Targeting CD38-exprimierender Tumorzellen mittels Nanobody-basierter Schwereketten-Antikörper, Nb-CARs und nano-BiKEs

Dissertation

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Abkürzungsverzeichnis

ADCC	Antibody-dependent cellular cytotoxicity
ADPR	Adenosindiphosphat-Ribose
ATP	Adenosintriphosphat
BiKE	Bispecific Killercell engager
BiKE-DCC	BiKE-dependent cellular cytotoxicity
Cas	CRISPR-associated
CAR	Chimäric antigen receptor
CAR-DCC	CAR-dependent cellular cytotoxicity
CD	Cluster of Differentiation
CDC	Complement-dependent cytotoxicity
CDR	contemplementary-determing regions
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Fc	Fragment crystallizable
FDA	U.S. Food and Drug Administration
hcAb	heavy chain antibody
HLE	Half-life extended
IL-15	Interleukin 15
KO	Knock-Out
NAD	Nicotinamidadenindinukleotid
nano-BiKE	Nanobody-basierter BiKE
HLE-nano-BiKE	Half-life extended nano-BiKE
Nb-CAR	Nanobody-basierter CAR
NK-Zelle	Natürliche Killer Zelle
MAC	membrane attack complex
scFv	Single chain variable Fragment
sog.	so genannten
VH	Variable Domäne der schweren Kette
VHH	Variable Domäne eines Schwereketten-Antikörpers
VL	Variable Domäne der leichten Kette
vWF	von-Willebrand-Factor

Aus Gründen der besseren Lesbarkeit wird auf die gleichzeitige Verwendung der Sprachformen männlich, weiblich und divers (m/w/d) verzichtet. Sämtliche Personenbezeichnungen gelten gleichermaßen für alle Geschlechter.

Zusammenfassung

Das Multiple Myelom ist eine der häufigsten hämatologischen Tumorerkrankungen. Trotz therapeutischer Erfolge in den letzten zwanzig Jahren gilt das Multiple Myelom jedoch weiterhin als unheilbar. Daher werden neue, effektive und praktikable Therapieansätze dringend benötigt. CD38 ist ein Ektoenzym, das auf Multiplen Myelomzellen überexprimiert wird. Deshalb bietet es sich als Target für die Immuntherapie an, wie der klinische Erfolg von CD38-spezifischen Antikörpern wie Daratumumab und Isatuximab beweist. Lamas produzieren so genannte Schwereketten-Antikörper (*heavy-chain antibodies, hcAbs*), die im Vergleich zu konventionellen Antikörpern keine leichte Kette enthalten. Die isolierte, das antigenbindende Paratop tragende, variable Immunoglobulin-Domäne dieser hcAbs nennt sich *Nanobody*. Nanobodies zeigen eine gute Löslichkeit und Reformatierbarkeit und eignen sich daher hervorragend als bindendes Element von rekombinanten Antikörper-Konstrukten. Durch Immunisierung von vier Lamas mit CD38 konnten wir 22 distinkte Familien CD38-spezifischer Nanobodies isolieren. Die Mehrheit dieser Nanobodies bindet an eines von drei verschiedenen Epitopen (E1, E2, E3) auf CD38. Drei dieser CD38-spezifischen Nanobodies (WF211 (Epitop 1), MU1067 (Epitop 2), JK36 (Epitop 3)) wurden in dieser Arbeit genutzt für die Generierung Nanobody-basierter humaner hcAbs, chimärer Antigenrezeptoren (*Nb-CARs*) und bispezifischer Killerzell Engager (*nano-BiKEs*). Diese CD38-spezifischen Nanobody-basierten Konstrukte zeigten eine effektive Lyse CD38-exprimierender Myelomzellen *in vitro*, *ex vivo* und *in vivo.* CD38-spezifische hcAbs, Nb-CARs und nano-BiKEs sind daher eine vielversprechende Option für die künftige Therapie des Multiplen Myeloms.

Abstract

Multiple myeloma is one of the most common hematological malignancies. However, despite significant therapeutic successes over the last twenty years, multiple myeloma is still considered incurable. Therefore, new effective and feasible therapies are urgently needed. CD38 is an ectoenzyme overexpressed on multiple myeloma cells. Thus, it is an ideal target for immunotherapy, as evidenced by the clinical success of CD38-specific monoclonal antibodies daratumumab and isatuximab. Besides conventional antibodies, llamas produce heavy chain antibodies (*hcAbs*), which do not contain a light chain. The independent variable immunoglobulin domain that carries the antigen-binding paratope of these hcAbs is called a *nanobody*. Nanobodies show good solubility and reformatability and therefore are a suitable binding element of recombinant antibody constructs. We immunized four llamas with CD38 and isolated 22 distinct families of CD38-specific nanobodies. Most of these nanobodies bind to one of three different epitopes (E1, E2, E3) on CD38. In the work described in this dissertation, three of these CD38-specific nanobodies (WF211 (epitope 1), MU1067 (epitope 2), JK36 (epitope 3)) were used for generating CD38-specific human hcAbs, chimeric antigen receptors (*Nb-CARs*), and bispecific killer cell engagers (*nano-BiKEs*). These CD38-specific nanobody-based constructs demonstrate effective lysis of CD38-expressing multiple myeloma cells *in vitro*, *ex vivo*, and *in vivo.* Nanobody-based CD38-specific hcAbs, CARs and BiKEs might therefore be a promising option for future therapies of multiple myeloma.

1 Einleitung

1.1 CD38-spezifische Antikörper für die Therapie des Multiplen Myeloms

Das Multiple Myelom ist eine hämatologische Krebserkrankung, bei der Plasmazellen unkontrolliert im Knochenmark des Patienten wachsen. Als Folge dieser Expansion der Plasmazellen treten platzbedingte Problematiken im Knochengewebe und Infektionen auf. Zusätzlich führt die Überproduktion von Antikörpern durch die Plasmazellen zur Schädigung der Nieren (1-3). Obwohl die Lebenserwartung von Patienten mit Multiplem Myelom durch Stammzelltransplantationen und neue Therapieansätze bereits deutlich zugenommen hat, gilt die Krankheit durch die hohe Rückfallrate als unheilbar (1, 4). Beinahe jeder achtzigste krebsbedingte Tod ist auf ein Multiples Myelom zurückzuführen (5). Effektivere Therapieansätze mit geringeren Nebenwirkungen werden daher dringend benötigt.

CD38 ist ein ca. 45 kDa großes Typ II Transmembran-Glykoprotein. Es wird auf verschiedenen Zellen des hämatopoetischen Systems wie T-, NK- und B-Zellen exprimiert. Auf Multiplen Myelomzellen und einigen anderen hämatologischen Tumorzellen wird CD38 überexprimiert (6). Da CD38 Teil der purinergen Signalkaskade ist, die extrazelluläres NAD+ und ATP zu immunosupressivem Adenosin umwandelt, kann eine hohe Expression von CD38 auf diesen Zellen zu einem anti-inflammatorischen Tumormilieu beitragen (7). Daher gilt CD38 als vielversprechendes Target für Immuntherapien.

Die CD38-spezifischen monoklonalen Antikörper Daratumumab und Isatuximab wurden bereits durch die FDA zur Behandlung des Multiplen Myeloms bei neu diagnostizierten und rückfälligen Myelom Patienten zugelassen (8). Mittlerweile gehören sie zu den Standardtherapeutika für Myelom-Patienten und ihr erheblicher Erfolg ist vielversprechend (4). Daratumumab wurde von der Firma Genmab aus IgH transgenen Mäusen isoliert, die mit CD38 immunisiert wurden (8, 9). Isatuximab ist ein humanisierter CD38-spezifischer Mausantikörper der Firma Sanofi (10). Auch andere CD38-spezifische Antikörper wie MOR202 und TAK-079 befinden sich zurzeit in der klinischen Testung (8, 11, 12).

1.2 Immunologische Effektorfunktionen von CD38-spezifischen Antikörpern

Über den Fc-Teil des Antikörpers vermittelte immunologische Effektorfunktionen können über Bindung des Antikörpers an CD38 auf den Multiplen Myelomzellen ausgelöst werden. Zu den Fc-vermittelten Effektorfunktionen zählen die Antikörper-vermittelte zelluläre Zytotoxizität (*Antibody-dependent cellular Cytotoxicity, ADCC*), Komplement-vermittele Zytotoxizität (*complement-dependent cytotoxicity*, *CDC*), Antikörper-vermittelte zelluläre Phagozytose und Apoptose.

Beim CDC bindet der Komplement-Faktor C1q an die Fc-Fragmente Targetzell-gebundener IgG1, IgG3 oder IgM Antikörper (13). Dies führt zur Aktivierung der Komplementkaskade und letztlich zur Formation des membrane attack complex (*MAC*). Dieser bildet eine Pore in der Zellmembran der Zielzelle, die zur Lyse der gebundenen Zelle führt. Spaltprodukte der Komplementfaktoren wie C3a und C4a locken zudem Phagozyten zum Ort der Entzündung.

Bei der ADCC wird der Fc-Teil eines gebundenen Antikörpers durch den Fcγ-Rezeptor FcγRIIIa (*CD16*) auf einer NK-Zelle gebunden. Durch die Bindung wird an der immunologischen „Synapse“ zwischen Zielzelle und NK-Zelle CD16 auf der NK-Zelle quervernetzt. Dies führt zur Aktivierung verschiedener Signalwege in der NK-Zelle und zur Phosphorylierung von intrazellulären ITAMs benachbarter Rezeptoren (**Abbildung 1**) (14-16). Der wichtigste Signalweg resultiert in der Exozytose von zytotoxischen Perforinen, die zur Lyse der gebundenen Zielzelle führen. Zudem werden pro-inflammatorische Zytokine freigesetzt, die weitere Immunzellen anlocken und Rezeptoren der TNF-Familie aktivieren, die ebenfalls zur Apoptose der Zielzelle führen können (15, 17).

Es wird vermutet, dass ADCC die für den therapeutischen Erfolg von einigen klinisch eingesetzten therapeutischen Antikörpern wichtigste immunologische Effektorfunktion ist (17-19).

Die therapeutischen Antikörper 4D5 (HER2-spezifischer monoklonaler Antikörper), Trastuzumab (p185HER-2/neu-spezifischer monoklonaler Antikörper) und Rituximab (CD20-spezifischer monoklonaler Antikörper) zeigen in Mäusen, die keine Fcγ-Rezeptoren exprimieren, keinen anti-Tumor Effekt (20). Der klinische Erfolg von Rituximab ist zudem statistisch höher in Patienten, die einen Fcγ-Rezeptor Polymorphismus aufweisen, der eine erhöhte Affinität zu IgG1 hat (21, 22).

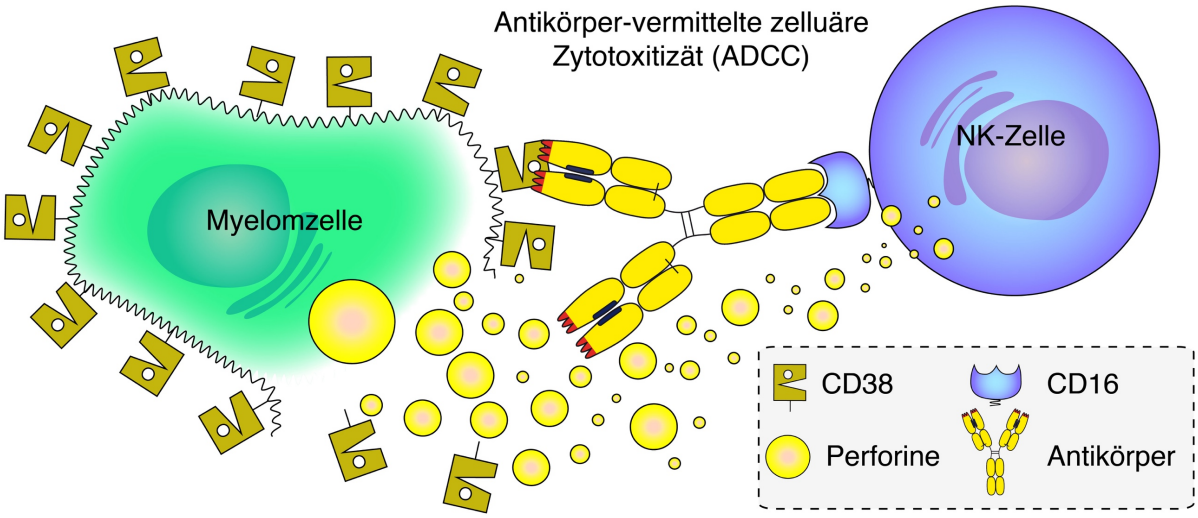

Abbildung 1: Schematische Darstellung der Antikörper-vermittelten zellulären Zytotoxizität (*Antibody dependent cellular cytotoxicity, ADCC*). Ein konventioneller Antikörper (gelb) bindet an ein membranständiges Antigen (CD38, oliv) auf einer Myelomzelle (grün). Durch Bindung des Fc-Rezeptors CD16 der NK-Zelle (blau) an den Fc-Teil des gebundenen Antikörpers (gelb) entsteht eine immunologische Synapse. Dies führt zur Ausschüttung von Perforinen durch die NK-Zelle und zur Lyse der Myelomzelle.

Daratumumab löst jedoch nicht ausschließlich direkte Effekte auf die Myelomzellen aus, sondern auch durch indirekte, immunmodulatorische Effekte wie das Targeting CD38-exprimierender regulatorischer T- und B-Zellen und myeloider Suppressorzellen (23, 24). Dies kann zu der Expansion von CD4+ und CD8+ T-Zellen und einer besseren tumorspezifischen Immunantwort führen (8, 23). Antikörper, die durch Bindung an CD38 zu einer Inhibition der enzymatischen Aktivität von CD38 führen, könnten zusätzlich das Tumormikroenvironment des Multiplem Myeloms weiter von einem kalten, immunosupressiven zu einem heißen, pro-inflammatorischen wandeln (25). Diese Effektormechanismen könnten ebenfalls für die Entwicklung anderer Therapien, die auf dem Targeting von CD38 basieren, relevant sein.

Die Gewichtung dieser einzelnen immunologischen Effektorfunktionen für den klinischen Erfolg von Daratumumab ist bislang unklar. Es ist jedoch wahrscheinlich, dass eine Kombination aus verschiedenen Effektorfunktionen relevant für die klinische Effizienz von Daratumumab ist.

CD38 wird auch auf vielen gesunden Zellen des lymphatischen Systems exprimiert. Bei der Behandlung mit Daratumumab ergeben sich daher so genannte Off-Target Effekte. NK-, B- und CD4+ T-Zellen, basophile Granulozyten und Monozyten zeigen in Myelom-Patienten nach der Therapie mit Daratumumab eine deutlich verminderte CD38-

Oberflächenexpression (26). Zudem ist der Anteil der immunregulatorischen $CD38^+$ T- und B-Zellen, basophilen Granulozyten und $CD56^{dim}$ NK-Zellen im Knochenmark dieser Patienten im Vergleich zu gesunden, unbehandelten Spendern deutlich vermindert (27). Spezifischere Immuntherapien mit geringeren Nebenwirkungen werden daher für die Therapie des Multiplen Myeloms dringend benötigt.

1.3 Antikörper-basierte Technologien

Neue Antikörper, die spezifisch an ein Antigen binden, können durch Immunisierung von Tieren mit diesem Antigen entwickelt werden. Da Fc-vermittelte immunologische Effektorfunktionen aber nur mittels eines endogenen Fc-Fragments ausgelöst werden können, müssen diese Antikörper für die klinische Verwendung im Menschen humanisiert werden. Dies kann wie bei Daratumumab durch Immunisierung von human IgH-transgenen Mäusen erfolgen, über den Austausch von Aminosäuren im Framework des Antikörpers wie bei Isatuximab oder durch genetische Fusion der bindenden Domäne des Antiköpers an den Fc-Teil eines humanen Antikörpers (**Abbildung 2A**) (28, 29). Das Risiko einer Immunreaktion des Patienten gegen den therapeutischen Antikörper wird hierdurch ebenfalls gesenkt (30).

Durch genetische Fusion mehrerer Bindungsfragmente aus verschiedenen Antikörpern können multivalente und multispezifische chimäre Antikörper-Konstrukte hergestellt werden (13, 31).

Der wissenschaftliche Fokus auf dem Gebiet der Immuntherapie entwickelt sich zunehmend von monoklonalen Antikörpern zu rekombinanten Antikörper-Technologien. Neue, vielversprechende immunologische Therapieansätze nutzen die Aktivierung von NK-Zellen um die Therapie des Multiplen Myeloms weiter zu verbessern (32). Zu diesen neuen Therapiemöglichkeiten zählen unter anderem rekombinante Antikörper-Konstrukte wie chimäre Antikörper, chimäre Antigenrezeptoren (*CARs*) und bispezifische Engager (6, 33). Diese Technologien basieren auf den spezifischen Bindungseigenschaften von Antikörpern. Die antigenbindende Domäne eines Antikörpers wird dabei isoliert und mit anderen NK-Zell stimulierenden Domänen verbunden (**Abbildung 2**).

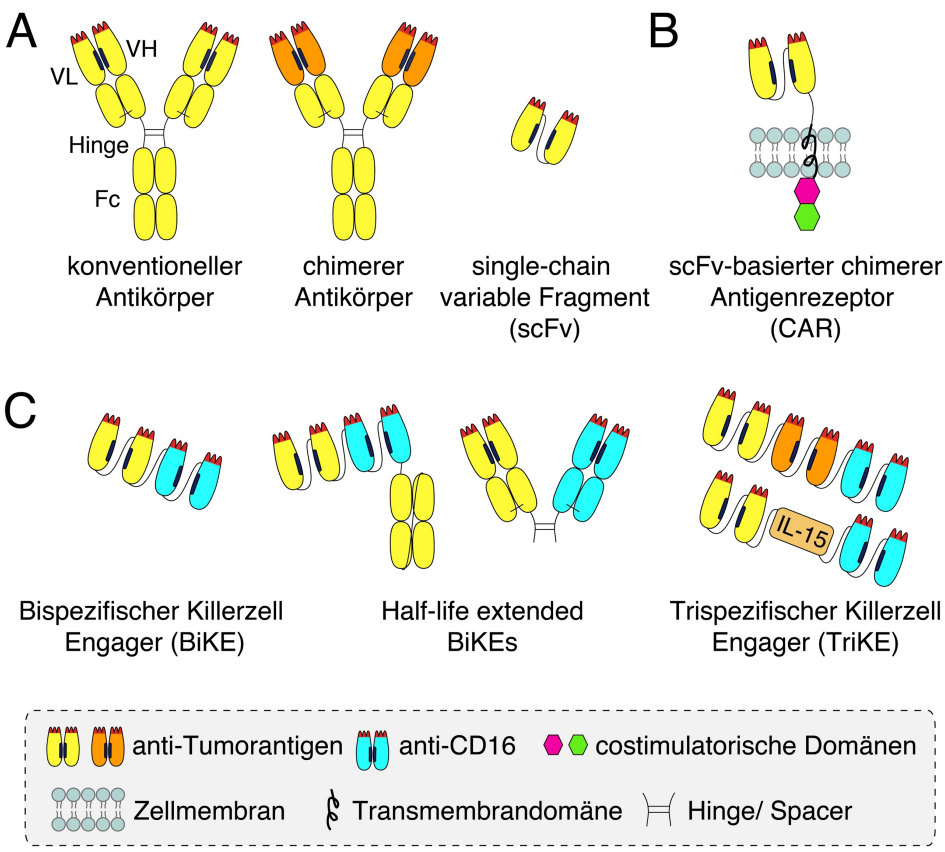

Abbildung 2: Schematische Darstellung rekombinanter Antikörper-Konstrukte, die auf konventionellen Antikörper basieren. (A) Konventionelle Antikörper (~150 kDa) bestehen aus zwei leichten und zwei schweren Ketten, die jeweils eine variable Region (*VL* bzw. *VH*) und eine konstante Region (*CL* bzw. *CH1*, *CH2* und *CH3*) enthalten. CH2 und CH3 der konstanten Region bilden den Fc-Teil (*Fragment crystallizable*) des Antikörpers. VL und VH sind über eine hydrophobe Region (blau) im Framework verpaart. Die jeweils drei CDR-Schlaufen (*complementarity determining regions*) der VL und VH bilden beim konventionellen Antikörper zusammen das Antigen-bindende Paratop (rot). Durch genetische Fusion des bindenden Elementes eines murinen Antikörpers (orange) mit der CH1, CH2 und CH3 Domäne eines humanen Antikörpers (gelb), können in Mäusen entstandene Antikörper für die Therapie von Menschen eingesetzt werden. Ein rekombinantes scFv (*single-chain variable Fragment*, ~25 kDa) entsteht durch genetische Fusion einer VL mit einer VH über einem Peptid-Linker. **(B)** Chimere Antigenrezeptoren (*CARs*) entstehen durch genetische Fusion eines Tumorantigen-spezifischen scFvs über einen extrazellulären Abstandhalter (*Hinge* oder *Spacer*) und einer Transmembran-Domäne (beide in schwarz) mit intrazellulären aktivierenden Domänen (*ITAMs*, *immunoreceptor tyrosine-based activation motifs*, magenta, grün). CARs können nach retroviraler Transduktion auf T- oder NK-Zellen (*CAR-T-Zellen* bzw. *CAR-NK-Zellen*) exprimiert werden. **(C)** scFv-basierte BiKEs (*bispecific killercell engager*, ~50 kDa) können mittels genetischer Fusion eines Tumorantigen-spezifischen scFvs an ein CD16-spezifisches scFv über einen Peptid-Linker erstellt werden. Die Halbwertszeit dieses Konstruktes *in vivo* kann durch genetische Fusion des BiKE an einen Fc-Teil oder durch Verknüpfung von zwei Fab-Fragmenten verlängert werden (*half-life extended BiKE, HLE-BiKE*). Ein trispezifischer Killerzell Engager (*TriKE*) entsteht durch Integration von costimulatorischen Domänen wie Interleukin-15 (*IL-15*) oder eines zweiten, tumorspezifischen scFv in einen BiKE.

1.4 Single-chain variable Fragments (scFvs)

Konventionelle Antikörper, wie sie in den meisten Säugetieren vorkommen, enthalten eine schwere und eine leichte Kette, deren variable Domänen (*VH* und *VL*) zusammen das antigenbindende Paratop bilden. Diese binden über die mittels genetischer Hypermutation spezifisch an das Antigen angepassten Komplementaritäts-bestimmende Regionen 1, 2 und 3 (*complementarity-determining regions*, *CDRs*) (34, 35). Die isolierte, mit einem Proteinlinker verbundene, variable VH- und VL-Domäne dieser Antikörper nennt sich single-chain variable Fragment (*scFv,* **Abbildung 2A**) (36). Da VH- und VL- Domänen im Antikörper mittels einer instabilen hydrophoben Interaktion verpaart sind, sind scFvs oft schlecht löslich und/oder weisen unspezifische Bindungseigenschaften auf (37). Zudem muss die optimale genetische Konfiguration von VL und VH im scFv evaluiert werden, was die Konstruktion von Antikörperformen, die auf einem scFv basieren, erschwert (34).

1.5 Nanobodies

Als *Nanobody* wird die isolierte, Antigen-bindende Immunglobulin-Domäne eines Schwereketten-Antikörpers bezeichnet (**Abbildung 3**). Diese finden sich in Lamas, Alpakas und anderen Kameliden (38). Schwereketten-Antikörpern fehlt durch eine Mutation, die vor über 50 Millionen Jahren auftrat, die CH1 Domäne und die leichte Kette (38). Somit ist auch das Antigen-bindende Fragment des Schwereketten-Antikörpers nicht aus zwei Domänen, sondern nur aus einer Domäne aufgebaut.
Nanobodies enthalten anstelle der in VH und VL enthaltenen hydrophoben Region eine hydrophile Region, sodass Nanobodies besser löslich sind als scFvs. Außerdem besitzen Nanobodies häufiger eine längere CDR3-Schlaufe als traditionelle Antiköper. Dadurch können Nanobodies auch schwer zugängliche Epitope eines Zielantigens binden, welche von konventionellen Antikörpern nicht erreicht werden können, wie zum Beispiel das aktive Zentrum eines Enzyms (34, 39).
Da Nanobodies nur aus einer Kette bestehen, kann die für den Nanobody kodierende DNA-Sequenz leicht in für andere Antikörper-Konstrukte kodierende Vektoren kloniert werden. Nanobodies bieten sich daher als Grundlage für die Konstruktion von chimären Antikörper-Konstrukten an (40). Nanobody-basierte Antikörper-Konstrukte sind kleiner als entsprechende Antikörper-Konstrukte, die auf konventionellen Antikörpern basieren. Dies trägt zu einer besseren Gewebepenetration *in vivo* bei (41).
In der Regel induzieren Nanobodies als variable Immunglobulin Domänen *in vivo* keine neutralisierende Antikörper gegen den Nanobody (6, 34).

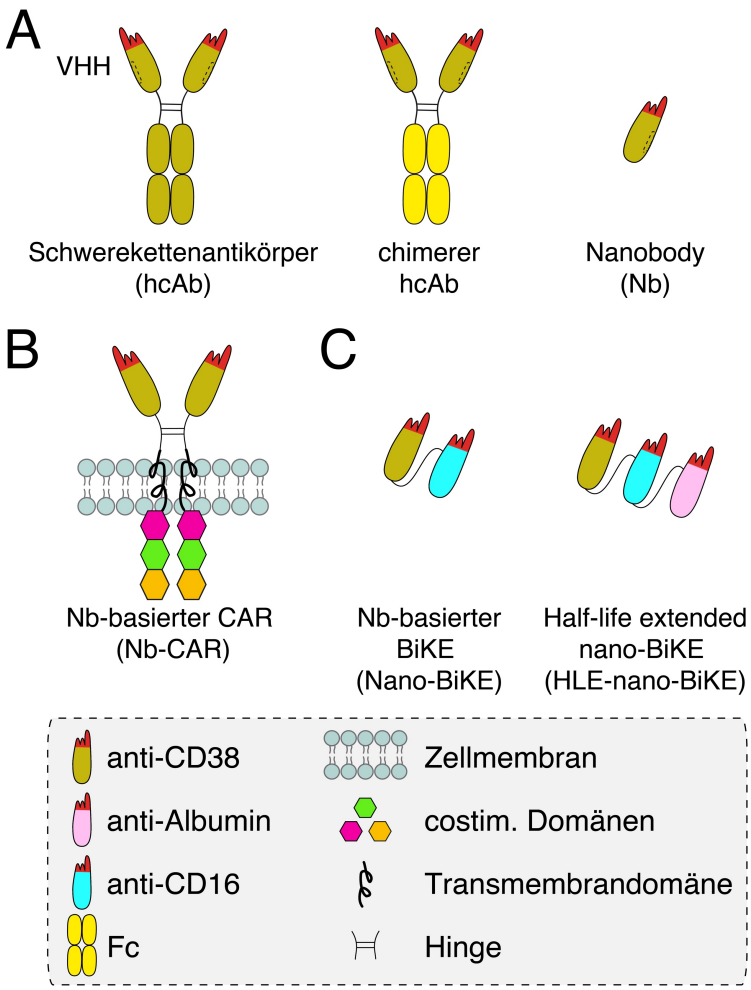

Abbildung 3: Nanobody-basierte rekombinante Antikörper-Konstrukte

(A) Schwereketten-Antikörper (*hcAbs*, ~75k Da) bestehen nur aus einer schweren Kette. Sie werden natürlicherweise von Kameliden neben konventionellen Antikörpern hergestellt. Nanobodies (*Nb*, ~15 kDa) sind die isolierte, Antigen-bindende Domäne (*VHH*) eines hcAbs. Statt der hydrophoben Region an der Interaktionsfläche von VH und VL findet sich an dieser Stelle im Nanobody eine hydrophile Region (gestrichelt). Dies erklärt die wesentlich höhere Löslichkeit und Stabilität eines Nanobodies im Vergleich zu VH und VL im scFv. Um *in vivo* immunologische Effektorfunktionen auslösen zu können, kann eine VHH genetisch an eine humane Hinge (schwarz) und Fc-Region (gelb) fusioniert werden. So entsteht ein humanisierter, chimerer Schwereketten-Antikörper. **(B)** Nanobody-basierte CARs (*Nb*-CARs) verwenden einen Tumorantigen spezifischen Nanobody als bindendes Element. **(C)** Ein Nanobody-basierter BiKE (*nano-BiKE*, ~30 kDa) entsteht durch genetische Fusion eines Tumorantigen spezifischen Nanobodies an einen CD16-spezifischen Nanobody. Die Halbwertszeit eines nano-BiKEs *in vivo* kann durch Fusion an einen Albumin-spezifischen Nb verlängert werden (*HLE-nano-BiKE*).

Das im Februar 2019 als erstes von der FDA und kurz darauf auch von der EMA zugelassene Nanobody-basierte Therapeutikum war Caplacizumab. Es bindet an ein

wichtiges Protein der Blutkoagulationskaskade, den van Willebrand Faktor (*vWF*). Bei Caplacizumab handelt es sich um einen Dimer aus zwei vWF-spezifischen Nanobodies, die mittels eines Alanin-Linkers verbunden sind (42). Es wird für die Therapie der erworbenen thrombotisch-thrombozytopenischen Purpura eingesetzt (43). Mit Ciltacabtagene Autoleucel (Cilta-cel, Carvykti®) wurde 2022 ein zweites Nanobody-basiertes Therapeutikum von der FDA und der EMA zugelassen. Dabei handelt es sich um einen biparatopischen CAR, der zwei BCMA-spezifische Nanobodies enthält. Cilta-cel zeigt herausragende Erfolge in der klinischen Anwendung (43, 44).

1.6 Chimäre Nanobody-basierte Konstrukte

Nanobodies bieten als Bindungselement für rekombinante Antikörper-Konstrukte eine erfolgversprechende Alternative zu scFvs. Tumorantigen-spezifische Schwereketten-Antikörper können in mit dem Tumorantigen immunisierten Lamas oder transgenen Mäusen gefunden bzw. produziert werden. Nanobodies und auch Schwereketten-Antikörper, die eine kamelide Fc-Domäne enthalten, können im Menschen *in vivo* aber keinen ADCC und CDC auslösen. Immunologische Effektorfunktionen von Antikörpern können nur mittels eines endogenen Fc-Fragments effektiv ausgelöst werden. Um diese Problematik zu umgehen, kann ein humanisierter Schwereketten-Antikörper (*hcAb*, **Abbildung 3A**) erstellt werden, indem ein Target-spezifischen Nanobody mit der Hinge, CH2 und CH3 eines humanen IgG1 Antikörpers genetisch fusioniert wurde. Diese chimären hcAbs können, wie andere therapeutisch eingesetzte, humane monoklonale Antikörper, Fc-vermittelte Effektorfunktionen *in vivo* auslösen (**Abbildung 4**). Durch die speziellen Bindungseigenschaften der Nanobodies können einige hcAbs zusätzlich die Enzymfunktion des gebundenen Targets beeinflussen (25, 45, 46).

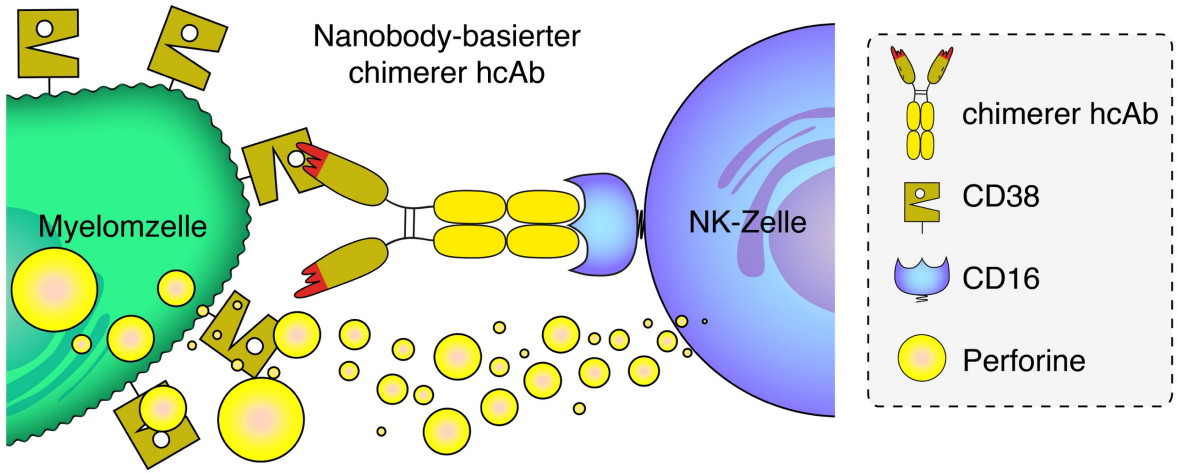

Abbildung 4: Durch einen humanisierten chimeren Schwereketten-Antikörper (*hcAbs*) ausgelöster ADCC. Der im humanisierten chimeren Schwereketten-Antikörper vorhandene Nanobody bindet an CD38 auf einer Myelomzelle. Der Fc-Teil des gebunden Antikörpers wird durch den Fc-Rezeptor CD16 auf der NK-Zelle gebunden, was zu einer immunologischen Synapse zwischen Myelomzelle und NK-Zelle führt. Durch die Kreuzvernetzung von CD16 an der immunologischen Synapse werden Perforine durch die NK-Zelle ausgeschüttet, die zur Lyse der Myelomzelle führen.

1.6.1 Chimäre Antigenrezeptoren (CARs)

NK-Zellen, die mit einem chimären Antigenrezeptor (*CAR*) ausgestattet sind, können unabhängig von Fc-Rezeptoren an Zelloberflächenantigene auf Tumorzellen binden und so CAR-vermittelte zelluläre Zytotoxizität (*CAR-DCC*) auslösen (47). Diese künstlichen Rezeptoren können sowohl auf T-Zellen als auch auf NK-Zellen exprimiert werden. CAR-basierte Therapien stellen eine vielversprechende Methode auf dem Gebiet der Immuntherapien dar (48). Derzeit werden weltweit über 2.000 klinische Studien zur Verwendung CAR-basierter Therapien durchgeführt (www.clinicaltrials.gov Stand: Januar 2023).

Der Aufbau eines CAR gleicht einem künstlichen Rezeptor: Eine Target-spezifische bindende Domäne, meist das scFv eines Antikörpers, wird mittels eines Peptid-Linkers und einer Transmembran-Domäne mit intrazellulären, costimulatorischen Domänen verbunden (**Abbildung 2B**). Bei Bindung des Rezeptors an das Target wird der CAR, ähnlich wie bei der ADCC, auf der T- oder NK- Zelle kreuzvernetzt (49). Diese Kreuzvernetzung führt zur Ausschüttung von Perforinen durch die CAR-exprimierende Immunzelle und zur Lyse der gebundenen Zielzelle. CAR-Zellen erster Generation enthielten nur das intrazelluläre Signalmodul von CD3ζ, während in neueren Generationen meist zwei oder mehr costimulatorische Domänen integriert werden, um die Effektivität der CAR-Zellen *in vivo* zu erhöhen (50).

Derzeit beinhalten CAR-basierte Therapien meist eine autogene Transplantation von genetisch veränderten, Patienten-eigenen T-Zellen (*CAR T-Zellen*). Ein erfolgreiches Beispiel eines CAR T Zell Therapeutikums ist Tisagenlecleucel, das für die Behandlung der akuten lymphatischen B-Zell Leukämie und dem diffusen großzelligen B-Zell Lymphom zugelassen ist (51). Bei der Therapie mit Tisagenlecleucel werden T-Zellen des Patienten mit lentiviralen Vektoren transduziert, um den CD19-spezifischen CAR zu exprimieren. Diese Zellen werden *ex vivo* expandiert und dem Patienten reapliziert. Ein Nachteil von CAR T-Zell basierten Therapien ist deren starke Abhängigkeit von der Qualität des Transplantats. Die Fitness und Quantität autologer Zellen ist oft stark reduziert, da sie von dem bereits erkrankten und vorbehandelten Patienten stammen (52, 53). Zudem müssen für jeden Patienten individuell neue CAR T-Zellen unter höchsten Sicherheitsstandards hergestellt werden, oft nur in von den pharmazeutischen Firmen zugelassenen Laboren. Dies macht die Therapie mit autologen CAR T-Zellen aufwendig, kostspielig und schlecht verfügbar (54, 55). CAR T-Zell-basierte Therapien haben zudem oft starke Nebenwirkungen, wie eine massiv erhöhte Zytokinausschüttung (*cytokine release syndrome*) sowie Transplantat-gegen-Patient Reaktionen (56).

NK-Zell basierte CAR-Zellen stellen hier eine Alternative dar, da sie bei der Transplantation oft geringere Nebenwirkungen auslösen als T-Zellen (47). NK-Zellen aus etablierten Zellkulturen oder allogenen Transplantaten lösen zudem geringere Transplantat-gegen-Patient Effekte aus (57).

Manche Antigene werden sowohl von der Zielzelle als auch der CAR-exprimierenden Zellen exprimiert. Beispiele hierfür sind CD38 und CD7 (58, 59). Dies führt zu schlechtem Wachstum der transduzierten Zellen durch eine gegenseitige Lyse der CAR-Zellen, dem sogenannten Fratrizid. Um dieses Phänomen zu vermeiden, kann das entsprechende Antigen in der Effektorzelle vor Transduktion mit dem CAR-Konstrukt mittels CRISPR-Technologie (*Clustered Regularly Interspaced Short Palindromic Repeats)* inaktiviert werden (58, 59). Dies ist aufwendig und muss bei autologen Transplantationen individuell für jeden Patienten durchgeführt werden. Auch hier bieten Zellkulturen einen enormen Kosten- und Zeit-Vorteil.

CAR NK-Zellen bieten sich also als *„Off-the-shelf“* Therapie an, da nicht individuell für jeden Patienten neue CAR-Zellen hergestellt werden müssen und geringere Nebenwirkungen zu erwarten sind (53, 60).

Obwohl scFvs als Bindungselement in CARs weit verbreitet sind, haben sie neben den oben genannten Nachteilen des scFvs-Formats auch im CAR Nachteile. Während der *ex vivo* Expansion der mit dem CAR transduzierten Zellen kann es zu Aggregation des CAR durch die schwachen, hydrophoben Bindungseigenschaften des als Bindungselement verwendeten scFvs kommen (61-63). Dies führt zu frühzeitiger Erschöpfung der CAR T-Zellen und kann zu einer verminderten Erfolgsrate der Therapie führen. Die Struktur des scFv verhindert zudem die Konstruktion von komplexeren CARs wie bispezifischen oder biparatopischen Dual-CARs, bei denen zwei scFvs im CAR hintereinandergeschaltet werden. Die potentielle falsche Verpaarung von VH und VL der beiden scFvs kann im Affinitätsverlust des CARs resultieren (64). Zudem kann die virale Verpackungskapazität des für die Transduktion verwendeten Virus durch die Integration von zwei scFvs (also vier variablen Domänen) überschritten werden (65). Nanobodies stellen daher auch bei der Konstruktion von CARs eine gute Alternative zu scFvs dar (**Abbildung 5**).

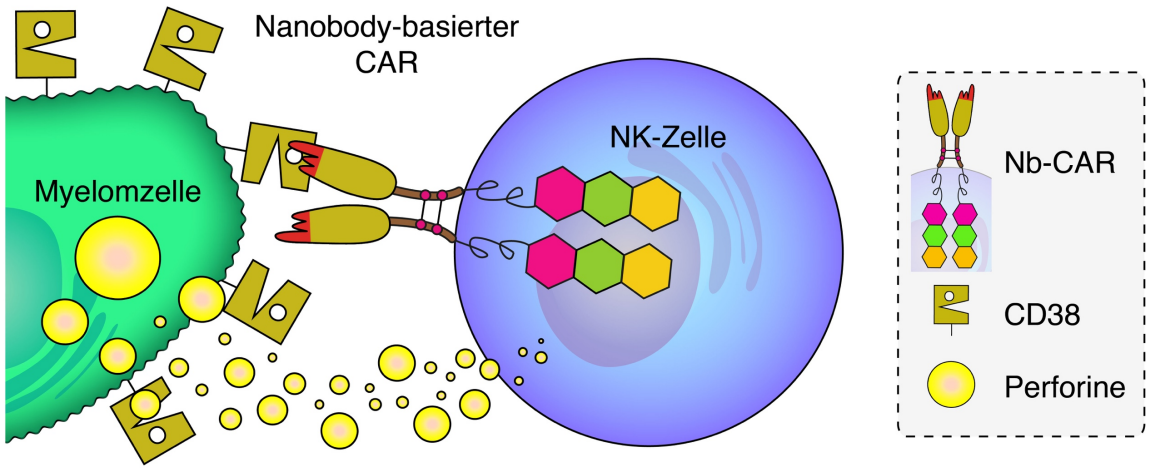

Abbildung 5: Nanobody-basierte CAR-abhängige zellvermittelte Zytotoxizität (*CAR-DCC*).
Durch Bindung des auf der NK-Zelle exprimierten, Nanobody-basierten CARs an CD38 wird der CAR auf der NK-Zelle kreuzvernetzt und die NK-Zelle aktiviert. Dies führt zur Ausschüttung von Perforinen durch die NK-Zelle und zur Lyse der gebundenen Myelomzelle.

Als erste Nanobody-basierte CAR T-Zell Therapie wurde 2022 Cilta-cel für die Therapie von rückfälligen Myelompatienten von der FDA zugelassen (66). Als Bindungselement wurden zwei BCMA-spezifische Nanobodies, die verschiedene Epitope auf BCMA erkennen, mittels eines Peptid-Linkers verbunden. So entsteht ein biparatopischer CAR (67). Cilta-cel zeigt beeindruckende Erfolge in der Therapie (44). Doch auch hier ist die Herstellung von individuellen, allogenen CAR T-Zell Transplantaten unter Einhaltung

der GMP-Reglungen notwendig, was zu einer aufwendigen und kostspieligen klinischen Testung und Anwendung führt. Durch die Herstellung in lizensierten GMP-Laboren sind die Herstellungskapazitäten begrenzt und die Therapie bisher für Patienten in Europa kaum verfügbar (54, 55). Diese Lücke kann nur geschlossen werden, indem Therapien entwickelt werden, die für alle Patienten verfügbar und finanzierbar sind. Daher ist sowohl die Entwicklung Protein-basierter Technologien, wie rekombinanter Antikörper und bispezifischer Engager, als auch allogen einsetzbarer CAR-Therapien relevant für die Therapie hämatologischer Erkrankungen.

1.6.1 Bispezifische Engager

Bispezifische Engager wie zum Beispiel bispezifische T-Zell Engager (*BiTEs*) stellen eine Erweiterung der Antikörper-basierten Therapieoptionen dar. Im klassischen BiTE wird ein für das Tumorantigen spezifisches scFv mit einem CD3-spezifischen scFv mittels eines Peptid-Linkers verbunden (**Abbildung 2C**) (68). CD3 ist eine Komponente des T-Zellrezeptor Komplexes. Die simultane Bindung des bispezifischen Engagers an das Zielantigen und CD3 führt zur Kreuzvernetzung von CD3 auf der T-Zelle an der "Synapse" zwischen Immunzelle und Zielzelle. Dies führt zur Ausschüttung von Perforinen und zur Aktivierung und Proliferation der T-Zelle (69, 70). Alternativ zu scFvs können als Bindungselemente auch chimäre Fab-Fragmente oder Nanobodies verwendet werden (71, 72).

Bispezifische Killerzell Engager (*BiKEs*) sind eine Weiterentwicklung des BiTE (73, 74). Hier wird ein Tumorantigen-spezifisches Bindungselement mit einem für den Fc-Rezeptor CD16-spezifischen Bindungselement verbunden. Die CD16-exprimierende NK-Zelle wird, wie bei der ADCC, zur Ausschüttung von Perforinen durch die Kreuzvernetzung von CD16 an der "Synapse" zur Tumorzelle angeregt. Der CD30-spezifische BiKE AFM13 zeigte in einer klinischen Phase 1b Studie an refraktären und rückfälligen Non-Hodgekin Lymphoma Patienten in Kombination mit dem Checkpoint Inhibitor Pembrolizumab eine response rate von 88% (75).

Die Bindungsaffinität von CD16 an den Fc-Teil des Antikörpers könnte im ADCC einen Einfluss auf die Effektivität desselbigen haben (76). Die Affinität von BiKEs zu CD16 ist über das CD16-spezifische Bindungselement steuerbar. So zeigen chimäre Antikörper, an deren Fc-Domäne ein CD16-spezifisches scFv fusioniert wurde, eine höhere Effizienz in ADCC-Assays als konventionelle Antikörper (77). Zudem ist die Effektivität von konventionellen Antikörpern *in vivo* limitiert durch die Prävalenz von physiologischem IgG im Plasma. Bereits im Serum enthaltene Antikörper konkurrieren

um die Bindung an Fc-Rezeptoren. Es werden hohe Level an therapeutischen Antikörpern benötigt, um diese Konkurrenz zu überwinden (78). Diese Problematik könnte durch die direkte Bindung des BiKE an CD16 umgangen werden (79).
Ein weiterer Vorteil gegenüber einem klassischen Antikörper (~150 kDa) besteht außerdem in der geringeren Größe des BiKE (~30-70kDa). Diese ermöglicht eine bessere Gewebepenetration und Biodistribution (41).
Die renale Ausscheidung dieser Proteine ist aufgrund der geringen Größe von scFvs schnell und damit die Halbwertszeit im Körper niedrig. Eine verlängerte Halbwertszeit kann durch Verwendung von Fc-basierten Fragmenten oder der Integration von Albumin-spezifischen Bindungselementen erreicht werden (79). Diese Konstrukte werden auch half-life extended genannt (*HLE-BiKE*). Eine klinische Studie mit dem EpCAM-spezifischen BiTE Catumaxomab, dessen Halbwertszeit mittels eines Fc-Fragments verstärkt wurde, zeigte jedoch Nebeneffekte, die möglicherweise aus dem integrierten Fc-Fragment resultieren (80, 81). Dennoch wurde Catumaxomab als erster bispezifischer Engager 2009 von der EMA zur intraperitonealen Behandlung der malignen Aszites zugelassen (82).
Trispezifische Killerzell Engager (TriKEs) sind eine Erweiterung des BiKEs, bei denen weitere tumorspezifische Bindungsfragmente oder costimulatorische Interleukine integriert werden (33, 73, 83, 84). TriKEs zeigen im Vergleich zu BiKEs oftmals eine gesteigerte Effizienz in der Lyse von Target-exprimierenden Zellen (33, 85).

Nanobody-basierte BiTEs (*nano-BiTE*) und BiKEs (*nano-BiKEs*) stellen eine effektive Alternative zu scFv-basierten BiKEs dar (**Abbildung 3C**). Van Fassen *et al*. verglichen verschiedene EGFR-, CD19- und HER2-spezifische Nanobody-basierte BiKEs in unterschiedlichen Konfigurationen. Eine hohe Effektivität und Spezifität konnte dabei für alle Targets festgestellt werden (86). Nanobodies bieten sich aufgrund ihrer guten Löslichkeit und Reformatierbarkeit als Grundlage für BiKEs an. Half-life extended Nanobody-basierte BiKEs (*HLE-nano-BiKEs*) können mittels Integration eines Albumin-spezifischen Nanobodies in den BiKE erstellt werden (**Abbildung 6**). So wird auch die oben beschriebene Problematik eines funktionellen Fc-Fragments zur Verlängerung der Halbwertszeit umgangen (80, 81). HLE-nano-BiKEs könnten durch ihre geringe Größe und Immunogenität und gute Gewebepenetration und Löslichkeit eine vielversprechende Alternative zu scFv-basierten BiKEs darstellen.

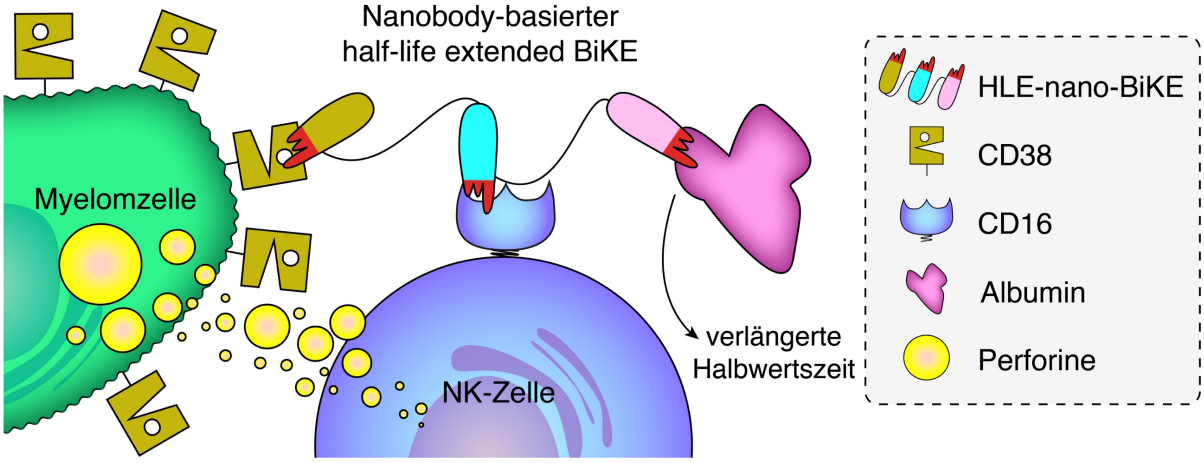

Abbildung 6: Nanobody-basierte BiKE-abhängige zellvermittelte Zytotoxizität (*BiKE-DCC*). Nb-basierte BiKEs führen nach Bindung des CD38-spezifischen Nanobodies an CD38 auf einer Myelomzelle und des CD16-spezifischen Nanobodies an den Fc-Rezeptor (CD16) auf einer natürlichen Killerzelle (NK-Zelle) zur Ausprägung einer immunologischen Synapse. Durch die Kreuzvernetzung von CD16 auf der NK-Zelle wird diese aktiviert, was zur Freisetzung von Perforinen und zur Lyse der gebundenen Myelomzelle führt. Durch Bindung des Albumin-spezifischen Nanobodies an Albumin wird die Halbwertszeit des Nb-BiKEs *in vivo* verlängert.

CD38-spezifische Nanobodies könnten also eine Grundlage bieten um das Potential Nanobody-basierter hcAbs, Nb-CARs und HLE-nano-BiKEs zu evaluieren und so das Multiple Myelom und andere CD38-assoziierte Erkrankungen zu heilen.

2 Zielsetzung der Arbeit

Vorarbeiten

Unsere Arbeitsgruppe konnte durch Immunisierung von Lamas mit rekombinantem, humanem CD38 und CD38-kodierenden cDNA Expressionsvektoren eine Bibliothek von 22 verschiedenen Familien CD38-spezifischer Nanobodies isolieren (45). Die Mehrheit dieser Nanobodies bindet an eines von drei voneinander unabhängigen Epitopen (E1, E2, E3) auf CD38. Die an E1 bindenden Nanobodies erkennen ein ähnliches Epitop wie Daratumumab, die an E3 bindenden Nanobodies ein ähnliches Epitop wie Isatuximab (**Abbildung 7**).

Eingereichte Publikationen

Für meine Arbeit wurden drei CD38-spezifische Nanobodies (WF211, MU1067, JK36) ausgewählt, die jeweils an eines der drei Epitope auf CD38 binden. Diese Nanobodies wurden in den publizierten Ergebnissen dieser Dissertation als Grundlage von CD38-spezifischen humanisierten Schwereketten-Antikörpern (*hcAbs*) (Schriewer *et al*. 2018 (28)), Nanobody-basierten chimären Antigenrezeptoren (*Nb-CARs*) (Hambach *et al*. 2020 (87)) und Halbwertszeit-verlängerten Nanobody-basierten bispezifischen Killerzell Engagern (*HLE-nano-BiKEs*) (Hambach *et al*. 2022 (88)) verwendet.

Ziel dieser Arbeit war es das Potential von CD38-spezifischen Nanobodies für die Generierung von rekombinanten Antikörper-Konstrukten zu evaluieren und so neue Therapieansätze für die Behandlung des Multiplen Myeloms zu evaluieren.

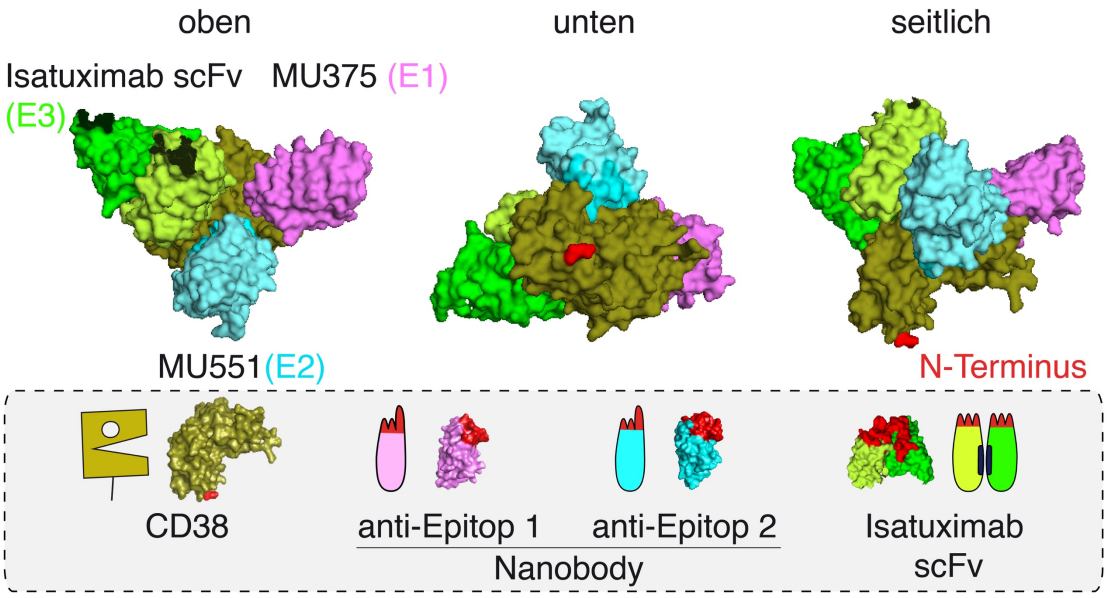

Abbildung 7: Die CD38-spezifischen Nanobodies WF211, MU1067 und JK36 binden an drei verschiedene Epitope auf CD38. 3D Modell der extrazellulären Domäne von CD38 (beige) im Komplex mit zwei Nanobodies (MU375 in magenta und MU551 in türkis) und dem scFv von Isatuximab (VH in dunkelgrün und VL in hellgrün). Die N-terminale Aminosäure (rot) von CD38 verbindet über die Transmembrandomäne die extrazelluläre Enzymdomäne mit der kurzen, intrazellulären Domäne des Proteins. Das linke Modell zeigt den Blick auf CD38 von der Membran-distalen Seite, das mittlere den Blick von der Membran-proximalen Seite und das rechte eine seitliche Ansicht des Enzyms. MU375 und Daratumumab binden ein überlappendes Epitop (E1), MU551 und Isatuximab binden von E1 unabhängige Epitope (E2 bzw. E3) (Abgeleitet aus Hambach *et al.* 2020 und Li *et al.* 2016 (87, 89)). Wie in Fumey *et al*. beschrieben, bindet WF211 dasselbe Epitop auf CD38 wie MU375 und Daratumumab (E1) und MU1067 bindet dasselbe Epitop wie MU551 (E2). JK36 bindet unabhängig von WF211 und MU1067 ein drittes Epitop, welches vermutlich mit dem von Isatuximab gebundenen Epitop übereinstimmt (E3) (Abgeleitet aus Fumey *et al*. 2017 (45)).

3 Publikationen

3.1 Nanobody-Based CD38-Specific Heavy Chain Antibodies Induce Killing of Multiple Myeloma and Other Hematological Malignancies

Zunächst soll die Publikation *„Nanobody-Based CD38-Specific Heavy Chain Antibodies Induce Killing of Multiple Myeloma and Other Hematological Malignancies“* vorgestellt werden, die 2020 im Journal *Theranostics* veröffentlicht wurde. Bei dieser Publikation habe ich Experimente für die Revision durchgeführt, die in Figure 4D-F und Table 2 dargestellt sind. Zudem habe ich an allen Abbildungen und der Korrektur des Manuskriptes mitgewirkt.

Titel: Nanobody-Based CD38-Specific Heavy Chain Antibodies Induce Killing of Multiple Myeloma and Other Hematological Malignancies

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Research Paper

Nanobody-based CD38-specific heavy chain antibodies induce killing of multiple myeloma and other hematological malignancies

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Nanobody® is a trademark of Ablynx. In this paper we use nanobody as the generic term for the recombinant VHH domain of a llama heavy chain antibody.

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Abstract

Rationale: CD38 is a target for the therapy of multiple myeloma (MM) with monoclonal antibodies such as daratumumab and isatuximab. Since MM patients exhibit a high rate of relapse, the development of new biologics targeting alternative CD38 epitopes is desirable. The discovery of single-domain antibodies (nanobodies) has opened the way for a new generation of antitumor therapeutics. We report the generation of nanobody-based humanized IgG1 heavy chain antibodies (hcAbs) with a high specificity and affinity that recognize three different and non-overlapping epitopes of CD38 and compare their cytotoxicity against CD38-expressing hematological cancer cells *in vitro*, *ex vivo and in vivo*.

Methods: We generated three humanized hcAbs (WF211-hcAb, MU1067-hcAb, JK36-hcAb) that recognize three different non-overlapping epitopes (E1, E2, E3) of CD38 by fusion of llama-derived nanobodies to the hinge- and Fc-domains of human IgG1. WF211-hcAb shares the binding epitope E1 with daratumumab. We compared the capacity of these CD38-specific hcAbs and daratumumab to induce CDC and ADCC in CD38-expressing tumor cell lines *in vitro* and in patient MM cells *ex vivo* as well as effects on xenograft tumor growth and survival *in vivo*.

Results: CD38-specific heavy chain antibodies (WF211-hcAb, MU1067-hcAb, JK36-hcAb) potently induced antibody-dependent cellular cytotoxicity (ADCC) in CD38-expressing tumor cell lines and in primary patient MM cells, but only little if any complement-dependent cytotoxicity (CDC). *In vivo*, CD38-specific heavy chain antibodies significantly reduced the growth of systemic lymphomas and prolonged survival of tumor bearing SCID mice.

Conclusions: CD38-specific nanobody-based humanized IgG1 heavy chain antibodies mediate cytotoxicity against CD38-expressing hematological cancer cells *in vitro*, *ex vivo and in vivo*. These promising results of our study indicate that CD38-specific hcAbs warrant further clinical development as therapeutics for multiple myeloma and other hematological malignancies.

Introduction

Multiple myeloma (MM) is a malignant plasma cell disorder with an incidence of 4-5 per 100,000 persons per year, causing 1% of all cancer-induced deaths [1, 2]. MM is characterized by bone, renal, hematological, and infectious complications due to accumulation of clonal plasma cells in the bone marrow and pathogenic antibody production [3]. Survival of MM patients has improved substantially with new drug classes such as proteasome inhibitors and immunomodulatory drugs when combined with autologous stem cell transplantation [4]. Despite this progress, the large majority of MM patients relapses and eventually dies from refractory disease. Moreover, current treatments are associated with severe side effects [5, 6]. This highlights the need for new, effective treatment options with higher specificity and fewer side effects [7-10]. Monoclonal antibodies targeting specific cell surface proteins represent an important new class of agents that may meet these needs [11-13].

The glycoprotein CD38 represents a particularly attractive target in MM as it is highly expressed on malignant plasma cells in all stages of the disease [13, 14]. Moreover, CD38 is overexpressed in the majority of acute lymphoblastic leukemia cases, in some acute myeloid leukemia cases, in non-Hodgkin's lymphoma, and in a subset of patients with chronic lymphocytic leukemia [15]. At the same time, CD38 is expressed only at low levels on mature lymphocytes and non-hematopoietic tissues. This expression pattern results in a favorable side-effect profile of CD38-targeting antibodies. Indeed, clinical studies have shown a marked activity of such antibodies in MM, studies in other hematological malignancies are ongoing [15]. CD38-specific antibodies may play a role in the treatment of diseases beyond hematological malignancies, including solid tumors and antibody-mediated autoimmune diseases [15, 16].

Most advanced in development is daratumumab, a monoclonal anti-CD38 IgG1 antibody generated by hybridoma technology after immunization of transgenic mice [17]. Daratumumab has single-agent activity and a limited toxicity profile, allowing favorable combination therapies with existing as well as emerging therapies [18]. Indeed, daratumumab has shown promising anti-myeloma activity in two late-stage clinical studies (GEN501 and SIRIUS) [7, 19]. Accordingly, daratumumab was approved by the US Food and Drug Administration for patients with MM who have received ≥3 prior lines of therapy [20].

Daratumumab kills MM cells via different mechanisms including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [17]. It has been proposed that these therapeutic effects are related to the specific epitope that is recognized by daratumumab on CD38 [17]. Binding at this unique epitope might position the Fc portion in an orientation that facilitates formation of IgG hexamers and activation of the complement cascade [21]. However, the unique epitope required for daratumumab binding raises the question of whether this might be a point of vulnerability for drug resistance [18]. Moreover, anti-idiotype antibodies could also neutralize the biological activity of daratumumab. To address this emerging limitation, alternative CD38-specific antibody constructs are required.

The discovery of single-domain antibodies (nanobodies, VHHs, 18 kDa) has opened the way for a new generation of antitumor therapeutics [22-30]. We generated 22 distinct families of llama-derived CD38-specific nanobodies and characterized their binding epitopes [31, 32]. We also generated humanized heavy chain antibodies (hcAbs, 80 kDa) by genetic fusion of CD38-specific nanobodies to the hinge- and Fc-domains of human IgG1 and characterized their CDC capacity *in vitro* [33]. Here, we compared the capacity of three CD38-specific hcAbs (WF211-hcAb, MU1067-hcAb, JK36-hcAb) that recognize three different epitopes of CD38 (E1, E2, E3) and of daratumumab (epitope E1) to induce CDC and ADCC in CD38-expressing tumor cell lines *in vitro* and in patient MM cells *ex vivo* as well as effects on xenograft tumor growth and survival *in vivo*. Our results underscore the potential of hcAbs for therapy of CD38-expressing hematological malignancies.

Materials and Methods

CD38-specific conventional and heavy chain antibodies

Daratumumab (Darzalex) was purchased from Janssen-Cilag, Neuss, Germany, to be used as positive control in our killing assays.

Llama-derived CD38-specific nanobodies WF211, MU1067, and JK36 and the (negative) control nanobody 1-15 were generated as described previously [31]. Nanobodies (18 kDa) are monovalent single domain antibody fragments derived from the heavy chain IgG antibodies naturally occurring in camelids [23, 34]. Nanobodies correspond to the variable domain (VHH) of these heavy chain antibodies. Their robust, soluble single domain format renders nanobodies amenable for genetic fusion to the hinge- and Fc-domains of other antibody isotypes [25, 35]. The resulting bivalent chimeric llama/human heavy chain antibodies (hcAbs) acquire the capacity to induce Fc-mediated effector functions (e.g CDC,

ADCC) at about half the size of a conventional monoclonal antibody (80 vs. 150 kDa) [35].

The heavy chain antibodies WF211-hcAb, MU1067-hcAb, and JK36-hcAb **(Figure 1A)** were generated by subcloning the coding region of the nanobodies upstream of the coding region for the hinge- and Fc-domains of human IgG1 in the pCSE2.5 vector (kindly provided by Thomas Schirrmann, Braunschweig, Germany) [33]. The Fc-domains of our hcAbs thus are the same (human IgG1) as that of daratumumab [17]. L-15 is a nanobody directed against the enzymatic subunit CDTa of *Clostridium difficile* [36], the corresponding heavy chain antibody (l-15-hcAb) served as the isotype control in all experiments.

Recombinant hcAbs were expressed in transiently transfected HEK-6E cells cultivated in serum-free medium. Six days post transfection, supernatants were harvested and cleared by centrifugation. HcAbs were purified by affinity chromatography using protein G-sepharose. Purity of antibody constructs was assessed by SDS-PAGE and Coomassie Brilliant Blue staining.

Cell lines

Daudi and CA46 lymphoma cell lines as well as the LP-1 myeloma cell line were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany).

Cell lines (CA46-luc, Daudi-luc, LP-1-luc) stably expressing the luc2 variant of *Photinus pyralis* luciferase (Promega, Madison, WI) under control of the spleen-focus-forming virus U3 region (SFFV promoter) were generated by lentiviral transduction. The vector was cloned by inserting the luc2 cDNA (Addgene plasmid #24337) in front of the internal ribosome entry site of the HIV-1 derived, 3rd generation, self-inactivating lentiviral vector LeGO-iG2-Puro+ co-expressing the fluorescent marker eGFP linked to a puromycin resistance by a 2A-sequence [37]. Production of lentiviral particles was performed as described [38]. Transduction of target cells was carried out in a 24-well plate with 50.000 cells in 500 µL medium per well by addition of 300 µL viral-particle containing supernatant in presence of 8 µg/mL polybrene and subsequent spin-inoculation for 1 hour at 1000×g and 25°C. Transduced cells were selected in culture medium containing 1 µg/mL puromycin. Stably transduced cells were FACS sorted (FACS Aria III, BD Biosciences, Heidelberg, Germany) based on eGFP expression.

Mouse Yac-1 lymphoma cells were transfected with an expression vector for human CD38 by electroporation (250 mV, 960 µF) using 3 µg DNA/10^7 cells in 400 µL RPMI and a Gene pulser (Bio-Rad GmbH, Munich, Germany). Stable transfectants (Yac-1-CD38) were obtained by selection in medium supplemented with blasticidin (10 µg/mL). Cells were subcloned by limiting dilution, and clones were analyzed for CD38 expression levels by flow cytometry. Cell lines were cultured in RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 2 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco) and 10% (v/v) fetal calf serum (Gibco).

NK-92, a human NK cell line, was obtained from DSMZ. NK-92 cells stably co-expressing GFP and human CD16 were obtained by retroviral

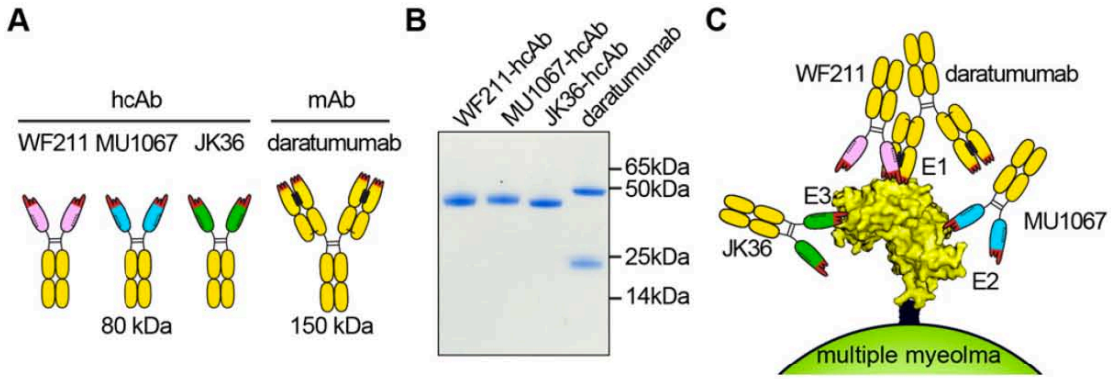

Figure 1. Structure, purity, and binding epitopes of CD38-specific antibody constructs. (A) Scheme of heavy chain antibodies (80 kDa). The single-domain antibody fragments (nanobodies) are indicated in pink (WF211), blue (MU1067), and green (JK36). The hinge and Fc-domains of human IgG1 are indicated in black and yellow, respectively. The three CDR loops of the antigen-binding paratope are indicated in red. Conventional antibody daratumumab (150 kDa) is indicated in yellow (heavy chains and light chains). Hydrophobic patches at the interface between the VH and VL domains are indicated in black. The corresponding hydrophilic patch in the nanobodies is indicated by dashed lines. **(B)** Coomassie-staining of an SDS-PAGE under reducing conditions with CD38-specific heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb and daratumumab verifies the purity and integrity of the constructs used in this study. Note that daratumumab consists of two heavy chains (50 kDa) and two light chains (25 kDa). The hcAbs consist of two identical heavy chains (42 kDa). Each lane contains 2 µg of the indicated construct. **(C)** The three hcAbs recognize three distinct epitopes of CD38. WF211-hcAb recognizes epitope 1 (E1), MU1067-hcAb recognizes epitope 2 (E2), and JK36-hcAb recognizes epitope 3 (E3). The binding epitope E1 of WF211-hcAb overlaps with the epitope of daratumumab.

transduction using the pSF91 retroviral vector [39]. The sequence for CD16, i.e. the ectodomain of FcγRIII fused to the transmembrane and cytosolic domains of FcεRI was kindly provided by Béatrice Clémenceau, Nantes, France [40]. The CD38 gene was inactivated in these cells using CRISPR/Cas9 technology (sc-401117-NIC, Santa Cruz Biotechnology). NK-92-CD16-GFP cells and NK-92-CD16-GFP-CD38ko cells were FACS-sorted and grown in alpha MEM culture medium (Gibco) supplemented with 12.5% FCS (Gibco), 12.5% horse serum (Gibco), 100 IU/mL IL-2 (Proleukin, Novartis, Nürnberg, Germany), and 2 mM L-glutamine (Gibco).

Binding affinities and epitope mapping of CD38-specific reagents

Binding specificities of hcAbs were assessed by staining of untransfected CD38-negative parental Yac-1 cells and their counterparts stably transfected with human CD38 with hcAbs WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (l-15-hcAb), or daratumumab. Binding was detected with a PE-conjugated donkey anti-human IgG-specific secondary antibody (Dianova, Hamburg, Germany). Control stainings were performed with the PE-conjugated secondary antibody alone. Cell-associated fluorescence was determined by flow cytometry.

Binding affinities of hcAbs were assessed by incubation of Yac1-CD38 cells with serial dilutions of antibodies. Cells were washed and incubated with PE-conjugated donkey anti-human IgG (Dianova, Hamburg, Germany). Cell-associated fluorescence was determined by flow cytometry.

The relative dissociation rates of Alexa^{647}-conjugated monovalent nanobodies and bivalent antibodies from cell surface CD38 were assessed by incubation of Yac1-CD38 cells with excess (100 nM) of fluorochrome-conjugated nanobodies, heavy chain antibodies or daratumumab for 30 min at 4°C. Cells were washed three times and then monitored for loss of cell-associated fluorescence over time at RT. An aliquot of CD38-expressing Yac-1 cells that had been labeled with the cell-tracking dye eFluor 450 was added at t = 0 as a sink for dissociated nanobodies or antibodies. Cell-associated fluorescence was determined by flow cytometry.

Epitope mapping was performed by incubation of CD38-expressing LP-1 lymphoma cells with a saturating concentration (100 nM) of unconjugated hcAbs WF211-hcAb, MU1067-hcAb, JK36-hcAb, or daratumumab for 30 min at 4°C before addition of Alexa^{647}-conjugated CD38-specific hcAbs or daratumumab (25 nM). Cells were further incubated for 30 min at 4°C, washed twice, and analyzed by flow cytometry. Percentage of cross-blockade was then calculated from mean fluorescence intensities (MFI) as follows:

$$\frac{MFI\ in\ absence\ of\ competing\ Abs\ -MFI\ in\ presence\ of\ competing\ Abs}{MFI\ in\ presence\ of\ competing\ Abs} \times 100.$$

CD38 expression analyses

Untransduced and GFP/luciferase-transduced CA46, Daudi, and LP-1 cells were incubated in PBS at 1x10^6 cells per assay with 1 µg/mL Alexa^{647}-conjugated MU1067-hcAb for 1 hour at 4°C. Samples were washed and analyzed by flow cytometry for expression of CD38 and GFP.

CDC of tumor cell lines

CA46-luc, Daudi-luc, or LP-1-luc cells were incubated with WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (l-15-hcAb), or daratumumab as positive control. The incubation was performed in the presence of 15% pooled human serum as source of complement for 60 min at 37°C. Heat inactivated (30 min at 56°C) serum was used as control to verify complement dependency. CDC was quantified by flow cytometric measurement of propidium iodide (PI) uptake. Percentage of lysed cells was defined as percentage of PI-positive cells.

ADCC of tumor cell lines

CA46-luc, Daudi-luc and LP-1-luc cells were incubated with serial dilutions of hcAbs or daratumumab. NK-92-CD16 cells were added as effector cells at an effector to target ratio [E:T] of 3:1.

Peripheral blood mononuclear cells (PBMCs) containing primary NK cells were obtained from buffy coats from healthy donors by Ficoll-Paque density gradient centrifugation and subsequent depletion of erythrocytes using lysis buffer (NH_4Cl + $KHCO_3$ + EDTA). To activate NK cells, PBMCs were incubated overnight in alpha MEM culture medium supplemented with 12.5% FCS, 12.5% horse serum, 100 IU/mL IL-2 (Proleukin, Novartis, Nürnberg, Germany), and 2 mM L-glutamine (Gibco). These cells were added as effector cells at an effector to target ratio [E:T] of 30:1.

Cells were then co-incubated for 4 h at 37°C. Synthetic D-luciferin (Biosynth, Staad, Switzerland) was added as substrate (150 µg/mL) for 20 min and

bioluminescence (BLI) was measured with a microplate reader (Victor³, Perkin Elmer, Boston, USA). Percentage of lysed cells was calculated as follows [41]:

$$1 - \frac{mean\ BLI\ of\ sample}{mean\ BLI\ of\ sample\ with\ control\ nanobody} \times 100$$

Mouse xenograft tumor model

Tumor xenograft experiments were conducted using female, 12-14 week old Fox Chase SCID mice (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl) obtained from Charles River Laboratories (Sulzfeld, Germany). Mice weighed 22 ± 2.5 g (range 20 to 25 g). Water and food were provided ad libitum. Mice were checked daily for signs of discomfort and for general appearance. Body weight was measured three times a week. Experiments were performed in accordance with international guidelines on the ethical use of animals and were approved by the local animal welfare commission (TVA 17/13).

4x10⁶ CA46-luc cells were intravenously injected in 200 µL saline solution. Mice were randomly grouped (n = 7 per group) and treated by weekly i.p. injections of 200 µL saline solution containing 50 µg (~2 mg/kg) of CD38-specific hcAbs WF211-hcAb, MU1067-hcAb, JK36-hcAb, the isotype control l-15-hcAb, or daratumumab. Weekly treatments started on day 7 after inoculation, i.e. at a time point where tumors were detected in all inoculated mice. Mice were treated 6 times until day 42 after tumor inoculation.

In vivo imaging was performed at weekly intervals starting one week after xenograft inoculation directly before the first antibody treatment. Mice were anesthetized with isofluorane and intraperitoneally injected with synthetic D-luciferin (6 mg in 200µL PBS). After 15 minutes, mice were positioned in the imaging chamber of the small-animal imaging system (IVIS-200, PerkinElmer, Boston, MA, USA). Luminescence was measured by counting photons emitted during an exposure period of 1 min. Under illumination, black-and-white images were made for anatomical reference. Rectangular regions of interest (ROIs) were placed around individual mice for quantitative analyses. Total flux [photons/sec] was determined with Living Image 4.2 software (PerkinElmer).

Animals were euthanized when turning moribund according to pre-defined criteria (weight loss >20%, loss of ability to ambulate, labored respiration, or inability to drink or feed) in order to avoid animal suffering.

CDC and ADCC of primary MM cells

Fresh primary MM cells were obtained from bone marrow aspirates after IRB-approved consent was obtained from all patients. Experiments were performed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration. The study was approved by the local IRB committee (PV5505). Bone marrow mononuclear cells (BM-MNCs) were prepared by Ficoll-Paque density gradient centrifugation of bone marrow aspirates and subsequent depletion of remaining erythrocytes using red blood cell lysis buffer (NH_4Cl + $KHCO_3$ + EDTA). Patient characteristics are provided in **Table 1.**

Table 1. Patient characteristics of Multiple Myeloma patients.

Parameter	MM-patients (n=8)	
Median age, years (range)	65	(50-75)
Sex, male, n (%)	5	(62.5%)
Serum analyses		
Median M-Protein in IgG subtype, g/L (range)	20	(17-24)
Median serum free light chain, mg/L (range)	62	(17-8393)
Subtype of immunoglobulins		
IgG κ, n (%)	5	(62.5%)
IgG λ, n (%)	1	(12.5%)
κ light chain only, n (%)	1	(12.5%)
λ light chain only, n (%)	1	(12.5%)
Bone marrow analyses		
Median bone marrow infiltration, % (range)	23%	(10-60%)
Previous therapy		
No prior treatment, n (%)	6	(75%)
Prior stem cell transplantation, n (%)	2	(25%)
Autologous, n (%)	2	(25%)
Allogeneic, n (%)	0	(0%)

For CDC assays, BM-MNCs were incubated in PBS+0.2% BSA and 100nM CD38-specific hcAbs, isotype control (l-15-hcAb), or daratumumab and 12.5% pooled human serum as a source of complement for 90 min.

For ADCC assays, BM-MNCs were incubated in alpha MEM and 100nM CD38-specific hcAbs, isotype control, or daratumumab and NK92 cells stably transduced with CD16 and GFP at an effector to target ratio [E:T] of 10:1 for 2.5 h.

For both assays, cells were then stained with a panel of antibodies (CD38, CD45, CD138/CD229/CD55/CD59, CD269/CD319/CD56, CD19) and analyzed via flow cytometry. Staining of CD38 was achieved with Alexa^{647}-conjugated Nbs that bind independently of the hcAb/mAb used for CDC/ADCC: JK36^{647} or MU523^{647} for daratumumab and WF211-hcAb, MU523^{647} for JK36-hcAb, and JK36^{647} or WF211^{647} for MU1067-hcAb. An FSC threshold was set to exclude debris while including the population of small CD19+ B cells. In case of ADCC, GFP-expressing NK-92 cells were excluded by gating. MM cells were identified by high co-expression of CD38 and CD138. Numbers of MM cells were determined using CountBright absolute

counting beads (Invitrogen). Percentage of surviving MM cells was calculated as follows: MM cell number of αCD38 hcAb or mAb treated sample / MM cell number of control hcAb treated sample x 100.

Statistical Analysis

For ADCC of tumor cell lines, a linear regression on the basis of a one-phase decay was performed and a one-way ANOVA was used to determine significant differences between the three hcAbs and daratumumab.

For xenograft tumors, a two-way ANOVA followed by a Bonferroni post hoc test was used to determine significant differences of light emissions between treatment groups. Survival curves were analyzed using Kaplan-Meier plots and strata compared using the log-rank test (GraphPad Prism).

For CDC and ADCC of primary MM cells, the number of MM cells per mL transformed to the natural logarithm was considered the dependent variable for statistical analysis using a mixed model approach (SPSS routine GENLINMIXED). A normal data distribution was assumed, and an identity link function was applied. Antibody-construct and cytotoxicity-assay were considered fixed effect variables in the model, patient-by-cytotoxicity-assay as random effect and antibody-construct within patient-by-cytotoxicity-assay as repeated measures. Model-estimated marginal means of constructs were compared pairwise within cytotoxicity-assays, with Sidak-adjusting of the alpha error for multiple comparisons. All tests were two-sided and a p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS v. 25.

Results

CD38-specific hcAbs show higher binding avidities than the respective monovalent nanobodies

We produced and purified three nanobody-based humanized heavy chain antibodies by genetically fusing nanobodies WF211, MU1067, JK36 to the hinge- and Fc-domains of human IgG1 **(Figure 1B)**. Competition binding studies with WF211-hcAb, MU1067-hcAb, and JK36-hcAb on Yac-1-CD38 cells confirmed specific binding to three distinct epitopes E1, E2, and E3 on the extracellular domain of CD38. Blocking experiments further revealed that the binding epitope of WF211-hcAb (E1) overlaps with the epitope of the conventional CD38-specific human IgG1 antibody daratumumab **(Figure 1C, Table 2)**.

Assessment of binding affinities using serial titration analyses of unconjugated antibodies revealed good and comparable binding of all three CD38-specific hcAbs, regardless of epitope specificity, to CD38-expressing Yac-1 cells **(Figure 2A)**. EC50 values of hcAbs were 0.90 nM for WF211-hcAb, 0.91 nM for MU1067-hcAb, and 0.89 nM for JK36-hcAb. Daratumumab showed slightly stronger binding with an EC50 of 0.45 nM. Isotype control heavy chain antibody l-15-hcAb did not show any detectable binding.

To assess the suitability of fluorochrome-conjugated nanobodies and heavy chain antibodies for flow cytometry, we analyzed their relative dissociation rates from CD38-expressing Yac-1 cells **(Figure 2B)**. Untreated, eFluor labeled cells were used as a "sink" for the dissociated antibodies. The results indicate that bivalent heavy chain antibody have increased avidity compared to the respective monovalent nanobody.

CD38-specific hcAbs do not effectively induce CDC of tumor cell lines *in vitro*

The ability of CD38-specific hcAbs to induce complement-dependent cytotoxicity (CDC) was tested with a human multiple myeloma cell line (LP-1) and two human Burkitt's lymphoma cell lines (Daudi, CA46). These cells express moderate to high levels of CD38 **(Figure 3A)** and low to moderate levels of the complement-inactivating surface proteins CD55 and CD59 **(Figure 3B)**. The three CD38-specific hcAbs induced little if any CDC in the three tested cell lines. In contrast, daratumumab induced varying degrees of complement-dependent lysis depending on the cell line **(Figure 3C)**. Daratumumab induced highest lysis (67 ± 1%) of Daudi cells, intermediate lysis (56 ± 3%) of LP-1 cells, and lowest lysis (15 ± 1%) of CA46 cells. Control experiments with inactivated serum resulted only in background levels of tumor cell lysis, confirming CDC as one mechanism of action of daratumumab. Similarly, experiments with the isotype control heavy chain antibody l-15-hcAb resulted only in background levels of tumor cell lysis, reflecting the inability of this antibody to bind to CD38.

CD38-specific hcAbs mediate effective ADCC of tumor cell lines *in vitro*

The ability of CD38-specific hcAbs to induce antibody-dependent cellular cytotoxicity (ADCC) was tested with luciferase-expressing tumor cell lines LP-1, Daudi, and CA46 as targets and CD16-transduced NK-92 cells or primary NK cells as effector cells. The three hcAbs effectively induced dose-dependent lysis of all three cell lines **(Figure 4)**.

Table 2. Epitope mapping of CD38-specific hcAbs and daratumumab.

	Epitope	1	1	2	3
Epitope	**Name**	daratumumab^{647}	WF211-hcAb^{647}	MU1067-hcAb^{647}	JK36-hcAb^{647}
1	daratumumab	**98***	86*	19	1
1	WF211-hcAb	97*	**87***	22	-6
2	MU1067-hcAb	95*	12	**98***	-3
3	JK36-hcAb	-2	-1	-2	**97***

Numbers indicate the percentage of cross-blockade by competing antibodies. Unconjugated blocking antibodies are indicated on the left, AF647-labeled detecting antibodies are indicated on the top. Self-blockade is indicated along the diagonal in bold fond and inhibition of binding by >85% is indicated by *. Negative numbers indicate enhanced labeling of cells in the presence of the competing nanobody. Data represent mean values from three independent experiments.

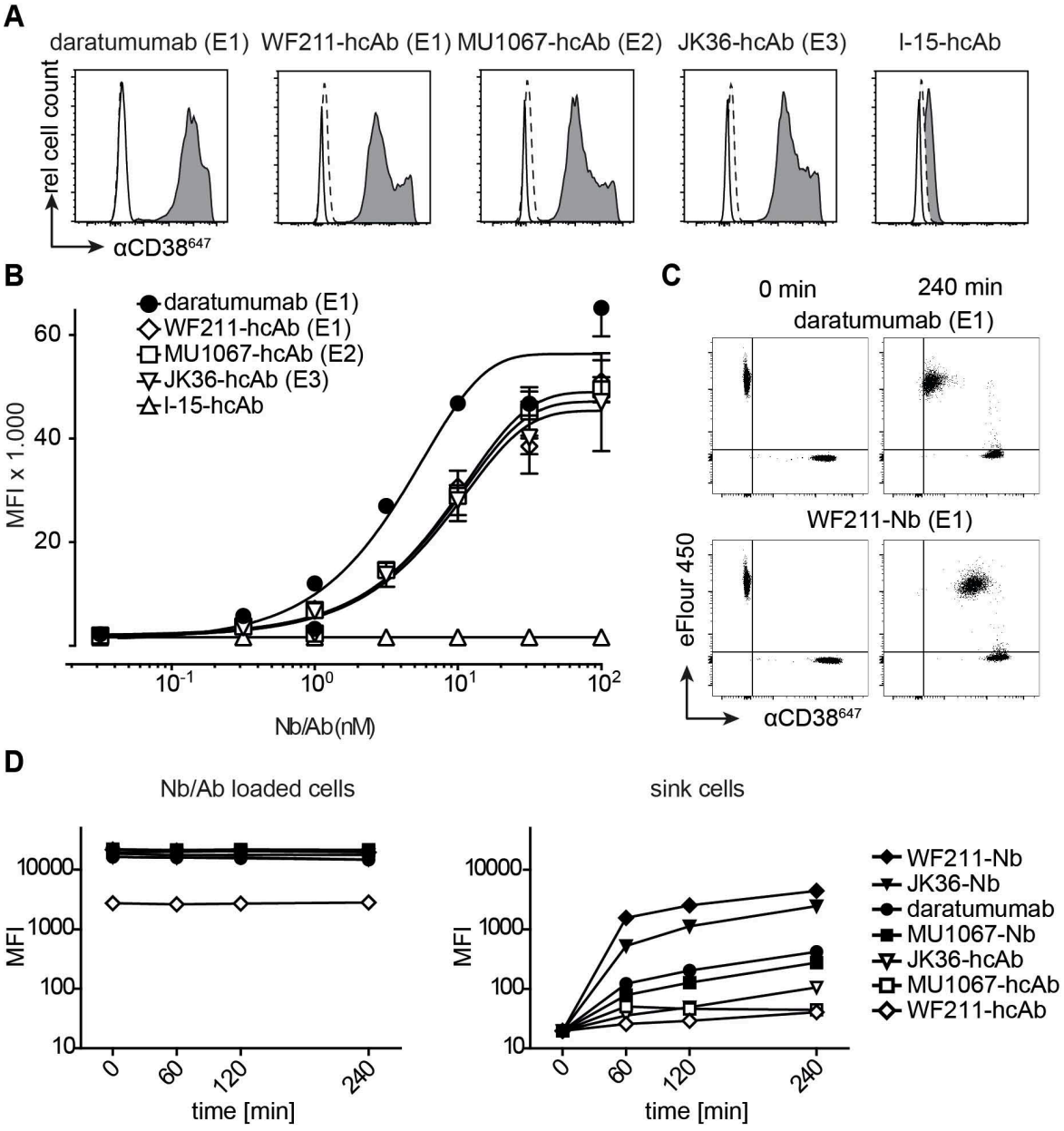

Figure 2. Binding of CD38-specific hcAbs to lymphoma cells. (A) Untransfected CD38-negative parental Yac-1 cells (open histograms) and their counterparts stably transfected with human CD38 (grey histograms) were stained with CD38-specific hcAbs WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab. Binding was detected with a PE-conjugated human IgG-specific secondary antibody. Control stainings were performed with the PE-conjugated secondary antibody alone (dashed lines). Epitope specificities of CD38-specific antibodies are given in parentheses. **(B)** Titration analysis of the binding of CD38-specific hcAbs to CD38-transfected Yac-1 cells. Cells were incubated with serial dilutions of CD38-specific hcAbs, isotype control (I-15-hcAb), or daratumumab. Binding was detected with a PE-conjugated human IgG-specific secondary antibody. Data represent mean ± SD from three independent experiments. MFI, mean fluorescence intensity. **(C)** Representative dot plots and **(D)** changes in mean fluorescence intensity over time of the dissociation of fluorochrome-conjugated monovalent nanobodies and bivalent hcAbs from CD38-transfected Yac-1 cells. Cells were incubated with excess (100 nM) Alexa^{647}-conjugated nanobodies, hcAbs or daratumumab for 30 min at 4°C. Cells were washed three times and then monitored for loss of cell-associated fluorescence over time at RT. An aliquot of CD38-expressing Yac-1 cells that had been labeled with the cell-tracking dye eFluor 450 was added at t = 0 as a sink for the dissociated antibody constructs.

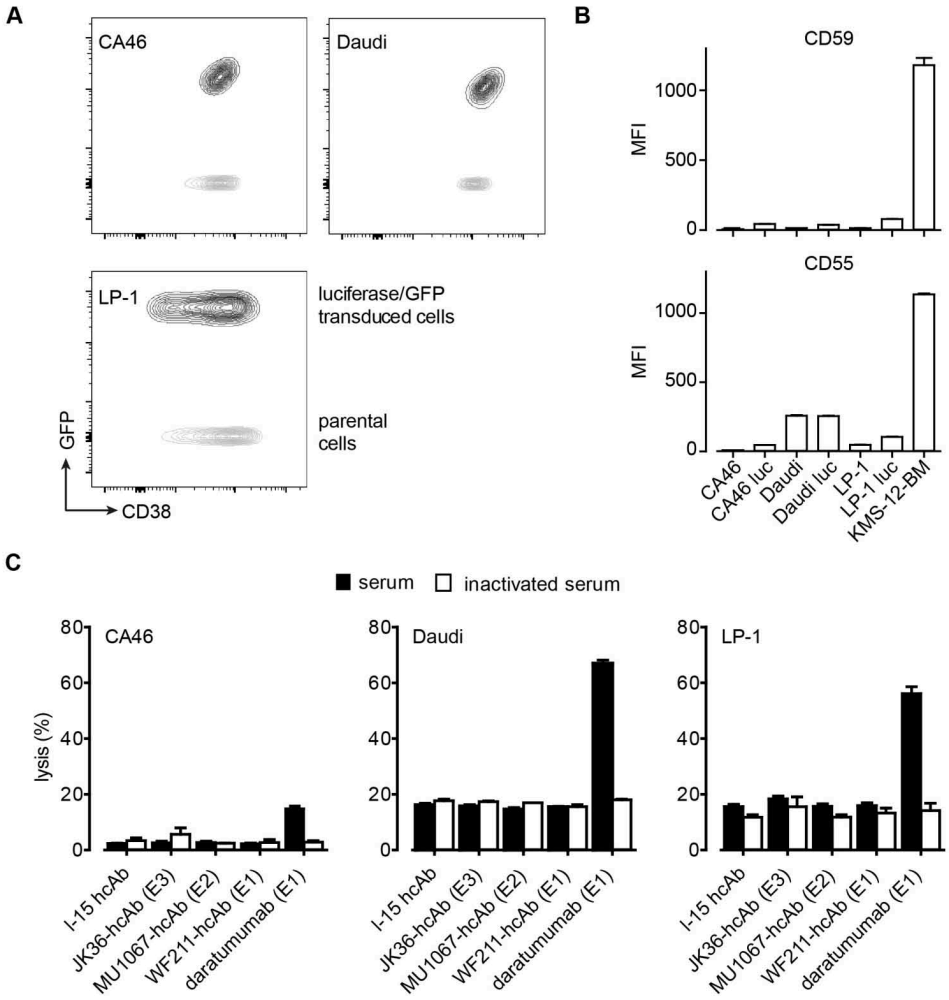

Figure 3. Cell surface expression of CD38, CD59, CD55 and CDC induction by CD38-specific hcAbs. (A) Cell surface expression of CD38 by human lymphoma cell lines (CA46, Daudi, LP-1) before and after transduction with GFP/luciferase. Non-transduced and GFP/luciferase-transduced cells were stained with Alexa^{647}-conjugated MU1067-hcAb and analyzed by flow cytometry. Dot plots were overlaid for direct comparison of CD38 expression levels by non-transduced parental cells (GFP-) and GFP/luciferase-transduced cells (GFP+). **(B)** Cell surface expression of CD55 and CD59 by CA46, Daudi, and LP-1 cells before and after transduction with GFP/luciferase. **(C)** CA46, Daudi, and LP-1 cells were incubated for 60 min at 37°C with CD38-specific heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab in the presence of 15% pooled human serum as source of complement. The same pooled serum pretreated for 30 min at 56°C (inactivated serum) was used as control. Cells were then stained with propidium iodide and analyzed by flow cytometry to determine the percentage of lysed cells. Epitope specificities of CD38-specific antibodies are indicated in parentheses. Data represent mean ± SD from three independent experiments.

In case of NK-92 effector cells (**Figure 4A-C**), highest maximal lysis with saturating doses of the three hcAbs (85-87%) was observed for Daudi-luc cells, without being statistically different from daratumumab (81%) (all $p>0.05$). In LP-1-luc cells, the maximal lysis of the three hcAbs ranged from 79-82% and was significantly higher than with daratumumab (68%) in the case of MU1067-hcAb and JK36-hcAb (both $p<0.05$), whereas there was no statistically significant difference between WF211-hcAb and daratumumab ($p>0.05$). Lowest maximal lysis of the three hcAbs was observed for CA46-luc cells (49-53%). Of note, the observed maximal lysis for all three hcAbs was significantly higher than for daratumumab (37%) (all $p<0.01$).

In case of primary NK effector cells (**Figure 4D-E**), highest maximal lysis with saturating doses of the three hcAbs (75-76%) was also observed in Daudi-luc cells, being statistically significantly higher for all three hcAbs than for daratumumab (58%) (all $p>0.001$). In CA46-luc cells, the maximal lysis for the three hcAbs ranged from 50-55% and was significantly higher than with daratumumab (40%) in the case of MU1067-hcAb ($p<0.001$) and WF211-hcAb ($p<0.01$), whereas there was no statistically significant difference between JK36-hcAb and daratumumab ($p>0.05$). Lowest maximal lysis for the three hcAbs was observed for LP-1-luc cells (27-34%), without being statistically significant different from daratumumab (21%) (all $p>0.05$).

Isotype control heavy chain antibody I-15-hcAb did not mediate any detectable lysis, reflecting its inability to bind CD38.

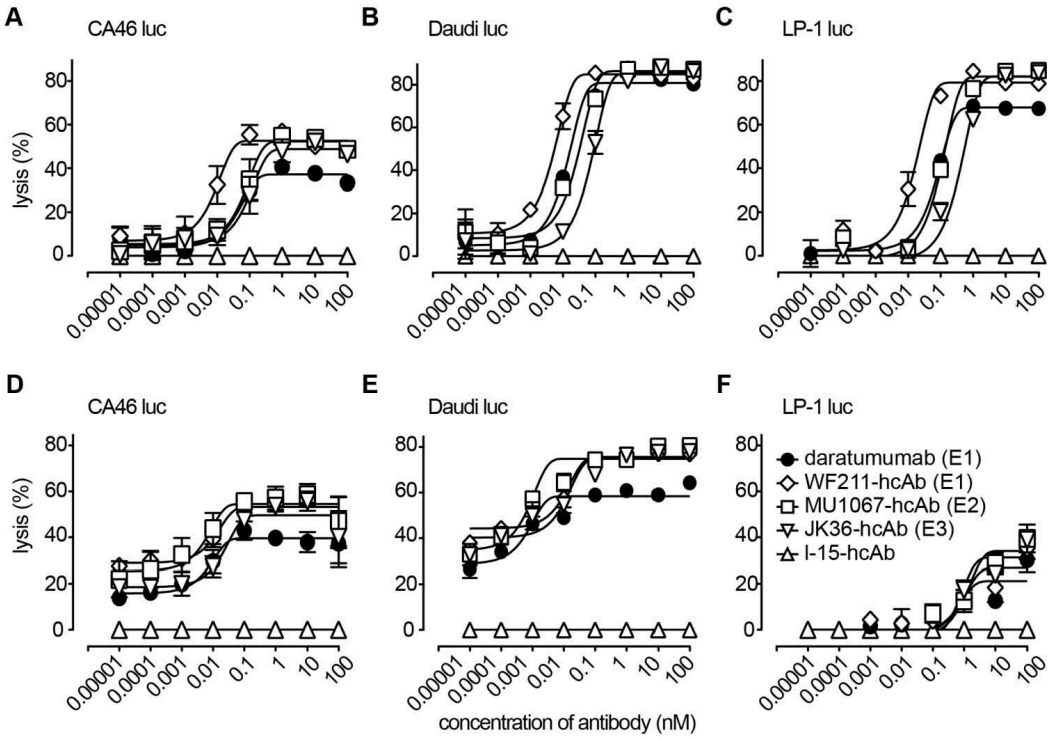

Figure 4. CD38-specific hcAbs induce ADCC. (A-C) CD16-transduced NK-92 effector cells were co-cultured for 4h at an effector to target ratio of 3:1 with luciferase (luc)-transduced lymphoma cells **(A:** CA46, **B:** Daudi, **C:** LP-1) in the presence of serial dilutions of heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab. **(D-F)** Primary NK effector cells were co-cultured for 4h at an effector to target ratio of 30:1 with luciferase (luc)-transduced lymphoma cells **(D:** CA46, **E:** Daudi, **F:** LP-1) in the presence of serial dilutions of hcAbs or daratumumab. Luciferin was added for 20 min at RT, and mean bioluminescence (BLI) was quantified to determine percentage of lysis. Epitope specificities of CD38-specific antibodies are indicated in parentheses. Data represent mean ± SD from three independent samples. Results are representative of three independent experiments.

CD38-specific hcAbs inhibit tumor growth in a mouse xenograft model

The ability of CD38-specific hcAbs to inhibit tumor growth *in vivo* was tested in mouse xenograft experiments after systemic administration of CA46-luc cells. CA46-cells were chosen because tumor growth *in vivo* with these cells showed less variability than with Daudi-luc or LP-1-luc cells. Treatment with hcAbs or daratumumab was initiated at day 7, i.e. when tumors became detectable by luminescent imaging. The results revealed effective tumor growth inhibition *in vivo* with all three hcAbs WF211-hcAb, MU1067-hcAb, and JK36-hcAb. **Figure 5A** shows representative images of *in vivo* luminescence tumor signals over time. Animals treated with the irrelevant isotype control heavy chain antibody showed unaffected tumor growth. In contrast, treatment with CD38-specific hcAbs or with daratumumab resulted in significant inhibition of tumor cell growth as compared with isotype control treatment from day 28 (all $p<0.001$, as compared with isotype control treatment from day 28) **(Figure 5B)**. Administration of hcAbs showed a trend toward stronger inhibition of tumor cell growth as compared to daratumumab, however without reaching a statistically significant difference (all $p>0.05$).

CD38-specific hcAbs improve survival in a mouse xenograft model

Mice with xenograft tumors receiving repeated treatment with any of the CD38-specific hcAbs (all $p\leq0.0001$) or daratumumab ($p=0.002$) demonstrated improved overall survival compared to mice receiving control treatment **(Figure 6)**. Median survival of SCID mice receiving control treatment was 50 days after intravenous injection of CA46-luc cells, which was significantly shorter compared to 75 days of mice receiving daratumumab ($p=0.002$) or mice treated with any of the CD38-specific hcAbs (all $p\leq0.0001$). The longest median survival was observed in the JK36-hcAb group (119 days) and WF211-hcAb group (92 days), which was significantly longer than median survival (75 days) of mice receiving daratumumab ($p=0.003$ and $p=0.031$, respectively). The group of mice receiving MU1067-hcAb had a median survival of 80 days, not significantly different from that of the daratumumab group ($p=0.110$).

CD38-specific hcAbs induce little CDC in primary MM cells *ex vivo*

The ability of CD38-specific hcAbs to induce

CDC was tested on fresh bone marrow samples from MM patients. The three CD38-specific hcAbs induced little CDC of primary MM cells **(Figure 7A)**. The percentage of surviving MM cells was 88% (CI: 78-99%) for JK36-hcAb, 83% (CI: 73-92%) for MU1067-hcAb, and 85% (CI: 67-104%) for WF211-hcAb. In contrast, daratumumab induced effective CDC resulting in 36% (CI: 14-57%) of surviving cells.

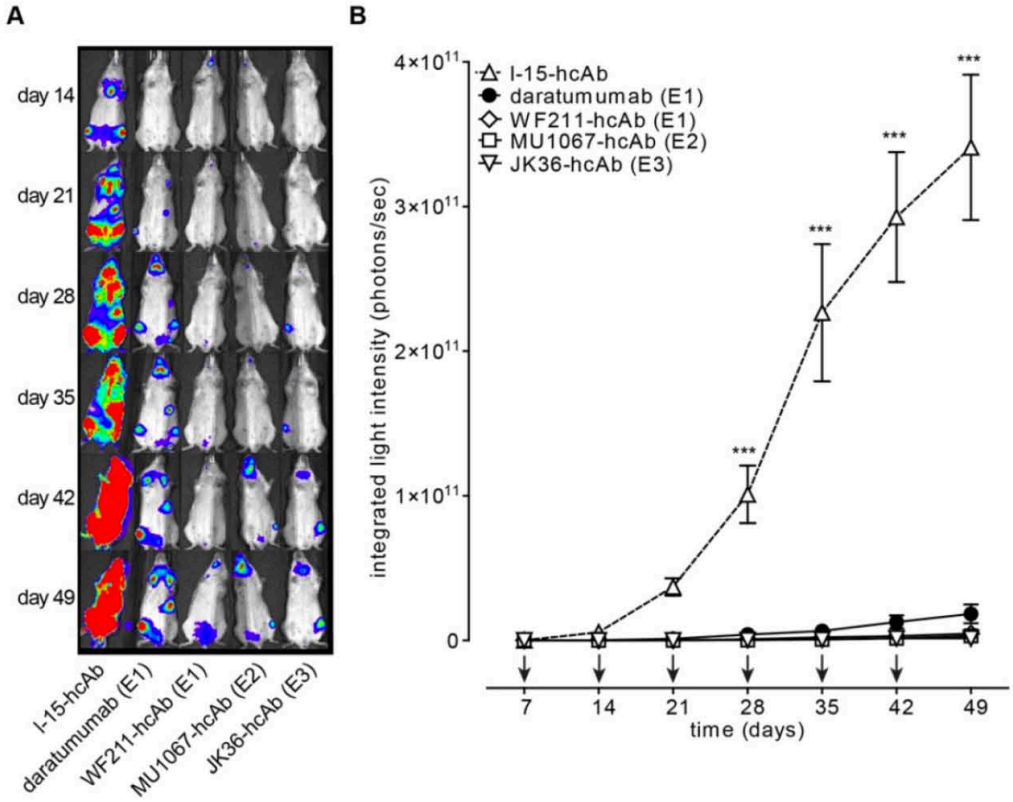

Figure 5. CD38-specific hcAbs inhibit the growth of CD38-expressing CA46 tumors *in vivo*. SCID mice (n = 7/group) were injected i.v. with luciferase-expressing CA46 cells (4 x 10^6 cells). Starting on day 7 after tumor injection, mice received weekly i.p. injections (2 mg/kg) of CD38-specific heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab. **(A)** At the indicated time points after tumor-injection, bioluminescent images of mice were obtained 15 min after i.v. injection of luciferin. Signal intensities of all mice and imaging time points are equally leveled ($1x10^8$ - $1x10^{11}$ photons/sec) to allow direct and fair visual comparison. **(B)** Light emission of tumors integrated over total body area plotted over time as a measure of tumor mass development. Arrows indicate antibody administrations. Data represent mean ± SD. From day 28 onward, tumor growth was significantly reduced in animals treated with hcAbs or daratumumab as compared to animals treated with isotype control (*** = $p<0.001$). Two-way ANOVA followed by a Bonferroni post hoc test was used for statistical analysis. Results are representative of three independent experiments. Epitope specificities of CD38-specific antibodies are indicated in parentheses.

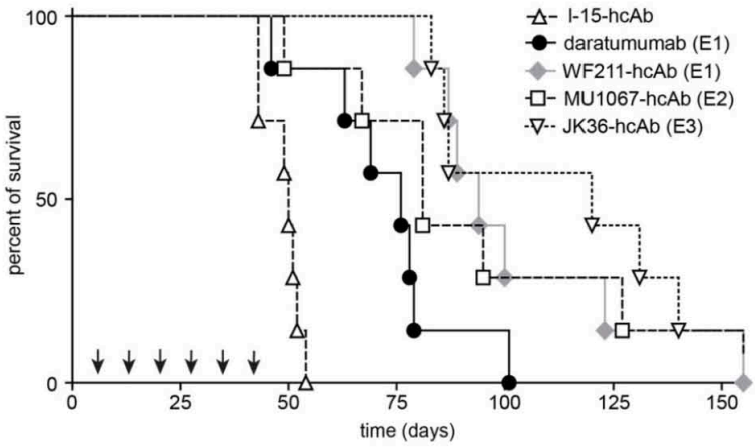

Figure 6. CD38-specific hcAbs prolong the survival of mice bearing CD38-expressing CA46 tumors. Kaplan-Meier plot of overall survival of SCID mice intravenously injected with CA46-luc cells. SCID mice (n = 7/group) received weekly i.p. treatments (2 mg/kg, arrows) with CD38-specific heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab as described in Fig. 5. Overall survival of mice treated with hcAbs or daratumumab was significantly longer as compared to mice receiving isotype control treatment ($p\leq0.0001$ and $p=0.002$, respectively). Overall survival of mice treated with the CD38-specific hcAbs was longer than that of mice receiving daratumumab treatment. This difference was significant for JK36-hcAb ($p=0.003$) and WF211-hcAb ($p=0.031$), but not for MU1067-hcAb ($p=0.110$). Log rank test was used for statistical analysis. Epitope specificities of CD38-specific antibodies are indicated in parentheses. Results are representative of three independent experiments.

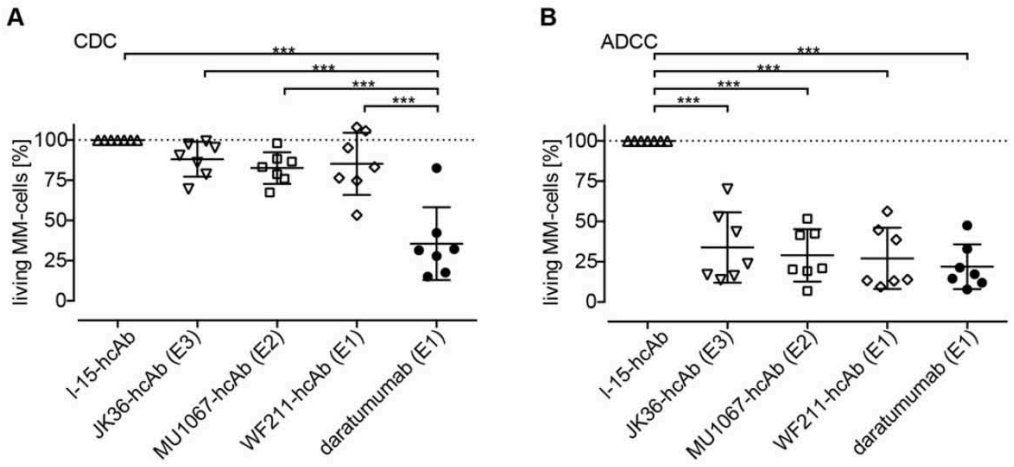

Figure 7. CDC and ADCC induction by CD38-specific hcAbs of primary myeloma cells obtained from MM patients. (A) Fresh bone barrow mononuclear cells from MM patients were incubated for 90 min with CD38-specific heavy chain antibodies WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab in the presence of 12.5% pooled human serum as source of complement. CDC was assessed by flow cytometric quantification of the number of living MM cells. **(B)** Fresh bone marrow mononuclear cells were incubated for 2.5 h with CD16- and GFP-transduced NK-92 effector cells at an effector to target ratio of approximately 10:1 in the presence of WF211-hcAb, MU1067-hcAb, JK36-hcAb, isotype control (I-15-hcAb), or daratumumab. ADCC was assessed by flow cytometric quantification of the number of living MM cells. Data are presented as % living MM cells relative to samples treated with the isotype control. Data represent means with 95%-confidence intervals. Sidak-adjusted p-values are indicated (***= p<0.001).

CD38-specific hcAbs mediate effective ADCC in primary MM cells *ex vivo*

The ability of CD38-specific hcAbs to induce ADCC was tested on fresh bone marrow samples from MM patients. All three hcAbs effectively induced lysis of primary MM cells **(Figure 7B)**. The percentage of surviving MM cells was 34% (CI: 14-55%) for JK36-hcAb, 29% (CI: 14-44%) for MU1067-hcAb and 27% (CI: 9-45%) for WF211-hcAb. The daratumumab-mediated ADCC was comparable resulting in 22% (CI: 9-35%) of surviving cells.

Discussion

We demonstrated the feasibility of using CD38-specific hcAbs to efficiently kill hematological cancer cells *in vitro*, *ex vivo* and *in vivo*. Specifically, we generated nanobody-based humanized IgG1 hcAbs with a high specificity and affinity that recognize three different and non-overlapping epitopes of CD38. CD38-specific hcAbs induced potent ADCC regardless of their epitope specificity, but failed to induce substantial CDC of tumor cell lines *in vitro* or primary MM cells *ex vivo*. CD38-specific hcAbs significantly reduced tumor growth *in vivo* and significantly prolonged survival of xenograft bearing mice.

All three CD38-specific hcAbs (WF211-hcAb, MU1067-hcAb, JK36-hcAb) had high and comparable binding affinities regardless of their epitope specificity. The stronger blockade of daratumumab^{647} by MU167-hcAb than vice versa is likely explained by a higher affinity of MU1067-hcAb^{647} for CD38: in cells that have been pretreated with unlabeled daratumumab, addition of MU1067-hcAb^{647} resulted in partial displacement of daratumumab. This interpretation is consistent with the faster dissociation of daratumumab from LP-1 cells than MU1067-hcAb.

However, despite binding with high affinity to CD38, the CD38-hcAbs showed little if any capacity to induce CDC of primary MM cells, confirming previous *in vitro* results using tumor cells lines [33]. Conversely, all three CD38-specific hcAbs effectively induced ADCC of tumor cell lines and primary MM cells with comparable potency to daratumumab. Interestingly, the heavy chain antibody WF211-hcAb that recognizes the same CD38 epitope (E1) as daratumumab showed the strongest ability to induce ADCC *in vitro*.

However, the observations made *in vitro* did not fully translate into the findings made in a systemic tumor xenograft mouse model *in vivo*. Despite the fact that CD38-hcAbs effectively induced ADCC but not CDC *in vitro*, all three hcAbs reduced tumor growth *in vivo* at least as effectively as daratumumab.

Previous studies have shown that CD38-targeting conventional monoclonal antibodies can mediate cytotoxicity against CD38-expressing hematological cancer cells via CDC, ADCC, antibody-dependent cellular phagocytosis (ADCP), direct induction of apoptosis, and modulation of CD38 ectoenzyme function [12, 42-44]. Although different conventional antibodies target the same antigen and induce similar degrees of ADCC, marked differences in CDC capabilities were observed when comparing different CD38-specific antibodies [42]. This is in line with our finding that all three CD38-specific hcAbs induce ADCC regardless of their

epitope specificity, but mediate little if any CDC.

The relative contributions of CDC and ADCC to the overall therapeutic activity of monoclonal antibodies are still unknown [18]. A combination of these mechanisms likely underlies the therapeutic efficacy. Our results show that CD38-specific hcAbs induce little CDC *in vitro* and *ex vivo* but mediate potent growth inhibition of systemic tumors in a mouse model. In this regard, our hcAbs have similar properties to another conventional CD38 antibody, MOR202, which does not induce CDC, but performs well in murine models, although not as well as daratumumab in clinical trials [45]. Future clinical studies are needed to assess whether the ADCC effect of CD38-specific hcAbs translates into high clinical efficacy.

Our study has important potential clinical implications, particularly for patients with reduced biological activity of daratumumab. The development of neutralizing anti-idiotype antibodies may reduce the biological activity of daratumumab. Moreover, the unique epitope recognized by daratumumab could be mutated so as to prevent its binding, presenting a point of vulnerability for drug resistance [18]. In such cases, the CD38-specific hcAbs described here that bind to alternative epitopes (MU1067-hcAb, JK36-hcAb) may provide alternative therapeutics. Moreover, they could be used to complement daratumumab in hematological cancer therapies.

Of note, CD38 expression levels on the surface of MM cells during daratumumab treatment are downregulated [46]. Progression of MM during daratumumab therapy may occur due to the reduced CD38 levels [47]. Targeting non-overlapping (MU1067-hcAb, JK36-hcAb) or overlapping CD38 epitopes (WF211-hcAb) with our nanobody-based hcAbs during this period may not be helpful and underlines the need for antibodies targeting alternative target-proteins such as BCMA and SLAMF7 on the surface of MM cells [12, 48].

Several questions could not be answered in our study and warrant further investigation: First, it will be interesting to assess the capability of our CD38-specific hcAbs to induce other effector functions, such as ADCP, direct induction of apoptosis, and modulation of CD38 ectoenzyme function. Second, binding and cytotoxicity assays involving other CD38 positive cells, including hematopoietic progenitor cells, activated immune effector cells, T regulatory cells, and endothelial cells are needed to gauge the potential therapeutic index. Third, future preclinical and clinical studies are warranted to assess the therapeutic efficacy of CD38-specific hcAbs in combination with other anti-MM agents and the efficacy in myeloma cell lines and patient cells resistant to conventional or novel therapies. Moreover, it will be of interest to explore the potential of CD38-specific hcAbs for treatment of diseases beyond hematological malignancies, including solid tumors and antibody-mediated autoimmune diseases [15, 16].

Conclusion

In conclusion, we show that CD38-specific nanobody-based humanized IgG1 heavy chain antibodies mediate cytotoxicity against CD38-expressing hematological cancer cells *in vitro, ex vivo and in vivo*. These promising results of our study indicate that CD38-specific hcAbs warrant further clinical development as therapeutics for multiple myeloma and other hematological malignancies.

Abbreviations

ADCC: Antibody-dependent cellular cytotoxicity; CDC: Complement dependent cytotoxicity; MM: Multiple Myeloma.

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Author Contributions

L.S., K.S. and K.P. contributed equally. L.S., K.S., K.P., J.H., W.F., J.K., N.B., S.M., B.R., J.L.R., K.R., J.S. N.B., B.A., and H.P. performed experiments and analyzed results. B.F., F.A., N.K., M.B., G.S., T.H., F.H., G.A., P.B., and F.K.-N. designed research and analyzed data. P.B. and F.K.-N. wrote the paper and share senior authorship.

Competing Interests

K.S., W.F., L.S., S.M., P.B., 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 and protein sales via MediGate GmbH, a wholly owned subsidiary of the University Medical Center Hamburg-Eppendorf.

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3.2 Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimäric Antigen Receptors (Nb-CARs)

In diesem Kapitel wird die Publikation *„Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimäric Antigen Receptors (Nb-CARs)“* vorgestellt, die im Jahr 2020 im Journal *Cells* publiziert wurde. In dieser Publikation wurden alle Experimente und Analysen von mir durchgeführt sowie alle Abbildungen von mir erstellt. Das Manuskript wurde von mir in enger Zusammenarbeit mit meinen Betreuern Prof. Dr. Peter Bannas und Prof. Dr. Friedrich Koch-Nolte erstellt. Aus diesem Grund bin ich in dieser Publikation die Erstautorin.
Teile dieser Arbeit wurden bereits in meiner Masterarbeit unter dem Titel *„Herstellung und Charakterisierung von CD38-spezifischen Nanobody-basierten Chimären Antigen Rezeptoren*“ diskutiert. Daher werden in dieser Dissertation nur Ergebnisse des WF211-basierten CARs Nb211-CAR, Figure 6 und Figure 7 diskutiert.

Titel: Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimäric Antigen Receptors (Nb-CARs)

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Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimeric Antigen Receptors (Nb-CARs)

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Abstract: The NAD-hydrolyzing ecto-enzyme CD38 is overexpressed by multiple myeloma and other hematological malignancies. We recently generated CD38-specific nanobodies, single immunoglobulin variable domains derived from heavy-chain antibodies naturally occurring in llamas. Nanobodies exhibit high solubility and stability, allowing easy reformatting into recombinant fusion proteins. Here we explore the utility of CD38-specific nanobodies as ligands for nanobody-based chimeric antigen receptors (Nb-CARs). We cloned retroviral expression vectors for CD38-specific Nb-CARs. The human natural killer cell line NK-92 was transduced to stably express these Nb-CARs. As target cells we used CD38-expressing as well as CRISPR/Cas9-generated CD38-deficient tumor cell lines (CA-46, LP-1, and Daudi) transduced with firefly luciferase. With these effector and target cells we established luminescence and flow-cytometry CAR-dependent cellular cytotoxicity assays (CARDCCs). Finally, the cytotoxic efficacy of Nb-CAR NK-92 cells was tested on primary patient-derived CD38-expressing multiple myeloma cells. NK-92 cells expressing CD38-specific Nb-CARs specifically lysed CD38-expressing but not CD38-deficient tumor cell lines. Moreover, the Nb-CAR-NK cells effectively depleted CD38-expressing multiple myeloma cells in primary human bone marrow samples. Our results demonstrate efficacy of Nb-CARs in vitro. The potential clinical efficacy of Nb-CARs in vivo remains to be evaluated.

Keywords: CD38; nanobody; chimeric antigen receptor; NK-92 cells; cellular cytotoxicity assays; luciferase; heavy chain antibody; multiple myeloma

1. Introduction

The NAD-hydrolyzing ecto-enzyme CD38 is overexpressed by multiple myeloma and other hematological malignancies [1–3]. CD38 has emerged as a promising target for therapy with cytotoxic antibodies. Three monoclonal antibodies have recently entered the clinic (daratumumab, isatuximab, and MOR202) for treatment of multiple myeloma (Figure 1a) [4–6].

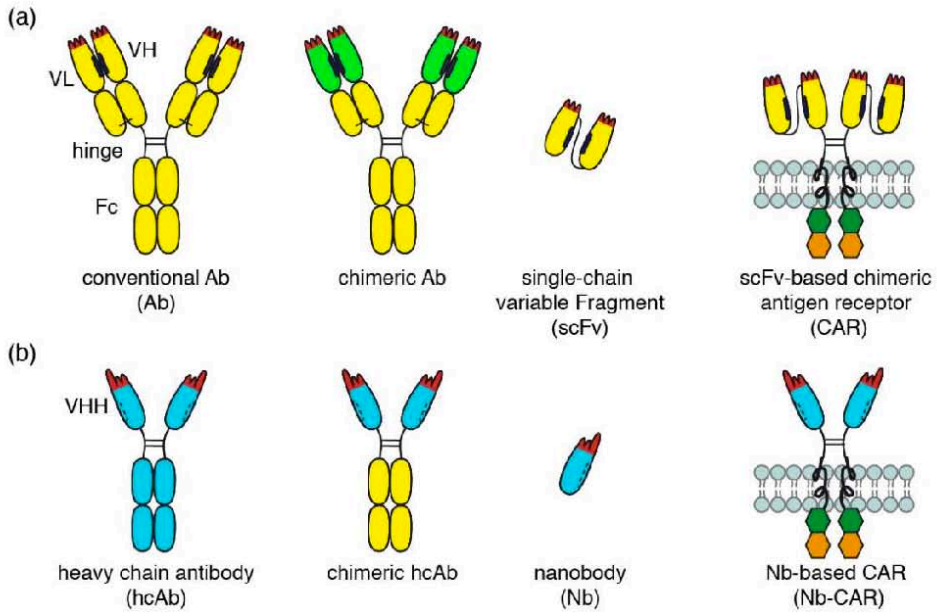

Figure 1. Comparison of conventional and nanobody-based chimeric antigen receptors. (**a**) Schematic diagrams of a conventional antibody (Ab), a chimeric antibody, a single chain variable fragment (scFv), and conventional scFv-based chimeric antigen receptor (CAR). Examples of conventional and chimeric CD38-specific Abs include daratumumab and MOR202 (conventional Abs) and isatuximab (chimeric Ab). (**b**) Schematic diagrams of a camelid heavy chain antibody (hcAb), a chimeric hcAb, a nanobody (Nb) and a Nb-based chimeric antigen receptor (Nb-CAR).

The variable domains of conventional antibodies (VH and VL) interact via hydrophobic patches (indicated in black) that help to correctly align the two domains for target binding. The hydrophobic nature of this interaction and its relatively low affinity, however, render scFvs unstable and sticky (Figure 1a). Nanobodies are single variable immunoglobulin domains derived from heavy chain antibodies (hcAbs) that naturally occur in llamas, dromedaries, and other camelids (Figure 1b) [7,8]. The mutation(s) that led to deletion of the CH1 domain in heavy chain antibodies must have occurred >50 million years ago, i.e., in a common ancestor of today's extant camelids (dromedary, camel, alpaca, llama) [9]. The cluster of V genes in the IgH locus of extant camelids encodes both, highly soluble (VHH) and less soluble (VH) domains. The former occur in the vast majority of camelid hcAbs, the latter in camelid conventional Abs akin to those of mice and men [9]. Camelid VHH domains apparently have been shaped by evolution for high solubility and stability as a single domain. This renders nanobodies particularly suited for reformatting in a LEGO-block like fashion into fusion proteins, including dimers or multimers of nanobodies, chimeric llama/human Nb-hcAbs, biparatopic hcAbs, and Nb-based CARs (Nb-CARs) [10,11]. Dimerization and multimerization of nanobodies has been used to increase the binding strength and the specificity of the constructs [10,11].

From immunized llamas, we previously isolated a panel of CD38-specific nanobodies [12,13]. We have shown that these nanobodies can be used to construct Nb-hcAbs, some of which exhibit more potent antibody-dependent cellular cytotoxicity (ADCC) than daratumumab (Figure 2) [10,14,15].

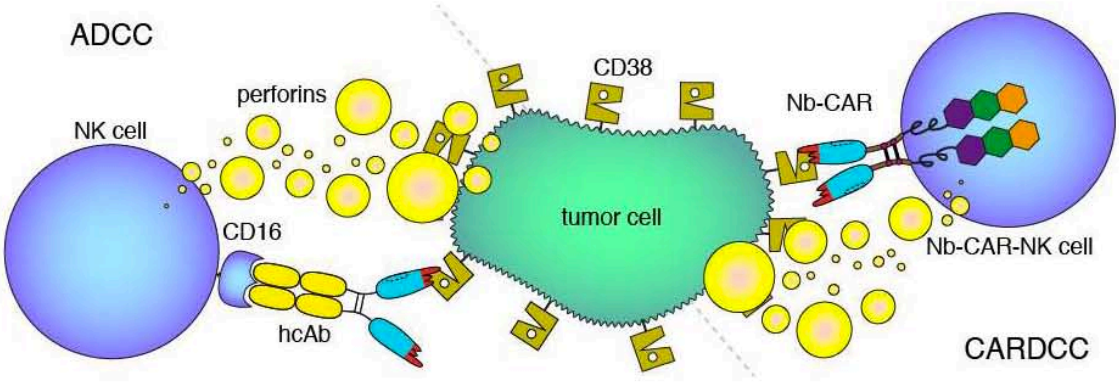

Figure 2. Antibody- and CAR-dependent cellular cytotoxicity. Schematic diagrams of antibody-dependent cellular cytotoxicity (ADCC, left) and chimeric antigen-receptor dependent cellular cytotoxicity (CARDCC, right) mediated by CD38-specific hcAbs and Nb-CARs, respectively. ADCC and CARDCC are mediated by perforins, pore-forming cytolytic proteins released from activated NK cells. Perforins are depicted here schematically as yellow bubbles.

The aim of the current study was to explore the utility of CD38-specific nanobodies as ligands for Nb-CARs. To this end, we stably transduced the human natural killer cell line NK-92 [16] with CD38-specific and control Nb-CARs. We chose NK-92 cells as effector cells rather than T cells because of their potential use as an "off-the-shelf" reagent. As target cells, we chose established multiple myeloma (LP-1, RPMI-8226) and Burkitt lymphoma cell lines (CA-46, Daudi) with known high cell surface levels of CD38. With these cells, we established CAR-dependent cellular cytotoxicity assays (CARDCC) (Figure 2), using luminescence and flow-cytometry to monitor the killing of target cells (see below). Our results indicate that Nb-CARs, indeed, may provide a basis for clinical development of novel therapeutics to target CD38-expressing tumor cells.

2. Materials and Methods

2.1. Cell Lines

The following human cell lines were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany): NK-92 (natural killer cell line), Daudi and CA-46 (Burkitt lymphoma cell lines), LP-1 and RPMI-8226 (myeloma cell lines). Cell lines (CA-46 luc, Daudi luc, LP-1 luc) stably expressing the luc2 variant of Photinus pyralis luciferase (Promega, Madison, WI, USA) under control of the spleen-focus-forming virus U3 region (SFFV promoter) were generated by lentiviral transduction as described [17,18]. Transduced cells were selected in culture medium containing 1 µg/mL puromycin and subsequently sorted by FACS based on eGFP expression (on a FACS Aria III, BD Biosciences, Heidelberg, Germany). Sorted cells were kept in culture and luciferase-expression was controlled regularly following addition of luciferin using a luminometric plate reader.

As specificity controls, we generated CD38-deficient variants of LP-1 luc, CA-46 luc, Daudi luc, and NK-92. In order to prevent expression of CD38, rather than to merely reduce its expression, we used CRISPR/Cas9 technology (sc-401117-NIC, Santa Cruz Biotechnology, Dallas, TX, USA) rather than shRNA technology. Cells were stained for CD38 using the AlexaFluor647-conjugated JK36 nanobody ($JK36^{AF647}$) and cells were FACS-sorted for lack of cell surface CD38. The sorted cells were maintained in culture and CD38 cell surface levels were controlled regularly using $JK36^{AF647}$.

2.2. Generation of Nb-CARs

The human CD38-specific nanobodies WF211, MU1067, JK36 and the ARTC2.2-specific control nanobody s-14 were generated from immunized llamas as described previously [13,19]. In the text, we use the full designation for these nanobodies. For the sake of clarity, we use abbreviated designations for Nb-CARs, e.g., Nb1067-CAR and Nb14-CAR instead of NbMU1067-CAR and Nbs-14-CAR. The nanobody coding region was fused by gene synthesis downstream of the signal sequence of VH and upstream of a strep-tag, the hinge region of human IgG4, the transmembrane and membrane-proximal ITAM domains of human CD28, the ITAM domain of human 4-1BB, and the C-terminal signaling domain of CD3ζ. This cassette was cloned into the gamma-retroviral vector pRSF91.iB.pre* (a derivative of pRSF91.GFP.pre* [20]) upstream of an EMCV internal ribosomal entry site (IRES) and the coding region for blue-fluorescent protein (mTagBFP). HEK293 cells were transiently transfected with the combination of the pRSF91.Nb-CAR.iB.pre* coding for the CAR and packaging plasmids pcDNA3.MLVgp [21] and phCMV-GALV$_{C4070A}$-env to obtain cell-free supernatants containing retroviral particles encoding Nb-CARs. Vector titers were then determined on HEK293T cells as described previously [18].

2.3. Stable Transduction of NK-92 Cells with Nb-CARs

NK-92 cells stably expressing Nb-CARs were generated by retroviral transduction. Transduction was carried out in a 24-well plate with 50,000 cells in 500 µL medium per well by addition of 300 µL viral particle-containing supernatant in the presence of 8 µg/mL hexadimethrine bromide and subsequent spin-inoculation for 1 h at 1000× *g* and 25 °C. Stably transduced cells were FACS-sorted based on mTagBFP-expression. CAR-expression by these cells was controlled regularly by staining of cells with AlexaFluor647-conjugated recombinant ectodomains of CD38 and ARTC2.2. The initial transduction efficiency was below 30%; cell sorting resulted in stable expression of the Nb-CAR by more than 95% of cells. The fluorochrome-conjugated ecto-domains of CD38 and ARTC2.2 served as both, positive and negative quality controls for determining the cell surface levels of target-specific Nb-CARs.

2.4. Production of Alexa Fluor 647-Labeled CD38 and ARTC2.2

The myc-his-tagged extracellular domains of CD38 (aa46–300) and ARTC2.2 (aa20–261) were produced in transiently transfected HEK-6E cells cultivated in serum-free medium. Six days post transfection supernatants were harvested and cleared by centrifugation. The myc-his-tagged proteins were purified by immobilized metal affinity chromatography using Ni-NTA agarose (Sigma, St Louis, MO, USA). Fluorochrome-labelling was performed using NHS esters according to the manufacturer's instructions (Alexa Fluor 647 Succinimidyl Ester, Invitrogen, Karlsbad, CA, USA).

2.5. Luminescence CARDCC Assays

CA-46 luc, Daudi luc, and LP-1 luc cells were co-incubated with NK-92-CAR for 4 h at 37 °C at the indicated ratios in αMEM culture medium supplemented with 10% fetal calf serum (FCS), 10% horse serum, 5 mM glutamine, and 5 ng/mL interleukin 2 (IL-2 Proleukin-S, Novartis, Basel, Switzerland). D-luciferin (Biosynth, Staad, Switzerland) was added as substrate (75 µg/mL) for 20 min and bioluminescence-intensity (BLI) was measured with a microplate reader (Victor^3, Perkin Elmer, Boston, MA, USA).

2.6. Flow Cytometric CARDCC Assays

Target cells were fluorescently pre-labeled by incubation with AlexaFluor647, effector cells by incubation with eFluor450. Cells were washed and co-incubated at the indicated E:T-ratios at 37 °C for the indicated time-periods. Dead cells were stained with propidium iodide (PI, Invitrogen, WA, USA) or Pacific Orange succinimidyl ester (PacO, Thermo-Fisher Scientific, Waltham, MA, USA) before analysis of cells by flow cytometry (BD FACS Celesta/Becton Dickinson). Percentage of cells was calculated as follows: % lysis [%] = 1 − (cells [sample]/ cells [sample with control CAR]) × 100%.

2.7. CARDCC Assays with Primary Human Bone Marrow Samples

Fresh bone marrow aspirates were obtained from patients after Institutional Review-Board-approved consent (PV5505). Bone marrow mononuclear cells (BM-MNCs) were prepared by Ficoll-Paque density gradient centrifugation of bone marrow aspirates and subsequent depletion of remaining erythrocytes using red blood cell lysis buffer (NH4Cl + KHCO3 + EDTA). BM-MNCs were co-incubated with eFluor450-labeled NK-92 Nb-CAR cells at an effector to target ratio [E:T] of 1:1 for 4 h at 37 °C in αMEM culture medium (see above). Cells were then stained with a panel of fluorochrome-conjugated antibodies (CD38, CD45, CD138/229, CD269/CD319/CD56, CD19) and PacO and analyzed via flow cytometry. We did not use CD138 in these four hour assays because of the known instability of this marker on the cell surface of MM cells [22]. Staining of CD38 was achieved with Alexa Fluor 647-conjugated nanobodies that bind independently of the nanobody contained in the CAR: $JK36^{AF647}$ or $MU523^{AF647}$ for Nb211-CAR, $MU523^{AF647}$ or $WF211^{AF647}$ for Nb36-CAR, and $JK36^{AF647}$ or $WF211^{AF647}$ for Nb1067-CAR. An FSC threshold was set to exclude debris while including the population of small $CD19^+$ B cells. NK-92 cells and dead cells were excluded via staining by eFluor450 and Pacific Orange, respectively. MM cells were identified by high co-expression of CD38 and CD56 or CD319. Numbers of MM cells were determined using CountBright absolute counting beads (Invitrogen, Karlsbad, CA, USA). Percentage of surviving MM cells was calculated as follows: Percent of survival [%] = (MM cell number per µL [NK-92-CAR-treated sample]/MM cell number per µL [untreated sample]) × 100%. Significance between CD38-specific Nb-CAR-NK and the control Nb-CAR-NK was calculated using unpaired T-test (GraphPad Prism, GraphPad Software, CA, USA).

3. Results

3.1. Generation of CD38-Deficient Cell Lines and Lentiviral Transduction of $CD38^+$ and $CD38^-$ Cell Lines with Luciferase

CD38 is overexpressed by several established human tumor cell lines, including LP-1 multiple myeloma, CA-46 and Daudi Burkitt lymphoma, and NK-92 natural killer cell lymphoma [1–3]. As specificity controls, we inactivated the CD38 gene in these cell lines using CRISPR/Cas9 technology. Cells were monitored for cell surface expression of CD38 using Alexa Fluor 647-conjugated CD38-specific nanobodies and CD38-deficient cells were FACS sorted for lack of CD38 cell surface expression (Figure 3a). The results confirm high expression of CD38 by parental cells and lack of CD38 expression by CD38ko cells. Similar results were obtained with Daudi luc cells (not shown).

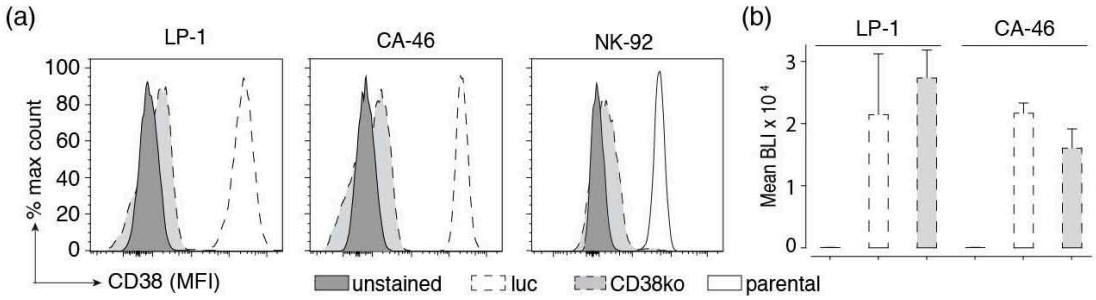

Figure 3. Inactivation of CD38 and stable expression of luciferase in established cell lines. (**a**) The CD38 gene was inactivated in the indicated tumor cell lines using CRISPR/Cas9 technology to provide specificity controls. Cells were FACS-sorted for lack of CD38 cell surface expression. Parental and CD38ko cells were incubated for 30 min at 4 °C with Alexa Fluor 647-conjugated Nb JK36 before analysis by flow cytometry. Results are representative for three similar experiments. (**b**) LP-1 and CA-46 cells and their corresponding CD38ko cell lines were lentivirally transduced with luc-GFP, encoding luciferase and GFP separated by an internal ribosomal entry site. GFP was used for cell sorting, luciferase was used for monitoring cell vitality by bio-luminometry. Cells were FACS-sorted for high expression of GFP. Triplicate samples of parental luc-GFP-expressing cells were incubated in a 96 well plate for 20 min at 24 °C with luciferin before analysis for luminescence with a plate reader. Results are representative for two similar experiments.

To permit luminescence-based cellular cytotoxicity assays, we stably transduced LP-1, CA-46, and Daudi cells with firefly luciferase and GFP. Cells were FACS sorted for high levels of GFP expression. Cells were monitored for luciferase activity following addition of luciferin using a luminescence plate reader. The results show lack of detectable bioluminescence by parental cells and very high levels of BLI signals in all luciferase-transduced cell lines ($CD38^+$ and CD38ko cells) (Figure 3b). Killing assays revealed a high dynamic range of BLI signals in all luciferase-transduced cell lines (see below). Similar results were obtained with Daudi luc cells (not shown).

3.2. Generation of Nanobody-Based Chimeric Antigen Receptors (Nb-CARs) and Transduction of NK-92 Cells with Nb-CARs

Nanobodies WF211, MU1067, and JK36 bind to three distinct, non-overlapping epitopes on CD38, designated E1, E2, and E3, respectively [13]. MU1067 inhibits the enzymatic activity of CD38, while WF211 and JK36 do not inhibit or enhance its activity [13]. The respective epitopes can be inferred from published crystal structures of CD38 in complex with nanobodies MU375 and MU551 and the Fab fragment of isatuximab (Figure 4a). WF211 competes for binding to CD38 with MU551 (E1), MU1067 competes for binding with MU375 (E2). JK36 binds to a third, non-overlapping epitope (designated E3) and competes neither with WF211 nor with MU1067 for binding to CD38. The Fab fragment of isatuximab also binds to an epitope that does not overlap with epitopes E1 and E2 (Figure 4a). It is possible, but not yet established experimentally that JK36 binds to a similar epitope as isatuximab.

For construction of Nb-CARs, we fused each of the three CD38-specific nanobodies and a control nanobody (the ARTC2.2-specific nanobody s-14) genetically to the components of a classic third-generation CAR, i.e., the hinge region of IgG4, the transmembrane and intracellular ITAM domains of CD28, and the cytosolic signaling domains of 4-1BB and CD3ζ (Figure 4b). We cloned these Nb-CAR encoding cassettes into a retroviral vector upstream of an IRES followed by the coding region for blue fluorescent protein (BFP). NK-92-CD38ko cells were stably transduced with these vectors and cells were sorted for high levels BFP expression and for high levels of cell surface Nb-CAR.

In order to detect cell surface levels of Nb-CARs, we monitored cell surface levels of the respective Nb-CARs by flow cytometry using the soluble ecto-domains of CD38 and ARTC2.2 conjugated to AlexaFluor647 (Figure 4c). The results show that $CD38^{647}$ specifically binds to NK-92 cells transduced with CD38-specific Nb-CARs but not to cells transduced with the ARTC2.2-specific Nb-CAR, whereas

ARTC2.2^{647} binds to NK-92 cells transduced with the ARTC2.2-specific Nb-CAR but not to NK-92 cells transduced with any of the three CD38-specific Nb-CARs.

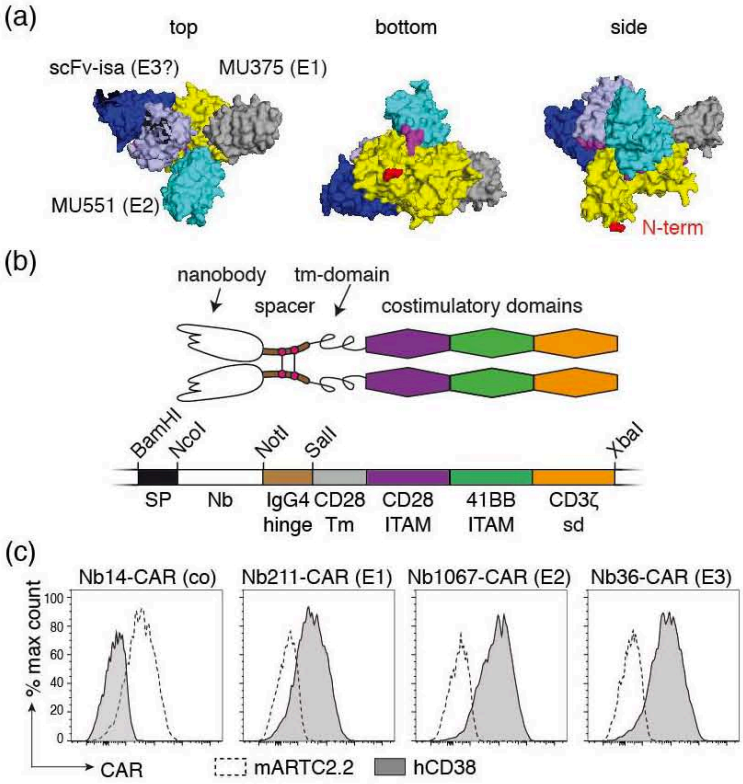

Figure 4. NK-92 cells stably expressing Nb-CARs. (**a**) 3D model of the extracellular domain of CD38 (yellow) in complex with two nanobodies (MU375 grey, and MU551 cyan) and the scFv of isatuximab (VH and VL in dark and light blue, respectively). The N-terminal amino acid of CD38 (depicted in red) connects to the membrane proximal amino acids and transmembrane domain. The model is presented from three points of view: the putative membrane-proximal side is designated "bottom," the membrane distal side "top" and the putative view parallel to the plane of the cell membrane "side." WF211 binds to the same epitope as MU375 (designated E1), MU1067 to the same epitope as MU551 (E2), and JK36 to a third epitope (E3). It is likely, but not yet confirmed experimentally, that JK36 and isatuximab bind to a similar epitope. (**b**) Schematic diagram of a Nb-CAR. The coding region for the Nb-CAR was assembled by gene synthesis. The indicated restriction enzyme recognition sites were incorporated to allow flexible exchange of the corresponding elements. SP: signal peptide; Nb: nanobody, tm: transmembrane; ITAM immunoreceptor tyrosine-based activation motif, sd: signaling domain. (**c**) NK-92 cells were stably transduced with the indicated Nb-CARs and sorted for high expression of blue fluorescent protein BFP, which was encoded downstream of an IRES sequence behind the coding region for the Nb-CAR. Cells were incubated with the fluorochrome-conjugated recombinant extracellular domains of mouse ARTC2.2 or human CD38 before analysis by flow cytometry. Results are representative for three similar experiments.

3.3. Luminescence-Based CAR-Dependent Cellular Cytotoxicity Assay (CARDCC)

In order to test whether Nb-CAR-NK cells specifically lyse CD38-expressing tumor cells, we co-incubated Nb-CAR-NK cells with luciferase-transduced CA-46 cells for 4 h at 37 °C before addition of luciferin. Luciferase activity was then monitored with a luminescence plate reader (Figure 5a). The results show that CD38-specific Nb-CAR-NKs effectively lyse CD38-expressing tumor cells but not the respective CD38ko daughter cell line. Time course analyses revealed that CARDCC happens in a time-dependent manner (Figure 5b). Similarly, the results of titration analyses show that CARDCC occurs in a dose-dependent manner.

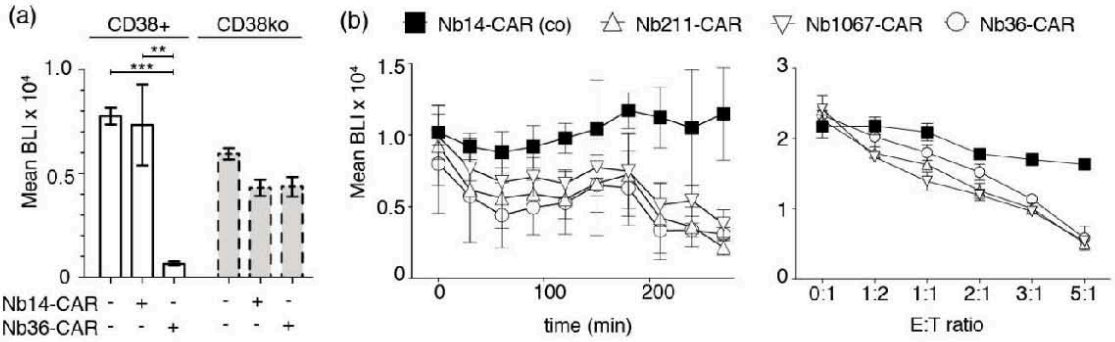

Figure 5. Luminescence-based CARDCC assay of CD38-expressing tumor cells. (**a**) CD38$^+$ or CD38ko CA-46 cells stably transduced with luciferase were incubated for 4 h at 37 °C in the absence (−) or presence (+) of NK-92 cells expressing either the control Nb14-CAR or the CD38-specific Nb36-CAR at an effector-to-target ratio of 4:1. Bioluminescence signal intensities (BLI) were measured 20 min after addition of luciferin. Significance between CD38-specific and control Nb-CAR-NK cells was calculated using unpaired T-test (**, $p < 0.01$; ***, $p < 0.005$). (**b**) The left and right panels show kinetic and dose response analyses of CARDCC toward luciferase-expressing CA-46 cells. Left panel: Nb-CAR-NK cells were co-incubated with CA-46 cells for the indicated times at 37 °C at an effector-to-target ratio of 1:1 before addition of luciferin and bio-luminometric analysis. Right panel: Nb-CAR-NK cells were co-incubated with CA-46 cells for 4 h at 37 °C at the indicated effector-to-target (E:T) ratios before bioluminometry. Results are representative for three similar experiments.

3.4. A Flow-Cytometric CARDCC

We next set out to monitor CARDCC by flow cytometry. As a means to distinguish target from effector cells, we labeled Nb-CAR-NK cells with the fluorescent dye eFluor450. To monitor cell death, we used the DNA staining dye propidium iodide, which is excluded by living cells. We co-incubated eFluor450-labeled Nb-CAR-NK cells with CA-46 cells for 4 h at 37 °C before addition of propidium iodide and flow-cytometric analysis (Figure 6a). The results confirm that NK cells expressing the CD38-specific Nb36-CAR, but not those expressing the control Nb14-CAR, indeed effectively lyse CD38-expressing tumor cells. CARDCC caused both—staining by propidium iodide and a decrease in forward light scatter (FSC). The latter likely reflects the shrinkage of lysed cells.

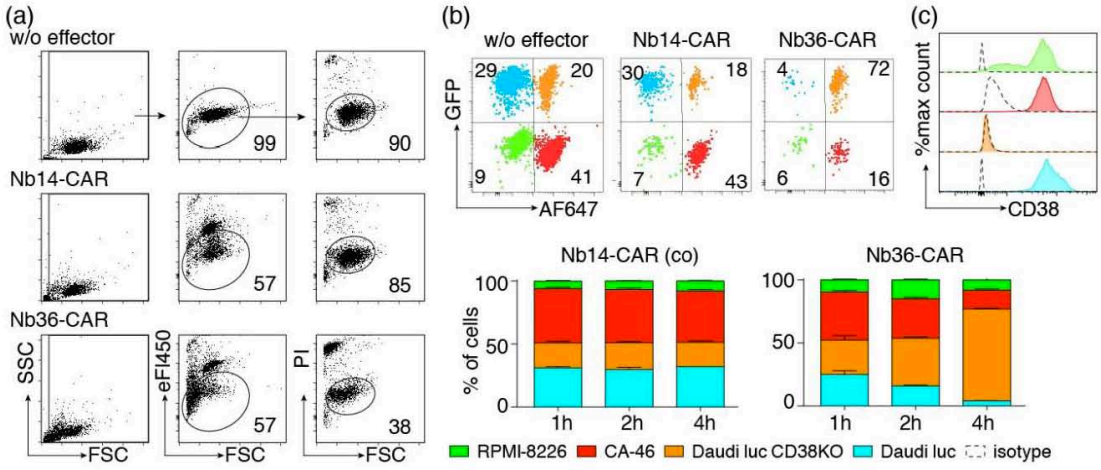

Figure 6. Flow-cytometric CARDCC assay of CD38-expressing tumor cells. (**a**) CA-46 cells were co-incubated for 4 h at 37 °C without (w/o) or with eFluor450-labeled NK-92 cells expressing either the control Nb14-CAR or the CD38-specific Nb36-CAR in an effector to target ratio of 1:1. Cells were analyzed by flow cytometry 10 min after addition of propidium iodide. Numbers indicate the percentage of cells in the gated population. (**b**) A mixed population of four color-coded cell lines was incubated for 1–4 h at 37 °C with or without (w/o) eFluor450-labeled Nb-CAR-NK cells at an effector-to-target ratio of 1:1. $CD38^{lo}$ RPMI-8226 cells were unlabeled. $CD38^{hi}$ CA-46 cells were pre-labeled with Alexa Fluor 647. $CD38^{+}$ and CD38ko Daudi luc cells were GFP positive, CD38ko Daudi cells were pre-labeled with AlexaFluor647. Gating was performed on live (PI-negative), target (eF450-negative) cells. (**c**) To detect cell surface levels of CD38, the target cells were stained with AlexaFluor647-conjugated MU1067-hcAb and with an AlexaFluor647-conjugated control hcAb (dashed lines). Color coding of cells is as in (b). Results are representative for three similar experiments.

We next set out to determine whether Nb-CAR-NK cells could specifically deplete CD38-expressing cells in mixed populations of $CD38^{+}$ and $CD38^{-}$ cells. To this end we co-incubated $CD38^{+}$ and CD38ko Daudi cells, CD38hi CA-46 cells and $CD38^{lo}$ RPMI-8226 cells for 1–4 h with eFluor450-labeled Nb-CAR-NK cells (Figure 6b). The four target cell lines were distinguished on the basis of GFP expression (Daudi luc and Daudi luc CD38ko) and pre-labeling of cells with Alexa Fluor 647 (CA-46 and Daudi luc cells). The results show that CD38-specific but not control CAR-NK cells specifically deplete CD38 expressing cells.

3.5. CD38-Directed Nb-CAR-NKs Specifically Deplete $CD38^{+}$/$CD56^{+}$ Myeloma Cells from Primary Human Bone Marrow Samples

In the final set of experiments we assessed CARDCC against primary multiple myeloma cells from bone marrow samples of eight human myeloma patients. Myeloma cells were identified by their high levels of cell surface CD38 (and CD56 or CD319). The percentage of such cells in the patient samples ranged from 1.4 to 16.9% (mean 7.1%). We excluded dead cells on the basis of low FSC and staining by PacO. Effector Nb-CAR-NK cells were excluded by staining with eFluor450. In order to permit quantification of absolute cell numbers we added counting beads to the samples (Figure 7a). We found that incubation of bone marrow samples with CD38-specific Nb-CAR-NK cells mediates a significantly higher loss of myeloma cells than incubation with control Nb-CAR-NK cells (Figure 7b).

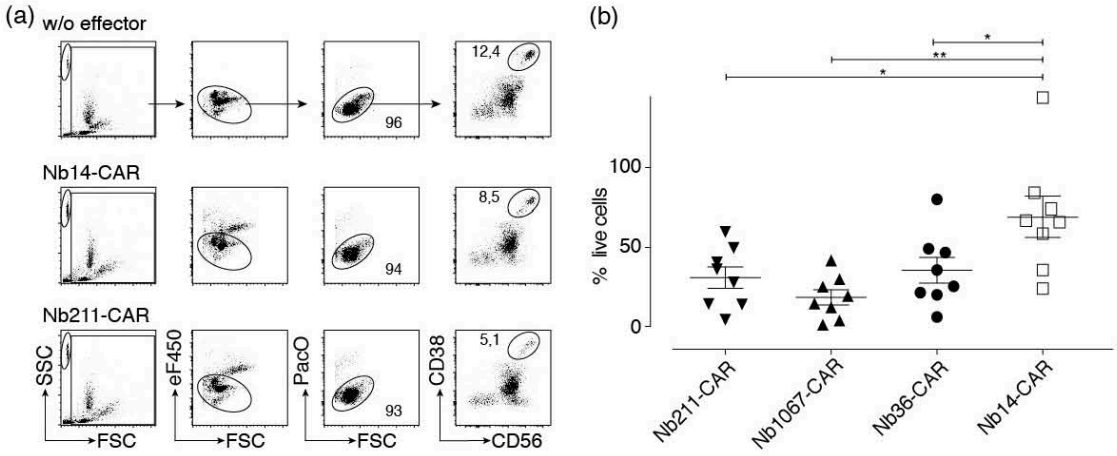

Figure 7. CD38-specific Nb-CAR-NKs deplete CD38$^+$ myeloma cells. Primary human bone marrow cells were co-incubated for 4 h at 37 °C without (w/o) or with eFluor450-labeled NK-92 cells expressing either a control CAR (Nb14) or a CD38-specific CAR (Nb211) in an effector to target ratio of 1:1. Cells were counterstained with fluorochrome conjugated αCD56, αCD319, and a CD38-specific Nb that binds a non-overlapping epitope to that of the Nb contained in the CAR before analysis by flow cytometry. (**a**) Beads (SSC^{hi}/FSC^{lo}), effector cells (eF450$^+$), and dead cells (PacO^{hi}/FSC^{lo}) were excluded by gating. Numbers indicate the percentage of cells in the gated population. Results are representative for eight similar experiments (one for each of eight patient samples). (**b**) The number of surviving myeloma cells (CD38$^+$/CD56$^+$ or CD38$^+$/CD319$^+$) were determined with the aid of cell counting beads. Percentages of surviving myeloma cells were calculated relative to the number of surviving myeloma cells in the absence of effector cells (set at 100%). Gating was performed as in (**a**). Significance between CD38-specific and control Nb-CAR-NK cells was calculated using unpaired T-test (*, $p < 0.05$; **, $p < 0.01$)

4. Discussion

Our results demonstrate that CD38-specific nanobodies provide effective ligands for chimeric antigen receptors. We show that the established human NK-92 cell line can be retrovirally transduced to stably express nanobody-based CARs. CAR-expressing NK cells, as a permanent cell line, have the potential to serve as an "off-the-shelf" reagent [16,23]. NK-92 cells were originally isolated from the peripheral blood of a 50-year-old patient with non-Hodgkin Lymphoma in 1992 [24]. These cells resemble the phenotype of an activated NK cell. In phase-I trials, NK-92 cells were found to have a high safety profile and to induce only mild graft-versus-host-disease [25,26].

Our results support previous studies demonstrating the potential of nanobody-based CARs [27–30]. Here we used CARs constructed from nanobodies directed against three different epitopes of CD38. All of these mediated effective CARDCC indicate that these epitopes of CD38 are accessible to the CARs. The results further suggest that there is not a preferred epitope of CD38 for CAR-mediated cytotoxicity.

Our data are also in line with those of previous studies using CD38-specific CARs based on scFvs and Nbs (Figure 8). Mihara et al. and Drent et al. fused the single-chain variable fragment of CD38-specific mAbs via the hinge and transmembrane domains of CD8a to the signal transduction domains of 4-1BB and CD3ζ [31,32]. An et al. used a similar architecture for a nanobody-based CAR [27]. More recently, Drent et al. replaced the 4-1BB costimulatory domain with a CD28-costimulatory domain and co-expressed 4-1BBL to provide additional 4-1BB signaling [33]. Our CAR-constructs in turn use the IgG4 hinge and the CD28 transmembrane region as a bridge to the signal transduction domains of CD28, 4-1BB, and CD3ζ. In all cases, cysteine residues in the extracellular linker likely mediated the formation of covalently linked dimers. It remains to be determined whether the efficacy of these constructs can be enhanced further by adjusting the length of the extracellular stalk, preventing disulfide formation, e.g., by site directed mutagenesis of cysteine to serine, and/or by replacing the

signal transduction modules derived from the T-cell surface proteins with signal transduction modules derived from NK cell surface proteins.

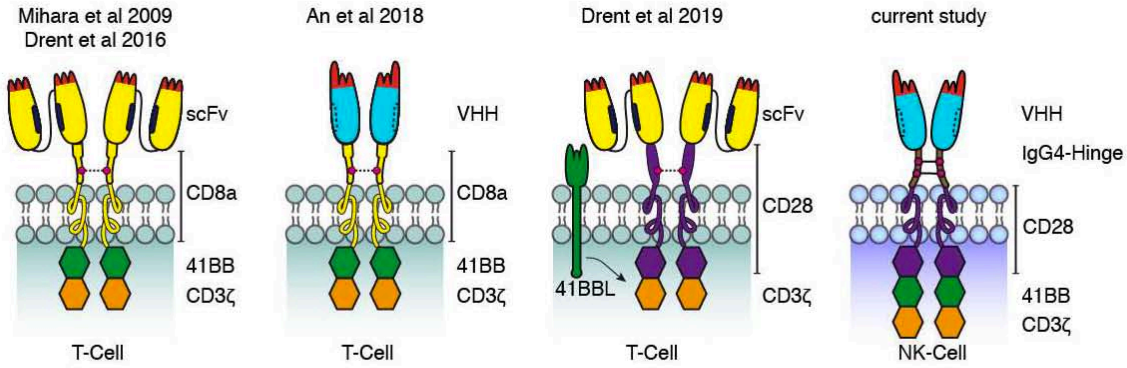

Figure 8. Comparison of CD38-specific CARs used in the current and previous studies. Schematic diagrams illustrating the structures of CD38-specific conventional and Nb-based CARs used in this study to transduce NK-92 cells and in previous studies to transduce T cells. The first conventional and Nb-based CARs reported consisted of a CD38-specific scFv or Nb connected via the hinge and transmembrane domains of CD8a to the signal transduction domains of 4-1BB and CD3ζ [31,32]. Recently, a more effective conventional CAR was reported carrying the hinge, transmembrane, and signal transduction domains of CD28 and a separate expression-cassette for 4-1BBL transduced into T cells. The Nb-CAR of the current study carries the IgG4 hinge and three cytosolic signal transduction domains.

The interaction between CAR-expressing effector cells and target cells expressing the cognate antigen presumably involves multivalent binding, i.e., the interaction of effector and target cells might encompass the simultaneous engagement of many CARs and cognate antigens on the respective surfaces of effector and target cells. Because non-tumor cells also express CD38, it would be beneficial for cancer therapy if Nb-CAR-NK cells would preferentially deplete $CD38^{high}$ cells. We found that RPMI cells with slightly lower levels of CD38 were more resistant to CARDCC after 2 h of co-incubation with Nb-CAR-NK cells. However, we cannot exclude that other cell surface proteins such as MHCI or inhibitory NK cell receptors influence the susceptibility to CARDCC.

The affinity of the ligand-binding domain of the CAR may also influence the relative cytotoxicity of CAR-expressing cells to target cells that express different levels of the target antigen [34–36]. A low-affinity CAR may preferentially direct CAR-expressing T cells or NK cell to tumor cells that express high surface levels of the target antigen, while sparing normal cells that express lower levels of the target antigen, thereby reducing the potential off target toxicity of CAR-T and CAR-NK cells. Since nanobodies consist of only a single immunoglobulin domain, it will likely be technically easier to modulate the affinity of a nanobody-based CAR than that of a scFv-based CAR [7,8,11].

The specificity may be enhanced by co-targeting of distinct targets, i.e., to two membrane proteins that are co-expressed on tumor cells [37]. This would reduce the unwanted binding of CAR-T or CAR-NK cells to normal cells that express only one of these two target proteins. Based on their excellent solubility and reformating capacity, nanobodies may prove more suitable than conventional scFvs for such approaches [38].

5. Conclusions

Nanobody-based chimeric antigen receptors (Nb-CARs) may provide a basis for the clinical development of novel therapeutics to target CD38-expressing tumor cells.

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3.3 Half-Life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers Induce Killing of Multiple Myeloma Cells

Als drittes soll in diesem Kapitel die Publikation „*Half-Life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers Induce Killing of Multiple Myeloma Cells“* vorgestellt werden, die 2022 im Journal *Frontiers in Immunology* veröffentlicht wurde. Bei dieser Publikation bin ich Erstautorin, da ich bis auf die in Figure 2C und Figure 4B gezeigten Ergebnisse alle Experimente durchgeführt, alle Abbildungen erstellt und das Manuskript maßgeblich mitverfasst habe.

Titel: Half-Life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers Induce Killing of Multiple Myeloma Cells

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Half-Life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers Induce Killing of Multiple Myeloma Cells

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CD38 is a target for immunotherapy of multiple myeloma. Llama-derived CD38-specific nanobodies allow easy reformatting into mono-, bi- and multispecific proteins. To evaluate the utility of nanobodies for constructing CD38-specific nanobody-based killer cell engagers (nano-BiKEs), we generated half-life extended nano-BiKEs (HLE-nano-BiKEs) by fusing a CD38-specific nanobody to a CD16-specific nanobody for binding to the Fc-receptor on NK cells and further to an albumin-specific nanobody to extend the half-life *in vivo*. HLE-nano-BiKEs targeting three different epitopes (E1, E2, E3) of CD38 were expressed in transiently transfected HEK-6E cells. We verified specific and simultaneous binding to CD38 on myeloma cells, CD16 on NK cells, and to albumin. We tested the capacity of these HLE-nano-BiKEs to mediate cytotoxicity against CD38-expressing multiple myeloma cell lines and primary myeloma cells from human bone marrow biopsies in bioluminescence and flowcytometry assays with NK92 cells as effector cells. The results revealed specific time- and dose-dependent cytolysis of CD38+ myeloma cell lines and effective depletion of CD38-expressing multiple myeloma cells from primary human bone marrow samples. Our results demonstrate the efficacy of CD38-specific HLE-nano-BiKEs *in vitro* and *ex vivo*, warranting further preclinical evaluation *in vivo* of their therapeutic potential for the treatment of multiple myeloma.

Keywords: bispecific engager, nanobody, multiple myeloma, BiKE, CD38, darzalex, daratumumab

INTRODUCTION

Multiple myeloma (MM) is a hematological disorder characterized by clonal expansion of plasma cells in the bone marrow. MM causes nearly one in eighty cancer-induced deaths worldwide (1, 2). MM leads to bone, renal, hematological, and infectious complications due to space constraints in the bone and the production of pathogenic antibodies (3). Survival of MM patients has improved with new drugs and autologous stem cell transplantation. Despite this progress, the majority of MM patients relapse (4), underlining the need for more effective treatment options with higher specificity and fewer side effects (5–7).

The NAD-metabolizing ecto-enzyme CD38 is overexpressed by MM cells and other hematological malignancies. This makes it a promising target for immunotherapies as illustrated by the approval of CD38-specific monoclonal antibodies daratumumab and isatuximab by the FDA for the treatment of relapsed MM patients (8–10).

With improvements in the field of antibody engineering, the scientific focus is shifting from conventional monoclonal CD38-specific antibodies towards recombinant antibody-based constructs, such as chimeric heavy-chain antibodies (11–14), bispecific or biparatopic constructs (15–17), chimeric antigen receptors (CARs) (18, 19), bispecific T cell engagers (BiTEs) (20, 21), and bispecific killer cell engagers (BiKEs) (22–26). BiKEs co-target the FcγRIII-Receptor CD16 on NK cells and a tumor cell surface protein, thereby engaging cytolytic NK cells to kill tumor cells (27–29).

Redirecting NK cells to MM cells via a CD38-specific BiKE therefore may provide an interesting option for the treatment of MM (25, 30). BiKEs often use two single chain variable fragments (scFvs) as binding motifs. The serum half-life of scFv-based BiKEs can be extended by adding an Fc part to the scFvs or using Fab-Fragments instead of the scFvs (HLE-BiKEs). By adding a second tumor-specific scFv or IL-15 to the BiKE, a trimeric killer cell engager (TriKE) can be generated (**Figure 1A**) (31).

Nanobodies are single variable immunoglobulin domains derived from heavy chain antibodies (hcAbs) that naturally occur in camelids (32, 33). Nanobodies show high solubility and are therefore particularly suited for reformatting in a Lego brick like fashion into fusion proteins (34, 35), including heavy chain antibodies and BiKEs (36). Due to their high solubility and small size, nanobody-based BiKEs (nano-BiKEs, ca. 30kDa) might provide better stability and higher tissue penetration in vivo than scFv-based BiKEs (ca. 50 kDa) (**Figure 1A**) (33, 37). However, the small size of nanobody dimers lies below that of the renal filtration barrier, accounting for a short serum half-life in vivo. This can be overcome by fusion of a nanobody-based BiKE to an albumin-specific nanobody (38–40), allowing nanobody-trimers, e.g. half-life extended nano-BiKEs (HLE-nano-BiKEs) (**Figure 1A**), to "piggy-back" on circulating albumin, thereby hindering renal filtration (41).

The aim of this study was to explore the utility of nanobodies recognizing three non-overlapping epitopes on CD38 for constructing CD38-specific HLE-nano-BiKEs and to evaluate their therapeutic potential *in vitro* and *ex vivo*.

MATERIALS AND METHODS

Cell Lines

Human cell lines NK92 (natural killer cell line), OPM-2 and LP-1 (myeloma cell lines), and CA-46 and Daudi (Burkitt lymphoma cell lines) were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). We generated OPM-2 luc, LP-1 luc, CA-46 luc, and Daudi luc cell lines stably expressing the luc2 variant of *Photinus pyralis* luciferase (Promega, Madison, WI) under control of the spleen-focus-forming virus U3 region (SFFV promoter) by lentiviral transduction (15). HEK-6E cells (42) were kindly provided by Yves Durocher, Ottawa, Canada.

Transduced cells were selected in culture medium containing 1 µg/mL puromycin and subsequently sorted by FACS based on eGFP expression (FACS Aria III, BD Biosciences, Heidelberg, Germany). Sorted cells were kept in culture and luciferase-expression was controlled regularly following addition of luciferin using a luminometric plate reader. NK92 cells were stably transfected with human CD16 and eGFP by retroviral transduction using the pSF91 vector. The sequence for CD16, i.e. the ectodomain of FcγRIII fused to the transmembrane and cytosolic domains of FcεRI was kindly provided by Béatrice Clémenceau, Nantes, France (43). The CD38 gene in NK92, NK92 hCD16, and LP-1 luc cells was inactivated using CRISPR/Cas9 technology (sc-401117-NIC, Santa Cruz Biotechnology) as previously described (15). Cell surface levels of CD38 were determined by staining for 30 min at 4°C with CD38-specific AlexaFluor647-conjugated JK36 hcAb or the ARTC2.2-specific s-14 hcAb as isotype control.

Generation of Nanobody-Based BiKEs

The human CD38-specific nanobodies WF211, MU1067, JK36 and the ARTC2.2-specific control nanobody s-14 (co) were generated from immunized llamas as described previously (44–46). WF211 binds Epitope 1 (E1), MU1067 binds Epitope 2 (E2), and JK36 binds Epitope 3 (E3) on CD38. For the sake of clarity, we use abbreviated designations for HLE-nano-BiKEs in the figures, e.g., HLE-nBiKE E1, HLE-nBiKE E2, and HLE-nBiKE E3 (**Figures 1B, C**).

The sequence coding for the CD16-specific nanobody c21 was obtained from published work by Ghislaine Behar, Paris, France (47). The sequence of the albumin-specific nanobody Alb11 was obtained from patent US20070269422A1. Nanobody Alb11 mediates *in vivo* half-life extension of monomeric and dimeric nanobodies by retarding renal filtration (48). The respective off-rates (k_{diss}) of these nanobodies have been published: WF211 $4.5x10^{-3}$ (s^{-1}), JK36 $2x10^{-4}$ (s^{-1}), MU1067 $1.2x10^{-4}$ (s^{-1}) (44), c21 $2.3x10^{-3}$ (s^{-1}) and Alb11 $9.8x10^{-4}$ (s^{-1}) (47, 48).

HLE-nano-BiKE E1, HLE-nano-BiKE E2, HLE-nano-BiKE E3, and isotype control HLE-nano-BiKE (HLE-nano-BiKE co) were generated by subcloning the coding region for the respective CD38-specific nanobody or control nanobody upstream of the coding regions for c21 and Alb11, with intervening, flexible gly-ser linkers and cloned into the pCSE2.5 vector [kindly provided by Tim Schirrmann, Braunschweig, Germany (49)]. HLE-nano-BiKEs were expressed in transiently transfected HEK-6E cells (50) cultivated in serum-free medium as described previously (51). Supernatants were harvested six days post transfection. HLE-nano-BiKEs were purified from the supernatants by affinity chromatography using protein A sepharose (51). Purity of antibody constructs was assessed by SDS-PAGE and InstantBlue™ Coomassie staining. Daratumumab (Darzalex) was purchased from Janssen-Cilag, Neuss, Germany, to be

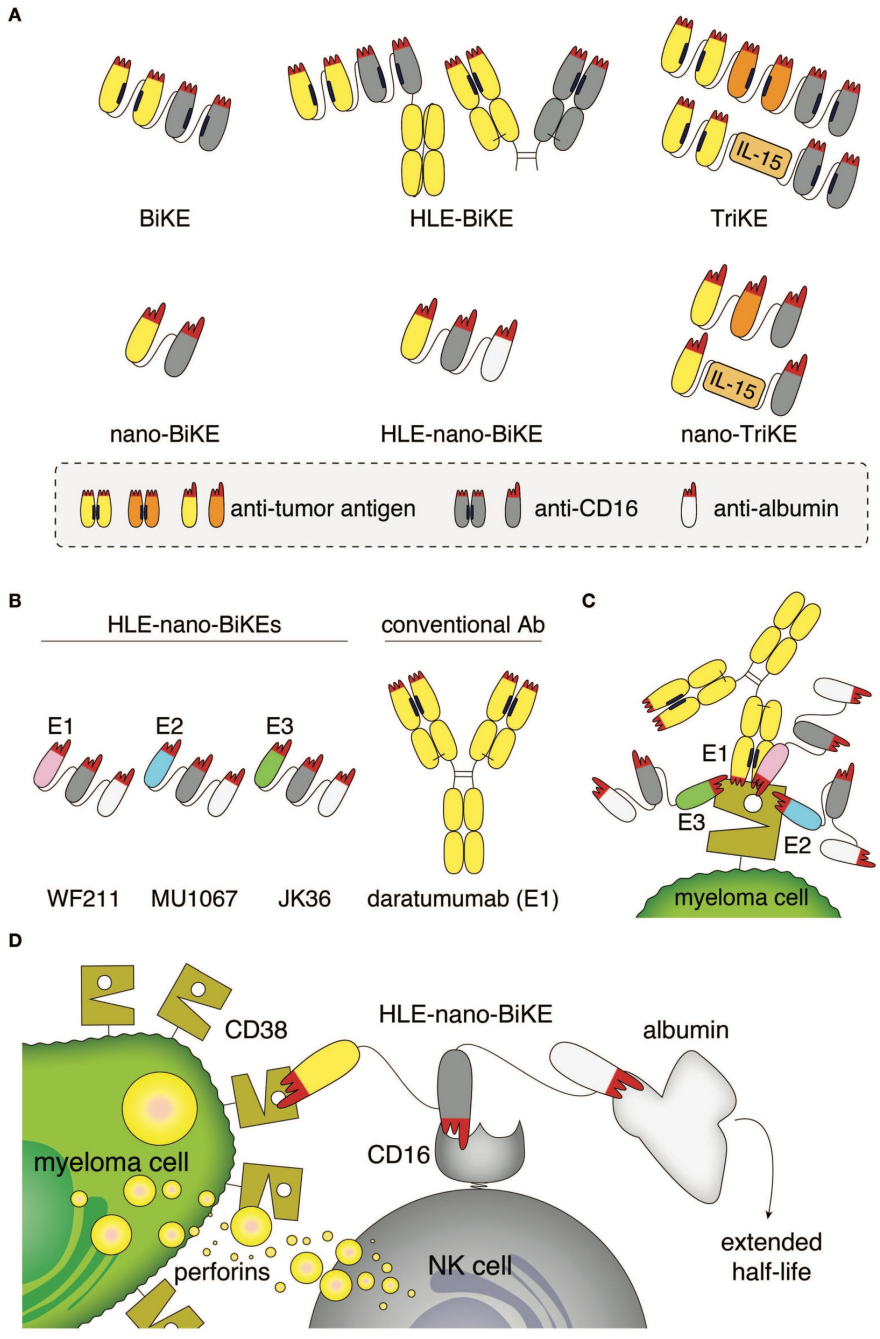

FIGURE 1 | Structure, binding sites, and mode of action of half-life extended CD38-specific nanobody-based bispecific killer cell engagers (HLE-nano-BIKEs). **(A)** Scheme of bispecific and trispecific killer cell engagers (BiKEs, TriKEs) based on scFvs (top) or nanobodies (bottom). Tumor-specific and CD16-specific modules are indicated in yellow/orange and grey, respectively. Half-life extension (HLE) can be mediated by fusion to an Fc-fragment (top) or an albumin-specific nanobody (white, bottom). The paratope of each variable domain (corresponding to the CDR loops) is shown in red. Nano-TriKEs could be generated in the future by fusion of a nano-BiKE to a second tumor-specific nanobody or IL-15. **(B)** Scheme of CD38-specific HLE-nano-BiKEs (45 kDa) consisting of three nanobodies linked *via* flexible glycine-serine linkers. The N-terminal nanobody in each CD38-specific HLE-nano-BiKE recognizes one of three distinct epitopes of CD38: WF211 (pink, epitope 1 [E1]), MU1067 (blue, epitope 2 [E2]), and JK36 (green, epitope 3 [E3]). The central nanobody (grey) recognizes CD16 (FcγIII receptor on NK cells) and the C-terminal nanobody recognizes albumin (white). Conventional human antibody daratumumab (150 kDa) is indicated in yellow. **(C)** Scheme of the binding sites of daratumumab and the three HLE-nano-BiKEs. WF211-based HLE-nano-BiKE E1 recognizes an epitope (E1) that overlaps with that of daratumumab, MU1067-based HLE-nano-BiKE E2 and JK36-based HLE-nano-BiKE E3 bind independent epitopes (E2, E3). **(D)** Scheme of the proposed mode of action of a CD38-specific HLE-nano-BIKE. The N-terminal nanobody (WF211, MU1067, or JK36) binds CD38 on the myeloma cell, the central nanobody binds and activates an NK cell by targeting CD16, and the C-terminal nanobody extends the half-life of the construct by binding to albumin.

used as positive control in our killing assays. Biotinylated human albumin (ab8033) was purchased from Abcam, Cambridge, United Kingdom.

Binding of BiKEs

Binding of HLE-nano-BiKEs was assessed by incubation of LP-1 luc cells or NK92 hCD16 CD38KO cells with 100 nM HLE-nano-BiKEs for 15 minutes. To detect specific binding of HLE-nano-BiKEs, biotinylated human albumin was added and detected with PE-Cy7 conjugated streptavidin (Becton Dickinson, NJ, USA). Control staining was performed with albumin and PE-Cy7-conjugated streptavidin alone. Cell-associated fluorescence was determined by flow cytometry.

Biolayer Interferometry

The extracellular domain of human CD38 (aa 46–300) was produced as a secretory protein with a His6x-Myc epitope tag in transiently transfected HEK-6E cells. The tagged protein was purified using immobilized metal affinity chromatography (IMAC). The purified protein was biotinylated using the EZ-Link™ Sulfo-NHS-LC-Biotin (A39257) from Thermo Fisher Scientific (Waltham, MA, USA) according to manufacturer's instructions. Recombinant CD16a (A42536) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human albumin (Alburex® 20) was purchased from CSL Behring (PA, USA).

Binding of the HLE-nano-BiKEs to CD38, CD16a, and albumin was determined by BLI-technology using a fortéBIO BLItz instrument. Assays were performed at 20°C with running buffer (PBS, 0.002% (v/v) Tween-20). Streptavidin sensors were hydrated in running buffer and loaded for 30 seconds with biotinylated CD38 (3.7 µM). After washing for 60 sec purified HLE-nano-BiKEs (E1, E2, E3, or control) (1.33 µM) were allowed to associate for 60 sec on immobilized CD38, followed by dissociation for 45 sec. Then, binding of CD16 (0.8 µM) to the bound BiKE was allowed for 60 sec followed by a 45 sec dissociation phase. Finally, albumin (6 µM) was added for 60 sec followed by 120 sec of dissociation. Curve fitting and affinity calculations were performed using Graph Pad Prism (version 7).

BiKE-Dependent Cellular Cytotoxicity (BiKE-DCC)

Cytotoxicity of CD38-specific HLE-nano-BiKEs was assessed using OPM-2 luc, LP-1 luc, CA-46 luc, or Daudi luc cells expressing CD38 and LP-1 luc CD38KO cells as negative control. NK92 hCD16 cells were used as effectors with NK92 cells as control for specificity of NK92 hCD16 cell activation through CD16. To distinguish cells in mixed populations, cells were strategically labeled with eFluor450 or eFluor670. LP-1 luc CD38KO cells were labeled with eFluor670, washed and then mixed with unlabeled LP-1 luc cells in a 1:1 ratio. Next, the indicated concentrations of HLE-nano-BiKEs were added and 15 min later, NK92 or NK92 hCD16 cells were added in the indicated effector to target ratio. Cells were incubated in αMEM culture medium supplemented with 10% fetal calf serum (FCS), 10% horse serum, 5 mM glutamine, and 5 ng/ml interleukin 2 (IL-2 Proleukin-S, Novartis, Basel, Switzerland) at 37°C for 3 h. Assays were performed without and in the presence of 16 mg/mL human albumin as indicated. Cells were then stained with 20 µg/mL propidium iodide (PI) to identify dead cells and analyzed by flow cytometry. Alternatively, D-luciferin (Biosynth, Staad, Switzerland) was added as substrate (75 µg/ml) for 20 min and bioluminescence-intensity (BLI) was measured using a microplate reader (Victor³, Perkin Elmer, Boston, USA). Percentage of lysed cells was calculated as follows:

$$percent\ of\ BLI\ [\%] = \left(\frac{BLI[sample]}{BLI[sample\ w/o\ BiKE]}\right) x100\,\%$$

For kinetic analyses, NK92 hCD16 cells were pre-incubated for 30 min with 100 nM HLE-nano-BiKEs or daratumumab and unbound constructs were removed by centrifugation. LP-1 luc were added at different ratios in αMEM culture medium supplemented with 10% FCS, 10% horse serum, 5 mM glutamine, and 5 ng/ml interleukin 2. D-luciferin (Biosynth, Staad, Switzerland) was added (75 µg/ml). Cells were incubated at 37°C and measurement of BLI was performed using a microplate reader (Victor³, Perkin Elmer, Boston, USA). Percentage of lysed cells was calculated as described above.

Fresh bone marrow aspirates were obtained from patients after Institutional Review-Board-approved consent (PV5505). Bone marrow mononuclear cells (BM-MNCs) were prepared by Ficoll-Paque density gradient centrifugation and subsequent depletion of remaining erythrocytes using red blood cell lysis buffer (NH4Cl + KHCO3 + EDTA). BM-MNCs were pre-incubated with 10 nM HLE-nano-BiKEs or daratumumab for 15 min before addition of NK92 hCD16 cells in an effector to target ratio of 3:1. Cells were then stained with a panel of fluorochrome-conjugated antibodies (CD38, CD45, CD138/229, CD269/CD319/CD56, CD19) and the viability dye Pacific Orange and analyzed *via* flow cytometry. Staining of CD38 was achieved with AlexaFluor647-conjugated nanobodies that bind independently of the nanobody contained in the HLE-nano-BiKE, i.e. $JK36^{AF647}$ or $MU523^{AF647}$ for HLE-nano-BiKE E1 and daratumumab, $MU523^{AF647}$ or $WF211^{AF647}$ for HLE-nano-BiKE E2, and $JK36^{AF647}$ or $WF211^{AF647}$ for HLE-nano-BiKE E3. An FSC threshold was set to exclude debris while including the population of small $CD19^{+}$ B cells. Dead cells were excluded using PacO staining. NK92 hCD16 cells were excluded by eGFP-expression. MM cells were identified by co-expression of CD138, and CD38. Numbers of MM cells were determined using CountBright absolute counting beads (Invitrogen). Percentage of lysed MM cells was calculated as follows:

$$percent\ of\ lysis\ [\%] - \left(1 - \left(\frac{MM\ cell\ number\ per\ \mu L[sample]}{MM\ cell\ number\ per\ \mu L[sample\ \ w/o\ \ BiKE]}\right)\right) x100\,\%$$

Significant differences in surviving cells treated with CD38-specific HLE-nano-BiKEs vs. control HLE-nano-BiKE was calculated using One-way ANOVA followed by a Holm-Sidak test (GraphPad Prism, GraphPad Software, CA, USA).

RESULTS

Specific and Simultaneous Binding of HLE-nano-BiKEs to CD38, CD16, and Albumin

We purified HLE-nano-BiKEs from the supernatants of transiently transfected HEK-6E cells using protein A and verified the integrity and purity of these constructs by SDS-PAGE and Coomassie staining (**Figure 2A**). Specific binding of HLE-nano-BiKEs to CD38 and CD16 was analyzed on CD38+/CD16- LP-1 cells and CD38-/CD16+ NK92 cells. Bound HLE-nano-BiKEs were detected with biotinylated albumin and PE-conjugated Streptavidin. This staining strategy allowed us to verify the functionality of the albumin-specific nanobody used in the trimeric construct (**Figure 2B**). Regardless of their epitope specificity (E1, E2, or E3), all three CD38-specific HLE-nano-BiKEs (E1, E2, and E3) bound CD38-expressing LP-1 luc cells. The control HLE-nano-BiKE co did not bind to CD38-expressing LP-1 luc cells, substantiating the specific binding of the CD38-specific HLE-nano-BiKEs *via* their CD38-specific nanobodies (WF211, MU1067, or JK36) to CD38 on the surface of LP-1 cells.

The three CD38-specific HLE-nano-BiKEs and the isotype-control HLE-nano-BiKE bound to NK92 hCD16 CD38KO cells. This verifies specific binding of the CD16-specific nanobody in the trimeric BiKE to CD16 on the effector NK92 cells. Further, the detection system with biotinylated human albumin confirmed the functionality of the albumin-specific nanobody in our HLE-nano-BiKEs.

Biolayer interferometry was used to determine whether HLE-nano-BiKEs allow for simultaneous binding of CD38, CD16, and albumin (**Figure 1D**). After binding of biotinylated CD38 to a streptavidin sensor, HLE-nano-BiKEs (E1, E2, or E3), recombinant CD16, and albumin were added sequentially. The results show successive incremental increases in signal intensities upon addition of a CD38-specific HLE-nano-BiKE, CD16, and allow for simultaneous binding of CD38, CD16 and albumin (**Figure 2C**). HLE-nano-BiKEs E2 and E3 showed comparable and relatively high signal intensities. Signal increase was lower for HLE-nano-BiKE E1. HLE-nano-BiKE E1 contains the CD38-

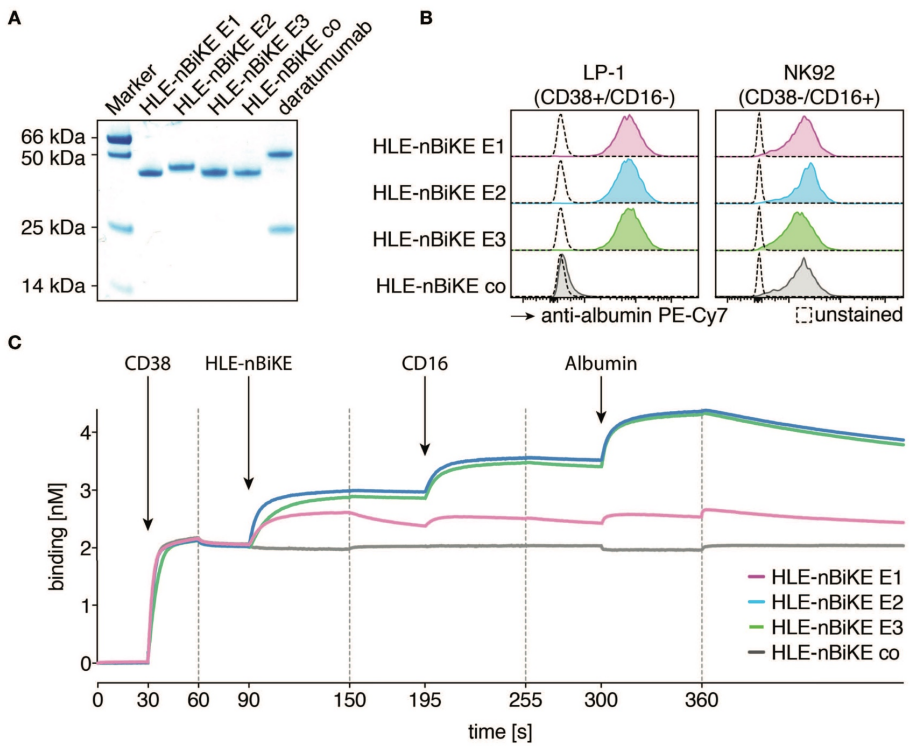

FIGURE 2 | Purified CD38-specific HLE-nano-BiKEs bind specifically and simultaneously to myeloma cells, NK cells, and albumin. **(A)** Purified HLE-nano-BiKEs and daratumumab (1 µg per lane) were size fractionated by SDS-PAGE under reducing conditions and visualized by Coomassie staining. **(B)** LP-1 luc myeloma cells (left panel) and NK92 hCD16 cells (right panel) were incubated with CD38-specific HLE-nano-BiKE E1, HLE-nano-BiKE E2, HLE-nano-BiKE E3, or an isotype control (HLE-nano-BiKE co). Bound HLE-nano-BiKEs were detected by flow cytometry using biotinylated human albumin and PE-Cy7-conjugated streptavidin by flow cytometry. Control stainings (unstained, dashed line) were performed with albumin and PE-Cy7-conjugated streptavidin alone. Results are representative of three similar experiments. **(C)** BLI analyses of the sequential binding of HLE-nano-BiKEs, CD16 and albumin to immobilized CD38. The biotinylated recombinant ectodomain of human CD38 was bound to a streptavidin-coated BLI sensor (30-90s). HLE-nano-BiKEs, recombinant ectodomain of human CD16, and human albumin were added at the times indicated. In each case binding was measured for 60s with a subsequent 60s dissociation phase. Arrows indicate the time point of protein addition; dashed lines indicate the start of the dissociation phase.

specific nanobody WF211, which has a lower affinity for CD38 than nanobodies MU1067 (HLE-nano-BiKE E2) and JK36 (HLE-nano-BiKE E3) (44). The isotype-control HLE-nano-BiKE showed no binding to CD38. These results demonstrate that CD38-specific HLE-nano-BiKEs can simultaneously bind CD38, CD16, and albumin.

CD38-Specific HLE-nano-BiKEs Specifically Induce NK92 Cell-Mediated Cytolysis of the CD38$^+$ LP-1 Myeloma Cell Line

The cytotoxic effect of CD38-specific HLE-nano-BiKEs was tested on LP-1 luc myeloma cells (**Figure 3**). We performed BiKE-dependent cellular cytotoxicity (BiKE-DCC) assays on a mixed suspension of GFP$^+$/CD38$^+$ and GFP$^+$/CD38KO LP-1 luc cells to control for specificity of BiKE-DCC to CD38 expressing target cells. Cytolysis was assessed by uptake and staining of cells for the DNA-binding dye propidium iodide. In parallel, we performed a BiKE-DCC assay with NK92 cells that lack cell surface CD16 to assess the dependency of BiKE-DCC on CD16.

The results reveal that all three CD38-specific HLE-nano-BiKEs specifically induce the killing of CD38-expressing LP-1 myeloma cells, but not of CD38KO LP-1 myeloma cells. Neither the isotype-control HLE-nano-BiKE co nor CD16-negative NK92 cells mediated killing of LP-1 myeloma cells. These results strongly suggest that the specific cytotoxic effect of our HLE-nano-BiKE is mediated by cross-linking CD38 on target myeloma cells with CD16 on NK92 effector cells.

HLE-nano-BiKEs Induce Cytolysis of Myeloma Cells by NK92 Cells in a Dose and Effector to Target-Ratio Dependent Manner

We next set out to assess the efficacy of BiKE-DCC and antibody-dependent cellular cytotoxicity (ADCC) mediated by daratumumab. For this we assessed the dependency of cytolysis on the concentration of HLE-nano-BiKEs or daratumumab and on the ratio of hCD16 effector cells to myeloma target cells (E:T-ratio). We used a luminescence-based assay with luciferase-transduced OPM-2 luc, LP-1 luc, CA-46 luc, and Daudi luc cells as target cells (**Figure 4**). These cell lines show moderate to high levels of CD38 on the cell surface (**Figure 4A**).

For assessment of BiKE-DCC, OPM-2 luc, LP-1 luc, CA-46 luc, and Daudi luc cells were incubated with increasing concentrations (0 nM to 10 nM) of HLE-nano-BiKEs or daratumumab and NK92 hCD16 cells at an E:T-ratio of 3:1. After 3h, luciferin was added and the BLI-Signal was measured (**Figure 4B**). The results show that the three CD38-specific HLE-nano-BiKEs mediate NK-cell cytotoxicity against all cell lines in a dose dependent fashion. BiKE-DCC and daratumumab

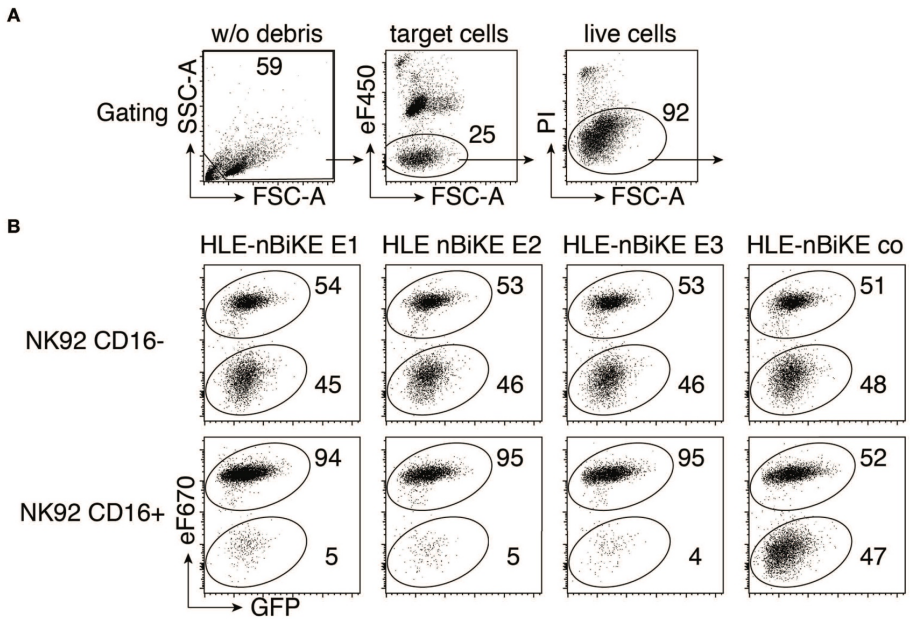

FIGURE 3 | CD38-specific HLE-nano-BiKEs specifically induce NK cell-mediated cytotoxicity toward CD38+ myeloma cells. **(A)** Effector NK92 cells were pre-labeled with eFluor450 (eF450). Gating was performed to exclude cellular debris (lower left corner) of panel 1, and then on target cells, identified as eF450-negative cells (panel 2). Dead cells were excluded by PI-staining. **(B)** CD38KO GFP+ LP-1 myeloma cells labeled with eFluor670 (eF670) (upper population) were mixed with unlabeled CD38-expressing GFP+ LP-1 cells (lower population) and the indicated HLE-nano-BiKEs before addition of eF450-labeled NK92 cells stably transfected with human CD16 (lower panels) or lacking CD16 (upper panels, negative control) at an effector-to-target ratio of 3:1. Cells were incubated for 3 h at 37°C and analyzed by flow cytometry in the presence of the DNA staining dye propidium iodide (PI). Loss of cells from the alive cells was used to monitor HLE-nano-BiKE-induced lysis of target cells. Gating was performed on target cells as described in **(A)** Numbers indicate percentage of cells in each quadrant. Results are representative of three similar experiments.

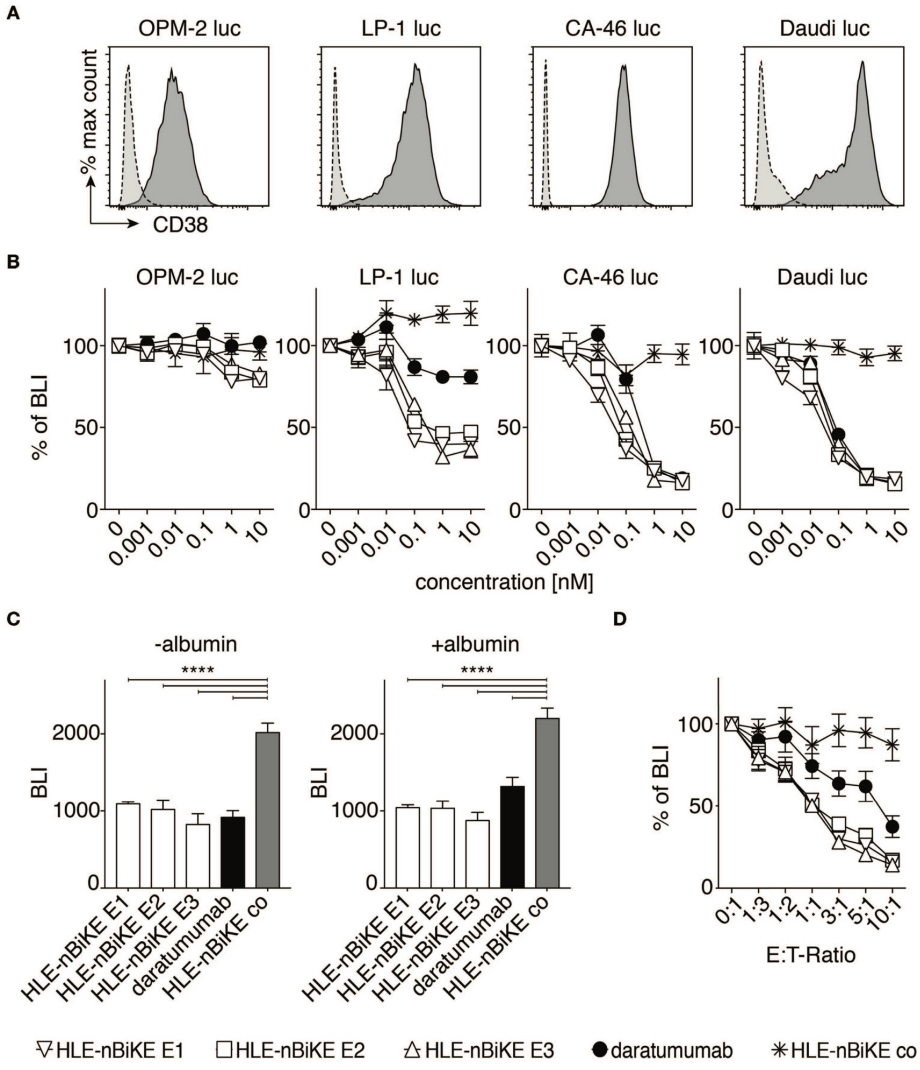

FIGURE 4 | Dose and effector to target-ratio (E:T-ratio) responses of BiKE-DCC. **(A)** Luciferase (luc) expressing OPM-2, LP-1, CA-46, and Daudi cells were incubated with AlexaFluor 647-conjugated CD38-specific JK36 hcAb (dark grey, solid line) or isotype control s-14 hcAb (light grey, dashed line) before analysis by flow cytometry. **(B)** NK92 hCD16 cells were incubated an E:T-ratio of 3:1 with OPM-2 luc, LP-1 luc, CA-46, or Daudi luc cells that had been pre-incubated with the indicated concentrations of HLE-nano-BiKEs or daratumumab for 3 h at 37°C before addition of luciferin and measurement of bioluminescence-intensity (BLI) with a plate reader. Data represent mean ± SEM of triplets and are representative for three independent experiments. **(C)** LP-1 luc cells were incubated for 15 min with 10 nM HLE-nano-BiKEs or daratumumab in the absence or presence of 16 mg/mL albumin. Then, NK92 hCD16 cells were added at an E:T-ratio of 3:1 and incubation continued for 95 min at 37°C before addition of luciferin and measurement of BLI. Bar diagrams illustrate the mean BLI of cells after incubation with HLE-nano-BiKEs or daratumumab. One-way ANOVA was performed and Holm-Sidak-adjusted p-values are indicated (****= $p < 0.0001$). **(D)** NK92 hCD16 cells were incubated at the indicated E:T-ratios (0:1-1:10) with LP-1 luc cells that had been pre-incubated with 10 nM HLE-nano-BiKEs or daratumumab and BLI was measured after 3 h of incubation at 37°C. Values indicate the mean BLI of cells incubated with 10 nM HLE-nano-BiKEs or daratumumab as percentage of the mean BLI of cells incubated in the absence of HLE-nano-BiKEs.

induced ADCC was lowest in OPM-2 luc cells, consistent with the relatively low cell surface levels of CD38 on these cells (**Figure 4A**).

Next, we aimed to determine whether the presence of albumin could impair HLE-nano-BiKE induced killing, i.e. by binding to the albumin-binding nanobody Alb11. LP-1 luc cells were incubated for 90 min with 10 nM HLE-nano-BiKEs or daratumumab and NK92 hCD16 cells at an E:T-ratio of 3:1 in the absence or presence of 16mg/mL albumin. The results show that all three CD38-specific HLE-nano-BiKEs (E1, E2, E3) as well as daratumumab effectively mediated NK-cell cytotoxicity against LP-1 myeloma cells. The presence of albumin impaired neither BiKE-DCC nor daratumumab mediated ADCC (**Figure 4C**).

We next compared the efficacies of BiKE-DCC vs. ADCC at different E:T-ratios using a saturating dose of 10nM BiKEs or daratumumab. The results again indicate that BiKE-DCC is more effective than ADCC (**Figure 4D**). The same degree of cytolysis was achieved at 3-10 fold lower E:T-ratios with CD38-

specific HLE-nano-BiKEs than with daratumumab. At this dose (10 nM) the isotype-control HLE-nano-BiKE co did not induce killing of myeloma cells, even at the highest E:T-ratio of 10:1.

Kinetics of CD38-Specific HLE-nano-BiKE-Induced Killing of Myeloma Cells

Next, we analyzed the kinetics of cytolysis induced by HLE-nano-BiKEs vs. ADCC induced by daratumumab using the saturating dose of 10 nM and E:T ratios of 5:1, 3:1, and 1:1 (**Figure 5**). The results reveal a much faster cytolysis induced by HLE-nano-BiKEs than by daratumumab. Again, the three CD38-specific HLE-nano-BiKEs recognizing different epitopes (E1, E2, E3) of CD38 induced cytolysis with a similar, time-dependent efficacy (**Figure 5**). Daratumumab also induced cytolysis in a time-dependent manner, but much delayed compared to the three HLE-nano-BiKEs, and again without reaching a maximal degree of cytolysis. Cells incubated with isotype-control HLE-nano-BiKE co and NK92 hCD16 cells showed a low degree of time-dependent cytolysis, especially at later time points, likely reflecting background (i.e. unspecific) cell death.

CD38-Specific HLE-nano-BiKEs Induce NK92 Cell Mediated Cytolysis of Primary Myeloma Cells

In a final set of experiments, we assessed the efficacy of BiKE-DCC compared to ADCC mediated by daratumumab against primary multiple myeloma cells from bone marrow samples of five myeloma patients, at 10 nM of HLE-nano-BiKEs or daratumumab and an E:T ratio of ~ 3:1 (**Figure 6**). MM cells were identified based on high cell surface levels of CD38 and CD138. The infiltration of MM cells varied from 3 to 35% (mean 13%). We excluded debris and dead cells based on low forward scatter and Pacific Orange-staining and NK92 hCD16 cells by their GFP-expression. Counting beads were added to the samples to permit quantification of absolute cell numbers. The results show that the CD38-specific HLE-nano-BiKEs and daratumumab induced NK92 hCD16 cell mediated cytolysis of primary CD138+/CD38+ MM cells with similar efficacies, while the isotype-control HLE-nano-BiKE co did not induce NK92 hCD16 cell mediated death of primary myeloma cells.

DISCUSSION

Our results demonstrate the feasibility of using CD38-specific HLE-nano-BiKEs to efficiently kill MM and Burkitt lymphoma cells. CD38-specific HLE-nano-BiKEs that recognize three different and non-overlapping epitopes of CD38 all induced potent cytotoxicity of tumor cell lines *in vitro* and of primary MM cells *ex vivo*.

Our CD38-specific HLE-nano-BiKEs all showed specific binding to CD38 on myeloma cells, CD16 on NK cells, and to human albumin. BiKE-DCC by HLE-nano-BiKEs was shown to rely on binding to both, CD38 on target myeloma cells and CD16 on effector NK cells. BiKE-DCC was dose- and time-dependent and effected by the E:T-ratio of NK effector cells to myeloma target cells. NK92 hCD16-mediated cytolysis was induced more effectively by our nanobody-based BiKEs (BiKE-DCC) than by the conventional CD38-specific antibody daratumumab (ADCC). Our results are in line with a recent publication by van Faassen et al., describing BiKEs directed against CD19, HER2 or EGFR that induced effective BiKE-DCC against cells that display the respective target on the cell surface (36).

Our trimeric HLE-nano-BiKEs were readily produced, purified and concentrated without showing any signs of aggregation. These features might overcome problems encountered previously with bispecific engagers constructed of scFvs, which are often difficult to produce, exhibit low solubility, and/or a tendency to aggregate, leading to developability issues (52–55). Key advantages of HLE-nano-BiKEs over scFv-based BiKEs are their high solubility, easy reformability, and small size. Moreover, our HLE-nano-BiKEs harness the advantages of the nanobody format for half life extension by linkage of the BiKE to

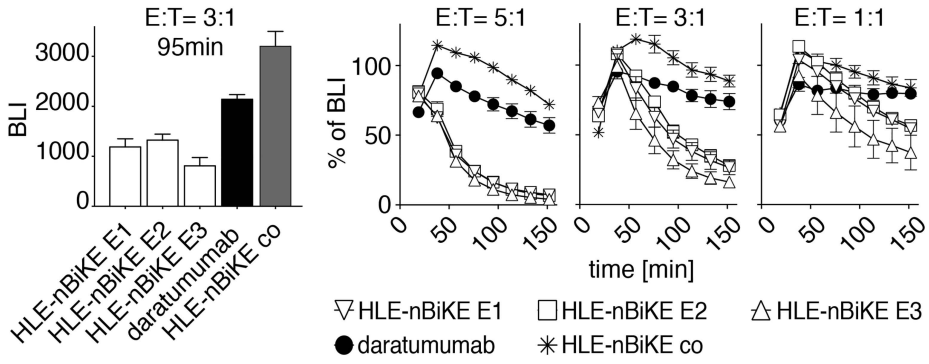

FIGURE 5 | Kinetics of CD38-specific HLE-nano-BiKE-induced killing of myeloma cells. $CD16^+$ NK92 hCD16 cells were incubated with nanobody-based CD38-specific HLE-nano-BiKEs (E1, E2, E3), isotype-control HLE-nano-BiKE co, or daratumumab and then added to luciferase-expressing LP-1 luc myeloma cells at the indicated effector to target ratios. Mean absolute BLI signal is shown in the left panel at an E:T-ratio of 3:1 after 95 min of incubation. At timepoint t= 0min NK cells were added to LP-1 cells preincubated with luciferin. Kinetics (0 - 150 min) of HLE-nano-BiKE-induced killing is shown as percentage of BLI in comparison to myeloma cells treated without HLE-nano-BiKEs in the right panel. Data represent mean ± SEM of triplets. Results are representative of three similar experiments.

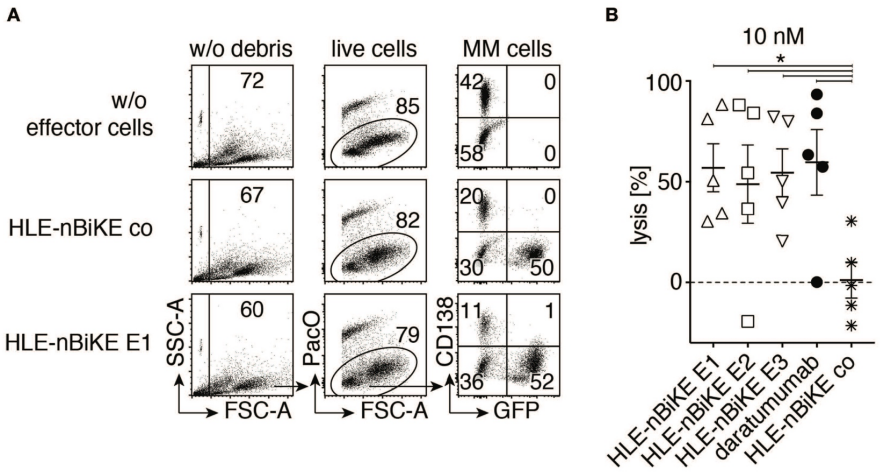

FIGURE 6 | CD38-specific HLE-nano-BiKEs deplete CD38+ primary myeloma cells from MM patients. Fresh primary human bone marrow mononuclear cells were incubated for 15 min in the absence or presence of 10 nM HLE-nano-BiKEs or daratumumab before addition of GFP-transfected NK92 hCD16 effector cells at an effector to target ratio of 3:1 and further incubation for 3 h at 37°C. Counting beads were added and cells were counterstained with Pacific Orange (PacO) and brilliant violet 421-conjugated CD138-specific mAb before analysis by flow cytometry. **(A)** Gating was performed on live cells by excluding beads (SSC-hi/FSC-lo) and debris (SSC-lo/FSC-lo) (left panels), doublets (not shown), and dead cells (middle panels). Myeloma cells and NK92 hCD16 effector cells were identified by their expression of CD138 and GFP, respectively. Numbers indicate the percentage of cells in the gated population. **(B)** The absolute number of surviving myeloma cells (GFP-/CD138+/CD38+) was determined with the aid of cell counting beads. Percentages of surviving myeloma cells were calculated relative to the number of surviving myeloma cells in samples incubated without HLE-nano-BiKEs (set at 100%) to calculate lysis. Data represent means ± SEM. One-Way ANOVA was performed and Holm-Sidak-adjusted p-values are indicated (*= $p < 0.05$).

an additional albumin-binding nanobody, rather than to a larger Fc-fragment (**Figure 1A**).

The albumin-specific nanobody in our HLE-nano-BiKE-constructs is meant to extend the half-life of the construct *in vivo* as shown in previous studies (38–40). Though the increased half-life of our CD38-specific HLE-nano-BiKEs *in vivo* remains to be shown, we could show that the albumin-specific nanobody used in our constructs binds to human albumin *in vitro* and that binding to albumin does not impair the capacity to mediate BiKE-DCC.

The three epitopes (E1, E2, E3) of CD38 on MM cells addressed by our HLE-nano-BiKEs have important clinical implications. Two of these HLE-nano-BiKEs (E2, E3) bind to epitopes on CD38 that are independent (E2, E3) of that of daratumumab (E1) (44). These two HLE-nano-BiKEs could therefore be used in MM patients that have been treated with daratumumab without being blocked from binding to CD38 by daratumumab (56, 57). Similarly, two of these HLE-nano-BiKEs (E1, E2) bind to epitopes on CD38 that are independent (E1, E2) of that of isatuximab (E3) and could therefore be used potentially in MM patients that have been treated with isatuximab.

Our HLE-nano-BiKEs are composed only of lama-derived variable immunoglobulin domains (VHH or nanobody). Similar to therapeutics that incorporate murine VH and VL domains such as blinatumumab, a CD19-CD3 bispecific T-cell engager composed of two murine scFv (58, 59) or rituximab (60), a chimeric antibody composed of murine VH and VL and human constant IgG1 domains, therapeutics composed of VHH domains such as caplacizumab (61) (a dimer of two llama VHH domains) show little if any immunogenicity in patients (62). Anti-drug antibodies in patients can abrogate the efficacy of antibody treatment; therefore antibody-constructs with a low immunogenicity are favorable (63–65). Moreover, the better solubility and the smaller size of an HLE-nano-BiKE (ca. 45kDa) in comparison to an scFv-based BiKE (ca. 55kDa) could additionally provide better tissue penetration *in vivo* (36, 37).

The major limitation of our proof-of-concept *in vitro* and *ex vivo* study is the lack of the assessment of cytotoxic effects of our CD38-specific HLE-nano-BiKEs in *in vivo* xenograft myeloma mouse models. These *in vivo* studies are warranted as follow-up experiments after further optimization of our HLE-nano-BiKEs *in vitro*. We aim to examine the effect of the length of the two linkers and the order of the three nanobodies on the effectiveness of the BiKE-DCC. Van Faassen et al. observed only small effects by changing the order of the nanobodies and the lengths of the linker in their nano-BiKEs (36). Notwithstanding, we hypothesize that the length of the linker may differentially affect the efficacy of HLE-nano-BiKEs that target different epitopes of CD38.

In summary, we here provide proof of principle for the efficacy of CD38-specific HLE-nano-BiKEs *in vitro* and *ex vivo*, warranting further preclinical evaluation *in vivo* of their therapeutic potential for the treatment of multiple myeloma.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hamburger Ärztekammer. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PB and FK-N conceived the project. All authors established experimental procedures. JH, PB, and FK-N wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: FK-N receives a share of antibody sales *via* MediGate GmbH, a wholly owned subsidiary of the University Medical Center Hamburg-Eppendorf. WF, FK-N, and PB are co-inventor on a patent application on CD38-specific nanobodies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Diskussion

In der vorliegenden Dissertation wurden CD38-spezifische Nanobodies als Grundlage für die Konstruktion von verschiedenen rekombinanten, Nanobody-basierten Biologika verwendet. Ziel war dabei die Evaluation von CD38-spezifischen Nanobody-basierten humanisierten Schwereketten-Antikörpern, CARs und BiKEs für die Therapie des Multiplen Myeloms *in vitro*, *ex vivo* und *in vivo*. Die dabei verwendeten CD38-spezifischen Nanobodies binden an drei voneinander unabhängige Epitope auf CD38, zwei davon uneingeschränkt von dem in der Klinik verwendeten CD38-spezifischen Antikörper Daratumumab.

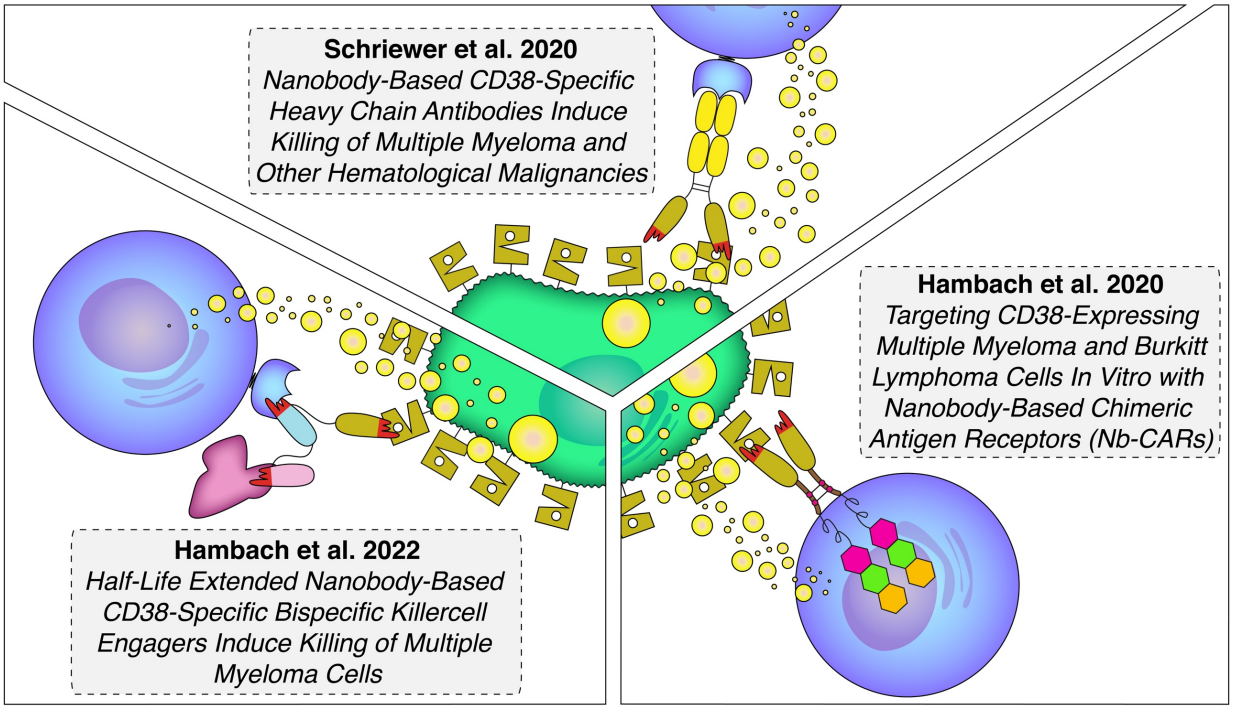

Abbildung 8: Schematische Darstellung der in dieser Dissertation behandelten Publikationen.

4.1 Nanobody-basierte CD38-spezifische Schwereketten-Antikörper

In der Publikation *„Nanobody-Based CD38-Specific Heavy Chain Antibodies Induce Killing of Multiple Myeloma and Other Hematological Malignancies“* beschreiben wir auf den CD38-spezifischen Nanobodies JK36, WF211 und MU1067 basierende chimere, humanisierte Schwereketten-Antikörper (*hcAbs*).

CD38-spezifische hcAbs wurden durch genetische Fusion der CD38-spezifischen Nanobodies an die Hinge-Region und Immunglobulin Domänen CH2 und CH3 von humanem IgG1 erstellt. Diese hcAbs zeigten eine hohe Spezifität und Affinität für humanes CD38-exprimierende Zellen (**Schriewer *et al.* 2020, Figure 2**).

4.1.1 CD38-spezifische hcAbs lösen *in vitro* effiziente ADCC aus

Die CD38-spezifischen hcAbs lösten *in vitro* effektive ADCC durch NK92 Zellen und primäre NK-Zellen gegen CD38-exprimierende Myelom- und Burkitt Lymphom Zelllinien aus, unabhängig von dem auf CD38 gebundenen Epitop (**Schriewer *et al.* 2020, Figure 4**). Auch *ex vivo* wurde effektive ADCC an primären Myelomzellen verschiedener Myelom Patienten festgestellt (**Schriewer *et al*. 2020, Figure 7B**). Die durch CD38-spezifische hcAbs ausgelöste ADCC war vergleichbar mit der durch den klinisch verwendeten Antikörper Daratumumab ausgelösten ADCC. WF211 hcAb, der an dasselbe Epitop wie Daratumumab bindet, löste die stärkste ADCC aus.

Die hier vorgestellten CD38-spezifischen hcAbs lösten trotz ihrer vergleichsweise hohen Affinität für CD38 sowohl *in vitro* als auch *ex vivo* kaum CDC aus (**Schriewer *et al*. 2020, Figure 3**). Dies entspricht Beobachtungen, die bereits zuvor in unserer Arbeitsgruppe gemacht wurden, bei denen CD38-spezifische hcAbs keine CDC an verschiedenen CD38-exprimierenden Tumorzelllinien *in vitro* auslösen konnten (13). CDC stellt bei den meisten klinisch eingesetzten Antikörpern keinen wichtige Wirkungsweise dar (90). Die Fähigkeit eines Antikörpers CDC auszulösen wird stark durch die Beschaffenheit des Fc-Teils beeinflusst. So können nicht nur Glykosylierungen des Fc-Teils, sondern auch terminale β-Galaktose und Glycane mit hohem Mannose-Gehalt die Bindung von C1q, dem ersten Teil der Komplementkaskade, verhindern (91, 92). Überraschenderweise konnte unsere Arbeitsgruppe zeigen, dass durch Kombination von zwei unabhängig voneinander bindenden CD38-spezifischen hcAbs erfolgreich eine CDC ausgelöst werden kann (13). Auch biparatopische hcAbs (Diabodies), bei denen zwei Nanobodies, die verschiedene Epitope auf CD38 binden, mittels eines Peptid-Linkers verbunden und an einen Fc-Teil

fusioniert wurden, lösten effektiv CDC aus. Vermutlich liegt dies an der Kreuzvernetzung von CD38 auf der Zellmembran durch die simultane Bindung von mehreren Antikörpern. Dies resultiert in der besseren Bindung des Hexamers C1q. Die Fähigkeit eines Antikörpers CDC auszulösen kann auch durch Mutation des Fc-Teils verbessert werden. Die Bildung von Antikörper-Hexameren kann durch Integration der Hexabody-Mutation E345R in den Fc-Teil des Antikörpers begünstigt werden, sodass C1q multivalent binden kann (93). Auch in den in dieser Arbeit vorgestellten hcAbs konnte eine verbesserte Auslösung von CDC durch Einführung der Hexabody-Mutation beobachtet werden (13).

4.1.2 CD38-spezifische hcAbs zeigen therapeutische Effekte *in vivo*

In einem *in vivo* Xenograft-Modell konnten die beschriebenen CD38-spezifischen hcAbs das Wachstum von CD38-exprimierenden Burkitt Lymphom-Tumoren reduzieren (**Schriewer *et al.* 2020, Figure 5**). Mit CD38-spezifischen hcAbs behandelte, Tumor-tragende Mäuse zeigten zudem signifikant längere Überlebensraten im Vergleich zur Isotyp Kontrolle (**Schriewer *et al.* 2020, Figure 6**). Dieser Effekt konnte bei allen verwendeten hcAbs beobachtet werden und überstieg teilweise sogar den Effekt von Daratumumab. Da hcAbs kleiner als konventionelle Antikörper wie Daratumumab sind, könnte dies ein Hinweis auf eine bessere Gewebepenetration durch hcAbs als durch konventionelle Antikörper sein. Diese Ergebnisse legen zudem nahe, dass ADCC als Antikörper-vermittelte immunologische Effektorfunktion eine größere Rolle spielen könnte als CDC, wie bereits in vorangegangenen Studien zur *in vivo* Wirkungsweise von anderen klinisch eingesetzten Antikörper beschrieben (17-19).

Neben ADCC und CDC können aber auch andere immunologische Effektorfunktionen durch therapeutische Antikörper ausgelöst werden, wie die Antikörper-vermittelte Phagozytose und die direkt induzierte Apoptose, die in dieser Studie nicht *in vitro* untersucht wurden. Einer der postulierten Mechanismen von CD38-spezifischen Antikörpern ist zudem die Hemmung der enzymatischen Aktivität von CD38 (90). Diese wurde in einer Veröffentlichung von Baum *et al*. für die hier beschriebenen hcAbs untersucht (40). Dabei wurde beobachtet, dass CD38-spezifische hcAbs, die an Epitop 2 auf CD38 binden, die ADPR (*Adenosindiphosphat-Ribose*) Cyclase- und cADPR (*cyclische ADPR*) Hydrolase-Aktivität von CD38 hemmen, Epitop 3 bindende hcAbs beeinflussen diese Aktivitäten ebenfalls in geringem Maß. Die untersuchten CD38-spezifischen hcAbs zeigten jedoch keinen Einfluss auf die NAD+ (*Nicotinamidadenindinukleotid*) Hydrolase-Aktivität, die den größeren Teil der

enzymatischen Aktivität von CD38 ausmacht (94, 95). Bisher ist die Gewichtung der Enzym-inhibierenden und immunologischen Effektorfunktionen von CD38-spezifischen Antikörpern für den therapeutischen Erfolg *in vivo* ungewiss. Es ist jedoch wahrscheinlich, dass wie bei Daratumumab eine Kombination aller Effektorfunktionen entscheidend ist.

4.1.3 CD38-spezifische hcAbs könnten zur Therapie von Patienten eingesetzt werden, die eine Resistenz gegen die Therapie mit Daratumumab entwickelt haben

CD38-spezifische hcAbs zeigten in dieser Arbeit mit anderen, sich bereits in der klinischen Testung befindlichen, CD38-spezifischen Antikörpern vergleichbare Effekte *in vitro*, *ex vivo* und *in vivo*. Dennoch sind weitere Studien notwendig, um zu bewerten, ob die Effektivität der CD38-spezifischen hcAbs in der hier vorgestellten Arbeit auch auf eine hohe Effektivität in klinischen Studien hinweist. CD38-spezifische hcAbs könnten besonders für Patienten Relevanz haben, bei denen Daratumumab nur geringe Effektivität zeigt. Die Bildung von neutralisierenden Antikörpern gegen das antigenbindende Paratop von Daratumumab kann ein Grund für das Scheitern der Therapie sein. Ebenso kann es durch Mutation zum Verlust des von Daratumumab gebundenen Epitops auf CD38 kommen (18). In diesem Fall könnten die hier beschrieben, CD38-spezifischen hcAbs zur Fortsetzung der Therapie eingesetzt werden, da diese alternative Epitope auf CD38 binden und präformierte, neutralisierende Antikörper gegen das antigenbindende Paratop von Daratumumab nicht an das Paratop der hcAbs binden. CD38 wird auf Myelomzellen während der Therapie mit Daratumumab oft herunterreguliert, ebenso wie auf B-, T- und NK-Zellen (90). Dies könnte auch zu einer Verminderung des therapeutischen Effekts durch Daratumumab beitragen. Diese Verringerung an CD38 hoch exprimierenden Zellen ist zum einen darauf zurückzuführen, dass auf Zellen mit einer hohen CD38-Expression Daratumumab verstärkt bindet. So werden diese schneller eliminiert als Zellen mit einer niedrigeren CD38-Expression (96). Zum anderen führt die Bindung von Daratumumab zum polaren Clustering von CD38 Molekülen auf der Zellmembran, sodass CD38 als Mikrovesikel freigesetzt wird und die CD38 Zelloberflächenexpression dieser Zellen sich verringert (97). Auch direkte Internalisierung und aktiver Transfer von CD38-Daratumumab-Komplexen von Myelomzellen zu Monozyten oder Granulozyten mittels Trogozytose könnte zur Reduktion der CD38-Expression auf Myelomzellen führen (96).

Die Reduktion von CD38 auf der Zelloberfläche von Myelomzellen durch Behandlung mit Daratumumab ist transient. Einige Monate nach Behandlung lässt sich feststellen, dass die CD38 Oberflächenexpression auf im Knochenmark verbleibenden Myelomzellen der Expression vor der Behandlung gleicht (98). Daher könnte es sinnvoll sein, Patienten, die refraktär oder rückfällig nach einer Therapie mit Daratumumab werden, mit einem anderen CD38-spezifischen Antikörper zu therapieren (90). Die hier vorgestellten CD38-spezifischen hcAbs bieten sich dafür an. Die verminderte Fähigkeit der CD38-spezifischen hcAbs CDC auszulösen könnte einen Hinweis darauf bieten, dass auch die Kreuzvernetzung durch CD38 geringer ist und somit eine geringere CD38-Reduktion durch Trogozytose und Mikrovesikel auf Myelomzellen *in vivo* zu erwarten wäre. HcAbs, deren Fc-Teil eine Hexabody Mutation enthält, die die Formation von Antikörper-Hexameren begünstigt, sowie biparatopische hcAbs könnten die CDC Wirkung verstärken (13).

CD38-spezifische Nanobodies oder hcAbs haben auch für das Imaging von CD38-exprimierenden Tumoren Vorteile gegenüber konventionellen Antikörpern. Fluorochrom-markierte CD38-spezifische Nanobodies konnten bereits für das spezifische Imaging von CD38-exprimierenden Tumoren in Mausmodellen verwendet werden (45, 99, 100). Es wurde dabei eine erfolgreiche Gewebepenetration des Tumors durch Fluorochrom-konjugierte CD38-spezifische Nanobodies festgestellt. Nanobodies oder hcAbs, die an Epitope auf CD38 binden, die unterschiedlich von dem von Daratumumab oder Isatuximab gebundenen Epitop sind, könnten für das Imaging in Patienten verwendet werden, die zuvor mit diesen monoklonalen Antikörpern behandelt wurden (100). Aufgrund der geringen Emission von Fluorochromen in tieferen Gewebeschichten müssten die hcAbs für die Verwendung im Menschen jedoch an radioaktive Isotope statt Fluorochrome gekoppelt werden, die in der SPECT- (*Single-Photon-Emissionscomputertomographie*) oder PET-Bildgebung (*Positronen-Emissions-Tomographie*) sichtbar gemacht werden können (101, 102). Alternativ könnten auch Isotope verwendet werden, die nicht nur für das Imaging der Tumore, sondern zeitgleich auch für die Chemotherapie verwendet werden können (103). So könnte der therapeutische Effekt der chimären CD38-spezifischen hcAbs zusätzlich zu ihren Fc-vermittelten und Enzym-inhibitorischen Effektorfunktionen um eine Target-orientierte Bestrahlung des Tumors erweitert werden.
CD38-spezifische hcAbs bilden daher eine vielversprechende Erweiterung der CD38-spezifischen Antikörper und sollten weitergehend klinisch untersucht werden.

4.2 Nanobody-basierte CD38-spezifische chimäre Antigenrezeptoren

In der Publikation *„Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimäric Antigen Receptors (Nb-CARs)“* stellt unsere Arbeitsgruppe eine weitere, auf den CD38-spezifischen Nanobodies basierte Technologie vor, die NK-Zellen unabhängig von ihrem Fc-Rezeptor aktiviert.

In dieser Arbeit wurden CD38-spezifische Nanobodies mittels der Hinge-Region von IgG4 mit intrazellulären ITAMS aus CD28, 41BB und CD3 zu einem Nanobody-basierten chimären Antigenrezeptor (*Nb-CAR*) verbunden (**Hambach *et al.* 2020, Figure 1, 4**). Dieser Nb-CAR wurde mittels retroviraler Transduktion in der NK-Zelllinie NK92 exprimiert.

NK92 Zellen exprimieren selbst CD38, wenn auch in geringerem Maße als die meisten Myelomzellen. Um eine unspezifische Aktivierung und gegenseitiges Killing der CD38-spezifischen Nb-CAR NK92 Zellen zu verhindern, wurde der für CD38 codierende Genlocus in diesen Zellen mittels CRISPR/Cas9 inaktiviert (**Hambach *et al.* 2020, Figure 3**).

Unsere Ergebnisse zeigen, dass NK92 CD38KO Zellen nach retroviraler Transduktion erfolgreich den CD38-spezifischen Nb-CAR exprimierten (**Hambach *et al.* 2020, Figure 4**). Die hier vorgestellten Nb-CAR NK92 Zellen enthielten Nanobodies, die an drei voneinander unabhängige Epitope auf CD38 binden. Alle hier vorgestellten CD38-spezifischen Nb-CAR NK92 Zellen lösten unabhängig vom auf CD38 gebundenem Epitop CAR-vermittelte Zellzytotoxizität (CAR-dependent cellular cytotoxicity, *CAR-DCC*) gegen CD38-exprimierende Zellen *in vitro* und *ex vivo* aus (**Hambach *et al.* 2020, Figure 5-7**) (Masterarbeit Julia Hambach 2019). Die Effektivität der Lyse war dabei abhängig von der eingesetzten Effektor- zu Target-Zell Ratio.

4.2.1 Aufbau des Nb-CARs

Verschiedene CD38-spezifische CAR Zellen wurden entwickelt (59, 104, 105), wovon einige sich bereits in der klinischen Evaluation befinden (NCT03473496, NCT03464916, NCT04861480, NCT04351022, NCT05442580, NCT04430530, NCT04016129, NCT03125577, NCT05239689, NCT03754764, NCT03767751, NCT03778346, NCT03222674. Stand: Januar 2023, clinicaltrials.gov) (**Hambach *et al.* 2020, Figure 8**). In den meisten dieser CARs wurde ein CD38-spezifisches scFv als bindende Domäne verwendet. So beschreiben zum Beispiel Mihara *et al.* und Drent *et al.* einen

scFv-basierten, CD38-spezifischen CAR, dessen bindendes Element über die Hinge und Transmembrandomäne von CD8a mit den ITAMs von 41BB und CD3ζ verbunden ist (59, 105). An *et al.* verwendeten ebenfalls einen CD38-spezifischen Nanobody als bindendes Element für CD38-spezifische CAR T-Zellen (106).

Nb211-CAR enthält die Hinge-Region von IgG4 als Spacer. Wie die Hinge von CD8 weist die Hinge-Region von IgG4 Cysteine auf, die die Formation von über Disulfidbrücken kovalent verbundenen Dimeren auf der Zelloberfläche hervorrufen (107). Long *et al.* beobachteten, dass eine chronische Aktivierung von CD19-spezifischen CAR T-Zellen durch Antigen-unabhängiges Clustering der als bindende Domäne enthaltenen scFvs zur frühzeitigen Erschöpfung dieser CAR T-Zellen führen kann (108). Singh *et al.* hingegen zeigten eine verbesserte klinische Effizienz von durch Homodimerisierung tonisch aktivierten, CD22-spezifischen CAR T-Zellen (109). Auch Hirobe *et al.* stellten ein besseres Killing durch EGFR-spezifische CAR T-Zellen fest, wenn der exprimierte CAR als Dimer vorlag im Vergleich zu einem monomeren CAR (110). Daher bleibt zu untersuchen, ob die Effizienz von Nb211-CAR durch die Optimierung des Linkers, zum Beispiel durch Änderung der Länge oder durch Substitution der Cysteine in der Hinge-Region verbessert werden kann (111). Auch die Verwendung von anderen, für NK-Zellen spezifischen, intrazellulären Signaldomänen könnte eine Optimierung der CAR-vermittelten zellulären Zytotoxizität bewirken.

4.2.2 NK92 Zellen als off-the-shelf Nb-CAR Zellen

Die Verwendung von NK-Zellen statt T-Zellen als Grundlage für CAR-exprimierende Zellen bringt einige Vorteile mit sich (47). Studien zeigen eine hohe klinische Sicherheit von NK-Zellen und CAR NK-Zellen (47, 112). Das Risiko von On-Taget/ Off-Tumor Toxizität gegen gesundes Gewebe ist durch die im Vergleich zu CAR T-Zellen geringere Überlebensdauer von CAR NK-Zellen *in vivo* vermindert (113). Aktivierte NK-Zellen produzieren zudem weniger Zytokine als CAR T-Zellen (113). Somit rufen CAR NK-Zell Transplantate geringere Hyperzytokinämie, Neurotoxizität und Transplantat-gegen-Patient Effekte hervor als CAR T-Zellen (114-116).

Die natürliche zytotoxische Aktivität von NK-Zellen gegen maligne Zellen über Rezeptoren wie NKp46, NKG2D und bestimmte aktivierende KIRs bietet einen weiteren Vorteil von CAR NK-Zellen (117). Diese ermöglichen eine zusätzliche, vom CAR unabhängige Aktivierung der NK-Zelle. Also könnten CAR-exprimierende NK-Zellen auch effektive Wirkung zeigen in Tumoren, die heterogen das Zielantigen

exprimieren (47). MHC-vermittelte Patient-gegen-Transplantat Effekte sind bei NK-Zell basierten Therapien gering (47, 118, 119) .
Die Verwendung von NK-Zellen aus bereits etablierten Zellkulturen wie NK92 bieten zusätzlich den Vorteil, dass die Zellen als *off-the-shelf* Therapeutikum verwendet werden können (120). Dies senkt die Kosten einer CAR-basierten Therapie im Vergleich zur autologen Transplantation beachtlich.
Die Zelllinie NK92 wurde 1992 aus dem Blut einer 50 Jahre alten Patientin mit non-Hodgekin Lymphoma isoliert. Ihr Phänotyp ist vergleichbar mit einer aktivierten NK-Zelle (120). In Phase-I Studien zeigten NK92 und CAR NK92 Zellen nur geringe *graft-versus-host* Effekte und eine gute Sicherheit der Anwendung (121, 122).

CD38 wird auf Multiplen Myelomzellen zwar überexprimiert, jedoch findet es sich auch auf gesunden NK-Zellen. Bei der Therapie mit Daratumumab wird eine Reduktion der NK-Zellen im Blut des Patienten beobachtet (123). Während der Therapie verbleibende NK-Zellen zeigen nur eine geringe CD38 Zelloberflächenexpression. Ein erneuter Anstieg der NK-Zell Population wird erst Monate nach der letzten Daratumumab Infusion beobachtet (123). In den hier vorgestellten CD38-spezifischen, Nb211-CAR-exprimierenden NK92 Zellen wurde vor der Transduktion mit dem CAR CD38 mittels CRISPR/Cas9 Technologie inaktiviert. Somit wird die Vitalität der Nb211-CAR NK92 Zellen nicht durch Fratrizid ("Brudermord") beeinflusst.

4.2.3 Off-Target Effekte von Nb-CAR NK-Zellen

Die Aktivierung der CAR-exprimierenden Zelle wird wie oben beschrieben ausgelöst durch die Kreuzvernetzung der CARs auf der CAR-exprimierenden Zelle (65). Diese Kreuzvernetzung entsteht an der „Synapse“ zwischen Zielzelle und Effektorzelle durch die multivalente Bindung des Targets durch mehrere CARs. Die unspezifische und übermäßige Aktivierung von CAR Zellen und die daraus resultierende übermäßige Zytokin-Ausschüttung im Patienten stellt eine zentrale Problematik von CAR-basierten Therapien dar (115, 124). CAR Zellen, die spezifisch nur Tumorzellen binden, die das Target stärker exprimieren als gesunde Zellen, könnten daher eine Lösung für diese Problematik darstellen.
Drent *et al.* verringerten die Affinität eines scFv-basierten, CD38-spezifischen CARs mittels light-chain exchange Technologie (104). Durch die geringere Affinität des scFv soll es erst bei simultaner Bindung von mehreren CAR-Rezeptoren an die Tumorzelle zur Aktivierung der CAR-exprimierenden Zelle kommen. Zellen, die viel CD38

exprimieren, sollen so spezifischer lysiert werden und Zellen mit niedriger bis moderater CD38 Expression überleben. Off-Target Effekte auf CD38-exprimierende Lymphozyten wie T-Zellen, B-Zellen und NK-Zellen durch CD38-spezifische, affinitäts-reduzierte CAR T-Zellen waren *in vitro*, *ex vivo* und *in vivo* geringer im Vergleich zu CAR T-Zellen, die höhere Affinität für CD38 aufwiesen. In einer kürzlich veröffentlichten Studie wurde dieser CD38-spezifische, affinitätsreduzierte CAR für die Therapie von akuter myoblastischer Leukämie (AML) *in vitro* und *ex vivo* evaluiert (125). AML Zellen zeigen oft eine geringere, heterogenere CD38 Zelloberflächenexpression als Multiple Myelomzellen (126). Durch die Affinitäts-Optimierung des CAR sollen somit Off-Target Effekte auf gesunde lymphatische Zellen verringert werden (125). Der affinitätsverringerte CAR wurde in primären NK-Zellen exprimiert, in denen - wie in unserer Arbeit - das CD38-kodierende Gen mittels CRISPR/Cas9 inaktiviert wurde. Diese CAR NK-Zellen zeigten von der Oberflächenexpression von CD38 auf der Zielzelle abhängige Lyse von AML-Blasten *ex vivo* und verminderten Fratrizid *in vitro* (125).

In dieser Arbeit konnte ich zeigen, dass die Effektivität des CAR-DCC durch unsere CD38-spezifischen Nb-CAR NK92 Zellen ebenfalls mit der CD38 Zelloberflächenexpression auf der Tumorzelle korreliert. Zellen, die kein oder wenig CD38 exprimierten, wurden nicht bzw. später lysiert als Zellen, die viel CD38 exprimierten (**Hambach *et al*. 2020, Figure 6**). Dies könnte einen Hinweis auf eine niedrigere CAR-DCC auf CD38-exprimierende, gesunde Körperzellen geben. WF211 hat wie zuvor beschrieben eine niedrigere Affinität für CD38 als JK36 und MU1067 (45). Es könnte daher sein, dass auf WF211 basierende Nb211-CAR NK92 Zellen geringere Off-Target Effekte auslösen, als Nb-CAR NK92 Zellen, die auf einem höher affinen Nanobody basieren. Dennoch könnte ein Effekt gegen CD38-exprimierende Immunzellen auftreten (90). Um diesen Effekt weiter zu reduzieren, könnte durch gezielte Mutation der CDR3 Schlaufe die Affinität des im Nb-CAR verwendeten Nanobodies WF211 zu CD38 weiter verringert werden.

Andererseits könnten auch Bindungselemente mit einer hohen Affinität für ihr Target von Vorteil für CAR-basierte Therapien sein. Der erst kürzlich für die Therapie des Multiplen Myeloms zugelassene BCMA-spezifische CAR Cilta-cel verwendet zwei BCMA-spezifische Nanobodies, die voneinander unabhängige Epitope auf BCMA binden (67). Im Vergleich zu CAR T-Zellen, deren CAR nur einen BCMA-spezifischen Nanobody enthielt, konnte bei der Behandlung mit Cilta-cel eine komplette Remission

bei dreifach refraktären Patienten schon mit nur einem Zehntel der transplantierten CAR-T Zellzahl erreicht werden (65, 127, 128). Dies könnte an der erhöhten Avidität des biparatopischen CARs für BCMA liegen. Auch die verbesserte Kreuzvernetzung von BCMA auf der Zielzelle durch die simultane Bindung von zwei Epitopen durch den CAR könnte zu einer erhöhten Aktivierung der Nb-CAR T-Zelle führen.

Eine weitere Möglichkeit die Spezifität von CAR-basierten Therapien für Tumorzellen zu erhöhen, ist die Integration von weiteren tumorspezifischen Bindungselementen in den CAR (129, 130). Bei diesen so-genannten Dual CARs werden zwei Elemente (Nbs oder scFvs), die unterschiedliche Antigene erkennen, mittels einen Peptid-Linkers miteinander verbunden und als bindendes Element des CARs verwendet. Durch simultanes Targeting von zwei tumorspezifischen Markern, die bestenfalls in dieser Kombination nur auf Tumorzellen zu finden sind, könnte die Spezifität des CAR für Tumorzellen verbessert werden (129). Entscheidend ist hier die bindenden Elemente des CARs so zu wählen, dass der CAR ausschließlich aktiviert wird, wenn er beide Targetproteine simultan bindet, jedoch nicht bei singulärer Bindung. Dies könnte zum Beispiel durch Bindungselemente mit einer geringen Affinität zum Target erfolgen. Somit würde die Aktivierung der CAR Zelle über die Avidität gesteuert. Aufgrund ihrer einfacheren Struktur ist die Konstruktion derartiger CARs mittels Nanobodies deutlich vereinfacht gegenüber der Konstruktion mittels scFvs (131).

Aus den hier vorgestellten Ergebnissen lassen sich die Nebenwirkungen einer Therapie von Multiplen Myelom Patienten mit Nb211-CAR NK92 Zellen noch nicht hinreichend abschätzen. *Ex vivo* Untersuchungen an gesunden Zellen aus Blut und Knochenmark und *in vivo* Versuche mit humanisierten Mäusen könnten Hinweise über mögliche On-Target Off-Tumor Effekte geben. Dennoch weisen die von uns gewonnen Erkenntnisse darauf hin, dass CD38-spezifische, Nanobody-basierte CARs eine vielversprechende Option in der Therapie des Multiplen Myeloms darstellen könnten.

4.3 Nanobody-basierte half-life extended CD38-spezifische BiKEs

Zur spezifischen Aktivierung von NK-Zellen können auch BiKEs verwendet werden. In der Publikation *„Half-life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers (HLE nano-BiKEs) Induce Killing of Multiple Myeloma Cells“* beschreiben wir auf CD38-spezifischen Nanobodies basierende, BiKEs mit verlängerter Halbwertszeit im Körper (*half-life extended nanobody-based BiKEs*, *HLE-nano-BiKEs*).

Gemäß ihrer Epitop-Spezifität haben wir den auf WF211 basierenden HLE-nano-BiKE hier E1-HLE-nano-BiKE genannt, den auf MU1067-basierenden BiKE E2-HLE-nano-BiKE, und den auf JK36 basierenden BiKE E3-HLE-nano-BiKE (**Hambach *et al*. 2022, Figure 1B**). Diese HLE-nano-BiKEs zeigten in Biolayer Interferometer Messungen spezifische, simultane Bindung an CD38, CD16 und Albumin (**Hambach *et al*. 2022, Figure 2**). Zudem vermittelten sie eine spezifische Dosis-, Effektor-zu-Target-Ratio und CD38-Oberflächenexpressions-abhängige Lyse von CD38-exprimierenden Myelom und Burkitt-Lymphom Zellen durch NK-Zellen *in vitro* und *ex vivo* (**Hambach *et al*. 2022, Figure 4-6**). Die BiKE-vermittelte zelluläre Zytotoxizität wird hier BiKE-DCC (*BiKE-dependent cellular cytotoxicity)* genannt. Der durch HLE-nano-BiKEs ausgelöste BiKE-DCC wurde in Abhängigkeit von CD38 auf der gebundenen Target Myelomzelle und CD16 auf der Effektor NK-Zelle ausgelöst (**Hambach *et al*. 2022, Figure 3**). Dieser BiKE-DCC war in unseren Experimenten zudem effektiver als der durch den konventionellen Antikörper Daratumumab ausgelöste, Fc-vermittelte ADCC (**Hambach *et al*. 2022, Figure 3-6**).

4.3.1 Vorteile eines Nanobody-basierten BiKEs gegenüber einem scFv-basierten BiKE

Unsere HLE-nano-BiKEs ließen sich gut in eukaryotischen Zellen exprimieren, aus Überständen mittels Affinitätschromatographie isolieren und aufkonzentrieren ohne zu aggregieren (**Hambach *et al*. 2022, Figure 2A**). Damit haben nano-BiKEs einen Vorteil gegenüber scFv-basierten BiKEs, die oft schwierig zu produzieren sind, eine geringe Löslichkeit zeigen und dazu tendieren zu aggregieren (86). Diese Eigenschaften erschweren die Testung und Produktion von scFv-basierten Konstrukten und benachteiligen damit die kommerzielle Produktion.

Weitere Vorteile von Nanobodies gegenüber scFvs für die Konstruktion von BiKEs sind die Verwendbarkeit von Nanobodies als Baukasten-System und ihre geringe Größe, die eine bessere Gewebepenetration ermöglicht. Die Halbwertszeit eines Nanobody-basierten BiKEs kann mittels Integration eines Albumin-spezifischen Nanobodies *in vivo*

verlängert werden (132, 133). Die Bindung des in den hier vorgestellten HLE-nano-BiKE enthaltenen Albumin-spezifischen Nanobodies an humanes Albumin zeigte keinen Einfluss auf die Fähigkeit des HLE-nano-BiKEs BiKE-DCC auszulösen (**Hambach *et al*. 2022, Figure 4C)**. Dies weist darauf hin, dass die Bildung der immunologischen „Synapse“ zwischen Zielzelle und Effektorzelle, die für den BiKE-DCC vonnöten ist, nicht beeinträchtigt wird durch die simultane Bindung des HLE-nano-BiKEs an Albumin.

4.3.2 CD38-spezifische Nanobodies im HLE-nano-BiKE

Bei nano-BIKES könnte das vom tumorspezifischen Nanobody gebundene Epitop eine Rolle spielen. Wie zuvor erwähnt binden zwei der drei im HLE-nano-BiKE verwendeten CD38-spezifischen Nanobodies ein von Daratumumab unabhängiges Epitop. Daher könnten diese, ähnlich wie die auf diesen Nanobodies basierenden hcAbs, für die Therapie von Patienten verwendet werden, die bereits mit Daratumumab vorbehandelt wurden oder sich noch in der Therapie mit Daratumumab befinden.

Auch die Affinitäten der einzelnen im nano-BiKE verwendeten Nanobodies könnten einen Einfluss auf die Effektivität des durch die BiKEs ausgelösten Killings *in vivo* haben. Bei HER2-spezifischen, scFv-basierten BiTEs hat eine Verminderung der Affinität des CD3-spezifischen scFv zu CD3 nur geringen Effekt auf die anti-Tumor-Aktivität des BiTE *in vivo* (134). Zudem konnte für PSMA-spezifische BiTEs gezeigt werden, dass die Gefahr eines Zytokin-Sturmes durch Verwendung eines niedrig affinen CD3-spezifischen Bindungselements vermindert werden kann (135). Auch bei nano-BiKEs könnte daher ein CD16-spezifischer Nanobody mit einer geringen Affinität für CD16 die unspezifische Aktivierung von NK-Zellen vermindern.

Wie im Falle von CARs, könnte die Affinität des Antigen-spezifischen Bindungselements auch die Effektivität eines BiKEs beeinflussen. HER2-spezifische BiTEs mit unterschiedlicher Affinität für das Tumorantigen zeigten auch unterschiedliche Effektivität *in vivo* (134). Eine geringe Affinität zu CD38 könnte im HLE-nano-BiKE von Vorteil sein, um eine erhöhte Spezifität für Zellen mit einer hohen CD38 Zelloberflächenexpression zu erreichen. Für die Konstruktion der hier vorgestellten HLE-nano-BiKEs wurden CD38-spezifische Nanobodies mit unterschiedlichen Affinitäten zu CD38 verwendet (**Hambach *et al*. 2022, Figure 2C**) (45). Es konnte aber *in vitro* kein entscheidender Unterschied im durch HLE-Nb-BiKEs ausgelösten NK-Zell vermittelten Killing von CD38-exprimierenden Zellen beobachtet werden (**Hambach *et al*. 2022, Figure 3-6**).

4.3.3 Aufbau des BiKEs

Die im BiKE verwendete Linkerläge und Reihenfolge der Nanobodies im BIKE ist ebenfalls interessant im Hinblick auf eine künftige Verbesserung der klinischen Effektivität der BiKEs. Van Faassen *et al.* beobachteten nur minimale Effekte auf die Effektivität und Bindung von EGFR-, CD19- und HER2-spezifischen nano-BiKEs durch Veränderung der Linkerlänge und Anordnung der Nanobodies im BiKE (86). Bei einigen scFv-basierten BiTEs wurde jedoch beobachtet, dass die Reihenfolge der scFvs im BiTE Einfluss auf die Effektivität der Aktivierung von T-Zellen haben könnte (136-138). Grund hierfür könnten aber auch Komplikationen bei der korrekten Paarung der in den scFvs vorhandenen VH- und VL- Domänen sein (139). Dennoch könnte für unsere HLE-nano-BiKEs die Reihenfolge der Nanobodies und Linkerlänge in Bezug auf das gebundene Epitop auf CD38 eine Rolle spielen.

Das co-Targeting eines weiteren Myelom-spezifischen Tumormarkers durch Integration eines weiteren Nanobodies in den HLE-nano-BiKE stellt eine weitere mögliche Weiterentwicklung dar. So entsteht ein Nanobody-basierter Trispezifischer Killerzell Engager (*nano-TriKE*) (79, 140, 141). Wie schon zuvor beschrieben, könnte eine hohe Spezifität für Zellen, die beide Targets exprimieren von Vorteil sein. Aber auch ein HLE-nano-TriKE, der Zellen bindet, die entweder das eine oder das andere Target exprimieren, könnte zum Beispiel in heterogenen Tumoren klinische Anwendung finden. Eine erhöhte Aktivierung von NK-Zellen könnte auch durch TriKEs erreicht werden, in die Interleukin-15 (*IL-15*) als costimulatorisches Element integriert wurde, wie bereits für verschiedene BiKEs gezeigt. Die Integration von IL-15 führt zur stärkeren Aktivierung der NK-Zelle und möglicherweise somit zu einer besseren Effektivität des TriKE *in vivo* im Vergleich zum BiKE ohne IL-15 (83, 84, 142).

Aufgrund der hier vorgestellten Ergebnisse sollten CD38-spezifische HLE-nano-BiKEs *in vivo* weitergehend getestet werden, da sie eine interessante Perspektive für die Therapie des Multiplen Myeloms darstellen.

4.4 Ausblick

Nanobodies zeigen evolutionär bedingt im Vergleich zu scFvs eine sehr gute Löslichkeit. Ihre geringere Bindungsfläche und die oft verlängerte CDR3-Schlaufe bieten ihnen ein weiteres Spektrum an Bindungsmöglichkeiten an ihr Ziel-Antigen als konventionelle Antikörper (34). So können einige Enzym-spezifische Nanobodies durch Blockade des aktiven Zentrums sogar die Enzym-Aktivität ihres Targets beeinflussen (25). Die geringe Größe, geringe Immunogenität und leichte Reformatierbarkeit der Nanobodies bieten zudem Vorteile für die Entwicklung von chimären Antikörper-Konstrukten (34). Zwei Nanobody-basierte Therapeutika wurden bereits für die klinische Anwendung zugelassen: Caplacizumab, ein vWF-spezifischer Homodimer und Cilta-cel, ein biparatopischer, BCMA-spezifischer CAR. Diese zeigen herausragende Erfolge in der klinischen Anwendung (43, 44).

In dieser Arbeit wurden CD38-spezifische Nanobodies als Grundlage für die Konstruktion von hcAbs, Nb-CARs und HLE-nano-BiKEs entwickelt. CD38 ist ein vielversprechendes Target für die Therapie des Multiplen Myeloms, da es auf Multiplen Myelomzellen überexprimiert wird. Durch simultanes Targeting von CD38-exprimierenden regulatorischen B- und T-Zellen könnte die Immunantwort gegen den Tumor verstärkt werden. Nanobodies, die die enzymatischen Funktion von CD38 beeinflussen, könnten zudem das Tumormikromileu pro-inflammatorisch verändern. Durch ihre baukastenartige Struktur können Nanobody-basierte Konstrukte hinsichtlich der Off-Target Effekte, Affinität, Avidität und Spezifität leicht verändert werden.

Die hier vorgestellten Publikationen zeigen vielversprechende, präklinische Ergebnisse durch CD38-spezifische hcAbs, Nb-CARs und HLE-nano-BiKEs und bieten eine spannende Grundlage um das Potential von CD38-spezifischen Nanobody-basierten Konstrukten für die Therapie des Multiplen Myeloms in klinischen Studien zu evaluieren.

4.4.1 Vorteile und Nachteile von Zell-basierten Therapien gegenüber Protein-basierten Therapien

Protein-basierte Therapien wie hcAbs und Nb-BiKEs sind in der Produktion günstiger und schneller herzustellen als Zell-basierte Therapien (33). HcAbs und BiKEs müssen nicht individuell für Patienten hergestellt werden und die Produktion von Proteinen unter GMP-Konditionen ist weniger aufwendig als die Produktion von Zellprodukten. Proteine können nach der Produktion über lange Zeiträume gelagert werden und benötigen keine kontinuierliche Pflege der Zellkultur. Protein-basierte Therapien erfordern jedoch die wiederholte Gabe des Proteins und Therapieerfolge enden oft nach der letzten

verabreichten Dosis. CAR-Zellen hingegen zeigen schnelle und sehr persistente Erfolge, da CAR-Zellen oft noch Jahre nach der Therapie im Körper des Patienten nachweisbar sind (143). Dies führt oft zu einem langfristigeren Erfolg als Protein-basierte Therapien. Protein-basierte Therapien eliminieren genauso wie CAR Zellen oft auch gesunde Zellen, die das Target-Antigen exprimieren (26, 27). Dies führt zum Verlust wichtiger Zellpopulationen. Besonders CAR T-Zellen stoßen zusätzliche immunstimulierende Substanzen aus, daher sind die Nebenwirkungen von CAR T-Zell Therapien oft stärker und Patienten sind zu Beginn der Therapie stationär zu beobachten (113, 124). Da insbesondere CAR T-Zellen im Körper verbleiben, werden auch gesunde, das Target exprimierende Zellen oft dauerhaft eliminiert. Somit ist sorgsam je nach Krankheit zu evaluieren, ob ein Protein-basierter oder Zell-basierter Ansatz für die Therapie sinnvoller erscheint. Im Falle des Multiplen Myeloms spricht der radikale Erfolg von Cilta-Cel für die Weiterentwicklung von CAR-basierten Therapien. Dennoch sind Protein-basierte Therapien wichtig um genügend Patienten zeitig, konstant und sicher mit Therapieoptionen zu versorgen.

4.4.2 Affinität und Avidität des Tumorantigen-spezifischen Nanobodies im hcAb, Nb-CAR und HLE-nano-BiKE

Off-Target Effekte auf gesunde, CD38-exprimierende Immunzellen führen zur Schwächung des Immunsystems des Patienten (90). Daher ist es wichtig durch Antikörper-basierte Therapien so spezifisch wie möglich nur maligne Zellen zu eliminieren, die zum Beispiel eine besonders hohe CD38 Zelloberflächenexpression zeigen. Affinität und Avidität eines rekombinanten Antikörper-Konstruktes beeinflussen die Spezifität und Effektivität sowohl von monoklonalen Antikörpern, als auch von BiKEs und CARs. Daher wäre es von Interesse, Affinitäts-verminderte und Affinitäts-erhöhte Versionen der hier vorgestellten hcAbs, Nb-CARs und HLE-nano-BiKEs zu erstellen. Da Nanobodies eine einfachere Struktur aufweisen als scFvs, ist dies weniger aufwendig als in konventionellen Antikörpern und auf scFvs-basierenden CARs und BiKEs. Mittels gezieltem Austausch von Aminosäuren in der CDR3 des Nanobodies kann seine Affinität gezielt gesteuert werden (144). Der biparatopische Nanobody-basierte BCMA-spezifische CAR Cilta-Cel zeigt vielversprechende Erfolg im Vergleich zu CARs, die nur einen Nanobody enthalten. Dies führt zu der Frage, ob biparatopische CARs generell mehr Effizienz zeigen als monovalente CARs (65). Es ist jedoch noch unklar, ob sich dieses Prinzip auch auf andere Targets als BCMA anwenden lässt. Dennoch wäre auch für die hier vorgestellten hcAbs, Nb-CARs und HLE-BiKEs die

Integration eines zweiten CD38-spezifischen Nanobodies, der ein anderes Epitop als der erste bindet, interessant, um die Avidität des Konstruktes für CD38 zu erhöhen. Alternativ können auch Nanobodies, die spezifisch sind für einen anderen Myelom-spezifischen Marker, zu einer verbesserten Spezifität des Konstruktes führen. Als zusätzlichen Myelom-spezifischen Marker bieten sich zum Beispiel das B-cell maturation antigen BCMA, der Plasmazellmarker CD138 oder das T-Lymphozyten-Oberflächenantigen CD229 an (145). Durch Integration eines zweiten, tumorspezifischen Nanobodies könnten auf der Grundlage von hcAbs, Nb-CARs und HLE-nano-BiKEs bispezifische Diabodies, Dual-Nb-CARs oder TriKEs erstellt werden. Mittels dieser Konstrukte könnten gezielt Myelomzellen gebunden werden, die beide Targets exprimieren und somit Off-Target Effekte auf andere Zellen minimiert werden. Grundlage hierfür könnte auch hier eine Affinitätsoptimierung beider verwendeter Nanobodies sein, sodass diese Konstrukte nur binden, wenn beide Targets auf der Zielzelle exprimiert werden. Auch die gezielte Auswahl von Nanobodies mit geringeren Affinitäten gegen ihr Target könnte sinnvoll sein. Viele Tumore sind heterogen und Tumorzellen, die das Target-Antigen nicht exprimieren oder in Folge der Therapie die Expression herunterregulieren, haben einen Wachstumsvorteil (146, 147). In diesem Falle könnte das simultane Targeting zweier Tumorantigene ein Tumor-Escape verhindern und die Rückfallrate senken (148).

Die Baukasten-Struktur der hier vorgestellten Konstrukte erlaubt einen einfachen genetischen Austausch des verwendeten Nanobodies und somit ein Screening verschiedenerer Varianten der Konstrukte. Somit bieten sich Nanobody-basierte Konstrukte für die Optimierung von Immuntherapien hinsichtlich ihrer Affinität und Avidität zu Vermeidung von Off-Target Effekten an.

4.4.3 Therapeutische Anwendungsmöglichkeiten für CD38-spezifische hcAbs, Nb-CAR NK92 Zellen und HLE-nano-BiKEs

CD38-spezifische hcAbs, Nb-CAR NK92 Zellen und HLE-nano-BiKEs stellen einen neuen, spannenden Ansatz für die Therapie des Multiplen Myeloms dar. CD38-spezifische hcAbs, Nb-CAR NK92 Zellen und HLE-nano-BiKEs könnten dabei auch als zusätzliche oder Folgetherapie nach einer Therapie mit einem anderen CD38-spezifischen Antikörper wie Daratumumab oder Isatuximab angewandt werden. Resistenzen gegenüber Daratumumab oder Isatuximab könnten durch die Verwendung von CD38-spezifischen Antikörper-Konstrukten, die von Daratumumab bzw. Isatuximab unabhängige Epitope auf CD38 binden und andere Effektorfunktionen erfüllen,

überwunden werden (18, 90). Daher könnten die hier gezeigten, auf CD38-spezifischen Nanobodies basierenden Konstrukte auch eine Option für die Therapie von Patienten darstellen, die Resistenzen gegen Daratumumab gebildet haben.
CD38-spezifische Therapien könnten auch für die Behandlung anderer CD38-exprimierender, maligner Zelltypen verwendet werden. CD38 wird auch in anderen hämatologischen Erkrankungen wie der akutem myeloiden Leukämie und chronischen lymphatischen Leukämie exprimiert (126, 149). Krebs-assoziierte Fibroblasten könnten durch eine erhöhte CD38-Expression eine pro-tumorale Aktivität in Melanomen haben (150). Auch in soliden Tumoren wie Lungenkrebs könnte die enzymatische Aktivität von CD38 zu einem für den Tumor förderlichen, anti-inflammatorischen Tumormikromilieu führen (151, 152). CD38-spezifische Konstrukte, die die enzymatische Aktivität von CD38 hemmen, könnten die anti-Tumor Aktivität des Immunsystems gegen diese Tumoren erhöhen. Da die CD38 Expression der Zielzellen einen entscheidenden Effekt auf den Therapie-Erfolg von CD38-spezifischen Antikörpern wie Daratumumab und Isatuximab hat, werden zunehmend auch Agenzien untersucht, die eine Erhöhung der CD38-Expression auf diesen Zellen bewirken (153, 154). Besonders von Interesse ist hier Tretinoin (all-trans-Retinsäure, ATRA), das sich strukturell von Retinol ableitet. Die CD38-Oberflächenexpression auf Lymphozyten wird unter anderem reguliert durch Retinolsäure-gesteuerte Elemente, die über ein im CD38-Gen vorhandenes Intron codiert werden (24, 155). Eine ATRA-vermittelte Steigerung der CD38 Expression steigerte nachweislich auch Daratumumab-vermittelte CDC und ADCC (153, 154). So könnte die Kombination von CD38-spezifischen Nanobody-basierten hcAbs, Nb-CAR NK92 Zellen und HLE-nano-BiKEs mit ATRA auch zu einer Verbesserung des Therapieerfolges führen. Das Targeting von CD38 bietet somit eine aussichtsreiche Grundlage nicht nur für die Therapie des Multiplen Myeloms, sondern auch für andere CD38-assoziierte Krankheiten.

Wir konnten zeigen, dass CD38-spezifische Nanobodies sich hervorragend für die Generierung von hcAbs, Nb-CARs und HLE-nano-BiKEs eignen. Die Vorteile von Nanobodies in Bezug auf Größe, Reformatierbarkeit, Produktionsaufwand, Kosten und Variabilität machen sie zu einer guten Alternative von scFvs. CD38-spezifische, Nanobody-basierte hcAbs, Nb-CAR NK92 Zellen und HLE-nano-BiKEs zeigten in den hier vorgestellten Arbeiten durchschlagende Effekte gegen Multiple Myelomzellen sowohl *in vitro*, als auch *ex vivo* und *in vivo*. Damit konnten wir ihr großes Potential für die Tumortherapie zeigen und legen damit den Grundstein für die Entwicklung neuer Nanobody-Konstrukte auch gegen andere Tumorentitäten.

5 Eidesstaatliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Ich versichere, dass dieses gebundene Exemplar der Dissertation und das in elektronischer Form eingereichte Dissertationsexemplar (über den Docata-Upload) und das bei der Fakultät (zuständiges Studienbüro bzw. Promotionsbüro Physik) zur Archivierung eingereichte gedruckte gebundene Exemplar der Dissertationsschrift identisch sind.

Hamburg, den 20.01.2023

6 Abgrenzung des eigenen Beitrags

An dieser Stelle soll mein Beitrag zu den in dieser Dissertation vorgestellten Publikationen im Detail spezifiziert werden.

6.1 Nanobody-based CD38-specific heavy chain antibodies induce killing of Multiple myeloma and other hematological malignancies

Manuskript: Korrektur und Revision

Figure 1: Grafische Darstellung

Figure 2: Grafische Darstellung

Figure 3: Grafische Darstellung

Figure 4: Panel A-F: Auswertung der Daten, grafische Darstellung
Panel D-F: Praktische Durchführung

Figure 5: Grafische Darstellung

Figure 6: Grafische Darstellung

Figure 7: Praktische Durchführung (in Zusammenarbeit mit Katharina Petry, Birte Albrecht, Jana Larissa Röckendorf und Natalie Baum) und grafische Darstellung

Table 1: -

Table 2: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

6.2 Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimäric Antigen Receptors (Nb-CARs)

Manuskript: Erster Entwurf, Recherche, Korrektur und Revision

Figure 1: Grafische Darstellung

Figure 2: Grafische Darstellung

Figure 3: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 4: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 5: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 6: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 7: Praktische Durchführung (in Zusammenarbeit mit Birte Albrecht, Katharina Petry, Jana Larissa Röckendorf und Natalie Baum), Auswertung der Daten und grafische Darstellung

6.3 Half-life extended Nanobody-based CD38-specific Bispecific Killercell Engagers (HLE nano-BiKEs) induce killing of Multiple Myeloma cells

Manuskript: Erster Entwurf, Recherche, Korrektur und Revision

Figure 1: Grafische Darstellung

Figure 2: *Panel A-C*: Grafische Darstellung
Panel A-B: Praktische Durchführung und Auswertung der Daten

Figure 3: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 4: *Panel A-D*: Auswertung der Daten, grafische Darstellung
Panel A, C, D: Praktische Durchführung

Figure 5: Praktische Durchführung, Auswertung der Daten und grafische Darstellung

Figure 6: Praktische Durchführung (in Zusammenarbeit mit Anna Josephine Gebhardt), Auswertung der Daten und grafische Darstellung

Hiermit bestätige ich, Prof. Dr. Friedrich Koch-Nolte, als Betreuer dieser Dissertation die Richtigkeit der hier gemachten Angaben zur Abgrenzung des eigenen Anteils.

7 Aus der Dissertation hervorgegangene Veröffentlichungen

7.1 In dieser Dissertation vorgestellte Publikationen

Schriewer L*, Schütze K*, Petry K*, **Hambach J**, Fumey W, Koenigsdorf J, Baum N, Menzel S, Rissiek B, Riecken K, Fehse B, Röckendorf JL, Schmid J, Albrecht B, Pinnschmidt H, Ayuk F, Kröger N, Binder M, Schuch G, Hansen T, Haag F, Adam G, Koch-Nolte F, Bannas P. *Nanobody-based CD38-specific heavy chain antibodies induce killing of multiple myeloma and other hematological malignancies.* **Theranostics. 2020** Feb 3;10(6):2645-2658.
doi: 10.7150/thno.38533. PMID: 32194826; PMCID: PMC7052896.

Hambach J, Riecken K, Cichutek S, Schütze K, Albrecht B, Petry K, Röckendorf JL, Baum N, Kröger N, Hansen T, Schuch G, Haag F, Adam G, Fehse B, Bannas P, Koch-Nolte F. *Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimeric Antigen Receptors (Nb-CARs).* **Cells**. **2020** Jan 29;9(2):321.
doi: 10.3390/cells9020321. PMID: 32013131; PMCID: PMC7072387.

Hambach J, Fumey W, Stähler T, Gebhardt AJ, Adam G, Weisel K, Koch-Nolte F, Bannas P. *Half-Life Extended Nanobody-Based CD38-Specific Bispecific Killercell Engagers Induce Killing of Multiple Myeloma Cells*. **Front Immunol. 2022** May 16;13:838406.
doi: 10.3389/fimmu.2022.838406. PMID: 35651607; PMCID: PMC9150782.
*= geteilte Erst-Autorenschaft

7.2 Publikationen

Baum N, Fliegert R, Bauche A, **Hambach J**, Menzel S, Haag F, Bannas P, Koch-Nolte F. *Daratumumab and Nanobody-Based Heavy Chain Antibodies Inhibit the ADPR Cyclase but not the NAD^+ Hydrolase Activity of CD38-Expressing Multiple Myeloma Cells.* **Cancers (Basel). 2020** Dec 30;13(1):76.
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Baum N, Eggers M, Koenigsdorf J, Menzel S, **Hambach J**, Staehler T, Fliegert R, Kulow F, Adam G, Haag F, Bannas P, Koch-Nolte F. *Mouse CD38-Specific Heavy Chain*

Antibodies Inhibit CD38 GDPR-Cyclase Activity and Mediate Cytotoxicity Against Tumor Cells. **Front Immunol. 2021** Sep 3;12:703574.
doi: 10.3389/fimmu.2021.703574. PMID: 34539634; PMCID: PMC8446682.

Hambach J, Mann AM, Bannas P, Koch-Nolte F. *Targeting multiple myeloma with nanobody-based heavy chain antibodies, bispecific killer cell engagers, chimeric antigen receptors, and nanobody-displaying AAV vectors*. **Front Immunol. 2022** Nov 2;13:1005800.
doi: 10.3389/fimmu.2022.1005800. PMID: 36405759; PMCID: PMC9668101.

Pape LJ, **Hambach J**, Gebhardt AJ, Rissiek B, Stähler T, Tode N, Khan C, Weisel K, Adam G, Koch-Nolte F, Bannas P. *CD38-specific nanobodies allow in vivo imaging of multiple myeloma under daratumumab therapy*. **Front Immunol. 2022** Oct 27;13:1010270.
doi: 10.3389/fimmu.2022.1010270. PMID: 36389758; PMCID: PMC9647632.

7.3 Abstracts bei Kongressen und Retreats

Schütze K, Petry K, **Hambach J**, Fumey W, Schriewer L, Röckendorf J, Baum N, Albrecht B, Eggers M, Haag F, Bannas P, Koch-Nolte F. *Fusion of two distinct CD38-specific nanobodies into a human IgG1 biparatopic heavy chain antibody (bi-hcAb) results in potent CDC against myeloma cells.* **XI. UCCH Research Retreat 2019**

Hambach J. *Targeting of CD38-expressing tumors using nanobody-based chimeric Antigen Receptors (CARs).* **UCCH Immuno-Oncology Symposium 2019**

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Hambach J, Riecken K, Albrecht B, Cichutek S, Jung M, Fehse B, Bannas P, Koch-Nolte F. *CD38^{+}-specific nanobody-based chimeric antigen receptors (CAR).* **1st Bonn Nanobody Symposium 2019**

Hambach J, Riecken K, Albrecht B, Cichutek S, Jung M, Fehse B, Bannas P, Koch-Nolte F. *CD38-specific nanobody-based Chimeric Antigen Receptors (CARs) targeting*

CD38 expressing multiple myeloma cells. **European School of Haematology - International Conference - Immune and Cellular Therapies: Focus on Immune Based Therapeutic Concepts 2019**

Hambach J, Khan C, Eggers M, Gebhardt AJ, Duttmann A, Fumey W, Bannas P, Koch-Nolte F. *Targeting CD38-expressing Multiple Myeloma Cells using Nanobody-based CD38-specific Bispecific Killer Cell Engagers (BiKEs).* **Nanobodies (Hybrid 2nd edition) 2021**

Hambach J, Riecken K, Eggers M, Albrecht B, Cichutek S, Jung M, Fehse B, Bannas P, Koch-Nolte F. *Targeting CD38-Expressing Multiple Myeloma and Burkitt Lymphoma Cells In Vitro with Nanobody-Based Chimeric Antigen Receptors (Nb-CARs).* **DFG Translational Immunology School 2021**

Hambach J, Gebhardt AJ, Pape L, Fumey W, Schütze K, Stähler T, Adam G, Weisel K, Koch-Nolte F and Bannas P. *Nanobody-based CD38-specific CARs and BiKEs sent into race against multiple myeloma.* **ESGCT 29th Congress in collaboration with BSGCT 2022**

Hambach J, Gebhardt AJ, Pape L, Duttmann A, Fumey W, Stähler T, Adam G, Weisel K, Koch-Nolte F, Bannas P. *CD38-specific nanobody-based half-life extended bispecific killer cell engagers (HLE-nano-BiKEs) for the treatment of multiple myeloma.* **EuroMAbNet 6th Antibody Validation Workshop and 12th Meeting 2022**

7.4 Poster

Schütze K, Petry K, **Hambach J**, Fumey W, Schriewer L, Röckendorf J, Baum N, Albrecht B, Eggers M, Haag F, Bannas P, Koch-Nolte F. *Biparatopic heavy chain antibodies (bi-hcAbs): fusion of two distinct CD38-specific nanobodies into a human IgG1 bi-hcAb results in potent CDC vs. myeloma cells.* **XI. UCCH Research Retreat 2019**

Hambach J, Riecken K, Albrecht B, Cichutek S, Jung M, Fehse B, Bannas P, Koch-Nolte F. *Engineering NK cells to express Nanobody-based Chimeric Antigen Receptors (CARs).* **ESGCT 27th Annual Congress Barcelona 2019**

Hambach J, Riecken K, Albrecht B, Cichutek S, Jung M, Fehse B, Bannas P, Koch-Nolte F. *CD38^{+}-specific nanobody-based chimeric antigen receptors (CAR).* **1st Bonn Nanobody Symposium 2019**

Hambach J, Khan C, Eggers M, Gebhardt AJ, Duttmann A, Fumey W, Bannas P, Koch-Nolte F. *Targeting CD38-expressing Multiple Myeloma Cells using Nanobody-based CD38-specific Bispecific Killer Cell Engagers (BiKEs).* **Nanobodies (Hybrid 2nd edition) 2021**

Hambach J, Gebhardt AJ, Pape L, Fumey W, Schütze K, Stähler T, Adam G, Weisel K, Koch-Nolte F and Bannas P. *Nanobody-based CD38-specific CARs and BiKEs sent into race against multiple myeloma.* **ESGCT 29th Congress in collaboration with BSGCT 2022**

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- frei aus Leonce und Lena von Georg Büchner

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