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Removal of Fc Glycans from [⁸⁹Zr]Zr-DFO-Anti-CD8 Prevents Peripheral Depletion of CD8⁺ T Cells

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Abstract

The *N*-linked biantennary glycans on the heavy chain of immunoglobulin G (IgG) antibodies (mAbs) are instrumental in the recognition of the Fc region by Fc-gamma receptors (FcγR). In the case of full-length mAb-based imaging tracers targeting immune cell populations, these Fc:FcγR interactions can potentially deplete effector cells responsible for tumor clearance. To bypass this problem, we hypothesize that the enzymatic removal of the Fc glycans will disrupt Fc:FcγR

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Author Contributions

JMW, OMK, and BEK performed experiments. JMW, OMK, BMZ, HMG, and NTV analyzed data. JMW, BMZ, HMG, and NTV wrote the manuscript. BMZ, HMG, and NTV conceptualized the studies. All authors edited and reviewed the manuscript prior to submission.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.0c00270>.

Tables comparing CD8⁺ T cell depletion, biodistribution, imaging dose tracer uptake; CD8⁺ T cell binding titration; isotype control imaged mice; CD45⁺/CD3⁺/CD4⁺ flow cytometric analysis of wt and deglycosylated tumors; spleen flow analysis (PDF)

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interactions and spare tracer-targeted immune cells from depletion during immunopositron emission tomography (immunoPET) imaging. Herein, we compared the *in vitro* and *in vivo* properties of ^{89}Zr -radiolabeled CD8-specific murine mAb (anti-CD8_{wt}, clone 2.43), a well-known depleting mAb, and its deglycosylated counterpart (anti-CD8_{degly}). Deglycosylation was achieved via enzymatic treatment with the peptide: *N*-glycosidase F (PNGaseF). Both anti-CD8_{wt} and anti-CD8_{degly} mAbs were conjugated to *p*-SCN-Bn-desferrioxamine (DFO) and labeled with ^{89}Zr . Bindings of both DFO-conjugated mAbs to Fc γ R and CD8⁺ splenocytes were compared. *In vivo* imaging and distribution studies were conducted to examine the specificity and pharmacokinetics of the radioimmunoconjugates in tumor-naïve and CT26 colorectal tumor-bearing mice. *Ex vivo* analysis of CD8⁺ T cell population in spleens and tumors obtained postimaging were measured via flow cytometry and qRT-PCR. The removal of the Fc glycans from anti-CD8_{wt} was confirmed via SDS-PAGE. A reduction in Fc γ R interaction was exhibited by DFO-anti-CD8_{degly}, while its binding to CD8 remained unchanged. Tissue distribution showed similar pharmacokinetics of [^{89}Zr]Zr-DFO-anti-CD8_{degly} and the wt radioimmunoconjugate. *In vivo* blocking studies further demonstrated retained specificity of the deglycosylated radiotracer for CD8. From the imaging studies, no difference in accumulation in both spleens and tumors was observed between both radiotracers. Results from the flow cytometry analysis confirmed depletion of CD8⁺ T cells in spleens of mice administered with DFO-anti-CD8_{wt}, whereas an increase in CD8⁺ T cells was shown with DFO-anti-CD8_{degly}. No statistically significant difference in tumor infiltrating CD8⁺ T cells was observed in cohorts administered with the probes when compared to control unmodulated mice. CD8 mRNA levels from excised tumors showed increased transcripts of the antigen in mice administered with [^{89}Zr]Zr-DFO-anti-CD8_{degly} compared to mice imaged with [^{89}Zr]Zr-DFO-anti-CD8_{wt}. In conclusion, the removal of Fc glycans offers a straightforward approach to develop full length antibody-based imaging probes specifically for detecting CD8⁺ immune molecules with no consequential depletion of their target cell population in peripheral tissues.

Graphical Abstract

Keywords

CD8 imaging; immunoPET; Fc glycan removal; immune cell depletion

■ INTRODUCTION

Advances in cancer immunotherapy created an immediate need to noninvasively monitor patient response and predict outcomes. This led to the development of immuno-positron emission tomography (immunoPET) tracers as companion diagnostic tools for assessing immune modulation. ImmunoPET uses monoclonal antibodies (mAbs) or their fragments for targeted delivery of PET radionuclides to cell surface-localized antigens or soluble proteins in the tumor microenvironment.^{1–4} These radioimmunoconjugates are popular imaging platforms due to streamlined and facile radiosynthetic protocols and the commercial availability of mAbs and long-lived radionuclides such as zirconium-89 (⁸⁹Zr, $t_{1/2}$ = 3.27 d).⁵ One pitfall of immunoPET, however, lies in the functional nature of the mAb, which can potentially modulate the activity of the target molecule or deplete the target cell. Depletion, which eliminates target cells, can be detrimental to immune cell populations with specific functions in immune oncology.^{6,7} Indeed, exposure to mAbs in picomolar to nanomolar concentrations can eradicate immune cells and effector molecules, negatively impacting tumor clearance.^{7,8}

A common approach to overcoming this drawback is the complete removal of the mAb's Fc region. The Fc region determines the effector function of an IgG mAb. Depending on the subclass, it can deplete the target cell by triggering antibody-dependent cell-mediated cytotoxicity (ADCC) through interaction with Fc-gamma receptors (Fc γ R) found on specific immune populations or by engaging complement proteins, resulting in complement-dependent cytotoxicity (CDC).^{8–10} Antibody engineering and fragmentation can be used to eliminate the Fc region. However, these processes are fraught with complications and can be time-consuming and expensive, hindering rapid development of immunoPET imaging agents for initial proof-of-concept studies. Therefore, more cost-effective and simpler methods are needed to prevent Fc:Fc γ R-mediated depletion without altering the pharmacokinetics or affinity of the antibody for its target.

One possible alternative strategy for developing immunoPET tracers targeting immune cells is the specific removal of the highly conserved N-linked glycans found on the C_H2 domain of the Fc region. These heavy chain glycans influence the binding of Fc γ R to the Fc region of IgGs,^{11,12} and their removal has been reported to disrupt Fc:Fc γ R binding,^{13,14} suggesting this approach may enable development of antibody-based immune cell imaging tracers while preventing cell depletion. Along these lines, it has been demonstrated that the deglycosylation of a desferrioxamine (DFO)-bearing immunoconjugate (i.e., DFO-trastuzumab_{degly}) dramatically attenuates its binding to both murine and human Fc γ RI.¹⁵ Moreover, it has been shown that a ⁸⁹Zr-labeled variant of this deglycosylated radioimmunoconjugate (i.e., [⁸⁹Zr]Zr-DFO-trastuzumab_{degly}) boasts significantly improved *in vivo* performance, specifically higher activity concentrations in the tumor with lower activity accumulation in the liver, spleen, and bones, versus its fully glycosylated counterpart ([⁸⁹Zr]Zr-DFO-trastuzumab).¹⁵ Similar *in vitro* and *in vivo* results have recently been reported for a second radioimmunoconjugate, a site-specifically labeled variant of [⁸⁹Zr]Zr-DFO-pertuzumab.¹⁶ These examples illustrate the benefits of Fc glycan removal within the context of improving the pharmacokinetics of a radiotracer for companion diagnostic purposes.

During tumor immunotherapy, infiltration of activated cytotoxic CD8⁺ T cells leads to tumor cell killing. As a result, monitoring these cells noninvasively via molecular imaging may indicate therapeutic response.^{17,18} With this in mind, we examined the effects of Fc glycan removal on the murine anti-CD8 mAb clone 2.43, a classic example of a CD8⁺ T cell-depleting mAb, with the goal of developing a full-length mAb immunoPET agent^{8,19,20} with no consequential depletion of its target CD8⁺ T cells. We deglycosylated the anti-CD8 2.43 (anti-CD8_{degly}) mAb via the peptide:*N*-glycosidase F (PNGaseF)-mediated cleavage of the *N*-linked oligosaccharides on its Fc region. Anti-CD8_{degly} was then titrated and incubated with splenocytes to examine CD8⁺ T cell binding. Its binding to murine FcγRI (mFcγRI) was further tested via ELISA. Changes in peripheral CD8⁺ T cell populations were measured via flow cytometry after administration of either anti-CD8_{degly} or unmodified wild-type mAb (anti-CD8_{wt}) in immunocompetent mice. To test their utility as imaging agents, both antibodies were conjugated with *p*-isothiocyanato-benzyl-desferrioxamine (DFO) and radiolabeled with ⁸⁹Zr. Biodistribution and competitive binding studies were performed in cohorts of mice administered with either the radiolabeled wt or deglycosylated mAbs to compare specificity and pharmacokinetics. Finally, we investigated the ability of both wt and deglycosylated tracers to detect CD8⁺ T cells in the immunogenic murine CT26 colorectal tumor model by immunoPET. Snap-frozen tumor tissue from imaged mice was evaluated for CD8 mRNA and IFN-γ transcripts after radioisotope decay. The impact of wt or deglycosylated mAb injection on peripheral spleen and tumor infiltrating CD8⁺ T cells was then examined by flow cytometry.

■ MATERIALS AND METHODS

Antibody Deglycosylation.

GlycoBuffer 2 (100 μL, 10× 0.5 M sodium phosphate, pH = 7.5) and Remove-iT PNGaseF (13 μL, 225 U/μL) were added to 2 mg of rat antimouse CD8α IgG2bκ clone 2.43 (BioXCell, West Lebanon, NH, BE0061), and to 2 mg of rat antimouse KLH IgG2bκ clone LTF-2 isotype control antibody (BioXCell, BE0090). The mixture was placed in an agitating thermomixer for 24 h at 37 °C and 500 rpm. The anti-CD8 2.43 PNGaseF reaction was then purified by incubation with magnetic chitin beads at 4 °C for 10 min, followed by magnetic rack separation. The solution was then concentrated by centrifugal filtration units with a 50,000 molecular weight cut off (MWCO, Amicon Ultra 2 mL, Millipore Corp.). The reaction was then analyzed by SDS-PAGE using 2 μg of each sample (0.5 mg/mL stock solutions of anti-CD8_{degly}, anti-CD8_{wt}, deglycosylation reaction and PNGaseF), with 18.5 μL of H₂O, 3 μL 500 mM dithiothreitol (NuPAGE 10× Sample Reducing Agent, Life Technologies), and 7.5 μL of 4× electrophoresis buffer (NuPAGE LDS Sample buffer, Thermo Fisher, Eugene, OR). The solution was then denatured by heating to 85 °C for 15 min with an agitating thermomixer. Finally, the samples were loaded along with a molecular weight marker (Novex Sharp Pre-Stained Protein Ladder, Life Technologies) onto a 1 mm, 10-well 4–12% Bis-Tris protein gel (Life Technologies) and run for ~7 h at 55 V in MOPS buffer. The completed gel was washed in triplicate with H₂O and stained with SimplyBlue SafeStain (Life Technologies) for 1 h and then destained overnight in H₂O. The gel was analyzed with an Odyssey CLx Imaging system (Li-Cor Biosciences).

Murine Fc γ RI ELISA.

An Immulon 4 HBX plate (Thermo Fisher Scientific, 3855) was coated with 4 μ g/mL recombinant mouse Fc γ RI (mFc γ RI, R&D Systems 2074-FC) in PBS and incubated overnight at 4 °C. After washing in triplicate with PBS-T (PBS + 0.05% Tween 20), the plate was blocked for 1 h at room temperature with 1% BSA in PBS-T with continuous shaking. After incubation, the plate was washed three times with PBS-T. The tested antibody (5 μ g/mL in 1% BSA in PBS-T) was added to the plate for 2 h, with shaking, followed by three washes with PBS-T. Then horseradish peroxidase-conjugated goat antirat IgG (H+L) in 1% BSA in PBS-T (1:5000) (Jackson ImmunoResearch Laboratories Inc., 112-035-003) was added to the plate for 1 h, with shaking, and subsequently washed three times with PBS-T. Next, 50 μ L/well of TMB-blotting solution (Thermo Fisher Scientific, 34018) was added, and the reaction was quenched after 5 min with 50 μ L/well 1 N HCl. The plate was read at 450 nm with a SpectraMax i3X (Molecular Devices).

Cell Lines and Xenografts.

CT26 cells were cultured in a sterile environment at 37 °C and 5% CO₂ using RPMI-1640 with Glutamax (Gibco) containing 10% FBS (Hyclone, SH30910.03, v/v) and 1% penicillin/streptomycin (Corning, 30-002-CI, v/v). All animal handling and experiments were conducted in accordance with the guidelines and regulations set by the Wayne State University Institutional Animal Care and Use Committee (IACUC). Male BALB/c mice (6–8 weeks, Charles River) were inoculated subcutaneously on the right shoulder with 1×10^5 CT26 tumor cells in 1:1 RPMI-1640 medium: Matrigel Matrix HC (Corning, 354262). The tumor growth was monitored by caliper measurement with tumor volume calculated as $V = L \times W \times H \times \pi/6$ until tumors reached a volume of ~150–250 mm³.

DFO Conjugation and Radiochemistry.

The anti-CD8_{wt}, anti-CD8_{degly}, and their respective IgG isotype control antibodies (IgG_{wt} and IgG_{degly}) were conjugated with DFO (Macrocyclics, LLC) (1:10 anti-CD8 mAb:DFO, 1:5 IgG:DFO). Briefly, a solution of DFO (66 nmol or 33 nmol of 20 mM stock in dimethyl sulfoxide) was added to 6.6 nmol of respective antibody in 1 $\times$ PBS, pH ~8.5–9. The solutions were incubated at 37 °C for 1.5 h. Following incubation, excess unbound DFO was removed via centrifugal filtration at 3000 rpm for 10 min using a 30 kDa MWCO filter with sterile saline as eluent (Vivaspin 500). [⁸⁹Zr]Zr⁴⁺ radiolabeling was conducted following previous protocols.⁴ Briefly, [⁸⁹Zr]Zr-oxalate (74 MBq, 2 mCi, 3D Imaging, LLC, Little Rock, AR) was diluted in saline and adjusted to pH ~7.0–7.5 with 1 M Na₂CO₃ in metal-free water. All antibodies were incubated (0.4 mg, 2.67 nmol) with the solution containing the radioisotope for 0.5 h at room temperature. Following labeling, 5 μ L of 20 mM EDTA was added to quench the reaction. The respective tracers were purified using a centrifugal filter (30 kDa MWCO, Vivaspin 500) to remove unbound radiometal and spun in triplicate at 3000 rpm for 10 min using sterile saline as the eluent. Radiochemical purity was analyzed by radio-instant thin layer chromatography (iTLC, Mini-Scan/FC, Eckert and Ziegler).

***In Vivo* CD8⁺ T Cell Depletion Analysis with DFO-mAb Conjugates.**

Naive BALB/c male mice ($n = 3/\text{group}$) were treated intraperitoneally (i.p.) with either 50 μg or 500 μg of DFO-anti-CD8_{wt} or DFO-anti-CD8_{degly}. In a separate study, CT26 colorectal tumor-bearing BALB/c mice were each administered 50 μg of either DFO-mAb conjugates by intravenous (i.v.) injection on the lateral tail vein. Spleens or tumor were removed at 48 h postinjection (p.i.) and dissociated for flow cytometry analysis.

Spleen and Tumor Dissociation.

Spleens were dissociated with 2 mL of 1 $\times$ PBS using frosted glass slides. The cells were pelleted by centrifugation at 4 $^{\circ}\text{C}$ for 5 min at 1500 rpm. The pellet was resuspended, and the red blood cells were lysed with 1 mL of H₂O followed by the addition of 1 mL of 2 $\times$ PBS. Large debris was allowed to settle. The supernatant was then transferred to a new tube, which was centrifuged to obtain a cell pellet. The splenocytes were resuspended with 1.5% FBS in 1 $\times$ PBS for flow cytometry. CT26 tumors from BALB/c mice were dissociated with the GentleMACs dissociator and mouse tumor dissociation kit (Miltenyi, Germany, 130–096–730) following the manufacturer's protocol.

Flow Cytometry.

All flow cytometry studies were conducted using a BD LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The following panel was used: BV605-conjugated CD19 (clone 6D5, Biolegend, 115540) for spleen cells, BV605-conjugated CD45 for tumor cells (clone 30-F11, BD Biosciences, 563053), APC efluor 780-conjugated CD3 (clone 17A2, Invitrogen, 47–0032–82), PerCP-Vio700-conjugated CD8 α (clone 53–6.7, BD Biosciences, 566410), Alexa Fluor-488-conjugated CD4 (clone RM4–5, Biolegend, 100532), and ghost viability dye Violet 510 (TONBO biosciences, 13–0870-T100). Spleen samples were gated based on viable CD3⁺/CD19[–]/CD4[–] cells and compared as % CD3; tumor samples were gated based on viable CD45⁺/CD3⁺/CD4[–] cells and compared as % CD45 and % CD3. In tissues administered with DFO-anti-CD8 conjugates, the detection of CD8⁺ T cells was indirectly measured as a % CD3⁺CD4[–] population due to the interference of the CD8 molecule by the imaging antibody. This method demonstrated the presence of the tracer bound to the CD8⁺ T cells evidenced by a population shift of CD8⁺ cells from the lower right quadrant to the lower left quadrant for the flow cytometry data.

CD8⁺ T Cell Binding of DFO-Anti-CD8_{wt} and DFO-Anti-CD8_{degly}.

Direct CD8⁺ T cell binding of both DFO-anti-CD8_{wt} and DFO-anti-CD8_{degly} was examined. Both immunoconjugates were labeled with cyanine-5.5 Mono-NHS Ester (cy5.5, GE Healthcare, PA15601,) at a 1:7 antibody:fluorophore mole ratio. Excess unbound cy-5.5 was removed by centrifugal filtration with a 30 kDa MWCO at 3000 rpm for 15 min. Binding to CD8 was analyzed via flow cytometry after incubating each immunoconjugate at different concentrations (100 pg/mL to 16 $\mu\text{g/mL}$) in separate wells containing 1×10^6 splenocytes.

PET Imaging.

Tumor-naïve and CT26 tumor-bearing BALB/c male mice were injected i.v. in the lateral tail vein with the radiolabeled mAbs (200–250 μ i/mouse, 7.4–9.4 MBq, 40–50 μ g). Images were acquired at 24–120 h p.i. while the mice were anesthetized with 2% isoflurane (Henry Schein) in oxygen using a Focus 220 microPET camera (Siemens Concord Medical Solutions). Images were reconstructed through filter back projection, decay-corrected to time of injection, and analyzed for uptake by manually drawing three-dimensional volumes of interest (VOI) to determine mean percent injected dose per gram (%ID/g) on specific tissues. Mice were euthanized after the last scan. Separate cohorts of mice were then injected with either of the nonradiolabeled tracers ($n = 5$ /group), or control ($n = 5$), and CD8⁺ T cells in the tumors and spleens were examined by flow cytometry.

Tissue Biodistribution and Competitive Inhibition Blocking Assay.

Mice were i.v. administered either a biodistribution dose (40–50 μ Ci, 1.48–1.85 MBq, 8–10 μ g) or an imaging dose of (200–250 μ Ci/mouse, 7.4–9.4 MBq, 40–50 μ g) of [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} or [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} ($n = 5$ /group) in the lateral tail vein, and euthanized via CO₂ asphyxiation at 48 h p.i. Separate cohorts of mice was coinjected with 10-fold excess (80–100, μ g) of either cold DFO-anti-CD8_{wt} or DFO-anti-CD8_{degly}. Radioactive tissues were harvested, weighed, and measured for bound activity via a gamma counter (PerkinElmer Wizard² 2480). Tracer uptake was measured as the percentage of radioactivity bound to tissue per gram of tissue weight (%ID/g).

Quantitative Reverse Transcribed PCR.

Tumor tissue from tracer injected mice ($n = 5$ /group) was snap-frozen in liquid nitrogen and homogenized in Trizol for RNA extraction per manufacturer protocol (Thermo Fisher, Waltham, MA). cDNA was synthesized with a ProtoScript First Strand cDNA synthesis kit (New England Biolabs, MA, E6300S). qRT-PCR was conducted with Taqman probes (Thermo Fisher) for CD8 α (mm01188922_m1), IFN- γ (mm01168134_m1), and GAPDH (mm99999915_g1) using a RNA equivalent of 10 ng of cDNA/well. Relative mRNA was calculated as ($2^{-\Delta CT}$) relative to GAPDH and control tumor expression for both CD8 and IFN- γ . Transcript levels were compared against control untreated mice ($n = 5$) and CT26 cells *in vitro*.

Statistical Analyses.

Statistical analyses were completed with GraphPad Prism 7.02 (GraphPad Software Inc., San Diego, CA, USA). Values are presented as mean $\pm$ standard deviation. All tests were conducted with a two-tailed Student's *t* test unless otherwise noted. A $p < 0.05$ was considered statistically significant.

RESULTS

Preparation and Characterization of Anti-CD8_{degly}.

Enzymatic removal of the *N*-linked glycans was performed using PNGaseF, an amidase that cleaves between the *N*-acetylglucosamine and asparagine residues of oligosaccharides. SDS-

PAGE confirmed enzymatic modification occurring only on the heavy chain of the mAb (Figure 1A). No detectable PNGaseF enzyme was present in the anti-CD8_{degly} final, purified product. We next examined the binding of each DFO-mAb to mFc γ RI via ELISA. Significantly decreased Fc:mFc γ RI binding was observed for DFO-anti-CD8_{degly} relative to DFO-anti-CD8_{wt} (Figure 1B). Finally, we evaluated the ability of DFO-anti-CD8_{degly} to bind CD8⁺ T cells via flow cytometry in a serial dilution assay. We found that deglycosylation did not significantly affect antibody binding compared to the wt mAb (Figure S1).

Deglycosylated Anti-CD8 mAb Does Not Deplete Peripheral CD8⁺ T Cells.

Flow cytometry was used to assess changes in CD8⁺ splenocyte populations, defined as CD3⁺/CD4⁺, in mice treated with 50 μ g (low) or 500 μ g (high) doses of either DFO-anti-CD8_{wt} or DFO-anti-CD8_{degly} (Figure 2A–E). Surprisingly, an increase in CD8⁺ T cells was detected in mice that received either low ($15.6 \pm 2.3\%$, $p < 0.0001$) or high ($13.1 \pm 1.5\%$) doses of DFO-anti-CD8_{degly} compared to the control untreated ($7.7 \pm 1.1\%$, $p = 0.0013$) and DFO-anti-CD8_{wt} treated cohorts (low: $p < 0.0001$, high: $p < 0.0001$). On the other hand, minimal viable CD8⁺ T cell populations remained in mice administered with both low (Figure 2B) and high-dose (Figure 2C) DFO-anti-CD8_{wt} compared to untreated mice (Figure 2F, Table S1), demonstrating the depleting capacity of the mAb at doses used for preclinical *in vivo* imaging.

[⁸⁹Zr]Zr-Anti-CD8_{degly} Exhibited Equivalent Splenic Uptake to wt Tracer.

Both wt and deglycosylated mAbs were radiolabeled with [⁸⁹Zr]Zr⁴⁺ to compare their utility as imaging tracers. A radiochemical purity of >99% was achieved for all probes based on iTLC. Specific activities of 185 ± 3.7 MBq/mg (5.0 ± 0.1 mCi/mg) and 185 ± 7.4 MBq/mg (5.0 ± 0.2 mCi/mg) were established for [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} and [⁸⁹Zr]Zr-anti-CD8_{degly}, respectively. Acquired PET images were utilized to examine the uptake of both radiotracers in the spleen, a CD8⁺ T cell-rich tissue in immunocompetent mice (Figure 3A). Surprisingly, while a significant difference in uptake was observed initially at 24 h p.i. in mice injected with [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} ($11.7 \pm 0.4\%$ ID/g) versus [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} ($8.5 \pm 0.3\%$ ID/g, $p = 0.0013$), no disparities were displayed at later time points. Maximum intensity projections (MIPs) and planar sections of images acquired with [⁸⁹Zr]Zr-anti-CD8_{degly} clearly displayed the spleen with decreased nonspecific uptake in background tissues compared to the wt probe (Figure 3B). In comparison, both [⁸⁹Zr]Zr-labeled nonspecific IgG_{wt} and IgG_{degly} showed poor delineation of the spleen (Figure S2). This study confirms that the specificity of the deglycosylated radiotracer is retained *in vivo*.

Comparison of Specificity and Pharmacokinetics.

Tissue biodistribution was performed at 48 h p.i. to evaluate the pharmacokinetics of [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} and examine its specificity for CD8. Nominal accumulation was displayed in the pancreas, bone, muscle, and heart (Figure 4A, Table S2). The liver, the main route of clearance for full-length mAbs, exhibited comparable radiotracer uptake. The highest accumulation was observed in the spleen (Figure 4A, inset) with both [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} ($35.5 \pm 8.1\%$ ID/g, Figure 4A) and [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} ($29.8 \pm 3.1\%$ ID/g, $p = 0.184$) displaying relatively similar accumulation. As expected, splenic

uptake of nonspecific isotype control mAbs [^{89}Zr]Zr-DFO-IgGwt ($2.5 \pm 0.9\%$ ID/g) and [^{89}Zr]Zr-DFO-IgG_{degly} ($2.1 \pm 0.3\%$ ID/g) was lower compared to the CD8-specific probes.

Each radiotracer's specificity was further investigated via coadministration of corresponding excess nonradiolabeled mAbs. A significant decrease in splenic accumulation was observed for both [^{89}Zr]Zr-DFO-anti-CD8_{wt} ($8.3 \pm 2.3\%$ ID/g, $p = 0.0008$) and [^{89}Zr]Zr-DFO-anti-CD8_{degly} ($4.9 \pm 0.8\%$ ID/g, $p < 0.0001$) (Figure 4A). The blocked cohorts exhibited decreased spleen uptake, which was accompanied by a concomitant increase in blood activity (Figure 4A, Table S2).

Discrepancies in tissue uptake between imaging and biodistribution doses were observed. Thus, we performed tissue biodistribution in mice injected with imaging doses (5fold higher) of either [^{89}Zr]Zr-DFO-anti-CD8_{wt} or [^{89}Zr]Zr-DFO-anti-CD8_{degly} (Figure 4B). Tracer uptake remained the highest in the spleen: $15.1 \pm 0.8\%$ ID/g for [^{89}Zr]Zr-DFO-anti-CD8_{wt} and $11.7 \pm 1.1\%$ ID/g for [^{89}Zr]Zr-DFO-anti-CD8_{degly} ($p = 0.005$, Table S3). The 2-fold lower splenic uptake in these cohorts compared to the results from the tissue distribution study is likely due to mass effects. Of note, tracer uptake in the spleen, lung, and gastrointestinal tissues (i.e., pancreas, stomach and small intestines) is significantly lower for the deglycosylated tracer, contributing to cleaner images with improved contrast (Figure 3B). Collectively, these studies confirmed identical specificity and similar pharmacokinetics of [^{89}Zr]Zr-DFO-anti-CD8_{degly} and the wt radiotracer.

Comparison of wt Versus Deglycosylated Anti-CD8 Tracers in Immunogenic CT26 Tumors.

We next sought to examine the deglycosylated radiotracer's potential to delineate intratumoral CD8⁺ T cell infiltration in BALB/c syngeneic CT26 colorectal tumors. These tumors are characteristically immunogenic with endogenous CD8⁺ T cell infiltrates present even without immune modulation. Uptake of both probes in the tumor was not significantly different (Figure 5A). Similar to earlier experiments (Figures 3 and 4), we also observed no difference in spleen uptake between the two tracers in tumor-bearing mice. To validate the presence of CD8 mRNA transcripts in tumors from this imaging experiment, we performed qRT-PCR on snap-frozen tumor sections, after tissues decayed to background, to measure transcript levels of CD8 and IFN- γ , a cytokine produced by activated CD8⁺ T cells, NIK cells, and the Th1 subset of CD4 T cells. Intratumoral transcript levels of CD8 in the [^{89}Zr]Zr-DFO-anti-CD8_{wt} cohort were similar to the control mice. Similar to the splenic flow cytometry with unlabeled wt or deglycosylated mAb (Figure 2), a marginal increase in CD8 transcript was observed in the [^{89}Zr]Zr-DFO-anti-CD8_{degly} cohort, which was not statistically significant when compared to control (Figure 5B, $p = 0.1$). Interestingly, higher transcript levels of IFN- γ were displayed by tumors administered with [^{89}Zr]Zr-DFO-anti-CD8_{degly} compared to control (Figure 5C, $p = 0.007$) and those injected with [^{89}Zr]Zr-DFO-anti-CD8_{wt} ($p = 0.006$). This study suggests the presence of CD8 in mice administered with either the wt or deglycosylated radiotracer.

We examined the peripheral and intratumoral depletion capacity of both conjugates in tumor-bearing mice by flow cytometric analysis. CT26 tumors and spleens from mice injected with nonradiolabeled DFO-anti-CD8 constructs were utilized to avoid the strikingly reduced viability we encountered arising from prolonged radiation exposure of dissociated,

frozen tissue samples during decay in storage (~1 month). There was no significant difference in % CD8⁺ T cells in the tumor (previously defined as % CD3⁺/CD4⁻), for untreated control, DFO-anti-CD8_{wt}, and DFO-anti-CD8_{degly} injected mice (Figure 5D, Figure S3A, % CD3: 7.5 ± 2.8%, 4.2 ± 1.8%, and 6.0 ± 2.7%, respectively). Additionally, there was no significant difference in CD8⁺ T cells as a percentage of the leukocyte common antigen, CD45 (Figure S3B,C, 1.7 ± 1.3%, 3.1 ± 0.8%, and 4.6 ± 3.1%, respectively). Consistent with earlier experiments, reduced percentages of CD8⁺ T cells were observed in the spleens of tumor-bearing mice administered with DFO-anti-CD8_{wt} (Figure 5E, 2.9 ± 0.5%) versus control (8.3 ± 0.7%, *p* < 0.0001). Higher CD8⁺ T cells were detected in mice injected with DFO-anti-CD8_{degly} (13.3 ± 1.7%) versus DFO-anti-CD8_{wt} (*p* < 0.0001) and control (*p* < 0.0001). These results suggest that target cell depletion in the CT26 microenvironment is less efficient than the periphery. Furthermore, we detected a similar increase in splenic CD8⁺ T cell percentages as in our initial depletion study with tumor-naïve mice and nonradiolabeled immunoconjugates (Figure 2). Collectively, the consistent increase in CD8⁺ cell density and enhanced IFN- γ transcript levels (Figure 5) of nondepleting anti-CD8_{degly} can imply that the engagement of the CD8 coreceptor with mAb 2.43 has stimulatory capacity.

DISCUSSION

Despite the nascent development of immune oncology drugs, only moderate outcomes are achieved, in part due to a dearth of reliable methods to monitor immune activity within the tumor bed. Patients receiving immunotherapy can experience a phenomenon known as pseudoprogression, marked by an observed increase in tumor volume or delay in apparent response due to an influx of immune cells. Pseudoprogression has led to premature classification of tumor progression by anatomical imaging modalities like MRI.²¹ This highlights the necessity of molecular imaging techniques, such as PET, to examine tumor response to treatment by specific molecular targeting. [¹⁸F]FDG imaging can universally identify all metabolically active cells but lacks the ability to interrogate specific immune cell populations that could influence response to immunotherapy. Recently, [¹⁸F]F-AraG has been developed to image activated T cells by accumulating through the deoxypurine salvage pathway.^{22,23} However, cytotoxic effects toward the T-lymphocyte and T-lymphoblastoid cell populations have been noted.²² This poses a concern, as the tracer lacks specificity for CD8⁺ cytotoxic T cells and can also adversely affect the immune response.

The most immediate approach to developing clinically relevant targeted imaging agents is via immunoPET, which relies on using full-length mAbs or their fragments as carriers of imaging reporters. However, their utility for imaging the immune system brings several challenges. Full mAbs are functional proteins with pharmacologic effects that can alter immune function, a downstream effect that is not warranted within the scope of imaging. A major drawback to full mAb use is the potential to deplete the target immune cell population. This can be problematic, especially for tracers directed at effector cells that are responsible for tumor clearance. One common approach to circumvent this issue is the creation of immunoconjugates without Fc regions.^{24,25} Along these lines, either antibody fragmentation can be used to produce Fab or F(ab')₂ fragments from full-length mAbs or genetic engineering can be used to create minibodies, diabodies, or scFv that lack the Fc

region.¹ However, these approaches have several disadvantages. For example, protocols for enzymatic cleavage are highly variable between mAbs, have been known to produce low yields, and may potentially affect immunoreactivity.^{26,27}

To date, most CD8 imaging studies have primarily utilized fragmented or engineered antibodies as radioisotope carriers to avoid Fc:Fc γ R-mediated cell depletion. Tavaré et al. developed CD8-specific minibodies and diabodies to monitor tumor response to several immunotherapies.^{3,8,28} Utility of these engineered mAb formats showed no notable effects on the CD8⁺ T cell population. Recently, the anti-CD8 minibody IAB22M2C completed Phase I clinical trials for first-in-human ⁸⁹Zr-radiolabeled PET/CT imaging of CD8⁺ T cells, which showed favorable uptake in CD8⁺ T cell-rich tissues and tumor.²⁹ In addition, a ⁸⁹Zr-radiolabeled single-domain antibody fragment (VHH-X118) successfully delineated CD8 expression following checkpoint blockade in both B16 melanoma and breast cancer models.³⁰ While the benefit of antibody fragmentation and engineering provides optimal pharmacokinetic properties such as decreased blood pool residency and faster tumor accumulation, from our perspective, the process is complex, time-consuming, and expensive. Thus, this is unfavorable especially during early stage tracer development.

Prior efforts from our group to develop an anti-CD8 radiotracer using the full length mAb YTS-105.18, previously reported to lack depleting effects,^{31,32} showed apparent reduction of the target cell population in vivo.⁴ This led us to explore simplified approaches to develop radiotracers with minimal to no impact on the target immune cells and their function. We found that removal of biantennary glycans can mitigate Fc:Fc γ R interactions and consequently prevent the disruption of immune activity within the tumor microenvironment. Herein, we report the effects of deglycosylation on mAb effector function within the context of immunoPET. To the best of our knowledge, this is the first study that has developed and evaluated a CD8-specific deglycosylated full mAb radiotracer.

We selected the CD8 antibody clone 2.43 due to its well-established depletion effects. We have shown that the deglycosylated immunoconjugate abolished binding to mFc γ RI, in contrast to the wt mAb, yet retained its affinity for the CD8 antigen. Administration of both DFO-mAbs in mice confirmed the reduction of peripheral CD8⁺ T cells by DFO-anti-CD8_{wt} even at low doses (~50 μ g) used for imaging, whereas the CD8⁺ lymphocyte population remained significantly higher in mice treated with DFO-anti-CD8_{degly}. For flow cytometric analyses, detection of CD8 was indirect (CD3+CD4⁻) due to the apparent blockade of the anti-CD8 mAb 53–6.7 binding epitopes by the 2.43 mAb. This is advantageous because it clearly indicates the presence of the 2.43 mAb on the CD8⁺ T cells that were evaluated. Biodistribution studies confirmed that the pharmacokinetic properties of the deglycosylated construct remained similar to that of the wt probe. Competitive blocking studies confirmed the specificity of both tracers. Furthermore, the in vivo specificity of [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} was demonstrated by comparison to the uptake of [⁸⁹Zr]Zr-DFO-IgG_{degly} probe. Interestingly, both wt and deglycosylated tracers marked CD8-rich tissues equally well despite the depleting nature of the wt probe. This result may likely be due to a number of reasons including residualization of ⁸⁹Zr, permitting a signal for PET imaging despite the elimination of the target. The presence of tissue resident effector cells can bind to the Fc region of the wt antibody, further contributing to tracer uptake and retention in the spleen, a

preferential site for accumulation of monoclonal antibodies.³³ Moreover, subtle but critical differences on the cellular level, as detected by flow cytometry, can be undetected by PET due to its limit of detection.

Comparable uptake of both tracers was observed in the tumor and spleen of PET imaged CT26-bearing BALB/c mice. The improved contrast of the MIPs observed in the [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} imaged mice can be due to a lack of nonspecific Fc:Fc γ R uptake. We then examined the effects of these radiotracers on intratumoral CD8 mRNA transcripts by qRT-PCR on snap-frozen tumor tissue collected after PET imaging. CD8 mRNA transcripts of mice injected with [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} remained unchanged, while the transcript levels from the [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} probe showed a nominal increase. The unchanged CD8 mRNA levels in the tumors of the [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} tracer injected mice identified an inability to deplete the target population. This approach was chosen to allow for the decay of the radionuclide prior to analysis. Unfortunately, we observed radiation-associated cell toxicity in dissociated frozen tumors after imaging, which confounded assessment of viable CD8⁺ T cell infiltrates. To further evaluate the impact of wt and deglycosylated tracers on intratumoral CD8⁺ T cells, we administered nonradiolabeled DFO-mAb conjugates in mice bearing CT26 tumors. The presence of viable CD8⁺ T cell population was scrutinized in both tumors and spleens. Interestingly, unlike the peripheral CD8 population where we saw similar depletion by the wt tracer as in nontumor-bearing mice, DFO-anti-CD8_{wt} did not noticeably affect the CD8⁺ T cell population in the tumor. While preservation of the infiltrating CD8⁺ population is important for maintaining antitumor immunity, depletion of peripheral CD8⁺ T cells is not ideal in a clinical setting, as these cells are important for normal immune homeostasis, including protection against infection. Additionally, the CT26 tumor microenvironment may abnormally lack sufficient infiltration of depletion-mediating effector cells, which may be more abundant or effective in other malignancies and lead to intratumoral CD8 depletion with a wt mAb.

We did not expect to find an apparent boost in the CD8⁺ T cell population in the spleen with the deglycosylated mAb. In contrast, the tumor bed showed comparable CD8⁺ T cell numbers and levels of CD8 mRNA transcripts with the wt tracer. However, IFN- γ mRNA transcripts were enhanced after exposure to DFO-anti-CD8_{degly}. These results suggest that CD8⁺ T cells may be activated by the 2.43 mAb in the absence of depletion, an effect of variable domain (Fv) binding at the target epitope that would otherwise not have been distinguished. This is not entirely surprising, as a tracer to the CD4 coreceptor has also shown stimulatory properties.³⁴ It is important to consider similar Fc-independent functional aspects when selecting a mAb for tracer development.

Taken together, our results underscore the utility of deglycosylation to produce antibody-based imaging tracers in a straightforward and cost-effective approach for preliminary tracer development studies. Deglycosylation at the highly conserved asparagine-297 residue in the Fc region of IgG antibodies attenuates Fc:Fc γ R binding for the development of any immunoPET tracer.^{35–38} While this study is centered on imaging CD8⁺ T cells, the concepts demonstrated here can be further utilized to develop imaging tracers for other cell populations. However, the impact of antibody deglycosylation on other targets should still be determined empirically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of anti-CD8_{degly}. (A) PNGaseF deglycosylation of anti-CD8 clone 2.43. Lane 1, MW ladder; 2, anti-CD8_{degly}; 3, anti-CD8_{wt}; 4, deglycosylation reaction; 5, PNGaseF; 6, MW ladder. (B) Optical density (OD) values at 450 nm were obtained in quadruplicate by ELISA to compare the Fc-FcγR binding following deglycosylation. In contrast to DFO-anti-CD8_{wt}, DFO-anti-CD8_{degly} had diminished binding to mFcγRI. *** $p = 0.0004$.

Figure 2.

DFO-anti-CD8_{degly} does not deplete the CD8⁺ T cell population. Flow cytometry analysis of spleens of (A) untreated mice, mice administered with DFO-anti-CD8_{wt} (B) 50 μ g or (C) 500 μ g, and mice treated with DFO-anti-CD8_{degly} (D) 50 μ g or (E) 500 μ g. In the untreated mice, a CD8⁺ T cell population was observed in Q3. The CD8⁺ T cell population was not present in the anti-CD8_{wt} mice as exhibited by the decrease in the cell population in Q4. The percentages noted in the quadrants for panels A–E are representative of one mouse. (F) CD8⁺ T cells analysis expressed as mean $\pm$ SD from one experiment ($n = 3$ mice/group) and analyzed by one-way ANOVA with a Tukey's posthoc analysis. **** $p < 0.0001$ versus wt. \$\$ $p = 0.0013$, \$\$\$ $p < 0.001$, \$\$\$\$ $p < 0.0001$ versus control.

Figure 3.

In vivo imaging of CD8 in BALB/c male mice. (A) Time activity curve of spleen uptake of both [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} and [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} identifies similar tracer uptake from 24 to 120 h p.i. ($n = 3$ mice/group, $n = 5$ mice/group at 48 h). (B) Maximum intensity projections (MIP, top panels) and planar sections (bottom panels) demonstrate overall distribution and uptake of both [⁸⁹Zr]Zr-DFO-anti-CD8_{wt} (left) and [⁸⁹Zr]Zr-DFO-anti-CD8_{degly} (right) at 48 h p.i. S = spleen. ** $p < 0.01$.

Figure 4.

Ex vivo validation of tracer specificity. (A) Tissue distribution of the $[^{89}\text{Zr}]$ Zr-DFO-anti-CD8 tracers ($n = 5$ /tracer for each time point) exhibits nominal uptake in organs with the highest uptake in the spleen (inset). Blocked cohorts ($n = 5$ /tracer) administered with 10-fold excess cold unmodified antibody respectively exhibited decreased tracer uptake in the spleen. (B) To examine mass effects on the pharmacokinetics of the tracers, an imaging dose of 200–250 μCi of tracer was intravenously injected. Splenic uptake remained the highest but displayed lower accumulation in comparison to the biodistribution dose (A). * $p < 0.05$,

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Stom. = Stomach, Sm. Int. = Small Intestines, Lg. Int. = Large Intestines, Panc. = Pancreas.

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Figure 5.

In vivo CT26 tumor imaging and *ex vivo* flow cytometric analysis. (A) Maximum intensity projections (MIP, top left) and planar sections (bottom right) of $[^{89}\text{Zr}]\text{Zr-DFO-anti-CD8}_{\text{wt}}$ ($n = 5$) and $[^{89}\text{Zr}]\text{Zr-DFO-anti-CD8}_{\text{degly}}$ ($n = 5$) in CT26 immunogenic colorectal tumors (T) showed no difference in uptake (right). (B) mRNA analysis of CD8 in the tumor showed a decrease in CD8 transcripts in the $[^{89}\text{Zr}]\text{Zr-DFO-anti-CD8}_{\text{wt}}$ imaged mice compared to the $[^{89}\text{Zr}]\text{Zr-DFO-anti-CD8}_{\text{degly}}$ mice. (C) IFN- γ transcripts were significantly lower in the $[^{89}\text{Zr}]\text{Zr-DFO-anti-CD8}_{\text{wt}}$ injected mice. mRNA studies were completed in triplicate from $n = 5$ mice/group and $n = 2$ samples from the CT26 cell line. Statistical analysis of mRNA was conducted by one-way ANOVA with a Tukey's *post hoc* analysis. (D) Flow cytometric analysis of dissociated CT26 from mice injected with nonradiolabeled tracers exhibited no difference in CD8 $^{+}$ T cells. (E) In spleens, DFO-anti-CD8 $_{\text{wt}}$ exhibited lower CD8 $^{+}$ T cells compared to control and DFO-anti-CD8 $_{\text{degly}}$; DFO-anti-CD8 $_{\text{degly}}$ exhibited significantly higher CD8 $^{+}$ T cells compared to control and wt.