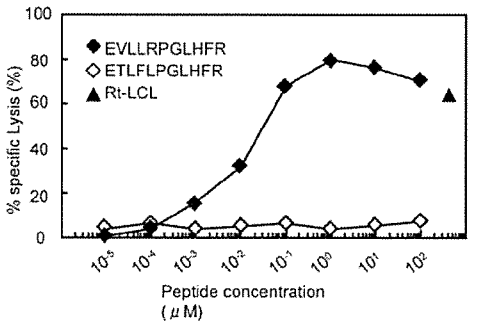

FIGURE 5. Evaluation of synthetic peptides for epitope reconstitution activity. Donor LCL (female) were labeled with ^{51}Cr, then pulsed with serial dilutions of either EVLLRPGLHFR or putative female X homologue ETLFLPGLHFR (the mismatched amino acids are underlined) and used as targets for CTL clone 1B6 in a standard ^{51}Cr release assay.

Moreover, when the whole TMSB4Y genomic region was analyzed using GENESCAN software (36) (http://genes.mit.edu/GENSCAN.html), no ORF other than the reported ORF encoding the 34-mer polypeptide was predicted with a risk of <1.6% of false negative. These results strongly suggest that theTMSB4Y/A33 minor H Ag is not derived from a functional polypeptide, but, rather, that it is a subsidiary translation product of the TMSB4Y transcript.

Defective ribosomal products (DRiPs) consist of prematurely terminated polypeptides and misfolded polypeptides produced from translation of genuine mRNAs in the proper reading frame or are produced entropically due to the inevitable imperfections inherent to protein synthesis or folding (37). DRiPs, which account for 30% of newly synthesized proteins, have been suggested to be a major source of peptides presented on the cell surface by class I MHC (38). TMSB4Y was expressed in normal cells as well as transformed cells when assessed by quantitative PCR specific for the region encoding the TMSB4Y/A33 epitope, and the full-length mRNA was readily detected (data not shown). According to the definition of DRiPs, which is defective products from genuine mRNAs in the proper reading frame, TMSB4Y/A33 should be one of epitopes derived from cryptic polypeptides rather than DRiPs. In any case, the identification of a minor H Ag encoded outside the conventional ORF has important implications for the identification of other minor H Ag epitopes using genetic linkage analysis. Recently, we identified two minor H Ags using a similar approach (29), where we looked for peptides with potential HLA-binding sequence motifs that spanned nonsynonymous single nucleotide polymorphisms in the conventional ORF. However, the results of the current study suggest that not only conventional ORFs but also regions other than conventional ORFs should be taken into consideration when attempting to identify the epitope within the region mapped by linkage analysis.

Although the function of TMSB4Y is not yet known, its X chromosome homologue, *TMSB4X*, also known as *thymosin β4*, encodes a protein that plays an important role in the organization of the cytoskeleton, which binds to and sequesters actin monomers (G actin), leading to inhibition of actin polymerization (22). As expected from its function, thymosin β4 is highly expressed in metastatic melanoma cells together with fibronectin and RhoC, a member of the Rho GTPase family (39). Because Rho-like GTPases are suggested to be linked with HA-1 (40) and HA-3 (41) proteins in cytoskeleton rearrangement and have myosin 1G encoding HA-2 minor H Ag (42) as one of the downstream effector proteins (43), TMSB4Y/A33 derived from the Y homologue of thymosin β4 may also be classified as one of malignancy-associated minor H Ags according to the recent proposal by Spierings et al. (43).

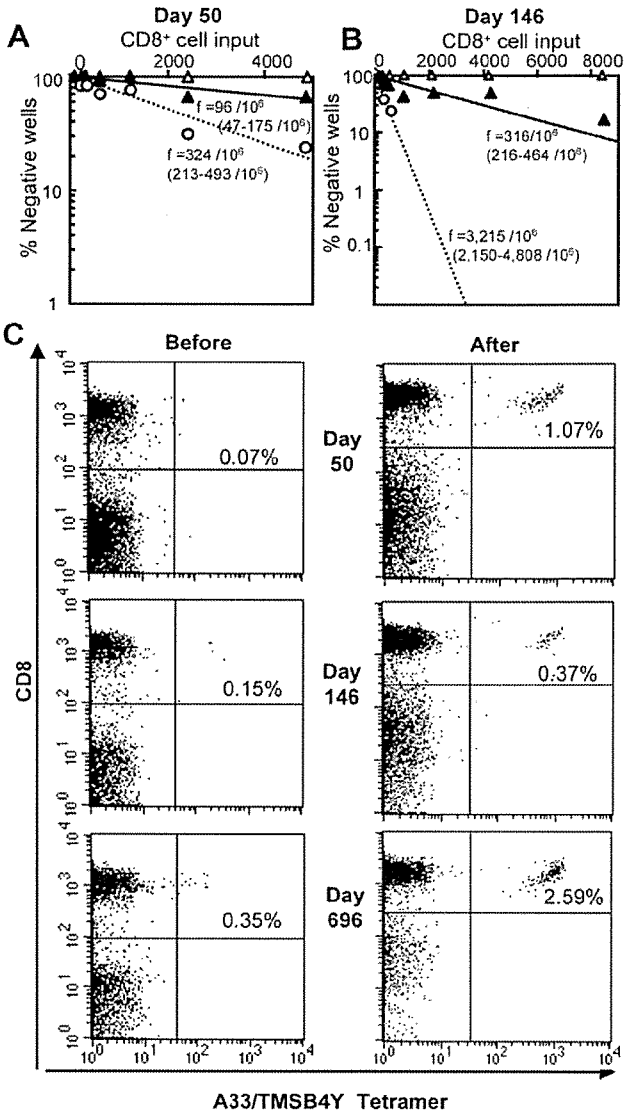

FIGURE 6. TMSB4Y/A33-specific CTLp frequency assay and tetramer analysis of post-HSCT PBMC. *A* and *B*, The proportion of CTL precursors specific for the identified TMSB4Y peptide among total CTLp against the recipient minor H Ags was quantitated using a standard limiting dilution assay. $CD8^+$ T cells from the PBMC on day 50 (*A*) or day 146 (*B*) post-HSCT were cultured at limiting dilution with irradiated CD40-B cells generated from pre-HSCT recipient PBMC. After three rounds of stimulation, a split-well analysis was performed for peptide-specific cytotoxicity against ^{51}Cr-radiolabeled recipient PHA blasts (○) or donor PHA blasts pulsed with TMSB4Y peptide (▲) or unpulsed (△). The wells were considered to be positive for lytic activity if the total cpm released by effector cells was >2.5 × SD above that in control wells (mean cpm released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated with L-Calc software. *C*, Thawed post-HSCT (days 50, 146, and 696) PBMCs and T cell lines generated by stimulating the PBMCs three times with irradiated CD40-B cells generated from pre-HSCT recipient PBMC were stained with a PE-conjugated HLA-A33 tetramer incorporating the TMSB4Y/A33 peptide. The percentage of tetramer-positive cells of the total $CD3^+CD8^+$ cells is shown.

CTLp frequency assays revealed that the magnitude of the CTL response to the TMSB4Y/A33 epitope was early after HSCT and represented one-quarter of the measurable donor CTL responses to

recipient minor H Ags in this donor/recipient pair. This illustrates the extent to which such cryptic peptides may contribute to the diversity and immunogenicity of the total class I MHC-associated peptide pool in normal cells. In this regard, the relative immunogenicity of another minor H Ag, HB-1, which is derived from a polypeptide whose translation is initiated at a CUG instead of a conventional ATG codon exclusively in transformed B cells (44, 45), should also be of interest. Recently, Schwab et al. (46) have shown that the insertion into a 3′UTR of a sequence encoding an antigenic peptide elicits T cells specific for this peptide in vivo, which recognize at least DCs, B cells, and fibroblasts from mice carrying the transgene. In contrast, analysis of >200 endogenously derived HLA-B*1801-associated peptides from a human B cell line revealed that all the peptides were encoded by conventional ORFs from a wide variety of cellular genes (47), suggesting that the frequency of cryptic peptides being presented on class I MHC molecules is <1/200. Identification of more minor H Ags may answer the question of the significance of cryptic peptides over conventional peptides.

A recent study has suggested that CTL responses against minor H Ags encoded or regulated by genes on the Y chromosome contribute to a selective GVL effect against myeloid and lymphoid leukemias after female into male HSCT, even though recipients of this combination experience increased GVHD (6). An HLA-B8-restricted minor H Ag encoded by *UTY* has been shown to be a potential target for immunotherapy against hematological malignancies (16). It is noted in this regard that clone 1B6, used for defining TMSB4Y, was isolated from a patient who did not develop acute GVHD, and that its lytic activity against nonhemopoietic cells, including dermal/oral fibroblasts and bone marrow stromal fibroblasts, was significantly lower than that against LCL and PHA blasts, suggesting that TMSB4Y/A33 is a potential target of immunotherapy like UTY. However, the expression of the *TMSB4Y* transcript was found to not be restricted to normal hemopoietic cells and leukemia/lymphoma cells. In addition, the increase in A33/peptide tetramer-positive cells observed late after HSCT during late GVHD may suggest that this minor H Ag could be related to chronic GVHD rather than the GVL effect. Thus, additional studies of the polypeptide expression level derived from the *TMSB4Y* 5′UTR in various types of cells need to be conducted to elucidate whether this cryptic product can serve as a target for GVL.

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Myeloma Cells Are Highly Sensitive to the Granule Exocytosis Pathway Mediated by WT1-Specific Cytotoxic T Lymphocytes

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ABSTRACT

Purpose: **Because WT1 is a universal tumor antigen, we examined the sensitivity of myeloma cells to WT1-specific cytotoxic T lymphocyte (CTL)-mediated cytotoxicity.**

Experimental Design: **WT1 expression in hematologic malignant cells was examined by quantitative reverse transcription-polymerase chain reaction. The cytotoxicity of a WT1-specific CTL clone against hematologic malignant cells, including myeloma cells, was examined by standard chromium-51 release assays. The extent of membrane damage induced by purified perforin was examined. Induction of WT1-specific CTLs from the patients with multiple myeloma (MM) was attempted, and we examined their function against myeloma cells.**

Results: **The expression levels of WT1 mRNA in myeloma and lymphoma cells were significantly lower than that in acute leukemia cells. Although the WT1 expression levels in myeloma and lymphoma cells were almost same, only myeloma cells were lysed efficiently by WT1-specific CTLs in a HLA-restricted manner. The amounts of interferon-γ produced by WT1-specific CTLs in response to stimulation with myeloma cells and with lymphoma cells were almost the same, suggesting that WT1 protein is processed and expressed in the context of HLA class I molecules similarly on both myeloma and lymphoma cells. The extent of membrane damage induced by purified perforin appeared to be significantly higher in myeloma cells than in lymphoma cells. WT1-specific CTLs appeared to be present in patients with MM.**

Conclusions: **The present study has shown that susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity and that WT1 is an ideal target antigen for cellular immunotherapy of MM.**

INTRODUCTION

Multiple myeloma (MM) is a fatal hematologic malignancy characterized by monoclonal growth of plasma cells (1, 2). Although recent therapeutic approaches for MM, including high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation, have improved the overall survival rate, MM is still an incurable disease. Recently, treatment strategies targeting mechanisms whereby myeloma cells grow and survive in the bone marrow, including thalidomide and its potent immunomodulatory derivatives and the proteasome inhibitor bortezomib, have been developed and are expected to improve the outcome of patients with MM resistant to conventional treatment (3, 4); however, the therapeutic efficacy of these agents is limited. Because cancer immunotherapy is tumor specific and less toxic, it seems an ideal therapeutic strategy for MM. The identification of target antigens that are expressed preferentially in tumor cells but not in normal cells and are recognized by T lymphocytes is essential to the development of efficacious cellular immunotherapy; however, to date, only a limited number of MM-associated antigens that are recognized by T lymphocytes have been identified.

The *WT1* gene encodes a zinc finger transcription factor (5), and WT1 binds to the early growth response-1 DNA consensus sequence present in various growth factor gene promoters (6). Although WT1 was initially shown to act as a transcriptional repressor, the specific functions of WT1 in normal and neoplastic tissues remain to be fully elucidated. During normal ontogenesis, the *WT1* gene is expressed in a time- and tissue-dependent manner, mainly in the fetal kidney, testis, ovary, and supportive structures of mesodermal origin (7). In contrast, in adults, *WT1* gene expression is limited to very few tissues, including the splenic capsule and stroma, the Sertoli cells of the testis, the granulosa cells of the ovary, the podocytes of the kidney, and $CD34^{+}$ hematopoietic progenitor cells (8–10). With regard to malignant cells, it has been reported that most cases of acute leukemia and blast crisis of chronic myelogenous leukemia aberrantly overexpress WT1 (11–15). Previous studies have shown that the expression level of WT1 in B-lymphoma cells is significantly lower than that in acute leukemia (16); however, the details of WT1 expression in MM and other types

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of mature B-cell malignancies have not been reported. In the present study, we addressed the question of whether WT1 is expressed abundantly in myeloma cells and whether WT1-specific cytotoxic T lymphocytes (CTLs) can exert cytotoxicity against myeloma cells in an antigen-specific and HLA-restricted manner.

Another question raised in the present study is the nature of the mechanisms determining the sensitivity of target cells to CTL-mediated cytotoxicity. The mechanisms of cytotoxicity mediated by CTLs have been examined extensively, mainly in murine systems using various mutant and knockout mice, and various pathways have been identified. Among these, the granule exocytosis pathway mediated by perforin/granzymes and the Fas/Fas ligand pathway are thought to be the main mechanisms of CTL-mediated antigen-specific cytotoxicity (17). We recently used a combination of Fas-deficient target cells and perforin-deficient effector T lymphocytes to show that the granule exocytosis pathway is important in antigen-specific cytotoxicity mediated by human $CD4^+$ as well as $CD8^+$ CTLs (18–20). CTLs lyse target cells via recognition of the complex of target antigen-derived peptide and HLA molecule. Therefore, the susceptibility of target cells to antigen-specific cytotoxicity mediated by CTLs is thought to depend primarily on their expression levels of target antigen and HLA molecules. In the present study, we examined the mechanisms of cytotoxicity against myeloma cells mediated by WT1-specific CTLs, focusing on the sensitivity of target cells to perforin-mediated cytotoxicity. The data obtained from the present series of experiments revealed that WT1 expression levels in myeloma cells and lymphoma cells were both significantly lower than that in acute leukemia cells; however, myeloma cells, but not lymphoma cells, were lysed efficiently by WT1-specific CTLs. The extent of membrane damage induced by purified perforin in myeloma cells appeared to be significantly higher than that induced in lymphoma cells. In addition, WT1-specific CTL precursors were detected in peripheral blood of the patients with MM. On the basis of the present data, we discuss the feasibility of targeting WT1 in cellular immunotherapy for MM.

MATERIALS AND METHODS

Cell Separation and Cell Lines. Bone marrow mononuclear cells were isolated from the patients with MM and healthy volunteers after obtaining informed consent and stored in liquid nitrogen until use. B-lymphoblastoid cell lines [B-(LCLs)] were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. LCLs were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The *HLA-A*2402* gene-transfected T2 cell line (T2-A24) was cultured in RPMI 1640 supplemented with 10% FCS and 800 μg/mL Geneticin (Life Technologies, Inc., Rockville, MD). All of the leukemia, myeloma, and lymphoma cell lines were cultured in RPMI 1640 supplemented with 10% FCS. All lymphoma cell lines used in the present study were established from patients with B-cell diffuse large non-Hodgkin's lymphoma or Burkitt's lymphoma. After obtaining consent from parents, cord blood was collected, and mononuclear cells were separated by Ficoll-Conray density gradient centrifugation. $CD34^+$ cells were isolated from cord blood mononuclear cells with immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA) coated with anti-CD34 monoclonal antibody (MoAb). Immunomagnetic separations were performed according to the manufacturer's instructions.

Generation of WT1 Peptide-Specific Cytotoxic T Lymphocytes. WT1 peptide-specific CTLs were generated as described below. Peripheral blood mononuclear cells isolated from five HLA-A24–positive MM patients and three healthy volunteers after obtaining informed consent were plated in 96-well round-bottomed plates at 1×10^5 cells per well in the presence of the WT1-derived peptide WT1-T2 (CMTWNQMNL, residues 235–243) at a concentration of 10 μmol/L in RPMI 1640 supplemented with 10% human AB-type serum, 5 ng/mL human recombinant interleukin (IL)-7 (Genzyme, Boston, MA), and 100 pg/mL human recombinant IL-12 (Genzyme). After culturing for 7 days, the cells were restimulated by adding autologous mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan)-treated peripheral blood mononuclear cells and the WT1-T2 peptide at 10 μmol/L. After an additional 7 days of culture, the cells were restimulated in the same way. The next day, IL-2 (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 10 units/mL. Ten days after the final stimulation, the cells in 20 μL (about 2×10^4 T lymphocytes per well) in each culture well were tested by ELISPOT assays for their antigen specificity. For expansion of WT1 peptide-specific bulk CTLs, the cells that showed specific spots in ELISPOT assays were stimulated by adding MMC-treated T2-A24 cells and 10 μmol/L WT1-T2 peptide, and then the specificity and cytotoxicity of the growing cells were examined by detection of interferon (IFN)-γ production by enzyme-linked immunosorbent assay (ELISA; Endogen, Rockford, IL) and chromium-51 release assay, respectively.

Establishment of a WT1 Peptide-Specific Cytotoxic T Lymphocyte Clone. A CTL clone designated TAK-1, which specifically recognizes WT1-T2 peptide in the context of HLA-A*2402, was established as described previously (16, 21). Briefly, $CD8^+$ T lymphocytes were stimulated repeatedly with WT1-T2 peptide-loaded dendritic cells. The cytotoxicity of the growing cells was examined, and cells that exerted a cytotoxic effect on a WT1-T2 peptide-loaded autologous B-LCL were cloned by a limiting dilution method as described previously (22).

Quantitative Analysis of WT1 Messenger RNA Expression. Total RNA was extracted from samples with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in a final volume of 50 μL with the One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reaction, carried out with 0.1 μg of total RNA from each sample, was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reverse transcription of the RNA was achieved at 48°C for 30 minutes, and polymerase chain reaction was performed with an enzyme activation step (10 minutes at 95°C) followed by 40 cycles of denaturation/annealing/extension (15 seconds at 95°C and 1 minute at 60°C). Sequences of primers and probes were as follows: *WT1* forward primer, 5′-CAACCACAGCACAGGGTACG-3′; *WT1* reverse primer, 5′-TCTG-

TATTGGGCTCCGCAG-3′; and probe, 5′-FAM-AGCGATA-ACCACACAACGCCCATCC-TAMRA-3′. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) quantitative analysis was performed with predeveloped TaqMan assay reagent target kits (Applied Biosystems). All analyses were performed in duplicate. To normalize differences in RNA degradation between the individual samples and in RNA loading for the RT-PCR procedure, the *WT1* expression level for a particular sample was defined as its *WT1* gene expression level divided by its *GAPDH* gene expression level (23). The *WT1* gene expression level of K562 leukemia cells, which strongly express *WT1*, was designated 1.0, and the levels for the experimental samples were calculated relative to this value (12).

Flow Cytometric Analysis. The expression levels of HLA class I molecules on myeloma and lymphoma cells were determined by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated anti–HLA-A, -B, -C MoAb (BD PharMingen, San Diego, CA). HLA-A24 expression on cells was examined by flow cytometry using an anti–HLA-A24 MoAb (One Lambda, Canoga Park, CA) with mouse IgG as the control. The stained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA). Measurement of mean fluorescence intensity and analysis of data were done with Cell Quest Software (Becton Dickinson).

Purification of Perforin. Human perforin was purified as described previously (24, 25). Briefly, perforin was extracted in 1 mol/L NaCl from granules of the human natural killer (NK) cell line YT and purified by ion metal affinity chromatography with an imidazole gradient in 10% betaine (IMAC; PerSeptive Biosystems, Cambridge, MA). Fractions with hemolytic activity as determined by sheep red blood cell assays were concentrated by Centricon ultrafiltration and stored in 2 mmol/L EDTA and 0.1% fatty acid-free bovine serum albumin until use. Purification of perforin was confirmed by SDS-PAGE and Western blotting (24).

Cytotoxicity Assays. ^{51}Cr release assays were performed as described previously (26). Briefly, 1×10^4 ^{51}Cr ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA)-labeled target cells and various numbers of effector cells in 200 μL of RPMI 1640 supplemented with 10% FCS were seeded into round-bottomed microtiter wells and incubated for 4 hours. In some experiments, the target cells were incubated with an anti-HLA class I framework MoAb (w6/32; American Type Culture Collection, Manassas, VA) or an anti–HLA-DR MoAb (L243; American Type Culture Collection) at an optimal concentration (10 μg/mL) for 30 minutes before adding effector cells to determine whether cytotoxicity was restricted by HLA class I. To determine whether WT1-specific CTLs lyse myeloma cells via recognition of the WT1 peptide, which is naturally processed in myeloma cells and expressed in the context of HLA-A24, cold target inhibition assay was performed. WT1 peptide-loaded and unloaded autologous LCL or HLA-A24–positive leukemia cell line MEG01, which was shown to be lysed by WT1-specific CTLs in a WT1-specific manner, was used as cold target cells. After incubation for 4 hours, 100 μL of supernatant were collected from each well. The percentage of specific lysis was calculated as follows: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm). Cytotoxicity mediated by purified perforin was measured by using 2-hour ^{51}Cr release assays and the trypan blue exclusion method. ^{51}Cr-labeled target cells were incubated with various concentrations of purified perforin in the assay buffer [150 mmol/L NaCl, 20 mmol/L HEPES, and 2.5 mmol/L $CaCl_2$ (pH 7.4)] for 2 hours at 37°C. After incubation, supernatants were harvested after centrifugation of the microtiter plates, and radioactivity was determined. For trypan blue exclusion, the cells incubated with or without purified perforin were stained with trypan blue, and the percentages of stained cells were determined.

Inhibition of Perforin-Mediated Cytotoxicity. To examine the Ca^{2+} dependency of the cytotoxicity, cytotoxicity assays were performed in the presence of EGTA (Sigma, St. Louis, MO) at various concentrations. To evaluate the role of perforin in CTL-mediated cytotoxicity, effector T cells were pretreated with concanamycin A (CMA; Wako Pure Chemical Industries, Osaka, Japan) at various concentrations for 2 hours and then incubated with the target cells in the presence of CMA. CMA is an inhibitor of vacuolar type H^+-ATPase that inhibits perforin-based cytotoxicity, mostly by accelerated degradation of perforin caused by an increase in the pH of lytic granules (27). Treatment with CMA at the concentration used in the present study showed no toxic effect against T lymphocytes and myeloma cells as determined from cell growth curves and ^{51}Cr release assays (data not shown).

Detection of Interferon-γ Production. The response of WT1-specific CTLs to various stimulator cells was examined by determining IFN-γ production. For the assays of IFN-γ production, 1×10^5 WT1-specific CTL clone cells or bulk CTLs and 5×10^4 MMC-treated tumor cells were suspended in 200 μL of RPMI 1640 supplemented with 10% FCS and cultured in flat-bottomed microtiter wells in the presence of 10 units/mL recombinant human IL-2. After 72 hours, the supernatants were collected from each well and assayed for IFN-γ production by ELISA.

ELISPOT Assays. ELISPOT assays were performed as described previously (28). Briefly, 96-well flat-bottomed MultiScreen-HA plates with a nitrocellulose base (Millipore; Millipore Corp., Bedford, MA) were coated with 10 μg/mL anti–IFN-γ MoAb (R&D Systems, Minneapolis, MN) and incubated overnight at 4°C. After washing with PBS, the plates were blocked with the culture medium for 1 hour at 37°C. T2-A24 cells (5×10^4 cells) were pulsed with 10 μmol/L WT1-T2 peptide or PBS alone in RPMI 1640 with 10% FCS for 1 hour at room temperature, and then responder cells were seeded in each well. The plates were incubated in a 5% CO_2 incubator at 37°C for 20 hours and washed extensively with PBS containing 0.05% Tween 20. A polyclonal rabbit anti–IFN-γ antibody (Endgen, Woburn, MA) was added to individual wells and left for 90 minutes at room temperature, followed by exposure to peroxidase-conjugated goat antirabbit IgG (Zymed, San Francisco, CA) for an additional 90 minutes. For visualization of IFN-γ–specific spots, 100 μL of 0.1 mol/L sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma) and 0.015% H_2O_2 were added to each well. After 40 minutes, the reaction was stopped by washing with water, and the plates were dried. Diffuse large spots were counted under a dissecting microscope.

Tetramer Production and Staining. HLA-A24/WT1 peptide tetramer was produced as described previously (28). Briefly, BL21(DE3) pLysS (Novagen, Madison, WI) competent cells were transformed with plasmid pET11d (Novagen) encoding HLA-A*2402 heavy chain or plasmid pET-3a (Novagen) encoding β_2-microglobulin to produce the recombinant proteins. Expression of the HLA heavy chain was limited to the extracellular domain, and the COOH terminus of the domain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA-peptide complexes were folded by adding the HLA protein to β_2-microglobulin in the presence of the modified 9-mer WT1-T2 peptide (CYTWNQMNL) or HIV-1 Env (RYLRDQQLL). Proteins were dialyzed against water and then concentrated. After purification by gel filtration, the complex was biotinylated using recombinant BirA enzyme (Avidity, Denver, CO) and then purified by gel filtration. HLA-peptide tetramers were made by mixing the biotinylated HLA with phycoerythrin-labeled streptavidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1. Tetramers were purified by gel filtration on a Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at 4°C until use. CTLs were stained with a mixture of the tetramer at 0.1 mg/mL and FITC-conjugated anti-CD8 MoAb (BD PharMingen) at 4°C for 20 minutes. After washing twice, the stained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson).

Statistical Analysis. Statistical evaluation was performed with the StatView 5.0 statistical software (Abacus Concept, Berkeley, CA). Differences in the expression of WT1 mRNA in leukemia, myeloma, and lymphoma cells; surface HLA class I molecules on myeloma and lymphoma cells; and perforin-mediated cytotoxicity against myeloma and lymphoma cells were compared using the Mann-Whitney *U* test for parallel groups. *P* values of <0.05 were considered statistically significant.

RESULTS

Expression of WT1 Messenger RNA and HLA Class I Molecules. WT1 mRNA expression levels in the human leukemia, myeloma, lymphoma cell lines, and bone marrow cells was determined by quantitative RT-PCR and calculated relative to that in the human leukemia cell line K562. Because relative WT1 expression levels in most normal tissues are $<10^{-6}$, levels of $>10^{-5}$ were considered positive. As shown in Fig. 1*A*, the relative WT1 expression levels in leukemia cell lines were 1.9×10^{-2} to 2.2×10. These values are significantly higher than those in myeloma and lymphoma cell lines [3.6×10^{-5} to 3.1×10^{-2} ($P < 0.001$) and 3.8×10^{-5} to 6.3×10^{-3} ($P < 0.001$), respectively]. No difference in WT1 expression levels between myeloma and lymphoma cell lines was detected ($P = 0.9$). The relative WT1 expression levels in bone marrow cells isolated from patients with MM and healthy volunteers were 4.9×10^{-4} to 3.9×10^{-3} and 1.2×10^{-4} to 4.7×10^{-3}, respectively, and no difference in WT1 expression levels between these two groups was detected ($P = 0.3$).

The surface expression of HLA class I on myeloma and lymphoma cell lines was determined by flow cytometric analysis as shown in Fig. 1*B*. All of the cell lines examined expressed surface HLA class I molecules, and the mean fluorescence intensities corresponding to HLA class I molecules on myeloma and lymphoma cell lines were not significantly different ($P = 0.6$).

Cytotoxicity of WT1-Specific Cytotoxic T Lymphocytes against Myeloma Cells and Lymphoma Cells. We previously established a WT1-T2 peptide-specific and HLA-A24–restricted CTL clone, designated TAK-1 (16). The TAK-1 clone cells had been stored frozen in liquid nitrogen and were thawed for use in the present study. To confirm that the freezing and thawing procedures had not affected the antigen specificity and HLA restriction of the TAK-1 cells, we first investigated their cytotoxic activity against peptide-loaded and unloaded cells. TAK-1 lysed autologous LCLs that had been loaded with the WT1-T2 peptide but was not cytotoxic to unloaded LCLs or to

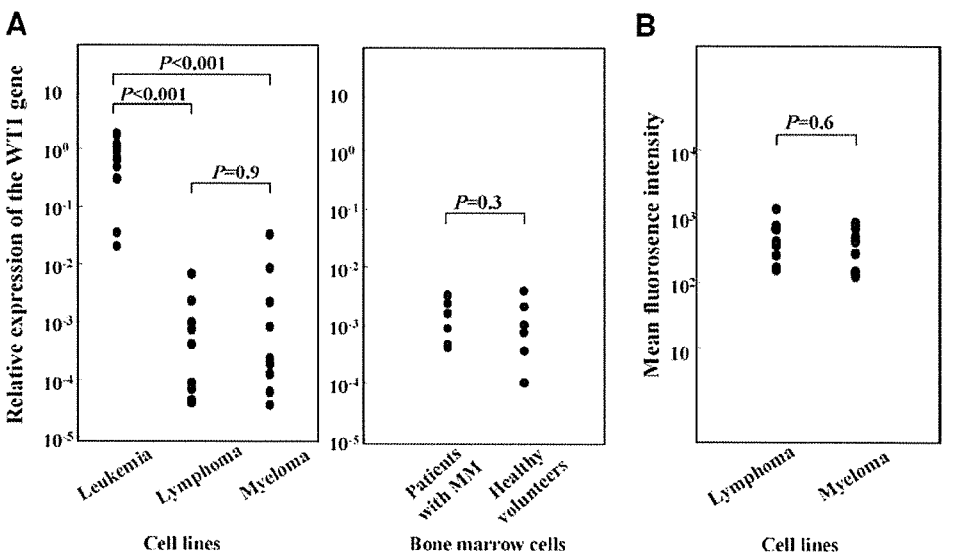

Fig. 1 Expression levels of WT1 mRNA and surface HLA class I molecules. *A*. Expression levels of WT1 mRNA in various leukemia ($N = 15$), lymphoma ($N = 10$), and myeloma ($N = 10$) cell lines; bone marrow cells isolated from patients with MM ($N = 6$); and healthy volunteers ($N = 6$) were determined by quantitative RT-PCR as described in Materials and Methods. *B*. Expression levels of surface HLA class I molecules on various lymphoma ($N = 10$) and myeloma ($N = 10$) cell lines were determined by flow cytometry.

those loaded with other WT1-derived peptides (data not shown). TAK-1 appeared to be cytotoxic only to HLA-A24–positive allogeneic LCLs and the HLA-A*2402 transfectant cell line T2-A24 (but not to its parent cell line, T2) in the presence of WT1-T2 peptide, as demonstrated previously. These data confirmed that TAK-1–mediated cytotoxicity is WT1-T2 peptide-specific and restricted by HLA-A24. The cytotoxicity of TAK-1 against the leukemia, lymphoma, and myeloma cell lines is shown in Table 1. As shown previously, TAK-1 exerted cytotoxicity against HLA-A24–positive leukemia cell lines that expressed WT1 abundantly, whereas no cytotoxicity against HLA-A24–negative leukemia cell lines was detected. TAK-1 appeared to have no cytotoxic activity against lymphoma cell lines that expressed WT1 at a low level, regardless of their HLA-A24 expression status. In contrast, TAK-1 exhibited strong cytotoxicity against HLA-A24–positive myeloma cell lines but not against HLA-A24–negative cells, although WT1 expression levels in myeloma cells and lymphoma cells were almost the same. The cytotoxicity of TAK-1 against bone marrow cells freshly isolated from patients with MM and healthy volunteers is also shown in Table 1. As observed with the myeloma cell lines, TAK-1 was cytotoxic to myeloma cells isolated from HLA-A24–positive patients. Although WT1 expression levels in bone marrow cells isolated from the healthy individuals and the patients with MM were almost the same, TAK-1 was not cytotoxic to normal bone marrow cells. Furthermore, TAK-1 did not appear to be cytotoxic to purified normal $CD34^+$ hematopoietic progenitor cells.

Table 1 Cytotoxicity of WT1-specific CTLs against various target cells

Target cells	HLA-A24	% of specific lysis* 20:1	10:1	5:1	% of myeloma cells†
Leukemia cell lines					
MEG01	+	48.3	35.8	28.3	
TK91	+	41.8	35.1	20.9	
K562	–	3.7	3.3	1.3	
NB4	–	5.0	3.3	2.8	
Lymphoma cell lines					
NAK	+	5.8	4.1	2.0	
IZU	+	3.6	2.6	1.4	
KUB	+	4.8	3.3	2.1	
DHL8	+	1.5	1.2	0.2	
Daudi	–	0.1	0.1	0.1	
Raji	–	1.0	0.3	0.0	
Namalwa	–	0.4	0.0	0.0	
Myeloma cell lines					
KMS-21BM	+	56.0	55.5	42.4	
KMS-34	+	61.9	58.8	50.9	
KMS-28BM	+	53.7	47.2	43.6	
KMS-18	+	38.9	33.0	13.5	
KMM-1	+	29.9	21.3	12.9	
KMS-11	+	27.2	24.5	15.8	
KMS-12PE	–	3.9	3.7	3.3	
KMS-20	–	3.0	3.4	2.0	
KMS-26	–	3.5	2.6	2.4	
Bone marrow cells isolated from MM patients					
Patient 1	+	22.3	19.7	13.7	78.3
Patient 2	+	21.8	15.0	11.5	Unknown
Patient 3	+	27.9	17.6	6.4	72.1
Patient 4	+	21.2	14.7	4.9	67.3
Patient 5	+	13.8	9.8	9.6	69.7
Patient 6	+	15.6	10.8	9.3	32.4
Patient 7	+	20.5	19.1	15.9	26.4
Patient 8	+	16.4	12.1	9.3	27.0
Patient 9	–	2.6	0.8	0.0	30.3
Patient 10	–	2.0	0.0	0.2	83.6
Patient 11	–	0.0	0.0	0.0	76.0
Bone marrow cells isolated from healthy volunteers					
Donor 1	+	0.9	0.8	0.5	
Donor 2	+	0.0	0.0	0.0	
Donor 3	+	1.1	0.4	0.0	
Donor 4	+	0.0	0.0	0.0	
Purified normal $CD34^+$ cells					
Donor 1	+	0.0	0.0	0.0	
Donor 2	+	0.0	0.0	0.0	

* The cytotoxicity of WT1 peptide-specific CTL clone TAK-1 against the various HLA-A24–positive and –negative cell lines, bone marrow cells isolated from patients with MM and healthy volunteers, and $CD34^+$ cells isolated from cord blood was determined by 4-hour ^{51}Cr-release assays at effector to target ratios of 20:1, 10:1, and 5:1.

† The percentages of myeloma cells contained in whole bone marrow cells of patients with MM were shown.

To confirm that the cytotoxicity of TAK-1 against myeloma cells is restricted by HLA-A24, inhibition assays using anti-HLA MoAbs were performed. As shown in Fig. 2*A*, the addition of an anti-HLA class I framework MoAb, but not an anti-HLA-DR MoAb, resulted in inhibition of the cytotoxicity mediated by TAK-1 against HLA-A24–positive myeloma cells. Taken together with the data shown in Table 1, these findings demonstrate that the cytotoxicity of TAK-1 against myeloma cells is restricted by HLA-A24.

To confirm that the cytotoxicity of TAK-1 against myeloma cells was mediated by specific recognition of the endogenously processed WT1, we performed cold target inhibition experiments. MEG01 is a leukemia cell line that has been previously shown by an antisense oligonucleotide method to be lysed by TAK-1 in a WT1-specific and HLA-A24–restricted manner (16). As shown in Fig. 2*B*, the addition of radioisotope-unlabeled MEG01 markedly decreased TAK-1–induced ^{51}Cr release from two myeloma cell lines, KMS-21BM and KMS-34, whereas the addition of unlabeled autologous LCL cells had no effect on cytotoxicity. Similarly, the addition of unlabeled WT1-T2 peptide-loaded autologous LCL cells decreased the cytotoxicity of TAK-1 against myeloma cell lines, as shown in Fig. 2*B*. These findings strongly suggest that WT1 is naturally processed in myeloma cells, expressed in the context of HLA-A24, and recognized by WT1-specific $CD8^+$ CTLs.

Granule Exocytosis Pathway of Cytotoxicity against Myeloma Cells Mediated by WT1-Specific Cytotoxic T Lymphocytes. We next examined the cytotoxic pathway of WT1-specific CTLs against myeloma cells. The perforin/granzyme pathway is known to be Ca^{2+} dependent, and recent studies have shown that extracellular Ca^{2+} is also necessary for the Fas/Fas ligand system (29). According to these findings, the cytotoxic activity of TAK-1 was examined in the absence of extracellular Ca^{2+}. As shown in Fig. 3*A*, the cytotoxicity of TAK-1 against myeloma cells was dramatically decreased in the presence of the Ca^{2+}-chelating agent EGTA. Thus, TAK-1-mediated cytotoxicity appears to be Ca^{2+} dependent.

Next, the significance of the granule exocytosis pathway was examined with an inhibitor of vacuolar type H^+-ATPase, CMA. Recent studies have shown that CMA is a selective

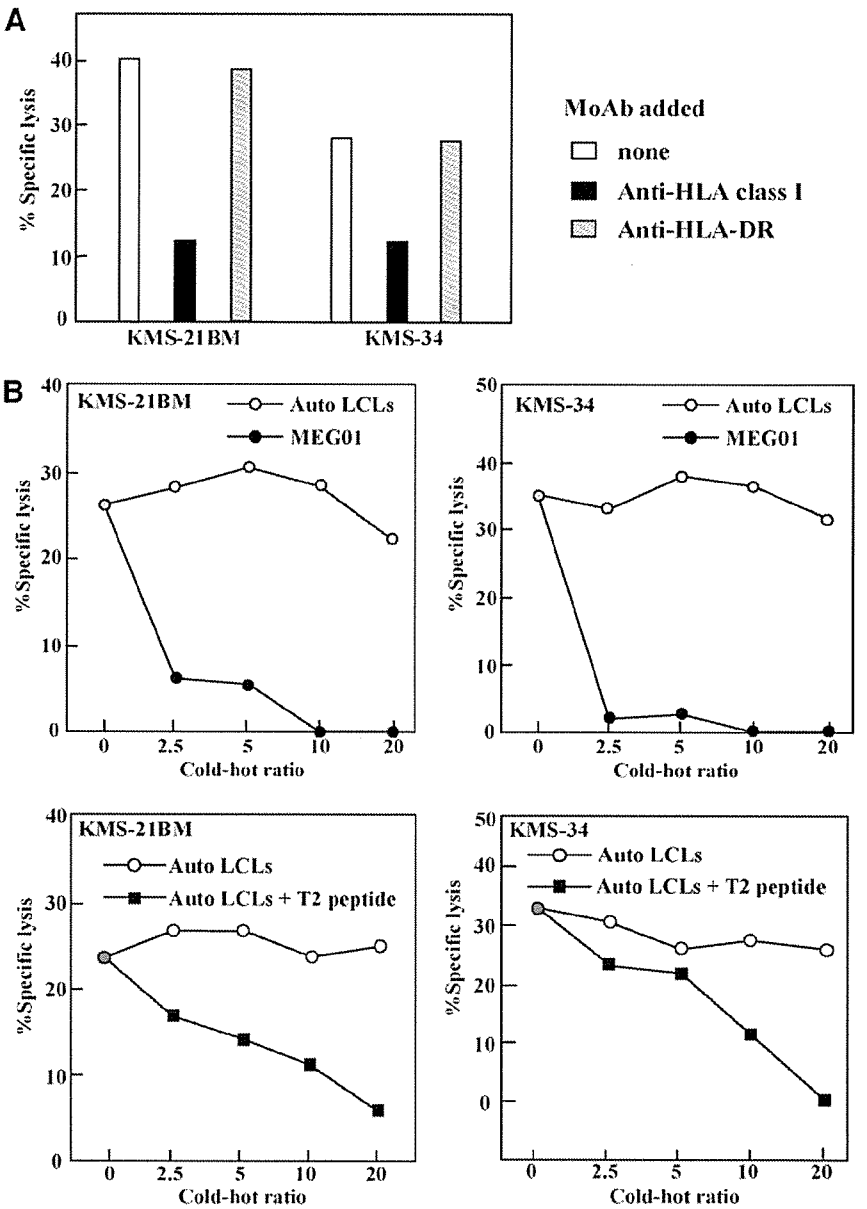

Fig. 2 HLA restriction and antigen specificity of cytotoxicity mediated by TAK-1 against myeloma cells. *A*, Inhibition of TAK-1–mediated cytotoxicity against myeloma cells by a HLA class I MoAb. The cytotoxicity of TAK-1 against the HLA-A24–positive myeloma cell lines KMS-21BM and KMS-34 was determined in the presence or absence of anti-HLA class I framework MoAb or anti-HLA-DR MoAb at an effector to target ratio of 10:1. The results shown represent the means of triplicate experiments. *B*, cold target inhibition assays. ^{51}Cr-labeled KMS-21BM and KMS-34 myeloma cells (1×10^4 cells) were mixed with various numbers of unlabeled autologous LCLs or MEG01 HLA-A24–positive leukemia cells, which have previously been reported to be lysed by TAK-1 in a WT1-specific manner. ^{51}Cr-labeled KMS-21BM and KMS-34 myeloma cells (1×10^4 cells) were also mixed with various numbers of unlabeled autologous LCLs or with WT1-T2 peptide-loaded autologous LCLs. The cytotoxicity of TAK-1 against the mixture of ^{51}Cr-labeled and unlabeled target cells was determined by 4-hour ^{51}Cr release assays at an effector to ^{51}Cr-labeled target cell ratio of 10:1. The results shown represent the means of triplicate experiments.

inhibitor that blocks perforin-based cytotoxicity, mostly as a result of accelerated degradation of perforin by an increase in the pH of lytic granules, but does not affect Fas-dependent cytotoxicity (27). The results shown in Fig. 3*B* demonstrate that pretreatment of TAK-1 with CMA at concentrations of >10 nmol/L resulted in inhibition of TAK-1 cytotoxicity against both KMS-21BM and KMS-34. These data suggest that the cytotoxicity of WT1-specific CTLs against myeloma cells is mediated through the granule exocytosis pathway.

High Sensitivity of Myeloma Cells to Perforin-Mediated Cytotoxicity. Because the expression levels of surface HLA class I molecules on myeloma cells and lymphoma cells appeared to be almost the same, the amounts of the WT1 peptide/HLA-A24 complex on myeloma cells and lymphoma cells after the addition of a WT1 peptide exogenously at the same concentration should also be equivalent. Therefore, comparison of the cytotoxic activities of WT1 peptide-specific CTLs against target cells loaded with a certain concentration of WT1 peptide may be a valid approach to determining the relative sensitivity of each target cell to perforin-mediated cytotoxicity. Accordingly, we compared the cytotoxic activities of TAK-1 against WT1 peptide-loaded myeloma cells and lymphoma cells at a low effector to target ratio. As shown in Fig. 4, TAK-1 was not cytotoxic to lymphoma cells loaded with WT1-T2 peptide at low concentrations; however, at these low concentrations, myeloma cells were lysed in a dose-dependent manner. At high concentrations of WT1 peptide, lymphoma cells were also lysed by TAK-1, although cytotoxic activity against WT1 peptide-loaded my-

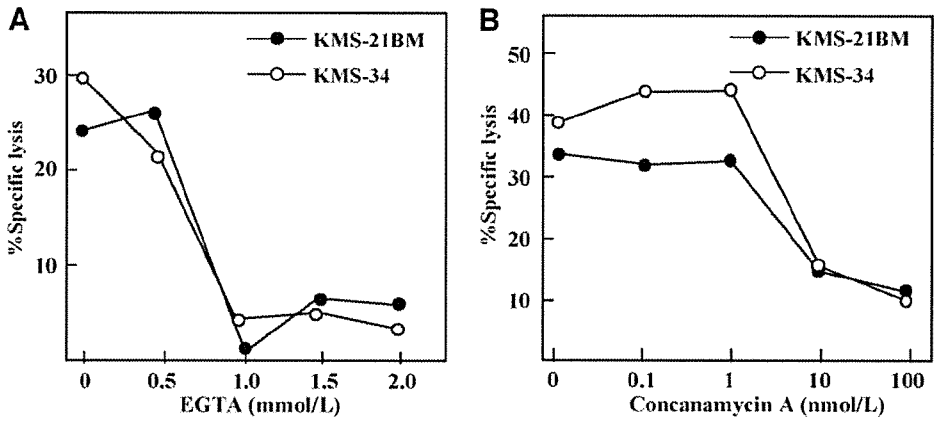

Fig. 3 Effects of EGTA and CMA on cytotoxicity of TAK-1 against myeloma cells. The cytotoxicity of TAK-1 against the HLA-A24–positive myeloma cell lines KMS-21BM and KMS-34 in the presence of EGTA or CMA at various concentrations was determined by 4-hour ^{51}Cr release assays at an effector to target cell ratio of 10:1. The results shown represent the means of triplicate experiments.

eloma cells was higher than that against WT1 peptide-loaded lymphoma cells. These data strongly suggest that myeloma cells are highly sensitive to the granule exocytosis pathway of WT1-specific CTLs.

We further compared the sensitivities of myeloma cells and lymphoma cells to perforin-mediated cytotoxicity by using purified perforin. As shown in Fig. 5, both the ^{51}Cr release assay and the trypan blue exclusion test revealed that myeloma cells are significantly more sensitive than lymphoma cells to cytotoxicity mediated by purified perforin.

Interferon-γ Production by WT1-Specific Cytotoxic T Lymphocytes Recognizing Myeloma Cells and Lymphoma Cells. We addressed the question of whether WT1-specific CTLs can recognize WT1 peptide/HLA-A24 complexes expressed on myeloma cells and lymphoma cells and produce cytokines equivalently in response to both types of malignant cell. To investigate this point, TAK-1 was stimulated with myeloma cells and lymphoma cells, and then IFN-γ production by TAK-1 was measured. As shown in Fig. 6, TAK-1 secreted IFN-γ equivalently in response to stimulation with HLA-A24–positive lymphoma cells and HLA-A24–positive myeloma cells; however, IFN-γ production by TAK-1 was not detectable after coculture with HLA-A24–negative cells. These data strongly suggest that although the production level of WT1 protein in myeloma cells and lymphoma cells is relatively low compared with that in acute leukemia cells, WT1 is efficiently processed in these mature B-cell malignant cells, and that WT1-specific CTLs can react equivalently to lymphoma cells and myeloma cells through the recognition of WT1 peptide in the context of HLA molecules.

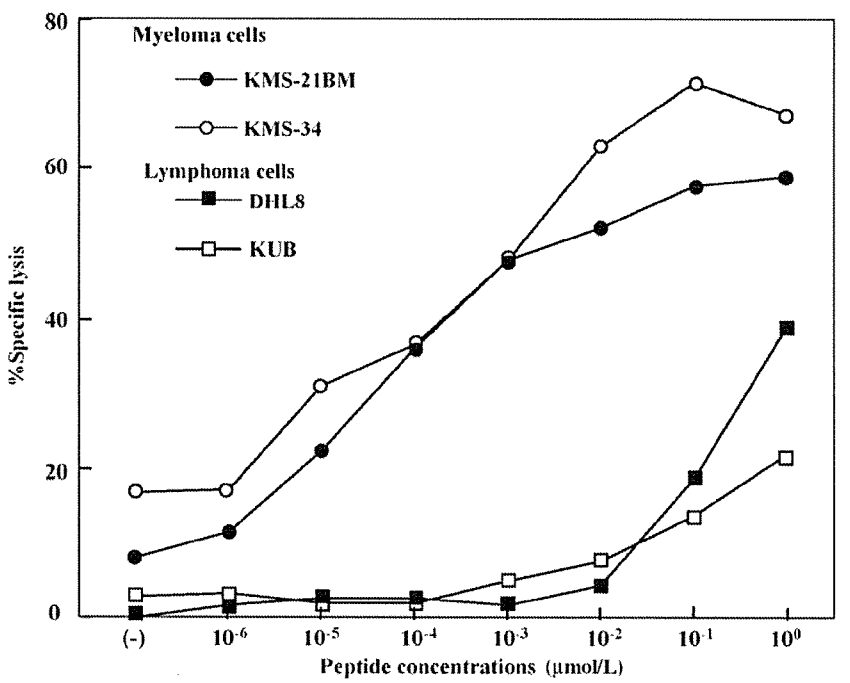

Fig. 4 Effect of WT1 peptide concentration on cytotoxicity of TAK-1 against myeloma cells and lymphoma cells. The cytotoxicity of TAK-1 against myeloma cell lines KMS-21BM and KMS-34 and lymphoma cell lines DHL8 and KUB, loaded with various concentrations of WT1-T2 peptide for 1 hour, was determined by 4-hour ^{51}Cr release assays at an effector to target ratio of 2:1. The results shown represent the means of triplicate experiments.

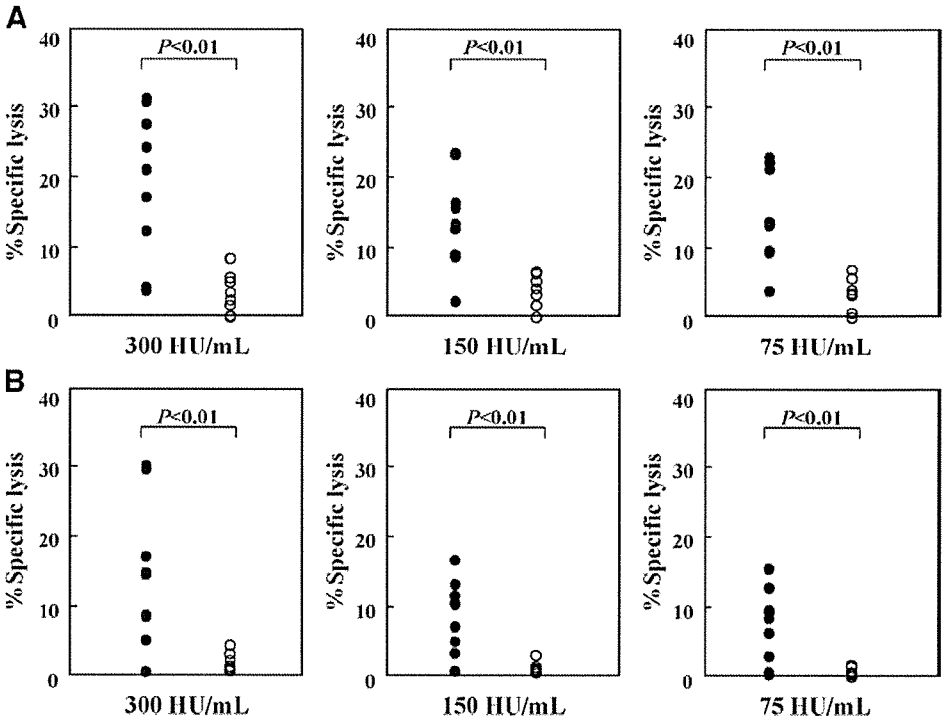

Fig. 5 Cytotoxicity of myeloma and lymphoma cells induced by purified perforin. *A*, trypan blue exclusion test. The myeloma cell lines (●; $n = 10$) and lymphoma cell lines (○; $n = 10$) were incubated with various concentrations of purified perforin for 2 hours at 37°C and then stained with trypan blue, and the percentages of stained cells were determined. *B*, ^{51}Cr release assay. ^{51}Cr-labeled myeloma cell lines (●) and lymphoma cell lines (○) were incubated with various concentrations of purified perforin. After 2 hours of incubation at 37°C, supernatants were harvested after centrifugation of the microtiter plates, and radioactivity was determined. The percentage of specific lysis was calculated as described in Materials and Methods. Each result represents the mean of triplicate experiments.

Generation of WT1 Peptide-Specific Cytotoxic T Lymphocytes from Patients with Multiple Myeloma. Finally, to investigate the feasibility of cellular immunotherapy for MM targeting WT1, we examined whether WT1 peptide-specific CTL precursors were present in the patients with MM. For detection of a small number of WT1 peptide-specific CTLs, we performed ELISPOT assays as described above. A T-cell response was considered positive if the number of spots in the presence of WT1 peptide-loaded T2-A24 cells was ≥3-fold higher than that in the presence of non–WT1 peptide-loaded T2-A24 cells and if there was a minimum of 20 peptide-specific spots in each well (after subtracting the number of spots observed in the presence of non–WT1 peptide-loaded T2-A24 cells). WT1 peptide-specific responses were detected in all five patients with MM and three healthy volunteers as shown in Fig. 7*A*. The percentage of positive wells in cultures of lymphocytes from the five patients with MM and three healthy volunteers ranged from 2.1% to 13.3% and from 4.2% to 12.5%, respec-

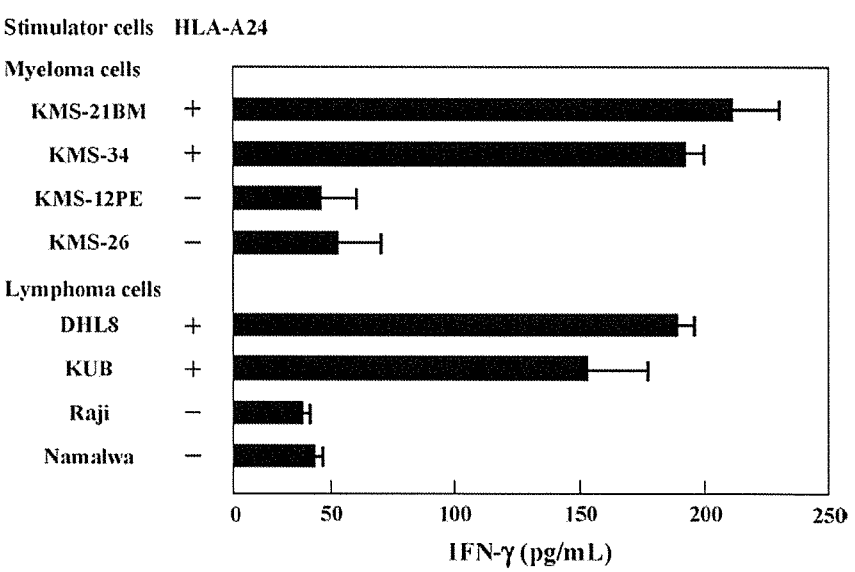

Fig. 6 Recognition of myeloma and lymphoma cells by TAK-1. TAK-1 cells were cocultured with various MMC-treated cell lines. After 3 days, the culture supernatants were harvested and assayed by ELISA for IFN-γ production. The data are expressed as the mean counts ± SDs of three wells.

tively, suggesting that there is no significant difference in the frequency of WT1-specific CTL precursors between patients with MM and healthy individuals.

To address whether WT1 peptide-specific CTLs generated from patients with MM can recognize WT1 peptide/HLA-A24 complexes expressed on myeloma cells and can lyse myeloma cells, IFN-γ production and cytotoxicity against myeloma cells were determined. As shown in Fig. 7*B*, WT1 peptide-specific bulk #3-B4 CTLs secreted IFN-γ in response to stimulation with HLA-A24–positive myeloma cells and WT1 peptide-loaded T2-A24 cells; however, IFN-γ production by #3-B4 CTLs was not detectable after coculture with HLA-A24–negative myeloma cells or non–WT1 peptide-loaded T2-A24 cells. Furthermore, as shown in Fig. 7*C*, #3-B4 CTLs exerted cytotoxicity against HLA-A24–positive myeloma cells and WT1 peptide-loaded T2-A24 cells. The #3-B4 CTLs were expanded by additional stimulation with MMC-treated WT1-T2 peptide-loaded T2-A24 cells. As shown in Fig. 7*D*, most #3-B4 CTLs were stained with WT1/HLA-A24 tetramer. These data strongly suggest that WT1-specific CTL precursors are indeed present in patients with MM and that immunotherapy for MM targeting WT1 might be feasible.

DISCUSSION

The new findings obtained from the present series of experiments are as follows. First, the expression level of WT1 mRNA in myeloma cells is relatively low compared with that in acute leukemia cells and is similar to that in B-lymphoma cells. Second, although the expression levels of WT1 in myeloma cells and lymphoma cells and surface HLA class I on myeloma cells and lymphoma cells are almost the same, only myeloma cells are lysed efficiently by WT1-specific CTLs in a HLA class I-restricted manner. Third, the cytotoxic pathway of WT1-specific CTLs against myeloma cells appears to be the conventional perforin-dependent granule exocytosis, and WT1 peptide-loaded myeloma cells are more susceptible to lysis by WT1-specific CTLs compared with WT1 peptide-loaded lymphoma cells. Fourth, WT1-specific CTLs produce IFN-γ at almost the

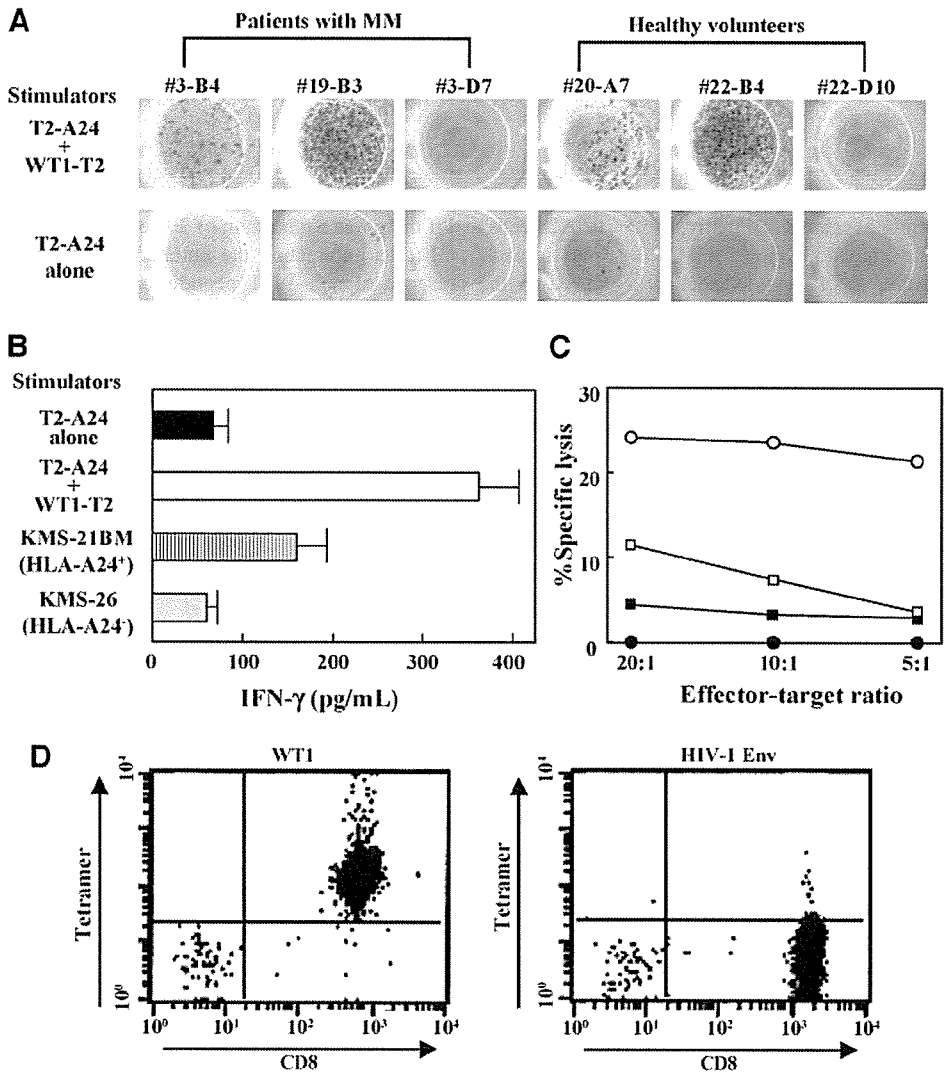

Fig. 7 Generation of WT1-specific CTLs from the patients with MM. *A*, ELISPOT assays. CTLs generated after the third stimulation with WT1-T2 peptide were tested for their specificity. ELISPOT assays were performed by incubating the CTLs with T2-A24 cells pulsed with 10 μmol/L WT1-T2 peptide or T2-A24 cells alone. These figures show examples of results considered positive (*#3-B4*, *#19-B3*, *#20-A7*, and *#22-B4*) or negative (*#3-D7* and *#22-D10*). *B*, ELISA for IFN-γ production. Bulk #3-B4 CTLs were cocultured with various MMC-treated cells with or without WT1-T2 peptide. After 3 days, the culture supernatants were harvested and assayed by ELISA for IFN-γ production. The data are expressed as the mean counts ± SDs of three wells. *C*, ^{51}Cr release assay. The cytotoxicity of the bulk #3-B4 CTLs against target cells, T2-A24 cells loaded with WT1-T2 peptide (○), T2-A24 cells alone (●), KMS-21BM (□), and KMS-26 (■) was determined by 4-hour ^{51}Cr release assays at various effector to target ratios. The results shown represent the means of triplicate experiments. *D*, tetramer staining. CTLs were stained with phycoerythrin-labeled tetramer in combination with FITC-conjugated anti-CD8 MoAb. #3-B4 CTLs were stained by the A*2402/WT1-T2 tetramer, but not by the irrelevant A*2402/HIV-1 env.

same level in response to stimulation with myeloma cells and lymphoma cells, suggesting that WT1 is processed and expressed similarly in the context of HLA class I molecules in myeloma cells and lymphoma cells. Fifth, and most importantly, the sensitivity of myeloma cells to cytotoxicity induced by purified perforin is significantly higher than that of lymphoma cells. In addition, WT1-specific CTL precursors appeared to be present in patients with MM as well as in healthy individuals.

Recently, various types of tumor-associated antigens have been identified. Some of them, including fusion gene products such as BCR-ABL (30–35) and ETV6-AML1 (36), are undoubtedly expressed only in tumor cells. However, the distribution of many tumor-associated antigens identified thus far is not strictly restricted to malignant cells, and normal cells also express these antigens at a relatively low level. The reason why only tumor cells and not normal cells are lysed by antigen-specific CTLs has been thought to be that the complex of tumor-associated antigen-derived peptide and HLA molecule is expressed on normal cells at too low a level to be recognized by CTLs. However, a previous study has revealed that a single peptide/major histocompatibility complex expressed on target cells can elicit a CTL response (37). These findings strongly suggest that protective mechanisms against CTL-mediated cytotoxicity must be present in normal cells, but the precise mechanism of this phenomenon is still obscure.

It is well known that CTLs are resistant to perforin-mediated cytotoxicity, for if they were susceptible, they would be killed by the perforin that they themselves release. Therefore, clarifying the mechanism of CTL resistance to perforin-mediated cytotoxicity might provide an insight into the cause of differential sensitivity to WT1-specific CTL-mediated cytotoxicity between myeloma cells and lymphoma cells. Previous studies have revealed that protective molecules specifically expressed on CTLs interact with perforin, thereby rendering CTLs resistant to perforin-mediated cytotoxicity (38, 39); however, the precise mechanism of the interaction between perforin and these lymphocyte membrane proteins is still unknown. Recently, it has been reported that proteolysis of perforin by surface cathepsin B provides self-protection to CTLs (40). The other molecular model that has been proposed for CTL self-protection involves the serpin granzyme B inhibitor PI-9 (41). Although these molecules were not studied here, it is possible that different expression levels of these inhibitors of perforin and granzymes determine the sensitivity of tumor cells to perforin-mediated cytotoxicity.

Resistance to perforin-mediated cytotoxicity possibly induced by membrane-stabilizing mechanisms has also been shown in human cytomegalovirus-infected fibroblasts (42). In addition, the human leukemia cell line ML-2 has been reported to be recognized by NK cells but resistant to NK cell-mediated cytotoxicity because of defective perforin binding (43). In that study, the binding of perforin to the leukemia cell membrane was examined by flow cytometry. However, it was argued that flow cytometry cannot assess surface binding of perforin to target cells (44). Indeed, binding of perforin to the cell membrane of target cells that are susceptible to granule exocytosis could not be detected by flow cytometry in our study (data not shown). Therefore, it will be necessary to use other experimental systems to clarify the precise mechanisms of susceptibility to perforin-mediated cytotoxicity.

Because the clinical outcomes of conventional chemotherapy for MM are not satisfactory, novel therapeutic approaches, including cellular immunotherapy, have been proposed. Although some potential target antigens for immunotherapy of MM have been identified, including idiotype (45–47), MUC1 (48), sperm protein 17 (49, 50), SPAN-Xb (51), and MAGE families (52), the number of suitable target antigens recognized by CTLs directed against myeloma cells is still limited (53). To the best of our knowledge, this is the first report to describe the efficacy of WT1-specific CTLs against MM. The present findings may contribute to the development of novel immunotherapeutic strategies for MM and suggest that vaccination with a WT1-derived peptide or WT1-coding DNA and adoptive immunotherapy using WT1-specific CTLs may provide an effective treatment option for MM as well as acute leukemia.

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Featured Article

The CC Chemokine Receptor 4 as a Novel Specific Molecular Target for Immunotherapy in Adult T-Cell Leukemia/Lymphoma

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm with dismal prognosis, and no optimal therapy has been developed. We tested the defucosylated chimeric anti-CC chemokine receptor 4 (CCR4) monoclonal antibody, KM2760, to develop a novel immunotherapy for this refractory tumor. In the presence of peripheral blood mononuclear cells (PBMCs) from healthy adult donors, KM2760 induced CCR4-specific antibody-dependent cellular cytotoxicity (ADCC) against CCR4-positive ATLL cell lines and primary tumor cells obtained from ATLL patients. We next examined the KM2760-induced ADCC against primary ATLL cells in an autologous setting. Antibody-dependent cellular cytotoxicity mediated by autologous effector cells was generally lower than that mediated by allogeneic control effector cells. However, a robust ADCC activity was induced in some cases, which was comparable with that mediated by allogeneic effector cells. It suggests that the ATLL patients' PBMCs retain substantial ADCC-effector function, although the optimal conditions for maximal effect have not yet been determined. In addition, we also found a high expression of FoxP3 mRNA and protein, a hallmark of regulatory T cells, in ATLL cells, indicating the possibility that ATLL cells originated from regulatory T cells. KM2760 reduced *FoxP3* mRNA expression in normal PBMCs along with *CCR4* mRNA by lysis of CCR4$^+$ T cells *in vitro*. Our data suggest not only that the CCR4 molecule could be a suitable target for the novel antibody-based therapy for patients with ATLL but also that KM2760 may induce effective tumor immunity by reducing the number of regulatory T cells.

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm that is characterized by highly pleomorphic lymphoid cells and is caused by human T lymphotrophic virus type I (HTLV-1; ref. 1). It has a very poor prognosis (1–3) because patients are usually highly immunocompromised and suffer from frequent severe infections and because tumor cells are usually resistant to conventional chemotherapeutic agents (4). Allogeneic stem cell transplantation may improve survival of ATLL patients only when an appropriate degree of graft *versus* host disease develops (5). However, only a small fraction of patients may benefit from allogeneic stem cell transplantation because ATLL has a long latency and occurs in elderly individuals with a median age of 55 years. Therefore, alternative treatment strategies for ATLL patients are needed to improve their prognosis.

The use of therapeutic monoclonal antibody for the treatment of cancer has become a promising approach over the last few years, as exemplified by the success of the anti-CD20 chimeric monoclonal antibody rituximab used for the treatment of B-cell non-Hodgkin's lymphoma (6–9). Other promising monoclonal antibodies are also emerging, such as Campath 1H (anti-CD52) for the treatment of B-cell chronic lymphocytic leukemia (10), anti-CD33 for acute myelocytic leukemia (11), anti-p185$^{HER2/neu}$ for breast cancer (12), and anti-vascular endothelial growth factor for colorectal cancer (13). In regard to ATLL, an anti-CD25 monoclonal antibody therapy has shown substantial effects, but the benefit over conventional chemotherapy has to be determined (14). Thus, development of promising monoclonal antibodies against dismal T-cell neoplasms, including ATLL, is an urgent issue.

We have recently developed a new chimeric monoclonal antibody, KM2760, that binds specifically to CC chemokine

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receptor 4 (CCR4) and whose Fc region is artificially defucosylated to enhance ADCC activity by increasing its binding affinity to FcγR on effector cells (15, 16). We have shown that KM2760 exhibits potent ADCC against non-ATLL CCR4-positive T-cell leukemia/lymphoma lines with human peripheral blood mononuclear cells (PBMCs) as effector cells both *in vitro* and *in vivo* mouse models (16). We have also shown that tumor cells obtained from a large majority of patients with ATLL express CCR4 and that the extent of CCR4 expression is significantly associated with skin involvement and poor prognosis (17). On the basis of these observations, we describe here the potent KM2760-induced ADCC against both ATLL cell lines and primary tumor cells obtained from patients with ATLL. In addition, we describe a correlation between *CCR4* and *FoxP3* gene expression, the latter of which is a hallmark of immunoregulatory T cells, suggesting that the KM2760 may provoke effective tumor immunity by reducing the number of immunoregulatory T cells.

MATERIALS AND METHODS

Chimeric Anti-CCR4 Monoclonal Antibody KM2760. We generated a chimeric anti-CCR4 IgG1 monoclonal antibody, KM2760, whose Fc region was defucosylated to enhance FcγR-mediated binding affinity with effector cells of ADCC (16).

Cell Lines and Patient Cells. HUT102, ATL102, and ATN-1 are human T-cell lines established from patients with ATLL (17, 18–20) with clonal integration of HTLV-1. MT-2 is a human T-cell line transformed by infection of HTLV-1 (17, 19–21). CCRF-CEM (16), PEER (16), TALL-1 (16), Jurkat (16), MOLT-4F (22), MOLT-3 (23), HPB-ALL (24), and NCU-LBL-1 are non-ATLL T-cell lines. NCU-LBL-1 was a human T lymphoblastic lymphoma cell line established in our laboratory. All cell lines were interleukin 2 independent and were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum. PBMCs containing $CD4^+CD25^+CCR4^+$ ATLL cells were isolated from ATLL patients' peripheral blood with Ficoll-Paque (Pharmacia, Uppsala, Sweden) and used as targets in ADCC assays. PBMCs from all healthy volunteer donors used throughout the study were prepared as above and used as effector cells. In the autologous setting, a CD3-positive subset was isolated from fresh PBMCs obtained from ATLL patients with antihuman CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions, and used as target cells. The remaining CD3-negative subset was used as effector cells. A CD3-negative subset was also isolated from one healthy adult volunteer (PBMC 1) and used as control effector cells. Patients and volunteers gave informed written consent before the sampling procedure and informed consent was provided according to the Declaration of Helsinki.

Flow Cytometry. Expression of CCR4 antigen on cell lines was examined by flow cytometry. One million cells were incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated mouse anti-CCR4 monoclonal antibody KM2160 at the final concentration of 10 μg/mL for 30 minutes. After washing twice, cells were analyzed by FACScan with the aid of CellQuest software (Becton Dickinson, San Jose, CA). In addition, the following antibodies were used: FITC-conjugated anti-CD55 (clone IA10), FITC-conjugated anti-CD59 (clone p282), FITC-conjugated anti-CD16 (clone 3G8), peridinin chlorophyll protein-conjugated anti-CD4 (clone SK3), and phycoerythrin-conjugated anti-CD25 (clone M-A251) with appropriate control monoclonal antibodies. All monoclonal antibodies, except for KM2160, were purchased from BD PharMingen (San Jose, CA).

Establishment of a CCR4-Expressing Stable Transfectant. A full open reading frame of CCR4 cDNA was cloned into the retroviral vector pLBPC [ref. 25; the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr. Garry Nolan, Stanford University (Stanford, CA)] with *Hin*dIII and *Not*I sites. CCR4-negative HUT102 cells (17) were transduced with retroviruses carrying either CCR4 or green fluorescent protein cDNA (26) and selected in the presence of puromycin (0.7 μg/mL) as described previously (25). The expression of CCR4 on the transduced HUT102 cells was verified by flow cytometry as described above and was 100% positive (data not shown).

ADCC Assay. ADCC was determined by a standard 4-hour chromium 51 release assay. Target cells (1×10^6 cells) were labeled with 1.5 kBq of $Na_2{}^{51}CrO_4$ (^{51}Cr) for 2 hours and kept for 15 minutes on ice and washed twice. Aliquots of the labeled tumor cells (2.5×10^3 cells/50 μL) were mixed with effector PBMCs (50 μL) and serial dilutions of monoclonal antibodies (100 μL) in 96-well U-bottomed plates and incubated at 37°C, 5% CO_2 for 4 hours. Effector PBMCs obtained from four healthy adult volunteers (numbered from 1 to 4) and ATLL patients were used at the fixed E:T ratio of 50:1. Then, supernatants were removed and counted in a gamma counter. The percentage of specific lysis was calculated according to the following formula: percentage of specific lysis = $(E - S)/(M - S) \times 100$, where E is the experimental release, S is the spontaneous release, and M is the maximum release by 1.5% Triton X-100. All expressed values were averages of triplicate experiments.

Complement-Dependent Cytotoxicity (CDC) Assay. The target cells were labeled in the same way as in the ADCC assay. Aliquots of the labeled cells were distributed into 96-well U-bottomed plates (5×10^3 cells/50 μL) and incubated with monoclonal antibodies (50 μL) serially diluted in RPMI 1640 supplemented with 40% of either heat-inactivated or intact pooled human serum (100 μL) obtained from 10 healthy adult volunteers. Pooled human serum, not heat-inactivated, was used as the source of complement. After 1.5 hours of incubation at 37°C, 5% CO_2, supernatants were removed and counted in a gamma counter, and the percentage of specific lysis was calculated in the same way as used in ADCC assays. In some experiments, blocking antibodies against CD55 (clone 1C6) and CD59 (clone 1F5) were used in the CDC assay to block the function of the CD55/59 (27, 28) at the final concentration of 10 μg/mL.

Cell Proliferation Assay. Cell proliferation was analyzed by the CEllTiter 96 AQueous One solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Aliquots of the ATLL cell lines or fresh ATLL cells were distributed into 96-well flat-bottomed microtiter plates and incubated with serial dilutions of KM2760 (0.1, 1.0, and 10.0 μg/mL) in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum. Combined 3-(4,5-dimethylthiazol- 2-yl)-5-(3-carboxymethoxy-phenyl)-

2-(4-sulfonyl)-2*H*-tetrazolium/phenazine methosulfate solution (provided with the kit) was added at 0, 24, 48, 72, and 96 hours to each well, and 2 hours (ATLL cell lines) or 4 hours (fresh ATLL cells) after incubation at 37°C, 5% CO_2, the absorbance at 490 and 650 nm were recorded with an ELISA plate reader. Subtraction of the 650-nm reference absorbance from the 490-nm absorbance, which is directly proportional to the number of living cells, provides elimination of background. Subtraction of the average absorbance of the three "no cell" control wells from all other experimental absorbance values is considered to yield corrected absorbance. All expressed values were averages of triplicate experiments. The influence of cytokines upon cell proliferation in the presence of KM2760 was also evaluated by adding recombinant human IFN-α-2b or recombinant human IFN-γ at the final concentration of 100 units/mL.

***FCGR3A* Genotyping.** Genotyping of the *FCGR3A* polymorphism was done by a reverse transcription-PCR followed by direct sequencing. Total RNA was prepared from the effector PBMCs obtained from four healthy adult volunteers and was reverse transcribed as a first-strand cDNA solution, and each aliquot was used to amplify *FcγRIIIa* mRNA. Nested PCR was used to produce a 928-bp fragment of the *FcγRIIIa* cDNA spanning the polymorphic site; primer pairs used were as follows: sense, 5′-CAGACTGAGAAGTCAGATGA-3′, and antisense, 5′-GTTCTATGTTTCCTGCTGCT-3′; and nested sense, 5′-GTTTACTTCCTCCTGTCTAG-3′, and nested antisense, 5′-TGAGGATGATAGGGTTGCAA-3′. This former primer set was designed not to amplify the *FcγRIIIb* cDNA. The nested PCR products were directly sequenced using the following internal primers: sense, 5′-AACACTGCTCTGCATAAGGT-3′, and antisense, 5′-ATATAGTCCTGTGTCCACTG-3′, with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Real-Time PCR for *CCR4* and *FoxP3*. Total RNA was prepared from fresh PBMCs of 8 patients with acute type ATLL, 1 HTLV-1 carrier, 11 healthy adult volunteers, and 6 ATLL cell lines (ATN-1, MT-2, ATL102, HUT102, CCR4-transduced HUT102, and green fluorescent protein-transduced HUT102) and 8 non-ATLL T-cell lines. Both $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were isolated with a human $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec) from fresh PBMCs of one healthy adult volunteer (PBMC 1), according to the manufacturer's instructions. $CD4^+CCR4^+$ and $CD4^+CCR4^-$ subsets were isolated in a similar way with biotin-conjugated anti-CCR4 monoclonal antibody (KM2160) and anti-biotin microbeads (Miltenyi Biotec). cDNA aliquots prepared from the purified $CD4^+CD25^+$, $CD4^+CD25^-$, $CD4^+CCR4^+$, and $CD4^+CCR4^-$ subpopulations were subjected to conventional RT-PCR for *CD4*, *CD25*, *CCR4*, and β-*actin*.

Fresh PBMCs from healthy adult volunteers were incubated with or without 10 μg/mL KM2760 in RPMI 1640 supplemented with 10% of heat-inactivated pooled human serum. After 6 hours of incubation at 37°C, 5% CO_2, total RNA was purified and incubated with DNase I and then reverse transcribed to first-strand cDNA. These aliquots were used to quantify *CCR4*, *FoxP3* , or β-*actin* mRNA. *CCR4* was PCR amplified with a primer set purchased from Roche Molecular Biochemicals (Mannheim, Germany), according to the manufacturer's instructions. *FoxP3* was amplified with the following exon-spanning primers: sense, 5′-GAGGACTTCCTCAAGCACT-3′, and antisense, 5′-TGCATGGCACTCAGCTTCT-3′. PCR was carried out with FastStart DNA master SYBR Green I (Roche Molecular Biochemicals) with the aid of a LightCycler Quick System 330 (Roche Molecular Biochemicals). β-*Actin* was used as an internal control (primer set was purchased from Roche Molecular Biochemicals). The standard curve for each gene was generated by amplifying serially diluted plasmids incorporating cDNA of the individual gene. The quantitative assessment of the mRNA of interest was done by dividing its expression level by that of β-*actin* and expressed as a copy-number ratio. All assays were conducted in triplicate, and the mean value was used as the mRNA level. Consequently, the *CCR4* copy number ratio 1 was defined as 4.48×10^{-4} copies of *CCR4* mRNA per β-*actin* mRNA. The *FoxP3* copy number ratio 1 was defined as 5.65×10^{-5} copies of *FoxP3* mRNA per β-*actin* mRNA.

Western Blot Analysis. As for FoxP3 protein expression, cell lysates extracted from PBMCs derived from the ATLL patients and healthy adult volunteers in addition to various cell lines, including ATLL and non-ATLL T cell lines, were used for Western blot analysis. Goat antihuman FoxP3 (Abcam, Cambridge, United Kingdom) and antigoat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) with the aid of the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, United Kingdom) were used. Goat anti-actin IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal control.

Statistical Analysis. The differences in the *CCR4/FoxP3* copy number ratio between the two groups was examined with the Mann-Whitney *U* test. Data were analyzed with the aid of StatView software, version 5.0 (SAS Institute, Cary, NC). In this study, $P < 0.05$ was considered as significant.

RESULTS

KM2760-Induced ADCC against ATLL Cell Lines. The dependence on CCR4 expression of KM2760-induced ADCC was verified with the CCR4-negative HUT102 cell line transduced with CCR4 cDNA or control green fluorescent protein cDNA by using a standard 4-hour ^{51}Cr-release assay. Only the CCR4-transduced HUT102 cell line was lysed effectively in the presence of PBMCs from four healthy adult donors (20 to 50% lysis in the presence of 1.0 μg/mL KM2760; data not shown). We next examined the KM2760-induced ADCC against four cell lines, including HUT102 as a negative control. Expression of CCR4 on these ATLL cell lines is shown in Fig. 1*A*. KM2760 induced a robust ADCC activity against CCR4-positive ATLL cell lines, but not HUT102, in a dose-dependent manner (Fig. 1*B*). Even in the absence of KM2760, 5 to 35% lysis by PBMCs, presumably due to natural killer (NK) cell activity, was observed; however, significant enhancement of lytic activity by KM2760 was obtained at a concentration as low as 0.1 μg/mL.

Susceptibility to lysis varied among cell lines and was influenced by the individual PBMCs used. Interestingly, the cellular composition of PBMCs from these healthy individuals thus might be associated with the ADCC activity. The percentages of $CD16^+$ cells in the PBMC 1, 2, 3 and 4 were 15.3, 10.7, 4.2, and 12.5%, respectively, and PBMC 3 showed a tendency

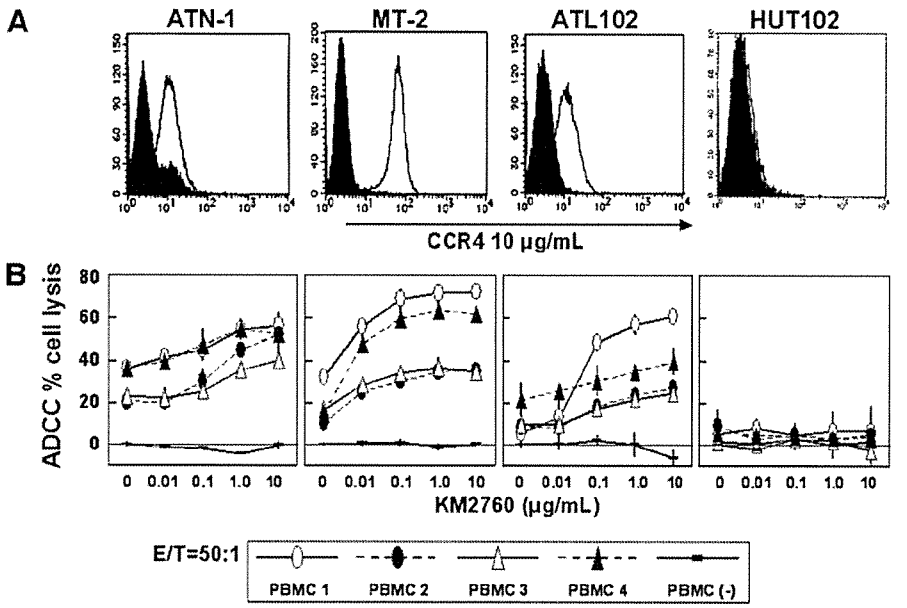

Fig. 1 CCR4 expression in ATLL cell lines and KM2760-induced ADCC. *A*. Four ATLL cell lines were stained with FITC-conjugated anti-CCR4 monoclonal antibody (KM2160) at the concentration of 10 μg/mL (blank histograms) or isotype control monoclonal antibody (filled histograms). *B*. ADCC against ATLL cell lines was measured by standard 4-hour ^{51}Cr release assay in the presence of effector PBMCs obtained from four normal volunteers and KM2760 at the concentrations indicated on the *X* axis. The percentages of $CD16^+$ cells in PBMC 1, 2, 3, and 4 were 15.3, 10.7, 4.2, and 12.5%, respectively. The E:T ratio was fixed at 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. Each result represents three independent experiments.

to reproducibly induce lower ADCC activity throughout the study. Genotyping results for the polymorphic status of amino acid position 158 in the *FCGR3A* encoding FcγRIIIa revealed that PBMC 1, 2 and 3 were *FCG3A-158 F/F* homozygous, and PBMC 4 was *FCG3A-158V/F* heterozygous.

KM2760-Induced ADCC against Freshly Isolated ATLL. Unlike established cell lines, tumor cells present in patients with ATLL may be heterogeneous and behave differently in KM2760-induced ADCC. To examine whether ATLL cells freshly isolated from patients also are susceptible to KM2760-induced lysis, we tested PBMC samples from three patients with refractory acute type ATLL. KM2760 induced ADCC against all fresh ATLL cells in a dose-dependent manner in the presence of PBMCs from four healthy adult donors (Fig. 2). The lysis ranged from 25 to 75% of $CD4^+CD25^+CCR4^+$ fresh ATLL cells was observed in the presence of 1.0 μg/mL KM2760, although the lytic activity varied among PBMC donors as observed in the case of ATLL cell lines.

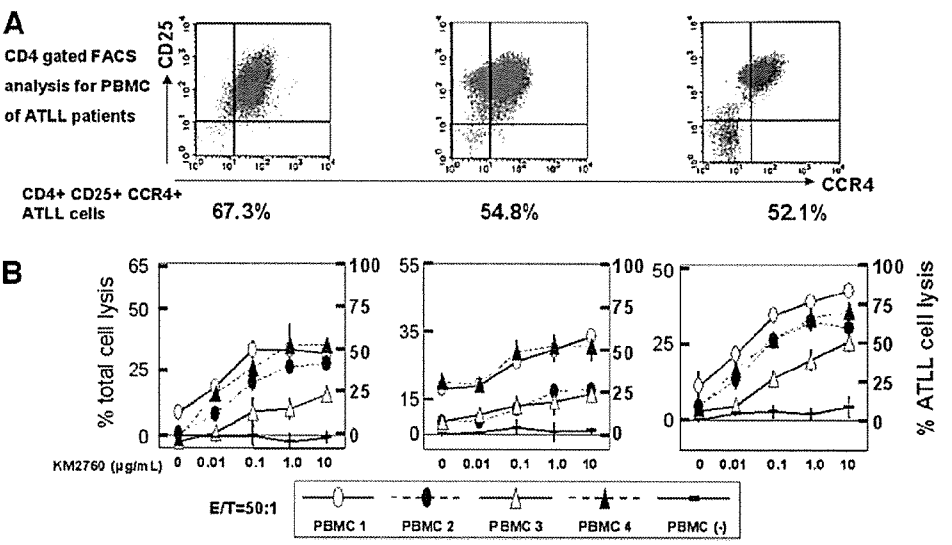

Fig. 2 KM2760-induced ADCC against ATLL cells obtained from patients. *A*, Freshly isolated PBMCs from three patients with refractory acute-type ATLL were analyzed by flow cytometry with peridinin chlorophyll protein-conjugated anti-CD4, phycoerythrin-conjugated anti-CD25 and FITC-conjugated KM2160 monoclonal antibodies. ADCC against the patient PBMCs containing ATLL cells was measured by a standard 4-hour ^{51}Cr release assay in the presence of KM2760 and effector PBMC obtained from four normal volunteers at the E:T ratio of 50:1. *B*, All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD.

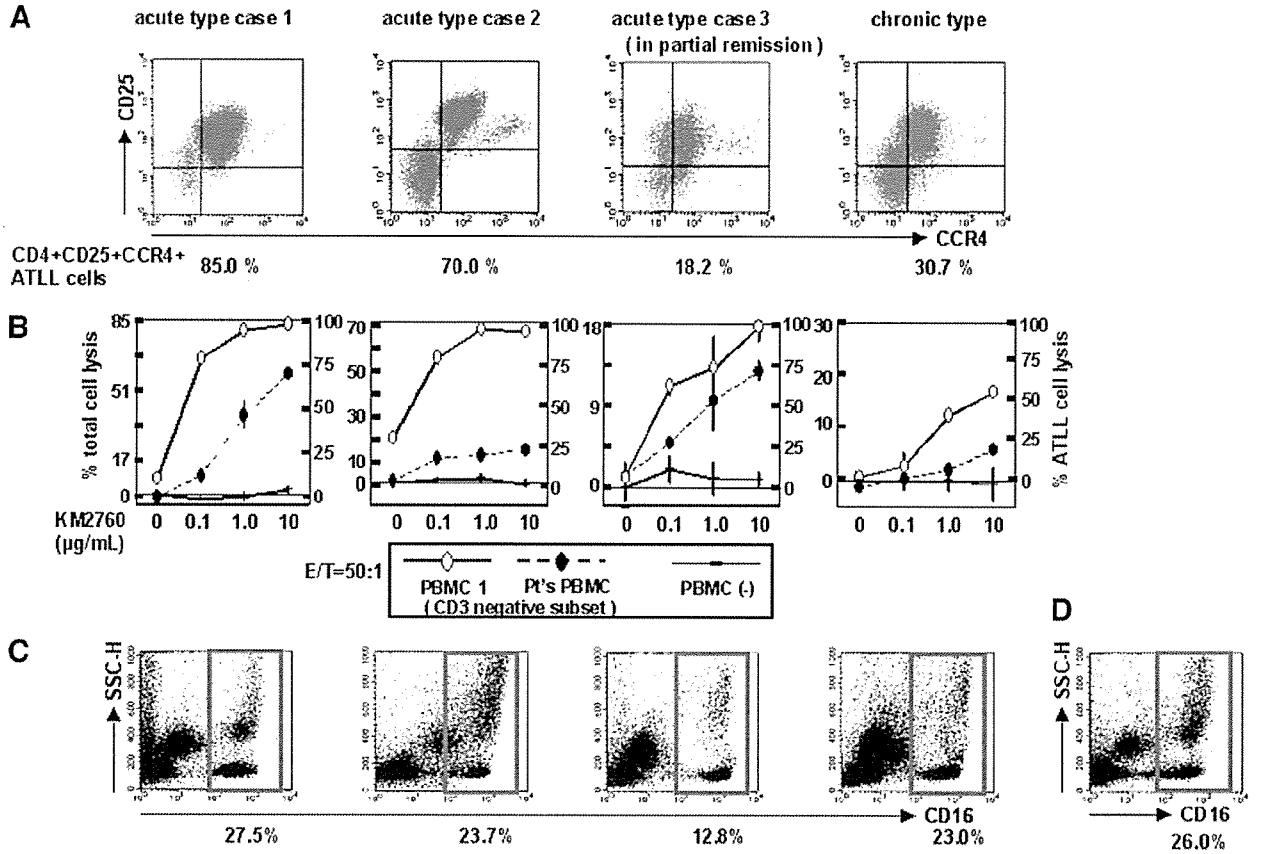

Fig. 3 KM2760-induced ADCC against ATLL cells obtained from patients tested in an autologous setting. Disease status of the patients analyzed are indicated above each data panel. *A*, target ATLL cells used in this study. Freshly isolated PBMCs from four patients with ATLL were sorted into CD3-positive (containing ATLL cells) subset and used as ADCC target cells. They were analyzed by three-color flow cytometry as in Fig. 2. The percentage of $CD4^+CD25^+CCR4^+$ cells (as ATLL cells) among the CD3-positive subset is indicated below. *B*, KM2760-induced ADCC activity mediated by autologous effector cells. KM2760-induced ADCC against the CD3-positive subset was measured by a standard 4-hour ^{51}Cr release assay in the presence of the CD3-negative subset at the E:T ratio of 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. *C*, autologous effector cells used in this study. The remaining CD3-negative subsets (containing NK cells and monocytes) of patient's PBMCs were used as autologous ADCC-effector cells. They were stained with FITC-conjugated anti-CD16 monoclonal antibody. The percentages of $CD16^+$ cells are indicated below each flow cytometry panel. *D*, allogeneic effector cells as control. A CD3-negative subset was also isolated from a healthy adult volunteer (PBMC 1) and used as control allogeneic ADCC-effector cells. It was stained with FITC-conjugated anti-CD16 monoclonal antibody. The percentage of $CD16^+$ cells is indicated below a flow cytometry panel.

The ultimate goal of immunotherapy is to obtain sufficient tumoricidal activity by simply administrating monoclonal antibodies *in vivo*; however, the therapeutic effect may be hampered by the immunocompromised situation that is common in patients with ATLL (please see the latter section). We thus next examined KM2760-induced ADCC in several patients with ATLL in an autologous setting. In contrast to the lysis induced by allogeneic PBMCs as effector cells, which is presumably due to NK cell activity, no lysis of fresh ATLL cells was induced by autologous PBMCs in the absence of KM2760. As shown in Fig. 3*B*, the extent of lysis of the fresh $CD4^+CD25^+CCR4^+$ ATLL mediated by autologous effector cells varied among the cases. In two acute-type ATLL patients (case 1 and case 3), a robust ADCC mediated by autologous cells was observed, which was comparable with that mediated by allogeneic cells. The latter patient, whose percentage of $CD16^+$ cells in the CD3-negative subset of PBMCs (12.8%, Fig. 3*C*) was about half of the $CD16^+$ cell percentage in the CD3-negative subset of allogeneic PBMC 1 (26.0%; Fig. 3*D*), was hematologically in partial remission after systemic chemotherapy. In the other two patients with acute- and chronic-type ATLL, a less efficient ADCC mediated by autologous cells was observed compared with that mediated by allogeneic ones. The percent $CD16^+$ cells in the CD3-negative subset of PBMCs in these two cases was ~23%, which was comparable with that in allogeneic PBMC 1 (26.0%; Fig. 3*D*). These data suggest that the ADCC-effector function of the PBMCs obtained from these two patients was suppressed to a greater extent.

Autologous KM2760-Induced ADCC against Freshly Isolated Peripheral T-Cell Lymphoma Cells. We have recently shown that nearly 40% of cases with peripheral T-cell lymphoma (PTCL) unspecified are positive for CCR4 (29). To test whether CCR4-positive PTCL, unspecified cells are susceptible to KM2760, we sought and found one patient diagnosed

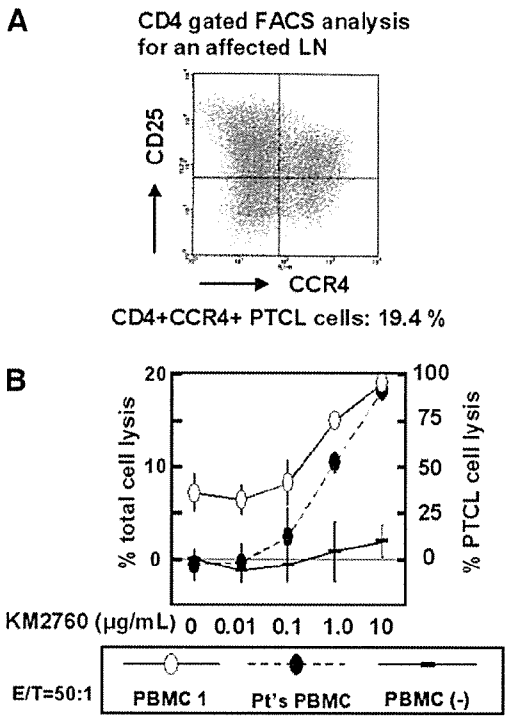

Fig. 4 KM2760-induced ADCC against PTCL, unspecified cells obtained from a patient. Mononuclear cells isolated from a biopsied affected lymph node (LN) of a patient with PTCL unspecified were analyzed as above. The LN contained 19.4% of $CD4^+$ $CCR4^+$ PTCL cells. KM2760-induced ADCC against the isolated mononuclear cells containing PTCL cells was measured by a 4-hour ^{51}Cr release assay in the presence of the autologous PBMCs that were free from tumor cell involvement or PBMCs from a normal donor as a control. The E:T ratio was fixed at 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD.

with CCR4-positive PTCL unspecified, who was seronegative for HTLV-1. Mononuclear cells containing tumor cells were obtained from one of the affected lymph nodes (tumor cells amounted to ~20% of all cells; Fig. 4, left panel) and used as target cells. Patient's PBMCs used as the effector cells were free from tumor cells. As shown in the right panel of Fig. 4, nearly the same level of KM2760-induced ADCC was observed with the patient's PBMCs and control PBMC 1, suggesting that KM2760 can effectively induce ADCC against primary tumor cells from patients with CCR4-positive PTCL.

Correlation between *CCR4* and *FoxP3* Expression in ATLL. Using quantitative reverse transcription-PCR, we analyzed *CCR4* mRNA level in PBMCs obtained from six healthy volunteers and eight acute-type ATLL patients. As shown in Fig. 5*A*, the *CCR4* copy number ratio of PBMCs obtained from six healthy volunteers, 5.0 ± 2.5 (average ± SD), were significantly lower than that of PBMCs obtained from eight ATLL patients, 101.8 ± 60.8 ($P = 0.0045$). It is generally known that the surface phenotype of ATLL cells is represented by CD4 and CD25, and we have recently reported that most ATLL cells also express CCR4 (17). Immunoregulatory T cells, which function by actively suppressing self-reactive T cells, exist in the $CD4^+CD25^+$ T-cell population. Immunoregulatory T cells engaged in the maintenance of immunologic self-tolerance (30–33) have been shown to be controlled by the transcription factor *FoxP3* (34). One of the clinical characteristics of ATLL is a highly immunocompromised state correlated with its poor prognosis; thus, we hypothesized that ATLL cells might originate from $CD4^+CD25^+$ ($CCR4^+$) immunoregulatory T cells. Three-color fluorescence-activated cell sorting analysis for PBMCs obtained from four healthy volunteers revealed that 44.4 ± 2.3% of $CD4^+CD25^+$ cells were positive for CCR4, and 70.0 ± 7.6% of $CD4^+CCR4^+$ cells were positive for CD25 (data not shown). These data indicate that the majority of $CD4^+CCR4^+$ T cells do express CD25 in the normal condition. As expected, *FoxP3* was expressed in the $CD4^+CCR4^+$ T cells at a level nearly nine times higher than that in $CD4^+CCR4^-$ T cells obtained from healthy volunteers (*FoxP3* copy number ratios were 92.2 and 10.1, respectively; Fig. 5*B*, left panel). In addition, there was a significant difference in the *FoxP3* copy number ratio of PBMCs obtained from eight ATLL patients and 11 healthy volunteers (82.0 ± 81.4 *versus* 9.0 ± 4.8; $P = 0.0003$; Fig. 5*B*, right panel), implying the presence of a profound immunosuppressive state in the patients with ATLL. In addition, expression of FoxP3 protein assessed by Western blot analysis was almost proportional to that of *FoxP3* mRNA level in each case (Fig. 5*C*). We also analyzed the *FoxP3* copy number ratio of six ATLL cell lines and eight non-ATLL T-cell lines. In contrast, all of them, irrespective of HTLV-1 involvement and CD4/CD25/CCR4 positivity, showed extremely low levels of the *FoxP3* copy number ratio, which ranged from 0 to 5.0 (data not shown). Corresponding to this result, Western blot analysis of FoxP3 protein in these cell lines detected no band or extremely faint bands compared with those of normal PBMCs. In any case, expressed level of FoxP3 protein correlated well with that of *FoxP3* mRNA level in each cell line (data not shown).

Finally, we examined whether KM2760 treatment would affect the mRNA expression of *CCR4* and *FoxP3* in fresh PBMCs from four healthy adult volunteers. A 6-hour KM2760 treatment reduced the *FoxP3* mRNA expression level in parallel with *CCR4* mRNA (Fig. 6). These results indicate that KM2760 induced ADCC against CCR4-positive T cells and that the majority of the lysed CCR4-positive T cells simultaneously expressed FoxP3.

KM2760-Induced CDC against ATLL Cell Lines and Fresh ATLL Cells. We next examined whether KM2760 possesses CDC activity; however, no activity was observed in either ATLL cell lines (Fig. 7*A*), or in fresh ATLL cells obtained from several acute type patients (data not shown). To investigate the underlying mechanism accounting for the lack of CDC activity, we analyzed the expression levels of complement inhibitors (CD55 and CD59) present on the surface of these ATLL cell lines (35). High levels of CD55 and CD59 expression were observed in ATLL cell lines (Fig. 7*B*). To confirm that CD55 and/or CD59 expression on the cell surface of the ATLL cell lines can inhibit CDC activity, we carried out blocking experiments in various combinations of complement, KM2760, and anti-CD55 and/or CD59 antibodies. As shown in Fig. 7*C*, the antibody treatments had no significant effect on ATN-1 and MT-2. On the other hand, the anti-CD55 but not anti-CD59