ImmunoPET of Malignant and Normal B Cells with $^{89}$Zr- and $^{124}$I-Labeled Obinutuzumab Antibody Fragments Reveals Differential CD20 Internalization In Vivo

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Abstract

Purpose: The B-cell antigen CD20 provides a target for antibody-based positron emission tomography (immunoPET). We engineered antibody fragments targeting human CD20 and studied their potential as immunoPET tracers in transgenic mice (huCD20TM) and in a murine lymphoma model expressing human CD20.

Experimental Design: Anti-CD20 cysteine-diabody (cDb) and cysteaminibody (cMb) based on rituximab and obinutuzumab (GA101) were radioiodinated and used for immunoPET imaging of a murine lymphoma model. Pairwise comparison of obinutuzumab-based antibody fragments labeled with residualizing ($^{89}$Zr) versus non-residualizing ($^{124}$I) radionuclides by region of interest analysis of serial PET images was conducted both in the murine lymphoma model and in huCD20TM to assess antigen modulation in vivo.

Results: $^{124}$I-GA1cDb and $^{124}$I-GAcMb produced high-contrast immunoPET images of B-cell lymphoma and outperformed the respective rituximab-based tracers. ImmunoPET imaging of huCD20TM showed specific uptake in lymphoid tissues. The use of the radiometal $^{89}$Zr as alternative label for GA1cDb and GA1cMb yielded greater target-specific uptake and retention compared with $^{124}$I-labeled tracers. Pairwise comparison of $^{89}$Zr- and $^{124}$I-labeled GA1cDb and GA1cMb allowed assessment of in vivo internalization of CD20/antibody complexes and revealed that CD20 internalization differs between malignant and endogenous B cells.

Conclusions: These obinutuzumab-based PET tracers have the ability to noninvasively and quantitatively monitor CD20-expression and have revealed insights into CD20 internalization upon antibody binding in vivo. Because they are based on a humanized mAb they have the potential for direct clinical translation and could improve patient selection for targeted therapy, dosimetry prior to radioimmunotherapy, and prediction of response to therapy.

Introduction

The CD20 antigen is expressed on the surface of B cells during all stages of B-cell development, except early pro-B cells and plasma cells. CD20 expression on B-cell malignancies such as lymphomas and leukemias has proven useful as target for antibody-based diagnosis and therapies. Rituximab was the first anti-CD20 antibody approved by the FDA (1997) for use in oncology and most lymphoma patients now receive rituximab during their treatment as monotherapy or in combination with chemotherapy (1–3). Rituximab is given to all patients with the same standard dose and scheduling established at licensing (375 mg/m$^2$ weekly × 4; ref. 4), regardless of factors known to affect pharmacokinetics and serum levels, such as CD20 expression level and density, tumor burden and localization, number of circulating B lymphocytes and antigen modulation (5). Although CD20 has long been thought to not shed, internalize or degrade upon antibody binding (6), several groups describe antigenic modulation, for example, internalization of Ag/Ab complexes (7, 8) and removal of Ag/Ab complexes from the tumor cell by monocytes, a process termed trogocytosis (9, 10). CD20 modulation might contribute to development of resistance to anti-CD20 mAb in B-cell lymphomas (11–13).

ImmmuPET, as a noninvasive molecular imaging method, combines the target specificity of antibodies with the sensitivity of positron emission tomography (PET; refs. 14, 15). Anti-CD20 immunoPET has the potential to enable personalized image-guided therapy by providing crucial information for diagnosis, prognosis, dosing, and evaluation of therapy response in lymphoma patients and could be especially informative as companion diagnostic for subsequent antibody-based therapy. Furthermore, the noninvasive detection of B...
Trasnlational Relevance

Diagnostic imaging for staging and response monitoring (FDG PET/CT) in lymphoma patients does not provide direct information about the prominent therapy target CD20. Immuno-positron emission tomography (immunoPET) represents a noninvasive approach to assess in vivo target expression and distribution. In this work, we evaluate novel antibody fragments targeting human CD20 and demonstrate high-contrast immunoPET imaging of human CD20-expressing murine lymphoma and of the normal B-cell compartment in transgenic mice. Pairwise comparison of $^{89}$Zr- and $^{124}$I-labeled antibody fragments by serial PET imaging allowed assessment of internalization of the radio-antibody in vivo. These obinutuzumab-based PET tracers have the potential for direct clinical translation and could provide useful information for diagnosis, therapy selection, dosing, or response to therapy.

cells in vivo could enable monitoring of immunotherapies, such as reconstitution after B-cell depletion, adoptive cell transfers or B-cell targeting vaccination and increase the understanding of B-cell-mediated disease states (e.g., rheumatoid arthritis, multiple sclerosis, diabetes; refs. 16, 17).

Radiolabeled rituximab has successfully been used for PET imaging of human CD20 expressing transgenic mice (18, 19), and both B-cell lymphomas and autoimmune disease in patients (20, 21). However, the long plasma half-life of full length IgGs requires delays between tracer administration and image acquisition in order to achieve sufficient clearance of the radio-antibody and therefore high target-to-background ratios. In contrast, engineered antibody fragments such as diabodies (scFv dimer) and minibodies (scFv-CN3) possess pharmacokinetics optimized for imaging. Removal of the constant domains (Cg2,Fc) abolishes effector functions and FcRn-mediated recycling. The reduction of the overall size results in improved tissue penetration and in accelerated blood clearance with half-lives that range from 2 to 5 hours for diabodies (renal clearance) and 5 to 12 hours for minibodies (hepatic clearance) in mice (22).

In this report, we present novel PET-imaging agents based on the humanized, type II anti-CD20 mAb obinutuzumab (GA101; ref. 23). Type II anti-CD20 antibodies bind CD20 in a different orientation from type I antibodies (rituximab) and are reported to internalize less rapidly (7, 24). Cell surface retention is preferable for imaging, allowing the use of radioiodinated tracers which show greatly reduced tissue background because iodine can diffuse from the cell upon internalization and degradation of the radioiodinated antibody. As a result we hypothesized that radioiodinated obinutuzumab-based antibody fragments should be superior to rituximab-based fragments for preclinical immunoPET imaging of lymphoma mouse models and ultimately for clinical translation.

To establish the optimal anti-CD20 immunoPET tracer, we compared different sized antibody fragments (cy-si, diabody, 55 kDa and cy-si minibody, 80 kDa) based on obinutuzumab (GA101) and Rituximab, radiolabeled with long-lived positron emitters iodine-124 ($^{124}$I, nonresidualizing, $t_{1/2}$ 4.18 days) and zirconium-89 ($^{89}$Zr, residualizing, $t_{1/2}$ 3.27 days). ImmunoPET tracers were evaluated in two preclinical mouse models expressing human CD20. First, in a subcutaneous B-cell lymphoma model (38C13-huCD20) and second, in transgenic mice expressing human CD20 on endogenous mature B cells. Furthermore, we utilized the differences regarding uptake and retention of $^{124}$I- and $^{89}$Zr-labeled GA101 antibody fragments to assess internalization of CD20/antibody complexes in vivo by comparing quantitative PET images over time, revealing differential internalization rates of healthy and malignant B cells.

Materials and Methods

Animals and cell lines

The murine B-cell lymphoma line 38C13 expressing human CD20 (38C13-huCD20) was previously described (25). Both 38C13 and 38C13-huCD20 were cultured in RPMI1640 (Life Technologies) supplemented with 50 μmol/L, 2-mercaptoethanol and 10% FBS.

Protocols for all animal studies were approved by the University of California Los Angeles (UCLA) Animal Research Committee. 6- to 8-week-old female SCID mice were purchased from Jackson Laboratory. Allografts were established by injecting 0.5 × 10^6 cells in 1:1 medium:Matrigel (BD Bioscience) subcutaneously into the shoulder region and were allowed to grow for 5 to 8 days. Human CD20 transgenic mice (hCD20TM) have been described previously (16), and were backcrossed onto BALB/c backgrounds and genotypes confirmed by PCR.

Generation of immunoPET tracers

Cloning, production, purification, and radiolabeling of obinutuzumab- and rituximab-based antibody fragments are described in Supplementary Data Methods. $^{124}$I-labeled antibody fragments were generated by direct radioiodination (Pierce Pre-Coated Iodination Tubes; Thermo Scientific). For site-specific $^{89}$Zr-radiolabeling, the chelator deferoxamine-maleimide (mal-DFO, Macrocytides) was conjugated to the reduced C-terminal cysteine of the antibody fragments and radiolabeling was performed as described by Vosjan and colleagues (26).

ImmunoPET/CT and biodistribution

Approximately 20 to 25 μg (~1.5–2.2 MBq) of $^{124}$I- or $^{89}$Zr-labeled antibody fragments were injected via tail vein into tumor bearing SCID mice or huCD20TM. Thyroid and stomach uptake of radioiodine was blocked with Lugol’s iodine and potassium perchlorate, respectively, as previously described (27). At 4, 8, and 24 hours postinjection, mice were anesthetized using 2% isoflurane and imaged with 10-minute acquisitions on an Inveon microPET scanner, followed by a microCT scan (Siemens). Following microPET/CT imaging, mice were sacrificed and organs and blood were collected, bilateral lymph nodes (mesenteric, inguinal, axillary, and brachial) were pooled, weighed, and gamma counted. The %ID/g was calculated based on a standard containing 1% of the injected dose.

Image quantitation and partial-volume correction

MicroPET images were reconstructed using non-attenuation filtered back-projection (FBP) or maximum a posteriori (MAP) reconstruction. MicroPET/CT overlay images are displayed as whole body maximum intensity projections (MIP). Image analysis was performed using AMIDE (28).

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$^{89}$Zr- and $^{124}$I-Anti-CD20 ImmunoPET
Tumor uptake values (%ID/gROI) and volumes were quantified by drawing ellipsoid regions of interest (ROI) over the entire tumor based on the microCT. The mean voxel value of the microPET scan was converted to %ID/g using the decay-corrected injected dose and empirically determined cylinder factors for $^{124}$I and $^{89}$Zr, respectively. Partial volume correction was based on the spheric approximation of the tumor volume (ROI) and previously determined recovery coefficient (RC) curves for $^{124}$I and $^{89}$Zr (29). Spleen uptake in huCD20TM was quantified by drawing small ellipsoid ROIs (~14 mm$^3$) within the tip of the spleen and %ID/gROI was calculated without partial volume correction, due to the complexity of organ shape, volume, and proximity to organs with high activity (liver, kidneys).

Data analysis
Data values are reported as mean ± SD unless indicated otherwise. Ex vivo biodistribution values are depicted as box-and-whiskers (min to max) graphs. For statistical analysis paired t tests and one-way ANOVA for comparing two or three groups, respectively, were performed using GraphPad Prism version 6.00.

Results
Generation of obinutuzumab (GA101) cys-diabody and cys-minibody
The cys-diabody (GAcDb, 54.5 kDa) and cys-minibody (GAcMb, 83.5 kDa) were generated based on the variable domains of obinutuzumab (GA101). Rituximab-based antibody fragments (RxcDb, RxcMb) were generated similarly for comparison. Antibody fragments contain two antigen-binding sites and a C-terminal cysteine for site-specific conjugation (Fig. 1A; Supplementary Figs. S1 and S2). Purity and correct assembly of purified proteins was confirmed by SDS-PAGE analysis and size-exclusion chromatography (SEC; Figs. 1B and C; Supplementary Fig. S1). Engineered antibody fragments retained the antigen binding characteristics of the parental antibodies. GAcDb and GAcMb reached maximal binding at lower levels than rituximab and showed apparent affinities in the low nanomolar range ($K_D$ ~4 nmol/L; Supplementary Fig. S3).

$I$-$124$ immunoPET and biodistribution in mice bearing subcutaneous B-cell lymphomas
Anti-CD20 cDb and cMb were successfully radiolabeled with $^{124}$I or $^{89}$Zr (results are summarized in Table 1). Radiochemical purity after size exclusion spin column was >98% for all tracers. Immunoreactivity of radiolabeled antibody fragments ranged from 65% to 85% (Supplementary Fig. S4).

In vivo targeting of the antibody fragments was evaluated in SCID mice bearing subcutaneous lymphoma tumors (38C13-huCD20). Mice received 20 to 25 μg (1.5–2.2 MBq) of labeled protein intravenously and were serially imaged at 4, 8, and 24 hours postinjection (p.i.). Figure 2 displays representative time series of PET/CT scans showing specific uptake in huCD20-positive tumors and fast clearance from blood and nonspecific tissues, resulting in high-contrast immunoPET images as early as 4 hours p.i. Background activity cleared by 24 hours p.i. with only antigen-positive tumors visible in the PET scan. Specificity of tumor uptake was confirmed in mice bearing 38C13-huCD20 tumors that were blocked by co-injection of 80 μg (4 mg/kg) cold antibody fragments and in mice bearing huCD20-negative 38C13 tumors. The comparison of

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Figure 1.
Production and characterization of engineered obinutuzumab (GA101) antibody fragments. **A**, Schematic representation of cys-diabody and cys-minibody antibody fragments. The heavy and light chain variable regions of obinutuzumab (GA101) are connected by glycine-rich linkers of 5 amino acid (G4S, diabody) and 15 amino acids ([G4S]$_3$, minibody) length, respectively. Downstream of the VH domain the human gamma 1 hinge and CH3 domain were inserted to form the minibody. Both antibody fragments contain a C-terminal histidine-cysteine tag (H6-GGC). **B**, SDS-PAGE analysis of purified GA101 cys-diabody and cys-minibody antibody fragments. The heavy and light chain variable regions of obinutuzumab (GA101) are connected by glycine-rich linkers of 5 amino acid (G4S, diabody) and 15 amino acids ([G4S]$_3$, minibody) length, respectively. Downstream of the VH domain the human gamma 1 hinge and CH3 domain were inserted to form the minibody. Both antibody fragments contain a C-terminal histidine-cysteine tag (H6-GGC). **C**, Size exclusion chromatography of the engineered GA101 antibody fragments in comparison to full length IgG.
\(^{124}\text{I}\)-GAcDb and \(^{124}\text{I}\)-RxcDb (Fig. 2A and B) showed that \(^{124}\text{I}\)-RxcDb cleared more quickly from the blood as indicated by decreasing activity in the heart and lower uptake in antigen-positive tumors. These observations were confirmed by \textit{ex vivo} biodistribution at 24 hours p.i. (Fig. 2B; Supplementary Table S1). Uptake of \(^{124}\text{I}\)-GAcDb in 38C13-huCD20 tumors was 4.6 ± 0.6 %ID/g at 24 hours p.i. and was significantly higher than in 38C13 control tumors (0.5 ± 0.1 %ID/g, \(P < 0.0001\)). \(^{124}\text{I}\)-RxcDb showed 1.8 ± 0.4 %ID/g uptake in 38C13-huCD20 tumors and 0.8 ± 0.1 %ID/g in 38C13 (\(P < 0.0001\)).

ImmunoPET with \(^{124}\text{I}\)-labeled GAcMb (Fig. 2C) showed higher activity in the blood (heart), likely due to the longer plasma half-life of the 80 kDa antibody fragment, resulting in higher tumor uptake compared with \(^{124}\text{I}\)-GAcDb. Uptake values as determined by \textit{ex vivo} biodistribution (Fig. 2D; Supplementary Table S1) were 9.2 ± 0.8 %ID/g in the 38C13-huCD20 tumors. Uptake in blocked tumors and in CD20-negative 38C13 tumors was significantly lower (3.8 ± 0.3 %ID/g, \(P = 0.0004\) and 1.9 ± 0.3 %ID/g, \(P < 0.0001\), respectively). \(^{124}\text{I}\)-RxcMb immunoPET (Fig. 2D) also showed tracer uptake in the positive tumors (1.2 ± 0.1 %ID/g), which could be blocked by co-injection of cold RxcMb (0.6 ± 0.2 %ID/g) and was not observed in negative tumors (0.45 ± 0.2 %ID/g). 38C13-huCD20 uptake values of \(^{124}\text{I}\)-RxcMb were considerably lower compared with \(^{124}\text{I}\)-GAcMb.

In summary, \(^{124}\text{I}\)-GAcDb and \(^{124}\text{I}\)-GAcMb outperformed the respective rituximab-based tracers (\(^{124}\text{I}\)-RxcDb and \(^{124}\text{I}\)-RxcMb) reaching higher tumor uptake and higher contrast microPET images (higher target-to-blood ratio). Furthermore, although \(^{124}\text{I}\)-GAcMb reached the highest tumor uptake, the faster clearing \(^{124}\text{I}\)-GAcDb reached the highest tumor-to-blood ratio at 24 hours p.i. (12.4 ± 2.5; Supplementary Table S1), resulting in better image contrast.

\(89\text{Zr}\)-ImmunoPET and biodistribution in mice bearing subcutaneous B-cell lymphomas

To investigate if a residualizing radiometal (\(^{89}\text{Zr}\)) may be advantageous in terms of greater tumor uptake and retention, mal-DFO was site-specifically conjugated to GAcDb and GAcMb for \(^{89}\text{Zr}\) immunoPET. Both \(^{89}\text{Zr}\)-GAcDb and \(^{89}\text{Zr}\)-GAcMb were used for serial immunoPET imaging of the 38C13-huCD20 lymphoma model and high-contrast images showing specific tumor uptake were obtained (Fig. 3). The residualizing nature of the \(^{89}\text{Zr}\) radio-metabolites caused higher nonspecific uptake, especially in organs of clearance (kidneys, liver, and spleen; Fig. 3A and B and Supplementary Table S1). Compared with the respective iodinated cys-diabody, \(^{89}\text{Zr}\)-GAcDb reached only slightly higher tumor uptake (4.9 ± 0.3 %ID/g by \textit{ex vivo} biodistribution), resulting in comparable tumor-to-blood ratios whereas the tumor-to-background (muscle) ratio was lower. \(^{89}\text{Zr}\)-GAcDb immunoPET images confirmed primarily renal clearance of the 55 kDa protein.

\(^{89}\text{Zr}\)-GAcMb immunoPET showed the highest uptake of all tested tracers in 38C13-huCD20 allogeneic grafts (21.8 ± 1.2 %ID/g) and primarily hepatic clearance. Blood activities were comparable to \(^{124}\text{I}\)-GAcMb, resulting in a higher tumor-to-blood ratio for \(^{89}\text{Zr}\)-GAcMb (11.0 ± 1.0) compared with \(^{124}\text{I}\)-GAcMb (4.0 ± 0.5; Supplementary Table S1). However, the tumor-to-muscle ratio was lower (41.1 ± 2.8 and 145.4 ± 19.3, respectively) caused by higher background retention of the residualizing \(^{89}\text{Zr}\) radio-metabolites.

I-124 and Zr-89 ImmunoPET of the normal B-cell compartment in huCD20 transgenic mice

To evaluate the \textit{in vivo} targeting ability of the obinutuzumab-based PET tracers in the context of normal tissue expression of CD20, a transgenic mouse model expressing human CD20 (huCD20TM) was used (16). PET/CT images of mice injected with \(^{124}\text{I}\)- and \(^{89}\text{Zr}\)-labeled GAcDb and GAcMb were obtained 4, 8, and 20 hours p.i. All tracers showed rapid and specific localization to the spleen and lymph nodes, where B cells reside (Fig. 4A and B; Supplementary Fig. S5). Specificity was confirmed in antigen-negative BALB/c mice (Supplementary Table S2).

The activity in the spleen decreased considerably more quickly with \(^{124}\text{I}\)-labeled cDb and cMb compared with \(^{89}\text{Zr}\)-labeled cDb and cMb. The mean spleen uptake values (%ID/g ± SEM) determined by \textit{ex vivo} biodistribution at 20 hours p.i. were 5.0 ± 0.4 for \(^{124}\text{I}\)-GAcDb compared to 17.8 ± 2.4 for \(^{89}\text{Zr}\)-GAcDb (\(P = 0.006\)) and 11.5 ± 1.2 for \(^{124}\text{I}\)-GAcMb compared to 38.9 ± 5.7 for \(^{89}\text{Zr}\)-GAcMb (\(P = 0.003\); Fig. 4C and D and Supplementary Table S2). Because of the partial volume effect (PVE), small structures such as lymph nodes were difficult to visualize, especially with the radioiodinated antibody fragments, although \textit{ex vivo} biodistribution confirmed tracer uptake in dissected lymph nodes (inguinal, axillary, mesenteric) to be 3.0 ± 1.0 %ID/g for \(^{124}\text{I}\)-GAcDb and 7.1 ± 0.3 %ID/g for \(^{124}\text{I}\)-GAcMb. Here, the use of residualizing \(^{89}\text{Zr}\)-labeled anti-CD20 antibody fragments was advantageous and resulted in clearly distinguishable lymph nodes (Supplementary Fig. S5, microPET 2 mm section, rescaled).

\textit{In vivo} internalization of CD20/antibody complexes from malignant and endogenous B cells

Pairwise evaluation of both \(^{124}\text{I}\)- and \(^{89}\text{Zr}\)-labeled antibody fragments over time provides an assessment of \textit{in vivo} binding and internalization of the antigen/antibody complexes (30). The \(^{124}\text{I}\)-labeled antibody fragments undergo intracellular catabolism and dehalogenation upon internalization and \(^{124}\text{I}\)-iodotyrosine diffuses rapidly from the cell. \(^{89}\text{Zr}\)-antibody fragments show identical target cell binding but noticeable differences in retention because the residualizing \(^{89}\text{Zr}\) radio-metabolites are trapped intracellularly (31).
Figure 2.

124I-obinutuzumab (GA101)-based antibody fragments outperform 124I-rituximab-based antibody fragments for in vivo immunoPET imaging of subcutaneous B-cell lymphoma. Representative serial immunoPET images of mice bearing s.c. 38C13-huCD20 tumors, blocked (bolus injection of cold antibody fragment), or CD20-negative 38C13 tumors at 4, 8, and 24 hours post injection of 20 to 25 μg of 124I-labeled antibody fragments. All anti-CD20 tracers show in vivo specificity to the CD20-expressing B-cell lymphoma. A, 124I-GA cDb, (B) 124I-Rx cDb, C, 124I-GA cMb, D, 124I-Rx cMb. Small-animal PET images (FBP reconstruction) are shown as full body maximum intensity projection overlaid with microCT. After the last microPET scan mice were sacrificed and ex vivo biodistribution performed (24 hours p.i., n ≥ 4). %ID/g values of each group are depicted as box-and-whisker plot (min-max).
ImmunoPET studies showed that both 124I- and 89Zr-anti-CD20 tracers result in high-contrast imaging, but differences were observed in uptake and retention of activity in the spleen of huCD20TM compared with the subcutaneous B-cell lymphoma model, suggesting differences in CD20 internalization or modulation between healthy B cells and B-cell lymphomas. Therefore, ROI quantification of serial microPET images and comparison of the change in radioactive signal was performed for 124I- and 89Zr-labeled GA101 antibody fragments in both the subcutaneously 38C13-huCD20 tumors and in huCD20TM. Peak uptake of the GAcMb appeared around 4 to 8 hours postinjection, whereas the smaller GAcDb reached peak uptake before the 4-hour time point (Fig. 5A). For both antibody fragments, the 89Zr-labeled and 124I-labeled versions showed similar uptake and retention in subcutaneously tumors, indicating no or very slow internalization of the radiotracers within 24 hours p.i.

Spleen uptake values in huCD20TM from 124I-labeled GAcDb and GAcMb decreased rapidly from the spleen, at a rate faster than that in the subcutaneous lymphoma model. In contrast, activity from 89Zr-labeled GAcDb and GAcMb was retained in the spleen, resulting in significantly different ROI values for the respective GAcDb (P = 0.0007) and GAcMb (P = 0.0003) tracers 24 hours p.i. (Fig. 5B). This observation indicates that endogenous B cells in the spleen internalize CD20/antibody complexes more rapidly than the B-cell lymphoma 38C13.

Discussion

In this study, we evaluated two obinutuzumab (GA101)-based antibody fragments (cys-diabody, GAcDb and cys-minibody, GAcMb) for immunoPET of CD20 expressing B cells using two alternative radiolabels (89Zr and 124I). To ensure a biologically inert imaging agent, the reformatted antibody fragments lack a full Fc region reducing the interaction with complement or FcRs (32–34). Both the cDb and the cMb retained binding characteristics (type II epitope and apparent affinity K_D ~4 nmol/L) of the parental mAb (23). Because the antibody fragments are based on a humanized mAb, translation into the clinic is facilitated. Clinical trials are of particular importance to establish pharmacokinetics and targeting in patients since clinical performance is difficult to predict based on preclinical studies in rodents.

Independent of the radiolabeling method (random iodination of tyrosine residues or site-specific conjugation of maleimide-DFO 89Zr to the reduced cys-tag), GA101-based fragments showed specific targeting to human CD20 expressing B cells as demonstrated in a subcutaneous B-cell lymphoma model (38C13-huCD20) and in a transgenic mouse model expressing human CD20 (huCD20TM). Rapid clearance of the antibody fragments enabled high-contrast small animal PET imaging at early time points.
Figure 4.
In vivo immunoPET imaging of the B-cell compartment in huCD20 Tm. (A, 124I- and \(^{89}\)Zr-GA101 cys-diabody (GAcDb) and (B) \(^{124}\)I- and \(^{89}\)Zr-GA101 cys-minibody (GAcMb) immunoPET in transgenic mice expressing human CD20 on mature B cells showed specific uptake in the spleen and lymph nodes. GAcDb showed high contrast images at earlier time points and renal clearance while GAcMb showed higher uptake in target organs and mainly hepatic clearance. Small-animal PET images (MAP reconstructions) are shown as full body maximum intensity projection overlaid with microCT. Ex vivo biodistribution 24 hours p.i. \(n \geq 3\). %ID/g values of each group (\(^{124}\)I and \(^{89}\)Zr-labeled) are depicted as box-and-whisker plot (min–max).
In the lymphoma model, the 124I-labeled GA101 cDb and cMb outperformed the respective rituximab-based antibody fragments, reaching higher tumor activity (4.6%ID/g vs. 1.8%ID/g for cDbs and 9.2%ID/g vs. 1.2%ID/g for cMbs at 24 hours p.i., respectively) and higher tumor-to-blood ratios. This finding was unexpected considering that type II mAbs (obinutuzumab, GA101) show binding at approximately half the surface density of type I binders (rituximab; ref. 24).

The low specific uptake of the rituximab-based fragments is in accordance with previously published anti-CD20 minibody, scFv-Fc and diabody immunoPET imaging of the same lymphoma model (35, 36). Furthermore, rituximab is described to have a faster off-rate than many other anti-CD20 antibodies, which might influence in vivo tumor retention (37, 38).

Compared with the 124I-labeled GaDb and cMb, the 89Zr-labeled antibody fragments achieved slightly higher tumor activity and retention due to the residualizing radiometal, resulting in higher tumor-to-blood ratios at 24 hours p.i.; at the same time, the tumor-to-background (muscle) ratio is lower caused by increased background activity. As expected, nonspecific uptake is most pronounced in organs of clearance (kidneys, liver, spleen), which in a clinical setting might impede detection of tumors or metastases in those organs. However, by selecting the antibody fragment according to size (diabody or minibody) the elimination pathway can be predetermined. The high uptake of 89Zr-labeled GaDb and GAcMb in the spleen of SCID mice is most likely caused by the abnormal structure and significantly smaller size (20–35 mg) and was not observed in BALB/c mice (39).

ImmunoPET of human CD20 transgenic mice (huCD20TM) showed specific tracer uptake in the spleen for both 124I- and 89Zr-labeled GaDb and GAcMb. Lymph nodes were difficult to visualize with 124I-labeled fragments while 89Zr-labeled antibody fragments showed retained activity indicating that naive B cells internalize CD20-antibody complexes. Values are represented as mean ± SD; *P < 0.05; **P < 0.0001; ns, not significant.

In summary, for B-cell lymphomas with cell surface retention of Ag/Ab complexes 124I-labeled obinutuzumab antibody fragments outperform rituximab-based fragments and the use of 124I is preferable to 89Zr as it results in higher image contrast due to lower background throughout the organism. However, for...
immunonePET imaging of small structures like lymph nodes or metastases and of internalizing antigens, the $^{89}$Zr-labeled antibody fragments are more suitable.

Recent studies in a similar transgenic mouse model using $^{89}$Zr-labeled intact rituximab showed high uptake in the spleen (103±12%ID/g at 24 hours p.i. from ex vivo biodistribution) but no uptake in lymph nodes (19). Possible explanations include the low injected protein dose (2–3 μg) resulting in rapid accumulation of the tracer in the spleen. The abundance of CD20 antigen throughout the body and high concentration of antigen in the spleen (antigen sink) makes imaging outside the sink challenging. In contrast to the antibody fragments presented in our study, the radiolabeled intact rituximab is biologically active and would deplete B cells if injected at higher doses. The ability of the obinutuzumab-based tracers to image small structures (lymph nodes) outside the antigen sink at low doses (1.5–20 μg protein/ dose) will be crucial for imaging in the context of normal tissue distribution of CD20.

Muylle and colleagues investigated the impact of preloading with unlabeled rituximab on tumor targeting using $^{89}$Zr-rituximab immunonePET in lymphoma patients and found impaired tumor targeting in the majority of patients (21). This study showed that tumor targeting is influenced by a variety of factors, for example, B-cell depletion, spleen volume, tumor heterogeneity, the site of tumor involvement, and preoad of unlabeled antibody. The authors emphasize the potential of immunonePET as an inherently quantitative imaging method, for dosimetry prior to radioimmunotherapy (RIT), better selection of patients for targeted therapy, and prediction of treatment outcome.

Dosimetry studies with $^{89}$Zr-labeled full-length antibodies in patients have suggested that nonspecific accumulation in the liver will be dose limiting, whereas for $^{124}$I-labeled tracers bone marrow exposure from blood activity is usually the limiting factor (42–44). A dosimetry study based on biodistribution data of $^{89}$Zr-rituximab in huCD20TM suggests liver (with pre-dose) and spleen (without pre-dose) as dose-limiting organs in humans (45). In both cases, the use of a smaller, faster-clearing antibody fragment like the Cy5-diobody and the Cy5-minibody could result in a reduction of the radiation exposure and absorbed dose to the patient.

In addition to confirming presence of target antigen in vivo, immunonePET has also been used to evaluate antibody modulation by pairwise comparison of residualizing and non-residualizing radiolabel conjugates to the same antibody. This was demonstrated for the slow internalization of prostate stem cell antigen (PSCA) bound by anti-PSCA minibody (124I-tosil-21F11 and $^{89}$Zr-tosil-21F11; ref. 29) and in a study by Cheal and colleagues using $^{124}$I-cG250 and $^{89}$Zr-cG250 to study in vivo receptor binding and internalization of carbonic anhydrase IX (30). In this study, serial ROI analysis of $^{124}$I-GAcDb and $^{124}$I-GAcMb immunonePET in huCD20TM showed that radioactivity decreased significantly faster from the spleen compared with the retention of activity using $^{89}$Zr-GAcDb and $^{89}$Zr-GAcMb. In contrast, the radiiodinated and radiometal labeled tracers demonstrated very similar retention in the subcutaneous lymphoma tumors. This suggests that CD20/GA101 complexes are indeed internalized and metabolized rapidly in huCD20 Tg B cells, compared to slower internalization in 38C13-huCD20 lymphoma cells. The results of our study are consistent with previous in vitro observations in the literature that tissue cultured B-cell lines and xenograft models show limited internalization whereas primary human B cells from healthy donors show more rapid internalization matching that seen in huCD20 Tg B cells (7, 8, 46). The differences in CD20 internalization seen in these two mouse models do not warrant general conclusions about CD20 modulation in normal and malignant cell line; however, this proof-of-principle study demonstrates the applicability of immunonePET to study the wide heterogeneity in CD20 modulation rates reported for primary lymphomas. Even though xenografts implanted into immunodeficient mice have major limitations and do not reflect the complexity of human disease, they do provide reproducible models and are widely used in the development of new antitumor therapies. ImmunonePET and the ability to assess internalization in vivo could therefore contribute to better understanding of those xenograft models and facilitate preclinical research and development of new therapies (47).

Although the literature regarding CD20 modulation is conflicting, it seems obvious that a substantial reduction of CD20 levels on target B cells and consumption of the therapeutic antibody would impair immunotherapy and might cause resistance. Beers and colleagues reported rapid CD20 internalization of type I mAb (rituximab) in human CD20Tg B cells (7), and little modulation of CD20 from the cell surface by type II anti-CD20 mAb (tositumomab). This process appears to be an intrinsic B cell phenomenon that does not require activatory Fc gamma R engagement by effector cells as shown in γ-chain −/− (activatory Fc gamma R-negative) mice. Internalization of CD20/type I mAb complexes occurred equally rapidly (within just 2 hours) in normal human peripheral blood B cells. The same group showed in a recent study that internalization of mAbs is antibody specificity and FcγRIIb dependent (46). Although most bound mAbs bind FcγRIIB, internalization of Ag/Ab/FcR complexes occurs only for a select subset (anti-CD20 type I). However, work by Taylor and colleagues (48) demonstrated that an endocytic process, termed trogocytosis, mediated by FcγR-expressing effector cells (monocytes, macrophages, or NK cells) might also play a role in promoting CD20/mbab loss in vivo. This study showed only modest internalization of rituximab from 38C13-huCD20 cells but substantial removal of CD20 and bound type I mAbs (rituximab and ofatumumab) upon incubation with THP-1 acceptor cells. Consistent with both studies, we found no tracer internalization in subcutaneous 38C13-huCD20 lymphomas but distinct internalization in the spleen (normal B cells) of transgenic mice. Although the rational for engineering antibody fragments based on a type II anti-CD20 mAb and without the Fc region was to ensure cell surface retention, our findings suggest that a fraction of CD20 internalization is independent of the epitope binding mode and FcγRIIB binding.

Future studies should include immunonePET of more physiologically relevant models, for example, syngeneic models of disseminated B-cell lymphoma in immunocompetent mice and imaging of tumors in the context of normal tissue expression in transgenic mice.

In conclusion, we presented novel anti-CD20 antibody fragments that specifically target human CD20 expressed on healthy and malignant B cells and produce high contrast immunonePET images. The combination of antibody fragments (size, plasma half-life, route of clearance) with radionuclides (residualizing vs. non-residualizing) offers optimized tracers for a variety of applications in B cell imaging. The ability to monitor CD20 internalization in vivo using noninvasive imaging could have an important impact on understanding B cell biology and support treatment.
design, especially when immunoPET tracers accompany subsequent antibody based therapies.

Disclosure of Potential Conflicts of Interest
J.M. Timmerman reports receiving commercial research grants from Bristol-Myers Squibb, Kite Pharma, and Valor Biotherapeutics, and is a consultant/advisory board member for Celgene and Seattle Genetics. A.M. Wu holds ownership interest (including patients) in and is a consultant/advisory board member for Imaginab, Inc. No potential conflicts of interest were disclosed by the other authors.

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References
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ImmunoPET of Malignant and Normal B Cells with $^{89}$Zr- and $^{124}$I-Labeled Obinutuzumab Antibody Fragments Reveals Differential CD20 Internalization In Vivo

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