CD26 is a potential therapeutic target by humanized monoclonal antibody for the treatment of multiple myeloma

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Abstract
CD26, a 110-kDa transmembrane glycoprotein that is expressed on several tumor cells including malignant lymphoma, has been implicated in tumorigenesis; however, little is known regarding its role in multiple myeloma (MM). Recently, we identified CD26 expression on human osteoclasts (OCs) and demonstrated that humanized IgG1 monoclonal antibody targeting CD26, huCD26mAb, inhibits human OC differentiation. Herein, we show that CD26 expression was present on plasma cells in the bone marrow tissues of MM patients. In vitro immunostaining studies revealed that although CD26 expression was low or absent on MM cell lines cultured alone, it was intensely and uniformly expressed on MM cell lines co-cultured with OCs. The augmented CD26 expression in MM cells was exploited to enhance anti-MM efficacy of huCD26mAb via a substantial increase in antibody-dependent cytotoxicity (ADCC) but not complement-dependent cytotoxicity (CDC). Moreover, huCD26mAb in combination with novel agents synergistically enhanced huCD26mAb induced ADCC activity against CD26⁺ MM cells compared with each agent alone. huCD26mAb additionally reduced the ratio of the side population (SP) fraction in CD26⁺ MM cells by ADCC. Finally, huCD26mAb significantly reduced the MM tumor burden and OC formation in vivo. These results suggest that CD26 is a potential target molecule in MM and that huCD26mAb could act as a therapeutic agent.

Introduction
Despite remarkable advances in the current treatment options, including proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) as well as high-dose chemotherapy followed by autologous stem cell transplantation, which have significantly improved the overall survival (OS) of multiple myeloma (MM) patients, most of them relapse or ultimately become refractory due to the residual disease within the MM microenvironment¹,². Therefore, the development of alternative therapeutic approaches, based on the understanding of the biology of the disease, is urgently required. Recently, a new generation of novel agents including PIs (carfilzomib and ixazomib)³–⁵, IMiDs (pomalidomide)⁶,⁷, and histone deacetylase inhibitors (HDACi: panobinostat)⁸ have emerged and are expected to further improve the clinical outcome of MM patients.

The use of immunotherapy in the treatment of cancers has been accelerating and increasing evidence has shown that antibody therapies can improve the outcome of patients with cancer⁹,¹⁰. Rituximab, a chimeric murine/human anti-CD20 monoclonal IgG1κ antibody targeting B cells, is currently indicated for the treatment of B-cell non-Hodgkin’s lymphoma (NHL) and chronic lymphocytic leukemia (CLL) and exerts significant activity, especially in combination with cytotoxic chemotherapy⁹. In contrast, clinical trials of rituximab therapy in MM have been disappointing, showing that few patients with MM achieve only minimal responses¹⁰ because only a small number of patients express CD20 in plasma cells¹¹,¹². Immunotherapeutic approaches for MM have been long awaited because of the significantly impaired
immune system due to the inhibition of normal plasma cells and the multiple mechanisms of immune evasion by MM cells, including the lack of unique targets that are highly expressed in MM cells but not normal cells, the enhanced expression of inhibitory ligands, such as programmed cell death ligand 1 (PD-L1), and the recruitment of regulatory T cells (Tregs). Recently, novel efficacious mAbs have been developed based on the identification of target antigens, such as elotuzumab, a humanized IgG1 monoclonal antibody targeting signaling lymphocyte activation molecule family member 7 (SLAMF7, CS1) and daratumumab, a humanized IgG1κ monoclonal antibody directed against CD38. These novel mAbs are effective for the treatment of MM patients who have received >3 prior lines of therapy or who were double refractory to a PI and an IMiD. These mAbs have become increasingly used in combination with bortezomib (BTZ)/dexamethasone (Dexa) or lenalidomide (Lena)/Dexa. These combinations have been shown to significantly improve overall response rates (ORR) and progression-free survival (PFS) in patients with MM compared with these agents alone.

CD26, a 110-kDa transmembrane glycoprotein with DPPIV activity, is widely expressed in a various normal cells, including T lymphocytes, natural killer (NK) cells, endothelial cells, and epithelial cells. Additionally, CD26 is expressed in several tumor cells and is involved in T-cell activation and tumorigenesis. However, its role in plasma cell malignancies has not been characterized yet. We recently identified that CD26 is intensely expressed in human osteoclasts (OCs) in osteolytic bone tumors, including MM, and that huCD26mAb, a humanized IgG1 monoclonal antibody that directly targets CD26, inhibits human OC differentiation. In addition, we detected that CD26 is expressed on MM cells in the bone marrow (BM) tissues of MM patient. In the present study, we showed that CD26 was intensely and uniformly expressed in MM cell lines co-cultured with OCs, while its expression was low or absent in those cultured alone in vitro. We further clarify CD26 as a potential target for the treatment of MM. We herein examine the therapeutic impact of novel huCD26mAb on MM cell growth, cell death via antibody-dependent cellular cytotoxicity (ADCC) and its associated osteolytic bone disease in vitro and in vivo and validate that huCD26mAb could be a promising immunotherapeutic option for MM.

Materials and methods

Cell lines

The cancer cell lines, U266, KMM1, KMS11, KMS12, KMS18, KMS20, KMS21, KMS34, and IM9, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), KMS26, KMS27, KMS28, and RPMI8226 were obtained from the National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN, Osaka, Japan). Nakadai was kindly gifted by Dr. Hattori’s laboratory. These cell lines were maintained in RPMI-1640 medium (Invitrogen, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Reagents and cells

Human bone marrow mononuclear cells (BM-MNCs) derived from MM patients and bone marrow stromal cells (BMSCs) were purchased from Lonza (Walkersville, MD, USA). Human NK cells were obtained from Biotherapy Institute of Japan (Tokyo, Japan). Recombinant human macrophage colony stimulating factor (M-CSF), soluble receptor activator of nuclear factor κ B ligand (sRANKL), Interleukin-6 (IL-6), stromal cell-derived factor (SDF)-1, tumor necrosis factor alpha (TNFα), B-cell activating factor of TNF family (BAFF), and a proliferation-inducing ligand (APRIL) were obtained from Peprotec (Rockyhill, NJ, USA). Levlimid® (lenalidomide: Celgene, Summit, NJ, USA), Velcade® (bortezomib: Millennium, Cambridge, MA, USA), and Decadron® (dexamethasone: Sigma Aldrich, St. Louis, MO, USA) were used as therapeutic agents. huCD26mAb, humanized IgG1, was generously provided by Y’s Therapeutics (Fig. 1a) (Tokyo, Japan). The huCD26mAb employed in the present study was generated by utilizing the complementarity-determining regions of the murine anti-human CD26mAb, 14D10, previously developed in our laboratory, which has no cross-reactivity to murine CD26. Isotype IgG1 (Sigma Aldrich) was used as a control.

Co-culture of MM cells and OCs

Human BM-MNCs (5 × 10⁵ cells per well) were cultured with human M-CSF (25 ng/ml; from day 0) plus sRANKL (50 ng/ml; from day 3) for 5 to 7 days in α-minimum essential medium (α-MEM) (Life Technologies) supplemented with 10% FBS, penicillin and streptomycin in type 1 collagen-coated 24-well plates (OC culture), described previously. After washing the OCs with phosphate-buffered saline (PBS) three times to detach and remove any non-adherent cells, a panel of MM cells was co-cultured with OCs in 24-well plates (1 ml per well) in triplicate for 72 h or cultured alone in the presence or absence of the indicated concentrations of huCD26mAb. In addition, MM cells were placed in 0.45 μm pore size transwell inserts (Corning, Corning, NY, USA) in wells containing OCs (co-culture without direct contact). At the end of each experiment, the MM cells were collected, counted with trypan blue staining and subjected to each assay. Details of the bone resorption...
assay are described in the Supplementary Materials and Methods.

**Immunohistochemistry and enzyme-histochemistry**

Bone marrow biopsy samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized and hydrated. Further details are provided in the Supplementary Materials and Methods.

**Antibody-dependent cellular cytotoxicity (ADCC) assay**

The effect of huCD26mAb to induce human NK effector cell-dependent lysis of MM cells was evaluated by calcein-AM release assay. Further details are provided in the Supplementary Materials and Methods.

**In vivo anti-MM therapy with huCD26mAb**

To assess the effect of huCD26mAb against tumorigenicity in MM in vivo, 2 xenograft models were
established either by subcutaneous inoculation of MM cells (NOD/SCID MM model) or direct intra-bone injection of MM cells into human bone grafts, subcutaneously implanted in 6-week female NOD/SCID mice, which were housed in our animal research facility (NOD/SCID-hu model)\(^{35-37}\). Further details are provided in the Supplementary Materials and Methods.

**Statistical analysis**

All statistical analyses were performed using Student’s \(t\)-test for two group comparisons and \(p\)-values \(<0.05\) were considered statistically significant. The data are presented as the mean values with 95% confidence intervals, and the results are representative of three independent experiments.

**Study approval**

Experimental procedures and study protocols were approved by the Ethics Committee of Keio University School of Medicine (permission ID number 2013-0034). Informed consent was obtained from all patients. All studies of human subjects were performed according to the principles outlined in the Declaration of Helsinki.

**Results**

**huCD26mAb inhibits OC differentiation derived from bone marrow mononuclear cells (BM-MNCs) from MM patients**

Following the 7-day culture of M-CSF/sRANKL-stimulated BMNCs derived from MM patients in type 1 collagen-coated 24-well plates (OC culture), a large number of multinuclear OCs formed that were positive for tartrate-resistant acid phosphatase (TRAP) and CD26 (Figure S1a). huCD26mAb induced a significant reduction in TRAP\(^+\) multinuclear mature OC numbers (>3 nuclei) in a dose-dependent manner (Figure S1b, c). Subsequently, it reduced bone resorptive activity in OCs derived from MM patients in a dose-dependent manner (Figure S1d).

**CD26 is intensely expressed in MM cells, co-cultured with OCs**

BM tissues of MM patient revealed that the CD26 \(+/CD138+\) plasma cells stained with CD138 with a membranous as well as a cytoplasmic expression pattern and CD26 with a cytoplasmic expression pattern (Fig. 1b). It has been reported that cellular components within the BM microenvironment, including OCs or BMSCs, enhance MM cell proliferation and survival by cell-to-cell contact between MM cells and the BM microenvironment. To further elucidate the role of CD26 in MM cells and the consequences of the interaction between MM cells and OCs, we established a MM cell-OC co-culture system. Initially, we examined CD26 expression in 14 MM cell lines (U266, KMM1, Nakadai, KMS11, KMS12, KMS18, RPMI8226, KMS20, KMS21, KMS26, KMS27, KMS28, KMS34, and IM9). Flow cytometry analysis showed that CD26 was barely or not detected in MM cell lines cultured alone (mono-culture) (Fig. 2a), but its expression increased in cell lines co-cultured with OCs (Fig. 2b). Immunostaining revealed that OCs express high level of CD26 compared with tested MM cell lines (U266, KMS18, KMS26, KMS27, and KMS28) cultured alone, which had low or slightly detectable level of CD26 (Fig. 2c). After co-culture, CD26 expression remained high in OCs and was upregulated in MM cell lines (Fig. 2c). Moreover, when subsequently cultured alone after removal of co-culture with OCs, CD26 expression in MM cell lines was downregulated again (Fig. 2c). In addition, CD26 protein expression was observed in MM cell lines co-cultured with OCs, while it was low or absent in those cultured alone according to immunoblot analysis (Fig. 2d). Further, to determine whether CD26 may be a potential biomarker for MM, we used an enzyme-linked immunosorbent assay (ELISA) to investigate whether there were detectable levels of CD26/DPPIV in the supernatant of MM cells or OCs grown in mono-culture and in that of the co-culture of MM cells with OCs. Consistently, the co-culture of MM cells with OCs significantly increased CD26/DPPIV secretion compared with the mono-culture of MM cells (Fig. 2e). The co-culture additionally increased CD26/DPPIV production 1.8- to 2.5-fold compared with OC culture (Fig. 2e). These results demonstrate that CD26 is overexpressed in both MM cells and OCs in their co-culture system. We also defined target genes in MM cell lines by real-time quantitative RT-PCR analysis. CD26 mRNA expression in MM cell lines co-cultured with OCs revealed 7- to 19.4-fold increase compared with those co-cultured alone (Fig. 2f).

Next, to test whether direct contact between MM cells and OCs is required for CD26 upregulation in MM cells, the co-culture system with or without direct contact was used. Immunohistochemistry showed that CD26 expression in MM cell lines (U266, KMS18, KMS26, KMS27, KMS28, and KMS34) co-cultured with OCs without direct contact was also increased as well as those in the co-culture with direct contact (Figure S2). Further, to elucidate the factors involved in the regulation of CD26 expression in MM cells, MM cell lines (KMS18, KMS26, and KMS28) were cultured under stimulation with anti-apoptotic cytokines, including IL-6, SDF-1, TNF\(\alpha\), APRIL, and BAFF, produced by the OCs or BMSCs in MM. and these cytokines enhanced CD26 expression in the MM cells (Figure S3). These results support that soluble factors such as IL-6 and TNF family cytokines may be certain factors responsible for CD26 upregulation in MM cells.
Fig. 2 (See legend on next page.)
Humanized anti-CD26 monoclonal antibody (huCD26mAb) inhibited the survival of CD26+ MM cells at higher concentrations

First, to clarify the role of CD26 in MM cell survival, we examined the impact of inhibition by huCD26mAb on MM cell growth. OCs derived from human BM-MNCs were co-cultured with the MM cells in 24-well plates for 72 h. Next, the cells were incubated in the presence of the indicated concentrations of huCD26mAb (0.1, 1.0, 10, 50, or 100 μg/ml) or isotype control IgG1 for 48 h, and viable MM cells were evaluated. huCD26mAb had little or no direct effect on CD26− MM cell lines cultured alone, but it directly and dose-dependently inhibited the growth of CD26+ MM cells co-cultured with OCs primarily at higher concentrations >10 μg/ml (Fig. 3a).

huCD26mAb revealed significant anti-MM efficacy by ADCC against CD26+ MM cells but not CD26− MM cells

Next, we conducted a calcein-AM release assay to examine the ability of huCD26mAb to lyse MM cells via ADCC. huCD26mAb-triggered ADCC against tested CD26+ MM cell lines (U266, KMS18, KMS26, and KMS28) co-cultured with OCs in the presence of NK effector cells from three different donors in an effector−target (E/T) ratio (0.2, 1.0, 5.0, 25, or 50)-dependent manner (Fig. 3b). At an E/T ratio of 20:1, huCD26mAb induced the lysis of CD26+ MM cell lines co-cultured with OCs, with lytic activity initiating at a huCD26mAb concentration of 0.0001 μg/ml and maximum lysis at 10 μg/ml by ADCC (Fig. 3c, S3). In contrast, huCD26mAb did not exhibit dose-dependent ADCC against CD26− MM cell lines cultured alone (Figure S4). These results demonstrated that the immune mechanisms of action of huCD26mAb though ADCC activity against CD26+ MM cells.

Treatment with huCD26mAb in conjunction with conventional or novel agents synergistically augmented ADCC against CD26+ MM cells, compared with huCD26mAb alone

We further explored whether pretreatment with conventional therapy (dexamethasone: Daexa) or novel agents (bortezomib: BTZ, lenalidomide: Lena) facilitates huCD26mAb-induced ADCC against CD26+ MM cells. CD26+ MM cell lines (KMS18, KMS26, and KMS28) were co-cultured with OCs and pretreated with Dexa (25 nM), BTZ (3 nM), and Lena (0.5 μM) for 24 h. Pretreatment with Dexa or BTZ significantly augmented subsequent MM cell lysis by NK effector cell-dependent ADCC by huCD26mAb (10 μg/ml) at the E/T ratio of 20 (Fig. 4a). Additionally, CD26+ MM.1R cell lysis, following pretreatment with Dexa, BTZ, and Lena (0.05, 0.5 μM) was enhanced by huCD26mAb-induced ADCC (Fig. 4b). Lenalidomide is an immunomodulatory drug that was previously shown to increase NK cell activity. Pretreatment of NK effector cells with Lena further augmented subsequent huCD26mAb-induced lysis of CD26+ MM.1R cells in a dose-dependent manner (Fig. 4c). These results suggest that huCD26mAb induces significant ADCC against MM cells, regardless of sensitivity or resistance to conventional or novel agents, and that the addition of novel agents to huCD26mAb increased sensitivity to huCD26mAb-induced ADCC against CD26+ MM cells.

huCD26mAb did not exhibit dose-dependent complement-dependent cytotoxicity (CDC) lysis against CD26+ MM cells

We evaluated the effect of CDC by huCD26mAb against CD26+ MM cells. In the presence of 50% human serum as a source of complement, human anti-HLA-DR induced
significant cell lysis, while huCD26mAb demonstrated a low or no potential to confer CDC against CD26+ MM cell lines (Fig. 5). Antibody-independent cytotoxicity (in the presence of serum without antibodies) was not observed. huCD26mAb treatment did not alter complement-regulatory protein expression, including CD55 or CD59, on these tested MM cell lines (data not shown).

**huCD26mAb blocked CD26+ MM cell adhesion to BMSCs**

To determine whether huCD26mAb exerts effects on CD26-mediated MM cell adhesion to BMSCs, we examined the impact of huCD26mAb on MM cell adhesion to BMSCs. Initially, MM cell lines (KMS18, 26, and 28) were co-cultured with BMSCs for 48 h. Subsequently, immunofluorescence staining showed increased CD26 expression in these cell lines (Fig. 6a). Next, calcein-AM labeled
Fig. 4 (See legend on next page.)
MM cell lines (KMS18, KMS26, and KMS28) were added to BMSC-coated plates in the presence of the indicated concentrations of huCD26mAb (0.1, 1.0, 10 μg/ml) or iso IgG1. The data represent the mean ± SE of triplicate wells (*p < 0.05). B CD26+ MM.1R co-cultured with OCs was pretreated with Dexa (25 nM), BTZ (3 nM), or Lena (0.5 μM). Next, huCD26mAb-triggered ADCC lysis against CD26+ MM.1R in the presence of NK effector cells was assayed by calcein-AM release assay. The data represent the mean ± SE of triplicate wells (*p < 0.05, **p < 0.01). C NK effector cells were pretreated with Lena (0.5 μM) for 24 h followed by huCD26mAb-induced ADCC against CD26+ MM.1R cells cultured with OCs. The data represent the mean ± SE of triplicate wells (*p < 0.05, **p < 0.01).

huCD26mAb inhibits both CD26+ MM cell growth and MM-related osteolytic bone disease in vivo

We further examined whether huCD26mAb can suppress MM tumor burden and its related bone disease in vivo. We used two xenograft models: a subcutaneous tumor model of MM and an intra-bone tumor model of MM. Initially, CD26+ KMS18 cells prepared by coculture with OCs in vitro were subcutaneously inoculated into the 6-week-old female NOD/SCID mice (Fig. 7a). Within 10 days of implantation, tumors became visible at
the injection site. The subcutaneous tumors decreased in both weight and size in the huCD26mAb-treated mice compared with those of the control mice (mean weight: 1.1 ± 0.5 versus 5.7 ± 0.6 g, p < 0.05, on day 29) (Fig. 7b, c).

Next, human bone grafts were subcutaneously implanted in the NOD/SCID mice, and KMS18 cells cultured alone were directly injected into the human bone grafts (NOD/SCID-hu mice) (Fig. 7d). CD26 expression in the KMS18 cells was increased through the interaction between the KMS18 cells and the BM microenvironment, including BM accessory cells such as OCs, osteogenic cells, endothelial cells, adipocytes, fibroblasts, and BMSCs.
Human Bone Implanted s.c. 5x10^6 KMS18 cells Injected into bone 4 weeks NOD/SCID mice huCD26mAb or PBS i.p. 3 times per week

Injected s.c. 5x10^6 CD26+KMS18 cells huCD26mAb or PBS i.p. 3 times per week

Control

huCD26mAb

Fig. 7 (See legend on next page.)
we analyzed the expression of the SP and main for the ATP-binding cassette (ABC) transporter, in conjunction with Lena affect SP fractions in CD26 as MP fractions (Fig. 8a). To determine whether mono-therapy with huCD26mAb, Lena, or huCD26mAb in conjunction with Lena affect SP fractions in CD26 + MM cells, we further investigated the impact of these reagents on the ratio of SP fractions in these cells by ADCC. RPMI8226 and KMS11, co-cultured with OCs were incubated with NK effector cells at the E/T ratio of 10 in the presence of Lena (5 μM), huCD26mAb (10 μg/ml), or both in combination for 24 h. Subsequently, SP analysis was performed by flow cytometry. Indeed, although Lena alone did not reduce the ratio of the SP fraction in CD26 + RPMI8226 or KMS11, huCD26mAb substantially reduced its ratio in both of MM cell lines. Moreover, the SP ratio was further decreased by combination therapy with huCD26mAb plus Lena (Fig. 8b, c). These results suggested that the intense CD26 expression on the SP fractions of CD26 + MM cells could be a therapeutic target by huCD26mAb.

**Discussion**

MM has a unique property to develop and expand within the BM and causes osteolytic bone destruction. OCs also support MM cell growth and protect MM cells from drug-induced apoptosis by creating a MM-specific BM microenvironment. Our recent work has revealed that CD26 is intensely expressed on activated OCs in MM, and huCD26mAb inhibits human OC differentiation. Furthermore, immunostaining on bone marrow biopsy specimens showed that CD26 is expressed on plasma cells around OCs in MM patient. Accordingly, the aims of this study were to examine in detail the expression profile of CD26 in MM cells through the interaction with the BM microenvironment and elucidate the anti-MM efficacy of huCD26mAb as a therapeutic antibody for MM. The current study is the first report to show CD26 as a potential novel antibody-based therapeutic target in MM.

Initially, the interaction of MM cells with OCs or BMSCs identified that CD26 is highly expressed and prevalent in both co-cultured MM cells and OCs as demonstrated by immunohistochemistry. Additionally, a high level of CD26/DPPIV expression was detected in the supernatants obtained from both co-culture of MM cells with OCs and OC culture compared with those obtained from the MM cell mono-culture. These results suggest
Fig. 8 (See legend on next page.)
the significance of CD26 in MM cells through the inter-
action with BM microenvironment. Importantly, ex vivo
experiments demonstrated that ADCC is the leading
mode of action of huCD26mAb in MM. huCD26mAb exerted potent anti-MM efficacy via immune effector
mechanism by ADCC in addition to direct on-tumor
effects, which resulted in potent cytotoxicity against CD26
+ MM cells. Moreover, the ADCC effect by huCD26mAb
was synergistically facilitated in conjunction with first-
generation novel agents, such as Lena and BTZ. NK
effector cells involved in ADCC were known to be clearly
inhibited by the exposure to steroids, while pretreatment
of the target cells with Dexe increased lysis by ADCC, and
the simultaneous treatment with Dexe plus rituximab did
not impair ADCC in malignant lymphoma.

These previous reports support our results showing cytotoxic
synergy with Dexe plus huCD26mAb in MM.

Moreover, a role for anti-MM efficacy by huCD26mAb
was supported by subsequent in vivo experiments. The
NOD/SCID-hu model is an in vivo system, which makes it
possible to evaluate both tumor burden and bone disease
via providing a favorable host environment for repro-
ducible growth of MM cells and mimicking the clinical
manifestations of MM. Thus, this model is widely used to
analyze MM biology and develop novel therapeutic stra-
gies for MM. This model is quite unique in the
point that both human hematopoietic cells and the
human hematopoietic microenvironment are engrafted in
the mice. Bone marrow accessory cells including OCs and
stromal cells in the human bone grafts can support sustained growth of MM cells. Moreover, human stromal
cells in the bone grafts can stimulate the proliferation and
differentiation of human stem cells through hema-
topoietic factors or physical interactions and multilineage
human hematopoiesis is maintained within implanted
human bone grafts of the mice for as long as 20 weeks
after implantation. Thus, human immune cells includ-
ing T lymphocytes or NK cells are considered to be able
to confer the inhibitory effects against CD26+ MM cells by
huCD26mAb as well as huCD26mAb exerts direct
inhibitory effects against CD26+ MM cells in the human
bone marrow of NOD/SCID-hu mice. In addition, our
results confirmed that huCD26mAb induced a significant
decrease in the number of TRAP+ OCs compared with
control mice in vivo. These results have clinical relevance,
implying that huCD26mAb appears to be an ideal therapeu-
tic antibody to target both CD26+ MM cells and OCs
for the treatment of MM.

Although immune-based approaches have not suc-
ceeded in MM until recently, these strategies have finally
attained an exciting breakthrough and represent a pro-
mising area for novel therapeutic options. Target antigens
for mAb therapy in MM have been either cell surface
proteins, cytokines, or chemokines, expressed or secreted
by MM cells, including growth factors, signaling mole-
cules, and adhesion molecules, which are involved in cell
growth, anti-apoptosis, angiogenesis, and cell-to-cell
contact between MM cells and BM accessory
cells. In addition to elotuzumab targeting CS1 and
daratumumab directed against CD38, other monoclonal
antibody targets include CD138, CD56, CD40, CD74, and
intercellular adhesion molecule-1 (ICAM-1) among cell
surface targets as well as IL-6, vascular endothelial growth
factor (VEGF), BAFF, and CXCR chemokine receptor 4
(CXCR4) for cytokine/growth factor-targeted molecules,
which have reached clinical trials.

CD26 regulates a variety of cytokines and chemokines
through the cleavage of N-terminal dipeptides from polypeptides, with proline or alanine in the penultimate
position. CD26 might be involved in regulating the activity of biopeptides to have a key role in tumor cell
survival and proliferation. Likewise, in our study, CD26
activity was important for both OC formation and OC-
induced MM cell growth. Therefore, the inhibition of
CD26 could be beneficial for the treatment of MM.

CD26 expression in tumor cells reveals varying results.
It acts as a tumor activator or tumor suppressor and may
either promote or suppress growth depending on the type
of malignancy. Indeed, the presence of CD26 is associated
with clinical aggressiveness in several tumors, while the

![Fig. 8 huCD26mAb, alone or in combination with Lena, reduced the SP ratio by ADCC. a SP fractions were determined by Hoechst 33342 dye and gated as indicated in both RPMI8226 and KMS11 co-cultured with OCs. Then, SP and MP fractions were isolated from RPMI8226 and KMS11 by cell sorter and the expression of CD26 in both SP and MP fraction was analyzed by flow cytometry. SP fractions equally expressed CD26 at a high level as MP fractions in both cell lines. b RPMI8226 and KMS11 co-cultured with OCs were mixed with NK effector cells from health donor at the E/T ratio of 10 in the presence of iso IgG1, Lena (5 μM) or huCD26mAb (10 μg/ml), alone or both in combination for 24 h. Thereafter, the ratio of SP fractions within the whole living cells was examined by flow cytometry. huCD26mAb reduced SP ratio in both MM cell lines through ADCC. In addition, it markedly decreased its ratio with Lena in combination. c The % change of SP fractions after the indicated treatments (monotherapy of Lena or huCD26mAb, or Lena plus huCD26mAb in combination), mixed with NK effector cells are revealed. The data represent the mean ± SD of three independent experiments (**p < 0.01).](image)
absence of CD26 results in distant metastasis in others. In hematological neoplasms, CD26 expression confers proliferative advantages or invasive properties in T-cell lymphoblastic lymphoma (LBL)/lymphoblastic leukemia, T-anaplastic large cell lymphoma (ALCL), and T-large granular lymphocyte lymphoproliferative disorder (T-LGL LPD)\(^{45-47}\). In addition, CD26 expression, together with CD38 and CD49d expression, identified B-CLL patients with an unfavorable clinical outcome\(^{48}\). In solid tumors, CD26 has a reliable biomarker of gastro-intestinal stromal tumor (GIST) risk grading\(^{49}\). Our previous studies showed that CD26 expression in mesothelioma cells as well as renal cell carcinoma (RCC) cells was associated with enhanced proliferative activity\(^{50-53}\). In addition, our previous reports showed that anti-CD26 mouse mAb (IgG1), IF7 or 14D10, inhibited tumor cell growth of CD26\(^{+}\) T-cell lymphoma or RCC\(^{50,54}\). huCD26mAb, constructed from a 14D10 coding sequence, has been shown to inhibit malignant mesothelioma cell growth\(^{51-56}\). On the other hand, the inhibition of CD26 triggers prostate cancer metastasis. The degradation of SDF-1/CXCL12, known to regulate prostate cancer cell metastasis by CD26, is involved in the metastatic cascade of prostate cancer\(^{67}\).

Moreover, CD26 has been indicated to have an important role in cell adhesion to the extracellular matrix (ECM) in selected conditions, which leads to the migration and metastasis of various types of tumors\(^{28,50,54}\). As a result of CD26 depletion through the transfection of interfering RNA, T-ALCL cells lost the ability to adhere to fibronectin and collagen I through the dephosphorylation of both integrin β1 and p38 mitogen-activated protein kinase (MAPK), which suppresses tumor development in in vivo xenograft models\(^{58}\). In addition to cytotoxic effects on MM cells, we validated that huCD26mAb induced inhibitory effects on CD26\(^{+}\) MM cell adhesion to BMSCs, which partly conferred to inhibit MM cell growth. Although huCD26mAb does not reduce expression of all adhesion molecules, it reduced the expression of membrane protein, CD49d in MM cells in the presence of effector cells.

The normal tissue profile of CD26 appears to be inevitably a major concern for tissue toxicities for an antibody therapy. A phase 1 clinical trial of huCD26mAb has been performed in patients with 33 cases of advanced CD26-expressing tumors, such as RCC, mesothelioma, and urothelial carcinoma\(^{59}\). In this trial, huCD26mAb was well tolerated with the major adverse events being infusion reactions. Owing to the expression of CD26 in normal hematopoietic cells, a decrease in the levels of peripheral lymphocytes, including CD26\(^{+}\) lymphocytes, was noted within a few days after the administration of huCD26mAb: however, this decrease was resolved within a month.

SP cells are defined as an enriched source of cancer-initiating cells with cancer stem cell (CSC) properties, which have been identified in several tumors\(^{38}\). We identified that the treatment of CD26\(^{+}\) SP fractions in both CD26\(^{+}\) RPMI8226 and KMS11 cells with huCD26mAb revealed sensitivity to ADCC and that huCD26mAb in conjunction with Lena resulted in additive cytotoxicity by ADCC, indicating that huCD26mAb could eradicate drug-resistant CD26\(^{+}\) CSCs in MM. Interestingly, CD26 has been validated as a novel marker of CSCs in several malignancies\(^{60,61}\). First, CD34\(^{+}\)/CD38\(^{+}\) leukemic stem cells (LSCs) derived from patients with the chronic phase (CP) of chronic myeloid leukemia (CML) expressed CD26 at high levels. Specifically, all CD34\(^{+}\)/CD38\(^{-}\}/CD26\(^{+}\) LSCs were positive for BCR/ABL, as determined by FISH, and exhibited CSC properties, such as long-term proliferation and repopulation activity in NSG mice, whereas CD26\(^{-}\) LSCs exhibited none of these functions\(^{60}\). Second, CD26\(^{+}\) colorectal cancer (CRC) cells led to the development of distant metastasis, which were associated with increased invasiveness and resistance to chemotherapy, while CD26\(^{-}\) CRC cells did not, suggesting the existence of CD26\(^{+}\) CSCs in CRC\(^{61}\).

In summary, we identified CD26 expression not only in activated OCs but also in MM cells in the bone marrow tissue of MM patient or MM cell lines co-cultured with OCs in vitro. Novel huCD26mAb, directly targeting CD26, elicited significant anti-MM efficacy by impairing both CD26\(^{+}\) MM cells and OCs in vitro and in vivo. Moreover, huCD26mAb facilitated its activity in conjunction with the existing standards of care. Our results strongly suggest that CD26 could be an ideal therapeutic target of antibody-based therapy in MM.

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H.N. designed the research, performed the experiments, analyzed the data and wrote the manuscript; M.H. analyzed the data; C.M., and M.S. contributed to the
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Conflict of interest

C.M. and T.Y. are consultants for Ys Therapeutics and Kissei Pharmaceutical Co. Ltd. The remaining authors declare that they have no conflict of interest.

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