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## A preclinical model of CD38-pretargeted radioimmunotherapy for plasma cell malignancies

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### Abstract

The vast majority of patients with plasma cell neoplasms die of progressive disease despite high response rates to novel agents. Malignant plasma cells are very radiosensitive, but the potential role of radioimmunotherapy (RIT) in the management of plasmacytomas and multiple myeloma (MM) has undergone only limited evaluation. Furthermore, CD38 has not been explored as a RIT target despite its uniform high expression on plasma cell malignancies. In this report, both conventional RIT (directly radiolabeled antibody) and streptavidin-biotin pretargeted RIT (PRIT) directed against the CD38 antigen, were assessed as approaches to deliver radiation doses sufficient for MM cell eradication. PRIT demonstrated biodistributions that were markedly superior to conventional RIT. Tumor-to-blood ratios as high as 638:1 were seen 24hr after PRIT, while ratios never exceeded 1:1 with conventional RIT. <sup>90</sup>Yttrium absorbed dose estimates demonstrated excellent target-to-normal organ ratios (6:1 for the kidney, lung, liver; 10:1 for the whole body). Objective remissions were observed within 7 days in 100% of the mice treated with doses ranging from 800  $\mu$ Ci to 1200  $\mu$ Ci of anti-CD38 pretargeted <sup>90</sup>Y-DOTA-biotin, including 100% complete remissions (no detectable tumor in treated mice compared to tumors that were 2982 $\pm$ 2834% of initial tumor volume in control animals) by day 23. Furthermore, 100% of animals bearing NCI-H929 multiple myeloma tumor xenografts treated with 800  $\mu$ Ci of anti-CD38 pretargeted <sup>90</sup>Y-DOTA-biotin achieved long-term myeloma-free survival (>70 days) compared to none (0%) of the control animals.

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#### Author Contributions:

D.J.G. designed experiments, performed experiments, wrote and revised the manuscript, analyzed results and designed the figures; N.N.O. performed experiments, analyzed results and contributed to figures; J.C.J. performed experiments and analyzed results; M.D.H. performed experiments and analyzed results; J.M.P. analyzed results; D.K.H. and D.S.W. produced essential reagents and analyzed results; Y.L. performed experiments, edited the manuscript and produced essential reagents; D.R.F. performed dosimetry analysis; A.L.K. and S.L.F. performed experiments; A.K.G. analyzed results and edited the manuscript; J.J.O. performed experiments and edited the manuscript; T.A.G. provided statistical support; B.L.W. performed experiments; W.I.B. analyzed results; O.W.P. designed experiments, performed experiments, analyzed results, revised the manuscript and edited figures.

## Keywords

Radioimmunotherapy; multiple myeloma; CD38; pretargeting; preclinical

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## INTRODUCTION

Bortezomib, lenalidomide and other novel agents have significantly improved the response rates, progression-free survival and overall survival for patients with multiple myeloma (MM) in recent years.(1, 2) Despite these advances, MM remains incurable. With currently available therapies the 77,000 MM patients living in the United States will almost universally relapse and die from progressive disease. Myeloma recurrence is presumably a function of malignant plasma cell clones, and possibly precursor stem cells, (3, 4) that evade or develop resistance to available therapies. The efficacy of radioimmunotherapy (RIT) in the treatment of hematologic malignancies is well established.(5–7) RIT selectively delivers radiation to target cells at multifocal disease sites and facilitates escalation to radiation doses not achievable through external beam therapy. The radiosensitivity of malignant plasma cells outside of the bone marrow has been well documented in clinical settings. Local recurrence of solitary extramedullary plasmacytomas occurs in less than 10% of cases after external beam radiation alone.(8) Radiation therapy is also effective as a palliative measure in patients experiencing pain or other sequelae resulting from MM-induced osteolysis. Steep dose response relationships have been demonstrated for most hematological malignancies, and the impact of radiation dose escalation may be of particular importance in the case of MM.(9)

A limited number of radionuclide based therapies have been explored in the treatment of MM.(10–14) While each of these radionuclide based approaches has theoretical promise, none have directly targeted radiation to the CD38 antigen on MM cells. The directed delivery of radionuclides to MM cells requires an antigen target that is specific, stable and uniformly expressed at high density. CD38 is a 45 kDa stable transmembrane glycoprotein receptor expressed at a high epitope density on 95–100% of malignant plasma cells.(15, 16) The CD38 antigen is expressed on activated T cells, monocytes and NK cells at relatively low levels when compared to plasma cells. Reports describing CD38 expression on cells that are not of hematopoietic origin have been inconsistent. Non-quantitative approaches have suggested that CD38 may be expressed in pancreatic, lung, brain and even skeletal muscle;(17) however, recent gene expression profiling of human tissues demonstrate that CD38 mRNA is largely restricted to cells of hematopoietic origin with minimal expression in non-hematopoietic derived tissue limited to the thymus and prostate. (18)

Unconjugated anti-CD38 monoclonal antibodies (mAbs) have induced myeloma cell killing both in culture and in xenograft mouse models.(19, 20) CD38 mAbs have reportedly been well tolerated in a small number of patients(21) and a phase I/II clinical trial with the anti-CD38 mAb daratumumab is currently accruing patients with relapsed or refractory MM. CD38 mAbs linked to cytotoxic agents have also demonstrated therapeutic activity in myeloma models.(22, 23)

While conventional one-step RIT with directly conjugated radiolabeled antibodies (Figure 1a) is effective at achieving disease control, disease eradication is thought to be limited by relatively low tumor-to-normal organ ratios of absorbed radioactivity (e.g. 1.5:1 for tumor-to-lung using <sup>131</sup>I-anti-CD20).(6) Multistep pretargeting methods can optimize delivery of the therapeutic radionuclide to tumor targets, while limiting normal organs from radiation exposure. Several approaches to pretargeting have been described.(24–26) One method employs an antibody-streptavidin (Ab-SA) construct, followed by administration of a small

molecule, radio-DOTA-biotin. In the first step, the tumor-reactive Ab-SA localizes to tumor sites without subjecting the rest of the body to non-specific irradiation. After maximal accumulation of Ab-SA in the tumor, a small molecular weight radioactive moiety (radio-DOTA-biotin) possessing a high affinity for the Ab-SA bound to tumor sites is administered as a second-step. Due to its small size, the second-step reagent penetrates tumors rapidly where it binds tightly to the pretargeted Ab-SA (Figure 1b). Unbound radio-DOTA-biotin molecules are cleared from the blood and excreted in the urine within minutes. This rapid clearance of the second step reagent limits the time during which normal tissues are exposed to non-specific radiation. Dissociating the slow target antigen binding distribution phase from the radionuclide delivery phase generates more favorable target-to-normal organ ratios. As a further refinement, a clearing agent (CA) can be injected shortly before the radiolabeled small molecule to remove unbound Ab-SA from the bloodstream and prevent it from complexing with the radiolabeled small molecule in the circulation.(26, 27)

This report describes a new approach to the treatment of MM. Anti-CD38 pretargeted radioimmunotherapy (PRIT) demonstrates superior biodistributions when compared with directly labeled conventional anti-CD38 antibody RIT and clear evidence of therapeutic efficacy which provides a compelling rationale for further study.

## MATERIALS AND METHODS

### Cell lines

The human MM cell lines NCI-H929 and L363 were a gift from David Maloney (obtained from American Type Culture Collection [ATCC, Bethesda, MD]). The human Ramos B-cell lymphoma and human RPMI 8226 MM cell lines were also obtained from ATCC. The MM1R cell line was a gift from Steven Rosen (Northwestern University, Chicago, IL) and was generated in his laboratory. Cells were grown in RPMI-1640 supplemented with 10% FBS, 50U/mL penicillin G and 50µg/mL streptomycin sulfate. Following two passages, cells were frozen and stored in liquid nitrogen for future use. For all studies a fresh vial of frozen cells was thawed and grown in culture for 7 to 21 days.

### Antibodies, antibody conjugates, fusion proteins and pretargeted reagents

The OKT10 hybridoma expressing murine IgG<sub>1</sub> anti-CD38 Ab and nonspecific IgG<sub>1</sub> control Ab, BHV1 (specific for bovine herpes virus 1), were obtained from ATCC. The OKT10-Ab and BHV1-Ab were produced from ascites in pristine-primed mice purified by HiTrap Protein G HP 5 mL column chromatography. DOTA-Ab reagents were generated as described previously.(27) Each Ab was also conjugated to streptavidin (SA) to form covalent synthetic chemical conjugates using previously described methods.(27) The CC49 scFv<sub>4</sub>SA-FP, which recognizes the TAG-72 antigen on human adenocarcinomas, was a gift from NeoRx (NeoRx, Seattle, WA). Expression, purification and characterization of CC49-FP has been previously described. (28, 29) To produce OKT10-CC, intact OKT10 IgG<sub>1</sub> anti-CD38 mAb was derivatized with either a DOTA chelate(27, 30) or SA using the heterobifunctional cross-linker SMCC using methods previously described.(27) Iminobiotin and cation exchange chromatography led to purities of >93%. The chemical conjugate (CC) harbors approximately one SA molecule conjugated to each OKT10 molecule as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and size exclusion HPLC (Figure 1d). OKT10-FP was generated through cloning Fv regions from the OKT10 anti-CD38 hybridoma which were fused to the full-length genomic SA gene of *Streptomyces avidinii*, and the resultant fusion genes (Figure 1c) expressed as soluble tetramers (174 kDa) in the periplasmic space of *E. coli*(29) (FP construction detailed in supplemental methods). Lineweaver-Burke and Scatchard cell binding assays confirmed that both OKT10-CC and OKT10-FP retain the full immunoreactivity and avidity of the parent OKT10-Ab; binding

specificity was confirmed by blocking with excess intact unconjugated mAb (Figure 2a,b). The biotin-binding capacity of the CC and FP were similar to recombinant SA by the HABA (4'-hydroxyazobenzene-2-carboxylic acid) assay (not shown).

### Radiolabeling

<sup>90</sup>Yttrium (<sup>90</sup>Y) and <sup>111</sup>Indium (<sup>111</sup>In) [Perkin Elmer, Waltham, MA] radiolabeling of intact DOTA-Ab for conventional RIT, and DOTA-biotin for pretargeted RIT, were conducted as described previously.(27, 30) Radiochemical purity was generally greater than 95% as determined by iTLC and avidin bead assay. DOTA-biotin was synthesized as described.(27)

### Trichloroacetic acid (TCA) precipitation

The amounts of intact and degraded radiolabeled antibody in culture supernatants were estimated by TCA precipitation as previously described (see Supplemental Methods Section for details).

### Competitive cell binding assay

L363 cells ( $0.5 \times 10^6$  cells per sample) were plated in a 96-well plate. Serial concentrations of either OKT10-Ab, OKT10 synthetic chemical conjugate (OKT10-CC), OKT10scFv<sub>4</sub> fusion protein (OKT10-FP), or isotype control, were added to each well followed by OKT10-APC (0.1µg, Alexa Fluor 647 Protein Labeling Kit from Molecular Probes [Invitrogen, Grand Island, NY]). The cells were incubated at 4°C for 45 minutes, washed and fluorescence intensity was measured on a flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

### In vitro binding characterization

The CD38 binding ability of OKT10 was assessed using Ramos targeted cells. Cells ( $0.1 \times 10^6$  per sample) were incubated with 30µL of 10µg/mL 1F5-Ab (positive control), OKT10-Ab, and BHV1-Ab (non-binding Isotype control) for 30 minutes at 4°C. Cells were washed and incubated with 25µL of 1:64 goat anti-mouse IgG (Fab specific) fluorescein isothiocyanate conjugate (Sigma-Aldrich, St. Louis, MO) in PBS. Cells were washed and fluorescence intensity was measured on a FACS Canto I flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Binding ability of OKT10 to L363 targeted cells was assessed using OKT10-APC.

### Cell binding assay

Anti-CD38 (OKT10-FP/OKT10-CC) [20µg/mL] or nonbinding control (CC49-FP/BHV1-CC) [20µg/mL] were added to  $1 \times 10^6$  cells pelleted in 96 well round bottomed plates on ice. Pellets were resuspended in the antibody solution, incubated at 4°C for 1hr, washed and resuspended in 200µL DMEM for 24hr and stored at 4°C or 37°C. Cells were pelleted and then resuspended in 25µL of <sup>111</sup>In-DOTA-biotin (100 ng/mL), incubated at 4°C for 1hr, washed twice, and activity was measured on a gamma counter.

### Mouse RIT and PRIT studies

Female athymic nude-*Foxn1*<sup>nu</sup> mice, aged 5 to 6 weeks, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). NCI-H929 and L363 cells ( $1 \times 10^7$ ) were injected subcutaneously into the right flank 9 to 11 days prior to study start dates. Mice bearing palpable plasmacytoma xenografts measuring  $100 \text{ mm}^3 \pm 10\%$  were selected for the studies and randomly assigned to experimental groups. MM tumor-bearing mice were placed on biotin-free diet for 5 days and injected with either 1.4nmol anti-CD38 OKT10-DOTA Ab or control BHV1-DOTA Ab each directly labeled with <sup>111</sup>In, or 1.4nmol of anti-CD38 OKT10 Ab-SA (OKT10-CC or OKT10-FP) or control Ab-SA (BHV1-CC or CC49 [recognizes the

irrelevant TAG-72 antigen on human adenocarcinomas]- scFv<sub>4</sub>SA-FP) followed 22hr later by 5.8nmol (50μg) CA and 2hr later by 1.2nmol (1μg) <sup>111</sup>In-DOTA-biotin for biodistributions or <sup>90</sup>Y-DOTA-biotin labeled with 400μCi (14.9 MBq), 800μCi (29.6 MBq), or 1200μCi (44.4 MBq) <sup>90</sup>Y for therapy studies. Mice were monitored thrice weekly for general appearance, tumor volume measurements, and body weight. Mice were injected with anti-asialoGM1 antiserum (200uL, WAKO, Richmond, VA) 9 days and 5 days prior to the injection of Ab-SA to abrogate natural killer cell activity and prevent spontaneous tumor regressions. Mice were euthanized when tumors reached a maximum bi-directional measurement of 20mm×20mm, when tumor ulceration occurred, or when mice lost >30% of baseline body weight, as required by institutional animal care guidelines.

### Blood clearance studies

Blood clearance studies were conducted according to the double-label method of Pressman. (31, 32) <sup>131</sup>Iodine (<sup>131</sup>I)-OKT10-CC (1.4nmol) and <sup>125</sup>Iodine (<sup>125</sup>I)-OKT10-FP (1.4nmol) were co-injected into mice via the tail vein (i.v.). NAGB (N-acetyl-galactosamine-biotin) CA (5.8nmol) was injected 24hr later. Venous sampling was conducted via the retro-orbital plexus at serial time points. <sup>125</sup>I and <sup>131</sup>I were counted on a gamma counter, and the %IDs/g of blood were calculated. Counts were corrected for <sup>131</sup>I crossover into the <sup>125</sup>I channel. Counts were also corrected for radioactive decay using an aliquot of the injectate.

### Dosimetry

Absorbed radiation doses to organs were calculated for <sup>90</sup>Y using beta kernel methods for localized beta dosimetry expressly developed for accurately calculating the radiation doses to small organs and tissues of the mouse. (33, 34) These methods account for energy losses by source and take into account the organ self-dose specific absorbed fractions and the beta-particle cross-organ dose contributions. Femoral bone marrow (BM) doses were determined using a model which incorporates Monte Carlo calculations of the energy absorbed fractions in the marrow shafts. (35) This model also accounts for the contributions of <sup>90</sup>Y on bone surfaces, if any, that may contribute to BM dose.

### Statistical Considerations

Differences in MM tumor xenograft volumes were compared by computing the means and standard deviations of each treatment group and employing Student's t-test to determine statistical significance. For relatively large differences in tumor volume, 8–10 mice per group were projected to provide adequate power to detect statistically significant differences. Only the detection of large differences between treatment groups was considered to be of clinical interest.

## RESULTS

### OKT10 anti-CD38 reagents are cell surface stable and enable excellent pretargeting

Experiments assessing binding and internalization of <sup>90</sup>Y labeled OKT10-Ab and FP were performed using 4 MM cell lines (L363, NCI-H929, RPMI-8226, MM1R), a CD38-expressing non-Hodgkin's lymphoma (NHL) line (Ramos), and 4 MM patient biopsy samples. These studies evaluated the degree of internalization, cell surface retention, passive dissociation ("shedding"), intracellular metabolism, and exocytosis of <sup>90</sup>Y-labeled metabolites, using flow cytometric, cell binding, and "acid-wash" methods as previously published for other targets.(36, 37) Only a minority (9±3%; Supplemental Figure 1) of CD38-targeted Ab or FP was internalized by L363 MM tumor cells after 24hr (the time interval between the 2 reagents in PRIT). After 24hr, ~60% of initially bound OKT10-Ab remained on the cell surface, while 30–40% dissociated passively from the cell surface into

the culture medium. A TCA precipitation assay was used to demonstrate that the radioactivity released into the culture medium reflected passive “shedding” of intact Ab rather than exocytosis of  $^{90}\text{Y}$ -labeled small molecular weight fragments from cells (detailed in supplemental materials). To demonstrate effective PRIT targeting of  $^{90}\text{Y}$ -DOTA-biotin, MM cells were labeled with Ab-SA, washed and incubated either at  $4^{\circ}\text{C}$ , (a temperature which completely inhibits endocytosis), or at  $37^{\circ}\text{C}$ . After 24hr of incubation,  $^{90}\text{Y}$ -DOTA-biotin was added and the amount of radioactivity targeted to MM cells was measured. The difference in cell associated radioactivity was minor, indicating that the magnitude of endocytosis of the 1<sup>st</sup> step reagent in 24hr was not sufficient to prevent effective targeting of the 2<sup>nd</sup> step reagent ( $^{90}\text{Y}$ -DOTA-biotin). Concordant results were seen for 3 other cell lines and the patient samples tested. These conclusions also pertain *in vivo* since favorable *in vivo* targeting of  $^{111}\text{In}$ -DOTA-biotin to xenografts is observed [Figure 3b–e].

### Comparative biodistributions of radioactivity demonstrate OKT10 PRIT improves the therapeutic index

Blood, tumor and nonspecific organ uptake of 1-step directly radiolabeled (conventional) OKT10 (OKT10-DOTA) was compared with multistep PRIT in 6 biodistribution experiments involving athymic nude mice bearing s.c. CD38+ human myeloma tumor xenografts (NCI-H929 and L363). Studies were designed to evaluate the relative merits of two distinct pretargeting constructs, OKT10-CC and OKT10-FP (administered at equimolar concentrations). Sixteen groups of 5 mice each were injected with 1.4nmol of OKT10-DOTA, OKT10-FP or OKT10-CC (including matched antibody isotope or FP matched controls). The PRIT reagents (OKT10-FP or OKT10-CC) were followed, 18–22hr later, by 5.8nmol CA and 2hr thereafter by 1.2nmol  $^{111}\text{In}$ -DOTA-biotin (1 $\mu\text{g}$ ). Groups of animals were euthanized at 2, 24, 48, and 96hr after injection of the radioactivity. Tumors excised from mice pretargeted with OKT10-CC contained  $8.8\pm 2.8\%$  of the injected dose of  $^{111}\text{In}$ -DOTA-biotin per gram(%ID/g) after 24hr compared to  $0.9\pm 0.4\%$  ID/g after 24hr in tumors excised from control mice pretargeted with a control Ab-SA chemical conjugate (BHV1-CC; all values are mean  $\pm$ S.D.) [Figure 3b]. The 24hr tumor-to-normal organ ratios of absorbed radioactivity were 10:1; 8:1; and 6:1 respectively for lung, liver and kidney in mice pretargeted with OKT10-CC; compared to < 1:1 for the lung, liver and kidney in control mice pretargeted with BHV1-CC.

Tumors excised from mice pretargeted with OKT10-FP demonstrated uptakes of radioactivity in tumor sites ( $6.4\%$  ID/g $\pm 2.3\%$  after 24hr) that were similar to those observed with the chemical conjugate while minimal tumor uptakes were seen in the controls treated with CC49-FP ( $0.55\%$  ID/g $\pm 0.21\%$  after 24hr; all values are mean  $\pm$ S.D.) [Figure 3c]. Tumor-to-organ ratios of absorbed radioactivity with OKT10-FP PRIT were 27:1; 10:1 and 3:1, respectively, for lung, liver and kidney at the 24hr time-point compared to 1:1 for the same organs in control mice pretargeted with the negative control CC49-FP. The minimal tumor uptake of radiobiotin detected in both the CC49-FP and BHV1-CC control groups demonstrates the specificity of targeting with OKT10-FP and OKT10-CC (Figure 3b,c).

While levels of radioactivity in tumor xenografts measured 24hr after administration of the radioactive species were similar in the OKT10-FP, OKT10-CC and OKT10-DOTA treated mice ( $6.4\pm 2.3\%$ ,  $8.8\pm 2.8\%$  ID/g, and  $9.4\pm 2.2\%$  respectively); PRIT (OKT10-CC and OKT10-FP) led to biodistributions of radioactivity that were far superior to the conventional (OKT10-DOTA) approach (Figures 3a–e). At 24hr following injection of the radiolabeled species, the tumor-to-blood ratio for the conventional RIT was <1:1, while for the OKT10-FP and OKT10-CC ratios were 638:1 and 9:1 respectively, confirming the capacity for PRIT to circumvent the tumor-to-normal organ distribution limitations seen with conventional 1-step RIT.(38, 39)

Excellent target specificity was demonstrated for both PRIT constructs. While tumor-to-blood ratios were most dramatic with the FP, this finding appears to be a consequence of very rapid circulatory clearance of the FP (see blood clearance studies below) and did not lead to superior overall biodistributions. For both constructs the kidney represented the normal organ with the largest non-specific radiation uptake. Tumor-to-kidney ratios were 6:1 and 3:1 for the CC and FP respectively 24hr after the  $^{111}\text{In}$ -DOTA-biotin infusion. Thus, the kidney was identified as the organ likely to define dose limiting toxicity in high dose therapy settings, and the findings supported an overall advantage for OKT10-CC pretargeting.

### Blood clearance studies favor OKT10-CC

Optimal tumor target accretion in PRIT models requires a sustained concentration of the first step SA reagent in the bloodstream during the biodistribution phase of the treatment algorithm, followed by rapid blood clearance after administration of biotinylated polymeric N-acetyl-galactosamine CA. The rapid clearance of OKT10-CC or FP complexed with CA is mediated by asialoglycoprotein receptors in the liver.(26) Detailed blood clearance studies conducted in athymic mice both when administered separately and co-injected(32) with both  $^{131}\text{I}$ iodine ( $^{131}\text{I}$ )-OKT10-CC and  $^{125}\text{I}$ iodine ( $^{125}\text{I}$ )-OKT10-FP at equimolar concentrations, revealed that 16% of the OKT10-CC remained in the circulation 24hr after injection, compared with only 1.4% of the OKT10-FP (not shown).

Synthetic CA reproducibly removed >85% of circulating OKT10-CC or OKT10-FP from the bloodstream within 60 minutes of administration in 5 blood clearance studies. In a representative experiment CA [5.8nmol] was injected 24hr after  $^{131}\text{I}$ -OKT10-CC (300 $\mu\text{g}$ ) into 5 athymic nude mice (i.v.). Retro-orbital venous sampling was conducted at serial time points up to 24hr and  $^{131}\text{I}$  was counted on a  $\gamma$  counter. After CA administration the concentration of  $^{131}\text{I}$ -OKT10-CC dropped from 22.8% $\pm$ 3.1%ID/g of blood to 3.2% $\pm$ 0.3%ID/g 60 minutes later [all values are mean  $\pm$ S.D.] (Figure 4). Consistent with prior studies(27), re-equilibration with conjugate in the extravascular space caused a slight rebound rise of the  $^{131}\text{I}$ -OKT10-CC during the subsequent 2hrs (Figure 4).

### Organ dosimetry

A detailed organ dosimetry analysis was performed to calculate  $^{90}\text{Y}$  absorbed dose estimates after OKT10-CC pretargeting (Figure 5). Radiation absorbed doses to tumors, whole body and nine normal tissues were determined based on integrating the areas under time-activity curves constructed by plotting the concentration of  $^{111}\text{In}$ -DOTA-biotin in tissues after various intervals of time.(33) The dosimetry method used accounted for organ self-dose absorbed fractions as well as beta-particle cross-organ dose contributions.(33) The  $^{90}\text{Y}$  absorbed dose estimates (Gy) per millicurie administered calculated from the  $^{111}\text{In}$  tracer generated tumor-to-normal organ ratios of 6:1 for the kidney, lung and liver; 15:1 for the spleen and 10:1 for the whole body.

### Therapy studies

#### **$^{90}\text{Y}$ -DOTA-biotin pretargeted to CD38 eradicates plasmacytomas in vivo—**

Therapy studies were performed in athymic mice (n=8–10/group) bearing s.c. xenografts of either L363 or NCI-H929 malignant plasma cells, producing tumors similar to human plasmacytomas. Reagent concentrations and time-points for administration of OKT10-CC, BHV1-CC and CA were identical to those reported for the biodistribution studies.

$^{90}\text{Y}$ -DOTA-biotin (2 $\mu\text{g}$ ) was labeled with 400, 800, or 1200 $\mu\text{Ci}$  per mouse in 3 OKT10-CC groups and 3 control groups (untreated control; 800 or 1200 $\mu\text{Ci}$   $^{90}\text{Y}$ -DOTA-biotin after BHV1-CC). All L363 tumor xenograft mice in the untreated control and BHV1-CC control

groups experienced exponential MM tumor growth and 78% of the untreated control animals required euthanasia within 17 days (Figure 6a). After 22 days, 100% of the 800 $\mu$ Ci and 89% of 1200 $\mu$ Ci BHV1-CC control groups had required euthanasia due to progressive tumor growth. All mice pretargeted with OKT10-CC prior to  $^{90}\text{Y}$ -DOTA-biotin demonstrated tumor shrinkage by day 6 at all dose levels (Figure 6a). After 20 days, 90% of the OKT10-CC treated animals in the 400, 800 and 1200 $\mu$ Ci groups remained alive. One animal treated with 1200  $\mu$ Ci was euthanized on day 10 due to weight loss (as required by institutional animal care guidelines); however the other 9 animals from that group were healthy, weighing  $106\pm 9\%$  (S.D.) of initial body weight on day 17 (Supplemental Figure 2). Objective remissions were observed within 6 days in 100% of the mice treated with OKT10-CC followed by 1200 $\mu$ Ci of  $^{90}\text{Y}$ -DOTA-biotin, including 100% complete remissions (no detectable tumor in OKT10-CC treated mice compared to tumors that were  $5240\pm 2495\%$  of initial tumor volume in untreated control animals) by day 17 ( $p < 0.0001$ , Student's t-test) [Figure 6a]. After 100 days, 70% of the OKT10-CC treated animals in the 1200 $\mu$ Ci group, 30% in the 800 $\mu$ Ci group and 20% in the 400 $\mu$ Ci group remained alive and tumor-free (Figure 6b). In mice bearing NCI-H929 MM xenograft tumors, 800 $\mu$ Ci pretargeted to OKT10-CC was sufficient to eradicate 100% of tumor xenografts within 23 days. At the identical time point, tumors progressed in all BHV1-CC treated controls receiving 800 $\mu$ Ci. Control tumors were  $2982\pm 2834\%$  of the initial tumor volume ( $p < 0.0001$ , Student's t-test) [Figure 6c]. Seventy days after treatment with 800 $\mu$ Ci of anti-CD38 pretargeted  $^{90}\text{Y}$ -DOTA-biotin, 100% of animals initially bearing NCI-H929 MM tumor xenografts were alive compared with no mice surviving in the control group [Figure 6d]. Hepatic and renal function was assessed through measurement of serum transaminases, blood urea nitrogen and creatinine levels 160 days after mice received  $^{90}\text{Y}$ -DOTA-biotin (800 $\mu$ Ci,  $n=5$ ). No significant toxicity was observed. The measured serum creatinine was  $< 0.4\text{mg/dl}$  in all animals, the average BUN, ALT and AST values were  $48\pm 33\text{mg/dl}$ ,  $46.8\pm 40$  and  $120.4\pm 116.7\text{U/L}$  respectively.

Tumor-to-normal organ ratios from the PRIT biodistribution studies described above were markedly superior to conventional RIT and provided the rationale for dose escalation up to 1200 $\mu$ Ci in PRIT therapy studies. In contrast to the efficacy and tolerability demonstrated with anti-CD38 PRIT, conventionally radiolabeled  $^{90}\text{Y}$ -OKT10-DOTA administered as a single step to athymic nude mice ( $n=10/\text{group}$ ) bearing s.c. NCI-H929 tumor xenografts was not well tolerated. Within 15 days of infusion, 100% of animals receiving 400 $\mu$ Ci of  $^{90}\text{Y}$ -OKT10-DOTA died from complications of radiation toxicity (weight loss, thrombocytopenia [petechiae], and failure to thrive). On day 8, these animals weighed  $79\pm 9\%$  of their initial body weight. When mice were treated with 200 $\mu$ Ci of  $^{90}\text{Y}$ -OKT10-DOTA, all tumor xenografts transiently decreased in size, but 6 of 10 animals exhibited tumor progression by day 40 and one died of radiation toxicity on day 19. All animals in the untreated and  $^{90}\text{Y}$ -BHV1-DOTA control groups (200 and 400 $\mu$ Ci) had either died of tumor progression or radiation toxicity (60%) or demonstrated tumor growth by day 40 (not shown).

To assess if any of the observed anti-tumor activity is attributable to the CD38 mAb in the absence of radiolabeled DOTA-biotin, mice bearing L363 MM xenografts ( $n=10$ ) were treated with OKT10-CC administered as a single agent without subsequent  $^{90}\text{Y}$ -DOTA-biotin infusion. These animals treated with OKT10-CC alone demonstrated no tumor response and 100% required euthanasia by day 17 for tumors that were  $7837\pm 3492\%$  of their initial volume. In contrast, a matched cohort of mice ( $n=10$ ) treated concurrently with identical doses of OKT10-CC followed by CA and  $^{90}\text{Y}$ -DOTA-biotin (800 $\mu$ Ci) demonstrated complete remissions by day 17 in 100% of animals and 70% of these animals were alive and tumor free at day 80 [Figure 7a,b].



## DISCUSSION

Plasma cell malignancies, including MM, are rarely cured. Despite the higher response rates and longer survival afforded by novel agents (2) the vast majority of patients with plasma cell malignancies die of progressive disease emphasizing the importance of continuing to investigate new treatment strategies. RIT is a therapeutic modality that is not cross-resistant with chemotherapy or “novel agents” and has been under-explored in this disease.

External beam radiation can cure isolated malignant plasmacytomas and radiation therapy is frequently used to palliate painful MM bone lesions. Sustained local disease control and durable symptom relief has been reported for 98% of lesions receiving >10Gy. (40) Further, the poor prognostic implications associated with high risk bone marrow cytogenetics in active MM are not predictive of a decrement in the very high rates of local control and cure after external beam radiation therapy is used to treat solitary extramedullary plasmacytomas with the same cytogenetic derangements. (41) This suggests that the unique attributes associated with the targeted delivery of radiation may augur clinical efficacy even among patients classified as “high risk”. When ionizing radiation has been combined with bortezomib, synergistic inhibition of MM cell proliferation has been reported. (12) It has also been suggested that the efficacy of bortezomib can be improved when combined with skeletally targeted <sup>153</sup>samarium lexidronam. (42) These findings raise the possibility that anti-CD38 RIT may be enhanced when combined with novel therapies.

Although several plasma-cell associated antigens have been identified, we elected to target CD38 because it has a high density and consistency of expression on clonal plasma cells. (15, 16) We demonstrate that the receptor has minimal internalization and is not shed from the plasma cell surface after binding to OKT10. The unmodified CD38 mAb daratumumab, which binds a unique epitope on the antigen receptor, has demonstrated anti-MM tumor cell activity both in vitro and in mouse xenografts; (19) however, perhaps as a consequence of binding a different epitope, OKT10-CC administered as a single agent without radioactivity demonstrates no anti-tumor effects. The promising results achieved with daratumomab however, complement our approach targeting CD38 for radionuclide delivery and validate the antigen as a desirable target. Despite the salient favorable attributes enumerated for CD38, no target antigen is perfect and it must be acknowledged that CD38 expression is not limited exclusively to malignant plasma cells. Nevertheless the expression of CD38 on normal plasma cells and some activated lymphoid cells must be viewed in the context of the successful selection strategies which have resulted in clinically approved RIT therapeutics for other hematologic malignancies, such as <sup>131</sup>Iodine-tositumomab (Bexxar) and <sup>90</sup>Yttrium-ibritumomab tiuxetan (Zevalin). The efficacy and favorable toxicity profiles of anti-CD20 RIT in B cell lymphoma have been clearly established despite expression of this antigen on normal B cells. (5–7) The CD38 antigen has many attributes parallel to CD20 to support its selection as viable target for RIT.

Several therapeutic radionuclides might be considered for RIT of MM. We selected <sup>90</sup>Y as the radioisotope for these studies because it is a pure, high-energy  $\beta$ -emitter that is readily complexed by the DOTA chelate and is commercially available in high specific activity and purity. The long path length of <sup>90</sup>Y's  $\beta$  particles permits the delivery of lethal radiation across several cell diameters, resulting in a “crossfire effect” that can eradicate antigen-negative MM cells (or MM cells that are in the center of a tumor cluster) by lethal radiation emitted from surrounding antibody-binding CD38+ MM cells. Furthermore, the current biotin reagents employed for PRIT can only accommodate radiometals, not <sup>131</sup>I, and attempts to produce effective <sup>131</sup>I-labeled biotin have generally been disappointing, re-enforcing the selection of <sup>90</sup>Y.

In this report, we demonstrate that the  $^{90}\text{Y}$ -based anti-CD38 PRIT approach chosen, rapidly and completely eradicates plasmacytoma xenografts with minimal toxicity. This therapeutic efficacy was predicted by anti-CD38 PRIT biodistributions that were markedly superior to those achievable by conventional RIT, as confirmed by radiation dosimetry estimates demonstrating excellent tumor-to-normal organ ratios. While both anti-CD38-SA constructs were effective, the rapid circulatory clearance of OKT10-FP resulted in a lower AUC during the distribution phase; and, despite excellent blood-to-tumor ratios of activity, the FP had lower tumor-to-kidney ratios of absorbed activity (3:1) when compared with OKT10-CC PRIT (6:1) after 24hr. Consistent with prior published reports, biodistribution studies predicted the kidney to be the dose limiting normal organ.(43) The renal biodistribution profile for the chemical conjugate therefore provided a rationale for selection of OKT10-CC as the best pretargeting reagent for subsequent therapy studies.

PRIT enables the administration of  $^{90}\text{Y}$  doses not attainable using conventional one-step RIT and appears to improve the therapeutic index, enhance efficacy and diminish toxicity. Removal of excess OKT10-CC from the vascular compartment by CA, combined with rapid urinary excretion of excess  $^{90}\text{Y}$ -DOTA-biotin, limited nonspecific radiation exposure to normal organs even at doses of 1200 $\mu\text{Ci}$  (three times the previously defined lethal doses in conventional  $^{90}\text{Y}$  labeled RIT).(44) Ninety percent of animals in the highest dose group demonstrated only mild transient weight loss, and regained their normal weight within 15 days (Supplemental Figure 2), while animals receiving either 400 or 800 $\mu\text{Ci}$  demonstrated no significant drop in weight. Among the OKT10-CC animals alive and disease free >70 days after therapy, no significant toxicity was detectable.

We elected to initiate preclinical studies of CD38-targeted RIT and PRIT using a plasmacytoma model because traditional radiation dosimetry and biodistribution methods can be most accurately performed using solid tumor nodules, and are much more difficult to apply in a quantitative fashion using marrow-based, disseminated disease. Tumor xenografts are therefore the standard for initial RIT validation studies (e.g. CD20 in NHL, CD45 in AML). (27) (45–48) In addition, xenograft models have played a vital role in validating the impressive efficacy of proteasome inhibitor therapy in MM and we have emulated this initial approach. (49)

Despite serving as a well validated model for the assessment of new RIT targets and MM therapeutics in large cohorts of animals, MM tumor xenografts do not fully recapitulate the clinical spectrum of the disease. (49, 50) The absence of human CD38 antigen expression in murine tissues is an inherent limitation of most xenograft models, and the xenograft model applies most directly to soft tissue plasmacytomas rather than disseminated MM. Furthermore xenografts do not provide a bone based platform that recapitulates the human marrow microenvironment. To address this concern we are conducting studies in a SCID-hu MM mouse model and have seen preliminary results that are encouraging, but beyond the scope of the current manuscript.

In conclusion, this is the first report of anti-CD38 RIT in MM and the first report documenting the efficacy of PRIT in this disease. The complete eradication of MM tumor xenografts provides a scientifically compelling rationale for further exploring high dose anti-CD38 PRIT as conditioning therapy prior to autologous stem cell transplant for MM. While xenograft tumors are not ideal, they have been used to accurately predict the value of CD20 and CD45 directed RIT or PRIT in lymphoma and leukemia mouse models, and have been used to establish the value of “novel agents” to treat MM despite the same limitations. Ultimately, clinical trials will be necessary to demonstrate safety and evaluate the potential for anti-CD38 PRIT to improve progression-free and long term survival among patients with MM.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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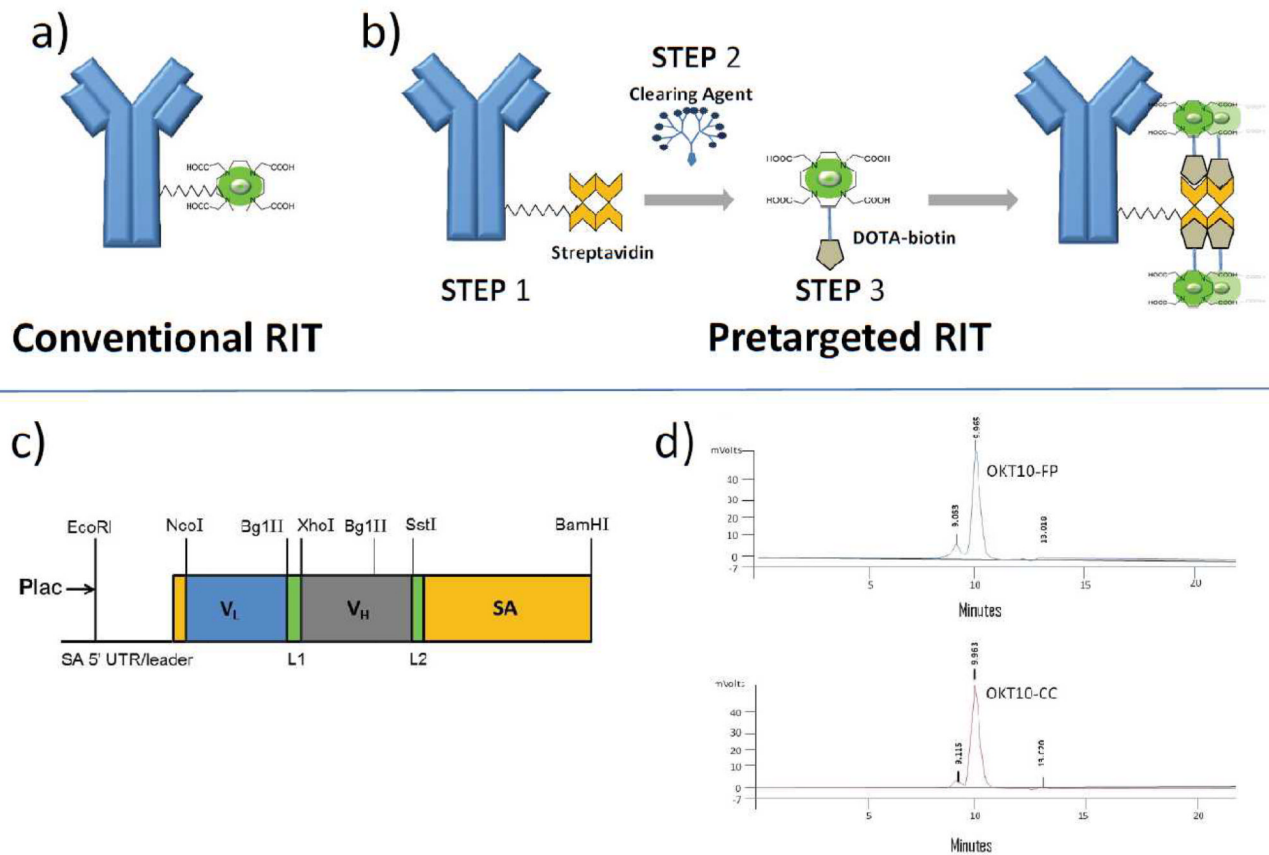
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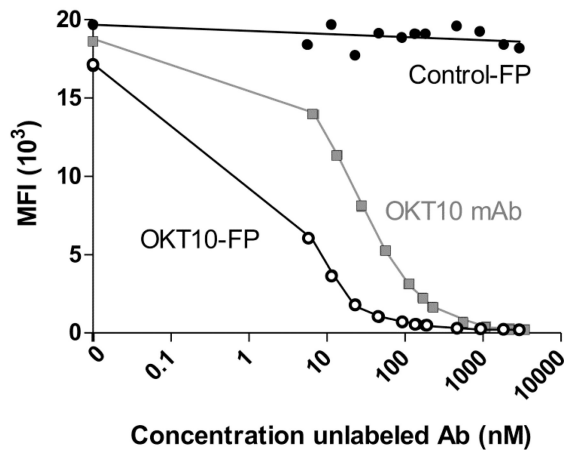
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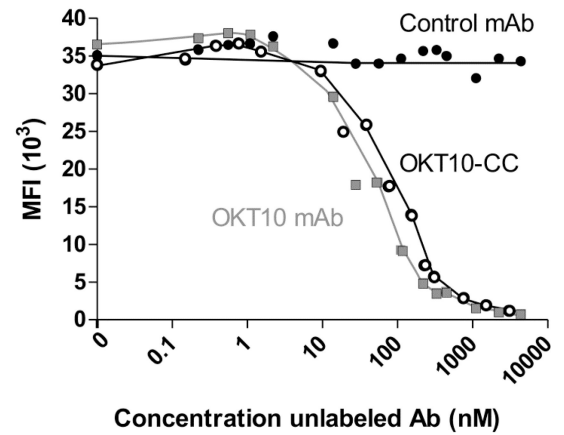


**Figure 1.** Components for pretargeted radioimmunotherapy (PRIT). Schema depicting (a) one-step conventional radioimmunotherapy and (b) multistep PRIT. PRIT involves infusion of the antibody-SA construct [Step 1], followed by injection of a synthetic N-acetylgalactosamine-containing clearing agent [Step 2] designed to facilitate hepatic clearance of excess antibody-SA from the bloodstream, and then infusion of the radiolabeled small molecule DOTA-biotin [Step 3]. (c) Schematic diagram of OKT10 scFv-SA fusion gene. (d) Size exclusion HPLC performed on OKT10-FP and OKT10-CC anti-CD38 streptavidin constructs demonstrate a retention time of 9.965 and 9.963 minutes respectively. A small amount of aggregate is present.

a)

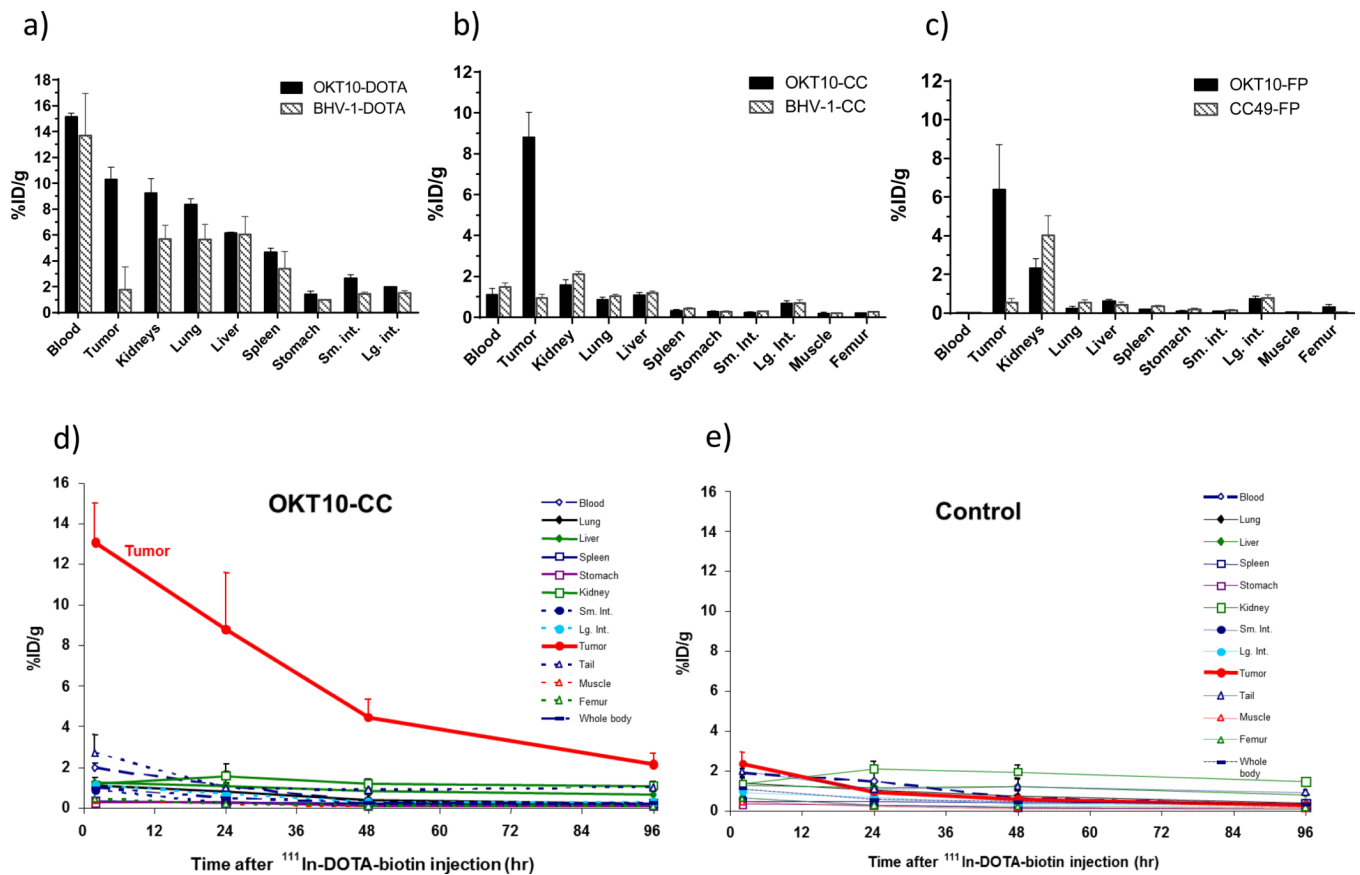


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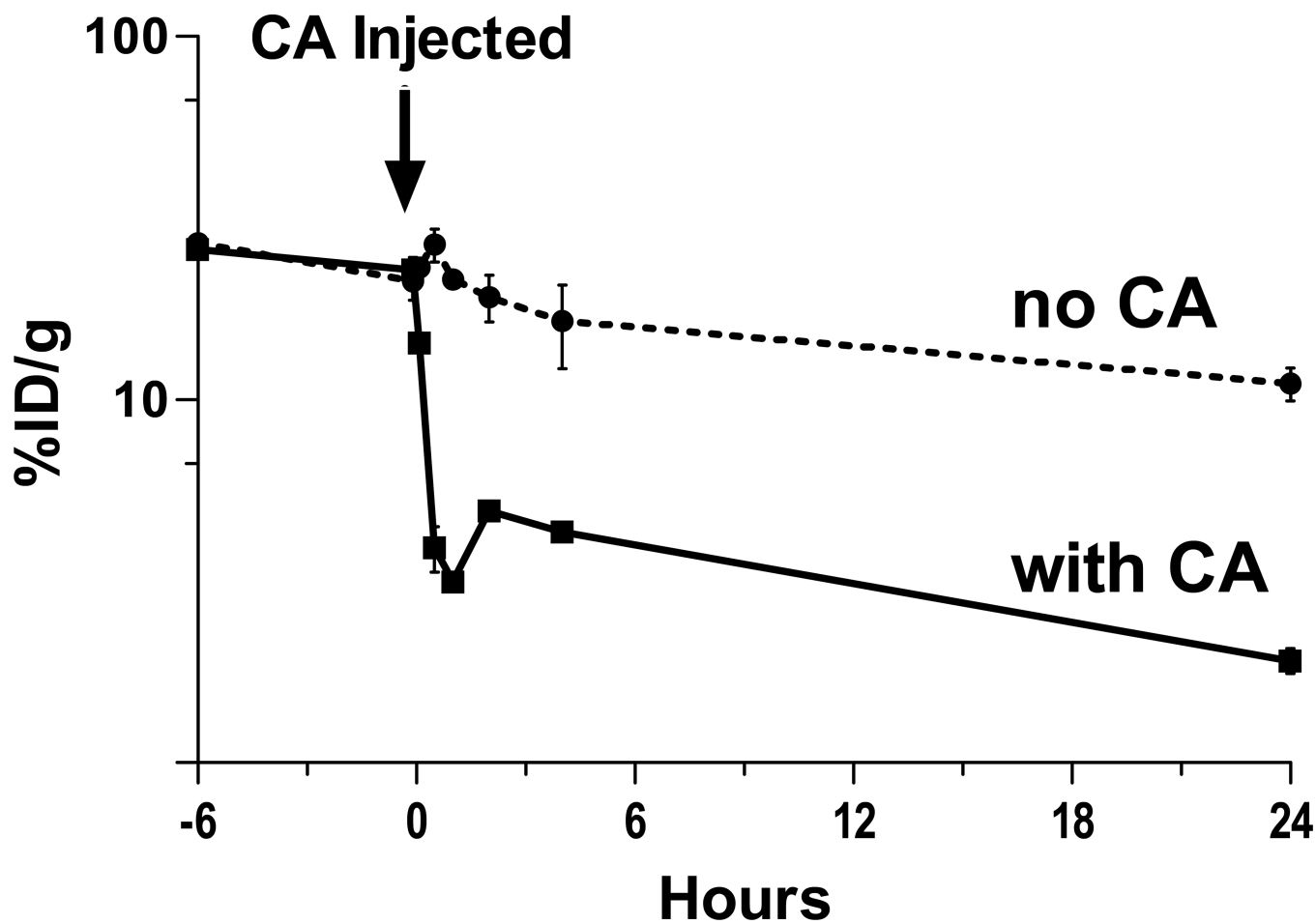
**Figure 2.**

Binding of Anti-CD38 antibody constructs to CD38 on malignant plasma cells. Competitive binding assays demonstrate blocking of Alexa-647 conjugated OKT10 mAb binding to CD38-expressing L363 MM cells with escalating concentrations of unlabeled (a) OKT10-FP or (b) OKT10-CC. No blocking is observed with escalating concentrations of nonbinding control Ab constructs.



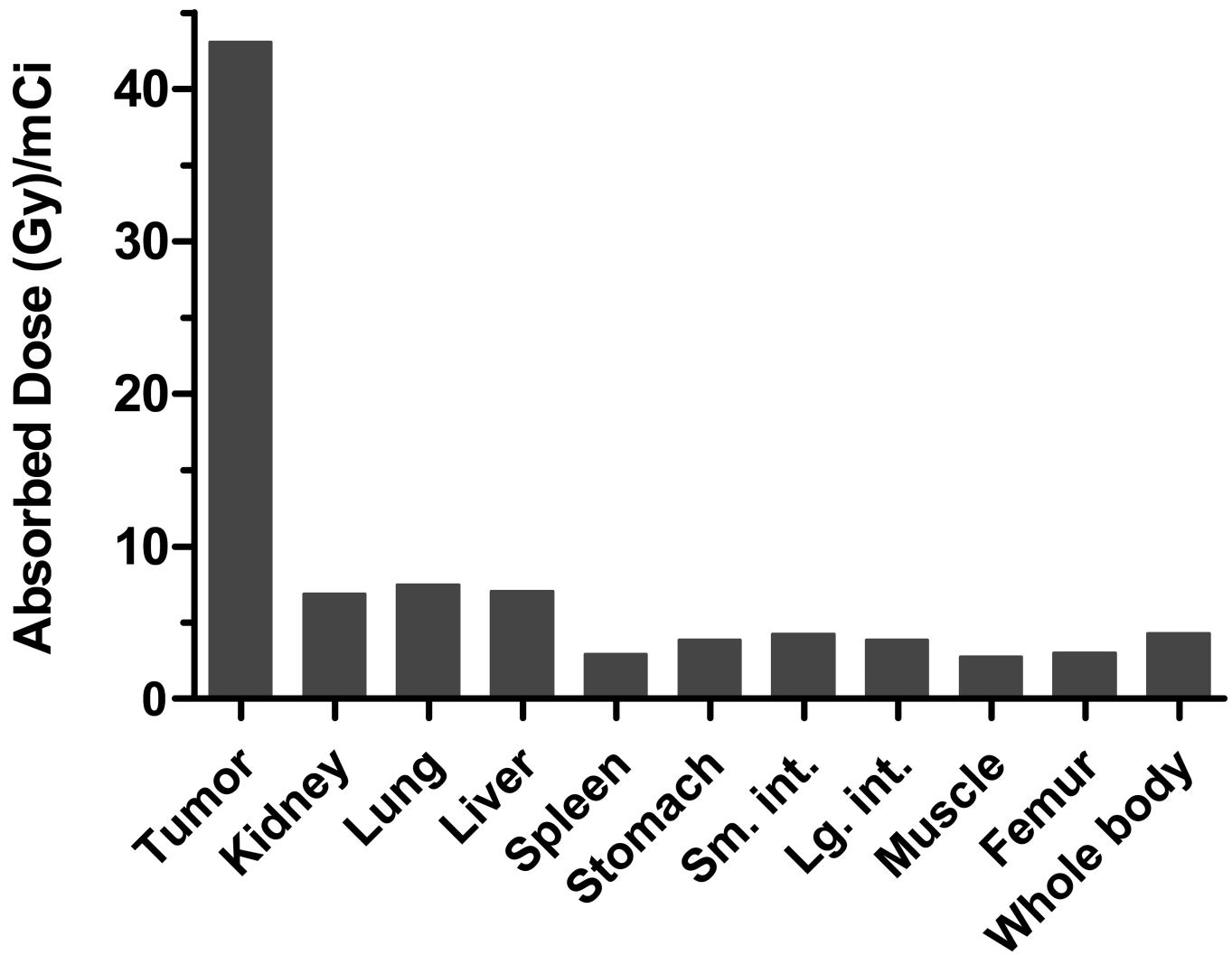
**Figure 3.**

Biodistributions of radioactivity in athymic nude-*Foxn1<sup>tmu</sup>* mice (n=5/group) bearing CD38+ MM xenograft tumors ( $1 \times 10^7$  cells injected into left flank). Blood, tumor and normal organ specimens were obtained 24hr after (a)  $^{111}\text{In}$ -OKT10-DOTA or  $^{111}\text{In}$ -BHV1-DOTA (control) (b)  $^{111}\text{In}$ -DOTA-biotin pretargeted by OKT10-CC or BHV1-CC (control CC) (c)  $^{111}\text{In}$ -DOTA-biotin pretargeted by OKT10-FP or CC49-FP (control FP). In PRIT animal studies (b,c) NAGB-clearing agent (5.8 nmol) was administered 2hr before the  $^{111}\text{In}$ -DOTA-biotin (1.2 nmol). Comprehensive tissue biodistributions of radioactivity obtained at sequential timepoints 2hr, 24hr, 48hr and 96hr following  $^{111}\text{In}$ -DOTA-biotin are shown for the mice from the OKT10-CC (d) and BHV1-CC (e) PRIT groups.

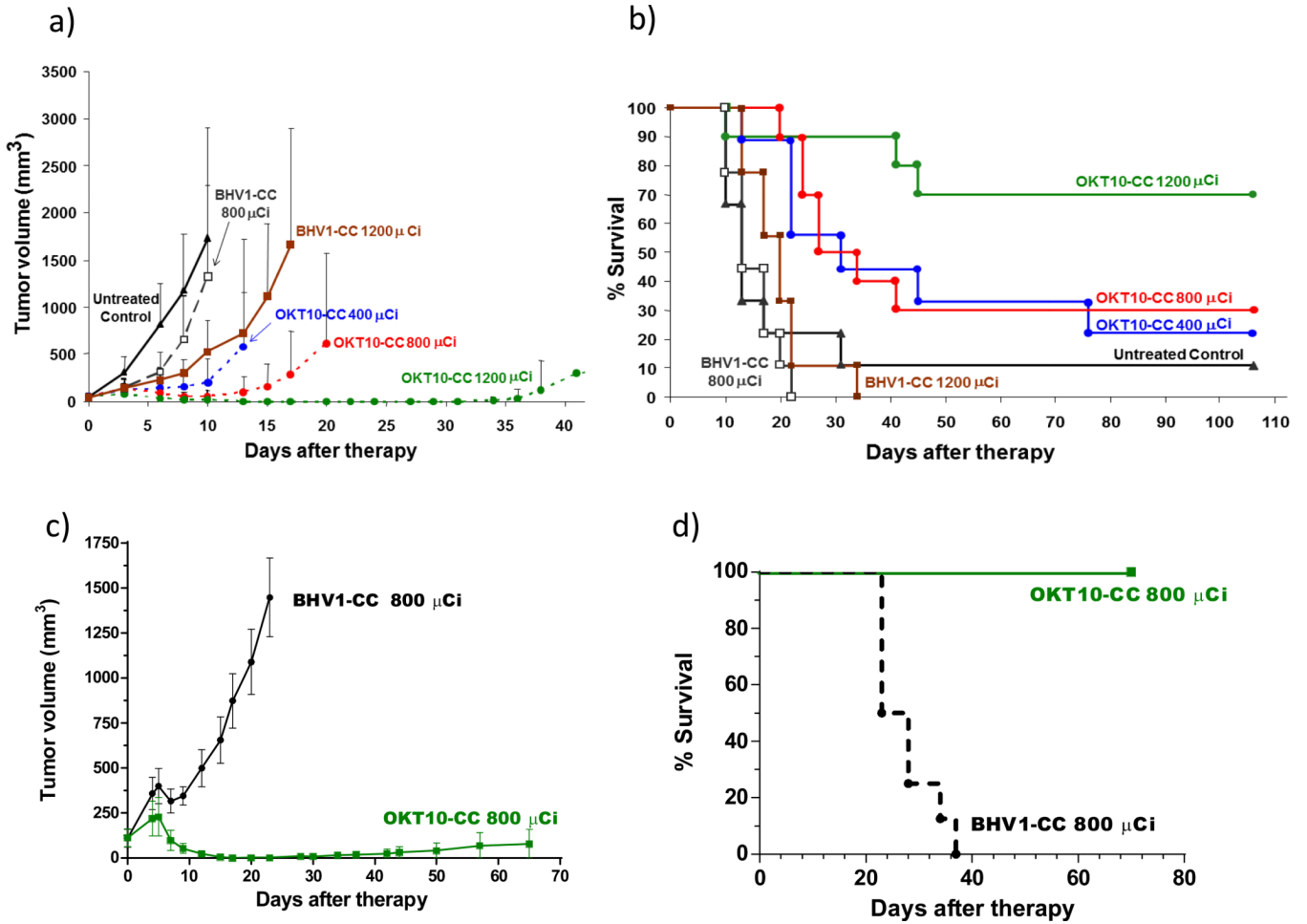


**Figure 4.** Effects of biotinylated N-acetyl galactosamine clearing agent (CA) on circulating OKT10-CC. CA [5.8 nmol] was injected 24hr after  $^{131}\text{I}$ -OKT10-CC (1.4 nmol) into 5 athymic nude mice (i.v.). Five control mice received  $^{131}\text{I}$ -OKT10-CC (1.4 nmol) and no CA. Retro-orbital venous sampling was conducted at serial time points up to 24 hr.

# $^{90}\text{Y}$ Absorbed Dose Estimates (Calculated from $^{111}\text{In}$ Tracer)

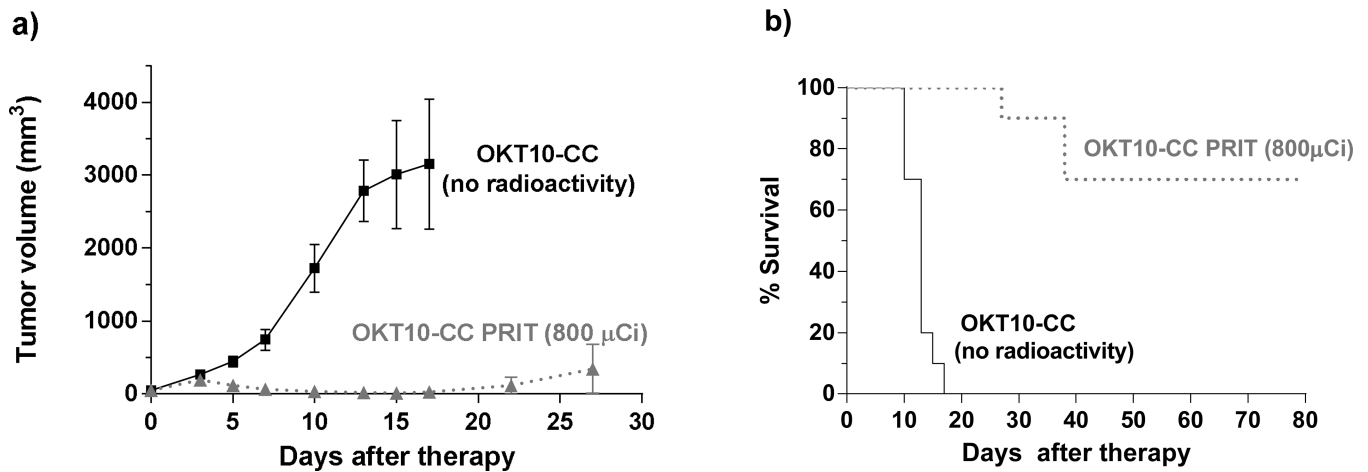


**Figure 5.**  $^{90}\text{Y}$  absorbed dose estimates (Gy/mCi) from an  $^{111}\text{In}$ -DOTA-biotin tracer. Radiation absorbed doses to tumor, whole body and normal tissues are estimated by integrating the areas under time-activity curves constructed by plotting the concentration of  $^{111}\text{In}$ -DOTA-biotin (after OKT10-CC pretargeting) measured in tissues by  $\gamma$ -counting at 2, 24, 48 and 96hr.(33)



**Figure 6.**

Tumor responses in athymic nude *mice* (n=8–10/group) bearing L363 (a, b) or NCI-H929 (c, d) right flank MM xenografts. Mice received 1.4 nmol (300 μg) of OKT10-CC or BHV1-CC (control) and CA [5.8 nmol (50 μg)]. Treatment groups received <sup>90</sup>Y-DOTA-biotin 24hr after the OKT10-CC at doses of 400 μCi (14.9 MBq), 800 μCi (29.6 MBq) & 1200 μCi (44.4MBq); and at doses of 800 μCi and 1200 μCi for the BHV1-CC control groups. (a) Mice were monitored thrice weekly for tumor volume measurements and were euthanized when tumors were 10% of body weight or when ulceration occurred as mandated by the institutional animal care committee. (Curves are truncated at the time the first animal was euthanized in each group). A dose dependent regression of MM tumor xenografts is demonstrated. (b) Kaplan-Meier analysis of cumulative survival of mice bearing L363 MM xenografts. (c) NCI-H929 treatment groups received 800 μCi (29.6 MBq) of <sup>90</sup>Y-DOTA-biotin 24hr after the OKT10-CC or BHV1-CC (control group). Complete regression of MM tumor xenografts in the OKT10-CC group is demonstrated. (d) Kaplan-Meier survival analysis of mice bearing NCI-H929 MM xenografts.



**Figure 7.**

Tumor responses in athymic nude-mice (n=10/group) bearing L363 right flank MM xenografts. All mice received 1.4 nmol (300 μg) of OKT10-CC as a single agent (no radioactivity) or followed by CA [5.8 nmol (50 μg)] and <sup>90</sup>Y-DOTA-biotin (800 μCi [29.6 MBq]) 24hr after the OKT10-CC. (a) Mice were monitored thrice weekly for tumor volume measurements and were euthanized when tumors were 10% of body weight or when ulceration occurred as mandated by the institutional animal care committee. (Curves are truncated at the time the first animal was euthanized in each group). Complete regression of MM tumor xenografts in the group receiving OKT10-CC followed by 800 μCi (29.6 MBq) is demonstrated. Animals receiving OKT10-CC as a single agent (no radioactivity) demonstrated rapid tumor progression. (b) Kaplan-Meier analysis of cumulative survival of mice bearing L363 MM xenografts.