FcRL5 as a Target of Antibody-Drug Conjugates for the Treatment of Multiple Myeloma

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Key words: FcRL5, FcRH5, IRTA2, antibody-drug conjugate, multiple myeloma

Abbreviations: Multiple myeloma (MM), antibody-drug conjugates (ADCs), Fc receptor-like 5 (FcRL5)

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Fc receptor-like 5 (FcRL5/FcRH5/IRTA2/CD307) is a surface protein expressed selectively on B-cells and plasma cells. We found that FcRL5 was expressed at elevated levels on the surface of plasma cells from the bone marrow of patients diagnosed with multiple myeloma (MM). This prevalence in MM and narrow pattern of normal expression suggest that FcRL5 could be a target for antibody-based therapies for MM, particularly antibody-drug conjugates (ADCs), potent cytotoxic drugs linked to antibodies via specialized chemical linkers, where limited expression on normal tissues is a key component to their safety. We found that FcRL5 is internalized upon antibody binding suggesting that ADCs to FcRL5 could be effective. Indeed, we found that FcRL5 ADCs were efficacious in vitro and in vivo but the unconjugated antibody was not. The two most effective consisted of our anti-FcRL5 antibody conjugated through cysteines to monomethylauristatin E (MMAE) by a maleimidocaproyl-valine-citrulline-p-aminobenzylxycarbonyl (MC-vcPAB) linker (anti-FcRL5-MC-vcPAB-MMAE) or conjugated via lysines to the maytansinoid DM4 through a disulfide linker (anti-FcRL5-SPDB-DM4). These two ADCs were highly effective in vivo in combination with bortezomib or lenalidomide, drugs in use for the treatment of MM. These data demonstrate that the FcRL5 ADCs described herein show promise as an effective treatment for MM.
Introduction

Multiple myeloma (MM) is a malignancy of plasma cells characterized by skeletal lesions, renal failure, anemia, and hypercalcemia. It is essentially incurable by current therapies. Current drug treatments for MM include combinations of the proteosome inhibitor bortezomib (Velcade), the immunomodulator lenalidomide (Revlimid), and the steroid dexamethasone. We have been searching for surface targets that could be used to develop antibody-based-therapies for MM. Unfortunately, unmodified antibodies to most surface targets have little if any efficacy. Therefore, in addition to the identification of potential targets, an appropriate technology to enhance the antibody anti-tumor efficacy needs to be identified as well. One approach to making effective antibody therapies is to conjugate the antibodies to cytotoxic drugs via specialized chemical linkers creating antibody-drug conjugates (ADCs). ADCs provide a means to target cytotoxic drugs to neoplastic cells reducing the non-specific systemic effects of the cytotoxic drug while also retaining any efficacy of the antibody (1, 2) and this technology has been applied to MM (reviewed in (3)).

One of the major obstacles to the development of antibody-drug conjugates for the treatment of MM is the selection of a suitable surface antigen. Expression of the target antigen on normal tissues can result in dose-limiting toxicities; thus, tumor specificity of the ADC target is desirable. Such tumor-specific targets are rare in practice, limiting choices to those with highly selective expression in tumor tissues and/or normal tissue expression either at low levels on non-vital normal tissues, or, at a minimum, expression on tissues that are not susceptible to the drug. Clinical experiences with ADCs and naked antibody therapies to B-lineage restricted targets (e.g. CD20 and CD22) that deplete normal B-cells have proven safe and effective (3). In principle, a target that was expressed on normal B-cells and plasma cells as well as MM cells could provide an appropriate target since
the normal cell types can be regenerated and in the case of targeted chemotherapies the normal tissue would be less sensitive to the toxin. The well-characterized B-cell specific antigens are lost when B-cells mature into plasma cells. Surface markers associated with MM such as CD38, CD138, and CD56 have relatively broad expression patterns including normal tissues that may cause target-dependent toxicity (3). However, several ADCs to some of these targets for the treatment of MM are in clinical development (see discussion).

Fc receptor-like 5 (FcRL5 also known as FcRH5 and IRTA2) belongs to a family of six recently identified genes of the immunoglobulin superfamily (IgSF). This family of genes is closely related to the Fc receptors with the conserved genomic structure, extracellular Ig domain composition and the ITIM and ITAM like signaling motifs (4). The ligand(s) for FcRL5 are unknown but FcRL5 has been implicated in enhanced proliferation and downstream isotype expression during the development of antigen-primed B cells (5). The FcRL genes are clustered together in the midst of the classical FcR genes, FcγRI, FcγRII, FcγRIII, and FcεRI, in the 1q21–23 region of chromosome one. This region contains one of the most frequent secondary chromosomal abnormalities associated with malignant phenotype in hematopoietic tumors, especially in MM (6). FcRL5 is expressed only in the B-cell lineage, starting as early as pre-B cells, but doesn’t attain full expression until the mature B-cell stage. Unlike all other B-cell specific surface proteins (e.g. CD20, CD19, and CD22) FcRL5 continues to be expressed in plasma cells while other B-cell specific markers are down-regulated (7). In addition, FcRL5 mRNA is over-expressed in multiple myeloma cell lines with 1q21 abnormalities as detected by oligonucleotide arrays (8). The expression pattern suggests that FcRL5 could be a target for antibody-based therapies for the treatment of MM. Here we show that FcRL5 has a high prevalence on the surface of MM, validate it as a target for the use of ADCs, characterize two ADCs that have the potential to be used in humans, and demonstrate that these anti-FcRL5 ADCs increase the effectiveness of current MM therapies in xenograft preclinical models.
Material and Methods

Antibodies

Antibodies to FcRL5 were generated and characterized as previously described (7). Antibody cross-reactivity to Cynomolgus monkey (cyno) FcRL5 was tested by flow cytometry using a stably transfected cell line expressing cyno FcRL5 (the clone a kind gift from Sothy Yi). Both Anti-FcRL5(13G9) and Anti-FcRL5(10A8) cross reacted to cyno FcRL5 and were humanized as previously described (9).

Cell lines

Because cultured MM cell lines down-regulate FcRL5, transgenic stable cell lines expressing human and cyno FcRL5 were established. The EJM and OPM2 MM cell lines (no authentication was done by the authors) were transfected with human FcRL5 using the Amaxa Nucleofector™ system. After puromycin selection the EJM pool was sorted for human FcRL5 expression by flow cytometry (Epics Elite; Beckman Coulter) resulting in the EJM-CMV.PD.FcRL5.LSP.2 (EJM-FcRL5) cell line, and the OPM2 pool was sorted for human FcRL5 expression using the MACS® Separation System (Miltenyi Biotec) resulting in the OPM2-CMV.PD.FcRL.SP.2 (OPM2-FcRL5) cell line. The SVT2 cell line was transfected with cyno FcRL5 using Lipofectamine™ 2000 (Invitrogen™). After G418 selection the SVT2 pool was sorted for cyno FcRL5 by flow cytometry resulting in the SVT2.MSCV.gD.cyFcRL5.SP.2 cell line.

Cell viability assay

The in vitro efficacy of anti-FcRL5(13G9) ADCs was determined using an ADC dosing titration on OPM2-FcRL5. Prior to ADC addition, cells were plated in quadruplicate at 75 x 10^3 per well in 384-well plates in RPMI containing 10 % fetal bovine serum and allowed to attach overnight. Anti-FcRL5(13G9) ADCs or the control anti-GP120 ADCs were added to experimental wells to final
concentration of 10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.014, 0.0046 or 0.0015 µg/ml, with "non-drug conjugate" control wells receiving medium alone. After 72 h incubation at 37 °C, cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp., Madison, WI). The concentration of anti-FcRL5(13G9) ADCs resulting in the 50% inhibition of cell viability was calculated from a four-variable curve analysis.

**Scatchard analysis**

To determine binding affinity (Kd), 0.5 nM ¹²⁵I labeled Hu Anti-FcRL5(10A8) was competed against unlabeled Hu Anti-FcRL5(10A8) respectively, ranging from 50 to 0.02 nM (12 step 1:2 serial dilution) in the presence of OPM2-FcRL5 or SVT2-MSCV.gD.cyFcRL5.SP.2 cells. After incubation at 4 °C for four hours, cells were washed, and cell pellet counts read by a gamma counter (1470 WIZARD Automatic Gamma Counter; Perkin Elmer). All points were carried out in triplicate and counted for 10 min. The average CPM were used for Kd calculation using the New Ligand (Genentech, South San Francisco, CA) program. Binding affinity for Hu Anti-FcRL5(13G9) was determined using the same protocol.

**Flow cytometry**

Bone marrow aspirates were collected from patients during their initial presentation for diagnosis of a suspected plasma cell disorder or follow up assessment. FcRL5 expression levels were determined on plasma cells within bone marrow aspirates from patients diagnosed as having multiple myeloma (MM, n=16) or monoclonal gammopathy of uncertain significance (MGUS, n=11). Additional data was collected from seven subjects that were ultimately diagnosed as having no evidence of bone marrow lymphoma (normal controls).

Leucocytes from bone marrow aspirates were prepared via ammonium chloride lysis of erythrocytes. In summary, a volume of the sample (between 0.5
and 1.5ml) was incubated with a 10-fold excess of ammonium chloride (8.6g/l in distilled H₂O, Vickers Laboratories, Pudsey, UK) for 5 minutes at 37°C. The leukocytes were then washed twice in buffer (FACSFlow (BD Biosciences, Oxford, UK) containing 0.3% bovine serum albumin (Sigma, UK). Leukocytes were stained with cocktails of Abs for 20 minutes at 4°C and washed twice in buffer prior to acquisition on a Canto II instrument (BD Biosciences). A minimum of 100,000 events were collected per sample. Fluorescently conjugated antibodies were used in combinations against the following markers: CD45 Pacific Orange (HI30, Invitrogen), CD20 Pacific Blue (B9E9, Beckman Coulter), CD19 PE-Cy7 (J3-119, Beckman Coulter), CD38 APC-AF750 (LS198-4-3, Beckman Coulter), CD138 APC (B-B4, Miltenyi), FcRL5 PE (10A8, mouse anti-human FcRL5, Genentech Inc), and CD56 PE (MY31, BD Biosciences).

Data analysis was carried out using FACSDiva software. Plasma cells were defined by strong CD138 and CD38 expression, CD45 Lo with light scatter characteristics of large mononuclear cells. Pan B-cells were identified as CD19+CD138-, CD45 Hi with lymphoid light scatter characteristics. The Median Fluorescence Intensity (MFI) for each antibody was derived for both cell populations: total plasma cells and pan B-cells. MFIs for FcRL5 were normalized by subtraction of the MFI of the appropriate conjugated isotype antibody (CD34PE) in Tube 2 within the same gated population. The isotype antibodies used in the panel were specific to non-B cell markers.

**Internalization studies**

OPM2 wild type or OPM2-FcRL5 were incubated on ice for 1h in complete carbonate-independent medium (Gibco, Carlsbad, CA) or at 37°C in growth media containing 3 µg/ml murine or humanized anti-FcRL5(10A8) or isotype controls (murine anti-gD tag or Trastuzumab hlgG1 respectively) and lysosomal
protease inhibitors (10 µg/ml leupeptin and 5 µM pepstatin (Roche; Indianapolis, IN). Cells were then washed twice, fixed with 3% paraformaldehyde (Electron Microscopy Sciences), quenched with 50 mM NH₄Cl (Sigma; St. Louis, MO), permeabilized with 0.4% Saponin/2% FBS/1% BSA, then incubated with 2 µg/ml Cy3 anti-mouse or anti-human (Jackson Immunoresearch; West Grove, PA). Where indicated, lysosomes were co-stained with 1:1000 mouse anti-human LAMP1 (BD Biosciences; San Jose, CA) and detected with FITC-anti-mouse (Jackson Immunoresearch; West Grove, PA). Cells were resuspended in 20 µl carbonate-independent medium and adhered to poly-lysine (Sigma; St. Louis, MO) coated slides prior to mounting coverslips in DAPI-containing VectaShield (Vector Laboratories; Burlingame, CA). Slides were imaged by epifluorescence microscopy using a 100x objective on a DeltaVision (Applied Precision LLC, Issaquah, WA) microscope powered by SoftWoRx (version 3.4.4) software. Figures were compiled using Photoshop CS (Adobe Systems, Inc., San Jose, CA).

**Antibody conjugation**

MCC-DM1 (Immunogen, Inc), MC-MMAF (Seattle Genetics, Inc), and MC-vc-PAB-MMAE (Seattle Genetics, Inc) ADCs were made as previously described (10, 11). N-succinimidyl-3-(2-pyridylthio)-butyrate-DM4 (SPDB-DM4) (Immunogen, Inc) conjugates were prepared using purified antibody in 50 mM potassium phosphate pH 7.5 containing 50 mM NaCl and 2 mM EDTA. SPDB was dissolved in 100 % ethanol and added to the protein solution in ratios of 6 to 8 moles of SPDB to 1 mole of antibody to achieve a final drug:antibody ratio of 3-4:1. The linker conjugation reaction was carried out at RT for 90 minutes followed by the addition of 1.7 fold excess DM4 over SPDB and an overnight reaction. The conjugates were purified using gel filtration, ion exchange chromatography or dialysis.
Animal studies

All animal studies were performed in compliance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Genentech, Inc.

To establish subcutaneous xenograft models, the tumor cells (2x10^7 cells in 0.2 mL Hank's Balanced Salt solution; Hyclone) were inoculated subcutaneously into the flanks of female CB17 ICR SCID mice (7-16 weeks of age from Charles Rivers Laboratories; Hollister, CA). When mean tumor size reached desired volume, the mice were divided into groups of 7-10 mice with the same mean tumor size and dosed intravenously (IV) via the tail vein with ADCs or antibodies.

Bortezomib (Velcade) was obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA), and dissolved in 0.9% sodium chloride at the appropriate concentration before drug injection. Mice were dosed by intravenous injection at 1 mg/kg twice weekly for two weeks.

Lenalidomide (Revlimid) was supplied by ECA International Corporation (Palatine, IL) and dissolved in DMSO. Immediately before use, lenalidomide was further diluted in 0.5% methylcellulose and 0.2% Tween 80, and administered intraperitoneally (IP) into mice at 50 mg/kg once a day for 13 days.

The SCID-rab model was performed as previously described (12). To establish SCID-rabbit bone model, the tibia, humerus, ulna and radius were harvested from New Zealand White rabbits (3-4 weeks of age from Myrtle Rabbitry; Thompson Station, TN), and fractioned into fragments (1-2 cm^3 size). The bone fragments were then surgically engrafted into the flanks of female CB17 ICR SCID mice (6-8 weeks of age from Harlan Sprague Dawley; Indianapolis, IN). At 6-8 weeks post bone engraftment, the LD cells (13) (1x10^6 cells in 50 µl phosphate-buffered solution) were injected directly into the engrafted bone. The LD line was maintained by serial passage because of poor in vitro growth.
samples were taken periodically to assess tumor burden by measuring the amount of human lambda light chain in serum. When the amount of human lambda light chain reached the desired serum level, mice were randomized into groups of 8-10 mice with the same mean serum concentration and received weekly intravenous injections of ADCs for two weeks.

RESULTS

Expression of FcRL5 in Multiple Myeloma

FcRL5 expression levels were assessed on B-cells and plasma cells within bone marrow aspirate samples from patients diagnosed with either MM (n=16) or MGUS (n=11) and compared to plasma cells (n=7) from normal control cases. FcRL5 expression was present on all plasma cells, however expression levels differed among groups. As shown in Figure 1A, the expression levels of FcRL5 appeared to be quite variable and elevated on bone marrow plasma cells derived from both MM and MGUS patients with MFI s ranging from 191-10654 and 490-6477 respectively compared to 289-897 on normal plasma cells from control cases. The expression levels were > 3 fold higher within the MM and MGUS groups versus normal plasma cells, with the group means being 1956 (+ 2456) on MM, 1860 (+1823) on MGUS and 559 (+195) on normal plasma cells respectively. These differences between MM or MGUS patients and normal controls were statistically significant, with the prob>chi squared values being 0.0092 and 0.0164 respectively as determined using the Wilcoxin (Kruskal-Wallis) test. Within the same subjects we found that CD19+ B-lymphocytes had either low or barely detectable FcRL5 expression across all subjects tested (data not shown).

Furthermore, it appears that FcRL5 staining in combination with CD38 staining can identify plasma cells or multiple myeloma cells as effectively as the
CD38/CD138 combination, currently used. The percentages of cells found in the CD138/CD38 gate were replicated with the CD38/FcRL5 and the CD138/FcRL5 combinations (Figure 1B). Furthermore, in samples that are CD138^{Hi} CD38^{Hi}, 100% of the cells from the FcRL5/CD38^{++} population were also CD138^{Hi}, indicating that the FcRL5/CD38 marker combination does not pick up non-plasma cells (Figure 1B). These observations suggest that addition of FcRL5 to the current CD138 and CD38 plasma cells markers could only improve the diagnostic sensitivity and accuracy of MM cell detection especially with aberrant immunophenotypes or should technical difficulties interfere with their detection.

One of the major obstacles of immunotherapy for multiple myeloma, especially with antibody-drug conjugates, is the selection of a suitable surface antigen. The fact that FcRL5 is elevated in MM or MGUS compared to normal plasma cells and that its levels are low on pan B-cells, render FcRL5 an excellent target for immunotherapy.

Validation of FcRL5 as an ADC target for the treatment of MM

The expression pattern of FcRL5 suggested that it would be a good target for the use of antibody or ADC therapy for the treatment of MM. To validate this hypothesis, we sought to identify cell lines where we could test potential anti-FcRL5 therapies. We identified 10 cell lines that expressed FcRL5 RNA but found none that express significant amounts of surface FcRL5. We have observed a similar down-regulation of other FcRL family proteins on cultured cells compared to the primary tissue expression levels (data not shown). To circumvent this problem we developed two transgenic MM cell lines, OPM2-FcRL5 and EJM-FcRL5, which stably express surface FcRL5 (see methods). During growth both in vitro and as xenograft models, OPM2-FcRL5 and EJM-FcRL5 cells express levels of surface FcRL5 that are equivalent to the higher end and the mean levels of expression found in MM patient cells respectively (data not shown).
Internalization upon antibody binding can be a key feature that determines whether a target will be more suited to a naked antibody approach, that uses antibody-dependent cell cytotoxicity (ADCC) and requires an extended duration of antibody binding on the cell surface, versus an ADC therapy where internalization to facilitate drug delivery is desirable. Anti-FcRL5 antibody bound specifically to OPM-FcRL5 cells versus controls (Figure 2A) and was internalized within two hours (Figure 2B). By 13 hours, anti-FcRL5 was almost completely delivered to lysosomes, as detected by colocalization with LAMP1 (Figure 2C-E). Uptake was specific, since an isotype control antibody gave no signal (Figure 2C-E insets) and no anti-FcRL5 signal was seen in OPM2 cells lacking FcRL5 (insets in A, B and data not shown). These data demonstrate that anti-FcRL5 is well internalized upon binding, and thus has properties that would make it an appropriate target for ADCs.

We sought to further test our hypothesis that FcRL5 would make a good target for ADCs by testing the effectiveness of four ADC formats all of which use potent inhibitors of microtubule polymerization as the active drug. We tested maytansinoid conjugates linked to the antibody through the ε-amino group of lysine with an uncleavable thioester linker (MCC-DM1) or a hindered disulfide reducible linker (SPDB-DM4). We also tested the monomethylauristatins linked to antibody cysteines with an uncleavable maleimidocaproyl (MC) linker (MC-MMAF) and a protease cleavable linker (MC-vc-PAB-MMAE) (see (14) and Figure 3) for detailed descriptions of these linker-drugs).

The anti-FcRL5-MC-vc-PAB-MMAE, anti-FcRL5-MC-MMAF, anti-FcRL5-SPDB-DM4 ADCs had similar efficacy in vitro, while anti-FcRL5-MCC-DM1 appeared less potent in comparison (Figure 4A). In vivo, only the cleavable linker ADCs, namely anti-FcRL5-MC-vc-PAB-MMAE and anti-FcRL5-SPDB-DM4 showed strong anti-tumor efficacy (Figure 4B), however, all the FcRH5 conjugates showed significant activity vs. vehicle and its corresponding control conjugates. The activity of the FcRH5 conjugates is also significantly different from each other (Figure S1). Unconjugated anti-FcRL5 antibodies did not have any efficacy in vitro or in vivo (Figure 5A and data not shown), consistent with
their rapid internalization and lack of ADCC induction. These data suggest that FcRL5 is a potential target for the treatment of MM with ADCs containing cleavable linkers.

**Generation of anti-FcRL5 ADCs suitable for treatment of humans.**

To generate ADCs suitable for the treatment of humans we tested our anti-FcRL5 antibodies for cross reactivity to cynomolgus monkeys with the goal of having FcRL5 ADCs that would be suitable for target-dependent safety studies in non-human primates (NHPs). We found that both anti-FcRL5(13G9) and anti-FcRL5(10A8) cross-reacted with cynomolgus monkey FcRL5 with high affinity (data not shown). Anti-FcRL5(13G9) and anti-FcRL5(10A8) were humanized using previously described methods to generate anti-FcRL5(hu13G9) and anti-FcRL5(hu10A8) (9). These humanized antibodies that cross reacts to cynomolgus monkey could serve as a basis for anti-FcRL5 antibody therapies to be tested in NHP for safety and subsequently used in humans in the clinic.

Next, we conjugated anti-FcRL5(hu10A8) to SPDB-DM4 and MC-vc-PAB-MMAE and tested the two resultant ADCs at several dose levels in our two xenograft models. In the OPM2-FcRL5 model a single dose of anti-FcRL5(hu10A8)-MC-vc-PAB-MMAE or anti-FcRL5(hu10A8)-SPDB-DM4 resulted in complete tumor remission (Figure 5A and 5B). Substantial responses included complete remissions with anti-FcRL5(hu10A8)-MC-vc-PAB-MMAE in the EJM-FcRL5 model (Figure 5C and 5D). In contrast, the unconjugated anti-FcRL5(hu10A8) or the negative control Trastuzumab ADCs had very little to no effect on the tumor growth, which indicates that the activity seen with the anti-FcRL5 ADCs was due to the target-specific delivery of cytotoxic drugs to the tumors, rather than ADCC. Interestingly, the EJM-FcRL5 model responded better than OPM2-FcRL5 to the FcRL ADCs despite having a lower expression level of FcRL5. In addition, anti-FcRL5-SPDB-DM4 was more effective than anti-FcRL5-MC-vc-PAB-MMAE in the OPM2-FcRL5 model whereas their efficacy was switched in the EJM-FcRL5 model. These data demonstrate that anti-FcRL5
ADCs have excellent preclinical efficacy in xenograft models of MM and that even in cases where one anti-FcRL5 ADC is not effective another one can be.

To further explore the effectiveness of our anti-FcRL5 ADCs we tested anti-FcRL5-SPDB-DM4 in a xenograft model that better recapitulates the biology of MM cells, such as the stromal-MM cell interactions and naturally expressed FcRL5. We used a SCID-rab model in which bone grafts were established subcutaneously in SCID mice and subsequently injected with LD tumor cells (12, 13). LD cells express FcRL5 and are dependent on stromal cells for survival. When co-cultured in vitro with stromal cells, LD cells could be maintained, but lost their FcRL5 expression, which led us to maintain them by serial passage in the in vivo SCID-rab bone MM model. Since the cells secrete Iga1, we monitored tumor growth by using ELISA for human lambda light chain (hulg). Once the average serum hulg concentration reached 594 ng/ml, the mice were grouped out and treated. Mice treated with vehicle and control ADC continued to have an increase in serum hulg, whereas the anti-FcRL5-SPDB-DM4 treated mice showed a reduction in the serum human Ig that later increased after treatment stopped (Figure 6). These data show that even in a higher bar in vivo model of MM, supported by host stromal interactions, anti-FcRL5 ADCs can be effective.

Combining ADC with current drug treatments of MM

MM is a difficult disease to treat; it is incurable and no single new treatment is likely to cure patients. Thus, it is important to establish whether anti-FcRL5 ADCs could combine with current therapies to improve treatment outcomes. We tested our two anti-FcRL5 ADCs in combination with bortezomib in the OPM-2-FcRL5 xenograft model. Biweekly doses of 1 mg/kg bortezomib (the maximum tolerated dose in this model) had similar efficacy as a single dose of 4 mg/kg of the anti-FcRL5-SPDB-DM4 or anti-FcRL5-MC-vc-PAB-MMAE, which slowed tumor growth without substantially regressing the tumors. However the
combination of anti-FcRL5-SPDB-DM4 and bortezomib resulted in 8 out of 9 complete tumor remissions (Figure 7A). Bortezomib plus anti-FcRL5-SPDB-DM4 showed significant activity vs. bortezomib alone (p< 0.0001, Figure S2), while bortezomib plus control-SPDB-DM4 did not (p=0.081). Anti-FcRL5-MC-vc-PAB-MMAE plus bortezomib resulted in 3 out of 9 complete tumor remissions (Figure 7B). Anti-FcRL5-MC-vc-PAB-MMAE alone showed same activity as bortezomib alone (p=0.7383). The combination was significantly more efficacious than either single agent alone (p< 0.0001, Figure S3). In addition, we tested the combination of anti-FcRL5-MC-vc-PAB-MMAE and lenalidomide in the OPM2-FcRL5 xenograft model. Thirteen daily doses of 50 mg/kg lenalidomide (the maximum tolerated dose in this model) had a similar efficacy to a single dose of 6 mg/kg anti-FcRL5-MC-vc-PAB-MMAE (Figure 7C); in both cases tumor growth slowed but did not substantially regress the tumors. The combination at the same doses regressed the tumors resulting in 8 of 8 tumors with partial remission. Anti-FcRL5-MC-vc-PAB-MMAE alone showed same activity as lenalidomide alone (p=0.5453) and the combination was more effective than either single agent alone (p<0.0001, Figure S4).

Mice receiving single-agent anti-FcRL5 ADCs continued gaining weights over time, tracking along with the vehicle group. In contrast, mice receiving bortezomib or lenalidomide had up to 5-10% weight loss. The combination groups also had 5-10% weight loss, indicating anti-FcRL5 ADCs combined well with bortezomib or lenalidomide, and did not appear to exacerbate the weight loss caused by bortezomib or lenalidomide.

These data demonstrate that anti-FcRL5 ADCs when used in combination with current treatments can lead to substantially improved therapeutic outcomes.
Discussion

In this work we have examined FcRL5 as a target for antibody based therapies. Our data characterizing FcRL5 as a marker suggest that addition of FcRL5 to the current CD138 and CD38 plasma cell markers can further improve the diagnostic sensitivity and accuracy of MM cell detection, especially in cases with aberrant immunophenotypes or when technical difficulties interfere with detection.

We detected FcRL5 expression on plasma cells from the vast majority of patients with multiple myeloma, which, combined with its restricted expression pattern and prevalence led us to the conclusion that FcRL5 could be a target for antibody-based therapies for MM. One aspect of FcRL5 biology that could slightly complicate the use of anti-FcRL5 antibody therapies is that there is a soluble form of FcRL5 in circulation that is elevated in MM patients (15). This circulating form of FcRL5 could interfere with antibody-based therapies at low doses of antibody therapy or in the first few treatments.

Our results suggest that, while unmodified anti-FcRL5 antibodies are not effective therapeutically, anti-FcRL5 ADCs show promising antitumor efficacy in multiple preclinical models. We report two anti-FcRL5 ADCs suitable for further testing in NHPs and use in humans. The linker-drugs we have used, MC-vc-PAB-MMAE and SPDB-DM4 are showing promise in the context of other ADCs in early clinical trials. Brentuximab vedotin (SGN-35, anti-CD30-MC-vc-PAB-MMAE) is currently under development for the treatment of Hodgkin Lymphoma (16) and CDX-011 (Anti-GPNMB MC-vc-PAB-MMAE) for the treatment of breast cancer and melanoma. In both cases the maximum tolerated dose was ~1.8 mg/kg every three weeks with the primary dose-limiting toxicities being diarrhea and leucopenia and, in the case of CR011, target-mediated skin toxicities (17). nBT062 (anti-CD138-SPDB-DM4) is being tested for the treatment of MM. CD138 is found on basolateral surface of epithelial cells, vascular smooth muscle cells, and the endothelium ((18) and references therein). nBT062 has promising preclinical activity in xenograft models of MM (19, 20) and based on these data a phase 1 clinical trial was started where patients were treated every three weeks.
at seven dose levels ranging from 10 mg/m\(^2\) to 200 mg/m\(^2\). Skin, eye, and gastrointestinal tract are the target organs for toxicity at high doses (21). The ocular toxicity is probably not target dependent as this has been observed with other ADC containing the SPDB-DM4 linker-drug (for example SAR3419, (22)). However, the skin and GI toxicity could be due to target expression in these tissues. In the 20 evaluable patients, 7 showed clinical benefit and 2 showed an objective response.

In addition to nBT062, an ADC targeting CD56, IMGN901 (huN901-DM1), is in Phase 1 clinical trials. IMGN901 (huN901-DM1) is a humanized anti-CD56 antibody with a linker cleavable by disulfide reduction (SPP) attached to the maytansinoid DM1, and it is also being tested in Phase 1 trials. CD56 is expressed in NK cells, a subpopulation of T-lymphocytes, neural tissue, and muscle. Although CD56 is not found in normal plasma cells it is expressed in 70% of MM (23). In the current Phase 1 trial, CD56\(^+\) relapsed or refractory MM patients received a single intravenous (IV) infusion of IMGN901 on 2 consecutive weeks every 3 weeks at doses ranging from 40 to 140 mg/m\(^2\). Headache, fatigue, and neuropathy were the major adverse events and one of 25 patients showed a partial response. That patient had been on treatment for over a year (24).

FcRL5 offers advantages over other ADC targets for the treatment of MM since the normal tissue expression of FcRL5 is limited to B-cells and plasma cells and it is found on most if not all MM. A broader expression pattern can lead to damage of normal tissues and more rapid clearance of the ADC due the antigen acting as a sink, limiting the therapeutic potential of the ADC. FcRL5-ADCs can overcome these problems and deliver the therapeutic agent selectively to the tumor, thus increasing therapeutic index. The promising clinical data with other ADCs using MC-vc-PAB-MMAE and SPDB-DM4, combined with our preclinical efficacy data in several MM preclinical models clearly demonstrate that both anti-FcRL5-MC-vc-PAB-MMAE and anti-FcRL5-SPDB-DM4 are potentially effective ADCs for the treatment of MM, both as single agents and especially in
combination with currently approved MM therapeutics.

Acknowledgements
We thank Cam Adams for the humanization of 10A8, Sothy Yi for cloning Cynomolgus FcRL5, and David Dornan for helpful advice and logistics.


Figure 1.
FcRL5 expression on plasma cells. A) FcRL5 is expressed on the surface of bone marrow resident plasma cells of MM or MGUS patients and normal subjects. FcRL5 expression levels were assessed by flow cytometry on plasma cells from bone marrow aspirates of MM (n=16) or MGUS cases (n=11) and controls (n=7). All MFIs were normalized by subtraction of the isotype control MFIs. B) FcRL5 can be used to identify plasma cells. FcRL5 expression was measured by flow cytometry using a conjugated murine anti-human FcRL5 monoclonal antibody. Plasma cell populations were gated using FSc/SSc and CD38, CD138, CD45, CD19 markers. Combinations of FcRL5 with CD138 or CD38 are shown as compared to the CD38/CD138 combination. The percentages of CD45lo plasma cells are shown in each combination from both a representative multiple myeloma patient (MM) and a normal control subject (Normal). The horizontal line indicates the sample median the box and lines represent the 25th and 75th quartiles respectively.

Figure 2
Anti-FcRL5 is internalized upon cell binding. Anti-FcRL5(m10A8) surface binding (A) or 2h uptake at 37°C (B) in FcRL5-OPM2 cells (insets show lack of signal in OPM2 wild-type cells), detected with Cy3-anti-mouse. C-E) Following 13h internalization of h10A8 (or isotype control, insets) detected with Cy3-anti-human (C and red channel in merge), OPM2-FcRL5 cells were co-stained with the lysosomal marker mouse anti-LAMP1 (D and green channel in merge) followed by FITC anti-mouse. Colocalization is evident from the yellow color in the merged panel (E), a subset of which is highlighted with arrows. Scale bar is 10 µm.

Figure 3. Structures of key small molecules. (A) Structure of MC-vc-PAB-MMAE attached to an interchain disulfide bond and (B) SPDB-DM4 attached antibody lysine. The drug to antibody ratio for the ADCs was 3 to 4. Only one linker drug is shown for clarity. (C) Structure of Bortezomib (Velcade). (D) Structure of Lenalidomide (Revlimid).
Figure 4. Efficacy of anti-FcRL5 ADCs with different linker-drugs. (A) In vitro killing of OPM2-FcRL5 cells with unconjugated anti-FcRL5(13G9) and anti-FcRL5(13G9) ADCs conjugated to different linker-drugs as indicated. (B) In vivo efficacy of OPM2-FcRL5 tumors with an average starting volume of 215 mm$^3$. Groups of 7 mice were IV dosed two times as indicated (arrows) with 10 mg ADC/kg mouse. Anti-gp120 is an isotype control antibody conjugated to the various linker-drugs a similar manner to anti-FcRL5. Error bars represent standard deviation.

Figure 5. Efficacy of anti-FcRL5(hu10A8) ADCs at various dose levels. (A) Anti-FcRL5(hu10A8)-MC-vc-PAB-MMAE inhibited the growth of OPM2-FcRL5 tumors with an average starting volume of 190 mm$^3$. Groups of 9 mice were IV dosed once as indicated (arrow) with 1, 3, 6, 9, or 18 mg ADC/kg mouse. (B) Anti-FcRL5(hu10A8)-SPDB-DM4 inhibited the growth of OPM2-FcRL5 tumors with an average starting volume of 185 mm$^3$. Groups of 9 mice were IV dosed once as indicated (arrow) with 0.5, 1, 2, 4, 8, or 12 mg ADC/kg mouse. (C) Anti-FcRL5(hu10A8)-MC-vc-PAB-MMAE inhibited the growth of EJM-FcRL5 tumors with an average starting volume of 134 mm$^3$. Groups of 8 mice were IV dosed once as indicated (arrow) with 1, 2, 4, or 8 mg ADC/kg mouse. (D) Anti-FcRL5(hu10A8)-SPDB-DM4 inhibited the growth of EJM-FcRL5 tumors with an average starting volume of 130 mm$^3$. Groups of 8 mice were IV dosed once as indicated (arrow) with 2, 4, or 8 mg ADC/kg mouse. Error bars represent standard deviation.

Figure 6. Anti-FcRL5-SPDB-DM4 is effective in the SCID-rab/LD model of MM. Anti-FcRL5-SPDB-DM4 treated mice showed reducing human Ig serum level in
the SCID-rab LD model with an average starting hulg concentration of 715 ng/ml. Groups of 6-8 mice were IV dosed two times as indicated (arrows) with 10 mg ADC/kg mouse.

Figure 7. Anti-FcRL5 ADCs showed greater activity when combined with standard of care therapeutics. OPM2-FcRL5 cell line (2 x 10^7) was injected into mice, and the resulting tumors were treated with (A) a single dose of 4 mg anti-FcRL5-SPDB-DM4/kg mouse or twice weekly doses as indicated (arrows) of 1 mg bortezomib/kg mouse as single-agent or in combination. Average starting volume was 306 mm^3 for the groups of 9 mice. (B) a single dose of 4 mg anti-FcRL5-MC-vc-PAB-MMAE/kg mouse or twice weekly doses as indicated (arrows) of 1 mg bortezomib/kg mouse as single-agent or in combination. Average starting volume was 331 mm^3 for the groups of 9 mice. (C) a single dose of 6 mg anti-FcRL5-MC-vc-PAB-MMAE/kg mouse and daily doses as indicated (line) of 50 mg lenalidomide/kg mouse as single-agent or in combination. Average starting volume was 446 mm^3 for the groups of 8 mice. Error bars represent standard deviation.
Figure 1

**A**

![Box plots showing FCRL5 expression (normalized MFI)](image)

- MGUS (n=11)
- MM (n=16)
- Normal (n=7)

**B**

![Flow cytometry plots for CD38, CD138, and FCRL5 expression in MM and Normal samples](image)

- CD38
- CD138
- FCRL5

*Figure 1*
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Molecular Cancer Therapeutics

FcRL5 as a Target of Antibody-Drug Conjugates for the Treatment of Multiple Myeloma

Kristi Elkins, Bing Zheng, MaryAnn Go, et al.

Mol Cancer Ther Published OnlineFirst July 17, 2012.

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