Antibodies are an attractive source of biotherapeutic agents due to their high affinity, exquisite target selectivity and extended half-life in vivo. Their development for therapeutic applications has been facilitated by hybridoma technology, antibody humanization and numerous in vitro antibody selection technologies that enable antibodies with desired biological properties to be engineered at will.

Co-incident with this increase in the in vitro development of antibodies for therapeutic applications has been the recognition of how antibodies evolve in vivo. Several studies have pointed to the relationship between antibody affinity and antigen specificity. The conformational flexibility of initial recombinant antibodies is considered to be an important feature of the immune system’s ability to generate antibodies against a broad spectrum of antigens. During antibody maturation, this structural plasticity is thought to be restricted through somatic hypermutation in vivo (and perhaps affinity maturation in vitro) leading to a reduced entropy cost for specific antigen binding and a corresponding increase in antigen specificity. The increase in antigen specificity helps to eliminate undesired off-target antibody interactions, and serves as part of immune system checkpoints designed to prevent autoimmune disease.

In contrast, antibodies that are generated in vitro lack any regulatory immune surveillance. For these, various screens utilizing protein chips and microarrays have been developed in order to evaluate or anticipate off-target interactions. In one study...
Humanization and characterization of LD1. The human chimeric antibody LD1 (chLD1) has been shown to bind human FGFR4, block signaling by FGF19 and other FGF ligands and suppress tumor growth in a HUH7 human hepatocellular carcinoma (HCC) xenograft model. As a first step in the humanization of LD1, the variable light and heavy domains of chLD1 were aligned with the human kappa I (huKI) and human VH subgroup III (huIII) variable domain frameworks used in trastuzumab (Fig. 1). Hypervariable regions from chLD1 were grafted into these human variable frameworks to generate a direct CDR-graft (hLD1.vA). When compared to chLD1 by surface plasmon resonance for binding FGFR4, the affinity of hLD1.vA was decreased by about 5-fold (not shown). Substitution of mouse sequence at various vernier positions in both the variable light and heavy domains was explored as a means to improve binding and led to the identification of three important mouse vernier positions in the LC: P44F, L461 and Y49S. These changes were incorporated in hLD1.vB that had an affinity for FGFR4 comparable to chLD1 (Table 1).

Surprisingly, despite similar FGFR4 binding affinity (Fig. 2A), hLD1.vB had diminished anti-tumor efficacy compared to chLD1 in the HUH7 human HCC xenograft model in nu/nu mouse (Fig. 2B). After 9 days, the HUH7 tumors of mice treated with PBS grew to an average volume of approximately 700 mm³. In the chLD1 treated group, average HUH7 tumor volume was approximately 400 mm³, representing a 43% inhibition of tumor growth compared to the tumor in the PBS-treated animals. However, the average tumor volume in mice treated with hLD1.vB was approximately 600 mm³, representing a 14% inhibition of tumor growth compared to the tumor in the PBS-treated animals.

A pharmacokinetic evaluation of chLD1 and hLD1.vB conducted in athymic NCR nude mice revealed rapid clearance for both chLD1 and hLD1.vB at 1 mg/kg IV (140 and 132 mL/day/kg, respectively), suggesting a target mediated clearance mechanism. This clearance mechanism appeared to be saturated for chLD1 at a higher dose of 20 mg/kg. At this dose the observed clearance (11.7 mL/day/kg; Fig. 2C) was within the range (6-12 mL/day/kg) of target-independent clearance observed for a typical humanized antibody in mouse (ref. 17; P. Theil, personal communication). However, hLD1.vB continued to be rapidly cleared (34.2 mL/day/kg; Fig. 2C). This suggested an additional clearance mechanism for hLD1.vB could be responsible for the apparent lack of efficacy in the mouse xenograft model.

Consistent with the pharmacokinetics (PK) finding, a biodistribution study using 125I-chLD1 and 125I-hLD1.vB revealed significantly different distribution profiles (Fig. 2D). 125I-chLD1 distributed rapidly and specifically to the liver due to the high expression of FGFR4 on hepatocytes while only a limited amount of 125I-hLD1.vB was found in liver at an equivalent dose by 2 h (~80 vs. 35% ID/g). In contrast, the observed distribution of these antibodies was reversed in blood suggesting that a competing interaction prevented distribution of hLD1.vB to the liver as opposed to a loss in antibody stability in vivo that would have led to a loss in overall radioactivity.
Identification of C3 interference. In an effort to reconcile the in vivo differences observed between chLD1 and hLD1.vB, we evaluated antibody stability in plasma as well as potential off-target plasma or tissue interactions that might affect their function. Plasma stability was evaluated by incubating chLD1 or hLD1.vB in mouse, rat, monkey or human plasma for 48 hours at 37°C followed by an assessment of both the FGFR4 binding activity and the total human IgG concentration. While the total chLD1 or hLD1.vB concentration as measured by the IgG ELISA did not change (not shown), the recovery of hLD1.vB detected by the FGFR4 ELISA was significantly reduced (by ~30%) in

Table 1. Binding kinetics of anti-FGFR4 variants

|   | LD1 Ab | kₐ (nM) | kₕ₋₀ (M·sec⁻¹) | kₕ₋₀ (sec⁻¹) |
|---|--------|---------|----------------|-------------|
| chLD1 | 0.45   | 3.82e5  | 1.73e-4        |             |
| hLD1.vB | 0.97   | 2.88e5  | 2.80e-4        |             |
| hLD1.v22 | 0.043  | 9.10e5  | 3.90e-5        |             |

The association and dissociation rates of human FGFR4 binding to immobilized antibody variants were measured using surface plasmon resonance.
mouse and rat plasma compared to a control incubation in PBS/BSA (Fig. 3A). In contrast, there was no loss of chLD1 FGFR4 binding activity in any condition tested. The significant reduction in hLD1.vB recovery, specifically from rodent plasma, suggested that the loss was not due to degradation, but more likely the formation of an interfering complex in rodent plasma. Since the interaction of hLD1.vB with mouse plasma might result in the generation of a higher molecular weight complex, iodinated chLD1 and hLD1.vB were also incubated in plasma and analyzed using size-exclusion HPLC. High molecular weight peaks were detected only in the mouse plasma samples containing 125I-hLD1.vB but not 125I-chLD1. In addition to the expected antibody peak at 150 kDa, peaks corresponding to ca. 270 and ca. 550 kDa were also detected initially (Fig. 3B), however by 48 h, only the 150 and 270 kDa peaks remained; the 550 kDa peak was no longer observed. These higher molecular weight peaks were not detected in cynomolgus monkey and human plasma or PBS/BSA containing hLD1.vB, or in any sample containing chLD1 (Sup. Fig. 1). Interestingly, the presence of these high molecular weight peaks correlated directly with the antibody recovery data obtained in the FGFR4 ELISA. Further, the presence of these peaks was diminished when the analysis was performed at pH 4.0 (Sup. Fig. 1), further supporting the hLD1.vB-dependent interaction with mouse serum.
frameworks. Further, the light and heavy chain variable domain frameworks used to humanize hLD1.vB share a very high degree of homology with several humanized antibodies, including trastuzumab that have not been reported to exhibit interactions with mouse serum proteins. The off-target interaction of hLD1.vB with mouse C3 thus most likely resulted from the particular combination of the mouse LD1 CDRs with the human variable domain frameworks.

We reasoned that some of the changes in the CDR sequences of hLD1.vB, which resulted from affinity maturation of the Fab fragment displayed on phage, could lead to improved affinity for FGFR4 with a concomitant loss in mouse C3 binding. Phage-selected variants were expressed as IgG and screened for FGFR4 binding affinity, as well as potential interaction with mouse C3, using an immunoprecipitation assay coupled with SDS-PAGE.

Immunoprecipitation of mouse plasma revealed a ca. 37 kDa protein that was selectively precipitated using hLD1.vB, but not chLD1 (Fig. 3C). Consistent with the findings from size-exclusion HPLC, this 37 kDa protein band was observed in rat but not cynomolgus monkey and human plasma samples (Sup. Fig. 1A–C). Furthermore, the protein was detected in the plasma from mice administered hLD1.vB (Sup. Fig. 2D). MS/Ms analysis of tryptic peptides derived from the 37 kDa mouse plasma protein identified this band as being derived from mouse complement C3 (Fig. 3D). A direct involvement of C3 was supported by the full recovery of hLD1.vB incubated in plasma from C3 knock out (ko) mice (Sup. Fig. 3).

Affinity maturation and re-evaluation of C3 binding. Both chLD1 and hLD1.vB share the same human constant regions and CDRs and thus differ only by their variable domain frameworks. Further, the light and heavy chain variable domain frameworks used to humanize hLD1.vB share a very high degree of homology with several humanized antibodies, including trastuzumab that have not been reported to exhibit interactions with mouse serum proteins. The off-target interaction of hLD1.vB with mouse C3 thus most likely resulted from the particular combination of the mouse LD1 CDRs with the human domain frameworks.

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average HUH7 tumor volume was approximately 350 mm$^3$. For both hLD1.v22 and chLD1 treated groups, this represents a 75% and 83% reduction in tumor size, respectively, compared to the PBS vehicle treatment group. The tumor doubling times of the groups treated with hLD1.vB (12.2 days), hLD1.v22 (15.8 days) or chLD1 (17.1 days) is significantly greater than that of the PBS-treated group (8.2 days). Additionally, the tumor doubling times of the hLD1.v22 or chLD1-treated groups were significantly longer than that for the hLD1.vB treated group. The similar in vivo performance of both hLD1.v22 and chLD1 compared to hLD1.vB, strongly implies that the specific off-target interaction with mouse complement C3 was causing the increased clearance resulting in lower exposure and reduced efficacy of hLD1.vB.

Discussion

As biotherapeutics, antibodies represent a promising class of molecules that offer high affinity, slow clearance and a high degree of specificity. With the advent of antibody engineering, the immune system can be bypassed thus enabling generation of a vast diversity of antibodies with novel properties. As a result, many therapeutically useful antibodies against human antigens have been generated as humanized antibodies derived from mouse hybridoma technology or selected directly using cloned or synthetic antibody repertoires expressed on phage and other display formats.18

Despite the power of the in vitro approach, engineered antibodies have an Achilles heel. Antibodies made in vitro suffer from the lack of in vivo selection and regulatory mechanisms. Host-generated antibodies that cross-react with self-antigens or interact broadly with normal tissues are normally eliminated to prevent autoimmune disease. In contrast, in vitro engineered molecules are not surveyed by this immune regulatory selection process.
In this work, we encountered an unexpected rapid clearance of a humanized antibody that was absent in the originating chimera. Although chLD1 and hLD1.vB had similar affinity and equally inhibited ligand binding to FGFR4 in vitro (Table 1 and Fig. 2A), hLD1.vB had diminished anti-tumor efficacy in vivo compared to chLD1 in the HUH7 HCC xenograft mouse model (Fig. 2B). A tissue distribution study comparing chLD1 and hLD1.vB revealed rapid and specific distribution of chLD1 to liver, a site with high FGFR4 expression, while hLD1.vB remained primarily in circulation (Fig. 2D). In addition, the recovery of hLD1.vB added to mouse or rat plasma in vitro was significantly reduced compared to the recovery of chLD1 or hLD1.vB added to plasma from cynomolgus monkey or human (Fig. 3A).

Analysis of mouse and rat serum samples by size exclusion HPLC revealed the presence of high molecular weight complexes specific to rodent serum samples containing hLD1.vB (Figs. 3B and Sup. Fig. 1). While the rapid clearance of hLD1.vB might be explained by potential plasma instability, aggregation or off-target binding, the lack of any low molecular weight peaks suggested degradation was unlikely and the lack of high molecular weight peaks in cynomolgus monkey and human samples, reduced the probability of aggregation, as one would expect aggregation to be species independent. Thus the generation of an hLD1.vB-plasma protein complex specifically in rodents seemed likely.

Immunoprecipitated samples corresponding to these high molecular weight peaks revealed an unexpected 37 kDa band in addition to bands corresponding to hLD1.vB. Mass spectral sequencing of peptides generated from the 37 kDa band indicated they were derived from mouse complement C3 and localized around a specific region encoding C3d (Fig. 3D and Sup. Fig. 2). Complement C3 is highly abundant in serum. Synthesized by hepatocytes, monocytes, macrophages and astrocytes, it normally plays an important role in identifying and clearing immune complexes from blood. Activation of C3 (185 kDa) by C3 convertases leads to the formation of C3b followed by further processing to form C3d, a 35 kDa proteolytic fragment of C3b. Although C3 fragments can form spontaneously within the circulation in vivo or immediately following blood draw and during ex vivo incubations, C3 fragments are not detected in circulation until complete blood stasis is attained. This is probably due to rapid clearance mechanisms or because the normal in vivo hydrolysis of C3 is thought to occur quite slowly. The 550 and 270 kDa peaks observed by size exclusion HPLC are consistent with the formation over time of an initial complex composed of two C3 molecules bound to hLD1.vB.
(ca. 520 kDa) followed by the conversion to two C3d molecules bound to hLD1.vB (ca. 220 kDa) over time (Fig. 3B). A direct role for mouse C3 in the clearance of hLD1.vB in NCR mice was supported by the improved serum recovery (Fig. 4A) and normal clearance of hLD1.vB relative to chLD1 in C3 ko mice (Fig. 5B).

Conversion of C3 to C3b results in the formation of a covalent thioester bond between C3b and its target proteins such as IgG. This thioester bond originates in the C3d region and links to IgG through the CH1 constant domain.23,24 The interaction of hLD1.vB with C3d in this instance appears to be exclusively through a unique non-covalent interaction as the observed complex was completely dissociated at lower pH and by SDS-PAGE (Figs. 3C and Sup. Fig. 1). In addition, the association of hLD1.vB with C3d only occurs in rodents, not in primates, despite the human constant domain usage in hLD1.vB. Finally, the interaction appears to involve the variable domains of hLD1.vB, since in serum, the presence of C3/C3d appears to block the interaction with FGFR4 (Figs. 3A and 4A).

The decrease in the ability of hLD1.vB to bind FGFR4 observed after incubation in rodent serum (Fig. 3A) suggests that at equilibrium only about 25% of the 1.3 μM hLD1.vB added is in complex with complement C3 despite the high C3 abundance (150 μg/ml or 0.8 μM) in mouse serum.25 This is indicative of a relatively weak interaction between hLD1.vB and mouse C3, although the impact of this interaction is significant in vivo. The generation of this C3/C3d-hLD1.vB complex appears to block interaction with FGFR4, cause rapid clearance and prevent distribution to the liver; all leading to a loss of efficacy in the HUH7 human HCC mouse xenograft model (Fig. 5C).

Although the C3/C3d-hLD1.vB complex is likely a limited problem specific to rodents, preclinical evaluation in a mouse xenograft model is often a necessary step in therapeutic antibody development. To eliminate this unintended interaction, we examined the differences between chLD1 and hLD1.vB (Fig. 1). The interaction with C3 cannot be ascribed to the CDR sequences since they are identical to that of chLD1. Further, the human variable light and heavy domain framework sequences are essentially identical to those used in many other humamized antibodies. Surprisingly, these features, when combined in the form of hLD1.vB, led to an interaction with mouse C3 and this interaction also blocked binding to FGFR4. Thus, if the interaction with C3 was dependent upon a combination between the CDR sequences from chLD1 and the human variable light and heavy domain frameworks (Fig. 1), we reasoned that amino acid changes in the CDRs obtained through affinity maturation of hLD1.vB on FGFR4 might disrupt C3 binding. Using serum recovery in the FGFR4 ELISA and immunoprecipitation as in vitro screens, we selected an affinity-maturated variant of hLD1.vB, hLD1.v22, which appeared to be stable in mouse serum and did not immunoprecipitate C3d (Fig. 4B). Subsequent in vivo studies demonstrated that the clearance and efficacy of hLD1.v22 was similar to that observed for chLD1 in normal mice (Fig. 5). The three amino acid changes in hLD1.v22, located in CDR-H2 of the heavy chain, both improve the affinity for FGFR4 (Table 1) and eliminate the interaction with mouse C3.

Recently, Wu et al. also described an affinity-maturated antibody variant derived from palivizumab that exhibited rapid clearance and limited bioavailability.10 Subsequent investigations revealed broad non-specific tissue binding as determined by immunohis-tochemistry on frozen tissue samples that resulted from specific mutations performed during affinity maturation of palivizumab. The authors report that reversion of three amino acid residues, introduced during the affinity maturation process, back to the original palivizumab sequence, led to a reduction in non-specific tissue binding and improved overall exposure and efficacy in vivo. In contrast to the observations of Wu et al. where the affinity maturation of palivizumab led to a broad non-specific tissue binding, this study reveals a specific off-target binding interaction between mouse C3 and hLD1.vB and the subsequent elimination of this interaction through affinity maturation. Affinity maturation thus resulted in an increase of non-specific off-target binding for palivizumab, but the loss of a specific off-target binding for hLD1.vB.

In contrast to polyreactive or multispecific antibodies typically associated with a much broader range of off-target antibody binding,1,3,7,10,26 this is the first reported instance of an antibody acquiring an unintended but highly specific off-target interaction. Several lines of evidence suggest that in early antibody development, polyreactive B-cells play an important role for providing a diverse antibody repertoire that can be recruited for the generation of high affinity antibodies against a diverse population of antigens. This multi-specificity is thought to result from conformational flexibility of the CDRs. During antibody affinity maturation through somatic mutation in vivo, this flexibility is thought to be reduced leading to increased affinity and specificity.4,6

With this in mind, one possibility is that conformational flexibility may have been introduced upon grafting the LD1 CDRs into the new human framework such that new interactions with C3d were enabled. Thus, rather than altering a direct interaction with C3 through affinity maturation, the changes made to CDR-H2 may have restricted the flexibility of this loop in a way that limited its interaction to FGFR4. The question of whether or not changes in CDR-H2 alter CDR flexibility or interact directly with FGFR4 will require structural conformation; however, from a kinetic standpoint, reduced flexibility would likely be reflected in an increased association rate. Compared to hLD1.vB, the association rate of hLD1.v22 was increased by 3-fold but this was only a minor component of the overall increase in affinity of 20-fold. We suspect therefore that the particular combination of LD1 CDR and human variable domain sequences led to the weak but specific interaction with mouse C3. These types of events are likely to be difficult to predict or eliminate in antibody development screening processes.

In the context of clinical development, toxicity resulting from off-target binding is an overriding concern. A therapeutic candidate that exhibits general tissue cross-reactivity might be flagged by in vitro screens such as protein microarrays,7,9,27,28 light directed peptide synthesis arrays,39 and tissue cross-reactivity assays.30-32 These screens along with preclinical evaluation in rodents and primates are important tools in the assessment of how well a molecular will behave in the clinic. But how can the risk of rare species-dependent highly specific off-target interactions be safeguarded? Further, the evidence leading to the identification of mouse C3 was facilitated by the presence of off-target binding in the blood compartment,
whereas off-target binding may just as easily occur in a particular organ or tissue. For clinical trials that fail due to unexplained toxicity or lack of efficacy, one may be left wondering if there might be a unique off-target interaction that had previously gone unnoticed.

**Materials and Methods**

FGFR4 was biotinylated using Sulfo-NHS-LC-biotin (Pierce; cat. 21335).

**Generation of chimeric LD1.** The extracellular domain of human FGF receptor 4 (FGFR4), expressed in CHO cells and purified as described in reference 14, was used to immunize balb/c mice. A hybridoma expressing LD1 was identified when clones were screened for the ability to block FGF19 binding to FGFR4 in a protein-based ELISA.

The murine LD1 variable domains were cloned from total RNA extracted from LD1 producing hybridoma cells using standard methods. The variable light (VL) and variable heavy (VH) domains were amplified using RT-PCR with degenerate reverse primers to the constant light (CL) and constant heavy domain 1 (CH1) and forward primers specific for the N-terminal amino acid sequence of the VL and VH regions. These variable domains were then cloned in-frame into vectors that contained the respective human light and heavy chain constant regions.

**Humanization and affinity maturation of LD1.** Hypervariable regions of LD1 were grafted onto the human kappa 1 (huKI) and human VH subgroup III (huIII) variable domain frameworks used in trastuzumab. Framework repair was used to optimize FGFR4 binding affinity through the addition of mouse vernier position until an minimum combination of framework changes was identified that fully restored FGFR4 binding affinity.33

hLD1.vB, displayed as a monovalent Fab-P3 fusion on phage, was affinity matured using a soft randomization strategy. Sequence diversity was introduced separately into each hypervariable region such that a bias towards the murine hypervariable region sequence was maintained using a poisoned oligonucleotide synthesis strategy.34 For each diversified position, the codon encoding the wild-type amino acid is poisoned with a 70-10-10-10 mixture of nucleotides resulting in an average 50% mutation rate at each position.

The hLD1.vB diversified phage libraries were panned using a soluble selection method.35 This approach relied upon a short rate at each position.

**Affinity determinations.** Affinity determinations were performed by surface plasmon resonance using a BIACore™-2000. Approximately 50 RU of hLD1.vB IgG was immobilized in 10 mM Sodium Acetate pH 4.8 on a CM5 sensor chip and serial 2-fold dilutions of the FGFR4 (0.48–1,000 nM) in PBST were injected at a flow rate of 30 µl/min. Each sample was analyzed with 4-minute association and 10-minute dissociation. After each injection the chip was regenerated using 10 mM glycine pH 1.7. Binding response was corrected by subtracting the RU from a flow cell with an irrelevant IgG immobilized at similar density. A 1:1 Langmuir model of simultaneous fitting of k_{on} and k_{off} was used for kinetics analysis.

**Xenograft experiments.** All animal protocols were approved by Genentech’s Institutional Animal Care and Use Committee. Female nu/nu (nude-CRL) mice at seven weeks of age were obtained from Charles River Laboratories International (strain code 088). Mice were maintained under specific pathogen-free conditions. HuH7 cells (5 x 10^6; Japan Health Science Research Resources Bank, cat. JCRB0403) were implanted subcutaneously into the flank of mice in a volume of 0.2 mL in HBSS/Matrigel (1:1 v/v; BD Biosciences, cat. 354234). Tumors were measured twice weekly with a caliper and tumor volume was calculated using the formula: \( V = 0.5 \times L \times W^2 \), where L and W are the length and width of the tumor, respectively. When the mean tumor volume reached 145 mm^3, mice were randomized into groups (n = 15) and treated once weekly with 0.2 mL intraperitoneal injections of vehicle (PBS), 30 mg/kg chLD1, 30 mg/kg hLD1.vB or 30 mg/kg hLD1.x22. Following treatment, tumor volumes were measured as described above. Percent tumor growth inhibition (% TGI) was calculated using the following formula, in which C = the mean tumor volume on Day 21 of the control vehicle group and T = the mean volume on Day 21 from each group of mice given the test treatment: %TGI = 100 x ((C - T)/C). Data were analyzed and log-rank tests were used to evaluate tumor doubling differences between groups with JMP software, Version 6.0 (SAS Institute). Data are presented as the mean tumor volume ± SEM.

**Pharmacokinetic studies in mice.** NCR nude mice were supplied by Taconic (cat. NCRNU). C3 knockout mice36 were back-crossed to C57BL/6 mice for at least 10 generations. Offspring were intercrossed to produce C3 knock-out mice and wild-type controls. In this study, they are referred to as C3 ko and C3 wt mice, respectively.

Mice weighing between 15.5 and 38.3 g were administered a 1, 5 or 20 mg/kg body weight IV bolus dose of anti-FGFR4 via the tail vein. Blood samples were collected via retro orbital bleed or cardiac stick (n = 3 mice per time point) and serum isolated at selected time points up to 28 day post dose. Serum samples were stored at -80°C until assayed for anti-FGFR4 serum concentrations using an ELISA.

**Radioiodinations.** Antibodies were radioiodinated using an indirect iodogen addition method.37 The radiolabeled proteins were purified using NAP5™ columns (GE Healthcare Life
in vitro incubations. For incubations in serum, antibodies were added to NCR nude, C3 ko or C3 wt mouse serum, 100 μl and PBS + 0.5% BSA at a final concentration of 200 μg/ml. Aliquots (100 μl) were made and incubated at 37°C with gentle rotation. The samples were transferred to dry ice at 0, 4, 8, 24, 48 and 96 h and stored at -70°C until ELISA analysis.

For incubations in plasma, antibodies were added to cynomolgus monkey, human, rat (Bioreclamation LLC, cat. CYNP4L11HP, HMPPL11HP and RATPL11HP, respectively) and NCR nude mouse plasma (Taconic, cat. NCRNUF). BSA and PBS + 0.5% BSA at a final concentration of 200 μg/ml ± 125I-antibodies at a final concentration of 5 x 10^6 CPM/ml. Aliquots (100 μl) were made and incubated at 37°C with gentle rotation. At 0, 24 and 48 h the samples were transferred to dry ice and stored at -70°C until analysis by size-exclusion HPLC (SE-HPLC) and subjected to protein-G extraction followed by SDS-PAGE (unlabeled antibody samples).

Tissue distribution study. Female NCR nude mice received 1 IV bolus dose of 125I-chLD1 at 300 μCi/kg ± unlabeled chLD1 at 20 μg/kg or 125I-hLD1.vB at 300 μCi/kg ± unlabeled hLD1.vB at 20 μg/kg. Blood was collected at 15 minutes and 2, 5, 24, 72 and 120 h post-dose and processed for serum. The serum was frozen at -70°C until analysis by size-exclusion HPLC and subjected to protein-G extraction followed by SDS-PAGE separation.

Mass spectrometric and bioinformatics analysis. Samples excised from SDS-PAGE were treated as previously described in reference 38. Briefly, following rapid in-solution microwave-assisted tryptic digestion peptides were separated by reverse phase chromatography and eluted directly into a nanospray ionization source with a spray voltage of 2 kV and were analyzed using an LTQ XL-Orbitrap mass spectrometer (ThermoFisher). Precursor ions were analyzed in the FTMS at 60,000 resolution. MS/MS was performed in the LTQ with the instrument operated in data dependent mode whereby the top 10 most abundant ions were subjected for fragmentation. Data was searched using the Mascot Search Algorithm (Matrix Sciences) or by de novo interpretation.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/mabs/15786

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