Defucosylated Anti–CC Chemokine Receptor 4 Monoclonal Antibody Combined with Immunomodulatory Cytokines: A Novel Immunotherapy for Aggressive/Refractory Mycosis Fungoides and Sézary Syndrome

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Abstract Purpose: Sézary syndrome (SS) and Mycosis fungoides (MF) in the advanced stage have dismal prognoses. Because CC chemokine receptor 4 (CCR4) has an important role in the skin-homing capacity of MF/SS cells, we postulated that anti-CCR4 monoclonal antibody (mAb) could represent a novel therapeutic agent against aggressive/refractory MF/SS.

Experimental Design: The defucosylated next-generation therapeutic mAb KM2760 induces enhanced antibody-dependent cellular cytotoxicity (ADCC). Here, we assessed the therapeutic potential of this antibody against aggressive MF/SS tumor cells in vitro and in animal models in vivo.

Results: KM2760 induced robust ADCC by peripheral blood mononuclear cell (PBMC) from healthy controls against a MF/SS cell line as well as against primary tumor cells from patients with aggressive MF/SS. KM2760 also showed significant antitumor activity in disseminated and nondisseminated MF/SS mouse models. In addition, ~ 30% of autologous MF/SS tumor cells were killed in in vitro assays of KM2760-induced ADCC mediated by patients’ PBMC after only 4 h, despite the low numbers of natural killer cells present in these PBMCs. It is also shown that ADCC induced by defucosylated therapeutic mAb can be greatly augmented by the immunomodulatory cytokines interleukin-12, IFN-α-2b, and IFN-γ.

Conclusions: The present study has encouraged us in the conducting of a phase I clinical trial of a completely defucosylated anti-CCR4 mAb in patients with CCR4-positive T-cell lymphomas, including aggressive MF/SS (ClinicalTrials.gov identifier: NCT00355472). In the near future, the efficacy not only of defucosylated anti-CCR4 mAb single-agent treatment but also of combination therapy with immunomodulatory cytokines will be clinically established to target aggressive/refractory MF/SS.

Mycosis fungoides (MF) is a mature T-cell lymphoma presenting in the skin, with a long natural history, including extracutaneous dissemination, occurring at advanced stages, mainly to lymph nodes (LN), liver, spleen, lung, and the blood.

In the terminal phase, transformation to a large T-cell lymphoma may be seen (1, 2). For early-phase patients with skin disease, treatment is usually limited to local topical therapeutic measures, whereas systemic chemotherapy is most appropriate for patients with extracutaneous disease [stage IV, according to the standard staging classification system for MF, the tumor-node-metastasis-blood system (3, 4), first proposed at the National Cancer Institute–sponsored Workshop on Cutaneous T-Cell Lymphomas (CTCL) in 1978]. However, these regimens generally provide temporary palliative control only. Patients with stage IV disease have a very poor prognosis, with a median survival of 13 months from the date of first treatment (5). Sézary syndrome (SS) is a generalized mature T-cell lymphoma characterized by the presence of erythroderma, lymphadenopathy, and neoplastic T lymphocytes in the blood and is regarded as a variant of MF (1). SS is an aggressive disease with an overall survival rate at 5 years of between 10% and 20% (2). As a terminal event, transformation to a large T-cell lymphoma may occur, as seen in MF. Therefore, we term stage IV MF and SS with large cell transformation “aggressive MF/SS” in the present article. No standard treatment strategies for patients with aggressive MF/SS have been developed thus far.
Defucosylated Anti-CCR4 mAb as a Novel Therapy for CTCL

Antibodies and flow cytometry. KM2760 has been described previously (17). The following mAbs were used for flow cytometry: FITC-conjugated anti-CCR4 mAb (KM2160), anti-CD16 mAb (clone 3G8), and anti-CD69 (FN50); phycoerythrin-conjugated anti-CD25 mAb (M-A251) and anti-CD56 (B159); peridinin chlorophyll protein– conjugated anti-CD4 mAb (SK3); and the appropriate FITC-conjugated, phycoerythrin-conjugated, and peridinin chlorophyll protein– conjugated isotype controls. KM2160 has also been described previously (17), and the other mAbs were purchased from BD PharMingen. Cells were analyzed by a FACS Calibur (Becton Dickinson).

CTCL cell line. Human CTCL cell lines HH, MJ, and HUT78, derived from peripheral blood of patients with MF, SS, and non-MF/SS aggressive CTCL, respectively (27–29), were obtained from the American Type Culture Collection.

Immunohistochemistry. Immunostaining for CCR4 was done on formalin-fixed, paraffin-embedded sections as described previously (15, 30). We immunostained for CCR4 in affected LN of three patients with aggressive MF/SS. All patients enrolled in the present study had suffered from disseminated skin plaque and received topical medications, such as steroid or skin-directed phototherapies, before LN biopsy. The patients gave informed written consent before this sampling procedure as well, provided according to the Declaration of Helsinki. All cases were diagnosed as having LN involvement of transformed large T cells with MF/SS according to the criteria of the WHO classification of malignant lymphoma (1) after precise immunohistochemical evaluation.

Animals. Male C. B-17/Icr-scid icl mice were purchased fromCLEA Japan, Inc. and used at 6 to 9 weeks of age. All of the in vivo experiments were done in conformity with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia (second edition).

CTCL mouse model. For the nondisseminated setting, 1.5 × 107 HH cells suspended in 0.2 mL RPMI 1640 were i.v. inoculated into 20 severe combined immunodeficient (SCID) mice via the tail vein. The tumor-bearing mice were divided into two groups of 10 mice each for KM2760 or control (saline) injections, respectively. KM2760 (20 mg/kg) or control injections into the tail veins of the mice were started 30 min after tumor inoculations and continued twice weekly for 4 weeks.

For the disseminated setting, 1.5 × 107 HH cells suspended in 0.2 mL RPMI 1640 were i.v. inoculated into 10 SCID mice via the tail vein. The tumor-bearing mice were divided into two groups of five mice each for KM2760 or control injections. The concentrations of lactate dehydrogenase in plasma were measured 7 days after tumor inoculations using LDH-J Reagent (Kainos Laboratories, Inc.) according to the manufacturer’s instructions. These were found to be identical in the two groups about to receive KM2760 or controls ([169 ± 48.8 (mean ± SD) and 168 ± 45.4 IU/L, respectively]). KM2760 (20 mg/kg) or control injections into the tail veins of the mice were started 7 days after tumor inoculations and continued twice weekly for 4 weeks.

ADCC assay. Standard 4-h 51Cr release assays were done as described previously (17). All experiments were done in triplicate and the percent cell lysis is presented as the mean value ± SD. The autologous ADCC in the patients with aggressive MF/SS was also done as described previously (17). Mononuclear cells from affected LN, the majority of which were tumor cells, were used as targets. PBMCs from the same patient, taken on the same day as the LN was biopsied and containing significant amounts of tumor cells, were used as the effectors. The autologous ADCC assay was done on the day when paired LN cells and PBMCs were freshly obtained. In some experiments, PBMCs obtained from healthy individuals were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum with or without human cytokines, such as recombinant IL-12 (169 IU/L, respectively), for 7 days to develop a new treatment strategy for these patients. In addition, we also investigated treatment with a combination of KM2760 and immunomodulatory cytokines, such as interleukin (IL)-12, IFN-α-2b, and IFN-γ, to further augment KM2760-induced ADCC.

Materials and Methods

Cells. PBMC or LN mononuclear cells were isolated from three patients with aggressive MF/SS using Ficoll-Paque (Pharmacia). PBMCs were also isolated in the same way from healthy individuals for use as effector cells in ADCC assays. All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study using human samples was approved by the institutional review board of Nagoya University Graduate School of Medical Sciences.

Therefore, alternative treatment strategies for these patients are urgently needed.

The use of therapeutic monoclonal antibody (mAb) for the treatment of cancer has become a promising approach over the last few years, as exemplified by the great success of mAbs such as rituximab (6, 7), trastuzumab (8), bevacizumab (9, 10), and cetuximab (11). Thus, development of candidate mAb for use against aggressive MF/SS should also represent a potentially fruitful exercise. The hallmark surface phenotype of MF/SS tumor cells is CD4+, CC chemokine receptor 4 positive (CCR4+), and cutaneous lymphocyte antigen positive, with both CCR4 and cutaneous lymphocyte antigen having important roles in the skin-homing capacity of the tumor cells (12–15). Thus, we postulated that the CCR4 molecule might represent an appropriate target for antibody-based immunotherapy, and to this end, we have developed a novel chimeric anti-CCR4 mAb, KM2760 (16). The major antitumor activity of KM2760 is mediated by antibody-dependent cellular cytotoxicity (ADCC); it does not exhibit any complement-dependent cytotoxicity or direct antiproliferative effects (17). ADCC is one of the most important mechanisms of action of therapeutic mAb against tumor cells (18–21). However, ADCC depends on the cytotoxic activity of effector cells, such as natural killer (NK) cells and monocytes/macrophages, but these cells are commonly qualitatively suppressed and quantitatively reduced in cancer patients. Indeed, several investigators have reported that the activity of NK cells, representative effector cells of ADCC, was decreased in patients with MF/SS (22–24). Thus, compromised NK cell activity is a critical issue for immunotherapeutic approaches to cancer treatment. To overcome this problem, the Fc region of KM2760 is defucosylated, which results in enhanced ADCC due to increased binding affinity to the FcγR on effector cells (25). We had previously confirmed that KM2760 induced significantly greater ADCC by human peripheral blood mononuclear cells (PBMC) than highly fucosylated, but otherwise identical, mAb (16). We therefore anticipated that the application of defucosylated antibodies would be a promising approach for developing next-generation therapeutic mAb with improved ADCC efficacy (18, 25, 26), even for cancer patients whose effector cells are qualitatively and quantitatively compromised.

Here, we tested this novel therapeutic approach using KM2760, a defucosylated chimeric anti-CCR4 mAb, against aggressive MF/SS tumor cells both in vitro and in animal models in vivo to develop a new treatment strategy for these patients. In addition, we also investigated treatment with a combination of KM2760 and immunomodulatory cytokines, such as interleukin (IL)-12, IFN-α-2b, and IFN-γ, to further augment KM2760-induced ADCC.
PBMCs were then mixed without washing with $^{51}$Cr-labeled target cells ($1 \times 10^6$) in the presence or absence of KM2760 at a final concentration of 10 $\mu$g/mL.

**Analysis of NK cell activation.** PBMCs from healthy individuals were incubated ($5 \times 10^5$ cells/mL) in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum at 37°C, 5% CO$_2$ for 72 h with or without IL-12 (1 ng/mL), IFN-α-2b (200 IU/mL), and IFN-γ (10 IU/mL) in 1.5 mL polypropylene tubes. Double staining with phycoerythrin-conjugated anti-CD56 and FITC-conjugated anti-CD69 mAbs was then done as described previously (31). Briefly, NK cells, which can be gated by their high expression of CD56 and relatively low side scatter compared with monocytes/macrophages, were assessed for their CD69 expression levels by flow cytometry. CD69 expression is reported to be a reliable activation marker for NK cells (32); hence, CD69-expressing NK cells are defined here as activated NK cells.

**Cell proliferation assay.** Proliferation of HH, HUT78, and MJ cells exposed to various concentrations of IL-12, IFN-α-2b, and IFN-γ for 72 h was measured using CellTiter 96 AQuueous One Solution Cell Proliferation Assay kits (Promega Corp.) as described previously (17).

**Statistical analysis.** The difference in the survival between KM2760-treated mice and the controls was analyzed by the Kaplan-Meier method and compared using the log-rank test. The differences in the percentage of NK cells in PBMC between aggressive MF/SS patients and healthy individuals were examined with the Mann-Whitney U–test. The significance of changes in the percent HH cell lysis and the percent activated NK cells in the absence or presence of the cytokine was examined using the Wilcoxon signed rank 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**

**KM2760 induces ADCC by human PBMC against a CTCL cell line.** A human CTCL cell line, HH was confirmed to be positive for CCR4 by flow cytometry (Fig. 1A). KM2760 induced robust ADCC activity against HH in the presence of PBMC obtained from all three healthy individuals tested at a fixed E:T ratio of 50:1 (Fig. 1B).

**KM2760 mediates potent antitumor activity in the CTCL mouse model.** A CTCL mouse model using HH was established in our laboratory. In this model, a few s.c. tumors appear on SCID mice 4 weeks after i.v. inoculation with HH. Subsequently, their number increases and each tumor enlarges rapidly, with the majority of hosts dying of probable cachexia 3 months after i.v. HH inoculation. Visceral involvement cannot be observed by macroscopic inspection in this CTCL mouse model.

In the nondisseminated setting, KM2760 treatment resulted in a significant prolongation of survival ($P = 0.0007$; Fig. 2A). None of the mice in the present study experienced toxicity caused by the KM2760 injections. In the disseminated setting, the KM2760-treated group survived significantly longer than the control group ($P = 0.0340$; Fig. 2B). In addition, none of these mice showed any signs of toxicity caused by KM2760 injections.

**KM2760 induces ADCC by autologous PBMC against freshly isolated aggressive MF/SS tumor cells.** We examined KM2760-induced ADCC in three patients with aggressive MF/SS in the autologous setting at a fixed E:T ratio of 50:1. The biopsy specimens taken from affected LN showed a diffuse infiltration of large atypical lymphoid cells with large atypical nuclei replacing the normal architecture of the LN (Fig. 3A, top).
Fig. 3. KM2760-mediated ADCC against autologous MF/SS tumor cells. A, MF/SS tumor cells obtained from the three patients assayed for ADCC: immunohistochemical images of affected LN from patients with aggressive MF/SS. Top, H&E staining; middle, CCR4 immunostaining; bottom, flow cytometric analyses of affected LN cells. Left, CD4 expression and SSC-H of whole LN cells, with the percentages of CD4+ cells in these LN cells presented below; right, dot plots of CCR4 and CD25 expression by CD4+ cells, with the percentages of CD4+ cells in each quadrant determined by CCR4 and CD25 expression presented below. B, surface phenotypes of the autologous effector PBMC from three patients with aggressive MF/SS patients used for the ADCC assays. Top left, CD4 expression and SSC-H of whole PBMC with the percentages of CD4+ cells presented below; right, dot plots of CCR4 and CD25 expression in the CD4+ cells with the percentages of CD4+ cells in each quadrant determined by CCR4 and CD25 expression presented below; bottom, CD16 expression and SSC-H of whole PBMC. The percentages of CD16+ NK cells in PBMC are presented on the right. C, CD16 expression and percent CD16+ cells in effector PBMC from three healthy controls used for the ADCC assay. D, KM2760-induced ADCC activity mediated by autologous or allogeneic effector cells against affected LN target cells, measured by a standard 4-h ^51Cr release assay at an E:T ratio of 50:1. All experiments were done in triplicate. Points, mean percent cell lysis; bars, SD.
Immunohistochemistry revealed that these MF/SS tumor cells were positive for CCR4 (Fig. 3A, middle). Flow cytometric analysis showed that the affected LN contained 41.5% (case 1), 69.4% (case 2), and 51.3% (case 3) of CD4+ cells, which were mostly CCR4+ tumor cells (86.0%, 60.9%, and 94.4% of these CD4+ cells, respectively; Fig. 3A, bottom).

Different proportions of atypical lymphoid tumor cells were present in the peripheral blood of these three patients (data not shown). Their PBMCs contained 49.8% (case 1), 27.8% (case 2), and 8.1% (case 3) of CD4+ cells (Fig. 3B, top), which were again mostly CCR4+ tumor cells (86.8%, 55.0%, and 70.4% of these CD4+ cell, respectively; Fig. 3B, top). CD16+ NK cells were also present in patient PBMC at 4.3%, 2.0%, and 0.8% in cases 1, 2, and 3, respectively (a mean of 2.4 ± 1.8% SD; Fig. 3B, bottom). The percentages of CD16+ NK cells in PBMC of healthy controls were 12.1%, 22.8%, and 19.6% in donors 1, 2, and 3, respectively (mean, 18.2 ± 5.5% SD; Fig. 3C). This difference in CD16+ NK cell numbers between advanced MF/SS patients and healthy individuals was statistically significant (P = 0.0495; Fig. 3B (bottom) and C). In two patients (cases 1 and 2), ADCC values mediated by autologous PBMC reached a maximum of ~30.0% in both, but in the third patient, very little ADCC was observed. In contrast, maximum ADCC values mediated by allogeneic control cells reached ~100% in all three individuals (Fig. 3D).

Effect of cytokines on cytotoxic activity against HH cells of PBMC in the presence or absence of KM2760. KM2760-induced ADCC against HH was measured in a standard 4-h 51Cr release assay using PBMC from seven healthy individuals as effector cells. The KM2760 concentration was fixed at 10 μg/mL. The E:T ratio was fixed at 5:1 to reflect the low level found in most CTCL patients. The mean percent cell lysis by KM2760-induced ADCC with non–cytokine-primed PBMC was 21.4 ± 12.1% (mean ± SD). The percent cell lysis by KM2760-induced ADCC with IL-12–primed (1 ng/mL), IFN-α-2b–primed (200 IU/mL), and IFN-γ–primed (10 IU/mL) PBMC from these seven healthy donors was 14.8%, 15.7%, and 15.7%, respectively (Fig. 4A). This difference in ADCC with or without cytokines was significant for IL-12 (P = 0.0180), IFN-α-2b (P = 0.0180), and IFN-γ (P = 0.0180; Fig. 4A).

The mean percent target cell lysis by non–cytokine-primed PBMC from these controls at a fixed E:T ratio of 5:1 was only 4.1 ± 3.2%. Lysis by IL-12–primed (1 ng/mL), IFN-α-2b–primed (200 IU/mL), and IFN-γ–primed (10 IU/mL) PBMC was 11.2 ± 6.7%, 8.3 ± 6.0%, and 5.5 ± 4.7%, respectively. The change in lysis with or without IL-12 was significant (P = 0.0180), as it was for IFN-α-2b (P = 0.0425), whereas IFN-γ did not facilitate lysis in the absence of antibody (P = 0.4990; Fig. 4B).

Effect of cytokines on NK cell activation. The percentage of activated NK cells found in PBMC from the seven healthy controls without cytokine stimulation was 6.7 ± 1.8%, which increased to 35.5 ± 20.7%, 34.2 ± 14.8%, and 15.7 ± 8.6% in the presence of IL-12 (1 ng/mL), IFN-α-2b (200 IU/mL), and IFN-γ (10 IU/mL), respectively. These increases were significant for IL-12 (P = 0.0180), IFN-α-2b (P = 0.0180), and IFN-γ (P = 0.0280; Fig. 5A). Flow cytometric analysis of NK cell activation in PBMC from one of these healthy individuals (PBMC 1) is shown in Fig. 5B.

Effect of cytokines on proliferation of CTCL cell lines. The percent viable cells of HH cells exposed to 1 and 10 ng/mL of IL-12, 200 and 2,000 IU/mL of IFN-α-2b, and 10 and 100 IU/mL of IFN-γ for 72 h compared with control HH (medium alone, 100.0 ± 4.0%) were 61.0 ± 2.0%, 50.6 ± 4.8%, 80.0 ± 5.1%, 74.8 ± 2.0%, 79.0 ± 5.1%, and 75.8 ± 7.3% (mean ± SD), respectively. For HUT78 cells, these values were 100.0 ± 2.2% (control) and 99.7 ± 1.9%, 103.6 ± 2.3%, 83.7 ± 0.9%, 71.7 ± 1.9%, 92.9 ± 2.6%, and 84.5 ± 2.2%, respectively, and finally for MJ cells, they were 100.0 ± 1.6% (control) and 103.6 ± 1.1%, 102.6 ± 8.2%, 81.3 ± 0.3%, 72.0 ± 3.0%, 96.8 ± 6.2%, and 95.6 ± 6.6%, respectively.

Discussion

Here, we showed that KM2760 induced robust ADCC by healthy donor PBMC not only against an established CTCL cell line (HH) but also against primary tumor cells obtained from patients with aggressive MF/SS. Furthermore, the present study also showed that this defucosylated mAb mediated marked antitumor activity in CTCL tumor-bearing SCID mice not only
in the nondisseminated but also in the disseminated setting. We have previously reported that KM2760 exerts its in vivo antitumor activity mainly via monocytes/macrophages as effector cells in SCID mice (33). Although the mouse effector system mediating the action of such therapeutic mAb may therefore differ from that of human (16, 33), the present findings indicate that the defucosylated anti-CCR4 mAb is likely to represent a promising novel therapeutic agent for patients with aggressive MF/SS.

The ultimate goal of immunotherapy is to achieve sufficient antitumor activity simply by administering mAb to humans in vivo. Thus, we next evaluated KM2760-induced ADCC in three aggressive MF/SS patients in an autologous setting. It was found that ~30% of fresh MF/SS tumor cells could be lysed by autologous PBMC in the presence of KM2760 after only 4 h in two of the three patients, despite the fact that the number of NK cells in the PBMC of these patients was significantly lower than in healthy controls and despite significant amounts of MF/SS tumor cells in the autologous PBMC. We believe that these findings have promise for the development of novel immunotherapies for patients with aggressive MF/SS. However, only low-level KM2760-induced ADCC was observed in one of the three patients; this patient had very few effector NK cells (0.8%) in the PBMC. This finding prompted us to seek ways in which to augment NK cell activity, aiming to further enhance KM2760-induced ADCC using immunomodulatory agents because of the importance of NK cells as ADCC effectors in humans (34). We focused on IL-12, IFN-α, and IFN-γ because of their current use in the clinical arena, as shown in trials of rituximab together with IL-12 (35) or IFN-α (36, 37), which suggested that these combination immunotherapies were clinically active. IFN-γ is already approved by the Ministry of Health, Labor, and Welfare, Japan for treatment of CTCL and cutaneous-type adult T-cell leukemia/lymphoma as an immunomodulatory agent. As expected, IL-12 and IFN-α-2b significantly augmented KM2760-induced ADCC against HH at the low E:T ratio of 5:1. The percent HH cell lysis by effector PBMC in the absence of KM2760, which is almost entirely due to NK cell-mediated lysis (34), was also significantly augmented by IL-12 and IFN-α-2b at this low E:T ratio. These observations are consistent with the fact that both IL-12 and IFN-α-2b significantly increased the percentage of activated NK cells in the PBMC. Although IFN-γ also significantly augmented KM2760-induced ADCC against HH, enhancement was less than with IL-12 or IFN-α-2b. IFN-γ also elevated the mean percent HH cell lysis by effector PBMC in the absence of KM2760, but this did not achieve statistical significance. This observation was also consistent with the fact that the extent of the increase of activated NK cells mediated by IFN-γ was smaller than that mediated by IL-12 or IFN-α-2b. Importantly, the significant enhancement of KM2760-induced ADCC was accomplished by IL-12, IFN-α-2b, or IFN-γ within their clinically achievable dose ranges (product information from Schering Corp. and Otsuka Pharmaceutical Co. Ltd.; ref. 38). In addition, these cytokines did not stimulate the proliferation of CTCL cell lines; rather, at least for IFN-α-2b, the percent viable cells of all CTCL cell lines tested were reduced. In contrast, IL-2 (1 ng/ml) and IL-15 (1 ng/ml), while also significantly augmenting KM2760-induced ADCC against HH, both increased proliferation of HH cells in a dose-dependent manner (data not shown). These observations suggest that great caution would need to be exercised were these two cytokines to be applied for treating aggressive MF/SS clinically. Collectively, these findings indicate that combination treatment with KM2760 and immunomodulatory agents, such as IL-12, IFN-α-2b, and IFN-γ, should overcome the highly immunocompromised situation, which is common in cancer patients, and should be a promising treatment strategy for patients with aggressive MF/SS.

Recently, it has become generally accepted that CD4+CD25+ regulatory T cells also express CCR4 (18, 39–43); we have found that KM2760 can deplete these CCR4+ regulatory T cells (17, 43). Other investigators recently reported that regulatory T cells directly inhibited not only other T cells but also NK cell effector function (44–46). Taking these important observations together, it is proposed that anti-CCR4 mAb would enhance NK cell mediation of ADCC by killing CCR4+ regulatory T cells, which would otherwise inhibit NK cell effector function, in humans in vivo.

In conclusion, the present study documents that the defucosylated chimeric anti-CCR4 mAb KM2760 mediates promising anti-MF/SS activity both in vitro and in vivo and provides a rationale for the eventual development of improved treatment strategies for patients with aggressive MF/SS.

Fig. 5. Effect of cytokines on NK cell activation. A, a comparison of the percent activated NK cells from seven healthy individuals incubated with or without IL-12 (left), IFN-α-2b (middle), or IFN-γ (right). NK cell activation was analyzed by flow cytometry, and CD69- and CD25-expressing NK cells were defined as activated NK cells. P value is indicated in each panel. Points, mean; bars, SD. B, an example of flow cytometric analysis of PBMC obtained from a healthy individual. PBMC 1. CD56-expressing NK cells were stained with anti-CD69 mAb (filled histograms) or isotype mAb (blank histograms).
Furthermore, the present study is the first to report that ADCC induced by next-generation defucosylated therapeutic mAbs, which mediate the strongest ADCC due to improved binding affinity to the FcγR on effector cells, can be greatly augmented by the immunomodulatory agents IL-12, IFN-α-2b, and IFN-γ, at least in vitro. These findings have encouraged us in the conducting of a phase I clinical trial of the completely defucosylated anti-CCR4 mAb in patients with CCR4-positive T-cell lymphomas, including aggressive MF/SS (ClinicalTrials.gov identifier: NCT00355472), to develop a novel treatment strategy for patients with aggressive/refractory T-cell lymphoma. In the near future, the efficacy not only of defucosylated anti-CCR4 mAb as a single agent treatment but also of combinations of this mAb with immunomodulatory agents, such as IL-12, IFN-α-2b, and IFN-γ, for aggressive/refractory MF/SS will be established in clinical trials in humans.

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References

1. Raffelaer E, Jaffé ES. Mycosis fungoides/Sézary syndrome. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. Pathology and genetics tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001. p. 216–20.
2. Kim YH, Hoppe RT. Mycosis fungoides and the Sézary syndrome. Semin Oncol 1999;26:276–89.
3. Bunn PA, Jr., Lamberg SI. Report of the Committee on Staging and Classification of Cutaneous T-Cell Lymphomas. Cancer Treat Rep 1979;63:729–83.
4. Kashiwagi S, Saito M, McMillan A, Zackheim HS. A modified staging classification for cutaneous T-cell lymphoma. J Am Acad Dermatol 2001;44:105–6.
5. de Coninck EC, Kim YH, Varghese A, Hoppe RT. Clinical characteristics and outcome of patients with extracutaneous mycosis fungoides. J Clin Oncol 2001;19:779–84.
6. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235–42.
7. Pfundenschuh M, Trumper L, Oosterbrug A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with diffuse large-B-cell lymphoma: a randomized controlled trial by the MA.\textsc{thera} International Trial (\textsc{miht}). Group. Lancet Oncol 2006;7:379–91.
8. Simon DJ, Leyland-Jones B, Shax S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–92.
9. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus interferon-α for metastatic colorectal cancer. N Engl J Med 2004;350:2339–44.
10. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 2006;355:2452–50.
11. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2004;350:567–78.
12. Girardi M, Heald PW, Wilson LD. The pathogenesis of mycosis fungoides. N Engl J Med 2004;350:1978–85.
13. Fesenczi K, Fuhlbirger RC, Pinkus LS, Pinkus GS, Kupper TS. Increased CCR4 expression in cutaneous T cell lymphoma. J Invest Dermatol 2002;119:1405–10.
14. Kim EJ, Hess S, Richardton SK, et al. Immunophenotyping and therapy of cutaneous Tcell lymphoma. J Clin Invest 2005;115:798–812.
15. Ishida T, Utsunomiya A, Iida S, et al. Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma. Arch Dermatol 2004;140:197–201. A close association with skin involvement and unfavorable outcome. Clin Cancer Res 2003;9:3625–34.
16. Niwa R, Shoji-Hosaka E, Sakurada M, et al. Defucosylated chimeric anti-CCR4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T cell leukemia and lymphoma. Cancer Res 2004;64:2127–33.
17. Ishida T, Iida S, Akutsuka Y, et al. The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-cell leukemia/lymphoma. Cancer Res 2004;64:7529–39.
18. Ishida T, Ueda R. CCR4 as a novel molecular target for immunotherapy of cancer. Sci Cancer Sci 2006;97:139–46.
19. Voso MT, Pantel G, Rutella S, et al. Rituximab reduces the number of peripheral blood B cells in vitro mainly by effecter cell-mediated mechanisms. Hae-matologica 2002;87:918–25.
20. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytokinesis against tumor targets. Nat Med 2001;7:443–46.
21. Carton G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polyclonality in IgF receptor-FcRnia gene. Blood 2002;99:754–8.
22. Laroche L, Kaiserlian D. Decreased natural killer cell activity in cutaneous T-cell lymphomas. N Engl J Med 1983;330:101–2.
23. Jensen JR, Kaltoft K, Bisslade S, Thstrup-Pedersen K. Natural and concanavalin A-induced cytotoxic activity towards continuously growing B lymphocytes derived from patients with cutaneous T-cell lymphoma. Arch Dermatol Res 1986;279:12–5.
24. Wood NL, Kittes EN, Lockblad WK. Depressed lymphomactivated killer cell activity in mycosis fungo- pois. A possible marker for aggressive disease. Arch Dermatol 1990;126:907–13.
25. Satoh M, Iida S, Shihara K. Non-fucosylated therapeutic antibodies as next-generation therapeutics. Expert Opin Biol Ther 2006;6:1611–73.
26. Carter PJ. Potent antibody therapeutics by design. Nat Rev Immunol 2006;6:343–57.
27. Starkebaum G, Loughran TP, Waters CA, Ruscetti FW. Establishment of an IL-2 independent human T-cell line possessing only the p70 IL-2 receptor. Int J Cancer 1991;42:46–53.
28. Popovic M, Saein PS, Robert-Gurroff M, et al. Isolation and transmission of human retrovirus (human T-cell leukemia virus). Science 1983;219:856–9.
29. Goobstenberg JE, Ruscetti FW, Mier JW, Gazdar A, Gallo RC. Human CTCL, and leukemia cell lines pro-duce and respond to T cell growth factor. J Exp Med 1981;154:1403–18.
30. Ishida T, Inagaki H, Ishii T, et al. The CCR4 as a novel molecular target for immunotherapy in adult T-cell leukemia/lymphoma. Leukemia 2002;6:2162–8.
31. North J, Bakhsh I, Marden C, et al. Tumor-primed human natural killer cells lyse NK-resistant tumor targets-regulatory T cells. J Exp Med 2001;194:847–53.
32. Davis TA, Maloney DG, Grillo-Lopez AJ, et al. The pathogenesis of TCL 4 expressing a transforming growth factor- \textalpha \textbeta \textdelta \textgamma \textepsilon \textzeta \textlambda \mu \nu \xi \omicron \pi \sigma \tau \upsilon \omega \alpha \beta \gamma \delta \epsilon \zeta \lambda \mu \nu \xi \omicron \pi \sigma \tau \upsilon \omega