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Defucosylated Anti-CCR4 Monoclonal Antibody Exerts Potent ADCC against Primary ATLL Cells Mediated by Autologous Human Immune Cells in NOD/Shi-scid, IL-2Rγnull Mice In Vivo

Asahi Ito,* Takashi Ishida,† Atae Utsunomiya,‡ Fumihiko Sato,*** Fumiko Mori,* Hiroki Yano,* Atsushi Inagaki,* Susumu Suzuki,* Hisashi Takino,‡ Masaki Ri,* Shigeru Kusumoto,* Hirokazu Komatsu,* Shinsuke Iida,* Hiroshi Inagaki,‡ and Ryuzo Ueda*

There is a lack of suitable small animal models to evaluate human Ab-dependent cellular cytotoxicity (ADCC) in vivo, because of the species incompatibility between humans and animals or due to nonspecific allogeneic immune reactions. To overcome these problems, we established a human tumor-bearing mouse model, using NOD/Shi-scid, IL-2Rγnull (NOG) mice as recipients, in which autologous human immune cells are engrafted and mediate ADCC but in which endogenous murine cells are unable to mediate ADCC. In the present study, we used NOG mice bearing primary adult T cell leukemia/lymphoma (ATLL) cells and a therapeutic chimeric anti-CCR4 mAb, the Fc region of which is defucosylated to enhance ADCC. We report significant antitumor activity in vivo associated with robust ADCC mediated by autologous effector cells from the same patients. The present study is the first to report a mouse model in which a potent antitumor effect of the therapeutic mAb against primary tumor cells is mediated by autologous human immune cells. Human autologous ADCC in mice in vivo was confirmed by the depletion of human immune cells before ATLL PBMC inoculation. In addition, NOG mice bearing primary ATLL cells presented features identical with patients with ATLL. In conclusion, this approach makes it possible to model the human immune system active in Ab-based immunotherapy in vivo, and thus to perform more appropriate preclinical evaluations of novel therapeutic mAb. Furthermore, the potent ADCC mediated by defucosylated anti-CCR4 mAb, observed here in vivo in humanized mice, will be exploited in clinical trials in the near future. The Journal of Immunology, 2009, 183: 4782–4791.

The use of therapeutic mAbs for the treatment of cancer has evolved into a promising approach over the last several years. Abs of the human IgG1 isotype are commonly used for therapeutic applications as they can mediate multiple effector functions including Ab-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and direct apoptosis induction (1–3). Of these, ADCC is an especially important mechanism of action of therapeutic mAb against tumor cells (4–7); therefore, a better understanding of ADCC will allow the development of novel, more effective treatment strategies using therapeutic mAbs. ADCC depends on the cytotoxic activity of immune effector cells, so to evaluate the antitumor effects of therapeutic mAb in a small animal model in vivo, the species incompatibility of the immune system between humans and animals is a critical issue. Indeed, we have previously reported that the mouse effector system mediating the antitumor action of therapeutic mAb does differ from the human (8–10). Thus, a current crucial problem in the field of human ADCC research is the lack of suitable small animal models. To overcome this, we have recently established “humanized mice,” in which human immune cells mediate the antitumor action of the therapeutic mAb, using NOD/Shi-scid, IL-2Rγnull (NOG) mice (11, 12) as recipients. In this model, we showed that human PBMC from healthy individuals functioned as ADCC effector cells against allogeneic tumor cell lines engrafted in the mice (13). Using this humanized mouse model, we now have the opportunity to perform more appropriate preclinical evaluation of many types of Ab-based immunotherapy, although in the initial study, we could not completely exclude nonspecific allogeneic immune responses because target and effector cells were obtained from different individuals. In addition, susceptibility to immunotherapy is likely to be different in established cell lines and primary tumor cells isolated directly ex vivo from patients, with the latter certainly being more relevant for evaluation of immunotherapeutic agents. Thus, the first aim of the present study was to establish the mouse model bearing human primary tumor cells, in which human autologous immune cells can engraft and function as ADCC effector cells.

In the clinical field of hematological malignancies, the development of the therapeutic mAb rituximab has changed the standard therapy in patients with B cell lymphomas and has markedly improved their prognoses (14, 15). In contrast, T cell lymphomas...
have very poor prognoses, and no standard treatment strategies for these diseases have been developed so far (16). Therefore, alternative treatment strategies for these patients are urgently needed. Because we previously found that CCR4 is expressed on certain types of these tumors (17, 18), we postulated that this molecule might represent a novel molecular target for immunotherapy against refractory T cell lymphoma. Accordingly, we have developed a next-generation chimeric anti-CCR4 mAb, KM2760, the Fc region of which is defucosylated, resulting in highly enhanced ADCC due to increased binding affinity to the FcγR on effector cells (4, 9, 19). Most importantly, based on our laboratory work on CCR4 (8–10, 20–22) and as an outcome of the success of this translational research, we have completed a phase I clinical trial of defucosylated humanized anti-CCR4 mAb in patients with CCR4-positive T cell leukemia/lymphoma in Japan (ClinicalTrials.gov identifier: NCT00355472), and are currently undertaking a phase II clinical trial. As the target disease representative of a refractory and incurable type of T cell lymphoma, we selected adult T cell leukemia/lymphoma (ATLL) (16, 23, 24) as the target disease.

Materials and Methods

ATLL cell lines

ATN-1, MT-2, and ATL102 were described previously (17). TL-Omi1 was kindly provided from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, Sendai, Japan.

Human cells

PBMC were isolated from patients with ATLL using Ficoll-Paque (Pharmacia Biotech) for use in cell proliferation assay or as effector cells in NK cells in NOG mice. All donors provided written informed consent before sampling in accordance with the Declaration of Helsinki, and the present study using human samples was approved by the institutional review board of Nagoya City University Graduate School of Medical Sciences.

Cell proliferation assay

Proliferation of the ATLL cell lines and primary ATLL cells cultured with different concentrations of KM2760 with or without goat anti-human IgG (Sigma-Aldrich) at a final concentration of 10 μg/ml for 48 h was assessed using the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega) as described previously (20). Primary ATLL cells were isolated from PBMC of five individual patients using anti-human CD4 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions.

Abs and flow cytometry

KM2760 has been described previously (9). The following mAbs were used for flow cytometry: MultiTEST CD3 (clone SK7) FITC/CD16 (B73.1) + CD56 (NCAM 16.2) PE/CD45 (2D1) PerCP/CD19 (SJ25C1) APC Reagent, allopregoycin-conjugated anti-human CD45 mAb (2D1), FITC-conjugated anti-CCR4 mAb (KM2160), PE-conjugated anti-CD25 mAb (M-A251), PerCP-conjugated anti-CD4 mAb (SK3), and the appropriate isotype control mAbs. KM2160 has been described previously (20), and the other mAbs were purchased from BD Biosciences. Whole blood cells from healthy individuals or mice were treated with BD FACS lysing solution (BD Biosciences) for lysing RBC. Cells were analyzed by a FACS Calibur (BD Biosciences) with the aid of FlowJo software (Tree Star).

Animals

NOG mice were purchased from the Central Institute for Experimental Animals and used at 6–8 wk of age. All of the in vivo experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia, Second Edition.

Primary ATLL cell-bearing mouse model, disseminated therapeutic setting

Whole PBMC (1 × 10^7) obtained from ATLL patient 1 and suspended in 0.2 ml of RPMI 1640 were i.p. inoculated into each of 10 NOG mice. The primary ATLL-bearing mice were divided into two groups of five for KM2760 or control (plain RPMI 1640) injections 7 days after inoculation, such that the mean serum lactate dehydrogenase (LDH) value was equal in each group. The concentration of LDH in serum was measured using LDH-J reagent (Kainos Laboratories) according to the manufacturer’s instructions. Thus, KM2760 (30 μg/mouse) and control i.p. injections were started 7 days after PBMC inoculations, when the mean serum LDH values were 2035 ± 110 (SD) and 2014 ± 582 Wroblewski unit/ml, respectively, and continued weekly for 4 wk. KM2760 therapeutic efficacy was evaluated 30 days after PBMC inoculation.

Primary ATLL cell-bearing mouse model, early therapeutic setting

Whole PBMC (1 × 10^7) obtained from ATLL patient 2 and suspended in 0.2 ml RPMI 1640 were i.p. inoculated into each of 10 NOG mice. The mice were also divided into two groups of five for KM2760 or control injections 2 days after PBMC inoculation. KM2760 (30 μg/mouse) or control medium was i.p. injected repeatedly into the mice after 2, 7, 12, and 17 days, and therapeutic efficacy was evaluated 29 days after the ATLL PBMC inoculation.

Primary ATLL cell-bearing mouse model, NK cell- or monocyte-depletion setting

PBMC containing 0.8 × 10^7 CD4-, CCR4-, and CD25-positive ATLL cells obtained from ATLL patient 3 and suspended in 0.2 ml RPMI 1640 were i.p. inoculated into each of 10 NOG mice. The mice were divided into two groups of five for KM2760 (group iv) or control (group i) injections 5 days after PBMC inoculation. KM2760 (30 μg/mouse) or control medium was i.p. injected repeatedly into the mice after 5, 10, 15, and 20 days, and therapeutic efficacy was evaluated 23 days after the ATLL PBMC inoculation. PBMC obtained from the same patient were depleted of NK cells using anti-human CD56 microbeads (Miltenyi Biotec), or of monocytes using anti-human CD14 microbeads (Miltenyi Biotec). The NK cell- or monocyte-depleted PBMC containing 0.8 × 10^7 ATLL cells were i.p. inoculated into each of five NOG mice (groups ii and iii), and they were treated with KM2760 in the same manner.

Immunopathological analysis

H&E staining and immunostaining using anti-CCR4 mAb (KM2160), CD4 (4B12, Novocastra), and CD25 (4C9, Novocastra) were performed on formalin-fixed, paraffin-embedded sections, as previously described (17, 18).

Soluble IL2R (sIL2R) measurement

The concentration of human sIL2R in mouse serum was measured by ELISA using the human IL-2 R immunoassay kit (R&D Systems) according to the manufacturer’s instructions.

Statistical analysis

The differences between groups regarding the percentage of ATLL cells in mouse whole blood cells, liver and spleen cell suspensions, and human sIL2R concentrations in mouse serum were examined with the Mann-Whitney U test. Data were analyzed with the aid of StatView software, version 5.0 (SAS Institute). In this study, p < 0.05 was considered significant.

Results

Effect of KM2760 on proliferation of ATLL cell lines and primary ATLL cells

We investigated whether KM2760 inhibited the proliferation both of CCR4-expressing ATLL cell lines (n = 4; ATN-1, MT-2, ATL102, and TL-Omi1) and primary ATLL cells obtained from five individual patients. No inhibitory effects of KM2760 on proliferation of any of these cells were observed, even when cross-linked with anti-human IgG (data not shown).

Characteristics of PBMC from ATLL patient 1

This patient’s PBMC for inoculation contained 75.1% ATLL cells (CD3-weakly positive and CD19/CD16/CD56-negative), 1.8% NK cells (CD16/CD56-positive and CD3-negative; Fig. 1, lower
left panel), 3.7% B cells (CD19-positive and CD3-negative), and 11.0% non-ATLL T cells (CD3-highly positive and CD19-negative; Fig. 1, lower right panel), and 5.1% monocytes determined by forward scatter height (FSC-H) and side scatter height (SSC-H) (Fig. 1, upper left panel). The patient’s PBMC contained 83.2% CD4-positive cells (data not shown), of which 90.0% were CD25- and CCR4-double positive. These CD4-, CCR4-, and CD25-positive cells were the ATLL tumor cells (Fig. 1, upper panels; Refs. 17, 20, 25).

KM2760 significantly decreased the percent ATLL cells in mice whole blood cells in the disseminated therapeutic setting

Thirty days after ATLL1 PBMC inoculation, the percentage of ATLL cells (both CD4- and CD25-positive) in the whole blood of control NOG mouse No. 1 was 14.8% (i.e., 41.3% (lymphocyte population) \( \times 35.9\% \) (human CD4- and CD25-positive cells) = 14.8%). In control NOG mice Nos. 2, 3, 4, and 5, and in anti-CCR4 mAb-treated NOG mice Nos. 1, 2, 3, 4, and 5, the percentages of ATLL cells in whole blood, calculated in the same manner, were 12.7, 22.5, 17.2, and 28.3%; and 0.38, 0.41, 0.41, 0.69, and 0.62%, respectively. Thus, KM2760 treatment significantly decreased the percentage of ATLL cells present in the blood of these mice (p = 0.0090; Fig. 2A).

CD45-positive cells among whole blood cells 30 days after ATLL1 PBMC inoculation were plotted according to CD3 and CD19 expression (Fig. 2B). The percentages of ATLL cells (CD3-weakly positive and CD19-negative, see Fig. 1, lower right panel) within the human CD45-positive cells in the blood of control and KM2760-treated NOG mice were 89.6 ± 2.70 (mean ± SD) and 48.5 ± 14.1% (Fig. 2B, lower panel), respectively. This difference was statistically significant (p = 0.0090).

KM2760 significantly decreased ATLL cells in mouse liver in the disseminated therapeutic setting

Thirty days after ATLL1 PBMC inoculation, the percentage of ATLL cells (CD4- and CD25-double positive) in the liver cell suspension of control NOG mouse No. 1 was 1.89% (i.e., 17.2% (blood nucleated cell population; Fig. 2C, upper panel) \( \times 11.0\% \) (CD4- and CD25-positive cells; Fig. 2C, lower panel) = 1.89%). The percentages of ATLL cells in liver cell suspensions from five control and five KM2760-treated NOG mice were 1.23 ± 0.71 and 0.40 ± 0.23%, respectively. This difference was statistically significant (p = 0.0472; Fig. 2C). These values were 3.64 ± 1.64 and 1.56 ± 2.34%, respectively, in the spleen (Fig. 2D). Although this latter difference was not statistically significant, a clear tendency can be observed. The percentage of ATLL cells in spleen-infiltrating blood nucleated cells of control and KM2760-treated mice was 14.5 ± 4.77 and 7.74 ± 3.93%, respectively, and this difference was statistically significant (p = 0.0472; Fig. 2D).

KM2760 decreased the ATLL lesions in different organs of NOG mice

Images of control mouse No. 1 and KM2760-treated mouse No. 2, 30 days after ATLL1 PBMC inoculation, are shown in Fig. 3A. Both liver and spleen of control NOG mouse No. 1 were enlarged and had a large number of tumor nodules. The kidneys were moderately enlarged. In contrast, liver, spleen, and kidney of KM2760-treated mouse No. 2 had no morbid lesions detectable macroscopically. Immunopathological analysis revealed that large atypical cells with irregular and pleomorphic nuclei proliferated with a multifocal pattern in the liver of control mouse No. 1 (Fig. 3B, left panel). These atypical cells were positive for CCR4 (Fig. 3B, middle panel), CD4, and CD25 (data not shown). However, the liver of treated mouse No. 2 had no atypical cell infiltration according to immunopathological analysis (Fig. 3B, right panel). The large majority of spleen cells in control mouse No. 1 had been replaced by large atypical cells with irregular and pleomorphic nuclei (Fig. 3C, left panel). These atypical cells were also positive for CCR4 (Fig. 3C, middle panel), CD4, and CD25 (data not shown). In treated mouse No. 2, atypical cells proliferated with a patchy pattern in the spleen (Fig. 3C, right panel), and were also positive for CD4 and CD25 (data not shown). In the lung, large atypical cells with irregular and pleomorphic nuclei proliferated with a patchy pattern in control No.1 (Fig. 3D, left panel), and were positive for CCR4 (Fig. 3D, middle panel), CD4, and CD25 (data not shown).
FIGURE 2. Flow cytometric analyses of each mouse 30 days after ATLL1 PBMC inoculation. A, Flow cytometric analyses of whole blood cells are presented. Lymphocytes are determined by FSC-H and SSC-H levels (upper panels) and plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells among lymphocytes is indicated above each panel (lower panels). B, Human CD45-expressing cells among mouse whole blood cells are plotted to show CD3 and CD19 expression. The percentage of ATLL (CD3-weakly positive and CD19-negative) cells among human CD45-positive cells is indicated above each panel. C, Blood nucleated cells infiltrating the liver, determined by FSC-H and SSC-H levels (upper panels), plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells is indicated above each panel (lower panels). D, Blood nucleated cells infiltrating the spleen, determined by FSC-H and SSC-H levels (upper panels), plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells is indicated above each panel (lower panels). n.s., Not significant.
In contrast, lung of treated mouse No. 2 had no atypical cell infiltration, as assessed by immunopathological analysis (Fig. 3D, right panel). In the kidney, CCR4-, CD4-, and CD25-positive atypical cells proliferated with a patchy pattern in the control but there was no atypical cell infiltration in the treated animal (data not shown). These two mice, control No. 1 and treated No. 2, were representative of the immunopathological observations made in each group of five animals. The immunopathological analysis of the liver and spleen of each mouse yielded results essentially consistent with the respective flow cytometric analysis.
KM2760 significantly decreased human sIL2R concentrations in serum in the disseminated therapeutic setting

We measured human sIL2R concentrations in serum as a reliable surrogate marker reflecting ATLL tumor burden (26) in the mice. Thirty days after ATLL1 PBMC inoculation, the serum sIL2R concentrations of the five KM2760-treated NOG mice (1477 ± 915 pg/ml) were significantly lower than in controls (23,436 ± 12,153 pg/ml; \( p = 0.0090 \); Fig. 4).

Characteristics of PBMC from ATLL patient 2

This patient’s PBMC contained 62.6% ATLL cells (CD3-weakly positive and CD19/CD16/CD56-negative), 6.5% NK cells (CD16/CD56-positive and CD3-negative), 4.0% B cells (CD19-positive and CD3-negative), and 21.8% non-ATLL T cells (CD3-highly positive and CD19-negative) (Fig. 5A, lower panels), and 0.9% monocytes determined by FSC-H and SSC-H (Fig. 5A, upper left panel).

FIGURE 4. sIL2R measurement by ELISA. The serum sIL2R concentrations of each primary ATLL-bearing NOG mouse are plotted. The anti-CCR4 mAb recipient NOG mice had significantly lower levels of sIL2R than control mice.

FIGURE 5. Therapeutic efficacy of anti-CCR4 mAb in the primary ATLL cell-bearing NOG mouse model in the early therapeutic setting. A, Flow cytometric analyses of whole blood cells obtained from ATLL patient 2. PBMC were isolated from whole blood and then inoculated into NOG mice. CD4-positive cells among PBMC are plotted to show CCR4 and CD25 expression (upper panels). PBMC are also plotted to show CD3 and CD16/56 expression (lower left panel) and CD3 and CD19 expression (lower right panel). B, Serum sIL2R concentrations of each primary ATLL-bearing NOG mouse. The anti-CCR4 mAb recipient NOG mice had significantly lower levels of sIL2R than control mice. C, Flow cytometric analyses of whole blood cells obtained from each mouse 29 days after ATLL2 PBMC inoculation. Lymphocytes were identified by FSC-H and SSC-H levels (upper panels) and then human CD45-expressing cells were gated and plotted according to CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells among human CD45-positive lymphocytes is indicated above each panel (lower panels).
The patient’s PBMC contained 69.5% CD4-positive cells (data not shown), of which 89.7% were CD25- and CCR4-double positive cells, representing the ATLL tumor cells (Fig. 5A, upper panels).

**KM2760 significantly decreased human sIL2R concentrations in serum in the early therapeutic setting**

The serum sIL2R concentrations of the KM2760-treated NOG mice (1815 ± 208 pg/ml) were significantly lower than in controls (7629 ± 2627 pg/ml; \( p = 0.0163 \)) 29 days after ATLL2 PBMC inoculation (Fig. 5B).

**KM2760 significantly decreased the percentage of ATLL cells within mouse whole blood cells in the early therapeutic setting**

Twenty-nine days after ATLL2 PBMC inoculation, the percentage of ATLL cells among whole blood cells of control NOG mouse No. 1 was 8.24% (i.e., 46.0% (lymphocyte population) of ATLL cells among whole blood cells of control NOG mouse within mouse whole blood cells in the early therapeutic setting PBMC inoculation (Fig. 5A, middle panels), of which 89.7% were CD25- and CCR4-double positive and CD19/CD16/CD56-negative), 3.52% NK cells (Fig. 6A, middle panel), and 0.9% monocytes determined by FSC-H and SSC-H (Fig. 6A, upper left panel). This patient’s PBMC contained 85.0% CD4-positive cells (data not shown), of which 91.1% were CD25- and CCR4-double positive cells, representing the ATLL cells (Fig. 6A, upper left and middle panels). The inoculum of CD56-depleted ATLL PBMC for the NOG mice (group ii) now contained only 0.07% NK cells (Fig. 6A, lower middle panel). The inoculum of CD14-depleted ATLL PBMC for the NOG mice (group iii) contained only 0.13% of the monocyte cell population (Fig. 6A, upper right panel), but had maintained the NK cell population (Fig. 6A, lower right panel). These results indicate that this manner of depleting each effector cell subset from whole ATLL PBMC was appropriate.

**KM2760 significantly decreases human sIL2R concentrations in serum after either NK cell or monocyte depletion**

The serum sIL2R concentrations of the NOG mice (group iv), inoculated with whole PBMC and treated with KM2760 (58 ± 66 pg/ml), were significantly lower than in controls without Ab (group i; 3276 ± 1289 pg/ml; \( p = 0.0088 \)). However, in animals inoculated with NK cell-depleted PBMC and treated with KM2760 (group ii), this value was 1521 ± 1137 pg/ml, \( p = 0.0088 \), and after monocyte depletion (group iii) it was 1027 ± 595 pg/ml, \( p = 0.0088 \) (Fig. 6B).

**KM2760 significantly decreases the percentage of ATLL cells in mouse whole blood in the NK cell- or monocyte-depletion setting**

Twenty-three days after inoculating ATLL3 PBMC, the percentage of ATLL cells (CD4- and CD25-double positive) in the whole blood of control NOG mouse No. 1 was 6.74% (i.e., 7.2% (human CD45-positive cells) \( \times 93.6% \) (human CD4- and CD25-positive cells; Fig. 6C, upper left panel) = 6.74%). In the other NOG mice, the percentages of ATLL cells in whole blood, calculated in the same manner, were 13.9 ± 5.98% for group i, 2.00 ± 1.29% for group ii, 0.73 ± 0.35% for group iii, and 0.19 ± 0.20% for group iv. The percentages of ATLL cells in group iv were significantly lower than in group i (\( p = 0.0090 \)), group ii (\( p = 0.0090 \)), or group iii (\( p = 0.0163 \)) (Fig. 6C).

The same assays were then conducted 23 days after ATLL3 PBMC inoculation for the liver cell suspensions of control NOG mouse No. 1. Here, the percentage of ATLL cells was 1.10% (i.e., 1.2% (human CD45-positive cells) \( \times 91.6\% \) (CD4- and CD25-positive cells, Fig. 6D, upper left panel) = 1.10%). In the other NOG mice, the percentages of ATLL cells in liver, calculated in the same manner, were 1.69 ± 0.95% for group i, 0.60 ± 0.53% for group ii, 0.11 ± 0.01% for group iii, and 0.04 ± 0.06% for group iv. The percentages of ATLL cells in group iv were significantly lower than in group i (\( p = 0.0088 \)) and group ii (\( p = 0.0465 \)), but not group iii (\( p = 0.1161 \)) (Fig. 6, D and F).

Finally, for spleen cell suspensions, 23 days after ATLL3 PBMC inoculation, the percentage of ATLL cells in control NOG mouse No. 1 was 1.10% (i.e., 1.6% (human CD45-positive cells) \( \times 68.6\% \) (CD4- and CD25-positive cells; Fig. 6D, upper left panel) = 1.10%). In the other NOG mice, the percentages of ATLL cells in the spleen cell suspension, calculated in the same manner were 4.13 ± 2.44% for group i, 2.57 ± 1.66% for group ii, 0.97 ± 0.48% for group iii, and 0.15 ± 0.21% for group iv. The percentages of ATLL cells in group iv were significantly lower than in group i (\( p = 0.0090 \)), group ii (\( p = 0.0090 \)), and group iii (\( p = 0.0163 \)) (Fig. 6, E and G).

**Discussion**

In the present study, we have achieved two goals: first, to establish novel, humanized mice using NOG recipients engrafted with primary ATLL cells together with autologous immune cells from the same patient, which functioned as ADCC effector cells; second, to document that the novel anti-T cell lymphoma agent KM2760 demonstrated significant therapeutic efficacy in these humanized mice. The latter was reflected in a reduction of the percentage of ATLL cells in blood, liver, or spleen, as well as a reduction in serum human sIL2R levels.

NOG mice have severe, multiple, immune dysfunctions, such that human immune cells engrafted into them retain essentially the same functions as in humans (11, 12). We have previously reported that endogenous immune cells in NOG mice are unable to mediate the antitumor action of KM2760 against established cell lines (13). In addition, KM2760 can induce only ADCC activity, but does not mediate complement-dependent cytotoxicity or direct antitumor activities (20). Here, we further confirmed that KM2760 had no direct antitumor activities against either CCR4-expressing, established ATLL cell lines or primary ATLL cells, even when it was cross-linked. Together, these results indicate that the KM2760-induced antitumor effects observed here in NOG mice bearing primary ATLL cells in vivo should be dependent on the activity of the engrafted human immune cells. To confirm this finding, we tested antitumor activity of KM2760 in NOG mice in vivo by the ex vivo depletion of each human immune cell subset before ATLL PBMC inoculation. Significant differences were observed regarding the therapeutic efficacy of KM2760 after NK cell-depletion, as demonstrated by its effect on the percentage of ATLL cells in the blood, liver, and spleen, and on serum sIL2R concentrations. This indicates that KM2760-induced ADCC is indeed mediated by human NK cells from ATLL patients, which remain able to kill autologous primary ATLL tumor cells engrafted and proliferating in mice in vivo. Significant differences in KM2760 therapeutic efficacy were also demonstrated by the effect on the...
FIGURE 6. Therapeutic efficacy of anti-CCR4 mAb in the primary ATLL cell-bearing NOG mouse model in the NK cell- or monocyte-depletion setting. A. Flow cytometric analyses of whole blood cells obtained from ATLL patient 3. PBMC were isolated from whole blood and inoculated into NOG mice (group i mice were controls without Ab and group iv mice were treated with KM2760). Whole PBMC are determined by FSC-H and SSC-H levels (upper left panel), and CD4-positive cells among whole PBMC are plotted to show CCR4 and CD25 expression (lower left panel). Whole PBMC are also determined by CD3 and CD16/56 expression (upper middle panel). PBMC obtained from the same patient were depleted of NK cells and inoculated into NOG mice (group ii). They are determined by CD3 and CD16/56 expression (lower middle panel). PBMC obtained from the same patient were depleted of monocytes and then inoculated into NOG mice (group iii). They are determined by FSC-H and SSC-H levels (upper light panel), and by CD3 and CD16/56 expression (lower right panel). B. The serum sIL2R concentrations of each primary ATLL-bearing NOG mouse are plotted. C, Human CD45-expressing cells among mouse whole blood cells are plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells among human CD45-expressing cells is indicated above each panel. D, Human CD45-expressing cells among mouse liver cell suspensions are plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells among human CD45-expressing cells is indicated above each panel. E, Human CD45-expressing cells among mouse spleen cell suspension are plotted to show CD4 and CD25 expression. The percentage of ATLL (CD4- and CD25-positive) cells among human CD45-expressing cells is indicated above each panel. The percentages of ATLL cells in the liver and spleen cell suspensions of each primary ATLL-bearing NOG mouse are plotted (F and G, respectively).
percentage of ATLL cells in the blood and spleen, and on serum sIL2R concentrations, after monocyte depletion, suggesting that human monocytes are also effector cells in vivo against autologous primary ATLL tumor cells engraved and proliferating in the mice. Most importantly, the present study is the first to report a mouse model in which a potent antitumor effect of the therapeutic mAb against primary tumor cells is mediated by autologous human immune cells. Although no statistically significant differences in KM2760 therapeutic efficacy after NK cell or monocyte depletion were observed in the present study, a clear trend emerged showing that monocyte-depleted mice retaining NK cells were more sensitive to KM2760 treatment than NK cell-depleted mice. This is consistent with the results of studies demonstrating the importance of NK cells as effectors for human ADCC (27–29), which we have previously confirmed in vitro (30), and in vivo in the humanized NOG mouse allogeneic system (13).

In the present study, inoculated PBMC of patient 1 consisted of 1.8% NK cells, 5.1% monocytes, and 75.1% ATLL cells, resulting in an ADCC E:T ratio of only 0.092 (1.85 : 51.75 : 0.092). For ATLL patients 2 and 3, the E:T ratio was low at all 0.118 and 0.074. To our great surprise, such small numbers of effector cells functions as ADCC effector cells and provided significant therapeutic benefit in KM2760-treated mice. The extremely enhanced ADCC observed here in mice in vivo was most likely due to the defucosylation (4, 9, 19) of the therapeutic Ab. Defucosylated human IgG1 mediates much higher ADCC (>50-fold) with human PBMC compared with fucosylated, but otherwise identical, Ab. Thus, defucosylated KM2760 needed far fewer effector cells to achieve the same level of ADCC as the fucosylated, but otherwise identical, IgG1 (9).

In the present system, primary ATLL cells expressing CD4, CD25, and CCR4 massively infiltrated into different organs such as spleen, liver, and lung in NOG mice, consistent with the report by Dewan et al. (31). Because NOG mice bearing primary ATLL cells present features very similar to patients with ATLL, this model should provide a powerful tool to understand the pathogenesis of ATLL, and furthermore, one which can be used not only to evaluate novel cytotoxic anti-ATLL agents, but also immunotherapeutic agents, including anti-CCR4 mAb, more appropriately, in vivo.

In conclusion, a novel primary tumor-bearing humanized animal model using NOG mice and PBMC from ATLL patients, in which human autologous ADCC can be evaluated, has been established. This model overcomes the limitations of preclinical in vivo investigations of ADCC caused by species incompatibility between humans and mice. In addition, this model completely excludes nonspecific allogeneic effects. This model makes it possible for human autologous ADCC to be evaluated, has been established.

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Disclosures
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References


