**SUPPLEMENTARY MATERIALS AND METHODS**

**Proteomics (OGAP)**

Tissue samples from patients diagnosed with breast cancer, TNBC, bladder cancer, pancreatic cancer, gastric cancer, colon cancer, multiple myeloma, were processed to generate cell membrane protein fractions and analysed by liquid chromatography-mass spectrometry using a Waters nanoACQUITY UPLC System fitted with a nanoACQUITY UPLC BEH 130 C18 column, 75 µm x 250mm (186003545) and a LTQ Orbitrap Velos (Thermo Fisher Scientific). Peptides were then analyzed using a LTQ Orbitrap Velos mass spectrometer. The raw data generated from the LTQ Orbitrap Velos were processed through the Mascot software (Matrix Science, UK) searching against the SwissProt (http://www.uniprot.org) sequence database. Peptides with an expectation value of 0.05% or less and an ion score of 28 or higher were loaded into our OGAP database to evaluate the proteomic expression. Proteomic expression for CD205 antigen and other proteins were then calculated using spectral counting and expressed as exponentially modified protein abundance index scores (emPAI) (1).

**Human tissue microarrays, IHC and visual scoring**

Formalin-fixed, paraffin-embedded (FFPE) human tumor tissue microarrays (TMAs) included both primary and metastatic tumor samples from ovarian, bladder, breast, gastric, pancreatic, lung, and colorectal cancers. Slides were dewaxed and rehydrated. Antigen retrieval was performed in a Tris pH 9.0 buffer and a standard IHC protocol was used to assess expression of the target antigen. Staining was performed using a mouse monoclonal primary antibody against CD205 (Leica; clone 11A10 [NC-L-DEC205]) and a mouse IgG isotype (R&D Systems; MAB002); concentration matched and used at a dilution of 1:80. The primary antibody was detected with an anti-mouse-HRP labeled secondary antibody polymer (Dako) and positive signal was detected with DAB chromogen. Alternately, the primary antibody was detected with an anti-mouse biotinylated secondary antibody (Dako), followed by incubation with streptavidin peroxidase (Dako) prior to detection with DAB chromogen. Slides were developed, counterstained, and visualized by brightfield microscopy. A scoring system was used to evaluate the CD205 antigen expression according to the observable staining. A score of 3+ denoted strong expression, 2+ indicated moderate expression and 1+ corresponded to weak expression. Samples that did not stain positively for CD205 were scored negative (0).

**RNA extraction and quantitative real time PCR detection**

Cell lysates and total RNA was prepared with RNeasy Mini Kit (Qiagen) according to manufacturer instructions. The corresponding cDNAs were synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To determine the expression levels of the CD205 transcript, duplicates of 1:5 dilutions of cDNA samples were amplified in 25 μl of TaqMan Universal PCR Master Mix using 1.25 μl of the 20X Inventoried Assay purchased from Applied Biosystem. The inventoried assays used were Hs00158966\_m1 (Human LY75) and Hs99999905\_m1 (human GAPDH as endogenous control), Applied Biosystem. Threshold cycle values (Ct) were selected from the geometric phase of the amplification curves and Ct values greater than 34 were considered not detectable. The amplification and quantitative real time detection was performed in a 7300 Real Time PCR system (Applied Biosystems). Data were presented as mean relative copy number (RCN) using RCN = 2-ΔCt , where ∆Ct is the Ct(target) – Ct(GAPDH).

**MEN1309/OBT076 binding to FcγRIIIA-158V**

An ELISA method was performed to measure the binding affinity of both unconjugated and conjugated MEN1309/OBT076 to FcγRIIIa-158V. Briefly, MaxiSorp 96-well plates (Roskilde, Denmark) were coated with 100 ml/well 2 μg/ml of anti-Tetra His antibody and incubated at 4°C overnight. The plates were washed 4 times with 300 µl/well PBS containing 0.05% Tween 20 and blocked with 100 µl/well PBS containing 0,5% BSA. After 1 hour incubation at room temperature and washig 100 µl/well of 1.5 µg/ml FcγRIIIa-158V (Sino Biologicals) were added to plates and incubated at room temperature for 1 hour. After washing, 100 µl/well of three-folds serial dilutions of antibodies were incubated at room temperature for 2 hours. Bound antibodies were detected using (1:40000) peroxidase-labeled goat antihuman IgG F(ab’)2 fragment specific (Jackson ImmunoResearch Laboratories) and 100 µl/well 3,3′,5,5′-Tetramethylbenzidine (TMB) as substrate (Thermo scientific, Waltham, MA, USA). The reaction was stopped with 100 µl/well Stop solution, and the adsorbance at 450 nm was measured on an Infinite M200 plate reader (Tecan, Switzerland).

**ADCC assay**

The ADCC assay was performed using the ADCC Reporter Bioassay from Promega, following the kit instructions with some modifications. Briefly, 4x106 THP-1 target cells were washed and re-suspended in 4 mL of ADCC buffer and were seeded on a white 96-well plate. Serial dilutions of antibodies, ranging from 0.3 ng/mL up to 1 µg/mL, were seeded adding a further volume of 25 µl/well. Jurkat effector cells were prepared by adding to 630 µl cells from the stock vial to 3600 µl of ADCC buffer. 25 ul of this dilution were added to each well obtaining a Effector / Target cell Ratio = 3 / 1 (75000 / 25000). Plates were incubated 6h at 37°C. After a second incubation at RT for 15 min, 75 ul of BioGlo reagent/well were added. Plates were maintained 1 min in the dark and then the developed luminescence was detected in the Infinite M200 reader.

**CDC assay**

The CDC assay was performed using purified human serum complement (Quidel, San Diego, CA). MEN1309 (100 μg/ml-0,00001 μg/ml) was diluted with RHB buffer (RPMI 1640 No Phenol Red, Life Technologies, 20 mM HEPES, pH 7.2, 2 mM glutamine, 0.1% BSA). Raji cells were washed in RHB buffer and resuspended at a density of 1x106 cells/ml. In a typical assay, 50 µl of a cell suspension (50,000 cells/well), 50 µl of diluted human complement (4%) and 50 μl of diluted MEN1309 were added to a flat-bottom tissue culture 96-well plate. A control curve with Rituximab antibody was added. The mixture was incubated for 3 hours at 37°C in a 5% CO2 incubator to facilitate complement-mediated cell lysis. Then, 50 μl of Alamar Blue (Accumed International, Westlake, OH) was added to each well and incubated overnight at 37°C. Fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results are expressed in relative percentage of cytotoxicity that is proportional to the number of viable cells. The percent CDC activity was calculated as follows: 100 - [(RFU test/RFU background) x100]

**In vivo study on PDX**

Briefly, in the Champions Oncology studies, immunocompromised female nude mice, from Harlan Laboratories (Indianapolis, IN, USA) between 5-8 weeks of age were housed on irradiated, Alpha-twist-enriched 1/8” corncob bedding (Sheperd) in individual HEPA ventilated cages (Innocage® IVC, Innovive USA) on a 12-hour light-dark cycle at 68-74°F (20-23°C) and 30-70% humidity. Animals were fed with water (reverse osmosis, 2 ppm Cl2) and an irradiated test rodent diet (Teklad 2919; 19% protein, 9% fat, and 4% fiber) ad libitum. Mice were implanted with tumor fragments from Champions TumorGraft® models CTG-1388 or CTG-1652. When the tumor volume (TV) reached approximately 150-300 mm3, animals were randomized into groups and treatments were initiated. Tumor growth was monitored twice a week using digital calipers and the TV was calculated using the formula (0.52 × [length × width2]). Body weight was determined twice a week using a digital scale. The studies were terminated when the mean tumor volume in the control group reached approximately 1500 mm3 or up to Day 60, whichever occurred first.

In Vall d’Hebron Institute of Oncology, 6 week-old female NOD/SCID mice (NOD.CB17/AlhnRj-Prkdc Scid, Janvier Labs) were orthotopically implanted into the mammary fad pad with breast tumor fragments or embedded in Matrigel and subcutaneously implanted with pancreatic cancer tumor pieces of approximately 3x3mm. Breast PDX models were maintained with 17-ß-estradiol (1μM) in the drinking water during all the experimental procedure. Tumor PDX were measured with calipers twice a week. Once tumors reached a volume of 150-200 mm3 mice were randomized and treated. Mice were maintained and treated in accordance with institutional guidelines of Vall d’Hebron University Hospital Care and Use Committee.

The response was also determined by comparing tumor volume change at time t to its baseline: % tumor volume change = ΔVolt = [100x(Volt – Voli) / Voli] for t = one week after the last treatment. We called this value Best Response and the average at the same t was called Best Average Response.

**Detailed IHC protocol and GLP tissue reactivity study**

To visualize CD205 expression, two IHC protocols were used – one developed at Menarini and one at Oxford BioTherapeutics. The protocol used at Oxford BioTherapeutics is as follows. CD205 IHC was performed on FFPE tumor sections cut at 4um thickness. Sections were deparaffinised and hydrated. Unmasking of antigenic sites was performed in a pressure cooker with Antigen Unmasking Solution pH 9.00 (Vector; H-3301) for 20 min. at high pressure (40 min retrieval required for xenografts). Sections were then incubated with peroxidase suppressing reagent (Thermo; 3500) for 5 minutes at room temperature to quench endogenous peroxidase, followed by 10% normal goat serum (NGS) (Thermo; 31873) for 30 minutes at room temperature to block non-specific protein interactions. Mouse monoclonal primary antibody CD205 (Leica; clone 11A10 [NC-L-DEC205]) and Mouse IgG Isotype (R&D Systems; MAB002) were concentration matched and diluted 1:80 in 10% NGS for 45 minutes. After incubation with primary antibody and isotype, tissue sections were incubated with EnVision+ System- HRP Labelled Polymer Anti-mouse (Dako; K4000). Staining was developed with Liquid DAB+ Substrate Chromogen System (Dako; K3468) for 10 minutes. Slides were counterstained with haematoxylin, dehydrated, and mounted. Sections were then visualized and scored. Menarini CD205 IHC was also performed on FFPE xenograft sections. Sections were deparaffinised and hydrated. Endogenous peroxidase was inhibited by incubation with H2O2 at 3%. Unmasking of antigenic sites was performed in microwave with Target Retrieval Solution pH 9.00 (S2368; Dako) for 6 min. in a microwave oven at 100C and 650 W. Sections were then incubated with mouse monoclonal primary antibody CD205 (clone 11A10 [NC-L-DEC205]; Leica) diluted at 1:80 in Phosphate Buffer Saline – PBS, for 45 min. at room temperature. After incubation with primary antibody, tissue sections were sequentially incubated with link biotinylated secondary antibodies (K5001; Dako REAL TM) and with streptavidin peroxidase (K5001; Dako REAL TM). Staining was developed with Di-amino-benzidine (K5001; DAB+Chromogen x50; Dako REAL TM) + Substrate (K5001; HRP Substrate Buffer; Dako REAL TM). Slides were counterstained with haematoxylin, dehydrated, and mounted. All masses were used also as negative controls and therefore PBS was used in place of primary antibody. To evaluate the immunostaining intensity, each slide was examined on Leica DMRB microscope. IHC scoring was performed using a semi-quantitative method considering the staining intensity of the immunoreactions. Each tumor was given a score according to the intensity of membrane staining as reported in below.

Scoring system

|  |  |  |  |
| --- | --- | --- | --- |
| Strong | Moderate | Weak | Negative |
| 3/+++ | 2/++ | 1/+ | 0 |

The GLP tissue cross reactivity study was conducted at Propath Ltd. (UK). An indirect IHC technique using an HRP detection method and a semi-automated method on the Ventana Discovery XT platform was selected. In summary, the IHC method consisted of the following steps: A) frozen tissue sections were fixed in Zinc formalin for 2 minutes at room temperature and blocked using 3% Hydrogen Peroxide in DPBS for 10 minutes, B) following a series of washes the slides were immunostained using a semi-automated protocol on the Ventana Discovery XT system, C) MEN1309, or the isotype control or buffer were manually applied to tissue sections and left to incubate for 52 minutes, D) an anti-DM4 secondary antibody was then manually applied to tissue sections for 28 minutes, E) the HRP detection reagents were applied following the manufacturer’s recommendations, F) slides were stained with hematoxylin and bluing reagent for 8 and 4 minutes respectively, G) slides were removed from the instrument, washed, dehydrated and mounted in a permanent mounting medium. Five serial sections for each human and cynomolgus monkey tissue were processed for the immunohistochemical assay. The IHC experiment included the following: A) incubation with MEN1309 at the lowest concentration of 0.125μg/mL B) incubation with MEN1309 at the intermediate concentration of 0.5μg/mL C) incubation with MEN1309 at the highest concentration of 2μg/mL D) incubation with the isotype control at the highest concentration of 2μg/mL E) incubation with buffer to replace MEN1309 or the isotype control. Positive control human thymus tissue was included to validate every IHC run and to ensure consistency. Inter-run reproducibility and variability were assessed by the study director through qualitative evaluation and comparison of the staining pattern and intensity in the positive and negative control slides. Slides were evaluated using light microscopy and a semi-quantitative method for the analysis of immunostaining. The number and intensity of cells with immunohistochemical staining was graded accordingly: 0 (-) negative (no stained cells) 1 (+) minimal (rare cells with low staining intensity) 2 (++) slight/moderate (many cells with medium/high staining intensity) 3 (+++) marked (significant numbers of cells with high staining intensity).

**Cross reactivity study of MBH1309/OBT076 in rat, mouse, cynomolgus and human whole blood**

Whole blood from rat and mouse has been supplied by Menarini RTC. Whole blood from cynomolgus monkeys (Macaca fascicularis) has been supplied by Covance Laboratories Gmbh, (Munster, Germany). Human whole blood from healthy donors was collected and analyzed at Dept. of Hematology-Policlinico Tor Vergata, Rome (Italy). Peripheral blood from all species was stored at room temperature before treatment. MBH1309/OBT076 (clone 16A5) was conjugated to Phycoerithrin (PE) at Biolegend, San Diego, CA. On the day of blood collection, blood samples were withdrawn and collected into tubes containing EDTA as anticoagulant. The antigen-binding activity of MBH1309/OBT076 on blood was analyzed by preparing a concentration-response curve with the PE-conjugated antibody in order to assess the saturating concentration on monocytic/DC, granulocytic and lymphocytic lineages. Briefly, 50 µl of mouse peripheral blood were transferred to flow cytometric tubes and incubated, depending on the species, with 5 µl of specific antibodies. Whole blood was incubated for 30 min at +4°C. After this step, red cells were lysed with 2 ml BD lysing solution 1X, for 10 min at room temperature, and then washed three times with staining buffer (PBS + 0.5% BSA). Samples were resuspended in 500 µl staining buffer and acquired on a BD FACSCanto II (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences).

**SUPPLEMENTARY REFERENCES**

1. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 2005; 4:1265-72.