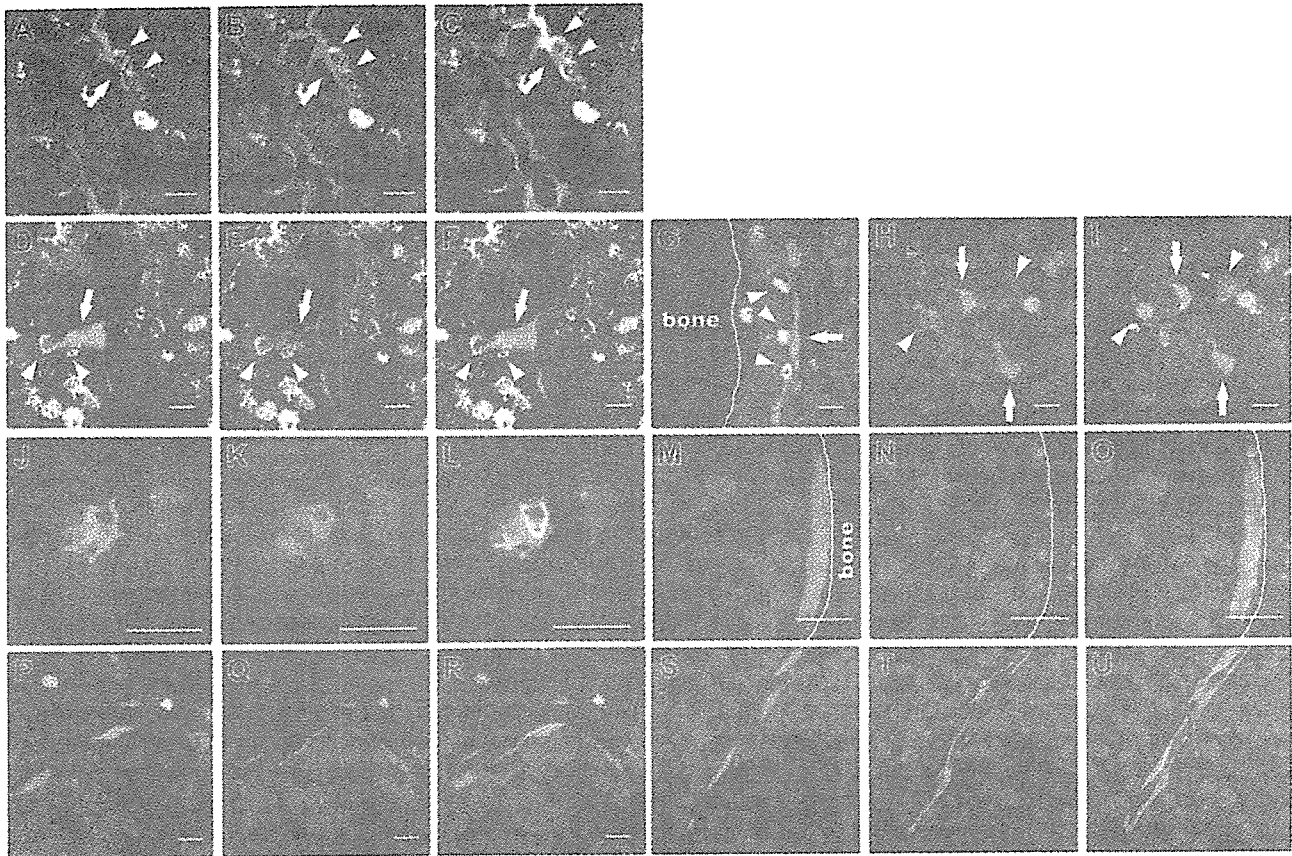
within 10 weeks after transplantation, human MSCs differentiated into pericytes, myofibroblasts, reticular cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells, which constitute the 3-dimensional structure of hematopoietic parenchyma and provide the milieu of hematopoiesis.<sup>26</sup> Therefore, we designated these cells as human MSC-derived hematopoietic microenvironment reconstituting cells (HMRCs).

### Engraftment of human hematopoietic cells

During analysis of the engraftment and differentiation of transplanted MSCs, we frequently observed physical contact between HMRCs and hematopoietic eYFP-cells (Figure 3A-I). In particular, eYFP-cells were intimately associated with ALP<sup>+</sup> eGFP-reticular cells, where eYFP-cells were almost surrounded by the thin delicate cytoplasm of the eGFP-reticular cells (Figure 3H-I). To address whether the presence of HMRCs in the murine HME contributes to the development of human hematopoietic cells, we analyzed the engraftment and differentiation of CBCD34 cells after transplantation with or without human MSCs. MSCs were injected into the right tibiae of irradiated NOD/SCID mice by IBMT, and then CBCD34 cells were administered intravenously into each mouse. Consistent with our histologic findings, more human CD45<sup>+</sup> cells were found in the tibiae into which MSCs had been injected than the tibiae that had not received injections. In addition, the presence of MSCs increased the percentage of CD34<sup>+</sup> cells



**Figure 2. In situ visualization of human MSCs and hematopoietic cells in the murine BM compartment at 4 weeks after IBMT or intravenous transplantation.** Transduction efficiencies of eYFP into CBCD34 cells and eGFP into MSCs, determined by flow cytometric analysis at the day of transplantation, were approximately 65% (A) and 99% (B), respectively. The shaded histograms indicate nontransduced cells. (C-E) The presence of human hematopoietic cells was determined by eYFP fluorescence and immunofluorescence staining with an anti-human CD45 antibody followed by Alexa-fluor 594 goat anti-mouse immunoglobulin secondary antibody. Cell nuclei were visualized by staining with TOTO3. Similar amounts of eYFP-cells expressing human CD45 (red) are present in BM after intravenous transplantation (C) or IBMT (D). (E) No eYFP- or CD45-reactive cells are present in the BM of noninjected mouse. (F) eGFP-MSCs are rarely seen in BM after intravenous transplantation (white arrowhead). After IBMT, eGFP-MSCs are easily identified in the BM cavity by eGFP fluorescence (G) and immunohistochemistry (H). (G-H) The same section was stained with an anti-GFP antibody after examining for eGFP fluorescence. All bars represent 10  $\mu$ m. Images in panels C-G were obtained using an LSM510 META confocal microscope; image in panel H was obtained using an Olympus AX80 microscope and a 20  $\times$ /0.7 NA UPlanApo objective lens.



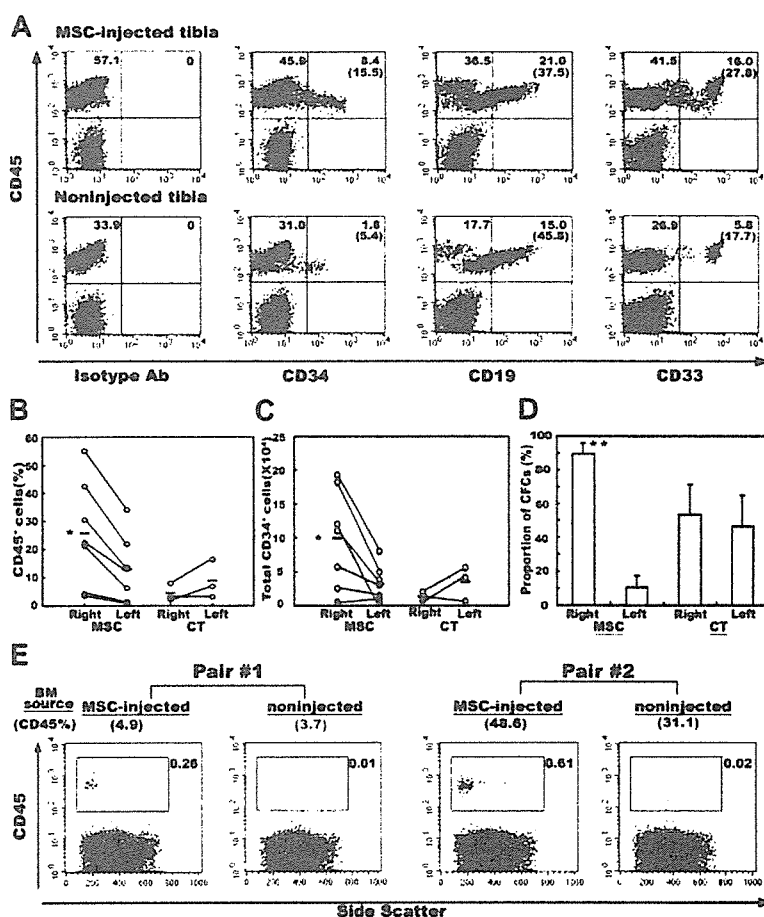
**Figure 3. Site-specific differentiation of human eGFP-MSCs in the murine BM compartment.** The engraftment and differentiation of transplanted eGFP-MSCs were determined by eGFP fluorescence and immunofluorescence staining for lineage-specific antigens. eGFP-cells were located on the abluminal side of endothelial cells (A) or lined the sinus wall (D). Those vasculature-associated eGFP-cells express  $\alpha$ -SM actin (red in panels B and E, and merged in panels C and F). (A-F) eYFP-cells reside next to eGFP-cells. (G) An elongated cell in the endosteal hematopoietic cord interacts with eYFP-cells. eGFP-reticular cells (H) with ALP<sup>+</sup> (red) cytoplasmic extensions (I), which haphazardly radiate into the hematopoietic parenchyma, interact with eYFP-cells. An eGFP-cell in the bone exhibits the morphology of authentic osteocytes with filopodial processes surrounded by bone matrix and extending into the canaliculi (J), and expresses osteocalcin (red, K-L). A bone-lining eGFP-cell (M) expresses N-cadherin (red, N-O). N-cadherin is localized to the cell surface. Vasculature-associated eGFP-cells (P,S) express CD31 (red, Q-R) and CD34 (red, T-U). Interactions between eGFP-cells (arrows) and eYFP-cells (arrowheads in A-I) are seen in the specimens from mice in which eGFP-MSCs and eYFP-CBCD34 were cotransplanted. All bars represent 10  $\mu$ m.

without affecting the proportion of CD19<sup>+</sup> B lymphocytes or CD33<sup>+</sup> myeloid cells (Figure 4A). There were 2-fold more human CD45<sup>+</sup> cells in the tibiae into which MSCs had been injected compared with the tibiae that had not received injections ( $25.7\% \pm 17.6\%$  versus  $12.8\% \pm 11.1\%$ ,  $P = .002$ , Figure 4B), and the absolute number of CD34<sup>+</sup> cells present in the tibiae into which MSCs had been injected was 3-fold higher than that in the tibiae that had not received injections ( $99\,296 \pm 68\,189$  versus  $31\,525 \pm 25\,224$ ;  $P = .007$ , Figure 4C). Control experiments, in which the same volume of PBS was injected into the right tibia, were conducted to exclude the possibility that this effect was due to injury from the injection. There were no significant differences in either the percentage of CD45<sup>+</sup> cells or the number of CD34<sup>+</sup> cells between the tibiae into which PBS had been injected and the contralateral tibiae (Figure 4B-C). We then examined the colony-forming ability of BM cells recovered from the mice that had received injections of MSCs and control mice given PBS. The cumulative numbers of colony-forming cells (CFCs) recovered from both tibiae of experimental animals were 2663 for the group that had received injections of MSCs and 1578 for the control group ( $n = 3$  for each group). The proportion of colony types formed from BM cells obtained from both groups was similar (data not shown). Because the numbers of CFCs recovered from each experimental animal varied considerably, we compared the proportion of CFCs present in the tibiae into which MSCs had been

injected with the tibiae that had not received injections over the total number of CFCs in each animal. In mice given MSCs, the tibiae into which MSCs had been injected contained the majority of CFCs ( $89.4\% \pm 6.7\%$  versus  $10.6\% \pm 6.7\%$ ,  $P = .007$ ), whereas CFCs were evenly distributed in the right and left tibiae ( $53.3\% \pm 18.1\%$  and  $46.7\% \pm 18.1\%$ , respectively) in control mice (Figure 4D). These results confirmed that the presence of human MSCs positively affected the engraftment of human HSCs.

To assess if there were any functional differences between human cells engrafted in the tibiae into which MSCs had been injected and tibiae that had not received injections, we transplanted BM cells obtained from each tibia of primary mice to separate secondary hosts. Two of 3 secondary host pairs showed human cell engraftment. A pair of recipients, which received cells from the primary host with minimal engraftment (CD45% content; 1.44% for tibiae into which MSCs had been injected and 0.52% for tibiae that had not received injections), failed to show engraftment. As expected, secondary hosts that received BM cells of the tibiae into which MSCs had been injected demonstrated a markedly higher level of engraftment than the hosts that received cells of tibiae that had not received injections (Figure 4E). Taken together, the presence of HMRCs in murine BM augmented not only the proportion of human cells but also the function of human cells engrafted in mouse BM.

**Figure 4. Effect of human MSCs on the engraftment of human hematopoietic cells.** (A) BM cells obtained from the MSC-injected or noninjected tibia from the same mouse were examined by flow cytometry at 6 weeks after transplantation. Representative flow cytometric profiles are shown. The relative frequencies of each population are shown at the corner of the respective quadrants. The numbers in parentheses indicate the proportion of CD45<sup>+</sup> cells positive for each marker. (B) Percentages of human CD45<sup>+</sup> cells in the right (injected) and the left (noninjected) tibia of the MSC-injected (MSC) and control mice (CT). (C) Absolute numbers of CD34<sup>+</sup> cells in the right and the left tibia of the MSC-injected and control mice. Each white circle represents one mouse. Values obtained from the same mouse are connected with lines. (D) Distribution of clonogenic progenitors into the right and the left tibia of the MSC-injected and control mice. The proportions of clonogenic cells present in the respective tibia over the total number of clonogenic cells recovered from both tibiae of each mouse are shown ( $n = 3$ ). \* $P < .05$  and \*\* $P < .01$  relative to the noninjected (left) tibia. (E) Secondary transplantation. BM cells obtained separately from the MSC-injected or noninjected tibia from the same mouse were transplanted into separate secondary hosts. Human cell engraftment was analyzed by CD45 expression at 6 weeks after transplantation. Results of 2 pairs of secondary host are shown. Numbers in parentheses above each flow cytometric profile show the percentage of CD45 cells in the BM cells of primary host. The relative frequencies of CD45 cells in secondary hosts are shown in each profile.



#### Interaction between human MSC-derived cells and human hematopoietic cells

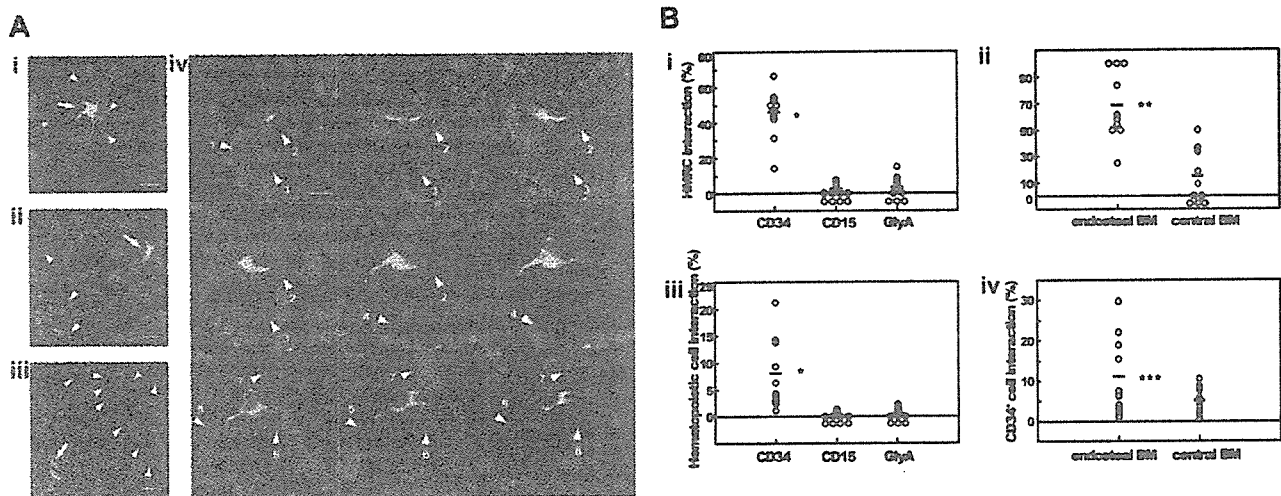
Having shown the effect of MSCs on human hematopoietic cell engraftment, we examined the underlying basis of increased engraftment in phenotypically and functionally primitive cells. First, we asked whether MSCs play a role in the migration and homing of HSCs to BM, critical steps in the engraftment and initiation of hematopoiesis after transplantation. Prior to intravenous administration of CBCD34, each mouse was given injections of MSCs into the right tibia and PBS into the left tibia. The percentages of CBCD34 cells homed to the tibiae into which MSCs and PBS had been injected were not different ( $0.05\% \pm 0.02\%$  and  $0.04\% \pm 0.01\%$ , respectively;  $n = 3$ ). Thus, MSCs did not seem to function in the initial homing of CBCD34 cells.

We then asked whether MSCs participate in the repopulation process of human hematopoietic cells in the murine HME. At 10 weeks after transplantation, human MSC-derived HMRCs and CBCD34-derived hematopoietic cells frequently interacted (Figure 3), suggesting the role of HMRCs in the maintenance of human hematopoiesis. When those hematopoietic cells were immunophenotyped, CD34<sup>+</sup> cells were often in contact or within close proximity to HMRCs (Figure 5Ai), contrasting to lineage-committed CD15<sup>+</sup> and GlyA<sup>+</sup> cells that were usually found away from HMRCs (Figure 5Aii-iii). Interestingly, one HMRC interacted with 7 CD34<sup>+</sup> cells (Figure 5Aiv). These results suggest that HMRCs interact specifically with primitive human hematopoietic cells; therefore, we quantitated HMRCs that were in physical contact with CD34<sup>+</sup>, CD15<sup>+</sup>, or GlyA<sup>+</sup> cells (Table 2; Figure 5B). Whereas approximately half of all HMRCs in BM were in contact

with CD34<sup>+</sup> cells, less than 4% of HMRCs were in contact with the lineage-committed cells (Figure 5Bi). Furthermore, the vast majority of HMRCs located in the endosteal region interacted with CD34<sup>+</sup> cells (Figure 5Bii). We then examined the human hematopoietic cells that were in contact with HMRCs. A significantly higher proportion of CD34<sup>+</sup> cells interacted with HMRCs compared to the lineage-committed cells (Figure 5Biii). The proportion of CD34<sup>+</sup> cells interacting with HMRCs in the endosteal region was 2-fold higher than that of CD34<sup>+</sup> cells in the central marrow (Figure 5Biv). The result suggests that CD34<sup>+</sup> cells in the endosteal region and the central marrow represent different populations of CD34<sup>+</sup> cells.

Because the heterogeneity among CD34<sup>+</sup> stem/progenitor cells is known,<sup>35-39</sup> we further examined the interaction between CD34<sup>+</sup> cells and HMRCs by transplanting SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup> cells or more mature CD34<sup>+</sup>CD38<sup>+</sup> progenitor cells together with MSCs (Figure 6). At 1 week after transplantation, there were no differences in the proportions of CD34<sup>+</sup> cells localized to the endosteal region ( $27.4\% \pm 7.8\%$  for the CD34<sup>+</sup>CD38<sup>-</sup> group and  $26.5\% \pm 12.1\%$  for the CD34<sup>+</sup>CD38<sup>+</sup> group) or CD34<sup>+</sup> cells interacting with HMRCs ( $8.1\% \pm 10.2\%$  and  $8.2\% \pm 9.7\%$ , respectively) between the 2 experimental groups. At 3 weeks after transplantation, the proportion of CD34<sup>+</sup> cells in the endosteal area was 2-fold higher in the group that had received transplants of SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup> cells ( $63.3\% \pm 7.9\%$  versus  $33.1\% \pm 8.3\%$ ;  $P < .001$ ). To our interest, a significantly higher proportion of CD34<sup>+</sup> cells were in contact with HMRCs in the group that had received transplants of CD34<sup>+</sup>CD38<sup>-</sup> cells ( $9.4\% \pm 4.8\%$  versus  $4.4\% \pm 4.0\%$ ;  $P < .05$ ). In the group that





**Figure 5. Interaction between HMRCs and CD34<sup>+</sup>, CD15<sup>+</sup>, or GlyA<sup>+</sup> cells.** (A) Bone slides were stained with either anti-CD34, anti-CD15, or anti-glycophorin A antibody followed by Alexa-fluor 594 goat anti-mouse immunoglobulin secondary antibody, and examined under fluorescent microscope. (Ai) CD34<sup>+</sup> cells are in physical contact with the cell body and cytoplasmic extensions of a HMRC. CD15<sup>+</sup> (Aii) or GlyA<sup>+</sup> cells (Aiii) are not found close to HMRCs. HMRCs and immunophenotyped cells are indicated by arrows and arrowheads, respectively. (Aiv) One HMRC in the hematopoietic parenchyma interacts with 7 CD34<sup>+</sup> cells (numbered arrowheads). Numerical letters at the left corner of each panel indicate the position of z-axis in the analytical planes. All bars represent 10  $\mu$ m. (B) Quantification of the interaction between HMRCs and CD34<sup>+</sup>, CD15<sup>+</sup>, or GlyA<sup>+</sup> cells. (Bi) Frequencies of HMRCs interacting with CD34<sup>+</sup>, CD15<sup>+</sup>, or GlyA<sup>+</sup> cells. (Bii) The vast majority of HMRCs located in the endosteal region interact with CD34<sup>+</sup> cells. (Biii) Frequencies of CD34<sup>+</sup>, CD15<sup>+</sup>, or GlyA<sup>+</sup> cells interacting with HMRCs. (Biv) In the endosteal region, 2-fold more CD34<sup>+</sup> cells interact with HMRCs than in the central marrow. Each white circle represents a value obtained by counting. \**P* < .005 relative to the CD15 and GlyA groups; \*\**P* < .005 and \*\*\**P* < .05 relative to the central group.

had received CD34<sup>+</sup>CD38<sup>+</sup> transplants, CD34<sup>+</sup> cells interacting with HMRCs became progressively rarer (8.2%  $\pm$  9.7% at 1 week and 4.4%  $\pm$  4.0% at 3 weeks) and were rarely observed at 6 weeks after transplantation (1.1%  $\pm$  1.0%). At both time points examined, the group that had received CD34<sup>+</sup>CD38<sup>-</sup> transplants contained higher proportions of CD34<sup>+</sup> cells interacting with bone-lining osteoblasts (9.3%  $\pm$  10.9% versus 2.7%  $\pm$  5.5% at 1 week and 32.4%  $\pm$  16.8% versus 17.4%  $\pm$  9.9% at 3 weeks). This tendency of CD34<sup>+</sup>CD38<sup>-</sup> cells, the preferential localization of CD34<sup>+</sup> cells in the endosteal area and their interaction with bone-lining osteoblasts, may reflect their high repopulation potential. In another words, SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup> cells localize to the endosteal area and interact with bone-lining osteoblasts as well as HMRCs, and progressively become distant to the endosteal area as they differentiate into progenitor cells and finally to mature cells. These results confirmed our earlier observation that HMRCs interacted with the primitive population of CD34<sup>+</sup> cells, which resulted in the enhanced engraftment of human hematopoietic cells.

**Molecular interaction of HMRCs and CD34<sup>+</sup> cells**

To determine how HMRCs participated in human hematopoietic cell repopulation in mice, we investigated the molecular interaction of HMRCs and CD34<sup>+</sup> cells. We found that CD34<sup>+</sup> cells adhered to HMRCs on the bone surface and appeared to proliferate along the endosteal surface, suggesting the existence of specific local signals between CD34<sup>+</sup> cells and bone-lining HMRCs (Figure 7A). Double staining for N-cadherin and CD34 demonstrated that bone-lining HMRCs associated with CD34<sup>+</sup> cells through the colocalization of N-cadherin (Figure 7B-E). In addition, an HMRC in the endosteal hematopoietic parenchyma expressed stromal cell-derived factor 1 (SDF-1) and interacted with a few eYFP-human hematopoietic cells (Figure 7F-H), although SDF-1 was not detected in ex vivo expanded MSCs by immunofluorescence analysis (data not shown). In BM, SDF-1 is constitutively expressed by osteoblasts, endothelial cells, and BM stromal cells.<sup>40</sup> In addition to its well-established role in homing and retention of HSCs in BM,<sup>41</sup> SDF-1 has been implicated

**Table 2. Interactions between HMRCs and human hematopoietic cells at 10 weeks after IBMT of eGFP-MSCs plus CB127**

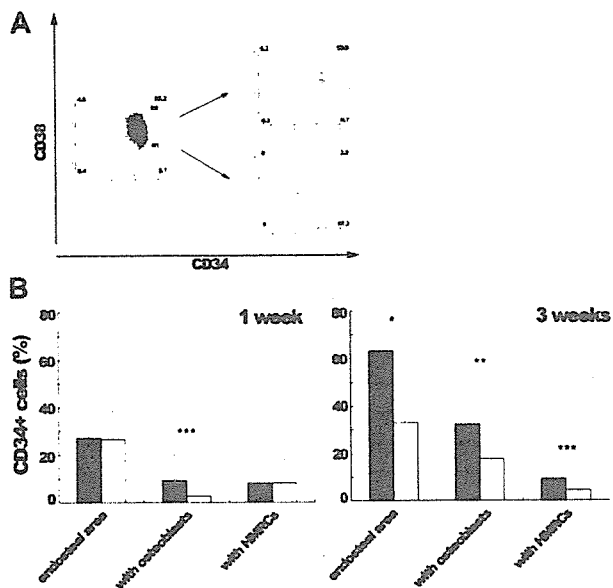
	HMRC interaction with hematopoietic cells			Hematopoietic cell interaction with HMRC		
	No. cells counted	HMRCs interacting		No. cells counted	Hematopoietic cells interacting	
		Total no.	Frequency, %		Total no.	Frequency, %
<b>CD34</b>						
Total	150	66	45.9 $\pm$ 13.5*	1609	76	8.0 $\pm$ 6.2*
Endosteal	81	49	68.0 $\pm$ 24.8†	1012	59	11.0 $\pm$ 9.4‡
Central	59	17	14.6 $\pm$ 17.9	597	17	5.0 $\pm$ 3.1
CD15	100	3	1.2 $\pm$ 2.4	2603	3	0.14 $\pm$ 0.28
GlyA	154	5	3.2 $\pm$ 4.5	1624	5	0.36 $\pm$ 0.49

BM sections stained with either anti-CD34, anti-CD15, or anti-glycophorin A (GlyA) antibody were examined under fluorescent microscope (10 slides from at least 5 different mice for each cell type). The total numbers of HMRCs and immunophenotyped hematopoietic cells present in the sections were counted, and individual cells were examined for physical contact between HMRCs and hematopoietic cells. Proportions of interacting cells/slide were calculated for each cell type and expressed as the means  $\pm$  SD. Interactions in CD34<sup>+</sup> cell group were further categorized into the endosteal and the central BM groups based on the histoanatomic location of cells.

\**P* < .005 relative to the CD15 and GlyA groups.

†*P* < .005 relative to the central group.

‡*P* < .05 relative to the central group.



**Figure 6.** In vivo localization of CD34<sup>+</sup> cells and their interaction with HMRCs after IBMT of CD34<sup>+</sup>CD38<sup>-</sup> or CD34<sup>+</sup>CD38<sup>+</sup> populations together with human MSCs. (A) Sorting profiles of CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> populations. The relative frequencies of each population are shown in the corner of the respective quadrants. (B) At 1 or 3 weeks after IBMT, bone sections were stained with an anti-CD34 antibody and examined for counting. Eighteen slides from 3 mice for each group were counted to obtain the proportion of CD34<sup>+</sup> cells localized to the endosteum (endosteal area) at the both time points. CD34<sup>+</sup> cells in the endosteal area were further categorized into cells attaching to bone-lining osteoblasts (with osteoblasts) and cells interacting with HMRCs (with HMRCs). The proportions of CD34<sup>+</sup> cells in each category are shown. At 1 week after IBMT, the proportions of CD34<sup>+</sup> cells both in the endosteal area and in contact with HMRCs were not different between the 2 groups. At 3 weeks after IBMT, the SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup>-transplanted group had the higher proportions of CD34<sup>+</sup> cells in the endosteal area as well as those interacting with HMRCs. At the both time points examined, the proportion of CD34<sup>+</sup> cells interacted with osteoblasts was higher in the SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup>-transplanted group. Bars represent the CD34<sup>+</sup>CD38<sup>-</sup>-transplanted group (■) and the CD34<sup>+</sup>CD38<sup>+</sup>-transplanted group (□). \**P* < .001, \*\**P* < .005, and \*\*\**P* < .05 relative to the CD34<sup>+</sup>CD38<sup>+</sup> group.

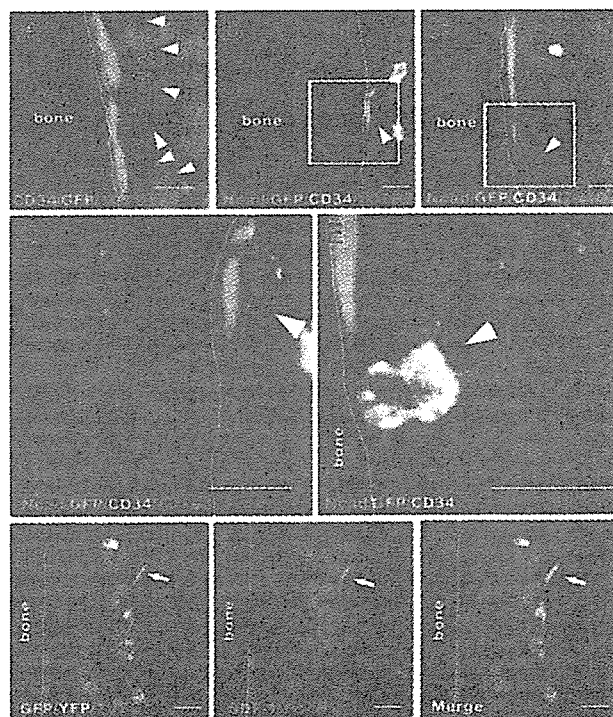
in regulating the status of primitive HSCs both in vitro and in vivo.<sup>42-44</sup> Therefore, HMRCs may contribute to the maintenance of primitive human HSCs through N-cadherin-mediated interactions and the production of SDF-1.

**Discussion**

Our study demonstrated that intramedullary transplanted human MSCs reconstituted the HME and provided direct evidence for a role of transplanted human MSCs in the enhancement of human hematopoietic cell repopulation in mice. The initial histologic analysis unveiled the integration of human hematopoietic cells into the specially and functionally compartmentalized HME of NOD/SCID mice. Based on this finding, we established a model system that enables the identification of the phenotype and function of human MSCs in vivo by directly injecting genetically marked human MSCs into the BM of NOD/SCID mice. Analogous to human HSCs, human MSCs persisted long-term in murine BM to at least 10 weeks after transplantation and were able to differentiate into the key components of the HME in the host. The presence of human MSCs in murine BM correlated with the increase in human hematopoietic cells that were phenotypically and functionally primitive. Engrafted human MSCs appeared to be involved in the maintenance of human hematopoiesis via secreted factors as well as by physically interacting with primitive hematopoietic cells.

The stem cell niche is a key determinant of stem cell development.<sup>45,46</sup> We are beginning to understand the murine HSC niche and the molecular mechanisms that govern the fate of murine HSCs,<sup>47</sup> but there exists a paucity of data on the cellular and molecular microenvironmental regulation of human hematopoiesis in vivo due largely to a lack of good experimental tools. Although the identification of SRCs has facilitated detailed characterization of human HSCs in vivo,<sup>20,48</sup> the key niches that function in human cell repopulation have not been identified. Our study has demonstrated for the first time that CD34-expressing stem/progenitor cells localize to the endosteal surface and mobilize toward the central marrow as they differentiate in BM. In addition, SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup> cells demonstrated a distinct trend to localize in the endosteal region and to interact with bone-lining osteoblasts, even at the early stage of hematopoietic reconstitution. This may be one of the reasons that CD34<sup>+</sup>CD38<sup>-</sup> cells have the high repopulation potential. We also found that human MSC-derived HMRCs locally created human HME in the murine environment. Visualization of human hematopoietic cells and human HMRCs in situ made it possible to elucidate the physical interaction between human hematopoietic cells and the human microenvironment. In this way, the SRC assay recapitulates human hematopoiesis in the murine environment both structurally and functionally and could serve as a good experimental system to study human hematopoiesis.

The presence of human MSCs in murine BM resulted in significantly more engraftment of phenotypically and functionally primitive human hematopoietic cells. With this newly established experimental system, we were able to present 3 lines of evidence that explain how human MSCs may facilitate human hematopoietic cell engraftment. First, the interaction of human MSC-derived HMRCs and human CD34<sup>+</sup> cells was mostly observed in the



**Figure 7.** Expression of N-cadherin and SDF-1 by HMRCs that interact with human hematopoietic cells. (A) CD34<sup>+</sup> cells (arrowheads) appear to colonize near the bone-lining HMRCs. (B-C) Bone-lining HMRCs colocalize with CD34<sup>+</sup> cells (arrowheads) through the asymmetrical expression of N-cadherin. (D-E) Higher magnifications of panels B and C. (F-H) An HMRC in the endosteal hematopoietic parenchyma (arrow) expresses SDF-1 and interacts with a few eYFP hematopoietic cells. All bars represent 10 μm.

endosteal region, at a significantly higher frequency than that of lineage-committed cells. When this interaction was examined further by using sorted populations of SRC-enriched CD34<sup>+</sup>CD38<sup>-</sup> cells and more mature CD34<sup>+</sup>CD38<sup>+</sup> progenitor cells, a preferential interaction of HMRCs with a primitive population of CD34<sup>+</sup> cells was evident. Whereas the proportion of CD34<sup>+</sup> cells interacting with HMRCs rapidly decreased in the group that had received CD34<sup>+</sup>CD38<sup>+</sup> progenitor cells, this frequency in the group that had received CD34<sup>+</sup>CD38<sup>-</sup> transplants did not change and was equivalent to the proportion of CD34<sup>+</sup> cells interacting with HMRCs at 10 weeks after transplantation. Considering that the proportion of CD34<sup>+</sup>CD38<sup>-</sup> cells originally contained in the CD34<sup>+</sup> cells (representing 5%-8% of CD34<sup>+</sup> cells in this study) was similar to HMRC-interacting CD34<sup>+</sup> cells (8.0% ± 6.2% at 10 weeks), transplanted human MSCs, which became an integral part of the functional HME, interacted with primitive cell populations, provided supportive environment of human hematopoiesis, and augmented human cell engraftment. Second, bone-lining HMRCs interacted with CD34<sup>+</sup> cells through the asymmetric expression of N-cadherin, similar to the way bone-lining osteoblasts maintain primitive HSCs in mouse. This specific interaction of CD34<sup>+</sup> cells with human MSC-derived bone-lining osteoblasts, a cellular component of the stem cell niche, indicates that similar regulatory mechanisms operate in human and murine hematopoiesis. Third, HMRCs in the endosteal hematopoietic parenchyma produced SDF-1 and interacted with human hematopoietic cells. This strengthens the previous observations that SDF-1 regulates the proliferation and survival of primitive HSCs and progenitor cells.<sup>12,42-44</sup> Taken together, these results suggest that human MSC-derived HMRCs contribute not only to the proliferation and differentiation of human hematopoietic progenitor cells that results in the increased chimerism but also to the maintenance of primitive human HSCs.

Embryonic development is strictly regulated through sequential and concerted events that are orchestrated by interactions between tissue stem cells and the microenvironment. This developmental

paradigm, including signaling molecules that regulate stem cell self-renewal,<sup>49</sup> is conserved for generating and maintaining specific tissues in adult life, and dysregulation of this process leads to pathologic conditions such as cancer.<sup>50</sup> Current empiric cancer studies have focused on identifying intrinsic genetic changes that lead to the aberrant proliferation of cells, and, as a result, therapeutic agents targeting the genetic mutations are emerging. However, our understanding of extrinsic, or microenvironmental, signals in the context of tumorigenesis has lagged behind. Because the microenvironment is responsible for homeostatic controls,<sup>45,51,52</sup> is there a specific microenvironment that permits, initiates, or complements tumorigenesis and supports progression of tumors? Could the microenvironment be a new target for cancer therapy? The experimental system we described here, which allows the visualization and reconstitution of a human microenvironment, provides a unique tool to study the maintenance of tissue homeostasis, which may lead to the elucidation of the tumorigenic microenvironment. In addition, the functional persistence of transplanted MSCs in the host environment means that MSCs may be used to deliver therapeutic genes or agents to target tissues.

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## References

- Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 1997;276:71-74.
- Pittenger MF, Mackay AM, Beck SC, et al. Multi-lineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
- Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol*. 1999;181:67-73.
- Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol*. 1998;176:57-66.
- Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol*. 2004;32:414-425.
- Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol*. 2000;18:307-316.
- Noort WA, Krusselbrink AB, in 't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp Hematol*. 2002;30:870-878.
- in 't Anker PS, Noort WA, Krusselbrink AB, et al. Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp Hematol*. 2003;31:881-889.
- Bensidhoum M, Chapel A, Francois S, et al. Homing of in vitro expanded Stro-1<sup>-</sup> or Stro-1<sup>+</sup> human mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. *Blood*. 2004;103:3313-3319.
- Davies SM, Bartholomew AM, Mahmud N, et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol*. 2001;29:244-255.
- Awaya N, Rupert K, Bryant E, Torok-Storb B. Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. *Exp Hematol*. 2002;30:937-942.
- Yahata T, Ando K, Sato T, et al. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. *Blood*. 2003;101:2905-2913.
- Wang J, Kimura T, Asada R, et al. SCID-repopulating cell activity of human cord blood-derived CD34<sup>+</sup> cells assured by intra-bone marrow injection. *Blood*. 2003;101:2924-2931.
- Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood*. 2004;104:3581-3587.
- Yahata T, Ando K, Miyatake H, et al. Competitive repopulation assay of two gene-marked cord blood units in NOD/SCID/gammac(null) mice. *Mol Ther*. 2004;10:882-891.
- Muguruma Y, Reyes M, Nakamura Y, et al. In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. *Exp Hematol*. 2003;31:1323-1330.
- Yahata T, Ando K, Nakamura Y, et al. Functional human T lymphocyte development from cord blood CD34<sup>+</sup> cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. *J Immunol*. 2002;169:204-209.
- Ito M, Hiramoto H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood*. 2002;100:3175-3182.
- Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood*. 2001;97:2293-2299.
- Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med*. 1996;2:1329-1337.
- Lambertsen RH, Weiss L. A model of intramedullary hematopoietic microenvironments based on

- stereologic study of the distribution of endocloned marrow colonies. *Blood*. 1984;63:287-297.
22. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109:625-637.
  23. Peled A, Zipori D, Abramsky O, Ovadia H, Shezen E. Expression of alpha-smooth muscle actin in murine bone marrow stromal cells. *Blood*. 1991;78:304-309.
  24. Charbord P, Taviani M, Humeau L, Peault B. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood*. 1996;87:4109-4119.
  25. Westen H, Bainton DF. Association of alkaline-phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. *J Exp Med*. 1979;150:919-937.
  26. Lichtman MA. The ultrastructure of the hemopoietic environment of the marrow: a review. *Exp Hematol*. 1981;9:391-410.
  27. Pereira RF, O'Hara MD, Laptev AV, et al. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A*. 1998;95:1142-1147.
  28. Nilsson SK, Dooner MS, Weier HU, et al. Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. *J Exp Med*. 1999;189:729-734.
  29. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 2003;425:836-841.
  30. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003;425:841-846.
  31. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*. 2004;118:149-161.
  32. Visnjic D, Kalajcic Z, Rowe DW, Katavic V, Lorenzo J, Aguilu HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood*. 2004;103:3258-3264.
  33. Stier S, Ko Y, Forkert R, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med*. 2005;201:1781-1791.
  34. Nilsson SK, Johnston HM, Whitty GA, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*. 2005;106:1232-1239.
  35. Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34<sup>+</sup>CD38<sup>-</sup> progenitor cells. *Blood*. 1991;77:1218-1227.
  36. Craig W, Kay R, Cutler RL, Lansdorp PM. Expression of Thy-1 on human hematopoietic progenitor cells. *J Exp Med*. 1993;177:1331-1342.
  37. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*. 1997;90:5002-5012.
  38. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A*. 1992;89:2804-2808.
  39. Murray LJ, Bruno E, Uchida N, et al. CD109 is expressed on a subpopulation of CD34<sup>+</sup> cells enriched in hematopoietic stem and progenitor cells. *Exp Hematol*. 1999;27:1282-1294.
  40. Ponomaryov T, Peled A, Petit I, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest*. 2000;106:1331-1339.
  41. Lapidot T. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. *Ann N Y Acad Sci*. 2001;938:83-95.
  42. Lataillade JJ, Clay D, Dupuy C, et al. Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood*. 2000;95:756-768.
  43. Lataillade JJ, Clay D, Bourin P, et al. Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. *Blood*. 2002;99:1117-1129.
  44. Cashman J, Clark-Lewis I, Eaves A, Eaves C. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. *Blood*. 2002;99:792-799.
  45. Watt FM, Hogan BL. Out of Eden: stem cells and their niches. *Science*. 2000;287:1427-1430.
  46. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001;414:98-104.
  47. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood*. 2005;105:2631-2639.
  48. Guenechea G, Gan OI, Dorrell C, Dick JE. Distinct classes of human stem cells that differ in proliferative and self-renewal potential. *Nat Immunol*. 2001;2:75-82.
  49. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. *Nature*. 2001;411:349-354.
  50. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414:105-111.
  51. Antonchuk J, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp Hematol*. 2001;29:1125-1134.
  52. Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer*. 2004;4:839-849.

## Efficient intervention of growth and infiltration of primary adult T-cell leukemia cells by an HIV protease inhibitor, ritonavir

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Adult T-cell leukemia (ATL), an aggressive malignancy of CD4<sup>+</sup> T cells associated with human T-cell leukemia virus type I (HTLV-I) infection, carries a very poor prognosis because of the resistance of leukemic cells to any conventional regimen, including chemotherapy. We examined the effect of ritonavir, an HIV protease inhibitor, on HTLV-I-infected T-cell lines and primary ATL cells and found

that it induced apoptosis and inhibited transcriptional activation of NF- $\kappa$ B in these cells. Furthermore, ritonavir inhibited expression of Bcl-x<sub>L</sub>, survivin, c-Myc, and cyclin D2, the targets of NF- $\kappa$ B. In nonobese diabetic/severe combined immunodeficient (NOD/SCID)/ $\gamma$ c<sup>null</sup> (NOG) mice, ritonavir very efficiently prevented tumor growth and leukemic infiltration in various organs of NOG mice at the same

dose used for treatment of patients with AIDS. Our data indicate that ritonavir has potent anti-NF- $\kappa$ B and antitumor effects and might be clinically applicable for treatment of ATL. These results would provide a new concept and novel platform for new drug development of leukemia and solid cancer as well. (Blood. 2006;107:716-724)

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### Introduction

Human T-cell leukemia virus type I (HTLV-I) is the causative agent of an aggressive form of CD4<sup>+</sup> T-cell leukemia designated adult T-cell leukemia (ATL).<sup>1,3</sup> ATL was first identified in Japan in 1977.<sup>4,5</sup> Common findings for patients with ATL include enlargement of peripheral lymph nodes, hepatomegaly, splenomegaly, hypercalcemia, and skin lesions. At present, there is no accepted curative therapy for ATL, and patients progress to death with a median survival duration of 13 months in aggressive ATL.<sup>6</sup> ATL has a poor prognosis mainly because of its resistance to conventional as well as high-dose chemotherapy.

ATL develops after a long period of latent infection. This long latency suggests that multiple genetic events, which accumulate in HTLV-I infected cells, are involved in the development of ATL. However, the precise molecular mechanism of leukemogenesis and the development of ATL after HTLV-I infection are not fully elucidated. A unique viral gene *Tax* is considered to play a central role in HTLV-I-induced transformation, which is responsible for transactivation of the HTLV-I long terminal repeat,<sup>7,8</sup> as well as numerous cellular genes involved in T-cell activation and growth, such as those encoding IL-2,<sup>9</sup> and the  $\alpha$ -chain of IL-2 receptor

(IL-2R $\alpha$ ) (CD25, Tac).<sup>10,11</sup> Induction of many cellular genes by Tax is mediated through the transcription factor NF- $\kappa$ B. The malignant cells associated with all phases of ATL express very high levels of IL-2R $\alpha$ <sup>12-14</sup> without expressing a significant amount of Tax.

HTLV-I-infected cell lines derived from a leukemic cell clone and primary ATL cells failed to express significant amounts of Tax and other viral proteins, suggesting that the expression of viral proteins is not always necessary for leukemic proliferation at the late stage of the disease. However, HTLV-I-infected cell lines and leukemic cells from patients with ATL display constitutive NF- $\kappa$ B binding activity and increased degradation of a specific inhibitor, I $\kappa$ B $\alpha$ .<sup>15</sup> In resting cells, NF- $\kappa$ B is sequestered as an inactive precursor by association with inhibitory I $\kappa$ Bs in the cytoplasm. On stimulation, I $\kappa$ Bs are rapidly phosphorylated, ubiquitinated, and degraded by a proteasome-dependent pathway, allowing active NF- $\kappa$ B to translocate into the nucleus where it can activate the expression of a number of genes.<sup>16</sup> NF- $\kappa$ B activation has been connected with multiple processes of oncogenesis, including control of apoptosis, cell cycle, differentiation, and cell migration<sup>16</sup>; therefore, inhibition of NF- $\kappa$ B was suggested to be a useful

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strategy for cancer therapy.<sup>17-20</sup> Despite the diversity in clinical manifestations of ATL, strong and constitutive NF- $\kappa$ B activation was reported to be a unique and common characteristic of ATL cells.<sup>15</sup> Thus, the indispensability of NF- $\kappa$ B for the maintenance of the malignant phenotype of HTLV-I provides a possible molecular target for ATL therapy.<sup>21-24</sup>

Ritonavir, a human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI), has been successfully used in clinical treatments of HIV infection, with patients exhibiting a marked decrease in HIV viral load and a subsequent increase in CD4<sup>+</sup> T-cell counts.<sup>25-28</sup> Evidence of other effects by ritonavir on cellular proteases, such as the cysteine proteases cathepsin D and E, was presented in the drug's original description, albeit at concentrations greater than 500-fold above the concentration required for inhibition of HIV protease.<sup>29</sup> PIs have also been shown to directly affect cell metabolism, interfere with host or fungal proteases, and block T-cell activation and dendritic cell function.<sup>30,31</sup> Recently, ritonavir has been shown to inhibit the chymotrypsin-like activity of the 20S proteasome, and it activates the chymotrypsin-like activity of the 26S proteasome conversely.<sup>30,32,33</sup> Ritonavir also has been reported to inhibit the transactivation of NF- $\kappa$ B induced by activators such as TNF $\alpha$ , HIV-1 Tat protein, and the human herpesvirus 8 protein ORF74. It is possible that inhibition of NF- $\kappa$ B activation by ritonavir is linked to additional pathways other than inhibition of proteasome.<sup>34</sup> PIs also have been shown to have direct antiangiogenic and antitumor activity.<sup>34,35</sup>

In this study, we investigated the antitumor effects of ritonavir on HTLV-I-infected cell lines and primary ATL cells. We found that ritonavir decreases NF- $\kappa$ B activity linked to the inhibition of I $\kappa$ B $\alpha$  phosphorylation and induces apoptosis of these cells. In addition, we established preclinical models to evaluate the efficacy of anti-ATL and anti-NF- $\kappa$ B therapies. In the ATL model, ritonavir potently inhibited the growth and infiltration of leukemic cells from patients at concentrations used for treatment of patients with AIDS.

## Materials and methods

### Cell lines

The T-cell leukemia cell line Jurkat, HTLV-I-infected T-cell lines MT-2,<sup>36</sup> MT-4,<sup>37</sup> C5/MJ,<sup>38</sup> SJL-1,<sup>39</sup> HUT-102,<sup>2</sup> MT-1,<sup>40</sup> and ED-40515(-),<sup>41</sup> and bcr-abl<sup>+</sup> leukemic cell line K562 were cultured in RPMI 1640 medium supplemented with 2% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 10  $\mu$ g/ml streptomycin. MT-2, MT-4, C5/MJ, and SJL-1 are HTLV-I-transformed T-cell lines. MT-1 and ED-40515(-) are T-cell lines of leukemic cell origin established from patients with ATL. The clonal origin of HUT-102 is unclear.

### Human specimens

Leukemic cells from 38 patients (8 patient samples for in vitro studies, 20 for establishment of ATL model, 10 for in vivo ritonavir studies) diagnosed as either acute type or chronic type were used in this study. The diagnosis of ATL was based on clinical features, hematologic findings, and the presence of anti-HTLV-I antibodies in the sera. Baseline characteristics for the patients who entered the study are shown in Table 1. Monoclonal HTLV-I provirus integration into the DNA of leukemia cells was confirmed by Southern blot hybridization in all cases (data not shown). All samples were collected after obtaining informed consent from patients. Peripheral blood mononuclear cells (PBMCs) from healthy volunteers and patients with ATL were purified by Ficoll-Hypaque gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and washed with RPMI 1640.

### Growth inhibition assay

The effect of ritonavir on cell growth was assayed by the WST-8 method as described previously.<sup>42</sup> The WST-8 Cell Counting Kit was obtained from Wako (Chemicals) (Osaka, Japan). Briefly,  $2 \times 10^5$  cells were incubated in a 96-well microculture plate in the absence or presence of various concentrations of ritonavir. After 72 hours of culture, 10  $\mu$ L WST-8 solution was added, and the cells were incubated for another 2 hours. The number of surviving cells was measured with a microplate reader at a reference wavelength of 655 nm and test wavelength of 450 nm. Cell viability was determined as the percentage of the control (ie, absence of ritonavir).

### Assay for apoptosis

Quantification of apoptosis was performed by immunostaining cells with Apo2.7, which specifically detects the 38-kDa mitochondrial membrane antigen 7A6 present only on the mitochondrial membrane of apoptotic cells, and so can be used as an early apoptotic marker in cells.<sup>43,44</sup> Cells cultured for 72 hours in the absence or presence of various concentrations of ritonavir were labeled with the Apo2.7-phycoerythrin-conjugated monoclonal antibody (Beckman-Coulter/Immunotech, Miami, Florida) or mouse IgG1 isotype control (Beckman-Coulter/Immunotech) and subsequently analyzed by flow cytometry.

### EMSA

Cells were placed in culture at  $1 \times 10^6$  cells/ml (cell line) or  $5 \times 10^6$  cells/ml (PBMCs) and examined for inhibition of NF- $\kappa$ B after exposure to ritonavir. Nuclear proteins were extracted, and NF- $\kappa$ B binding activities to  $\kappa$ B element were examined by electrophoretic mobility shift assay (EMSA) as described previously.<sup>15</sup> In brief, 5  $\mu$ g nuclear extracts were preincubated in a binding buffer containing 1  $\mu$ g poly(dI:dC) (Amersham Biosciences), followed by addition of <sup>32</sup>P-labeled oligonucleotide probe containing NF- $\kappa$ B element ( $5 \times 10^4$  cpm). These mixtures were incubated for 15 minutes at room temperature. The DNA-protein complexes were separated on a 4% polyacrylamide gel and visualized by autoradiography. To examine the specificity of the NF- $\kappa$ B element probe, unlabeled competitor oligonucleotides were preincubated with nuclear extracts for 15 minutes before incubation with probes. The probe or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: a typical NF- $\kappa$ B element from the *IL2RA* gene, 5'-gatcCGGCAGGG-GAATCTCCCTCTC-3'; and AP-1 element of the *IL8* gene, 5'-gatcGTGAGACTCAGGTTT-3'. Underlined sequences represent the NF- $\kappa$ B or AP-1 binding site. To identify NF- $\kappa$ B proteins in the DNA protein complex revealed by EMSA, we used antibodies specific for various NF- $\kappa$ B proteins, including p65, p50, c-Rel, and p52 (Santa Cruz Biotechnology, Santa Cruz, CA), to elicit a supershift DNA protein complex formation. These antibodies were incubated with the nuclear extracts for 45 minutes at room temperature before incubation with radiolabeled probes.

### Western blot analysis

Treated cells were solubilized at 4°C in lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the following specific antibodies: polyclonal antibodies against I $\kappa$ B $\alpha$ , cIAP2, survivin, cyclin D2 (Santa Cruz Biotechnology), Bel-X<sub>1</sub> (Transduction Laboratories, San Jose, CA), and c-Myc (NeoMarkers, Fremont, CA) and monoclonal antibodies against phospho-I $\kappa$ B $\alpha$ , hyperphosphorylated form of pRb (Cell Signaling Technology, Beverly, MA), Bel-2, and actin (NeoMarkers). The protein bands recognized by the antibodies were visualized using the enhanced chemiluminescence system (Amersham, Piscataway, NJ).

### Plasmids and transfection

Reporter plasmid  $\kappa$ B-1-UC (kindly provided by I. Fujisawa, Kansai Medical University, Osaka, Japan) is a luciferase expression plasmid controlled by 5 tandem repeats of a NF- $\kappa$ B binding site from the *IL2RA*

**Table 1. Clinical characteristics of patients**

Patient	Age, y/sex	Diagnosis	WBC count, cells $\times 10^9/L$	Lymphocytes, %	Atypical cells, %	Treatment status
1	54/M	Acute	192.80	65.0	91.0	Untreated
2	77/F	Acute	186.0	41.0	70.0	Untreated
3	67/F	Chronic	10.40	38.0	89.0	Treated
4	58/M	Acute	67.30	71.0	80.0	Treated
5	71/M	Acute	19.70	51.0	61.0	Treated
6	66/F	Chronic	29.40	49.0	75.0	Untreated
7	48/F	Chronic	9.40	29.0	55.0	Untreated
8	69/F	Acute	53.80	44.0	95.0	Treated
9	58/M	Chronic	11.30	59.0	70.0	Treated
10	60/M	Chronic	9.12	61.0	80.0	Treated
11	65/F	Acute	29.40	25.0	60.0	Untreated
12	49/F	Chronic	15.10	48.0	75.0	Treated
13	57/M	Chronic	10.00	50.0	57.0	Treated
14	72/F	Acute	39.00	70.0	80.0	Treated
15	79/F	Chronic	10.10	47.0	27.0	Untreated
16	68/F	Acute	7.00	86.0	86.0	Treated
17	59/F	Chronic	8.99	74.5	40.0	Treated
18	70/M	Acute	31.60	71.7	68.0	Treated
19	49/M	Acute	5.00	19.5	78.0	Treated
20	44/M	Chronic	36.40	33.0	43.0	Untreated
21	65/F	Chronic	14.70	76.0	22.0	Untreated
22	63/F	Acute	12.40	71.0	82.0	Untreated
23	56/F	Chronic	7.20	46.0	15.0	Treated
24	78/F	Acute	94.50	63.3	49.0	Treated
25	62/F	Acute	10.10	53.0	27.0	Untreated
26	66/M	Chronic	38.10	63.0	39.0	Treated
26	39/F	Chronic	18.50	16.0	57.0	Untreated
28	48/F	Acute	53.50	39.0	64.0	Untreated
29	75/F	Acute	15.00	72.0	65.0	Untreated
30	84/F	Acute	14.40	69.0	61.0	Treated
31	73/M	Chronic	7.80	59.0	47.0	Untreated
32	43/F	Chronic	18.60	63.0	43.0	Untreated
33	54/F	Acute	69.00	49.0	50.0	Untreated
34	66/F	Acute	10.20	38.0	51.0	Untreated
35	73/F	Chronic	15.70	58.0	39.0	Untreated
36	63/F	Acute	32.90	71.0	95.0	Treated
37	44/F	Chronic	22.60	51.0	45.0	Untreated
38	68/M	Acute	30.00	79.0	81.0	Untreated

WBC indicates white blood cells.

gene. Reporter plasmid AP-1-LUC (kindly provided by N. Mukaida, Kanazawa University, Kanazawa, Japan) is a luciferase expression plasmid controlled by 2 copies of the AP-1 binding site from the IL-8 promoter. The expression plasmid for HTLV-1 Tax has been described previously.<sup>45</sup> Transient transfections were performed in Jurkat and HUT-102 cells by electroporation using  $5 \times 10^6$  cells and reporter and effector plasmids. To normalize transfection efficiencies, a thymidine kinase (TK) promoter-driven Renilla luciferase plasmid (phRL-TK; Promega, Madison, WI) was cotransfected as an internal control plasmid. Then, 16 hours after transfection, ritonavir was added to the cultures at various concentrations, and cells were further cultured for 24 hours for assay of luciferase activity. Transfected cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and lysed in reporter lysis buffer (Promega). Lysates were assayed for reporter gene activity with the dual-luciferase reporter assay system (Promega).

#### Inoculation of ATL cells and collection of samples

NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the Animal Center of National Institute of Infectious Diseases (Tokyo, Japan). The Ethical Review Committee of the Institute approved the experimental protocol. Mice were anesthetized with ether, and cells were inoculated either intraperitoneally in

abdominal region or subcutaneously in the postauricular region of NOG mice without injection of human recombinant IL-2 at a dose of 1 to  $2 \times 10^7$  cells per mouse. All mice were killed 30 or 60 days after inoculation with primary ATL cells. Blood was collected from the tail to make a smear, as well as from the heart with heparinized syringes. PBMCs and splenocytes were isolated by density gradient concentration with Ficoll-Hypaque. Blood smear slides were fixed in methyl alcohol for May-Grunwald and Giemsa staining. PBMCs and splenocytes were stored at  $-80^\circ\text{C}$  for further experiments. Tissues and various organs of mice were collected and fixed with Streck Tissue Fixative, then processed to paraffin wax-embedded sections for staining with hematoxylin and eosin (HE) and immunostaining.

#### Treatment of ATL mice with ritonavir

Ritonavir was obtained from Abbott Labs, North Chicago, IL. Primary ATL cells ( $2 \times 10^7$ ) from 10 patients were inoculated subcutaneously in the postauricular region of NOG mice. One day after inoculation of ATL cells, mice were treated with either RPMI 1640 (control mice) or drug (ritonavir 30 mg/kg/d) intraperitoneally daily for 30 days followed by observation for another 30 days without treatment. ATL cell growth and progression were monitored by observation of physical condition of mice during a 2-month follow-up period.

**Immunohistochemistry**

Paraffinized cryosections of various organs were deparaffinized and hydrated in xylenes or clearing agents and graded alcohol series, then rinsed for 5 minutes in water. Deparaffinized samples were incubated with 0.025% trypsin/PBS for 30 minutes followed by washing, and then incubated with 0.3% methanol for 30 minutes at room temperature and washed 2 times with PBS. Immunostaining was done as described previously<sup>16</sup> using Vector MOM immunodetection kit (Vector Labs, Burlingame CA) for ATL cells with a 1:500 dilution of primary mouse monoclonal antibody specific for human CD4 and CD25 (Dako, Caterpillar, CA). This was followed by washing in PBS and incubation with a secondary antibody MOM biotinylated anti-mouse IgG, after which cells were again washed in PBS and incubated with VECTASTAIN Elite ABC for 20 minutes at room temperature. Positive staining was visualized after incubation of these samples with a mixture of 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer and 0.01% hydrogen peroxide for 5 minutes. The samples were counterstained with hematoxylin for 2 minutes, hydrated completely, cleaned in xylene, and then mounted. Photographs were taken by light microscopy (BX41, Olympus, Tokyo, Japan) using UplanF1 lenses (DP70, Olympus; magnification ×40).

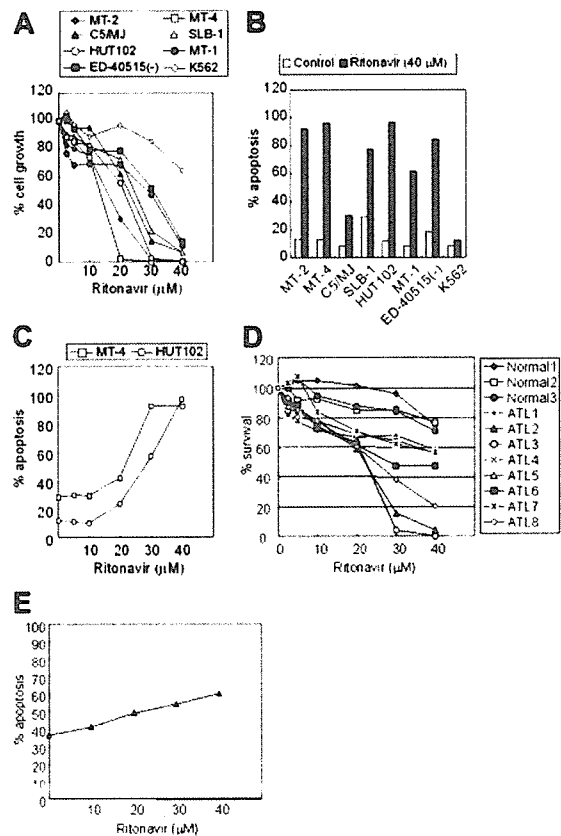
**Results**

**Ritonavir reduces cell growth and induces apoptosis of HTLV-I-infected cell lines and primary ATL cells**

Ritonavir was examined for its effect on proliferation of HTLV-I-infected cell lines (Figure 1A). Ritonavir effectively inhibited the proliferation of HTLV-I-infected cell lines as measured by WST-8 on the third day of culture in a dose-dependent manner, but not that of K562 cells. Further experiments using Apo2.7 showed that ritonavir caused apoptosis of HTLV-I-infected cell lines in a dose-dependent manner, but not that of K562 cells (Figure 1B-C). In addition, we explored the anti-ATL effect of ritonavir on freshly isolated ATL cells from patients. In all ATL cases, ritonavir reduced the survival of ATL cells in a dose-dependent manner (Figure 1D). Ritonavir also caused apoptosis of ATL cells (Figure 1E). In contrast, ritonavir hardly affected the survival of peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers as measured by WST-8 (Figure 1D).

**Ritonavir suppresses constitutive NF-κB expressed by HTLV-I-infected cell lines and primary ATL cells**

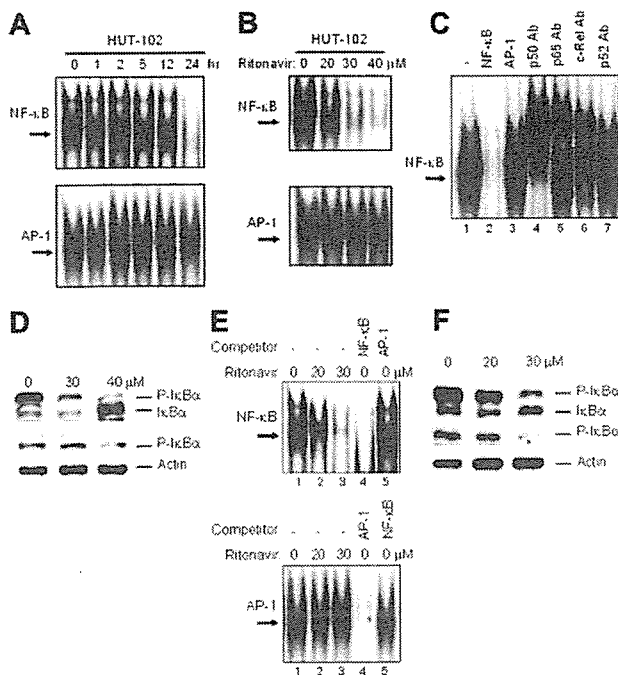
To examine the effect of ritonavir on NF-κB DNA binding, electrophoretic mobility shift assay (EMSA) was performed. We first examined the minimum duration of exposure to ritonavir required for suppression of NF-κB. For this, HUT-102 cells were incubated with 40 μM ritonavir for different periods of time, and nuclear extracts were prepared and examined for NF-κB by EMSA. Down-regulation of NF-κB occurred at 24 hours in HUT-102 cells. However, no change in binding activity of AP-1 was observed (Figure 2A). The observed protein/DNA binding was specific for NF-κB, because the binding was effectively competed and abrogated by excess unlabeled NF-κB oligonucleotide but not by AP-1 (Figure 2C). The NF-κB complex contained p50, p65, and c-Rel (Figure 2C). Forty micromolar concentration of ritonavir was sufficient to suppress constitutive NF-κB activation in residual HTLV-I-infected T-cell lines (data not shown). It should be noted that K562 cells did not show constitutive NF-κB activation (data not shown). We also found a concentration-dependent inhibitory effect of ritonavir on the constitutive increase of NF-κB DNA binding activity (Figure 2B). Twenty micromolar concentration of



**Figure 1. Effect of ritonavir on the growth and induction of apoptosis of HTLV-I-infected cell lines and freshly isolated ATL cells.** (A) Dose-response effect of ritonavir on the growth of HTLV-I-infected cell lines. Cells (10<sup>5</sup>/mL) were cultured for 72 hours in the presence of various concentrations (2.5-40 μM) of ritonavir. Cell growth was assessed by the water-soluble tetrazolium (WST)-8 method and is expressed as a percentage of control (untreated cells) and represents the mean of triplicate cultures. (B) Effect of ritonavir on induction of apoptosis of HTLV-I-infected cell lines. Cells were cultured for 72 hours with ritonavir (40 μM), and apoptosis was measured by Apo2.7 immunostaining. Data represent the mean percentages of apoptotic cells from both untreated (□) and ritonavir-treated (■) cells. (C) Dose-response effect of ritonavir on induction of apoptosis of MT-4 and HUT-102 cells. (D) Dose-response effect of ritonavir on the cell viability of freshly isolated ATL cells. Cells (10<sup>5</sup>/mL) were cultured for 72 hours in the presence of various concentrations (2.5-40 μM) of ritonavir. (E) Dose response effect of ritonavir on induction of apoptosis of ATL cells.

ritonavir caused only a partial inhibition of NF-κB/DNA binding, whereas strong inhibition was observed at 30 and 40 μM in HUT-102 cells. However, AP-1 binding was not inhibited. This inhibition coincided with an accumulation of unphosphorylated IκBα and a decrease of the slower-migrating form of phosphorylated IκBα, a prerequisite for its subsequent degradation (Figure 2D, top). Thirty micromolar concentration of ritonavir caused only a partial decrease of the slower-migrating form of phosphorylated IκBα, whereas significant decrease of the slower-migrating form of phosphorylated IκBα and an accumulation of unphosphorylated IκBα were observed at 40 μM. We determined the alteration of phosphorylation of IκBα using antibody against phosphospecific IκBα. Results in Figure 2D (middle) show that 40 μM ritonavir decreased the phosphorylated IκBα content. We also determined whether the same results were obtained in primary ex vivo ATL specimens. As shown in Figure 2E, the amount of NF-κB that translocates to the nucleus is also decreased, as determined by EMSA. Twenty micromolar concentration of ritonavir caused only a partial inhibition of NF-κB/DNA binding, whereas strong inhibition was observed at 30 μM in primary ATL cells from acute





**Figure 2. Ritonavir inhibits constitutive NF- $\kappa$ B activation.** (A) HUT-102 cells treated with ritonavir (40  $\mu$ M) for the indicated time were evaluated for NF- $\kappa$ B and AP-1 activation. (B) HUT-102 cells treated with the indicated concentration of ritonavir for 24 hours were evaluated for NF- $\kappa$ B and AP-1 activation. (C) Cold competition using 100-fold molar excess of unlabeled NF- $\kappa$ B and AP-1 oligonucleotides (lanes 2 and 3) demonstrated the specificity of the protein/DNA binding complexes. Specificity of NF- $\kappa$ B binding was also determined by using antibodies to the NF- $\kappa$ B components p50, p65, c-Rel, and p52, resulting in supershift (lanes 4-7). (D) HUT-102 cells were treated with the indicated concentration of ritonavir for 24 hours, and cell lysates were immunoblotted for I $\kappa$ B $\alpha$  (top) and phospho-I $\kappa$ B $\alpha$  (middle). Actin immunoblots confirm that similar amounts of cell extracts were analyzed (bottom). (E) Primary acute-type ATL cells treated with concentrations of ritonavir as indicated for 24 hours were evaluated for NF- $\kappa$ B and AP-1 activation. Where indicated, 100-fold excess amounts of competitor oligonucleotides were added to the reaction mixture (lanes 4 and 5). (F) The bands of phosphorylated I $\kappa$ B $\alpha$  were down-regulated by ritonavir treatment. Detection of actin expression was used as an internal control.

type. However, AP-1 binding was not inhibited. Ritonavir abolished proximal signaling events leading to I $\kappa$ B $\alpha$  phosphorylation (Figure 2F). Twenty micromolar concentration of ritonavir caused only a partial decrease of the slower-migrating form of phosphorylated I $\kappa$ B $\alpha$ , whereas significant decrease of the slower-migrating form of phosphorylated I $\kappa$ B $\alpha$  and an accumulation of unphosphorylated I $\kappa$ B $\alpha$  were observed at 30  $\mu$ M (top). Thirty micromolar concentration of ritonavir abolished the phosphorylated I $\kappa$ B $\alpha$  content (middle). We obtained similar results using another acute-type ATL cells (data not shown).

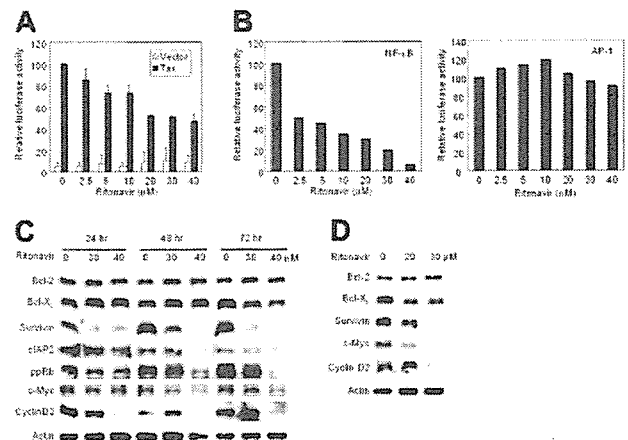
#### Ritonavir represses Tax-induced and constitutive transcriptional activity of NF- $\kappa$ B

We examined whether ritonavir inhibits the transcriptional activity of NF- $\kappa$ B. First, we tested the effect of ritonavir on Tax-induced NF- $\kappa$ B transcriptional activity in Jurkat cells (HIV-1-uninfected cell line) transfected with Tax expression plasmid. Ritonavir caused only a partial inhibition of proliferation in Jurkat cells cultured for 24 hours even at the highest dose. Tax-induced NF- $\kappa$ B transcriptional activation was suppressed by ritonavir in a dose-dependent manner (Figure 3A). Next, we determined the effect of ritonavir on constitutively activated NF- $\kappa$ B and AP-1 transcriptional activity in an HIV-1-infected cell line HUT-102. Ritonavir inhibited the constitutively activated transcriptional activity of NF- $\kappa$ B in a dose-dependent manner, but not that of AP-1 (Figure

3B). Twenty micromolar concentration of ritonavir caused only a partial inhibition of NF- $\kappa$ B/DNA binding (Figure 2B), whereas strong inhibition of NF- $\kappa$ B transcriptional activity was observed at the concentration less than 20  $\mu$ M. This discrepancy might derive from differences in sensitivity between these 2 assays. These results indicate that ritonavir inhibits both Tax-induced and constitutive NF- $\kappa$ B transcriptional activity.

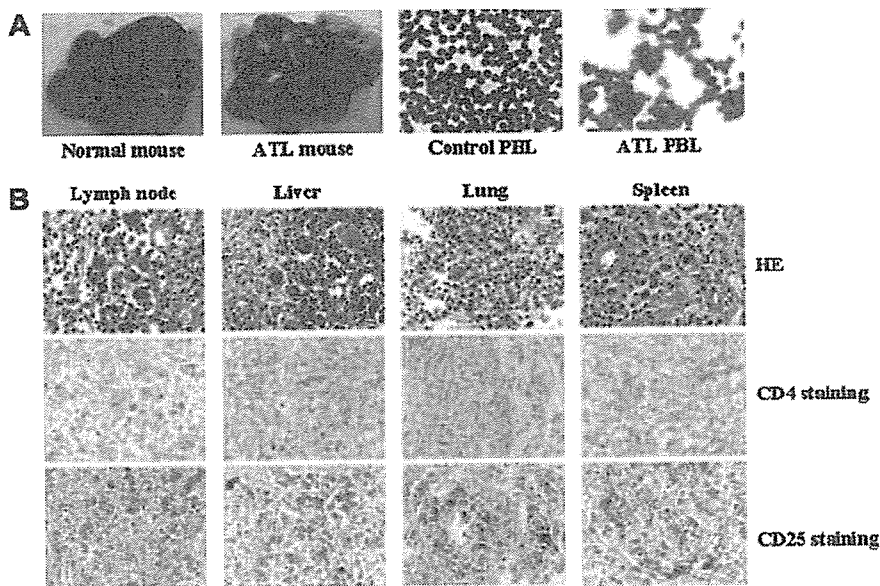
#### Ritonavir down-regulates the expression of NF- $\kappa$ B-regulated gene products

The antiproliferative and proapoptotic effects of ritonavir were explored by examining the level of intracellular regulators of cell cycle and apoptosis after exposure to ritonavir (Figure 3C). Ritonavir down-regulated levels of Bcl-X $_1$ , survivin, cIAP2, c-Myc, cyclin D2, regulated by NF- $\kappa$ B,<sup>47-51</sup> and the phosphorylated form of pRb in HUT-102 cells cultured with 40  $\mu$ M ritonavir for 72 hours. Thirty micromolar concentration of ritonavir caused only a partial down-regulation of Bcl-X $_1$  and the phosphorylated form of pRb, whereas significant down-regulation of survivin was observed in HUT-102 cells cultured for 72 hours, suggesting that NF- $\kappa$ B-regulated genes have the differential sensitivity to ritonavir. However, ritonavir did not modulate levels of Bcl-2 and Tax proteins in these cells (Figure 3C; data not shown). We also explored the effect of ritonavir on NF- $\kappa$ B-regulated gene products in ATL cells freshly isolated from an acute-type patient. As shown in Figure 3D, Bcl-X $_1$ , survivin, cyclin D2, and c-Myc, but not Bcl-2, showed a decline. Thus, expression of NF- $\kappa$ B regulated genes, the induction of which are involved in antiapoptosis (Bcl-X $_1$ , survivin, and cIAP2) and cell cycle (cyclin D2 and c-Myc), apparently had been down-regulated in the presence of ritonavir. We obtained a similar result using other acute-type ATL



**Figure 3. Ritonavir inhibits NF- $\kappa$ B transcriptional activation and expression of apoptosis- and cell-cycle-associated proteins.** (A) Ritonavir inhibits Tax-induced NF- $\kappa$ B transcriptional activation.  $\kappa$ B-LUC was transfected into Jurkat cells with Tax-expressing plasmid (■) or empty vector (□). After transfection, cells were treated with increasing concentrations of ritonavir. Luciferase activity is expressed relative to the basal level measured in cells transfected with the reporter plasmid and Tax-expressing plasmid without further treatment, which was defined as 100. Data represent the mean  $\pm$  SD from 3 independent experiments. (B) Ritonavir inhibits constitutive active NF- $\kappa$ B transcriptional activity in HUT-102 cells.  $\kappa$ B-LUC and AP-1-LUC were transfected into HUT-102 cells. After transfection, cells were treated as in panel A. Luciferase activity was normalized, based on the Renilla luciferase activity from pRL-TK. Relative luciferase activity is expressed relative to the basal level measured in cells transfected with the reporter plasmid without further treatment, which was defined as 100. (C-D) Western blot analyses. HUT-102 cells (C) and primary acute-type ATL cells (D) were cultured with the indicated concentration of ritonavir for 24 to 72 hours (HUT-102 cells) and 24 hours (ATL cells). Cells were harvested and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with indicated antibodies.

**Figure 4. Successful engraftment and massive infiltration of primary ATL cells into various organs of NOG mice inoculated with PBMCs from patients with ATL.** (A) Photographs of whole organs of normal and ATL cell-challenged mice with enlarged spleen, liver, and lungs (left 2 panels). Right 2 panels show May-Grunwald and Giemsa staining of PBMCs collected from normal and ATL cell-challenged mice, 2 months after inoculation of ATL cells, respectively. (B) HE (top) and immunohistochemical staining of various organs of NOG mice inoculated with ATL cells. Immunohistochemical staining using anti-CD4 (middle) and anti-CD25 (bottom) was conducted on various organs of mice tissues 2 months after inoculation of ATL cells. Magnification × 40.



cells (data not shown). Taken together these data suggest that constitutively high NF-κB activity in ATL cells is indispensable for their survival, and that specific inhibition of this activity by a clinically available drug results in the induction of apoptosis.

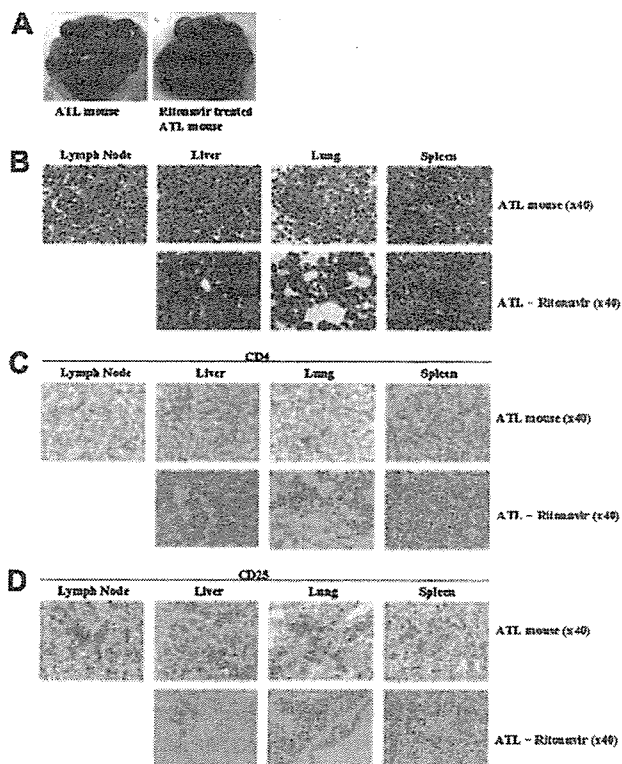
**Establishment of a novel ATL model**

PBMCs from patients with ATL were inoculated either intraperitoneally into the abdominal region or subcutaneously in the postauricular region of unconditional NOD/SCID/γc<sup>null</sup> (NOG) mice. All mice developed clinical sign of near-death, such as piloerection, weight loss, and cachexia 6 to 8 weeks after inoculation of ATL cells in addition to the enlargement of lymph nodes, spleen, lungs, and liver, whereas no tumors were found in the postauricular region or abdominal cavity where primary ATL cells were inoculated (Figure 4A). There was no difference in respect to the successful engraftment of ATL cells inoculated either intraperitoneally or subcutaneously in NOG mice. Histologic analysis of ATL-bearing mice showed massive infiltration of leukemic cells in various organs of NOG mice that were efficiently expressing human CD4 and CD25 molecules (Figure 4B). A higher level of IL-2Rα (CD25) expression was observed on the surface of malignant cells associated with all stages of ATL,<sup>12-14</sup> as well as ATL cells infiltrated into various organs of patients.<sup>52,53</sup> Thus, results from this model indicated successful engraftment and massive infiltration of primary ATL cells in various organs of NOG mice, like leukemia but without producing tumors at the sites of inoculation.

**Ritonavir inhibits ATL cell growth and infiltration in NOG mice**

To study the effect of ritonavir on ATL, we injected primary ATL cells ( $2 \times 10^7$ ) from 10 patients subcutaneously into the postauricular region of NOG mice. One day after inoculation, mice were treated with either RPMI-1640 (as control) or ritonavir (30 mg/kg/d) intraperitoneally daily for 30 days followed by observation for another 30 days without any treatment. ATL cell inoculation promoted the development of piloerection, weight loss, and cachexia, all of which are signs of near-death, in addition to the enlargement of lymph nodes, spleen, lungs, and liver in all control mice 2 months after inoculation (Figure 5A). In contrast, ritonavir-treated mice appeared to be healthy and had almost no enlargement of these organs (Figure 5A). Clinical evaluation of organ invasion 2

months after injection of primary ATL cells showed that ritonavir treatment inhibited their infiltration into lymph nodes, spleen, lungs, and liver (Figure 5B-D). Samples from 7 patients of 10 injected in mice treated with ritonavir presented substantial inhibition of organ invasion, and 2 (patients 5 and 7) showed partial inhibition, whereas one sample (patient 6) failed to do so (Table 2).



**Figure 5. Effect of ritonavir on ATL cell growth and infiltration.** (A) Mice were injected with ATL cells ( $2 \times 10^7$  cells) subcutaneously in the postauricular region. One day after inoculation, the mice were administered either RPMI 1640 or ritonavir (30 mg/kg/d) intraperitoneally every day for 30 days followed by observation for another month without therapy. Photographs of whole organs of RPMI 1640-treated ATL mice (left) and ritonavir treated ATL mice (right) with enlarged spleen, liver, and lungs. (B) HE staining (B) and immunohistochemical staining using anti CD4 (C) or anti-CD25 (D) in various organs of NOG mice 2 months after inoculation of ATL cells. Upper panels show various organs of mice treated with RPMI 1640, and lower panels represent various organs of mice treated with ritonavir. Magnification × 40.

**Table 2. Effect of ritonavir on infiltration of ATL cells in various organs of SCID mice**

Patient no. and treatment	Liver	Spleen	Lung	Lymph node
1				
Control	+++	+++	+++	+++
Ritonavir	+	-	-	NF
2				
Control	+++	+++	++	+++
Ritonavir	+	-	-	NF
3				
Control	+++	+++	+++	+++
Ritonavir	+	-	+	NF
4				
Control	+++	+++	+++	+++
Ritonavir	+	+	+	NF
5				
Control	+++	+++	+++	+++
Ritonavir	++	++	++	NF
6				
Control	+++	+++	+++	+++
Ritonavir	+++	++	+++	++
7				
Control	+++	+++	+++	+++
Ritonavir	++	+	++	NF
8				
Control	+++	+++	+++	+++
Ritonavir	+	+	+	NF
9				
Control	+++	+++	+++	++
Ritonavir	++	+	+	NF
10				
Control	+++	+++	++	+++
Ritonavir	+	+	+	NF

PBMCs from patients with ATL patients were inoculated subcutaneously into postauricular region of NOG mice, which were treated with ritonavir or RPMI-1640 as a control 1 day after inoculation for 1 month. Various organ infiltration was evaluated 2 months after inoculation.

NF indicates no formation of new lymph node; + + +, massive; + +, marked; +, slight; +, focally present or no infiltration; -, no infiltration.

In contrast, all control mice showed formation of new lymph nodes and infiltration with ATL cells into various organs (Figure 5B-D; Table 2). Organ infiltration of primary ATL cells was analyzed and evaluated by HE staining and immunostaining of CD4 and CD25. Together, these data indicate that ritonavir significantly inhibits ATL cell growth and infiltration in various organs of NOG mice (Figure 5).

## Discussion

ATL is a malignancy of CD4<sup>+</sup> T-lymphocytes etiologically linked to a retrovirus, HTLV-I.<sup>1-3</sup> The malignant cells associated with all phases of ATL express very high levels of H-2R $\alpha$  (CD25).<sup>12-14</sup> The median survival duration of all patients with aggressive ATL was 13 months, and overall survival at 2 years was estimated to be 31.3%.<sup>6</sup> The various chemotherapies so far developed have not increased significantly the survival of patients with ATL. Given disappointing results using conventional chemotherapy, new approaches for the treatment of ATL are required. Previous reports have shown that primary ATL cells proliferate and infiltrate into various organs of SCID mice.<sup>54-56</sup> Our ATL model was consistent with others, but it

represented more aggressive features about cell growth and infiltration in SCID mice. The tumor cells massively infiltrated into various organs in a manner similar to a leukemia-expressing T-cell marker CD4 and an activating marker CD25, especially into the spleen, lymph nodes, liver, and lungs of NOG mice. Our NOG ATL model presents many features 6 to 8 weeks after inoculation of ATL cells such as the clinical signs observed in patients with ATL. Two clinical types, acute and chronic, carry very different prognosis. However, no difference of cell growth, surface phenotype, and NF- $\kappa$ B activity is observed in primary leukemic cells from patients with acute- and chronic-type ATL. Therefore, the same characteristics of freshly isolated ATL cells with acute- and chronic-type were observed in NOG mice. Thus, it represents a novel model to evaluate tissue toxicity and the efficacy of therapeutic agents directed toward the treatment of ATL.

Constitutive NF- $\kappa$ B activation was demonstrated not only in HTLV-I-infected cell lines but also on fresh ATL cells regardless of Tax expression,<sup>15</sup> which could contribute to the drug-resistance of ATL cells through overexpression of Bcl-X<sub>L</sub>.<sup>51</sup> We and others have previously reported that suppression of high NF- $\kappa$ B activity inhibited cell growth and induced apoptosis of HTLV-I-infected cell lines and primary ATL cells both in vitro and in vivo.<sup>21-24</sup> Recently, ritonavir has been shown to inhibit NF- $\kappa$ B activity induced by activators such as TNF $\alpha$ , Tat, and ORF74.<sup>34</sup> This led us to investigate whether this drug exhibits anti-ATL effects in vitro and in our preclinical murine ATL model. In the present study, we revealed that ritonavir treatment of HTLV-I-infected cell lines and ATL cells inhibited phosphorylation of I $\kappa$ B $\alpha$ , allowing suppression of NF- $\kappa$ B activity. Our results also suggest that inhibition of NF- $\kappa$ B activity by ritonavir reduced cell growth and induced apoptosis of these cells. This is consistent with down-modulation of NF- $\kappa$ B-regulated genes such as antiapoptotic (Bcl-X<sub>L</sub>,<sup>50,51</sup> cIAP2,<sup>57</sup> and survivin<sup>49</sup>) and cell-cycle-related (cyclin D2<sup>48</sup> and c-Myc<sup>47</sup>) genes. We also examined the effect of ritonavir on the proliferation of an HTLV-I-negative T-cell line, Jurkat, in vitro. Exposure of ritonavir for 72 hours reduced the rate of proliferation (data not shown). Because ritonavir is suggested to affect proteasomal proteolysis,<sup>33</sup> it may effect a stabilization of p21, p27, and p53 proteins. Like proteasome inhibitors, ritonavir may also affect multiple pathways critical for survival of HTLV-I-positive and -negative malignant T cells. Additional experiments will be necessary to elucidate the mechanisms of anti-ATL activity.

In the therapy of HIV infection, the blood plasma ritonavir concentrations are between 5 and 15  $\mu$ M,<sup>58</sup> but much higher maximal concentrations (up to 46  $\mu$ M) have been determined in individual patients.<sup>59</sup> In the present study, we used the concentration of ritonavir for doing in vitro experiments from 0 to 40  $\mu$ M and in vivo 30 mg/kg/d used for treatment of patients with AIDS. Our murine ATL model clearly indicates that 30 mg/kg/d of ritonavir significantly inhibits ATL cell growth and infiltration into various organs of NOG mice. The plasma exposure produced by this dose in mice is only approximately one half of the plasma exposure observed with the licensed dose of ritonavir in humans (600 mg twice daily). In our NOG ATL model, ritonavir at this treatment dosage is well tolerated without severe adverse effects observed in the mice during the treatment period. These data strongly suggest that the HIV PI, ritonavir, is a promising antitumor agent against ATL and could be used clinically for ATL regimens. Ritonavir exhibited anti-ATL activity against leukemic cells from patients

with acute- and chronic-type ATL in vitro and in vivo. The expression of CD25 and NF- $\kappa$ B activity do not differ between acute and chronic ATL cells.<sup>12-15</sup> These results suggest that anti-ATL activity of ritonavir correlates with suppression of NF- $\kappa$ B activity. It is also of interest to note that HTLV-I uses an aspartic protease analogous to HIV protease in its replication. To our knowledge, the activity of ritonavir against HTLV-I protease has not been assessed. Although Tax expression is very low or undetectable in ATL malignancy, a direct antiviral effect of ritonavir cannot be ruled out at this time.

In summary, using a large number of patient samples we have established a novel NOG ATL model that presents features similar to patients with ATL. These results also indicate that the HIV PI, ritonavir, showed antitumor and anti-NF- $\kappa$ B activity against primary ATL cells. Finally, our results strongly suggest that NF- $\kappa$ B serves as a potential molecular target to treat ATL, and that ritonavir might be used clinically as a single compound or in combination with the reducing dose of chemotherapeutic agents for treatment of patients with ATL.

## References

- Hinuma Y, Nagata K, Hanaoka M, et al. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci U S A*. 1981;78:6476-6480.
- Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A*. 1980;77:7415-7419.
- Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A*. 1982;79:2031-2035.
- Takatsuki K, Uchiyama T, Sagawa K, Yodoi J. Adult T-cell leukemia in Japan. In: Seno S, Takaku F, Irino S, eds. *Topics in Hematology*. Amsterdam, The Netherlands: Excerpta Medica; 1977:73-77.
- Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*. 1977;50:481-492.
- Yamada Y, Tomonaga M, Fukuda H, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol*. 2001;113:375-382.
- Felber BK, Paskalis H, Kleinman-Ewing C, Wong-Staal F, Pavlakis GN. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science*. 1985;229:675-679.
- Sodroski JG, Rosen CA, Haseltine WA. Transacting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science*. 1984;225:381-385.
- Maruyama M, Shibuya H, Harada H, et al. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded p40x and T3/Ti complex triggering. *Cell*. 1987;48:343-350.
- Ballard DW, Bohnlein E, Lowenthal JW, Wano Y, Franza BR, Greene WC. HTLV I tax induces cellular proteins that activate the  $\kappa$ B element in the IL-2 receptor  $\alpha$  gene. *Science*. 1988;241:1652-1655.
- Cross SL, Feinberg MB, Wolf JB, Holbrook NJ, Wong-Staal F, Leonard WJ. Regulation of the human interleukin-2 receptor  $\alpha$  chain promoter: activation of a nonfunctional promoter by the transactivator gene of HTLV-I. *Cell*. 1987;49:47-56.
- Uchiyama T, Broder S, Waldmann TA. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells, I: production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J Immunol*. 1981;126:1393-1397.
- Uchiyama T, Hori T, Tsudo M, et al. Interleukin-2 receptor (Tac antigen) expressed on adult T cell leukemia cells. *J Clin Invest*. 1985;76:446-453.
- Waldmann TA, Greene WC, Sarin PS, et al. Functional and phenotypic comparison of human T cell leukemia/lymphoma virus positive adult T cell leukemia with human T cell leukemia/lymphoma virus negative Sezary leukemia, and their distinction using anti-Tac. Monoclonal antibody identifying the human receptor for T cell growth factor. *J Clin Invest*. 1984;73:1711-1718.
- Mori N, Fujii M, Ikeda S, et al. Constitutive activation of NF- $\kappa$ B in primary adult T-cell leukemia cells. *Blood*. 1999;93:2360-2368.
- Baldwin AS. The NF- $\kappa$ B and I $\kappa$ B proteins: new discoveries and insights. *Annu Rev Immunol*. 1999;14:649-681.
- Watanabe M, Dewan MZ, Okamura T, et al. A novel NF- $\kappa$ B inhibitor DHMEQ selectively targets constitutive NF- $\kappa$ B activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. *Int J Cancer*. 2005;114:32-38.
- Adams J, Palombella VJ, Elliott PJ. Proteasome inhibition: a new strategy in cancer treatment. *Invest New Drugs*. 2000;18:109-121.
- Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res*. 1999;5:2638-2645.
- Hideshima T, Chauhan D, Richardson P, et al. NF- $\kappa$ B as a therapeutic target in multiple myeloma. *J Biol Chem*. 2002;277:16639-16647.
- Dewan MZ, Terashima K, Taruishi M, et al. Rapid tumor formation of human T-cell leukemia virus type 1-infected cell lines in novel NOD-SCID<sup>g $\gamma$ c<sup>null</sup></sup> mice: suppression by an inhibitor against NF- $\kappa$ B. *J Virol*. 2003;77:5286-5294.
- Kitajima I, Shinohara T, Blitakovics J, Brown DA, Xu X, Nerenberg M. Ablation of transplanted HTLV-I Tax-transformed tumors in mice by antisense inhibition of NF- $\kappa$ B. *Science*. 1992;258:1792-1795.
- Mori N, Yamada Y, Ikeda S, et al. Bay 11 7082 inhibits transcription factor NF- $\kappa$ B and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. *Blood*. 2002;100:1828-1834.
- Tan C, Waldmann TA. Proteasome inhibitor PS-341, a potential therapeutic agent for adult T-cell leukemia. *Cancer Res*. 2002;62:1083-1086.
- Collier AC. Efficacy of combination antiretroviral therapy. *Adv Exp Med Biol*. 1996;394:355-372.
- Collier AC, Coombs RW, Schoenfeld DA, Bassett R, Baruch A, Corey L. Combination therapy with zidovudine, didanosine and saquinavir. *Antiviral Res*. 1996;29:99.
- Collier AC, Coombs RW, Schoenfeld DA, et al. Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. *N Engl J Med*. 1996;334:1011-1017.
- Markowitz M, Saag M, Powderly WG, et al. A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. *N Engl J Med*. 1995;333:1534-1539.
- Kempf DJ, Marsh KC, Denissen JF, et al. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. *Proc Natl Acad Sci U S A*. 1995;92:2484-2488.
- Andre P, Groettrup M, Klennerman P, et al. An inhibitor of HIV-1 protease modulates proteasome activity, antigen presentation, and T cell responses. *Proc Natl Acad Sci U S A*. 1998;95:13120-13124.
- Liang JS, Distler O, Cooper DA, Jamil H, Deckelbaum RJ, Ginsberg HN, Sturley SL. HIV protease inhibitors protect apolipoprotein B from degradation by the proteasome: a potential mechanism for protease inhibitor-induced hyperlipidemia. *Nat Med*. 2001;7:1327-1331.
- Schmidtko G, Holzhutter HG, Bogoy M, et al. How an inhibitor of the HIV-1 protease modulates proteasome activity. *J Biol Chem*. 1999;274:35734-35740.
- Gaedicke S, Firat-Geier E, Constantiniu O, et al. Antitumor effect of the human immunodeficiency virus protease inhibitor ritonavir: induction of tumor-cell apoptosis associated with perturbation of proteasomal proteolysis. *Cancer Res*. 2002;62:6901-6908.
- Pati S, Pelsier CB, Dufraigne J, Bryant JL, Reitz MS Jr, Weichold FF. Antitumor effects of HIV protease inhibitor ritonavir: inhibition of Kaposi sarcoma. *Blood*. 2002;99:3771-3779.
- Sgadan C, Barillari G, Toschi E, et al. HIV protease inhibitors are potent anti-angiogenic molecules and promote regression of Kaposi sarcoma. *Nat Med*. 2002;8:225-232.
- Miyoshi I, Kubonishi I, Yoshimoto S, et al. Type C virus particles in a cord T-cell line derived by cocultivating normal human cord leukocytes and