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

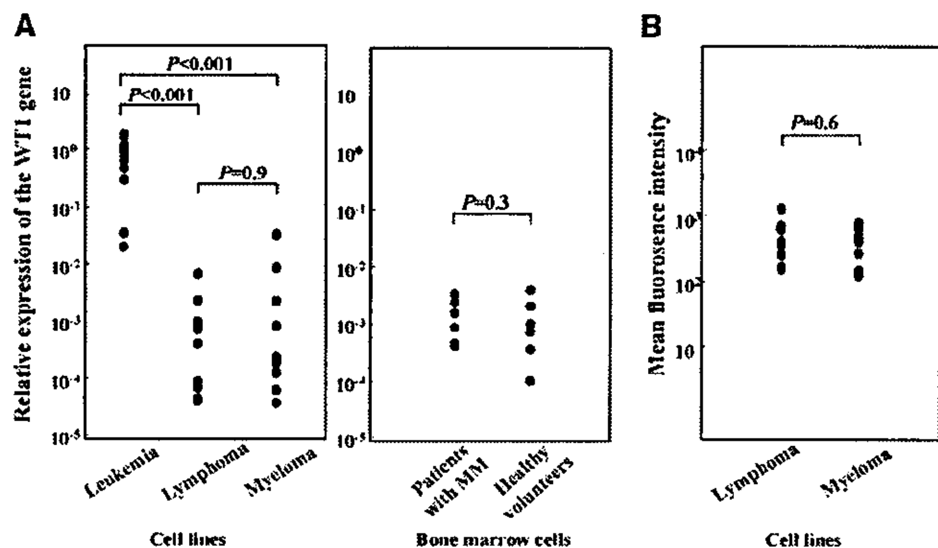

Fig. 1 Expression levels of WT1 mRNA and surface HLA class I molecules. *A*. Expression levels of WT1 mRNA in various leukemia (N = 15), lymphoma (N = 10), and myeloma (N = 10) cell lines; bone marrow cells isolated from patients with MM (N = 6); and healthy volunteers (N = 6) were determined by quantitative 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*			% of myeloma cells†
		20:1	10:1	5:1	
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

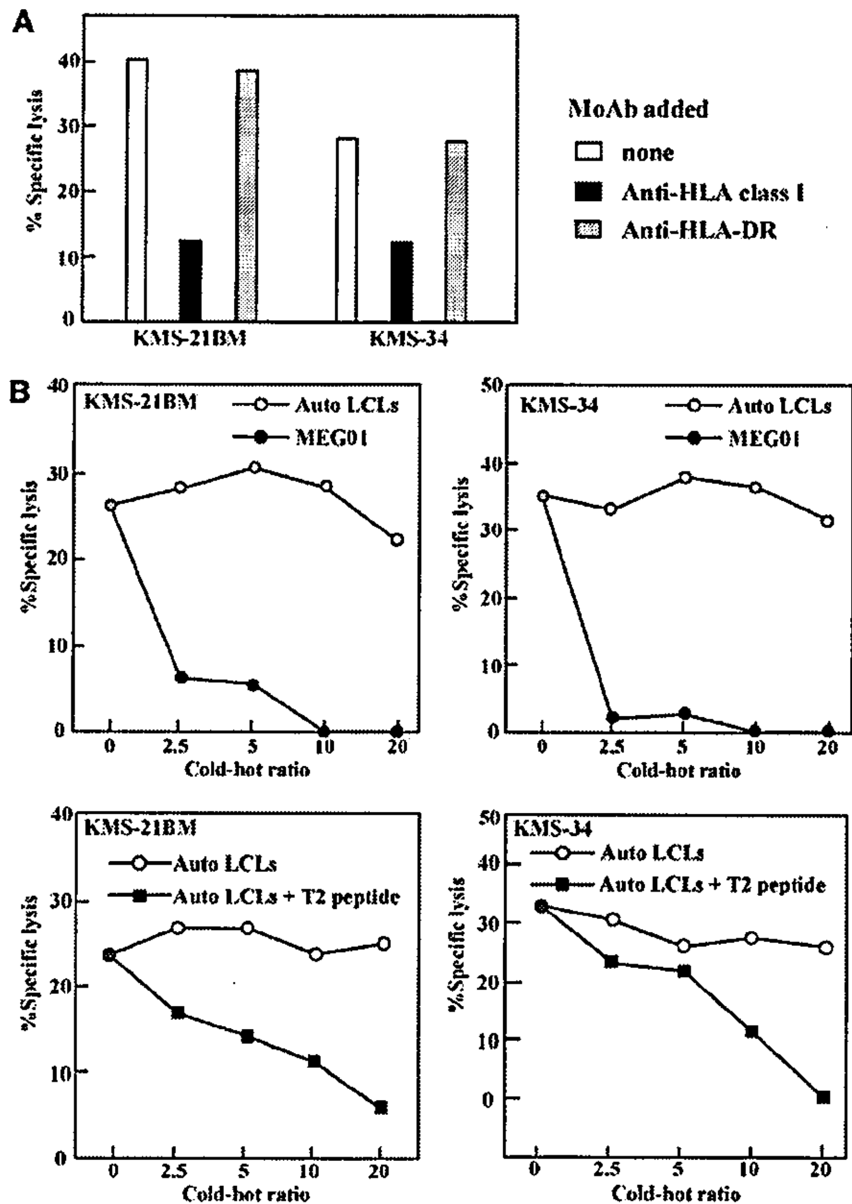

Fig. 2 HLA restriction and antigen specificity of cytotoxicity mediated by TAK-1 against myeloma cells. *A.* Inhibition of TAK-1–mediated cytotoxicity against myeloma cells by a HLA class I MoAb. The cytotoxicity of TAK-1 against the HLA-A24–positive myeloma cell lines KMS-21BM and KMS-34 was determined 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-

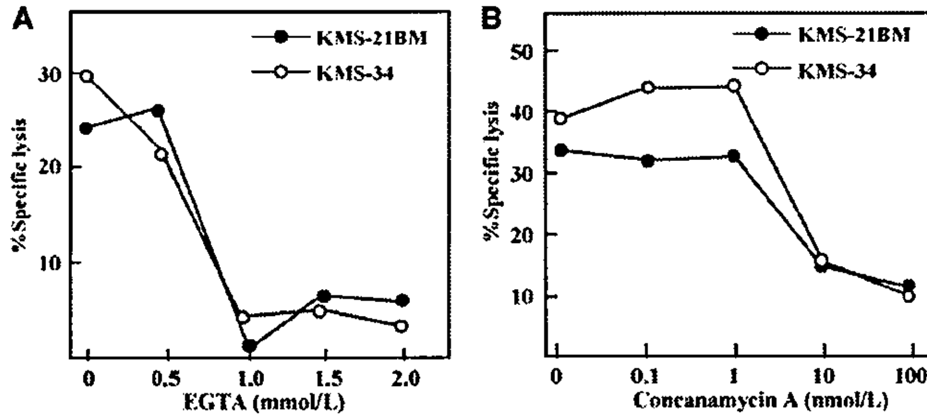

Fig. 3 Effects of EGTA and CMA on cytotoxicity of TAK-1 against myeloma cells. The cytotoxicity of TAK-1 against the HLA-A24-positive myeloma cell lines KMS-21BM and KMS-34 in the presence of EGTA or CMA at various concentrations was determined by 4-hour ^{51}Cr release assays at an effector to target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

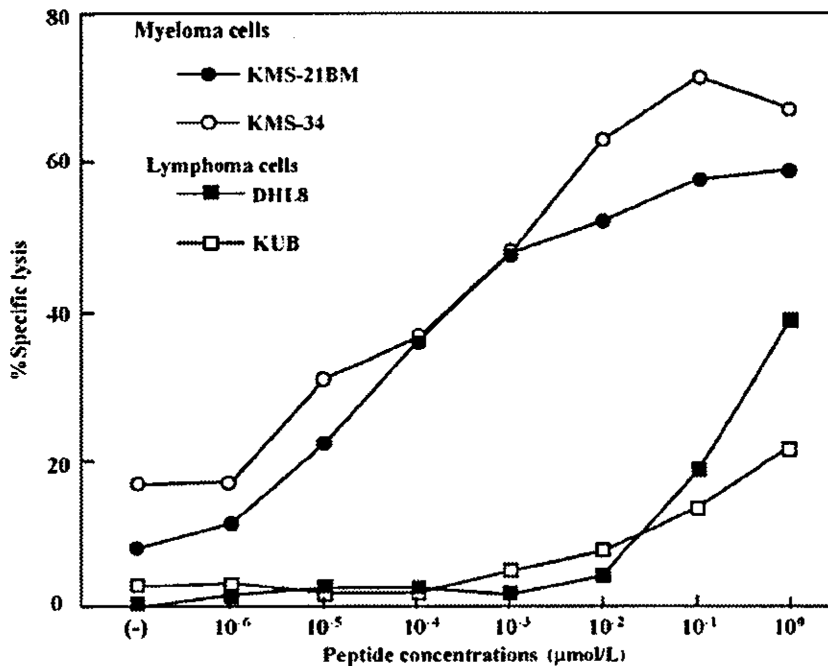

Fig. 4 Effect of WT1 peptide concentration on cytotoxicity of TAK-1 against myeloma cells and lymphoma cells. The cytotoxicity of TAK-1 against myeloma cell lines KMS-21BM and KMS-34 and lymphoma cell lines DHL8 and KUB, loaded with various concentrations of WT1-T2 peptide for 1 hour, was determined 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.

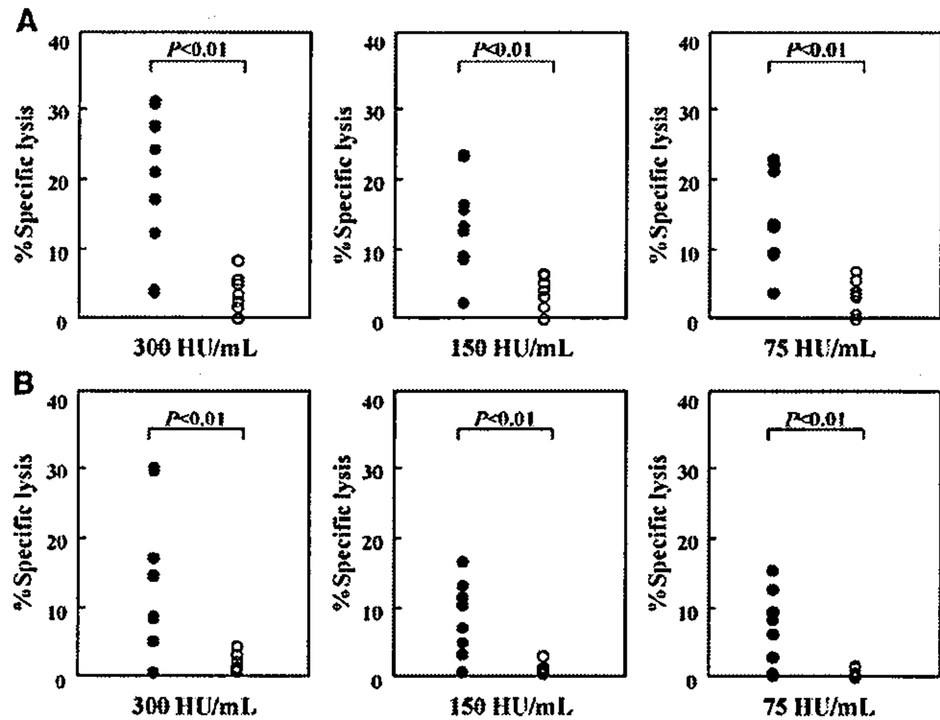

Fig. 5 Cytotoxicity of myeloma and lymphoma cells induced by purified perforin. *A*, trypan blue exclusion test. The myeloma cell lines (●; n = 10) and lymphoma cell lines (○; n = 10) were incubated with various concentrations of purified perforin for 2 hours at 37°C and then stained with trypan blue, 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-

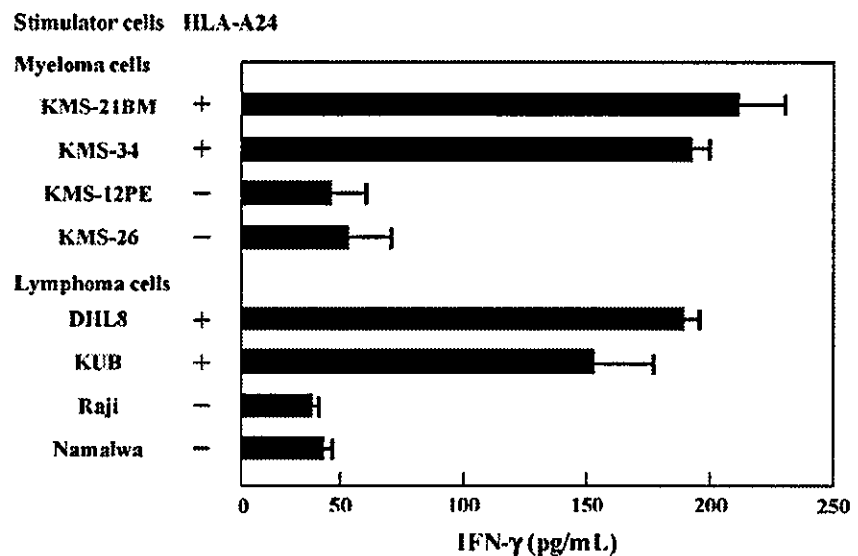

Fig. 6 Recognition of myeloma and lymphoma cells by TAK-1. TAK-1 cells were cocultured with various MMC-treated cell lines. After 3 days, the culture supernatants were harvested and assayed by ELISA for IFN-γ production. The data are expressed as the mean counts ± SDs of three wells.

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

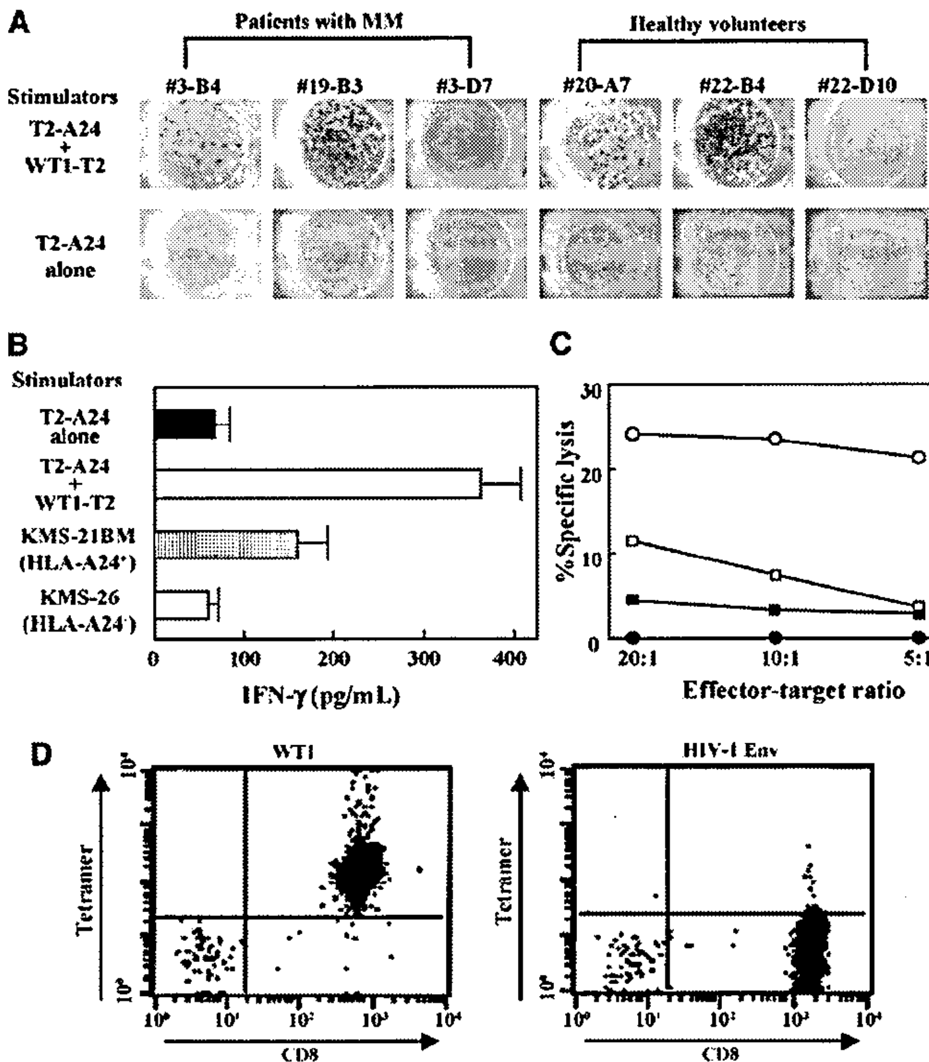

Fig. 7 Generation of WT1-specific CTLs from the patients with MM. *A*, ELISPOT assays. CTLs generated after the third stimulation with WT1-T2 peptide were tested for their specificity. ELISPOT assays were performed by incubating the CTLs with T2-A24 cells pulsed with 10 μmol/L WT1-T2 peptide or T2-A24 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 1 (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′-GAGGACTTCCTCAAG-CACT-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

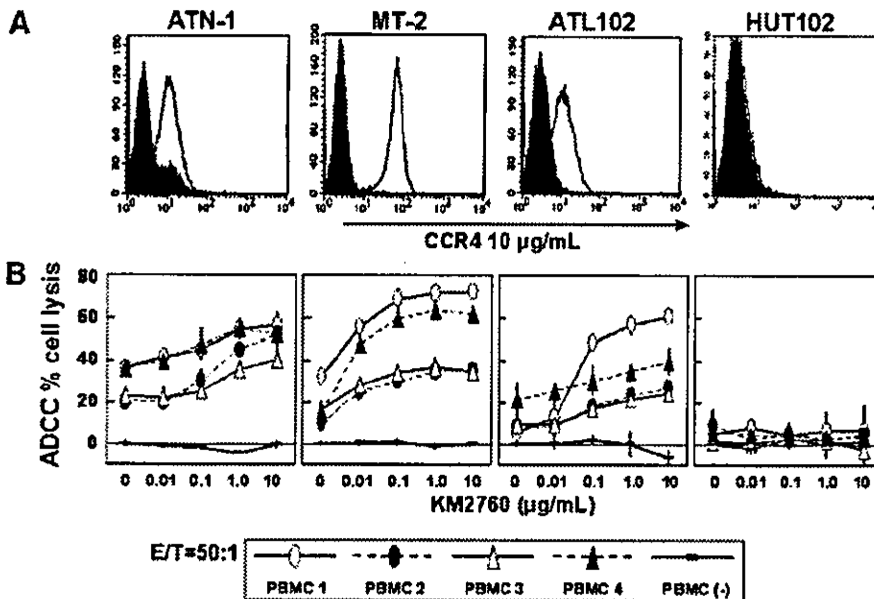

Fig. 1 CCR4 expression in ATLL cell lines and KM2760-induced ADCC. *A*. Four ATLL cell lines were stained with FITC-conjugated anti-CCR4 monoclonal antibody (KM2160) at the concentration of 10 μg/mL (blank histograms) or isotype control monoclonal antibody (filled histograms). *B*. ADCC against ATLL 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.

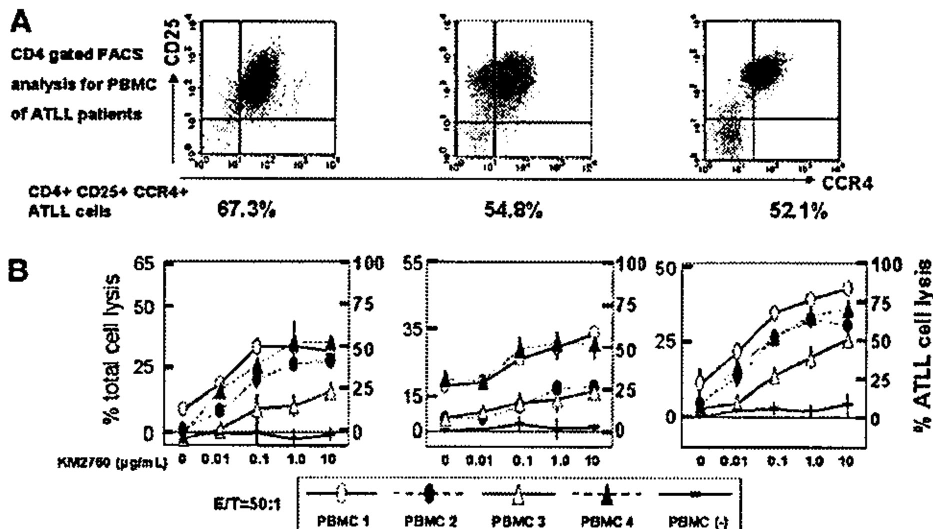

Fig. 2 KM2760-induced ADCC against ATLL cells obtained from patients. *A*. Freshly isolated PBMCs from three patients with refractory acute-type ATLL were analyzed by flow cytometry with peridinin chlorophyll protein-conjugated anti-CD4, phycoerythrin-conjugated anti-CD25 and FITC-conjugated KM2160 monoclonal 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.

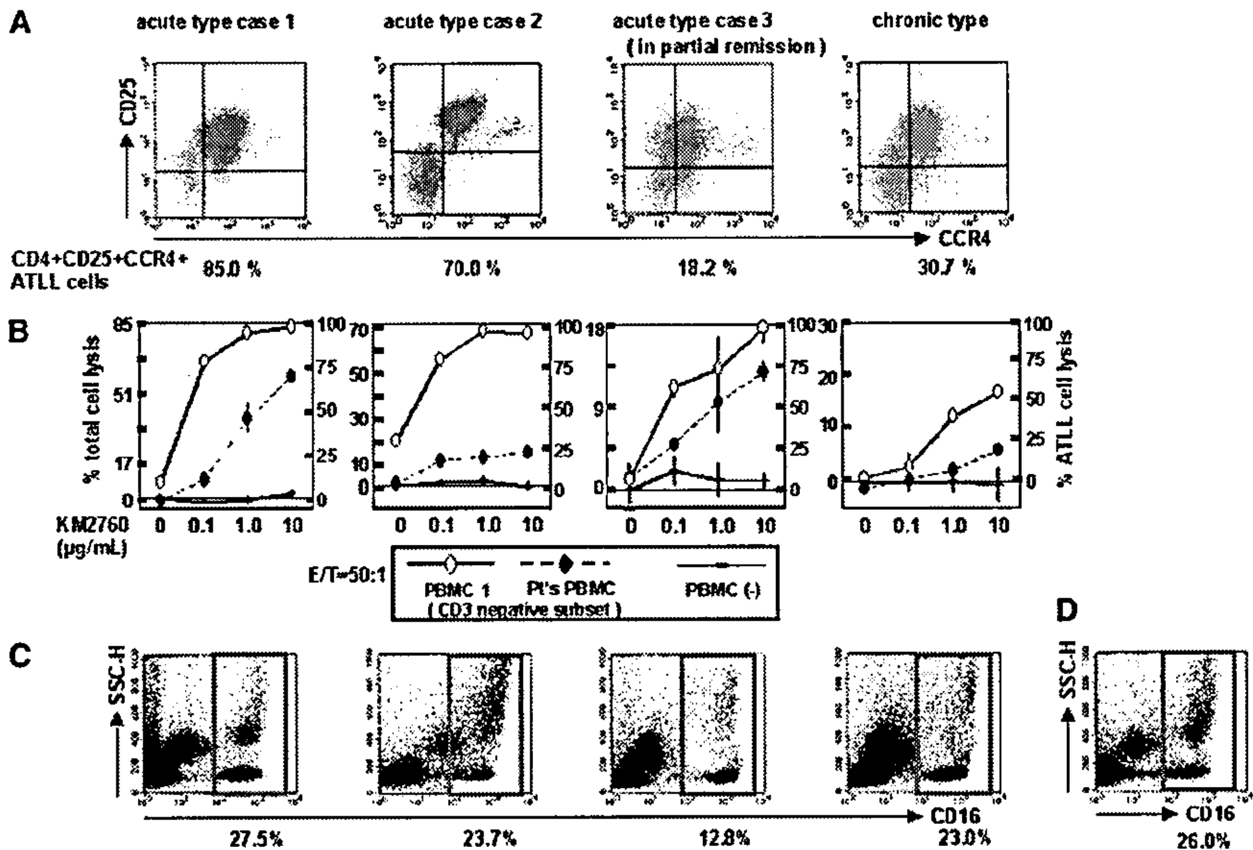

Fig. 3 KM2760-induced ADCC against ATLL cells obtained from patients tested in an autologous setting. Disease status of the patients analyzed are indicated above each data panel. *A*, target ATLL cells used in this study. Freshly isolated PBMCs from four patients with ATLL were sorted into CD3-positive (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

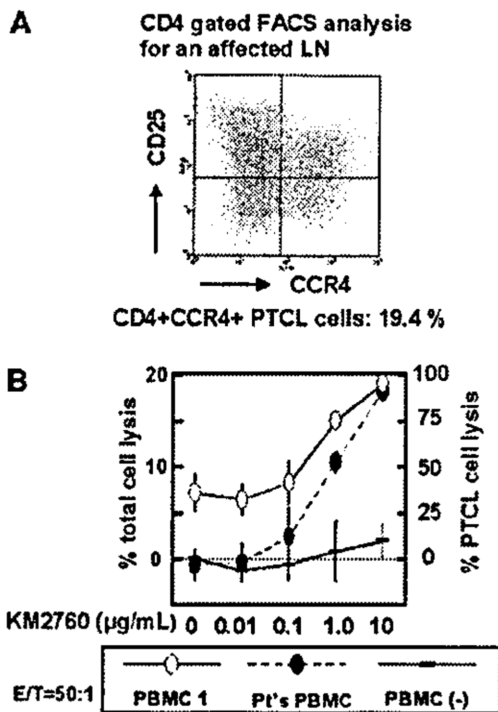

Fig. 4 KM2760-induced ADCC against PTCL, unspecified cells obtained from a patient. Mononuclear cells isolated from a biopsied affected lymph node (LN) of a patient with PTCL unspecified were analyzed as above. The LN contained 19.4% of $CD4^+$ $CCR4^+$ PTCL cells. KM2760-induced ADCC against the isolated 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

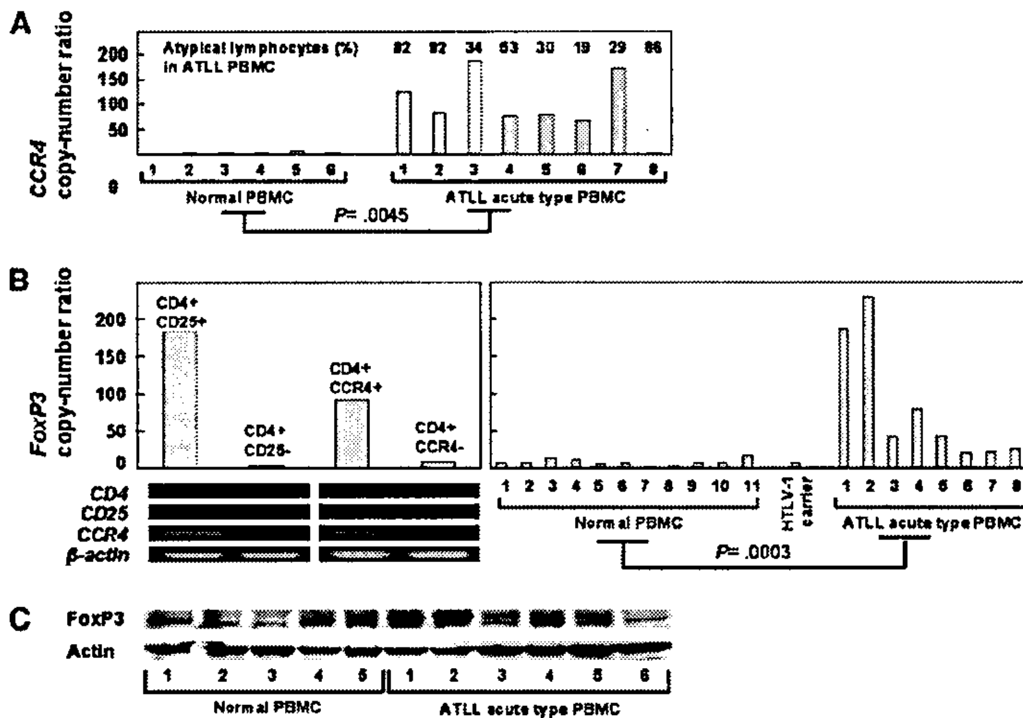

Fig. 5 Expression of CCR4 and FoxP3 in PBMCs from normal volunteers or patients with ATLL. *A*, quantification of the relative *CCR4* mRNA levels in PBMCs obtained from six normal volunteers and eight patients with acute-type ATLL. Serially diluted cDNA aliquots from these PBMCs were analyzed with primer sets for *CCR4* and β-*actin* in a 20-μL reaction mixture containing FastStart DNA master SYBR Green I with the aid of a LightCycler Quick System 330. The quantitative assessment of the mRNA of interest was done by dividing the *CCR4* expression level by that of β-*actin* and expressing the result as a copy-number ratio. *B*, expression of *FoxP3* in CD4+ T-cell subpopulations of PBMCs obtained from a normal volunteer. cDNA aliquots prepared from the purified $CD4^+CD25^+$, $CD4^+CD25^-$, $CD4^+CCR4^+$, and $CD4^+$ $CCR4^-$ subpopulations were subjected to conventional reverse transcription-PCR for *CD4*, *CD25*, *CCR4*, and β-*actin* (*left panel*). Quantification of the relative *FoxP3* mRNA levels was carried out for cDNA prepared from PBMCs of 11 normal volunteers, 1 HTLV-1 seropositive carrier, and 8 patients with acute-type ATLL (*top right panel*). *C*. Expression of FoxP3 protein was analyzed in PBMC extracts obtained from six patients with acute-type ATLL and 5 healthy adult volunteers by Western blot analysis.

antibody increased the CDC lysis of ATL102 irrespective of the presence of KM2760, indicating that ATL102 can be lysed with complement alone if CD55 is blocked functionally. In addition, KM2760 did not induce CDC activity against any of eight non-ATLL T-cell lines irrespective of the presence of CD55 and/or CD59 (data not shown).

Effect of KM2760 on Proliferation of ATLL Cell Lines and Fresh ATLL Cells. We investigated whether KM2760 could inhibit proliferation of both ATLL cell lines and fresh ATLL cells obtained from several acute type patients. No inhibitory effect induced by KM2760 on proliferation of ATLL cell lines or fresh ATLL cells was observed (data not shown). Addition of recombinant human IFN-α or recombinant human IFN-γ did not affect the proliferation in any of them, whereas IFN-α alone induced growth inhibition of MT-2 and all fresh ATLL cells (data not shown).

DISCUSSION

In the present study, we extended our previous observations of potent KM2760-induced ADCC activity against CCR4-positive non-ATLL T-cell lines (16) to ask whether tumor cells from patients with ATLL and PTCL also could be good targets in KM2760-based immunotherapy. Our data clearly showed the potent KM2760-induced ADCC against both ATLL cell lines and fresh ATLL cells obtained from patients. This KM2760-induced ADCC was completely dependent on the cell surface expression of CCR4 on the target cells. However, the observed ADCC activity differed in individual PBMCs used for the assay and in the cell lines tested. There are several potential explanations to account for these observations: CCR4 expression level, percentage of $CD16^+$ cells, and genotype of the *FCGR3A* polymorphism. Firstly, the cell surface expression level of CCR4 seemed to be an important factor for better ADCC activity as MT-2 cell line, which was stained the best with CCR4 antibody, showed the highest ADCC activity. Secondly, the percentage of $CD16^+$ cells among PBMCs is most likely critical because it was almost reproducibly correlated with ADCC activity, as reported in myeloma study with a plasma cell-specific antibody (36). However, it is of note that one of the normal donors (PBMC 3) who had only 4.2% of $CD16^+$ cells in PBMCs still showed sufficient ADCC activity against CCR4-positive ATLL cells, supporting our previous report that defucosylated chimeric anti-CCR4 monoclonal antibody needed much fewer effector cells to achieve the same cytotoxicity as

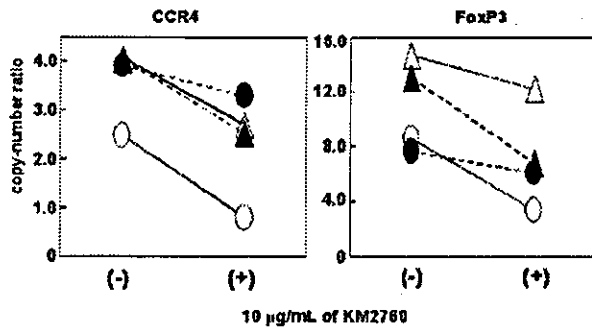

Fig. 6 Reduction of *CCR4* and *FoxP3* mRNA levels in PBMCs treated with KM2760. Fresh PBMCs from four normal volunteers were incubated in the presence (+) or absence (−) of 10 µg/mL KM2760 in RPMI 1640 supplemented with 10% of heat-inactivated pooled human serum. After 6 hours of incubation at 37°C, 5% CO_2, cDNA was prepared from harvested cells and assessed for the expression of *CCR4* and *FoxP3* by quantitative reverse transcription-PCR, and the copy number ratio for each was calculated. ○, PBMC 1; ●, PBMC 2; △, PBMC 3; and ▲, PBMC 4.

that shown by nondefucosylated antibody (16). This feature also should be therapeutically beneficial because the number of effector cells capable of penetrating into tumor masses could be much less than that of tumor cells in the clinical settings. Finally, it has been shown that human IgG1 binds more strongly to FcγRIIIa on NK cells homozygous for *FCGR3A-158V* allele than to those homozygous or heterozygous for *158F* alleles (37, 38). FcγRIIIa is expressed on both NK cells and monocytes, which are the most important natural cytotoxic effectors. Homozygosity for the *158V* allele is associated with better clinical and molecular responses to the chimeric anti-CD20 IgG1 monoclonal antibody rituximab in follicular lymphoma (39) but not in chronic lymphocytic leukemia (40). The *FCGR3A* genotype of the effector PBMCs used throughout the current study was examined. Unfortunately, PBMCs from donors homozygous for the *158V* allele were not found among our donors; nevertheless, a robust ADCC activity was observed irrespective of the absence of donors homozygous for the *158V* allele. We have recently shown that defucosylated chimeric IgG1 monoclonal

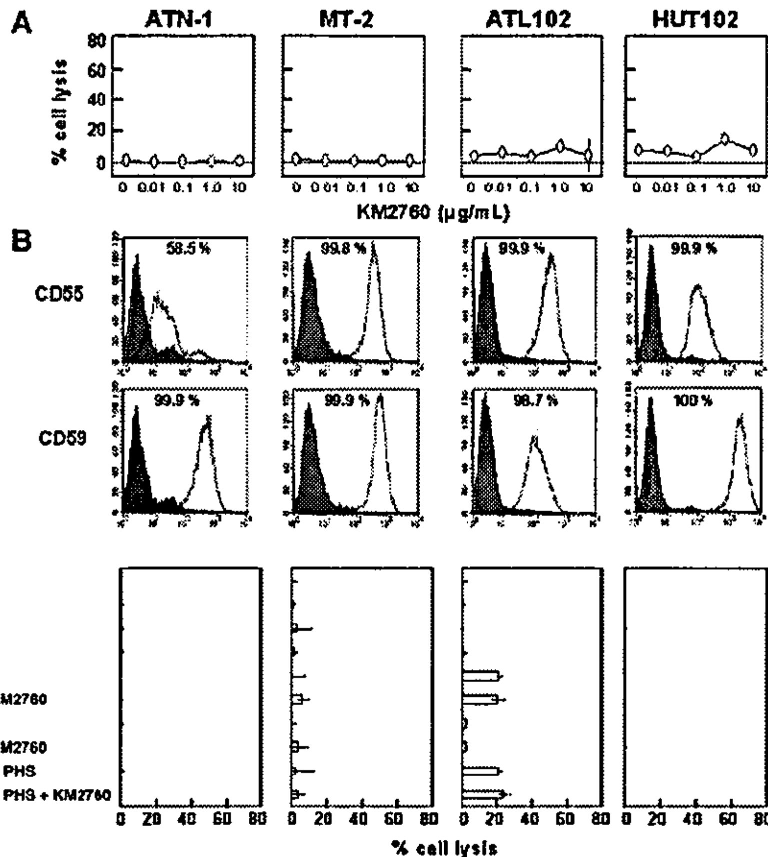

Fig. 7 CDC activity of KM2760 against ATLL cell lines and effect of CD55 and CD59 blocking. *A*. CDC activity against ATLL cells was measured by 1.5 hours of ^{51}Cr release assay. Cells were incubated with the indicated concentrations of KM2760 in RPMI 1640 supplemented with intact pooled human serum (PHS) at the final concentration of 20%. All experiments were done in triplicate, and the percent lysis is presented as the average ± SD. *B*. ATLL cell lines were stained with FITC-conjugated anti-CD55 or anti-CD59 monoclonal antibodies (blank histograms). Staining with isotype control monoclonal antibody is shown as filled histograms. The percentage of positive cells is indicated in each panel. *C*. Blocking antibodies against CD55 (1C6) and CD59 (1F5) were added to the CDC assay to block the function of the CD55 and CD59, individually or in combination. The concentration of KM2760, anti-CD55, and anti-CD59 monoclonal antibodies were fixed at 10 µg/mL. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. *A* and *C* each represents three independent experiments.

antibodies, anti-CD20 and anti-CCR4, can induce much stronger ADCC than nondefucosylated ones (15, 16). These findings can probably explain why the PBMCs, even from donors homozygous or heterozygous for the *158F* allele, induced remarkable antibody-dependent cellular cytotoxicity activity in the presence of defucosylated chimeric anti-CCR4 monoclonal antibody KM2760. This feature should be therapeutically beneficial because a comparable ADCC activity of KM2760 can be expected irrespective of *FCGR3A* genotype.

From the clinical point of view, KM2760 induced a potent ADCC activity at concentrations that are considered to be clinically attainable (1 to 10 μg/mL) against freshly isolated tumor cells in the presence of allogeneic PBMCs as effector cells. Antibody-dependent cellular cytotoxicity against freshly isolated ATLL cells mediated by autologous effector cells was generally lower than that mediated by allogeneic control ones. However, a robust activity, which was comparable with that mediated by allogeneic effector cells, was induced in two of the four cases. It has been shown that HTLV-1 can infect many human cell types other than CD4-positive T-lymphocytes such as NK cells but not hematopoietic progenitor cells (41–47). Previous reports have revealed that the NK cell activity was significantly decreased in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis, although the underlying mechanism remains unknown (48, 49). If HTLV-1 integration into effector cells is the direct reason for their impaired effector function, newly generated cells from the hematopoietic progenitor cells would be free from the virus and expected to show normal function until they become infected. Because close cell-to-cell interaction is required to transmit viruses from HTLV-1-bearing cells to noninfected cells (50), and this process should be time-consuming, there must be an open-window period in which to repopulate normal effector cells. Alternatively, effector cells differentiated or remaining in an immunocompromised environment caused by the copresence of ATLL cells might have impaired cytolytic function (51). In any case, our observation, that one patient in partial remission after chemotherapy showed much better ADCC activity despite the lower percentage of autologous $CD16^+$ effector cells in the CD3-negative subset of PBMCs, indicates that chemotherapy before KM2760 administration could be an optimal choice for patients with large tumor burdens in peripheral blood. In addition, treatment with KM2760 for patients with lymphoma type ATLL or PTCL, the latter of which was shown in this study, could be clinically effective because these patients are free from peripheral blood involvement of the tumor cells.

In this report, we showed not only that *FoxP3* mRNA was expressed in the $CD4^+CCR4^+$ T-cell subset at a much higher level than that in the $CD4^+CCR4^-$ T-cell subset obtained from normal volunteers but also that FoxP3 was highly expressed in freshly isolated ATLL cells. It is known that the surface phenotype of ATLL cells is represented by positivity for CD4, CD25, and CCR4. Our novel findings suggest that ATLL cells might originate from $CD4^+CD25^+$ ($CCR4^+$) immunoregulatory T cells. Thus, it can be envisaged that *FoxP3*-expressing ATLL cells would give rise to a profound immunosuppressive environment around themselves so that they can escape from the host's immunosurveillance. Interestingly, all established ATLL cell lines expressed extremely low or no detectable FoxP3 when assessed by quantitative PCR and Western blot analysis. We surmise that they do not need to express FoxP3 because there exists no effector T cells that attack the ATLL cell lines in culture. In addition, the suppression of the host's normal effector T cells by these ATLL cells can result in a severe immunocompromised state, which is one of the clinical characteristics of patients with ATLL. A similar situation has been shown in Hodgkin's lymphoma (52). Moreover, it has been shown that tumor cells in Hodgkin's lymphoma express thymus and activation-regulated chemokine, which is one of the specific ligands for CCR4, and that reactive lymphocytes surrounding the tumor cells do express CCR4 (53). These studies support our hypothesis of the close relationship between FoxP3 and CCR4 expressions in ATLL cells. Because KM2760 reduced the *FoxP3* mRNA expression level, presumably by specifically killing FoxP3-coexpressing $CCR4^+$ T cells in PBMCs obtained from normal volunteers, KM2760 may be beneficial in reducing the immunosuppressive effect of not only FoxP3-expressing ATLL cells but also FoxP3-expressing normal immunoregulatory T cells and subsequently provoke effective tumor immunity or restore the host's profound immunosuppressive state. Collectively, our findings strongly suggest that KM2760 also can be used as a potential immunomodifier.

KM2760 showed no CDC activity against CCR4-positive target cells. ATLL cell lines showed high expression of complement inhibitors such as CD55 and CD59, whereas addition of blocking antibodies against CD55 or CD59 did not induce CDC activity by KM2760. Besides CDC, we did not detect any direct inhibitory effect on proliferation of the ATLL cell lines or fresh ATLL cells, even when combined with IFN-α or IFN-γ, which have been used for the treatment of several hematologic malignancies, including ATLL. Collectively, we conclude that the major antitumor activity of KM2760 is mediated by ADCC.

In conclusion, the present study shows a promising ADCC activity of the defucosylated chimeric anti-CCR4 monoclonal antibody, KM2760, against tumor cells of ATLL and PTCL, although the optimal conditions for obtaining maximal effector function of autologous effector cells from patients still need to be explored. Moreover, the ability of KM2760 to act as an immunomodifier and break tolerance to tumor cells is also encouraging. As rituximab, a chimeric anti-CD20 monoclonal antibody, has changed the standard therapy in elderly patients with diffuse large B-cell lymphoma (54), now KM2760 could be an ideal treatment modality against patients with ATLL and CCR4-positive PTCL.

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Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases)

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Abstract

To ascertain the consequences of severe leukopenia and the tempo of recovery, we studied the immunity of 56 adult patients treated for multiple sclerosis or systemic sclerosis with autologous CD34 cell transplantation using extremely lymphoablative conditioning. NK cell, monocyte, and neutrophil counts recovered to normal by 1 month; dendritic cell and B cell counts by 6 months; and T cell counts by 2 years posttransplant, although CD4 T cell counts remained borderline low. Initial peripheral expansion was robust for CD8 T cells but only moderate for CD4 T cells. Subsequent thymopoiesis was slow, especially in older patients. Importantly, levels of antibodies, including autoantibodies, did not drop substantially. Infections were frequent during the first 6 months, when all immune cells were deficient, and surprisingly rare (0.21 per patient year) at 7–24 months posttransplant, when only T cells (particularly CD4 T cells) were deficient. In conclusion, peripheral expansion of CD8 but not CD4 T cells is highly efficient. Prolonged CD4 lymphopenia is associated with relatively few infections, possibly due to antibodies produced by persisting pretransplant plasma cells.
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Introduction

Autoimmune diseases may be caused by a one time failure of negative selection leading to the generation of an autoreactive T or B cell clone. This hypothesis lead to the development of clinical trials of extremely lymphoablative therapy, typically with autologous CD34 cell transplantation to minimize hematological toxicity [1]. The aim was to eliminate the autoreactive T or B cell clone and hope that the error in negative selection would not be repeated. The trials have provided a unique opportunity to study the consequences of severe leukopenia (in particular, lymphopenia) and homeostatic recovery in humans. The conditioning used in our trials [2,3] consisted of total body irradiation and cyclophosphamide administered from day 5 to day 2 and anti-thymocyte globulin (ATG) administered from day 5 to day 5; this resulted in severe lymphopenia (significantly more severe than after autologous transplantation for cancer using

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