Antitumor effect of novel anti-podoplanin antibody NZ-12 against malignant pleural mesothelioma in orthotopic xenograft model

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Abstract

Podoplanin (PDPN)/Aggrus is highly expressed in several types of cancers including malignant pleural mesothelioma (MPM). Previously, we developed a rat anti-human podoplanin monoclonal antibody NZ-1 and a rat-human chimeric anti-human podoplanin antibody NZ-8 derived from NZ-1, which induced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against podoplanin-positive MPM cell lines. In this study, we demonstrated the antitumor effect of NZ-1, NZ-8 and NZ-12, a novel rat-human chimeric anti-human podoplanin antibody derived from NZ-1, in MPM orthotopic xenograft SCID mouse model. Treatment with NZ-1 and rat NK (CD161a+) cells could inhibit growth of tumor weight and production of pleural effusion in NCI-H290/PDPN or NCI-H226 orthotopic xenograft model. NZ-8 and human NK (CD56+) cells also inhibited tumor weight and pleural effusion in MPM orthotopic xenograft model. Furthermore, NZ-12 induced significantly potent ADCC mediated by human MNC compared with either NZ-1 or NZ-8. Effectively antitumor effect was observed by treatment with NZ-12 and human NK (CD56+) cells in MPM orthotopic xenograft model. In addition, the combined immunotherapy based on the ADCC activity of NZ-12 mediated by human NK (CD56+) cells with pemetrexed led to enhance antitumor effects in MPM orthotopic xenograft model. These results strongly suggested that combination therapy both the podoplanin targeting immunotherapy using NZ-12 and pemetrexed might become hopeful therapeutic strategy against MPM.
Introduction

Expression of podoplanin (PDPN)/Aggrus, which is a transmembrane sialomucin-like glycoprotein, was detected on some of normal tissues such as kidney podocyte, endothelium of lymphatic vessels and type I alveolar epithelium (1-3) and many types of cancers including malignant brain tumor, oral cancers, esophageal cancers, squamous carcinoma, testicular seminomas, bladder cancers, fibrosarcomas, and malignant pleural mesothelioma (MPM) (4-9). Function of podoplanin, which bind to the platelet aggregation-stimulating (PLA2G) domain of PDPN–C-type lectin-like receptor-2 (CLEC-2) in platelet, is induction of platelet aggregation and that leads to cancer metastasis (8, 10). Furthermore, high expression of podoplanin in cancer-associated fibroblasts was reported to be related to severe malignancy and poor prognosis in cancer patients (11-14). Therefore, it has been expected that podoplanin will become a target for cancer therapy and diagnosis.

MPM, which is mainly caused by exposure of asbestos, develops in pleural cavity with high-grade malignancy (15). It is expected that number of MPM patients will increase from 2030 to 2040 in Asia and from 2010 to 2020 in Europe (16, 17). The standard therapy of MPM is based on combination of surgical operation, radiation therapy, and systemic chemotherapy. However, the prognosis of MPM is very poor because MPM is one of the progressive cancers and resist against these therapies (18, 19). Though treatment of pemetrexed, which is only validated chemotherapy drug for MPM, combined with cisplatin is used as standard chemotherapy against MPM patients, the combination therapy prolonged progression-free survival only 2.8 months relative to without pemetrexed (20). Therefore, development of novel therapy for MPM is desired to improve the prognosis.

Immunotherapy using therapeutic antibodies against tumor-associated antigens (TAAs) or antigenic peptides derived from TAAs has been considered as novel mechanism therapy against various cancers including MPM (21, 22). Several therapeutic antibodies such as trastuzumab and rituximab have already been used in clinical practice. It is well known that important mechanisms of clinical antitumor efficacy of therapeutic antibodies are antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (23). Previously, we generated a rat anti-human podoplanin monoclonal antibody NZ-1 (24-26) and a rat-human chimeric anti-human podoplanin antibody NZ-8 derived from NZ-1 (9, 27). These anti-podoplanin antibodies induce potent ADCC and CDC activity against podoplanin positive MPM cell lines in vitro and have significant antitumor effect in MPM subcutaneous transplantation SCID mouse model. However, these anti-podoplanin antibodies have a therapeutic effect in MPM orthotopic xenograft model which is more similar to MPM clinical presentation, is still unknown. In the present study, we investigated whether anti-human podoplanin antibodies NZ-1, NZ-8 and NZ-12, which is a novel rat-human chimeric anti-human podoplanin antibody derived from NZ-1, can induce antitumor effects in MPM orthotopic xenograft SCID mouse model. Furthermore, we also evaluated combination antitumor effect of both anti-human podoplanin antibody based immunotherapy and pemetrexed.

Materials and Methods

Cell line

In this study, we used 4 human MPM cell lines. NCI-H290, ACC-MESO-1, and ACC-MESO-4 were provided by Dr. Yoshitaka Sekido (Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya, Japan) (28). NCI-H226 and Chinese hamster ovary (CHO) were purchased from American Type Culture Collection (ATCC, Rockville, MD). NCI-H290 cells were transfected with appropriate amounts of pcDNA3/human podoplanin (PDPN) or pcDNA3/mock plasmids, using Metafectene (Nippon Genetics Co. Ltd., Tokyo, Japan) according to description of the manufacturer’s instructions. Stable transfectants (NCI-H290/PDPN and NCI-H290/mock) were selected by culture in 0.5 mg/ml Geneticin (Thermo Fisher Scientific Inc., Waltham, MA) containing medium. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (CRPMI1640) (Thermo Fisher Scientific Inc.), 100 U/mL penicillin, and 100 µg/mL streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) in 5% CO₂ at 37 °C.

Antibodies and Reagents

A rat anti-human podoplanin monoclonal antibody (mAb) NZ-1 and a rat-human chimeric anti-human podoplanin antibody NZ-8 were developed as described previously (24, 27). For the generation of rat-human chimera anti-human
incubation, cells were washed with PBS and subcloned into the pCAG-Neo (Wako Pure Chemical Industries Ltd., Osaka, Japan), and V\(_H\) of a rat NZ-1 antibody and C\(_\beta\) of human IgG\(_\lambda\) were subcloned into pCAG-BLE vectors (Wako Pure Chemical Industries Ltd.). The cDNAs coding for the V\(_H\) and V\(_\lambda\) (lambda chain) regions were constructed by a PCR-based method. Antibody expression vectors were transfected into CHO cells using Lipofectamin LTX kit (Thermo Fisher Scientific Inc.). Stable transfectants of CHO/NZ-12 were selected by cultivating the transfectants in medium containing 1 mg/ml Geneticin and 0.5 mg/ml Zeocin (Thermo Fisher Scientific Inc.). CHO/NZ-12 cells were cultivated in CHO-S-SFM II medium (Thermo Fisher Scientific Inc.). The media containing NZ-12 were centrifuged, and the obtained supernatant was applied to a column of protein G-Sepharose (GE healthcare Bio-Sciences, Pittsburgh, PA). After extensive washing with phosphate-buffered saline (PBS), NZ-12 was eluted using 0.1 M glycine and 0.15 M NaCl (pH 2.8; Thermo Fisher Scientific Inc.), and then neutralized with 1 M Tris pH 10.0. The antibodies were dialyzed against PBS. Expression and purity of the proteins were confirmed by SDS-PAGE using 5-20% gradient gels (Wako Pure Chemical Industries Ltd.). Rat IgG was purchased from Southern Biotechnology (Birmingham, AL). Human IgG was purchased from Cappel (Cochranville, PA). Pemetrexed was obtained from Eli Lilly Japan (Indiana police, IN).

**Animals**

Five to six weeks old male SCID mice and six weeks old male Wistar rats were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions throughout this study. All animals were acclimatized for at least one week before experiments. All experiments were performed in accordance with the guidelines of University of Tokushima, Committee on Animal Care and Use.

**Flow cytometry**

Expression of podoplanin was detected by flow cytometry as described previously\(^6\). Cells (5 × 10\(^6\)) were washed with PBS and stained with NZ-1 (1 µg/ml) or rat IgG (1 µg/ml). After 30 min incubation, cells were washed with PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat F(ab\(^\)'\)\(_2\) fragment anti-rat IgG (H+L) antibody (Beckman Coulter Inc., Fullerton, CA). The cells were washed again and resuspended in PBS. FACS Calibur flow cytometer with CellQuest software (BD Biosciences, Franklin Lakes, NJ) was used for the analysis.

**Preparation of effector cells**

Preparation methods of effector cells were previously described\(^9\), 29, 30). Rat splenocytes were harvested from Wistar rat spleens. Spleens were homogenized in RPMI1640 and centrifuged. To deplete red blood cells, the cell pellet was suspended in red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO). After wash and resuspension in CRPMI1640, splenocytes were used as effector cells. To separate rat NK cells from rat splenocytes, a magnetic cell-sorting system was used. Splenocytes were incubated with FITC-conjugated anti-CD161a antibody (BD Biosciences), after that, with anti-FITC mAb-coupled super-paramagnetic microbeads (Miltenyi Biotec, Auburn, CA). CD161a-positive selection was conducted using an autoMACS (Miltenyi Biotec). Isolated CD161\(^+\) cells that yielded purity ≥90% as determined by flow cytometry were used in experiments. Human peripheral blood mononuclear cells (MNC) were obtained from leukocytes in lymphocyte separation medium (Litton Bionetics, Kensington, MD). Leukocytes were separated from peripheral blood of healthy donors using an RS-6600 rotor of a Kubota KR-400 centrifuge (Kubota, Tokyo, Japan). CD56\(^+\) cells were purified from human MNC to use autoMACS and CD56 microbeads (Miltenyi Biotec). Human MNC were treated with CD56 microbeads, and then cells were separated by autoMACS. The purities of CD56\(^+\) cells were ≥90%. The human study was approved by the ethics committee of University of Tokushima, and written informed consent was obtained from all donors.

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

ADCC was determined with \(^{51}\)Cr release assay\(^9\), 29, 31). Target cells were incubated with 0.1 µCi of \(^{51}\)Cr-sodium chromate at 37°C for 1 h. After wash with CRPMI1640 three times, \(^{51}\)Cr-labeled target cells were placed in 96-well plates in triplicate. Effector cells and anti-human podoplanin antibody or control IgG were added to the plates. After 6 h incubation, \(^{51}\)Cr release of the supernatant from each well (100 µL) was
measured using a gamma counter (PerkinElmer, Waltham, MA). Percent of cytotoxicity was calculated from the following formula: \( \% \text{ Specific lysis} = \frac{(E - S)(M - S)}{M} \times 100 \), where \( E \) is the release in the test sample, \( S \) is the spontaneous release, and \( M \) is the maximum release.

**Complement-dependent cytotoxicity (CDC)**

CDC was evaluated by \( ^{51} \text{Cr} \) release assay as described previously (6, 32). Target cells were incubated with \( ^{51} \text{Cr} \)-sodium chromate (0.1 \( \mu \)Ci) for 1 h at 37°C. After that, the cells were washed by CRPMI1640. \( ^{51} \text{Cr} \)-labeled cells were incubated with baby rabbit complement (Cedarlane, Ontario, Canada) at a dilution of 1:4 and NZ-12 (1 \( \mu \)g/mL) or control human IgG (1 \( \mu \)g/mL) for 6 h in 96-well plates. After incubation, the supernatant including \( ^{51} \text{Cr} \) was measured using a gamma counter. Percent of cytotoxicity was calculated as above.

**Animal experiments**

SCID mice were injected into the thoracic cavity with NCI-H290/PDPN (1.0 \( \times 10^6 \) cells) or NCI-H226 (1.0 \( \times 10^6 \) cells) on day 0. Intrathoracic administration or intraperitoneal injection (i.p.) of anti-human podoplanin antibody or control IgG was started on day 0, and continued twice a week for 2-3 weeks. Rat CD161a\(^+\) cells (1.0 \( \times 10^6 \) cells), human CD56\(^+\) cells (1.0 \( \times 10^6 \) cells), or control normal saline were injected into thoracic cavity started on day 3, and continued weekly for 2-3 weeks. SCID mice of pemetrexed combination group were treated with pemetrexed (100 mg/kg, i.p.) on day 4, 5, 6, 11, 12, and 13. Three weeks (NCI-H290/PDPN) or 9 weeks (NCI-H226) after tumor cell inoculation, the mice were sacrificed, and then the thoracic tumors were weighed and the volume of pleural effusion was measured using a 1 mL syringe.

**Statistical analyses**

The statistical significance of differences in *in vitro* and *in vivo* data was analyzed by standard Student’s t test and one-way ANOVA. In this study, \( P \)-values less than 0.05 were considered significant in all experiments.

**Results**

**A rat anti-human podoplanin antibody NZ-1 induces ADCC against podoplanin-transfected MPM cell lines**

First, we confirmed that NZ-1 induces ADCC against NCI-H290/PDPN as target cells. NCI-H290, one of the podoplanin-negative MPM cell lines, develops thoracic tumors and pleural effusion on orthotopic xenograft SCID mouse model (33). We generated NCI-H290/PDPN to transfected pcDNA3/podoplanin into NCI-H290. As shown in Fig. 1A, NCI-H290/PDPN expressed podoplanin was determined by flow cytometry. Using NCI-H290/PDPN as target cells, we could detect ADCC induced by NZ-1 with rat splenocytes (Fig. 1B). Fig. 1C shows that E:T and dose-dependent ADCC activities against NCI-H290/PDPN were also induced by NZ-1. Moreover, NZ-1 exhibited significant ADCC activity against NCI-H290/PDPN and NCI-H226 when rat NK cells used as target cells (Fig. 1D).

**Antitumor activity of NZ-1 in MPM orthotopic xenograft model**

Previously, we reported that injection of both NZ-1 and rat NK cells inhibited the growth of podoplanin-positive MPM cells inoculated subcutaneously for SCID mouse (39). To determine whether NZ-1 also induced antitumor effect in orthotopic xenograft model, firstly, we evaluated the local administration effect of NZ-1 in NCI-H290/PDPN orthotopic xenograft mouse model. NZ-1 or control IgG was injected into the thoracic cavity twice a week, and rat NK (CD161a\(^+\)) cells or PBS was injected into the thoracic cavity weekly for two weeks. Three weeks after tumor cell inoculation, both NZ-1 and rat NK cells administration group almost completely inhibited the growth of tumor weight and the production of pleural effusion (Fig. 2). In contrast, administration of NZ-1 alone did not induce antitumor effect in our model. Next, we demonstrated whether systemic treatment of NZ-1 induces antitumor effect in orthotopic xenograft SCID mouse model. NZ-1 or rat IgG was injected i.p. twice a week, and rat NK cells or PBS was injected into intrathoracic cavity weekly for three weeks. In both NCI-H290/PDPN and NCI-H226 inoculated mice, systemic administration of NZ-1 with rat NK cells also significantly inhibited growth of intrathoracic tumor and production of pleural effusion compared with either NZ-1 or rat NK cells administration (Fig. 3A, 3B).

**Antitumor activity of a rat-human chimeric anti-human podoplanin antibody NZ-8 in MPM orthotopic xenograft model**

We previously reported that NZ-8 induced ADCC activity mediated by human NK cells (39).
To evaluate the antitumor effect of NZ-8 with human NK cells in MPM orthotopic xenograft model, we used NCI-H290/PDPN orthotopic xenograft model. As shown in Fig. 6A, ADCC activity against NCI-H290/PDPN was observed by treatment with NZ-8 and human MNC. Injection of NZ-8 (i.p.) twice a week and human NK (CD56⁺) cells in intrathoracic cavity weekly for 2 weeks significantly inhibited tumor weight and pleural effusion production compared with administration of NZ-8 or human NK cells alone (Fig. 4B).

**In vitro and in vivo antitumor effect of a novel rat-human chimeric anti-human podoplanin antibody NZ-12**

Because it was demonstrated that NZ-1 and NZ-8 induced antitumor effect in MPM orthotopic xenograft model, to establish more potent target therapy to podoplanin, we generated a novel rat-human chimeric anti-human podoplanin antibody NZ-12 derived from NZ-1. As shown in Fig. 5A, NZ-12 induced a significant level of ADCC mediated by human MNC against podoplanin-positive MPM cells. ADCC activity induced by NZ-12 was significantly higher than that of NZ-1 or NZ-8. NZ-12 also induced CDC activity against podoplanin-positive MPM cells (Fig. 5B). Moreover, ADCC activity of NZ-12 was mediated by human NK (CD56⁺) cells (Fig. 5C). To use NCI-H290/PDPN orthotopic xenograft SCID mouse model, the tumor weight and production of pleural effusion were significantly inhibited by injection of NZ-12 (i.p.) twice a week with human NK (CD56⁺) cells into intrathoracic cavity weekly for 2 weeks (Fig. 5D, 5E). By contrast, treatment with NZ-12 alone did not inhibit the tumor growth.

**Effect of combination treatment with NZ-12 and pemetrexed**

Our result suggested that administration of NZ-12 with human NK cells inhibited tumor growth on MPM orthotopic model. Therefore, for further evaluation of efficacy of NZ-12 against MPM, we demonstrated combination effect of both NZ-12 and pemetrexed treatment in MPM SCID mouse model. As shown in Fig 6A, expression of podoplanin in NCI-H290/PDPN was not changed by 72 h incubation with pemetrexed (0.1 µM). On the other hand, the same treatment condition of pemetrexed inhibited the proliferation of NCI-H290/PDPN in vitro (data not shown). Furthermore, treatment with pemetrexed for target cells did not inhibit ADCC activity induced by NZ-12 with human MNC (Fig. 6B). Using the orthotopic xenograft SCID mouse model, administration of NZ-12 (i.p.), human NK (CD56⁺) cells injected into thoracic cavity, and pemetrexed (i.p.) significantly reduced intrathoracic tumor growth and production of pleural effusion compared with the immunotherapy based on NZ-12 (NZ-12 with human NK cells) or pemetrexed alone (Fig. 6C).

**Discussion**

In the present study, we have demonstrated that anti-human podoplanin antibodies, NZ-1, NZ-8, and NZ-12 possess therapeutic antitumor effect in MPM orthotopic xenograft SCID mouse model. Furthermore, NZ-12 induced effectively ADCC activity compared with NZ-1 or NZ-8. In addition, we have also shown that combination treatment with the immunotherapy based on NZ-12 and pemetrexed mediated significant antitumor activity compared with the single therapy. These data strongly suggested that NZ-12 is an effective antibody against MPM.

We previously established human MPM orthotopic xenograft SCID mouse model, which inoculated MPM cells into thoracic cavity (34, 35). Several weeks after inoculation, the mice would die caused by increase of thoracic tumor and production of bloody pleural effusion inside the thoracic cavity of the mice. Because the disease state was similar to that of human MPM patient, this model could use evaluation of the therapeutic effect against MPM (33, 36). Though our previous study has only showed antitumor effects of anti-human podoplanin antibody with NK cells in MPM s.c. xenograft model (9), in this study, administration of anti-human podoplanin antibodies NZ-1, NZ-8, and NZ-12 with NK cells reduced growth of intrathoracic tumor and production of pleural effusion in MPM orthotopic xenograft model. By contrast, no antitumor effects were observed by the injection of the anti-human podoplanin antibody alone in this experimental condition. These results indicated that antitumor effect of anti-human podoplanin antibodies was caused by NK cells mediated ADCC in MPM orthotopic xenograft mouse model. Furthermore, cancer progression was reduced by not only intrathoracic injection of the anti-human podoplanin antibody but also i.p. injection. Because the drug injection into thoracic cavity introduced the several side effects, such as pneumothorax, inflammation, and infection.
systemic administration was preferred in clinical practice. Therefore, in the current study, it seemed that systemic treatment of the anti-human podoplanin antibodies would be useful for podoplanin-positive MPM patients. We have demonstrated that NZ-12, a novel rat-human chimeric anti-human podoplanin antibody generated from NZ-1, with human NK cells induced antitumor effects against MPM in vitro and in vivo. ADCC activity of NZ-12 mediated by human NK cells was much higher than that of NZ-1 or NZ-8. In our previous study, NZ-1 could only induce ADCC activity by rat NK cells because NZ-1 was rat anti-human podoplanin antibody (19). On the other hand, NZ-8, another rat-human chimeric anti-human podoplanin antibody generated from NZ-1, could induce ADCC activity by human NK cells as effector cell. The reason why ADCC activity of NZ-12 is significantly higher than that of NZ-8 is still unknown. However, it might be related to difference in the development method between NZ-12 and NZ-8. In development of the human chimeric antibody, kappa chain was generally used as the antibody light chain, and NZ-8 was also established by using kappa chain. In contrast, because NZ-1 has lambda chain of rat antibody, NZ-12 was also generated using human lambda chain. Therefore, it might be considered that potent ADCC activity of NZ-12 compared with NZ-8 was caused by difference between the binding affinity of NZ-12 against podoplanin and that of NZ-8 due to the development method. Treatment of pemetrexed combined with cisplatin is used as the standard chemotherapy against MPM patients. However, the difference of survival between pemetrexed with cisplatin and cisplatin alone was only 2.8 month (20). Therefore, development of novel therapeutic strategy in combination with pemetrexed is needed to improve therapeutic outcome for MPM. In this study, prolonged treatment of pemetrexed did not change the expression level of podoplanin in MPM cell, suggesting that ADCC activity of NZ-12 against MPM was not inhibited by pemetrexed in vitro. In addition, the combined immunotherapy based on the ADCC activity of NZ-12 mediated by human NK cells with pemetrexed injection led to enhance antitumor effects in MPM orthotopic xenograft model. Though many anti-cancer chemotherapy drugs suppress the immune function due to myelosuppression, in contrast, several chemotherapy drugs including gemcitabine mediate combination effect with immunotherapy caused by modulate the immune function (37, 38). Pemetrexed which is an antifolate antimetabolite agent, is likely to induce myelosuppression. Nevertheless, our results suggested that treatment of both NZ-12-mediated immunotherapy and pemetrexed would induce combined effects against intrathoracic MPM, which is probably an additive effect because pemetrexed had no effect the expression of podoplanin and ADCC activity of NZ-12 mediated by human NK cells. In conclusion, we found that anti-human podoplanin antibodies possess antitumor effect based on ADCC activity in MPM orthotopic xenograft model. Furthermore, treatment of both NZ-12, which could especially induce potent ADCC activity mediated by human NK cells, and pemetrexed induced combined antitumor effects in MPM orthotopic xenograft model. These findings suggested that combination therapy both the podoplanin targeting immunotherapy using NZ-12 and pemetrexed will become the promising therapeutic strategy against MPM

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Disclosure Statement
The authors have no conflict of interest.

References


FIGURE LEGENDS

Fig. 1. ADCC activity of a rat anti-human podoplanin antibody NZ-1 against NCI-H290/PDPN in vitro. (A) Expression of podoplanin was detected by FACS analysis. (B) ADCC activity of NZ-1 using rat splenocytes against NCI-H290/PDPN or NCI-H290/Mock was evaluated by 6-h 51Cr release assay in the presence of 1 µg/ml antibody at the E/T ratio of 100. (C) E/T ratio., antibody dose-dependent effects of ADCC against NCI-H290/PDPN mediated by NZ-1 with rat splenocytes were demonstrated by 51Cr release assay. (D) Rat NK (CD161a+) cells were isolated from rat splenocytes by autoMACS. ADCC activity of 1 µg/ml NZ-1 mediated by NK (CD161a+) cells was evaluated by 6-h 51Cr release assay at the E/T ratio of 10. **P < 0.01 versus control (values are means ±S.E.).

Fig. 2. Antitumor effect of NZ-1 injected into thoracic cavity in NCI-H290/PDPN orthotopic xenograft model. SCID mice (n=5) were injected into the thoracic cavity with 1.0 × 10^6 NCI-H290/PDPN cells. NZ-1 (100 µg) or control rat IgG (100 µg) intrathoracic injection was started on day 0, and continued twice a week for 2 weeks. Rat NK (CD161a+) cells (1.0 × 10^6 cells) or control normal saline intrathoracic injection continued weekly for 2 weeks. 3 weeks after tumor cell inoculation, the mice were sacrificed, and weight of thoracic tumors and the volume of pleural effusion were measured. *P < 0.01 (values are means ±S.E.).

Fig. 3. Antitumor effect of NZ-1 intraperitoneal injection in MPM orthotopic xenograft model. SCID mice (n=5) were injected into the thoracic cavity with NCI-H290/PDPN (A) or NCI-H226 (B) (1.0 × 10^6 cells). Intraperitoneal injection of NZ-1 (100 µg) or control rat IgG (100 µg) was started on day 0, and continued twice a week for 3 weeks. Intrathoracic injection of rat CD161a+ cells (1.0 × 10^6 cells) or control normal saline started on day 3, and continued weekly for 3 weeks. 3 weeks (NCI-H290/PDPN) or 9 weeks (NCI-H226) after tumor cell inoculation, the mice were sacrificed. *P < 0.05, **P < 0.01 (values are means ±S.E.).

Fig. 4. Antitumor activity of a rat-human chimeric anti-human podoplanin antibody NZ-8 in NCI-H290/PDPN orthotopic xenograft model. (A) ADCC activity against NCI-H290/PDPN was determined with 6-h 51Cr release assay at the E/T ratio of 100 in the presence of control human IgG (1 µg/ml), NZ-1 (1 µg/ml), or NZ-8 (1 µg/ml) with human MNC. (B) NCI-H290/PDPN (1.0 × 10^6 cells) was inoculated into the thoracic cavity on day0 (n=5). NZ-8 (100 µg) or control human IgG (100 µg) injection (i.p.) was started on day 0, and continued twice a week for 2 weeks. Human NK (CD56+) cell (1.0 × 10^6 cells) injection intrathoracic cavity or control normal saline started on day 3, and continued weekly for 2 weeks. Mice were sacrificed 3 weeks after tumor cell inoculation. *P < 0.05, **P < 0.01 (values are means ±S.E.).

Fig. 5. Antitumor effect of novel rat-human chimeric anti-human podoplanin antibody NZ-12 in vitro and in vivo. (A) ADCC induced by human MNC against MPM cell lines, ACC-MESO-1, ACC-MESO-4, NCI-H226,
and NCI-H290/PDPN, was evaluated by 6-h $^{51}$Cr release assay at the E:T ratio of 100 in the presence of control human IgG (1 µg/ml), NZ-1 (1 µg/ml), NZ-8 (1 µg/ml), and NZ-12 (1 µg/ml) with human MNC. (B) CDC activity was demonstrated by 6-h $^{51}$Cr release assay in the presence of 1 µg/ml NZ-12 or control human IgG with baby rabbit complement at a dilution of 1:4. (C) Human NK (CD56$^+$) cells were isolated from human MNC by autoMACS. ADCC activity of 1 µg/ml NZ-12 mediated by human NK (CD56$^+$) cells was evaluated by 6-h $^{51}$Cr release assay at the E/T ratio of 5, 10 and 25. (D, E) SCID mice (n=5) were injected intrathoracic cavity $1.0 \times 10^6$ NCI-H290/PDPN cells. NZ-12 (100 µg) or human IgG (100 µg) injection (i.p.) was continued twice a week for 2 weeks. Human NK (CD56$^+$) cell ($1.0 \times 10^5$ cells) injection into the thoracic cavity continued weekly for 2 weeks. 3 weeks after tumor cell inoculation, the mice were sacrificed. *P < 0.05 , **P < 0.01 (values are means ±S.E.).

Figure 6. Combination effect of treatment with the immunotherapy based on NZ-12 and pemetrexed in vivo. (A) NCI-H290/PDPN was incubated with pemetrexed (MTA) 0.1 µM. After 72 h incubation, expression of podoplanin was evaluated by FACS analysis. (B) ADCC activity of NZ-12 (1 µg/ml) against NCI-H290/PDPN mediated by human MNC was evaluated by 6-h $^{51}$Cr release assay (E:T ratio 100) in the presence of MTA (0.1 µM) or not. NCI-H290/PDPN treated with MTA (0.1 µM) for 72h was also used as target cells. (C) SCID mice (n=5) were injected into the thoracic cavity with NCI-H290/PDPN ($1.0 \times 10^6$ cells). NZ-12 (100 µg) or human IgG (100 µg) was injected i.p. twice a week for 2 weeks. Human NK (CD56$^+$) cells ($1.0 \times 10^5$ cells) or normal saline was injected into the thoracic cavity weekly for 2 weeks. Pemetrexed (100 mg/kg, i.p.) was treated on day 4, 5, 6, 11, 12, and 13.
Fig. 1.

Panel A: Flow cytometry histograms showing FL1-H fluorescence for NCI-H290/Mock and NCI-H290/PDPN.

Panel B: Bar graph showing cytotoxicity (%) for NCI-H290/Mock and NCI-H290/PDPN with treatments rlgG and NZ-1.

Panel C: Graphs showing cytotoxicity (%) vs E/T ratio for Rat splenocytes treated with rlgG and NZ-1.

Panel D: Graphs showing cytotoxicity (%) vs Ab (µg/mL) for Rat splenocytes treated with rlgG and NZ-1.

Panel E: Bar graph showing cytotoxicity (%) for NCI-H290/PDPN and NCI-H226 for Rat NK cells with treatments rlgG and NZ-1.
Fig. 2.
Fig. 3.

A  NCI-H290/PDPN

B  NCI-H226

![Graphs showing tumor weight and pleural effusion for NCI-H290/PDPN and NCI-H226 cell lines with different treatments.](image-url)
Fig. 4.

A) E: Human MNC

B) Tumor Weight (g)

C) Pleural Effusion (mL)
Fig. 6.

A

Control  MTA (0.1μM 72h)

B

E: Human MNC

N.S.

Cytotoxicity (%)  

Control  With MTA  (6h)  After 72h treatment of MTA

N.S.

hlgG  ∇  NZ-12

C

Tumor Weight (g)

rlgG  MTA  NZ-12 + NK  MTA + NZ-12 + NK

N.S.

*  **  +

Pleural Effusion (mL)

rlgG  MTA  NZ-12 + NK  MTA + NZ-12 + NK

N.S.

*  **