Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of CD38+ tumors in mouse models in vivo

William Fumey1,2, Julia Koenigsdorf1,2, Valentin Kunick1,2, Stephan Menzel1, Kerstin Schütze1,2, Mandy Unger1, Levin Schriewer1,2, Friederich Haag1, Gerhard Adam2, Anna Oberle3, Mascha Binder3, Ralf Fliegert3, Andreas Guse3, Yong Juin Zhao3, Hon Cheung Lee3, Fabio Malavasi4, Fernando Goldbaum7, Rob van Hegelsom8, Catelijne Stortelers8, Peter Bannas2 & Friedrich Koch-Nolte1

The cell surface ecto-enzyme CD38 is a promising target antigen for the treatment of hematological malignancies, as illustrated by the recent approval of daratumumab for the treatment of multiple myeloma. Our aim was to evaluate the potential of CD38-specific nanobodies as novel diagnostics for hematological malignancies. We successfully identified 22 CD38-specific nanobody families using phage display technology from immunized llamas. Crossblockade analyses and in-tandem epitope binning revealed that the nanobodies recognize three different non-overlapping epitopes, with four nanobody families binding complementary to daratumumab. Three nanobody families inhibit the enzymatic activity of CD38 in vitro, while two others were found to act as enhancers. In vivo, fluorochrome-conjugated CD38 nanobodies efficiently reach CD38 expressing tumors in a rodent model within 2 hours after intravenous injection, thereby allowing for convenient same day in vivo tumor imaging. These nanobodies represent highly specific tools for modulating the enzymatic activity of CD38 and for diagnostic monitoring CD38-expressing tumors.

CD38 is a 43 kDa type II transmembrane ecto-enzyme that is highly expressed in hematological malignancies including multiple myeloma1,2. CD38 consists of a short intracellular N-terminal domain, a transmembrane helix and a long C-terminal extracellular catalytic domain3. CD38 is a multifunctional enzyme that catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from extracellular NAD4,5. The presence of CD38 is routinely used as a marker for chronic lymphocytic leukemia (CLL) where high levels of CD38 correlates with a poor prognosis6. Binding of CD38 to its ligand CD31 enhances the proliferation and migration of CLL cells6,7. It has been proposed that the enzymatic activity of CD38 contributes to a microenvironment favorable for tumor survival in the bone marrow niche8. CD38 represents a promising target for monoclonal antibody (mAb)-based immunotherapy of multiple myeloma9-12. Daratumumab, a human IgG1 antibody generated by immunization of a human IgH-transgenic mouse, has shown promising results13,14. However, the use of mAbs has disadvantages that include the limited tissue penetration due to their large size of approximately 150 kD15,16.

Nanobodies are the smallest antigen-binding domains derived from naturally occurring heavy chain antibodies from camelids. These single domain antibodies have several advantages over conventional antibodies,
including the capacity to bind and block functional epitopes such as the active site cleft of enzymes, better tissue penetration in vivo, and the facile construction of bi- or multi-specific biologicals by genetic fusion15–18.

Binding of Daratumumab to CD38 prevents subsequent binding of many commercially available antibodies, which makes them unsuitable for plasma and myeloma cell identification in patients treated with daratumumab19. Thus, nanobodies that bind independently of daratumumab might be useful as companion diagnostic for identifying CD38 expressing cells in patients treated with daratumumab. Moreover, the enzymatic activity of CD38 may contribute to immune suppression observed in myeloma patients and to a microenvironment that is favorable for tumor survival in the bone marrow niche4. Thus, nanobodies that modulate the enzymatic activity of CD38 could have a therapeutic application for counteracting the immunosuppressive and tumor promoting activities of CD38.

Our goal was to evaluate the potential of CD38-specific nanobodies as novel diagnostics for hematological malignancies. Using phage display technology we successfully generated human CD38-specific nanobodies from immunized llamas. We characterized these nanobodies in terms of CD38-binding on cells, modulation of enzymatic activity, affinity, epitope specificity, complementary binding to daratumumab and targeting of CD38-expressing tumors in a mouse model. The results suggest that some of these nanobodies hold promise for detecting and monitoring CD38-expressing tumors.

Results

Panning of VHH-phage display libraries from immunized llamas on CD38-transfected cells yields 22 distinct families of CD38-specific nanobodies. Two llamas were immunized with recombinant nonglycosylated CD38 ecto-domain (aa 46–300) and two llamas were immunized with a cDNA expression vector encoding full length CD38 (Figure S1). Phage display libraries were generated by PCR-amplification of the VHH-repertoire from blood lymphocytes obtained 4–10 days after the last boost immunization18,20. CD38-specific nanobodies were selected by binding of phages to CD38-transfected lymphoma cells. Selected clones were sequenced and clones that were found more than once, or a plurality of clones with one or a few amino acid substitutions in the CDR regions, were defined as a family. The results revealed selection of clones derived from 22 distinct nanobody families, with CDR3 lengths ranging from 3 to 21 amino acid residues. FACS analyses performed with crude periplasmic lysates from E. coli to detect nanobodies that bound to CD38-transfected but not to untransfected cells, confirmed the specificity of the selected nanobody families for CD38 (Figure S2a).

Table 1 provides an overview of the CD38-specific nanobodies. For each family, the number of isolates (ranging from 1–27) and the number of variants within a family (ranging from 1–6) and the variant amino acid positions within the CDR3 region are indicated. Some nanobodies showed only little if any intrafamily variation, while others contained members with highly divergent amino acid sequences. Families 5, 14, and 20 contain the three nanobodies (MU375, MU1053, MU551) described in our previous study reporting the 3D-structures of these nanobodies in complex with CD3821.

Characterisation of monovalent CD38-specific nanobodies carrying a C-terminal His6-c-Myc tag. For each nanobody family, we subcloned the member that had shown the highest staining intensity of CD38-transfected cells in the periplasmic screening assay (Figure S2b). To circumvent the problem of endotoxin contamination of nanobodies inherent to the expression system, we recloned the nanobody encoding region into a eukaryotic expression vector (pCSE2.5) optimized for secretory protein production in suspension cultures of HEK-6E cells in serum free medium22–24. SDS-PAGE analyses of HEK cell culture supernatants harvested 6d after transfection revealed consistent production levels of ~50 µg nanobody per ml of HEK-6E supernatant (Figure S3).

Specific binding of purified CD38 nanobodies were determined by off-rate analysis on real time bio-layer interferometry (BLI) analysis using the immobilized ectodomain of human CD38 (Table 1), revealing dissociation rates ranging from 7.8 × 10⁻³ to 6.5 × 10⁻³ s⁻¹. Several nanobodies had very slow off-rates below the detection limit of the instrument (WF121, WF139, MU1105 and WF124). As reference, the single chain variable fragment (scFv) of Daratumumab (see below) was included (kd of 4.4 × 10⁻³ s⁻¹). In addition, qualitative comparisons of the dissociation rates were performed using fluorochrome-conjugated CD38 nanobodies bound to CD38-transfected cells by flow cytometry over a timeframe of 16 hours (Figure S4). The results confirm the strong binding and slow dissociation from native CD38 on the cell-surface by monovalent CD38-specific nanobodies.

Three nanobody families inhibit and two nanobody families stimulate the enzymatic activity of CD38. Nanobodies directed to enzymes reportedly show a propensity to block enzymatic activity25,26. CD38 catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from NAD⁺ and the synthesis of cyclic GDP-ribose (cGDPR) from nicotinamide guanine dinucleotide (NGD⁺)27. Since the latter can be monitored conveniently by fluorimetry, we used this GDPR-cyclase assay to analyze the capacity of CD38-specific nanobodies to modulate the enzymatic activity of CD38. CD38-specific nanobodies from 22 families were analysed for their capacity to modulate the GDPR-cyclase activity of CD38 (Fig. 1). Three nanobodies (JK2, MU1067, MU523, families 4, 20, 19) inhibited the conversion of NGD⁺ to cGDPR by recombinant CD38 in a dose-dependent manner. Two other nanobodies (WF14 and MU738, families 7 and 9) enhanced CD38-catalyzed synthesis of cGDPR.

Crossblockade analyses reveal binding of nanobodies to three non-overlapping epitopes. Next, we aimed to assess whether the selected nanobodies recognize overlapping or distinct epitopes on CD38. To this end, we performed crossblockade flow cytometry analyses with Alexa647-conjugated nanobodies from nine different families in the presence of excess unlabeled nanobodies (Table 2, Figure S5). The results allowed grouping of the selected nanobodies into three distinct non-overlapping bins. Group 1 nanobodies block binding of nanobodies MU1068 (family 12), WF211 (family 17), and MU274 (family 13). These nanobodies recognize
Daratumumab in a monovalent scFv format, designated Dara scFv (Figure S6). We performed cross-blockade analyses with Alexa 647 conjugated Dara scFv to determine which of the CD38-specific nanobodies could bind to CD38 in the absence (−) or presence (+) of a disulfide bond connecting CDR2 and CDR3, and the length of the CDR3 within a family are indicated in italic. Names indicate the presence of a short (s) or long hinge (l). The number of amino acid substitutions (max diff) and variant amino acid positions in the CDR3 lengths. Isolate indicates the number of clones selected per family, variant indicates the number of families. Table 1.

Table 1. Characteristics of CD38-specific nanobody families. Families were designated in order of increasing CDR3 lengths. Isolate indicates the number of clones selected per family, variant indicates the number of clones carrying distinct but evidently related amino acid sequences, max diff indicates the maximum difference between two members of a family in number of amino acid substitutions. Variant amino acid positions in the CDR3 within a family are indicated in italic. Names indicate the presence of a short (s) or long hinge (l), the absence (−) or presence (+) of a disulﬁde bond connecting CDR2 and CDR3, and the length of the CDR3 in numbers of amino acid residues. Kdis shows off-rates determined by SPR analyses on the immobilized glycosylated extracellular domain of CD38. Epitopes are numbered arbitrarily, with nanobodies that block the binding of one another considered to recognize the same or overlapping epitopes.

Four nanobody families bind CD38 independently of daratumumab. In order to perform comparative binding analyses with monovalent CD38 nanobodies, we cloned the antigen-binding domain of daratumumab as a tandem construct (Figure 1), and hence it is conceivable that these sensitize CD38 by stabilization of a more active conformation.

These results of in-tandem epitope binning analyses of Dara scFv with CD38-specific nanobodies confirmed the independent binding of Dara scFv and nanobody families 1, 2, and 4. Moreover, three distinct members of family 22 were shown to bind CD38 in conjunction with Dara scFv, irrespective of the order of injection, while binding of family 7 was only observed when Dara scFv was allowed to bind first, in support of a conformational
mechanism (Figure S7, Table S1). Hence within epitope group 3, families 1, 2 and 22, and within epitope group 2, family 4, represent subgroups that bind to an epitope that is non-overlapping with Dara scFv. Taken together, CD38 nanobodies from 4 distinct families and two non-overlapping epitope groups are capable of binding CD38 in conjunction with Dara scFv.

Nanobodies bind to human CD38 on lymphoma cell lines, peripheral blood NK and B cells, and primary myeloma cells. Next, purified fluorochrome-conjugated monovalent anti-CD38 nanobodies were analyzed for binding to native CD38 on the cell surface of human tumor cells, NK cells and B cells (Fig. 3). The results confirm high level of CD38 expression by established tumor cell lines derived from multiple myeloma (LP-1) and Burkitt’s lymphoma (CA46, Daudi) (Fig. 3a). On peripheral blood leukocytes of normal donors, all
nanobodies showed high level staining of CD16+ NK cells and a subset of CD19hi B cells and much lower staining of T cells and CD19int B cells, consistent with the known expression of CD38 by these cells (Fig. 3b). The same staining pattern was observed with the conventional mAb LS198–4–3 that is commonly used for routine diagnostics. We further analyzed the utility of the nanobodies to detect tumor cells in primary bone marrow samples from patients with multiple myeloma. The results show specific discrimination of myeloma cells (CD45lo/CD56 hi) with CD38-specific nanobodies (Fig. 3c).

We next set out to determine whether the nanobodies that bind independently of Dara scFv to a non-overlapping epitope could also stain tumor cells in a therapeutic setting, i.e. when saturated with intact daratumumab. To this end, we preincubated LP-1 myeloma cells with a large excess of Darzalex® before incubation with fluorochrome conjugated nanobodies (Fig. 4). The results show that nanobodies JK2 and JK36 effectively stain cell surface CD38 even after opsonization with daratumumab.

Specific detection of CD38+ tumors in vivo with nanobody MU1067 conjugated to the near infrared dye Alexa680. Next we determined whether CD38-specific nanobodies could be used as imaging agents to detect CD38 expressing tumors in vivo (Fig. 5). To this end we used a two-sided tumor model in nude mice bearing untransfected and CD38-transfected lymphoma cells injected subcutaneously in the left and right flanks. In order to allow in vivo imaging with the IVIS200 system, nanobody MU1067 was conjugated to the near infrared dye Alexa680 and specific binding of Alexa680-MU1067 to CD38-expressing cells was confirmed by flow cytometry. Seven days after injection of tumor cells, Alexa680-MU1067 (50 µg/mouse, 2.5 mg/kg) specifically detected CD38+ tumors in vivo already within 1 hour after nanobody injection (Fig. 5a,b). At this time point very strong signals were also detected in the kidneys, consistent with renal excretion of excess unbound nanobody (15 kD). At 2 hours after injection, signals from the CD38+ tumor exceeded those of the kidneys. At the time of sacrifice (48 h post injection) the CD38+ tumors continued to show high signals, while signals in other tissues returned to background levels (Fig. 5c), with low fluorescent signals still detectable in kidneys. While the liver itself showed only background fluorescence, fluorescent signals in the gall bladder at the time of sacrifice likely reflect biliary excretion of fluorochromes. In conclusion, in the time window from 2–24 h post injection, high tumor/background ratios were observed in all animals (Fig. 5b).
Figure 3. Fluorochrome-conjugated nanobodies detect CD38 on the surface of lymphoma cell lines, peripheral blood lymphocytes, and primary myeloma cells. (a) Untransfected mouse lymphoma cell lines Yac-1 and DC27.10 and their counterparts stably transfected with human CD38 (top row of panels) and human lymphoma cell lines (bottom row) were stained with Alexa647-conjugated nanobody MU1067 or an irrelevant control nanobody. (b) Blood samples from normal donors were incubated with fluorochrome-conjugated antibodies against CD45 (pan-lymphocytes), CD56 (NK cells), CD19 (B cells) and CD3 (T cells) and CD38-specific mAb LS1983-4-3, Nb JK36, or a control nanobody for 30 min at RT. Erythrocytes were lysed and cells were analyzed by flow cytometry. Gating was performed on CD45+ lymphocytes. (c) Bone marrow samples from two myeloma patients were incubated with fluorochrome-conjugated antibodies against CD45 and CD56 and nanobody MU1067 for 30 min at RT. Erythrocytes were lysed and cells were analyzed by flow cytometry. Gating was performed on lymphocytes. Myeloma cells in these patients express CD56 but do not express CD45.
Discussion

The goal of this study was to generate nanobodies directed against the cell surface ecto-enzyme CD38 as new diagnostic and potential therapeutic tools for hematological malignancies. We successfully identified 22 families of CD38-specific nanobodies from phage display libraries generated from immunized llamas. Our results show...
that some of these nanobodies modulate the enzymatic activity of CD38 and allow specific detection of CD38 expressing tumors in vivo.

11 of 22 nanobody families were obtained from protein-immunized llamas after panning on the aglycosylated CD38 ectodomain, the other 11 nanobody families were obtained from cDNA-immunized llamas by binding to CD38-transfected cells in solution. Since the four llamas used were outbred and genetically diverse, it is not possible to conclude that one or the other strategy is better. However, it is perhaps noteworthy that three of four families that bind CD38 independently of daratumumab (families 1, 2, and 4) were derived from genetic immunizations whereas the clone with the highest affinity (MU523, family 19) was derived from a llama immunized with protein.

All CD38 nanobodies bind to three independent non-overlapping epitopes (Fig. 6a). Interestingly, all nanobodies from epitope binning group 1 and many nanobodies from epitope groups 2 and 3 interfered with binding of Dara scFv. The nanobody CDR3 loop can fold over a side of the variable domain to increase the interaction surface with the antigen and the solvent accessible surface area of a nanobody can be as large as that of a VH-VL pair. However, the size of the pair of variable domains of Dara scFv is roughly twice as large as that of a single variable domain of a nanobody. Consistently, the results of the tandem epitope binning analyses by Octet show that the binding site of Dara scFv is larger than that of the CD38-specific nanobodies. Although there is no structural data available on the binding of Daratumumab, it presumably uses both its VH and VL domains for binding to CD38, i.e. it can be expected to cover roughly twice as large a surface area of CD38 than the nanobodies. One nanobody family of epitope group 1 (JK2, family 4) and a subgroup of three nanobody families within epitope group 3 (WF9, JK36, and MU1105, families 1, 2 and 22) bound CD38 independently of daratumumab. Nanobodies JK2 and JK36 effectively recognize cell surface CD38 even after opsonization with saturating doses of daratumumab. These nanobodies could potentially be used to monitor expression of CD38 on the cell surface of lymphocytes and tumor cells in daratumumab-treated patients.

We have previously determined the precise epitopes of three different CD38 nanobodies within epitope groups 1 and 2 by co-crystallisation with the CD38 ectodomain (Fig. 6b). Structural information for nanobodies MU375 (cyan, family 5), MU551 (grey, family 20), or the Fab fragment of mAb hb7 (red) or of mAb sar650984 (blue) were aligned with PyMOL using PDB-IDs 2i65, 5f21, 5f10, 3raj, and 4cmh, respectively. Mutation of serine 274 (green) to phenylalanine abolishes binding of daratumumab.

Previous studies have uncovered a striking propensity of nanobodies from immunized llamas to bind to the active site of enzyme antigens. In our study, three of 22 nanobody families, i.e. all epitope 2 nanobodies, blocked CD38-catalyzed conversion of NGD+ to cyclic GDPR in a dose dependent fashion, whereas two nanobody families (families 7 and 9 from epitope groups 3 and 1, respectively) potentiated CD38 enzyme activity. In this context it is also of interest to note that only one of well over 100 monoclonal antibodies generated against CD38 has been shown to inhibit the enzyme activity of CD38. The crystal structure of this mAb sar650984 in complex with CD38 revealed binding of sar650984 far away from the active site crevice, implying an allosteric mode of action. It seems likely that the enzyme-inhibiting nanobodies similarly act in an allosteric fashion, considering that the binding site of the MU551 family 20 nanobody is also located away from the active site crevice. Similarly, the nanobodies that were found to sensitize the catalytic activity of CD38 may act in an allosteric manner, given the observation of conformational constraints for these nanobodies in the tandem binding studies. It has been suggested that metabolites of NAD+ generated by CD38 in the tumor microenvironment can promote tumor growth and immunosuppression. Thus, it is conceivable that blocking the
enzymatic activity of CD38 may be of therapeutic benefit in cancer. If so, this could influence the choice of nano-
bodies as therapy candidates for pre investigational new drug experiments. In particular, use of the antagonistic
nanobody family 4 that binds independently of daratumumab, would be feasible even in patients undergoing
daratumumab treatment. It will thus be interesting to determine whether allosteric modulation of CD38-enzyme
activity in vivo by CD38 nanobodies can counteract its purported immunosuppressive and tumor promoting
effects in the tumor microenvironment 8.

In a subcutaneous xenograft tumor model in nude mice, we examined the capacity of CD38-specific nano-
bodies to specifically target CD38-expressing tumor cells. In this model, the subcutaneous location and the nude
skin minimized quenching of fluorescent signals from the NIRF-conjugated nanobodies by muscle, bone or hair
and thus facilitated in vivo imaging. Moreover, since the nanobodies do not cross react with mouse CD38, the
mouse model provided a clear background. The results of these experiments clearly demonstrate the capacity of
nanobodies to specifically target CD38+ vs. CD38− tumors. In a clinical setting, radionuclide-labeled nanobodies
can be expected to provide higher sensitivity at lower doses 29, but higher background signals due to binding of
endogenously expressed CD38 in healthy tissues. Besides the high specificity and affinity to CD38, the
efficient imaging of CD38+ tumors can be attributed to the small size of the nanobody which allows excellent
tissue and tumor penetration15,16,29 and fast clearance of excess unbound nanobodies from the circulation by renal
excretion30. Hence the current panel of high affine monovalent CD38-specific nanobodies are attractive for use as
companion diagnostic for anti-CD38 therapies.

For therapeutic applications, nanobodies can readily be humanized, e.g. by fusion to the hinge and Fc-domains
of human IgG1 31. Moreover, the VHH domain itself can be humanized by substituting framework residues to
more closely resemble those of human VH domains 32. This is done routinely for llama-derived nanobodies in
clinical development33,34.

Table 2. Epitope mapping of CD38-specific nanobodies. CA46 lymphoma cells were preincubated for 30 min at
4 °C with unconjugated nanobodies (indicated on the left) before addition of Alexa647-conjugated nanobodies
(indicated on top). Cells were further incubated for 30 min at 4 °C, washed and analyzed by flow cytometry.
Numbers indicate the percentage maximal blockade of the mean fluorescence intensity of cells labeled in the
absence of competing nanobodies, negative numbers indicate enhanced labeling of cells in the presence of the
competing nanobody. Inhibition of binding by 50–80% is highlighted in light grey, inhibition of binding by
>80% in dark grey. Self-blockade by the nanobody used for labeling is indicated by highlighted boxes in the
diagonal.

| ep | fam | name | JK36 | WF100 | JK19 | WF211 | MU274 | MU1068 | MU523 | MU1067 | JK2 | Dara |
|----|-----|------|------|--------|------|--------|------|--------|------|--------|-----|------|
| 3  | 18  | WF121| 99   | 99     | 99   | 71     | 10   | 59     | -13  | 5      | 16  | 99   |
| 3  | 21  | WF124| 100  | 99     | 97   | 88     | 15   | 64     | -9   | 3      | 22  | 99   |
| 3  | 3   | WF42 | 99   | 97     | 99   | 3      | 7    | 23     | -2   | 4      | 14  | 97   |
| 3  | 1   | WF9  | 49   | 66     | 65   | -16    | 5    | 18     | -13  | 3      | 22  | -23  |
| 3  | 2   | JK36 | 97   | 96     | 97   | -13    | 16   | 22     | -13  | 4      | 18  | -9   |
| 3  | 22  | WF100| 90   | 80     | 88   | 9      | 0    | 27     | -7   | 6      | 52  | 46   |
| 3  | 15  | JK19 | 99   | 98     | 97   | 12     | 8    | 39     | -17  | 1      | -35 | 86   |
| 3  | 7   | WF14 | 62   | 69     | 69   | 16     | 6    | 25     | -12  | 2      | 26  | 32   |
| 1  | 9   | MU738| 39   | 33     | -8   | 64     | 53   | 74     | -15  | 6      | 8   | 87   |
| 1  | 11  | JK22 | 27   | 5      | 29   | 97     | 96   | 97     | -8   | 0      | 23  | 99   |
| 1  | 8   | JK28 | 74   | 60     | 96   | 94     | 94   | 95     | -9   | 8      | -10 | 98   |
| 1  | 17  | WF211| 18   | 4      | 52   | 97     | 96   | 97     | -9   | 8      | 12  | 99   |
| 1  | 14  | MU1053| 10  | 12     | 38   | 94     | 93   | 95     | -10  | 6      | -11 | 98   |
| 1  | 5   | MU370| 24   | 26     | 31   | 89     | 80   | 86     | -5   | 5      | -8  | 97   |
| 1  | 13  | MU274| 36   | 45     | 14   | 98     | 99   | 99     | 2    | 7      | -32 | 99   |
| 1  | 12  | MU1068| 46  | 35     | 36   | 86     | 81   | 90     | -7   | 5      | 0   | 97   |
| 1  | 6   | JK29 | 33   | 36     | 1     | 97     | 98   | 98     | -4   | 4      | 9   | 99   |
| 1  | 16  | MU415| 37   | 47     | 18   | 98     | 98   | 98     | -11  | 7      | 36  | 98   |
| 1  | 10  | JK44 | 45   | 28     | -6   | 87     | 83   | 90     | -13  | 3      | 10  | 95   |
| 2  | 19  | MU523| 43   | 40     | 8    | 30     | 20   | 71     | 98   | 98     | 99  | 99   |
| 2  | 20  | MU1067| 26  | 48     | 34   | 31     | 8    | 79     | 99   | 99     | 99  | 99   |
| 2  | 4   | JK2  | 9    | 20     | 9    | -2     | 3    | 15     | 16   | 17     | 56  | -1   |
In conclusion, our results underscore the potential of nanobodies for modulating the enzymatic activity of CD38 and for specific in vivo detection of CD38+ tumors. Importantly, we describe four nanobody families that bind independently of daratumumab, which could potentially be valuable for monitoring the efficacy of daratumumab therapy since they can still detect CD38 after binding of daratumumab. The nanobodies reported here thus hold promise as new diagnostic and potential therapeutic tools for multiple myeloma and other CD38-expressing malignancies.

Methods

Protein production and llama immunizations. The extracellular domain (aa 46–300) of a variant of CD38 in which the three potential N-linked glycosylation sites were inactivated was produced as a secretory protein in yeast cells and purified as described previously. The extracellular domain of CD38 (aa46–300) with intact glycosylation sites was produced as a secretory protein with a chimeric His6x-Myc epitope tag in the pCSE2.5 vector (kindly provided by Dr. Thomas Schirrmann, Braunschweig). For cDNA immunization the full-length reading frame of CD38 was cloned into the pEF-DEST51 expression vector. Two llamas (Lama glama) (designated 10, 25) were immunized subcutaneously with purified recombinant aglycosylated protein emulsified with Specol adjuvant (240 µg in 500 µl total volume). Two llamas (designated 538 and 539) were immunized by ballistic cDNA immunization. The humoral immune response was monitored in serially diluted serum by ELISA on microtiter plates (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, MA) coated with recombinant CD38, using monoclonal antibodies directed against llama IgG2 and IgG3 kindly provided by Dr. Judith Appleton, Cornell University, NY. Animals were bled 4–18 days after the 3rd or 4th boost.

Cells. The Yac-1 and DC27.10 mouse lymphoma cell lines were transfected with linearized full-length human CD38 expression vector pEF-DEST51. Stable transfectants were selected in medium containing blasticidin and by fluorescence activated cell sorting. Human multiple myeloma (RPMI-8266, U266, LP-1) and Burkitt’s lymphoma (CA46, DAUDI) cell lines were obtained from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Bone marrow aspirates of patients MM123 and MM129 were obtained after written informed consent as approved by the ethics committee (Ethikkommission der Ärztekammer Hamburg, PV4767).

Construction of phage display library and selection of CD38-specific nanobodies. Mononuclear cells were isolated from 120 ml of blood by Ficoll-Paque® (GE Healthcare, Chalfont St Giles, UK) gradient centrifugation. RNA purified from these cells by TRIZOL reagent (Invitrogen, Carlsbad, CA) was subjected to cDNA synthesis with random hexamer primers. The VHH coding region was amplified by PCR with degenerate VH-H-specific primers. PCR products were purified from agarose gels, digested sequentially with SfiI and NotI (NEB, Ipswich, MA) and cloned into the pHEN2 phagemid vector downstream of the PelB-leader peptide and upstream of the chimeric His6x-Myc epitope tag. Transformation into XL1-Blue E.coli (Stratagene, La Jolla, CA) yielded libraries with sizes of 4.0 × 10^11–10^12 clones. Phage particles were precipitated with polyethylene glycol from culture supernatants of E.coli transformants infected with a 10-fold excess of M13K07 helper phage (GE Healthcare, Chalfont St Giles, UK).

Panning of specific phage was performed using either the recombinant aglycosylated human CD38 ectodomain immobilized on microtiter plates (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, MA) or in solution with CD38-transfected Yac-1 cells. Phage particles (1.6 × 10^11) were incubated with recombinant CD38 or CD38-transfected cells for 60 min with agitation at room temperature in PBS, 10% Carnation non-fat dry milk powder (Nestlé, Glendale, CA). Following extensive washing, bound phages were eluted from ELISA plates with 50 mM diethylamine and neutralized with 1 M Tris-HCl pH 8. Phages were eluted from transfected cells by trypsinization. Eluted phages were triturated and subjected to one or two rounds of panning, following the same procedure. Phage titers were determined at all steps by infection of TG1 E.coli cells (Stratagene, La Jolla, CA).

Plasmid DNA was isolated from single colonies and subjected to sequence analyses using pHEN2-specific forward and reverse primers.

Production and reformattting of nanobodies. Monomeric nanobodies were expressed in HB2151 E.coli cells (GE Healthcare, Chalfont St Giles, UK). Protein expression was induced with IPTG (Roche, Rotkreuz, Switzerland) when bacterial cultures had reached an OD_600 of 0.5 and cells were harvested after further cultivation for 3–4 h at 37°C. Periplasmic lysates were generated by osmotic shock and removal of bacterial debris by high speed centrifugation. Nanobodies were readily purified from E.coli periplasmic lysates by immobilized metal affinity chromatography (IMAC).

The coding region of selected nanobodies was subcloned using NcoI/PciI and NotI upstream of a chimeric His6x-Myc epitope tag into the pCSE2.5 vector (kindly provided by Thomas Schirrmann, Braunschweig). Daratumumab scFv was generated by gene synthesis using the published sequence (WO 2011/154453) by fusing the VH domain and the VL domain via a 15GS linker flanked by Ncol and NotI sites and cloned upstream of a chimeric His6x-Myc epitope tag into the pCSE2.5 vector.

Recombinant myc-his tagged nanobodies and Dar scFv were expressed in transiently transfected HEK-6E cells cultivated in serum-free medium. Six days post transfection, supernatants were harvested and cleared by centrifugation. Nanobodies in cell supernatants were quantified by SDS-PAGE and Coomassie staining relative to marker proteins of known quantities: 10 µl samples of the supernatant were size fractionated side by side with standard proteins (albumin 4 µg, IgH 2 µg, IgL 1 µg, lysozyme 0.4 µg; albumin 1 µg, IgH 0.5 µg, IgL 0.25 µg, lysozyme 0.1 µg). Yields of recombinant nanobodies typically ranged from 0.5–3 µg/10 µl. Myc-His tagged nanobodies were purified by immobilized metal affinity chromatography using Ni-NTA agarose (Sigma, St Louis, MO).
ELISA. Recombinant CD38 (100 ng/100 μl PBS/well) was adsorbed to 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific, Waltham, MA) at 4 °C over night. Wells were washed twice with PBS and blocked for 2 hours with PBS containing 5% nonfat powdered milk at room temperature. Wells were incubated for 30 min with llama pure and immune serum (diluted 1:100 in PBS). Following washing with PBS/0.05% Tween 20, bound antibodies were detected with llama IgG-specific mAbs followed by peroxidase-conjugated anti-mouse IgG (Jackson) and (TMB) (Sigma, St Louis, MO) as substrate. The absorbance at 450 nm was measured using a Victor3 ELISA-reader (Perkin-Elmer, Waltham, MA).

Off-rate determination. Off-rates of CD38 nanobodies were determined by BLI technology, using an Octet RED384 instrument (ForteBio). As running buffer HBS-EP + (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20) was used. Assays were performed at 25 °C. The shake speed during the biosensor preparation and off-rate determination was set at 1000 rpm. Amine reactive 2nd Generation (AR2G) biosensors (ForteBio) were activated for 10 minutes with EDC20 mM)/NHS10 mM) and recombiant human CD38 was loaded at 10 μg/ml in 10 mM sodium acetate pH 6 for 15 min. After immobilization, surfaces were deactivated with 1 M ethanamine (pH 8.5) for 10 min. During off-rate screening 100 nM and 1 μM nanobody were allowed to associate during 5 min on immobilized human CD38 followed by a 10 min dissociation. After each cycle the human CD38 surfaces were regenerated via 5 short pulses of 5 s of 100 mM HCl and running buffer. Data processing and off-rate determination was performed with ForteBio Data Analysis Software Version 9.0.0.12. Sensorgrams were double referenced by subtracting 1) running buffer on reference biosensor containing only human CD38 and 2) nanobody interaction on parallel reference biosensors on which no human CD38 was immobilized. Processed curves were evaluated via a fitting with the model ‘1:1’.

CD38 epitope binning. In-tandem epitope binning of CD38-specific nanobodies and Dara scFv was performed on an Octet RED384 instrument (ForteBio). As running buffer HBS-EP + (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20) was used. Experiments were performed at 20 °C. The shake speed during the biosensor preparation and epitope binning was set at 1000 rpm. Amine reactive 2nd Generation (AR2G) biosensors (ForteBio) were activated for 10 min with EDC(20 mM)/NHS(10 mM) and human CD38 protein was loaded at 10 μg/ml in 10 mM sodium acetate pH16 for 15 min. After immobilization, surfaces were deactivated with 1 M ethanamine (pH 8.5) for 10 min. In the epitope binning, 100 nM nanobody 1 was loaded during 3 min on immobilized CD38 to saturate all available epitopes. Nanobody 2 was presented after a 10 s dip in running buffer for 3 min followed by a 1 min dissociation. After each cycle the human CD38 surfaces were regenerated via 5 short pulses of 5 s each of 100 mM HCl followed by running buffer. Data was processed with ForteBio Data Analysis Software Version 9.0.0.12. Binding levels of nanobody 2 were determined at the end of the 3 min association and compared to levels at baseline (beginning of association). Irrelevant nanobody controls were included. The binding level of nanobody 2 for each nanobody 2 - nanobody 1 pair was divided by the binding response of nanobody 2 on a CD38 surface saturated with nanobody 2 (self-binning). Normalized data was hierarchically clustered using Ward’s method (distance measure: half square Euclidian distance; scale: logarithmic) and visualized in Spotfire (TIBCO Software Inc.).

Fluorimetric enzyme assay. CD38 catalyzes both, the synthesis of cADPR and nicotinamide from β-NAD+, and the fast hydrolysis of cADPR to ADPR. A fluorimetric enzyme assay with slower kinetics has been developed using nicotinamide guanine dinucleotide (NGD+) as substrate. NGD+ is converted to cyclic GDP-ribose (cGDR) and nicotinamide phosphorylated by a very slow hydrolysis of cGDP to GDP, leading to accumulation of the fluorescent product cGDR. Enzymatic production of cGDR from NGD+ (80μM, Sigma, St Louis, MO) was monitored continuously for 50 min at 410 nm (emission wavelength) with an excitation wavelength set at 300 nm, using a Hitachi F-2000 fluorimeter. Anti-CD38 nanobodies were pre-incubated at a final concentration of 400, 40, 4, and 0.4 nM with 5 nM recombinant glycosylated extracellular domain of CD38 for 15 min at RT before addition of NGD+ and further incubation in the dark at RT in triplicate wells for each treatment. Readings (EX300/EM410) from wells without CD38 were subtracted from all sample readings and were plotted for each nanobody concentration in Relative Fluorescence Units (RFU) vs. time. The rate of cGDR production was calculated as the slope of these curves (RFU/s) during the linear phase of the reaction, i.e. between t = 10 min and t = 20 min.

Flow cytometry. Untransfected Yac-1 cells and Yac-1 cells stably transfected with human CD38 were incubated for 30 min with nanobody-containing periplasmic lysates (diluted 1:10 in PBS). Following washing with PBS/0.1% BSA, bound nanobodies were detected with FITC-conjugated anti-c-Myc mAb 9E10 (Sigma, St Louis, MO). Human tumor cell lines, peripheral blood leukocytes, and bone marrow cells were incubated for 30 min with fluorochrome-conjugated nanobodies and monoclonal antibodies directed against CD3 (SK7), CD16 (3G8), CD19 (HIB19), CD45 (HI30), and CD38 (LS198–4–3) (BD Biosciences, Heidelberg and Beckman-Coulter, Krefeld). Darzalex® was purchased from Janssen-Biologics, Leiden. For binding stability analyses, CD38-transfected cells were incubated for 60 min with serial dilutions of monovalent nanobodies for 15 min at RT before addition of NGD+ and further incubation in the dark at RT in triplicate wells for each treatment. Readings (EX300/EM410) from wells without CD38 were subtracted from all sample readings and were plotted for each nanobody concentration in Relative Fluorescence Units (RFU) vs. time. The rate of cGDR production was calculated as the slope of these curves (RFU/s) during the linear phase of the reaction, i.e. between t = 10 min and t = 20 min.

For nanobody dissociation analyses, two separate aliquots of CD38-transfected cells were incubated either with Cell Proliferation Dye eFluor® 450 (eBioscience) or with Alexa® 647-conjugated nanobodies for 20 min at 4 °C. Cells were washed four times, mixed at a 1:1 ratio and further incubated at 4 °C or at 37 °C for 0.5, 2, or 16 h before FACS analyses. The dissociation of nanobodies from the target cells and association with the eFluor® 450 labeled cells was analyzed using the FlowJo software (Treestar).
Cross-blockade analyses. For epitope analyses, cells were preincubated with excess monovalent nanobodies or Dara scFv (2 µg/100 µl PBS/0.1% BSA) for 30 min at RT before addition of fluorochrome-conjugated nanobodies (500 ng in 0.5 µl PBS) and further incubation for 20 min at RT. Cells were washed and analyzed by flow cytometry on a BD-FACS Canto. Data were analyzed using the FlowJo software (Treestar).

In vivo and ex vivo imaging. Tumor graft experiments were conducted using athymic nude mice (NMRI-Foxn1nu) obtained from Charles River Laboratories (Sulzfeld, Germany). Experiments were performed in accordance with international guidelines on the ethical use of animals and were approved by the animal welfare commission (Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen Hamburg, Nr. 17/13). Prior to optical molecular imaging in vivo, 8–10-week-old mice were kept on an alfalfa-free diet for 7 d to minimize autofluorescence of the intestine. For generation of tumor grafts, mice were injected s.c. on the right side with 1 × 10⁶ CD38-transfected DC27.10 cells and on the left side with 1 × 10⁶ untransfected DC27.10 cells, each in 0.2 ml of a 50:50 mix of RPMI medium and Matrigel (BD Biosciences, Franklin Lakes, USA). After 7 d, i.e. when tumors reached ~8 mm in diameter, 50 µg of Alexa647-conjugated nanobody MU1067 was injected i.v. via the tail vein. Similar doses have been found to yield good tumor to background ratios in previous studies using nanobodies conjugated to near infrared fluorochromes. Optical molecular imaging was performed before injection and at indicated time points after injection. For optical molecular imaging, mice were anesthetized with isoflurane and positioned in the imaging chamber of a small animal imaging system (IVIS-200, Caliper Life Sciences, Hopkinton, Massachusetts, USA). After qualitative imaging in vivo, quantitative analyses were performed by placing ROIs around the CD38-positive tumors, the CD38-negative tumors (negative control) and the hind limb (background signal). Total radiant efficiency was determined with Living Image 4.2 software (Caliper Life Sciences). The tumor-to-background ratio was calculated by dividing the tumor uptake value by the background value. For ex vivo validation of in vivo measurements, animals were sacrificed 48 h post-injection. Tumors and organs (spleen, lungs, liver, kidneys, stomach, ileum, and muscle) were dissected and imaged with the IVIS-200.

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Author Contributions
WF, J.K., S.M., and V.K., contributed equally and share first authorship. PB and F.K.-N. share senior authorship. WF, J.K., V.K., K.S., L.S., S.M., M.U., A.O., R.F., and R.v.H. performed experiments and analyzed results. F.H., G.A., M.B., A.G., Y.J.Z., H.C.L., F.M., F.G., C.S., P.B., and F.K.-N. designed research and analyzed data. F.K.-N. and FG, are co-founders of Inmunova, a wholly owned subsidiary of the University Medical Center Hamburg-Eppendorf. F.G. is co-founder of Inmunova, a wholly owned subsidiary of the Instituto Leloir, which provides molecular assembly vaccines and llama immunizations.

Additional Information
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Competing Interests: WF, K.S., PB., S.M., C.S., and F.K.-N. are co-inventors on a patent application on CD38-specific nanobodies. F.H. and F.K.-N. receive a share of antibody sales via MediGate GmbH, a wholly owned subsidiary of the University Medical Center Hamburg-Eppendorf. F.G. is co-founder of Immunoiva, a wholly owned subsidiary of the Instituto Leloir, which provides molecular assembly vaccines and llama immunizations.

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