Influence of Fc Modifications and IgG Subclass on Biodistribution of Humanized Antibodies Targeting L1CAM

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Immuno-PET is a powerful tool to noninvasively characterize the in vivo biodistribution of engineered antibodies. Methods: L1 cell adhesion molecule–targeting humanized (HuE71) IgG1 and IgG4 antibodies bearing identical variable heavy- and light-chain sequences but different fragment crystallizable (Fc) portions were radiolabeled with 89Zr, and the in vivo biodistribution was studied in SKOV3 ovarian cancer xenografted nude mice. Results: In addition to showing uptake in L1 cell adhesion molecule–expressing SKOV3 tumors, as does its parental counterpart HuE71 IgG1, the afucosylated variant having enhanced Fc-receptor affinity showed high nonspecific uptake in lymph nodes. On the other hand, aglycosylated HuE71 IgG1 with abrogated Fc-receptor binding did not show lymphoid uptake. The use of the IgG4 subclass showed high nonspecific uptake in the kidneys, which was prevented by mutating serine at position 228 to proline in the hinge region of the IgG4 antibody to mitigate in vivo fragment antigen-binding arm exchange. Conclusion: Our findings highlight the influence of Fc modifications and the choice of IgG subclass on the in vivo biodistribution of antibodies and the potential outcomes thereof.

Key Words: immuno-PET; aglycosylated antibody; afucosylated antibody; Fab arm exchange

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Monoclonal antibodies rank among the most sought-after class of pharmaceuticals being developed for the treatment of several diseases in humans (1). Their increasing utility has bolstered antibody-engineering efforts to improve efficacy and mitigate toxicities (2,3). Altering the glycosylation status, introducing point mutations in the fragment crystallizable (Fc) region, and changing the immunoglobulin G (IgG) subclass are common strategies whereby the binding of an IgG to Fc y-receptors (FcγR) on immune effector cells can be modulated (4–7). However, the impact of these modifications on antibody biodistribution has not been adequately examined. Arguably, most therapeutic antibodies are unnaturally engineered biomolecules synthesized using recombinant technologies; hence, their in vivo biodistribution cannot be taken for granted. Intriguingly, of all the Food and Drug Administration (FDA)–approved antibodies, only a few have dynamic time-dependent in vivo biodistribution and pharmacokinetics data profiled in patients (8). Furthermore, only a handful of these antibodies have had preclinical biodistribution analysis before or after FDA approval (9). Longitudinal imaging by immuno-PET can fill this existing knowledge gap by enabling quantitation of the in vivo pharmacokinetics and biodistribution of antibodies while delineating their on-target binding and off-target disposition. Critically, immuno-PET and biodistribution studies performed in relevant preclinical animal models early in antibody drug development campaigns can serve as a harbinger for clinical translation and success of antibody therapeutics in human patients (10,11).

Most FDA-approved antibody therapeutics belong to the fully human or humanized IgG1 subclass. In addition to target-specific binding at the fragment antigen-binding end of the IgG molecule, human or humanized IgG1 antibodies bind strongly to activating FcγRs such as FcγRIIIa, which is expressed on immune effector cells such as natural killer cells to mediate antibody-dependent cellular cytotoxicity (ADCC), a key mechanism of action of several therapeutic antibodies. Furthermore, afucosylated IgG1 antibodies lacking a core fucose in the N-linked biantennary oligosaccharide units of the Fc region have stronger Fc–FcγRIIIa binding, leading to enhanced ADCC activity (Fig. 1) (12). On the other hand, aglycosylated IgG1 antibodies lacking the N-linked biantennary oligosaccharide unit in the Fc region have abrogated Fc–FcγR interactions (Fig. 1) (13). Of late, IgG4—–the least abundant IgG in human serum—has emerged as a subclass of choice for the development of therapeutic antibodies, including those used for immunotherapy (14). The low affinity of IgG4 antibodies for activating FcγRs but high affinity for the inhibitory FcγRIIB renders them relatively benign for ADCC. In fact, IgG4s are considered antiinflammatory antibodies because of their ability to dampen immune responses against repetitive allergen exposure (15). These

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properties make IgG₄ a subclass of choice for the design of immuno-therapeutics such as nivolumab and pembrolizumab, which bind to programmed cell death protein 1 on effector T cells in the tumor microenvironment and render efficacy without eliciting secondary immune mechanisms such as ADCC (5,7,16). Collectively, all the aforementioned features highlight the importance of in vivo biomolecular interactions along the Fc–Fc receptor axis that are worth considering during the design and development of therapeutic antibodies (17).

In the work at hand, we asked 3 questions fundamental to the molecular composition of humanized IgGs targeting the cell surface glycoprotein L1 cell adhesion molecule (LICAM), without interfering with the antibody’s ability to bind its cognate antigen or interact with the neonatal Fc receptor. Foremost, we asked how enhancement of Fc–FcγR affinity by afcosylation impacts the in vivo distribution of humanized IgG₁. Next, we were curious to know how Fc silencing via antibody aglycosylation, which abrogates Fc–FcγR interaction, influences the in vivo distribution of humanized IgG₁. Lastly, we wanted to know how the choice of IgG subclass—switching from IgG₁ to IgG₄ with and without fab arm exchange (FAE), and loss of most Fc functions—affects antibody distribution in vivo. To that end, we developed a panel of humanized antibodies (Fig. 1; Table 1) targeting human LICAM, which is overexpressed in several malignancies (18,19). To noninvasively visualize the antibodies in vivo, we radiolabeled them with ⁸⁹Zr and used immuno-PET in athymic nude mice bearing subcutaneously implanted LICAM-expressing SKOV3 tumors.

**MATERIALS AND METHODS**

### Animal Model

All animals were treated as per guidelines approved by the Research Animal Resource Center and Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center. Female athymic nude (Nu/Nu) mice 8–10 wk old were purchased from Charles River Laboratories. Animals were housed in ventilated cages, given food and water ad libitum, and allowed to acclimatize for 1 wk before inoculation of tumor cells. SKOV3 tumors were induced on the right shoulder via subcutaneous injection of 5 million cells in a 150-μL cell suspension of a 1:1 (v/v) mixture of fresh medium and Matrigel (BD Biosciences). The xenografted mice were used for in vivo studies when the tumor volumes reached approximately 300 mm³.

### PET Imaging

PET imaging was conducted using a mouse hotel on an Inveon PET/CT scanner (Siemens Healthcare) (20). SKOV3-xenografted mice were intravenously administered ⁸⁹Zr-labeled antibodies (8 MBq; 45 μg suspended in 150 μL of phosphate-buffered saline per mouse; n = 2 mice per antibody variant). Animals were scanned under the influence of anesthesia by inhalation of 2% isoflurane (Baxter Healthcare) and medical air. PET data for each mouse were recorded via static scans at 48, 96, and 144 h after injection. The PET/CT images were calibrated and cropped before analysis and scaled using AMIDE software (Stanford University). The images were rendered using VivoQuant (Invicro).

### Biodistribution

Ex vivo biodistribution analysis was performed on a separate cohort of SKOV3-xenografted mice that were intravenously administered 1.15 MBq (6.4 μg of each ⁸⁹Zr-labeled antibody variant suspended in 150 μL of phosphate-buffered saline per mouse). Six animals were used per antibody variant, wherein 3 animals were injected with ⁸⁹Zr-labeled antibody alone and 3 animals were injected with a mixture of ⁸⁹Zr-labeled antibody and a 38-fold excess (mass) of the unlabeled antibody variant. Animals were euthanized by CO₂ asphyxiation at 144 h after injection. After euthanasia, tissues of interest were harvested via necropsy, weighed, and assayed for radioactivity on a γ-counter calibrated for ⁸⁹Zr. Counts were converted into activity using a calibration curve generated from known standards. Count data were background-and decay-corrected to the time of injection, and the percentage injected dose (%ID) per gram for each tissue sample was calculated by normalization to the total activity injected.

### Statistics

All data are expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism, version 9.1.0. Statistical comparisons of radioactivity concentrations in each organ across the various groups in the ex vivo biodistribution studies were done using nonparametric multiple Mann–Whitney tests to compare ranks. The Holm–Sidák multiple-comparison test was applied, and the threshold for P value comparison was set to 0.05.
**RESULTS**

A panel of IgG1 and IgG4 antibodies having identical variable heavy- and light-chain sequences targeting human L1CAM but modified Fc regions was generated (Table 1) to gain insights into the influence of Fc modifications and subclass on the in vivo biodistribution of IgG1 and IgG4 antibodies, respectively.

After purification of the various 89Zr-labeled antibodies, we obtained radioimmunoconjugates having an average molar activity of 26.6 MBq/nmol. A cell-based immunoreactivity assay confirmed the ability of the various radioimmunoconjugates to bind L1CAM-expressing SKOV3 cells (Table 1; Supplemental Fig. 1; supplemental materials are available at http://jnm.snmjournals.org) (21). Incubation of the radioimmunoconjugates in serum and evaluation by radio–instant thin-layer chromatography demonstrated less than 4% demetallation up to 7 d after radiosynthesis, suggesting high stability of the radioimmunoconjugates in a biologically relevant medium (Supplemental Fig. 2). Size-exclusion high-performance liquid chromatography of the 89Zr-labeled antibodies incubated without a radioprotectant in chelaxed phosphate-buffered saline at 37°C showed more than 80% of the radioimmunoconjugates being stable and existing as monomers up to 6 d after radiosynthesis (Supplemental Figs. 3 and 4).

Athymic nude mice were used in our studies because of their ability to grow tumors from implanted human cancer cell lines and the presence of functional innate immune cells such as macrophages, dendritic cells, and natural killer cells in this strain. Macrophages and natural killer cells comprise 2 main Fc-dependent effector cells responsible for eliciting antibody-dependent cellular phagocytosis and ADCC, respectively (22). Furthermore, despite only 60%–70% homology between mouse and human FcγRs, human IgGs are reported to bind orthologous mouse FcγRs with similar strength, suggestive of potentially similar downstream biologic activities mediated by human Fc–murine FcγR interactions in mice (23). Immuno-PET imaging of the three 89Zr-labeled IgG1 variants—humanized (HuE71)-IgG1, HuE71-IgG1-Afuco, and HuE71-IgG1-Aglyco—demonstrated uptake of radioactivity in SKOV3 tumors (Figs. 2A–2C). However, the three IgG1 variants yielded distinct vivo distribution patterns of radioactivity. SKOV3-xenografted mice injected with 89Zr-HuE71-IgG1 showed persistence of radioactivity in blood up to 96 h after injection, suggesting slow in vivo clearance of L1CAM-targeted antibodies in this model (Fig. 2A). Besides target-specific tumoral uptake of radioactivity, nonspecific uptake was found in the liver and joints of the long bones of mice. Similarly, 89Zr-HuE71-IgG1-Afuco yielded uptake of radioactivity in the tumor, liver, and joints of the long bones. However, this variant revealed high-intensity bilateral hot spots corresponding to the axillary and cervical lymph nodes (Fig. 2B) and demonstrated increased clearance from circulation relative to 89Zr-HuE71-IgG1. Additionally, 89Zr-HuE71-IgG1-Afuco outlined the spleen and showed a higher radioactivity concentration in long bone joints and the vertebral column. The faster clearance and elevated nonspecific tissue uptake patterns observed for 89Zr-HuE71-IgG1-Afuco may be attributed to enhanced binding of the afucosylated Fc with mouse FcγRIV-expressing immune cells in the lymph nodes and reticuloendothelial system. The latter is exemplified by results from the analysis of Fc–FcγR binding by surface plasmon resonance (Table 2). Notably, conjugation of desferrioxamine to lysine residues distributed randomly in the Fc region of IgG1 molecules did not impact binding to murine FcγRIV and human FcγRIIIa-158V.

Previous studies found significant increases in the binding affinity of afucosylated IgG1 antibody to human FcγRIIIa but no change in the binding affinity to human FcγRI and human neonatal Fc receptor (12,24,25). So, we focused our surface plasmon resonance (SPR) analysis of the differentially glycosylated IgG1 Fc variants to human FcγRIIIa. Murine FcγRIV was included in the surface plasmon resonance assay since it is a functional ortholog of human FcγRIIIa, and binding to murine FcγRIV may contextualize findings from in vivo studies performed in mice (26). Notably, ADCC in humans is mediated via interaction of the Fc of target antigen-bound IgG1 and human FcγRIIIa expressed on immune effector cells. Furthermore, afucosylated human or humanized IgG1 antibodies have been shown to target murine FcγRIV for enhanced tumor therapy by ADCC in mice (27). SPR analysis of the anti-L1CAM IgG1 variants used in our study demonstrated 3-fold higher affinity of the HuE71-IgG1-Afuco for murine FcγRIV than of the HuE71-IgG1. Interestingly, HuE71-IgG1-Afuco also showed a similar 3-fold higher binding to the 158V isofrom of human FcγRIIIa. On the other hand, and as expected, HuE71-IgG1-Aglyco showed no binding to either mouse or human FcγRs. Lastly, immuno-PET of 89Zr-HuE71-IgG1-Aglyco in SKOV3-xenografted mice displayed tumoral uptake of radioactivity with a relatively lower concentration in the liver and bone joints and no detectable uptake in lymph nodes (Fig. 2C).

**TABLE 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HuE71-IgG1</th>
<th>HuE71-IgG1-Afuco</th>
<th>HuE71-IgG1-Aglyco</th>
<th>HuE71-IgG4</th>
<th>HuE71-IgG4-M</th>
<th>Hu3F8-IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>L1CAM</td>
<td>L1CAM</td>
<td>L1CAM</td>
<td>L1CAM</td>
<td>L1CAM</td>
<td>GD2</td>
</tr>
<tr>
<td>Immuneactive fraction (%)</td>
<td>93.1 ± 2.2</td>
<td>89.5 ± 1.5</td>
<td>85.8 ± 2.9</td>
<td>86.7 ± 0.2</td>
<td>88.6 ± 0.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

Antibodies Used in This Study and Their Biochemical and Functional Characteristics

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89Zr-HuE71-IgG1-Aglyco demonstrated high liver uptake (10.8 ± 2.1 %ID/g) compared with the other two variants, which showed less than 6 %ID/g in this tissue. Most other nontarget tissues showed remarkable differences in uptake of radioactivity between the three Fc-modified IgG1 variants. However, axillary lymph nodes isolated from SKOV3-xenografted mice injected with 89Zr-HuE71-IgG1-Aglyco yielded a significantly lower radioactivity concentration in this tissue. Unlike PET images, only mice injected with 89Zr-HuE71-IgG1 demonstrated a significantly higher radioactivity concentration in the bone (femur) than did xenografts injected with 89Zr-HuE71-IgG1-Afuco. Indeed, SKOV3 tumors showed high and specific uptake of radioactivity for all three 89Zr-labeled L1CAM-targeted IgG1 variants. However, the tumoral uptake values (%ID/g) in mice dosed with the unblocked L1CAM-targeted 89Zr-radioimmunoconjugates demonstrated significantly decreased uptake of 89Zr-HuE71-IgG1-Afuco compared with radioimmunoconjugates of the other two IgG1 variants. The relatively low tumoral uptake (7.7 ± 2 %ID/g) of 89Zr-HuE71-IgG1-Afuco may be attributed to concentration of a significant proportion of the radioactivity or antibody in the liver and lymph nodes of SKOV3-xenografted mice. Determining the %ID taken up in the various tissues revealed that despite having the highest radioactivity concentration (%ID/g) for 89Zr-HuE71-IgG1-Afuco, the axillary lymph nodes had less than 2% of the total injected radioactivity at 144 h after injection. Instead, the liver accumulated more radioactivity (17.6 ± 3.4 %ID) and turned out to be a major sink for the afucosylated IgG1 variant.

Importantly, the histopathologic examination of lymph nodes harvested from SKOV3-xenografted mice injected with 89Zr-HuE71-IgG1-Afuco showed no morphologic evidence of infiltrating neoplastic cells. Instead, these nodes demonstrated reactive hyperplasia characterized by marked paracortical and medullary histio- and plasmacytosis (Fig. 2E). The latter was a unique feature relative to lymph nodes harvested from SKOV3-xenografted mice and tumor-naïve mice that never received 89Zr-HuE71-IgG1-Afuco. Along those lines, axillary lymph nodes harvested from SKOV3-xenografted mice injected with 89Zr-HuE71-IgG1 and 89Zr-HuE71-IgG1-Aglyco showed minor sinus histiocytosis but displayed normal lymphoid tissue architecture (Fig. 2E).

Next, we studied the influence of IgG subclass on the in vivo biodistribution of antibody drugs. To delineate the in vivo biodistribution of IgG antibodies, we generated a humanized IgG4 variant of the L1CAM-targeting antibody and conducted serial PET imaging.
imaging studies in SKOV3-xenografted mice. Serial PET imaging of $^{89}$Zr-Hu-E71-IgG$_4$ revealed slow in vivo clearance of the antibody from blood and a high radioactivity concentration in the liver (Fig. 3A). In addition to tumoral uptake of radioactivity, $^{89}$Zr-Hu-E71-IgG$_4$ showed a radioactivity concentration in the kidneys and axillary lymph nodes of SKOV3 xenografts. We hypothesized that uptake of radioactivity in the kidneys may be attributed to FAE, leading to instability of the antibody in vivo. FAE is an intrinsic property of the IgG4 subclass whereby two half-molecules (heavy-chain–light-chain pair) of the antibodies dissociate from one another at the hinge and recombine spontaneously with other IgG4 half-molecules in serum to form monovalent bispecific antibodies in vitro and in vivo (28). Introducing a point mutation from serine to proline at position 228 (S228P) in the hinge region of IgG4 antibodies has been shown to mitigate the propensity of FAE (6). To validate our hypothesis, an S228P hinge-mutated IgG4 variant—$^{89}$Zr-Hu-E71-IgG$_{4M}$—was synthesized and evaluated in vivo. $^{89}$Zr-Hu-E71-IgG$_{4M}$ demonstrated gradual accretion of radioactivity in the SKOV3 tumor while showing little to no radioactivity in the kidneys (Fig. 3B). Note, $^{89}$Zr-Hu-E71-IgG$_{4M}$ faintly highlighted the liver, axillary lymph nodes, and bone joints in this model.

The use of an IgG4 variant of the anti-GD2 antibody, Hu3F8, as an isotype control and the similarity in radioactivity uptake in the kidneys of SKOV3-xenografted mice injected with $^{89}$Zr-Hu3F8-IgG$_4$ and $^{89}$Zr-Hu-E71-IgG$_4$ further validated that the abnormal kidney uptake is attributed to FAE intrinsic to IgG4 antibodies (Fig. 3C). Further, results from an ex vivo biodistribution analysis performed on SKOV3-xenografted mice injected with the three $^{89}$Zr-labeled IgG4 antibodies corroborated findings from immuno-PET studies (Fig. 3D). Importantly, $^{89}$Zr-Hu-E71-IgG$_{4M}$ yielded a significantly lower radioactivity concentration (2.5 ± 0.56 %ID/g) in the kidneys than did $^{89}$Zr-Hu-E71-IgG$_4$ (7.4 ± 2.32 %ID/g; P = 0.02). Furthermore, blockade of tumoral uptake of radioactivity by coinjection of a 0.25 mg/kg dose of $^{89}$Zr-labeled IgG4 antibody with a 38-fold excess (mass) of unmodified L1CAM-targeted IgG4 antibodies in ex vivo biodistribution studies confirmed target-mediated uptake in SKOV3 tumors (Fig. 3D). Notably, $^{89}$Zr-Hu-E71-IgG$_{4M}$ demonstrated increased tumoral uptake of radioactivity (16.1 ± 4.26 %ID/g) compared with $^{89}$Zr-Hu-E71-IgG$_4$ (5.5 ± 2.4 %ID/g; P = 0.03) and $^{89}$Zr-Hu3F8-IgG$_4$ (3.6 ± 1.76 %ID/g; P = 0.02). Data from PET studies showed that targeting with $^{89}$Zr-Hu-E71-IgG$_{4M}$ yielded a high radioactivity concentration in the kidneys (15.2 ± 5.14 %ID/g) and showed between 1 and 8 %ID/g in most healthy tissues. Tumoral uptake (4.9 ± 0.46 %ID/g) of the isotype antibody may be attributed to enhanced permeability and retention in this compartment. Lastly, the high radioactivity concentration in multiple tissues harvested from mice injected with $^{89}$Zr-Hu-E71-IgG$_{4M}$ blocking dose arm is most likely a result of persistence of $^{89}$Zr-Hu-E71-IgG$_{4M}$ in the blood at 144 h after injection.

**DISCUSSION**

Recent insights into pharmacologic modulation at the Fc–FcγR axis have made this molecular interaction an important consideration in the development of antibody-based drugs for cancer immunotherapy (16,17,29). Furthermore, single-nucleotide polymorphisms in FcγR-encoding genes have been implicated in disease etiology and clinical responses (30). Specifically, patients carrying the 158V/V genotype showed improved outcomes from rituximab therapy due, in part, to improved ADCC activity in vivo (31). Additionally, we have previously shown that stronger in vitro Fc–FcγR binding for an afucosylated variant of the humanized anti-GD2 IgG1 antibody yielded improved preclinical efficacy because of enhanced ADCC in vivo (25).

Our current findings with the afucosylated anti-L1CAM IgG1 variant concur broadly with two $^{89}$Zr-immuno-PET studies done using HER3-targeted humanized IgG1 antibodies—GSK2849330 and RG7116—which were Fc-glycoengineered for enhanced ADCC activity (32,33). The high uptake of radioactivity in the liver and spleen of xenograft models developed in immunodeficient SCID mice used in those studies was attributed to enhanced binding of the antibodies with FcγRs expressed on tissue-resident auxiliary immune cells in the reticuloendothelial system (32,33). However, neither of those antibodies showed elevated radioactivity concentrations in lymph nodes. The latter may be due, in part, to the higher immunodeficient status of SCID mice used in those studies and the presence of functional natural killer cells in athymic

**TABLE 2**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>KD (M) murine FcγRIIa-158V</th>
<th>Relative murine FcγRIIa-158V binding</th>
<th>KD (M) human FcγRIIa-158V</th>
<th>Relative human FcγRIIa-158V binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuE71-IgG$_1$</td>
<td>7.24E−07</td>
<td>1.00</td>
<td>8.73E−07</td>
<td>1.00</td>
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<tr>
<td>Desferrioxamine-HuE71-IgG$_1$</td>
<td>5.39E−07</td>
<td>1.34</td>
<td>6.64E−07</td>
<td>1.31</td>
</tr>
<tr>
<td>HuE71-IgG$_1$-Afuco</td>
<td>2.27E−07</td>
<td>3.19</td>
<td>2.86E−07</td>
<td>3.05</td>
</tr>
<tr>
<td>Desferrioxamine-HuE71-IgG$_1$-Afuco</td>
<td>2.19E−07</td>
<td>3.31</td>
<td>2.94E−07</td>
<td>2.96</td>
</tr>
<tr>
<td>HuE71-IgG$_1$-Aglyco</td>
<td>NB</td>
<td>—</td>
<td>NB</td>
<td>—</td>
</tr>
<tr>
<td>Desferrioxamine-HuE71-IgG$_1$-Aglyco</td>
<td>NB</td>
<td>—</td>
<td>NB</td>
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</tr>
</tbody>
</table>

**KD (M) = equilibrium dissociation constant; NB = no binding.**
nude mice used in our study. Although suggestive of an Fc-mediated phenomenon, the pronounced lymph node uptake of radioactivity in mice injected with the afucosylated variant warrants further validation in immunocompetent syngeneic tumor models or xenograft models developed in mice reconstituted with a functional human immune system. Admittedly, immunodeficient mice impact the in vivo biodistribution of exogenously injected human or humanized IgG1 because of relatively low titers of endogenous IgG and the availability of unoccupied high-affinity FcγRs on tissue-resident immune cells in the liver, spleen, and bone marrow (34). This phenomenon is exacerbated in highly immunodeficient mouse strains developed on the NOD-SCID background (35). However, low levels of serum IgG2a in athymic nude mice have also been implicated in the rapid clearance of exogenously injected human IgG1 and mouse IgG2a (36).

Along these lines, a comparison of the afucosylated versus parental L1CAM-targeting IgG1 in tumor-naïve athymic nude mice revealed a lower radioactivity concentration of 89Zr-HuE71-IgG1-Afuco in the blood at 120 h after injection, suggesting faster in vivo pharmacokinetics (Supplemental Fig. 5), which is consistent with our findings for this variant in SKOV3-xenograft mice. Furthermore, the relatively high radioactivity concentration in the long bone joints of mice injected with 89Zr-HuE71-IgG1-Afuco is also indicative of faster in vivo catabolism of the radioimmunoconjugate, leading to the release of 89Zr for in vivo uptake and complexation with hydroxypatite in the bone joints. When target expression is absent in the bones, radioactivity uptake in this tissue is commonly attributed to the in vivo catabolism of desferrioxamine-conjugated 89Zr-labeled antibodies in mice and the osteophilic nature of 89Zr (37). Intriguingly, there was no significant difference between radioactivity concentrations in the liver joints of SKOV3-xenografted mice injected with L1CAM-targeted 89Zr-labeled IgG4 antibodies than with their Fc-active IgG1 counterparts. Case in point, femurs harvested from SKOV3-xenografted mice injected with 89Zr-HuE71-IgG4 and 89Zr-HuE71-IgG4M showed 4.0 ± 0.74 %ID/g (P = 0.03) and 4.4 ± 1.58 %ID/g (P = 0.047), respectively, compared with 89Zr-HuE71-IgG1, which yielded 8.3 ± 2.35 %ID/g in this tissue. Furthermore, radioactivity concentrations of 89Zr-labeled L1CAM-targeted IgG4 variants were comparable to that yielded by the Fc-silent IgG1 variant—89Zr-HuE71-IgG1-Afuco (3.7 ± 0.75 %ID/g)—in this tissue. The latter is suggestive of slow in vivo catabolism and low nonspecific uptake in healthy nontarget tissue. Lastly, the nonspecific hepatic uptake of radioactivity highlights a plausible contribution of Fc-γR interactions between Fc-active radiolabeled IgG1ς and parenchymal and nonparenchymal cells in the liver (38). It is known that the liver is involved in the in vivo catabolism of radiometal-labeled antibodies, leading to initial accumulation of 89Zr-radiometabolites and subsequent complexation of free 89Zr in the long bone joints of mice (37,39,40).

Highlights aside, a limitation of the current work is that it uses antibody variants developed for a single tumor-associated antigen in a singular xenograft model developed on an immunodeficient background. Additionally, identification of cells having elevated expression of murine FcγRIV in lymph nodes leading to the manifestation of reactive hyperplasia, and pinpointing cells in the liver that bind ADCC-enhanced IgG1 antibodies to impact in vivo pharmacokinetics, are outstanding questions that warrant further investigation.

CONCLUSION

Collectively, our findings highlight the influence of Fc-glycosylation status and choice of IgG subclass on the in vivo
biodistribution of the most widely used human or humanized antibody subclasses (IgG1 and IgG4) approved as therapeutics for human use. Our results demonstrate that deglycosylated IgG1 antibodies yield low nonspecific off-target uptake in healthy tissues, whereas S228P hinge-mutated IgG4 antibody eliminates Fc-mediated renal uptake of radioactivity. Importantly, this work illustrates the value of immuno-PET in delineating the in vivo biodistribution of ADCC-enhanced IgG1 antibodies and in macroscopically highlighting potential nontumor tissue depots. Doing so can inform antibody drug development efforts to uncover mechanisms leading to in vivo therapeutic benefit or toxicity. From a theranostic perspective, our results suggest that developing immuno-PET agents using ADCC-enhanced tumor-targeting IgG1 antibodies may yield false-positive results in lymph nodes because of Fc–FcγR interactions in vivo. Similarly, immuno-PET agents developed using tumor-targeting wild-type IgG4 antibodies may yield false-positive results from nonspecific uptake of radioactivity in the kidneys while grossly understimating tumor burden because of loss of the radiotracer to in vivo FAE. In sum, we hope that the results described herein further motivate the use of molecular imaging to inform the preclinical development of novel antibody-based theranostic agents.

DISCLOSURE

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No other potential conflict of interest relevant to this article was reported.

KEY POINTS

QUESTION: What is the impact of Fc modification and choice of IgG subclass on the in vivo pharmacologic profile of humanized antitumor antibodies?

PERTINENT FINDINGS: Humanized IgG1 antibodies yield differential in vivo pharmacokinetics and biodistribution based on the glycosylation status of the Fc. Afucosylated IgG1 antibodies with enhanced Fc–FcγR binding and ADCC activity yield faster in vivo pharmacokinetics and show nonspecific Fc-mediated sequestration in lymph nodes and the reticuloendothelial system. Aglycosylated IgG1 antibodies with abrogated Fc–FcγR binding yield lesser nonspecific uptake of the antibody and related radiocatabolites in vivo, yielding stealth targeting vectors. S228P hinge-mutated IgG4 antibodies overcome in vivo FAE to yield a better radiopharmacologic profile by eliminating uptake of antibody and associated radioactivity in the kidneys.

IMPLICATIONS FOR PATIENT CARE: Using immuno-PET to characterize the in vivo pharmacokinetics and biodistribution to uncover potential mechanism of action or toxicity of engineered antibodies can yield better and safe antibody-based drugs to improve patient care.

REFERENCES


