CS1, a Potential New Therapeutic Antibody Target for the Treatment of Multiple Myeloma

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Abstract

Purpose: We generated a humanized antibody, HuLuc63, which specifically targets CS1 (CCND3 subset 1, CRACC, and SLAMF7), a cell surface glycoprotein not previously associated with multiple myeloma. To explore the therapeutic potential of HuLuc63 in multiple myeloma, we examined in detail the expression profile of CS1, the binding properties of HuLuc63 to normal and malignant cells, and the antimyeloma activity of HuLuc63 in preclinical models.

Experimental Design: CS1 was analyzed by gene expression profiling and immunohistochemistry of multiple myeloma samples and numerous normal tissues. HuLuc63-mediated antimyeloma activity was tested *in vitro* in antibody-dependent cellular cytotoxicity (ADCC) assays and *in vivo* using the human OPM2 xenograft model in mice.

Results: CS1 mRNA was expressed in >90% of 532 multiple myeloma cases, regardless of cytogenetic abnormalities. Anti-CS1 antibody staining of tissues showed strong staining of myeloma cells in all plasmacytomas and bone marrow biopsies. Flow cytometric analysis of patient samples using HuLuc63 showed specific staining of CD138+ myeloma cells, natural killer (NK), NK-like Tcells, and CD8+ Tcells, with no binding detected on hematopoietic CD34+ stem cells. HuLuc63 exhibited significant *in vitro* ADCC using primary myeloma cells as targets and both allogeneic and autologous NK cells as effectors. HuLuc63 exerted significant *in vivo* antitumor activity, which depended on efficient Fc-CD16 interaction as well as the presence of NK cells in the mice.

Conclusions: These results suggest that HuLuc63 eliminates myeloma cells, at least in part, via NK-mediated ADCC and shows the therapeutic potential of targeting CS1 with HuLuc63 for the treatment of multiple myeloma.

Multiple myeloma is a malignant disease of plasma cells, occurring in adults with an incidence of ~14,000 new cases per year in the United States. The median survival from diagnosis is ~3 years (1, 2). Despite advances in therapy including stem cell transplantation and new biological agents, such as bortezomib and immunomodulatory agents, multiple myeloma is still considered an incurable disease (2–6). Thus, new therapies are needed.

Monoclonal antibody (mAb) therapy has made a major effect in the area of B-cell non–Hodgkin lymphoma therapy. In particular, anti-CD20 (rituximab) has become a standard therapeutic agent in B-cell lymphomas and has improved outcome in these diseases (7–17). However, no effective immunotherapeutic option exists yet in multiple myeloma. In an effort to develop new immunotherapeutics for the treatment of multiple myeloma, we identified CS1 (CD2 subset-1, CRACC, SLAMF7, and CD319), a member of the signaling lymphocyte activating-molecule–related receptor family (18), as a cell surface antibody target selectively expressed in plasma cells. Other members of the signaling lymphocyte activating-molecule–related receptor family include signaling lymphocyte activating–molecule (CD150), 2B4 (CD244), CD84, NTB-A (Ly-108), and Ly-9 (CD229; ref. 19). These molecules are characterized by two or four extracellular immunoglobulin (Ig)-like domains and an intracellular signaling domain with immune receptor tyrosine-based switch motifs with the consensus amino acid sequence TxYxxV/I (20, 21).

In this study, we show that normal plasma cells and multiple myeloma cells express high levels of CS1 mRNA and protein. Other normal lymphocytes subsets [natural killer (NK), NK-like T cells, CD8+ T cells, activated monocytes, and dendritic cells]

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also express CS1, albeit at generally lower levels than plasma cells. We generated a panel of murine and humanized mAbs to human CS1 to validate this protein as a potential target for the treatment of multiple myeloma. We show that the humanized anti-CS1 mAb HuLuc63 has potential as an immunotherapeutic by binding to multiple myeloma cells and mediating antibody-dependent cellular cytotoxicity (ADCC) with effector cells isolated from multiple myeloma patients. In addition, HuLuc63 significantly decreased established tumors *in vivo* in a myeloma xenograft model, an activity that seemed to be dependent on the presence of functional NK cells.

Materials and Methods

Identification of CS1 and gene expression profiling. CS1 was identified using representational difference analysis done by subtracting naïve B-cell ($CD19^+IgD^+CD38^{int/-}$ $CD27^-$) cDNA from a memory B cell and plasma cell ($CD19^{+/lo}$ $IgD^-CD38^{int/-}CD27^+$) cDNA library (22, 23). Using standard molecular biology techniques, the cDNA subtraction library was ligated into a standard plasmid vector and transformed into electrocompetent *Escherichia coli* (DH-10B) cells. Single bacterial colonies, each representing one specific insert, were amplified using standard colony PCR. The cDNA for CS1 was identified to be preferentially expressed in the memory B cell and plasma cell cDNA library. To confirm expression of CS1 in normal plasma cells and examine expression in diseased plasma cells from patients with monoclonal gammopathies of undetermined significance and multiple myeloma, gene expression profiling was done on CD138-purified plasma cells as described by Zhan et al. (24). Gene expression profiling of nonmalignant adult tissues and cells was done using the Eos Hu03, a customized Affymetrix GeneChip, as previously described (25).

Tissue specimens and cell lines. This study was done with approval of the Institutional Review Boards of the Cleveland Clinic and University of Arkansas for Medical Sciences with informed consent. Frozen and paraffin-embedded tissues were obtained from the archives of the Division of Pathology and Laboratory Medicine of the Cleveland Clinic. Frozen tissues were maintained at -80°C. Tissue microarrays were constructed from representative tumor areas using a manual tissue microarrayer (Beecher Instruments) with two or three cores per case (1.5 or 1.0 mm cores). Multiple myeloma cell lines (L363 and OPM2) were obtained from German Collection of Microorganisms and Cell Cultures. Fresh normal donor blood, blood/bone marrow samples from multiple myeloma patients, and excess stem cell apheresis samples were used for flow cytometry and antibody dependent cellular cytotoxicity studies.

Antibodies. Female BALB/c mice (Taconic Farms) were immunized with purified CS1 protein using standard immunization schedules. mAbs were generated by standard techniques with spleen cells being fused to the P3X63Ag8 fusion partner (American Type Culture Collection). Anti-CS1 specific mAbs were identified using a variety of techniques including ELISA for CS1 protein, Western blotting, and flow cytometry analysis on CS1-expressing cell lines. Specific anti-CS1 mAbs were selected for further studies, including MuLuc63 ($IgG2_a$) and MuLuc90 ($IgG2_b$), both of which recognize the extracellular region of CS1, and 1G9 (IgG1), which recognizes the intracellular region of CS1.

HuLuc63 is the humanized version of the mouse MuLuc63 mAb. Humanization was done using methods described by Queen et al. (26) and in the Supplementary Data. A low fucose version of HuLuc63 (HuLuc63-LF) was generated by stably transfecting the rat myeloma YB2/0 cell line with the HuLuc63 plasmids (27). The carbohydrate structure of HuLuc63-LF was confirmed by mass spectrometry on purified protein. YB2/0 cells have been shown to produce low fucose antibodies with enhanced binding to Fc γ-receptors and enhanced ADCC activity (28). To make a variant of HuLuc63 with decreased ADCC activity (HuLuc63-AA), a variant expression plasmid with two mutations in IgG1 CH2 domain (L234A/L235A) was used; these mutations decrease ADCC via decreased Fc γ-receptor binding (29).

MSL109, the control IgG1 antibody used in all experiments, is a fully human anticytomegalovirus mAb (30).

Immunohistochemistry. Immunohistochemistry (IHC) was done with MuLuc63 or 1G9 using an automated immunostainer with 3,3′-diaminobenzidine detection (Ventana Medical Systems and Dako Autostainer; Dako North America). The 1G9 antibody and heat-induced epitope retrieval using CC1 retrieval solution (30 min, 95°C; Ventana) was used for paraffin tissues. 1G9 staining in tumor tissues was scored as negative (0-10%), 1+ (11-25%), 2+ (26-50%), 3+ (51-75%), and 4+ (76-100%).

Flow cytometry. Flow cytometry was done using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest acquisition and analysis software (Becton Dickinson). Directly conjugated anti-CS1 (FITC) was used in multiparameter flow cytometry with commercially available mAbs (Becton Dickinson) conjugated to phycoerythrin (PE), peridinin-chlorophyll protein (PerCP), or allophycocyanin (APC) to allow specific lymphocyte subset, plasma cell, blast, or stem cell gates. For stem cell analysis, at least 100 $CD34^+$ events were collected using standardized methods (31). For assessing binding of HuLuc63 to NK (CD16+CD56+), NK-like T cells (CD16+CD56+CD3+), and T cells (CD3+CD8+ or CD3+ CD8-), the antibody cocktails used were as follows: CD3 APC/CD8 PerCPCy5.5/CD16 (16+56) PE/HuLuc63 FITC, or MSL109 FITC. For assessing of HuLuc63 FITC to CD20+HLA-DR+ B cells and CD14+HLA-DR+ monocytes, the antibody cocktails used were as follows: CD20 APC/HLA-DR PerCP/CD14 PE/HuLuc63 FITC, or MSL109 FITC. For assessing HuLuc63 binding to granulocytes (CD13+ CD45+), the antibody cocktails used were as follows: CD3 APC/CD13 PE/CD45 PerCP/HuLuc63 FITC, or MSL109 FITC. CD138+ plasma cells were identified using the following antibody cocktail: CD138 PE/CD45 PerCP/CD3 APC/HuLuc63 FITC or MSL109 FITC. CS1 expression in cell populations was assessed as percentage positive cells (relative to isotype control) or as mean fluorescence intensity (MFI, mean channel number HuLuc63-FITC antibody/mean channel isotype control).

Whole blood lymphocyte depletion experiment. Whole blood from healthy volunteers was collected in sodium heparin anticoagulant Vacutainer tubes (BD Biosciences). The samples were kept at room temperature before use and were used on the same day. Samples were incubated for 24 h with HuLuc63 (100 and 200 μg/mL), MSL109 (100 and 200 μg/mL), rituximab (40 μg/mL; Genentech/Biogen Idec), alemtuzumab (100 μg/mL; Berlex laboratories/ILEX Pharmaceuticals/Genzyme corporation), or PBS at 37°C, 5% CO2. For each experimental condition, absolute cell counts were measured using TruCOUNT tubes (BD Biosciences) using the following combinations of antibody fluorochrome conjugates: Mix-1: CD3 FITC (Clone SK7; BD Biosciences), CD16-PE (B73.1; BD Biosciences), CD56-PE (MY31; BD Biosciences), CD19-APC (SJ2SC1; BD Biosciences), and CD45-PerCP (Clone 2D1; BD Biosciences); Mix-2: CD3 FITC, CD8-PE (SK1; BD Biosciences), CD4-APC (SK3; BD Biosciences), and CD45 PerCP; Mix-3: CD3 FITC, CD27-PE (M-T271; BD Biosciences), CD45 PerCP, and CD19 APC. Whole blood staining was done according to manufacturer's specifications (BD Biosciences). Samples were analyzed using a BD FACSCalibur flow cytometer, and data analysis was done according to the manufacturer's recommended procedure for TruCount combined with Multitest reagents.

ADCC. ADCC was assessed in standard 4-h $^{51}CrO_4$ release assays. NK cell effectors were purified from healthy allogeneic donors or multiple myeloma patients using CD56 antibody–coated magnetic beads (Miltenyi Biotech) and primary myeloma cell targets were purified from multiple myeloma patients using CD138 antibody–coated beads (Miltenyi Biotech). Chromium-labeled targets were incubated with HuLuc63 or MSL109 antibody at 10 μg/mL for 30 min before adding to wells containing effectors. For blocking studies, FcR antibody or isotype control antibody (Serotec) was added to wells at 10 μg/mL. Percent-specific lysis was calculated as: [experimental

release - spontaneous release]/[maximum release - spontaneous release] × 100%.

In vivo *myeloma xenograft model.* The antitumor activity of HuLuc63 was evaluated in 6- to 8-wk-old female IcrTac:ICR-Prkdcscid mice (Taconic Farms), severe combined immunodeficient (SCID)-beige mice (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7; Taconic Farms), and NOD-SCID mice with a knockout in IL-2Rg (NOD-SCID IL2Rγ<null>; Jackson Laboratory). Mice were inoculated with 1×10^7 OPM2 (DSMZ-German Collection of Microorganisms and Cell Cultures) cells into the lower right flank. Tumor measurements were done twice weekly by caliper as described previously (32). When tumors reached an average size of ~100 mm^3, animals were randomized into groups of 8 to 10 mice each and were injected i.p. with 5 to 10 mg/kg of antibody thrice a week for a total of 9 doses. For depletion of NK cells, ICR-SCID mice were treated with 0.1 mL of rabbit anti–asialo-GM1 antibody (Wako Chemicals USA, Inc.) 3 d before the start of antibody treatment, then twice per week for the duration of the study. Peripheral blood was monitored for NK cell depletion by staining with anti–CD45-PerCP and DX5-FITC (BD Biosciences). Tumor growth was monitored for a period of ~1.5 mo. One-way ANOVA with a Tukey posttest was used to compute differences between groups. Experiments were terminated once tumors reached a size of >2,000 mm^3. Animal work was carried out under NIH guidelines ("Guide for the Care and Use of Laboratory Animals") using protocols approved by the Institutional Animal Care and Use Committee at PDL BioPharma.

Results

CS1 is highly expressed in plasma cells and multiple myeloma. To identify plasma cell–specific antibody targets, a cDNA library was constructed by subtractive hybridization of naïve B-cell cDNA from memory B-cell and plasma cell cDNA. The aim was to identify gene products up-regulated in plasma cells that localize to the cell surface for antibody accessibility but have little to no expression in vital organs to minimize undesirable side effects of a targeted antibody. Genes with the desired expression profile were triaged by extensive bioinformatic analysis to determine their structural and functional classification, as well as their potential for cell surface localization. *CS1*, a gene encoding a cell-surface protein previously associated with NK cells (33, 34), was one of the genes that seemed to be highly expressed in plasma cells.

To examine the plasma cell expression in detail, gene expression profiling was done on CD138-purified plasma cells using the Affymetrix DNA microarray technology. High *CS1* expression was revealed in plasma cells from healthy donors, patients with monoclonal gammopathies of undetermined significance, smoldering myeloma, and >500 patients with frank multiple myeloma at diagnosis (Fig. 1A). In comparison to the plasma cell samples, NK cells expressed substantially lower levels of *CS1* mRNA. Using average intensity units (AIU) as an arbitrary measure of expression level, the median AIU for NK cells was 2,662, whereas the median AIU values for the plasma cell samples were 7,886 for multiple myeloma, 9,241 for smoldering myeloma, 6,207 for monoclonal gammopathies of undetermined significance, and 7,783 for normal plasma cells. We also observed that primary myeloma cells expressed higher levels of *CS1* than human myeloma cell lines (median AIU, 4,867). In contrast, 15 normal nonlymphoid tissues (lung, uterus, kidney, stomach, brain, breast, spleen, prostate, skeletal muscle, testis, thymus, liver, ovary, heart, and small intestine) tested negative and exhibited a median AIU of 152 (range, 6-741; data not shown).

CS1 expression was observed in multiple myeloma from all patient populations, including high-risk and low-risk molecular profiles and those with and without cytogenetic abnormalities. *CS1* expression also seemed high in all seven molecular subtypes of multiple myeloma, which included groups characterized by activating translocations involving c-MAF/MAFB, CCND1, CCND3, and MMSET/FGFR3, as well as groups characterized by hyperdiploidy, low-bone disease, and higher gene expression associated with proliferation (24). In an analysis of 256 Total Therapy 2 patients at baseline, median *CS1* expression seemed highest in the low-bone disease subgroup, followed by MMSET/FGFR3, CCND3, hyperdiploidy, higher gene expression associated with proliferation, CCND1, and c-MAF/MAFB (ordered by highest to lowest expression; Fig. 1B). In addition, *CS1* expression was maintained in multiple myeloma patients after relapse from therapy. A paired analysis of a subset of multiple myeloma patients showed that *CS1* gene expression was detectable in patients both at diagnosis and at relapse (Fig. 1C). Although *CS1* expression was lower at relapse in some of the patients, in all cases, expression levels were still high (median AIU, 2,386; range, 895-15,738).

To examine the expression of *CS1* in normal tissues in more detail, we compared gene expression levels across 332 normal adult tissue samples representing 86 different organs, as well as 148 leukocyte samples representing 12 different cell types using a customized DNA microarray (35). *CS1* expression was restricted to leukocytes and was not expressed in any major body organs (data not shown). Specifically, *CS1* was expressed in NK cells, as previously published, as well as a subset of T cells, activated monocytes, and activated dendritic cells. The restricted expression profile of *CS1* in normal cells and tissues, as well as the high uniform expression of *CS1* in multiple myeloma suggest that CS1 may be a potential therapeutic antibody target for the treatment of multiple myeloma.

CS1 protein is highly expressed in normal and neoplastic plasma cells but not in normal tissue parenchyma or in a variety of solid tumors. To study CS1 protein expression, a panel of anti-CS1 mAbs was generated. Three mAbs were selected for further study by virtue of their specificity to CS1 (Fig. 2). All three recognized CS1 protein from cell extracts by Western blotting. Although the mAb clones MuLuc63 and MuLuc90 recognize distinct epitopes in the extracellular region of CS1, clone 1G9 is specific for the intracellular domain of CS1 (data not shown). IHC investigation showed that MuLuc63 and MuLuc90 stained only fresh frozen tissues, whereas 1G9 stained formalin-fixed paraffin-embedded samples.

To examine the expression pattern of CS1 in normal tissues, a panel of normal cryopreserved samples was stained with MuLuc63. Pericellular staining, indicating cell-surface expression, was detected only on infiltrating leukocytes in the following organs: mammary gland, tonsil, lymph node, spleen, stomach, duodenum, ileum, cecum, colon, bladder, ureter, urethra, kidney, cervix, uterus, prostate, ovary, vagus nerve, trigeminal ganglia, liver, and thyroid (see Fig. 3A for staining in cecum). Tissues double-stained by IHC showed that the majority of CS1-positive infiltrating cells also express CD138, a marker for plasma cells (data not shown). In lymphoid tissue, staining was observed in reactive germinal centers and in subepithelial areas. Rare small lymphocytes

were also seen that expressed CS1 both within and outside germinal centers. No staining with MuLuc63 was detected in the epithelia, smooth muscle cells or vessels of heart, liver, lung, kidney, colon, small intestine, lymph node, spleen, tonsil, peripheral nerve, brain, spinal cord, aorta, adrenal, thyroid, pituitary, pancreas, parathyroid, bladder, cervix, ovary, endometrium, mammary gland, testes, retina, prostate, skeletal muscle, and skin.

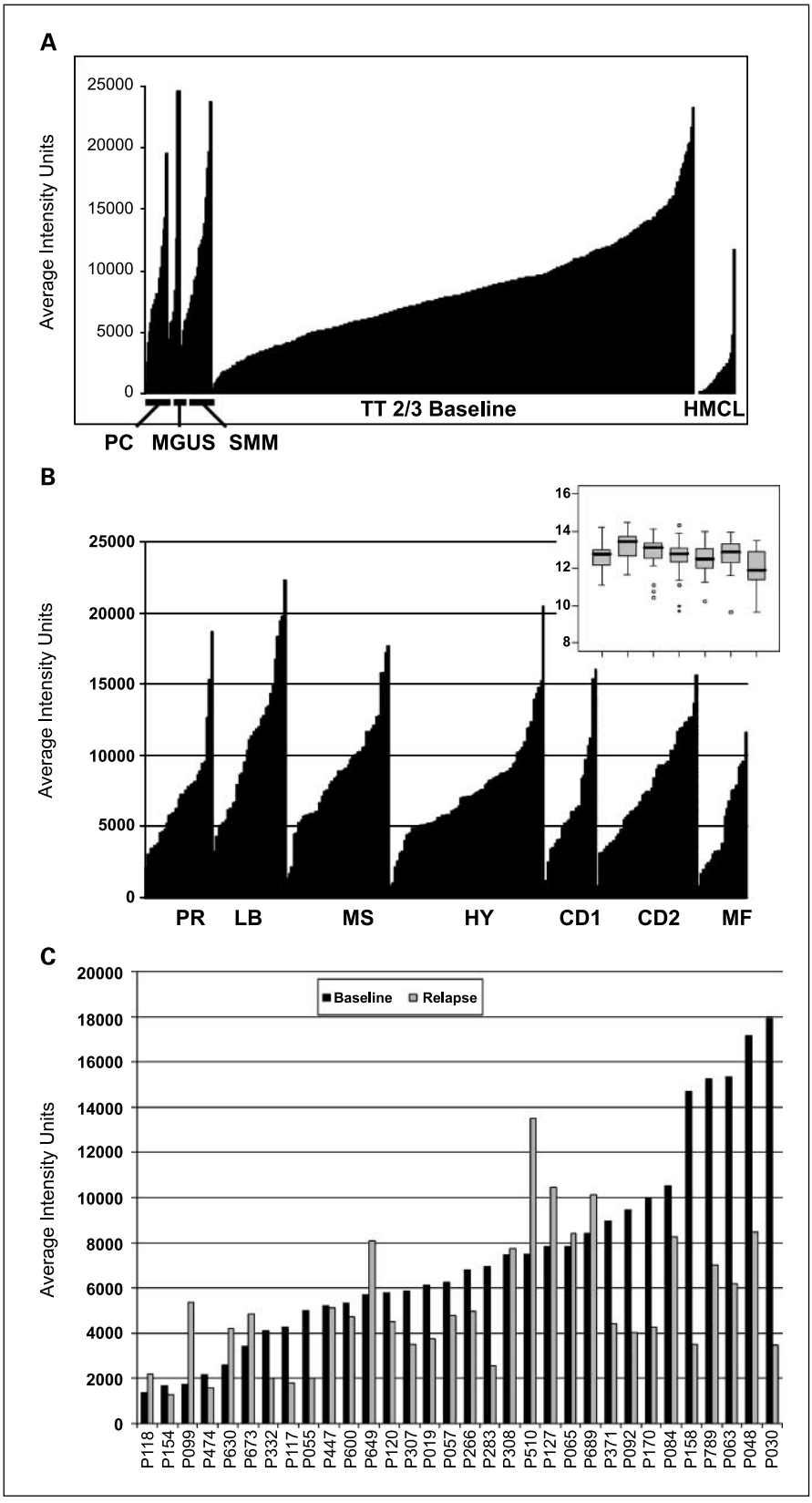

Fig. 1. Expression of *CS1* in normal, benign, and malignant plasma cells (PC). *A, CS1* gene expression was quantified using probeset 219159 from the Affymetrix gene chip U133 in plasma cell samples from 24 healthy donors, 14 monoclonal gammopathies of undetermined significance (MGSUS), 35 smoldering myeloma (SMM), 532 multiple myeloma [MM; Total Therapy (TT) 2/3 baseline], and 45 human myeloma cell lines (HMCL). The expression values ranged from 900-19,548 AIU for normal plasma cells, 1,771-24,632 AIU for monoclonal gammopathies of undetermined significance, 426-2,3697AIU for smoldering myeloma, 258-2,3727 AIU for multiple myeloma, and 28-11,698 AIU for human myeloma cell lines. *B, CS1* gene expression in 256 multiple myeloma patients at baseline before Total Therapy 2 and ordered by molecular classification based on Zhan et al. (24). Inset, median AIU values, showing the low-bone disease (LB) group with the highest median expression level. *C,* paired gene expression analysis at time of diagnosis (*black*) versus at time of relapse after Total Therapy 2 (*gray*) shows that *CS1* expression is retained at significant levels at relapse. PR, higher gene expression associated with proliferation; MS, MMSET/FGFR3; HY, hyperdiploidy; CD1, CCND1; CD2, CCND3; MF, c-MAF/MAFB.

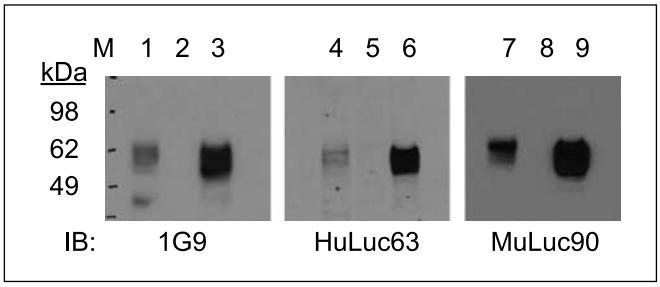

Fig. 2. Western blot analysis using anti-CS1 mAbs 1G9, HuLuc63, and MuLuc90 show specificity for CS1. Cell extracts were made from OPM2 multiple myeloma cells (*lanes 1, 4*, and *7*), parental 293 cells (*lanes 2, 5*, and *8*), and 293 cells transfected with a *CS1* expression vector (*lanes 3, 6*, and *9*). Immunoblots probed with anti-CS1 mAbs 1G9, HuLuc63, and MuLuc90 show a broad reactive protein band(s) of ~60 kDa in the OPM2 and the CS-transfected 293 cells. M, markers in kDa.

We also stained a variety of normal and neoplastic tissues using the formalin-fixed paraffin-embedded reactive 1G9 antibody. Normal tissue IHC showed that all tissues were negative with the exception of plasma cells and scattered lymphocytes. Tissues (five examples of each type) included brain, breast, colon, heart, kidney, liver, lung, ovary, pancreas, prostate, skin, small intestine, spleen, stomach, testis, tonsil, uterus, and urinary bladder. IHC analyses of multiple solid tumors were negative for anti-CS1 staining. The tumor samples (n = 5 for each type) included ductal breast adenocarcinoma, lobular breast adenocarcinoma, renal cell carcinoma, prostate carcinoma, endometrial adenocarcinoma, gastric adenocarcinoma, urothelial carcinoma, small cell lung carcinoma, non–small cell lung carcinoma, ovarian carcinoma, testicular germ cell tumor, head and neck squamous cell carcinoma, melanoma, and pancreatic adenocarcinoma.

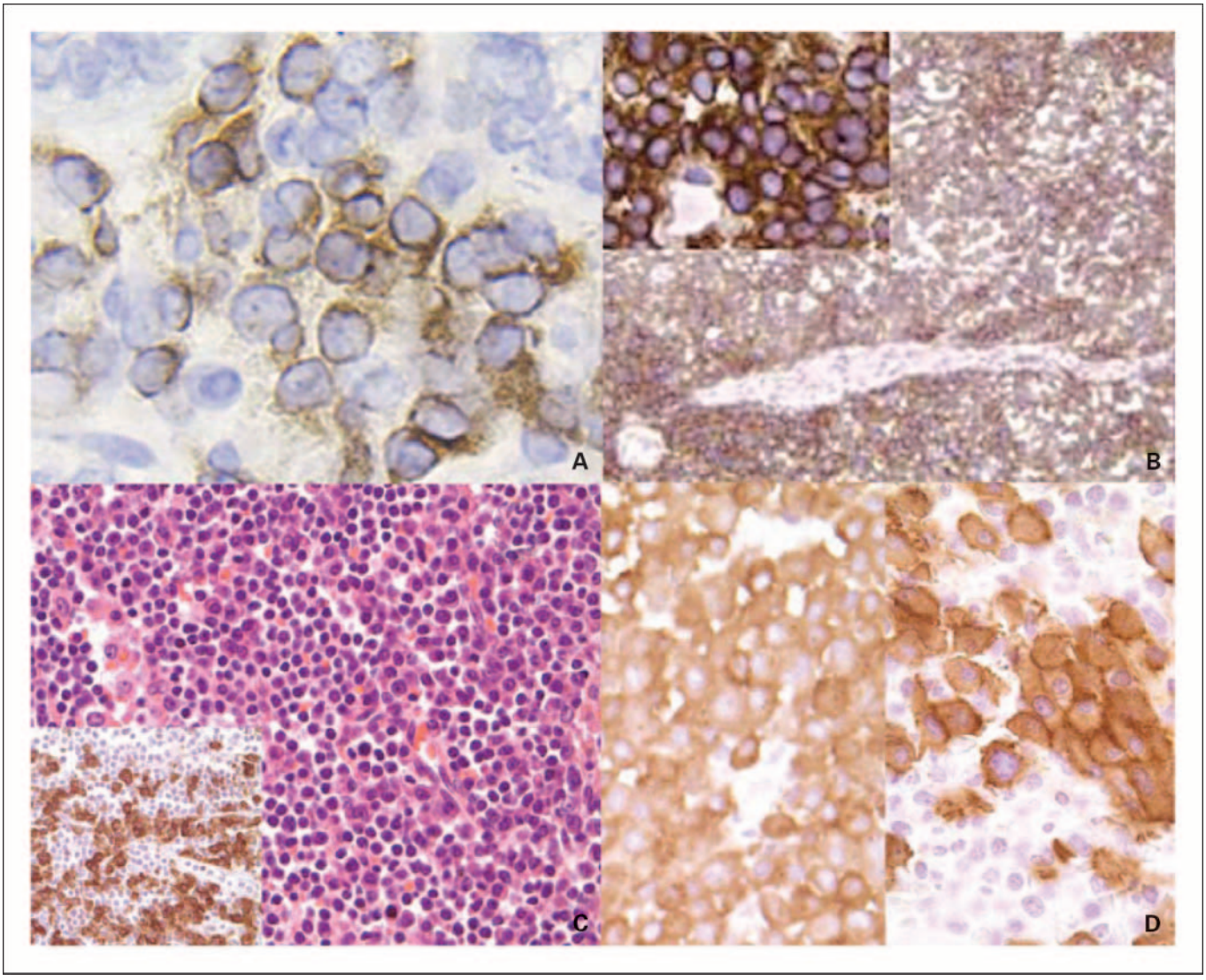

Fig. 3. CS1 protein expression is restricted to normal and malignant plasma cells in tissues. Frozen section IHC stained with MuLuc63: (*A*) plasma cell staining in the lamina propria of cecum (×1,000); *B,* malignant plasma cell staining in extramedullary plasmacytoma (×100; inset, ×400). Paraffin section IHC stained with 1G9; *C,* a case of lymphoplasmacytic lymphoma (H&E, ×200) showing CS1 expression (inset, ×200) in neoplastic cells with plasmacytic maturation; *D,* two examples of bone marrow with multiple myeloma show typical strong plasma cell staining (×400).

Table 1. CS1 (1G9) expression in lymphoma, leukemia, and myeloma

	CS-1 IHC*				
	Neg	**1+**	**2+**	**3+**	**4+**
Myeloma (20)					
Multiple myeloma in BM (20) †	0	0	1	0	19
Plasmacytoma (8)	0	0	1	2	5
T-cell lymphoma (37)					
Peripheral T-cell, unspecified	28	0	4	3	2
B-cell lymphoma (113)					
Follicular lymphoma (44)	42	2	0	0	0
Diffuse large B-cell lymphoma (39) ‡	37	1	0	1	0
Mantle cell lymphoma (6)	6	0	0	0	0
Marginal zone lymphoma (5)§	3	2	0	0	0
Small lymphocytic lymphoma (5)	5	0	0	0	0
Lymphoplasmacytic lymphoma (5)	2	1	1	1	0
Burkitt lymphoma (5)	5	0	0	0	0
Lymphoblastic lymphoma (5)‖	5	0	0	0	0
Hodgkin lymphoma (10)	10	0	0	0	0
Acute leukemia (66)					
Acute myeloid leukemia (37)	37	0	0	0	0
Acute lymphoblastic leukemia (29)¶	29	0	0	0	0

Abbreviations: Neg, negative; BM, bone marrow.
*Neg, 0% to 10% tumor cells; 1+, 11% to 25%; 2+, 26% to 50%; 3+, 51% to 75%; 4+, >75%.
†20 cases from 14 patients.
‡The positive cases were T-cell–rich large B-cell lymphoma histology.
§Four splenic marginal zone, one mucosa-associated lymphoid tissue type.
‖Two B precursor, Three T cell.
¶Twenty-five B precursor, 4 precursor T.

Analysis of a series of lymphomas and leukemias (Table 1) showed that CS1 expression was absent in the vast majority of acute leukemias, B-cell lymphomas, and classic Hodgkin lymphomas. In contrast, CS1 seemed to be strongly expressed in extramedullary plasmacytomas (8 of 8 cases were 2+ to 4+; Fig. 3B), in the neoplastic plasma cell component of 3 of 5 cases of lymphoplasmacytic lymphoma (Fig. 3C), and in the vast majority of tumor cells in multiple myeloma bone marrow cores (19 of 20 cases, 4+; Fig. 3D). Interestingly, the multiple myeloma case exhibiting less reactivity (scored 2+) expressed the mature B-cell marker CD20 as well as CD138 but was negative for *IGH/CCND1* translocation. Two of five cases of splenic marginal zone lymphoma also expressed CS1 in a minority of cells. Approximately 25% of peripheral T-cell lymphomas expressed moderate levels of CS1, perhaps reflecting an origin from T cells naturally expressing CS1.

HuLuc63, a humanized anti-CS1 mAb, binds to lymphocyte subsets and to neoplastic plasma cells by flow cytometry. The gene and protein expression data suggest that CS1 is a potential therapeutic antibody target for the treatment of multiple myeloma. To investigate this possibility and in anticipation of treating multiple myeloma patients with an anti-CS1 antibody, the MuLuc63 anti-CS1 mAb was selected for humanization. As part of a safety evaluation of the humanized mAb, HuLuc63, we examined its binding profile to various leukocyte subsets in blood and bone marrow samples using flow cytomety. In normal donors, HuLuc63 stained primarily CD3+CD16+CD56+ NK-like T cells, CD3-CD16+CD56+ NK cells, and approximately half of CD3+CD8+ T cells. In contrast, substantially lower binding was detected on CD3+CD8- T cells, and no significant binding of HuLuc63 occurred on monocytes (CD14+HLA-DR+), B cells (CD20+HLA-DR+), or granulocytes (CD13+CD45+; Table 2).

HuLuc63 binding data were observed on similar subsets of leukocytes in bone marrow samples from multiple myeloma patients, except that binding to these subsets in bone marrow was found to be more variable between subjects (Table 2). In addition, the bone marrow samples contained between 1% and 20% CD138+ plasma cells, the target cells for the anti-CS1 antibody. The great majority of plasma cells in multiple myeloma stained strongly positive with HuLuc63, the staining intensity being substantially higher than the staining intensity on normal NK cells (Table 2).

Because multiple myeloma is often treated with autologous stem cell transplantation, we examined the potential concern of HuLuc63 binding to CD34+ stem cells. Flow cytometry analysis of CD34+ stem cells from apheresis samples collected for the purposes of autologous stem cell transplantation (six lymphoma patient samples) and two multiple myeloma patient bone marrow aspirate samples showed that HuLuc63 did not significantly bind stem cells (data shown only for the multiple myeloma samples in Table 2).

To determine the effects of HuLuc63 on cell counts of lymphocytes and their major subsets, whole blood samples from 8 healthy individuals were incubated with HuLuc63 (100 or 200 μg/mL) for 24 hours at 37°C. Several control antibodies were used in this study, including MSL109 as a negative control, rituximab (Rituxan; Genentech/Biogen Idec), an anti-CD20 antibody that depletes B cells *in vitro* and *in vivo* (36, 37), and alemtuzumab (Campath1H; Berlex/ILEX Oncology Inc/Genzyme), an anti-CD52 antibody that depletes various lymphocytes *in vivo* (38) as positive controls. Samples were tested for depletion of T, NK, B, and memory B cells as measured by a decrease in absolute cell counts by flow cytometry. The results indicated no apparent effect of HuLuc63

on total lymphocytes, T-, and B-cell counts (data not shown). On average, NK cell counts were decreased by 20% at both doses of HuLuc63, although the observed decline was variable between donors. In contrast, rituximab treatment resulted in an average decrease of 70% of all B cells, and alemtuzumab treatment resulted in depletion of all lymphocyte subsets (range of 65-100%). Overall, these data show that HuLuc63 treatment of normal healthy blood did not result in substantial depletion of major lymphocyte subsets.

HuLuc63 mediates ADCC and* in vivo *antimyeloma activity that is dependent on NK cells. Having shown expression of CS1 on the surface of neoplastic plasma cells at relatively high levels compared with normal NK and T cells, we sought to determine whether HuLuc63, a humanized IgG1 antibody, could mediate ADCC toward primary myeloma cells isolated from multiple myeloma patients. The results showed that HuLuc63 induced specific myeloma-cell lysis in multiple assays using purified NK cells from healthy allogeneic donors (five assays) or autologous NK cells from multiple myeloma donors (six assays) as effectors (Fig. 4A). Similar results were obtained by Tai et al. (32) using peripheral blood mononuclear cells (PBMC) as effectors. Blocking the Fc receptor (CD16) on NK cells with an anti-CD16 antibody significantly inhibited this activity (Fig. 4A). In separate experiments using human PBMC effectors, depletion of NK cells significantly impaired HuLuc63-mediated ADCC toward the OPM2 myeloma cell line (Fig. 4B). In contrast, depleting T cells, B cells, or monocytes from a PBMC effector population had no effect on HuLuc63-mediated ADCC (data not shown), suggesting that NK cells are the dominant effectors for this activity.

In parallel with our studies, Tai et al. (32) showed that HuLuc63 exhibits significant dose-dependent antitumor activity using myeloma xenograft models *in vivo* in mice. Because our *in vitro* data suggested that HuLuc63-mediated ADCC activity was dependent on NK cell and Fc receptor function, we tested the contribution of the Fc region of HuLuc63 to *in vivo* activity. Two modified forms of HuLuc63 were produced; one form had low-fucose content (HuLuc63-LF) and bound with high affinity to human CD16a (affinity constant (K_D), 0.6 μmol/L compared with 2.1 μmol/L for wild-type HuLuc63), whereas the second form contained a double L234A/L235A mutation (HuLuc63-AA) and had a lower affinity toward human CD16a (KD, 7.6 μmol/L). The modified forms of HuLuc63 were tested with wild-type HuLuc63 in the OPM2 xenograft model for *in vivo* antitumor activity. The results show that higher antitumor activity correlated with increased affinity of antibody to the Fc receptor. Treatment with wild-type HuLuc63 resulted on average in a 70% decrease in tumor volumes compared with control isotype antibody treatment, whereas HuLuc63-LF caused an average of 85% decrease in tumor volume, although these groups were not significantly different from each other (Fig. 4C). In contrast, HuLuc63-AA did not exhibit any antitumor activity compared with wild-type HuLuc63 (Fig. 4C). These results combined with the *in vitro* ADCC results suggest that HuLuc63 exerts antimyeloma activity through NK cell–mediated ADCC via binding of CD16 expressed on the surface of NK cells.

To further explore this hypothesis, HuLuc63-mediated antitumor activity was explored in mice that had been either depleted of NK cells or are deficient in NK cell function or numbers. In a single study, NK cell depletion abolished the HuLuc63-mediated antitumor effect (Fig. 4D). Similarly, there was no statistically significant difference between HuLuc63 and isotype control on antitumor activity in the NK cell–deficient NOD-SCID/IL-2Rγ knockout mice (Fig. 4D; ref. 39).

Table 2. Summary of *in vitro* binding of FITC-labeled HuLuc63 to leukocyte subsets in human whole blood and bone marrow samples

Cell type	% HuLuc63+ in WB of Healthy donors, mean ± SD (*n*)	% HuLuc63+ in WB of multiple myeloma donors, mean ± SD (*n*)	% HuLuc63+ in BM of multiple myeloma donors, mean ± SD (*n*)
Plasma cells	Not determined	73.1 ± 20.3 (5)* MFI, 5.6 ± 3.9	92.2 ± 7.7 (11) MFI, 10.1 ± 5.2
CD8-T	9.8 ± 5.7 (10)	12.2 ± 16.2 (18)	15.1 ± 8.8 (13) MFI, 1.4 ± 0.4
CD8+T	55.9 ± 21.8 (10)	59.6 ± 27.7 (18)	71.7 ± 19.3 (13) MFI, 4.5 ± 2.1
NK/T	82.7 ± 21.6 (10)	74.9 ± 27.6 (18)	84.8 ± 12.3 (13) MFI, 6.1 ± 2.9
NK	96.3 ± 4.7 (10)	97.1 ± 2.2 (18)	70.1 ± 31.6 (13) MFI, 4.9 ± 2.8
B-cell	3.8 ± 6.5 (10)	12.4 ± 12.5 (18)	16.0 ± 17.2 (12) MFI, 2.0 ± 1.0
Monocytes	7.9 ± 7.1 (10)	9.2 ± 7.0 (18)	13.3 ± 13.8 (13) MFI, 1.6 ± 0.4
Granulocytes	0.9 ± 1.7 (10)	2.7 ± 2.6 (16)	8.9 ± 11.0 (13) MFI, 1.6 ± 0.7
Stem cells	Not determined	Not determined	0.74, 2.3 (2)†

NOTE: *n*, number of individuals tested; MFI, MFI by flow cytometry (relative to isotype control).
Abbreviation: WB, whole blood.
*Plasma cell leukemia samples.
†Values for % HuLuc63 staining on stem cells are shown for two donors. The values for each donor were similar to isotype control staining indicating that HuLuc63 does not bind stem cells.

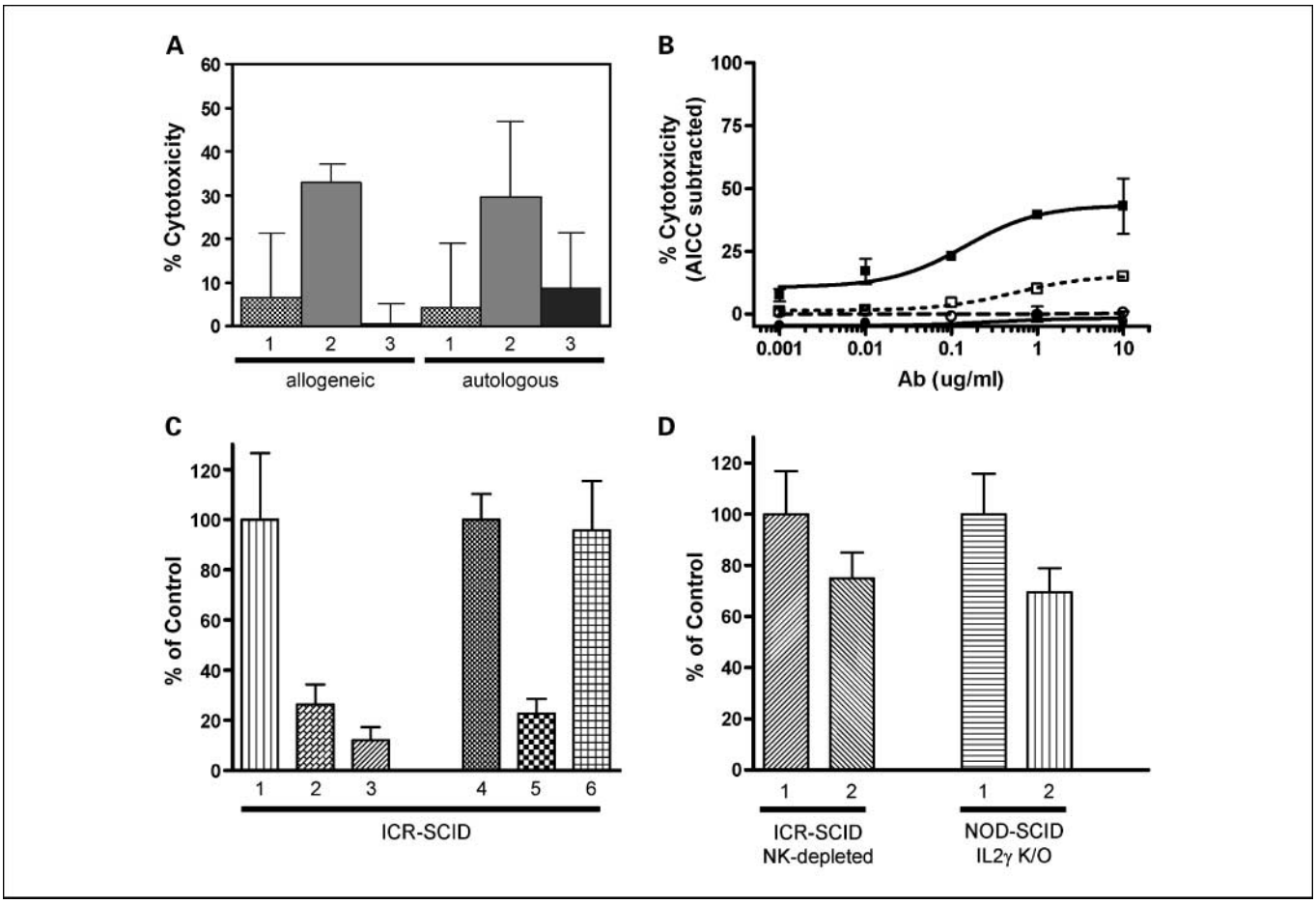

Fig. 4. HuLuc63 mediates ADCC *in vitro* and inhibits myeloma tumor growth *in vivo*. *A,* HuLuc63-mediated ADCC of Cr-51 – labeled primary myeloma cells by purified allogeneic or autologous NK cells. Shown is the average specific lysis from multiple chromium release assays using as effectors purified NK cells from healthy allogeneic donors (5 assays) or autologous NK cells (6 assays) in the presence of (*1*) control isotype antibody (10 μg/mL), (*2*) HuLuc63 (10 μg/mL), or (*3*) HuLuc63 (10 μg/mL) and anti-CD16 Ab (10 μg/mL). The effector to target ratio was 30:1. *B,* significant ADCC of Cr-51 – labeled OPM2 cells by human PBMCs occurred in the presence of increasing concentrations of HuLuc63 (*closed squares, solid line*) compared with isotype control Ab (*solid circles, solid line*). Depletion of NK cells from the same PBMC preparation significantly reduced HuLuc63-mediated lysis (*open squares, dashed line*). Control Ab exhibited no lysis activity with NK-depleted PBMCs (*open circles, dashed line*). *C* and *D,* mice with established OPM2 xenograft tumors (average of ~100 mm^3) were randomized into groups 2 to 3 wk after inoculation and were then treated with either humanized IgG1 control antibody or HuLuc63. In all experiments, the average tumor volumes at necropsy (on study days 40-45) were plotted as percent control for comparisons between the different animal models. *C,* antitumor activity of HuLuc63 was compared with HuLuc63-LF (both dosed at 5 mg/kg) and then to the CD16-binding mutant HuLuc63-AA (both dosed at 10 mg/kg). Compared with isotype control (*1*), HuLuc63 (*2*), and HuLuc63-LF (*3*) caused significant tumor growth inhibition ($P < 0.01$ and $P < 0.003$, respectively) but were not significantly different from each other. In the second experiment, HuLuc63 (*5*) exhibited significant antitumor activity compared with control (*4*; $P < 0.00001$), whereas HuLuc63-AA (*6*) did not inhibit tumor growth ($P = 0.4$). *D,* compared with isotype control (*1*), HuLuc63 (*2*) at 10 mg/kg per dose did not exhibit significant antitumor activity in ICR-SCID mice that were depleted of NK cells with an anti – asialo-GM1 antibody ($P = 0.1$) or in NOD-SCID/IL-2Rγ knockout mice ($P > 0.05$), which lack NK cells.

Discussion

We have identified CS1, a member of the signaling lymphocyte activating – molecule family of cell surface receptors, as a potential novel target for the treatment of multiple myeloma. We also generated a humanized anti-CS1 mAb, HuLuc63 that recognizes the extracellular region of CS1 and targets myeloma cells for ADCC. The goals of this study were to examine in detail the expression of CS1, to characterize the cellular binding properties of HuLuc63, and examine the potential of HuLuc63 as a therapeutic antibody candidate for the treatment of multiple myeloma.

Our gene expression profiling data showed that CS1 was highly prevalent in multiple myeloma. The expression did not correlate with any of the molecular classifications in multiple myeloma (24), suggesting that this target is not restricted to any particular multiple myeloma subgroup. A separate gene expression study, done in parallel, was in agreement with our results that *CS1* was highly expressed and prevalent in multiple myeloma (32). In addition, multiple myeloma patients that had been treated with either Total Therapy 2 (40) or Total Therapy 3, continued to express *CS1* at the time of relapse, indicating that significant chemotherapeutic regimen do not markedly affect the expression of *CS1* in the myeloma cells. Using IHC, we also showed high-level expression in all cases of multiple myeloma and plasmacytomas. In addition, cases of secondary plasma cell leukemia were also positive. Thus, plasma cell malignancy in bone marrow, tissue, and blood all seemed to express high levels of CS1.

Examination of a series of B-cell lymphomas also showed that CS1 expression was not observed at any appreciable degree in mature B-cell neoplasms without plasmacytic differentiation

(small lymphocytic lymphoma, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma). Cases of lymphoplasmacytic lymphoma showed CS1 expression in the neoplastic plasmacytic components, whereas small mature B-cells lacked CS1. Marginal zone lymphomas, which may also show plasmacytic differentiation, weakly expressed CS1 Thus, even in neoplastic lymphoid tumors, CS1 was seen only in terminally differentiated plasma cells. Interestingly, a subset of T-cell lymphomas also expressed CS1, possibly reflecting derivation from a normal counterpart expressing CS1 or perhaps an activated state. Using this method, we were unable to show significant expression in small lymphocytes in B- and T-cell zones of lymphoid tissues, possibly due to decreased sensitivity of this assay compared with flow cytometry.

The expression of CS1 in malignant plasma cells, combined with the lack of expression in the parenchyma of any major organs, makes CS1 an attractive target for the treatment of multiple myeloma. In comparison, other potential antibody targets for multiple myeloma treatment such as CD138 (syndecan-1) and CD40 are expressed in other tissues (41–49). The normal tissue profile of CS1 suggests that there does not seem to be major concern for tissue toxicity and/or a sink for an antibody-directed therapy. Importantly, hematopoietic stem cells, which are often used in the treatment of multiple myeloma in the form of autologous stem cell transplantation, do not express CS1, allowing the possibility of combining a potential HuLuc63 treatment with autologous stem cell transplantation.

In a study exploring potential efficacy, we showed that HuLuc63 can elicit allogeneic and autologous NK cell–mediated ADCC toward primary myeloma cells from multiple myeloma patients. In a parallel study, HuLuc63 elicited ADCC using PBMC effector cells but does not elicit complement-dependent cytotoxicity (32). A role for ADCC was supported by our *in vivo* xenograft studies in which OPM2 tumor growth inhibition with HuLuc63 treatment was dependent on Fc–Fc-receptor interaction, as well as the presence of functional NK cells. HuLuc63 was also reported to have direct effects on the survival of primary myeloma cells *in vitro*, presumably by inhibiting interaction with bone marrow stromal cells (32). However, a direct effect on the *in vitro* survival and/or proliferation of OPM2 and L363 myeloma cells was not observed in our study (data not shown). Therefore, although other potential mechanisms of action cannot be ruled out, our data supports the hypothesis that the primary mechanism of action of HuLuc63 involves NK-mediated cell killing.

In summary, CS1 is expressed at high levels in benign and neoplastic plasma cells derived from multiple myeloma patients. Importantly, CS1 is expressed in multiple myeloma samples from extensively treated patients on currently approved therapies. HuLuc63, a humanized anti-CS1 mAb, mediates *in vitro* and *in vivo* antimyeloma activity and, therefore, shows promise as a therapeutic agent. Currently, HuLuc63 is in a phase 1 safety trial for the treatment of relapsed/refractory multiple myeloma.

Disclosure of Potential Conflicts of Interest

B. Balasa., A. Draksharapu, B.P. Shum, M. Huseni, D. Powers, A. Nanisetti, Y. Zhang, A.G. Rice, A.V. Abbema, M. Wong, G. Liu, M. Dillon, S. Chen, S. Rhodes, F. Fuh, N. Tsurushita, S. Kumar, V. Vexler, D.E.H. Afar, and M.B. Williams are current or past employees and shareholders of PDL BioPharma, Inc.

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