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Strategien der Optimierung von Immuntherapien: CD16-CAR-T-Zellen und neue TLR-7 Agonisten

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Severin Johannes Jacobi

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Berichterstatter:	Prof. Dr. Stefan Endres
Mitberichterstatter:	Prof. Dr. Louisa von Baumgarten
	Prof. Dr. Andreas Humpe
	Prof. Dr. Marion Subklewe
Mitbetreuung durch den promovierten Mitarbeiter:	Prof. Dr. Sebastian Kobold PD Dr. Gabriela Wiedemann
Dekan:	Prof. Dr. med. Thomas Gudermann

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1 Affidavit

Eidesstattliche Versicherung

Jacobi, Severin

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

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München, 21.01.2025

Severin Jacobi

2 Inhaltsverzeichnis

3 Abkürzungsverzeichnis

ADCC	*Antibody-dependent cytotoxicity*
APC	*Antigen-presenting cell*
CAR	*Chimärer Antigenrezeptor*
CDC	*Complement-dependent cytotoxicity*
CDR	*Complementarity determining regions*
CRS	*Cytokine release syndrome*
EGFR	*Epidermal growth factor receptor*
Fab	*Fragment antigen binding*
Fc	*Fragment crystallizable*
FcγRIII	Fcγ-Rezeptor III (CD16)
GE	*Glykoengineering*
ICANS	*Immune effector cell-associated neurotoxicity syndrome*
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IL-12	Interleukin-12
IRF	Interferonregulationsfaktor
MDSC	*Myeloid-derived suppressor cells*
MHC	*Major histocompatibility complex*
modCAR-T-Zellen	Modulare CAR-transduzierte T-Zellen
MyD88	*Myeloid differentiation factor 88*
NF-κB	*Nuclear factor kappa B*
NK-Zellen	Natürliche Killerzellen
PD-L1	*Programmed death-ligand 1*
PRR	*Pattern-recognition receptors*
R848	Resiquimod
TCR	*T-cell receptor*
TDL	Tumor-drainierende Lymphknoten
TIL	Tumorinfiltrierende Lymphozyten
TLR	Toll-*like*-Rezeptor
TME	*Tumor microenvironment*
TNF-α	Tumornekrosefaktor-α
TRAIL	*Tumor necrosis factor-related apoptosis inducing ligand*

4 Veröffentlichungen

Die Ergebnisse dieser Arbeit wurden in folgenden internationalen Fachzeitschriften als Originalarbeiten veröffentlicht:

1. Rataj F, **Jacobi SJ**, Stoiber S, Asang F, Ogonek J, Tokarew N, Cadilha BL, van Puijenbroek E, Heise C, Duewell P, Endres S, Klein C, Kobold S.
High-affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy.
British Journal of Cancer 2019 Jan; 120(1):79-87.

2. Wiedemann GM*, **Jacobi SJ***, Chaloupka M*, Krächan A, Hamm S, Strobl S, Baumgartner R, Rothenfusser S, Duewell P, Endres S, Kobold S.
A novel TLR7 agonist reverses NK cell anergy and cures RMA-S lymphoma-bearing mice.
Oncoimmunology 2016; 5(7):e1189051

5 Einleitung

5.1 Grundlagen der Immuntherapie

5.1.1 Das Konzept des Immunoediting

Das Immunsystem erkennt körpereigene sowie körperfremde Einflüsse, welche die Intaktheit des Gewebes stören, und reagiert auf diese (Mujal et al. 2021). Auch die maligne Entartung von Zellen kann als „verändertes Selbst“ durch das Immunsystem detektiert und bekämpft werden. Eine wesentliche Rolle spielen dabei Mutationen, die sich im Rahmen der Tumorentstehung anhäufen und das Proteom sowie das Verhalten der Zellen verändern (Burnet 1957, Dunn et al. 2004). Proteine, die dabei einer Immunreaktion zugänglich sind, können als Tumorantigene bezeichnet werden (Kufe et al. 2003). Trotz einer möglichen Erkennung durch das Immunsystem kann es zu Entwicklung und Fortschreiten von Tumorerkrankungen kommen. Das Modell des „*Immunoediting*“ beschreibt die Interaktion von Immunsystem und Tumor dabei als dreistufigen Prozess: Eliminierung, Equilibrium und Evasion (Dunn et al. 2004).

Während der Phase der Eliminierung erkennen und vernichten angeborenes und adaptives Immunsystem Zellen, die den zellintrinsischen Mechanismen der Tumorsuppression entkommen sind. Im Laufe der weiteren Tumorentwicklung werden vereinzelt T-Zellen dieser Eliminierung entgehen, jedoch wird die Entstehung eines Tumors durch den ständigen Druck des Immunsystems verhindert. In dieser Situation des Gleichgewichts der beiden Kräfte, die Jahre andauern kann, erwerben die Tumorzellen durch weitere Mutationen und die immunologische Selektion zunehmend Mechanismen der Resistenz. Schlüsselrollen spielen dabei die verminderte Präsentation von Antigenen über den *major histocompatibility complex* (MHC) und die Expression immunsuppressiver Oberflächenmoleküle, wie zum Beispiel dem *programmed death-ligand 1* (PD-L1) (O'Donnell et al. 2019). Auch die Umgebung der Tumorzellen (*tumor microenvironment*, TME) wird dabei zunehmend immunsuppressiv. Im Vordergrund steht dabei neben einem besonderen Milieu von Metaboliten und Zytokinen die Ansammlung bestimmter Zellpopulationen wie regulatorischer T-Zellen oder myeloider Suppressorzellen (*myeloid-derived suppressor cells*, MDSC) (Naing 2021). Wird schließlich der Status des Gleichgewichts durchbrochen, kommt es zu einem klinisch apparentem Tumorwachstum (Schreiber et al. 2011, Sun et al. 2018).

Immuntherapeutische Ansätze versuchen an den Evasionsmechanismen des Tumors anzugreifen, die anti-tumoralen Eigenschaften des Immunsystems zu nutzen und eine langfristige Eliminierung zu erreichen. In den letzten Jahren hat der therapeutische

Einsatz von Checkpoint-Inhibitoren, die Schlüsselstellen immunsuppressiver Signalwege blockieren, für klinische Erfolge bei einer Reihe von Tumorentitäten gesorgt (Brahmer et al. 2012, Hodi et al. 2010, Yarchoan et al. 2017).

5.1.2 Sekundäre Evasion als Herausforderung der Immuntherapie

Bei der Therapie mit Checkpoint-Inhibitoren sowie anderen immuntherapeutischen Ansätzen werden zum Teil langfristige Erfolge mit der Etablierung eines immunologischen Gedächtnisses beobachtet. Dennoch zeigt ein großer Teil der Patienten eine primäre Resistenz oder ein sekundäres Fortschreiten der malignen Erkrankung trotz anfänglichem Ansprechen (Fares et al. 2019, Haslam et al. 2019). Der sekundäre Progress kann dadurch erklärt werden, dass ein Tumor therapeutisch zunächst in eine Phase des Equilibriums überführt wird, bei der er nicht vollständig vernichtet wird. Aufgrund neuerlicher Selektionsmechanismen entgeht der Tumor jedoch der immunologischen Kontrolle, schreitet im Sinne der Evasion fort und zeigt dadurch eine erworbene Resistenz (O'Donnell et al. 2019, Schreiber et al. 2011). Für viele Entitäten konnte gezeigt werden, dass die Kombination unterschiedlicher immuntherapeutischer Ansätze die Wahrscheinlichkeit einer Tumorelimination erhöhen kann und somit ein verbessertes Überleben ermöglicht (Sharma et al. 2017, Zappasodi et al. 2018). Die vorliegende Arbeit beschreibt zwei Ansätze, die den Mechanismen der Immunevasion durch Modulation der angeborenen sowie erworbenen Immunantwort begegnen. Zunächst werden einige, für die Fragestellung relevante, Elemente des Immunsystems im Hinblick auf ihre Bedeutung in der Tumortherapie dargestellt.

5.2 Antikörpertherapie

5.2.1 Stellenwert von Antikörpern in der Krebstherapie

Als durch die Entwicklung der Hybridomtechnologie die Herstellung monoklonaler Antikörper möglich wurde, revolutionierte dies nicht nur die immunologische Forschung, sondern auch die moderne onkologische Therapie (Köhler et al. 1975). Heute stellen monoklonale Antikörper die größte Klasse der klinisch angewandten Biotherapeutika dar. So wurden mittlerweile über 100 monoklonale Antikörper in den USA für verschiedene Erkrankungen, darunter auch diverse Tumorerkrankungen, zugelassen (Mullard 2021).

Die Wirkung von Antikörpern unterscheidet sich durch das gewählte Antigen sowie die Struktur des Antikörpers. Um eine direkte anti-tumorale Wirkung zu erzielen, können Elemente von Signalwegen, die in der malignen Transformation dereguliert sind, als

Zielstrukturen gewählt werden. Dabei können onkogene Signalwege der Tumorzelle sowie wachstumsfördernde Prozesse der Tumorumgebung, zum Beispiel die Angiogenese, blockiert werden (Nelson et al. 2010). Durch die Bindung an Rezeptoren von Wachstumsfaktoren können so proliferative, anti-apoptotische und invasive Signale inhibiert und die Apoptose der Zielzelle ausgelöst werden (Redman et al. 2015). Als Beispiel hierfür kann der Einsatz von Cetuximab in der Therapie des Kolonkarzinoms angeführt werden. Dieser Antikörper bindet an den Rezeptor des *epidermal growth factor* (EGFR) und hemmt damit die Aktivierung dessen Signalwegs, der bei Kolonkarzinomen dereguliert sein kann (Karapetis et al. 2008, Li et al. 2005).

Des Weiteren kann durch Bindung der Antikörper an Antigenstrukturen der Tumorzellen indirekt eine zytotoxische Wirkung vermittelt werden. Dabei werden im Wesentlichen drei Mechanismen unterschieden. Bei der komplement-vermittelten Zytotoxizität (*complement-dependent cytotoxicity*, CDC) wird die Signalkaskade des Komplementsystems aktiviert, die in der Formierung eines membran-attackierenden Komplexes mit folgender Lyse der Zielzelle mündet. Zudem führen Elemente des Komplementsystems durch Chemotaxis zu einer Rekrutierung einer zellulären Immunreaktion (Dunkelberger et al. 2010). Die Antikörper-abhängige zelluläre Zytotoxizität (*antibody-dependent cytotoxicity*, ADCC) wird durch Bindung des kristallisierbaren Fragments des Antikörpers (*fragment crystallizable*, Fc) an Rezeptoren von Effektorzellen, vor allem NK-Zellen, ausgelöst. Nach Aktivierung dieser Zellen kommt es zur Ausschüttung und Oberflächenexpression von Molekülen, die die Apoptose der Zielzelle einleiten (Scott et al. 2012, Weng et al. 2003). Als dritter Mechanismus wird die Phagozytose durch Makrophagen beschrieben, die ebenfalls über einen Fc-Rezeptor vermittelt wird (Gül et al. 2014).

Ein weiterer erfolgreicher Ansatz in der Antikörpertherapie ist die Verstärkung der bestehenden körpereigenen anti-tumoralen Immunantwort. Dies kann durch blockierende Antikörper von Immuncheckpoints (z.B. Ipilimumab) sowie bispezifische Antikörper, die Tumorzellen mit T-Zellen verbinden und Letztere dadurch aktivieren, erreicht werden (Baeuerle et al. 2022, Hodi et al. 2010, Staerz et al. 1985).
An tumorspezifische Antikörper können zudem therapeutische Moleküle wie Radionuklide oder Zytostatika sowie Zytokine gebunden werden, die so gezielt zu ihrem Wirkort an der Tumorzelle gebracht werden können (Jin et al. 2022).
Eine schwache Effektorfunktion, eine unvollständige Penetration des Tumors durch Antikörper sowie Nebenwirkungen durch Bindung und Aktivierung von

Effektormechanismen abseits von Zielzellen stellen Herausforderungen der onkologischen Antikörpertherapie dar (Scott et al. 2012).

5.2.2 Strukturmodifikationen therapeutischer Antikörper

Um den anti-tumoralen Effekt therapeutischer Antikörper zu verstärken, kann deren Struktur modifiziert werden (Jin et al. 2022). Antikörper bestehen aus zwei identischen schweren und zwei identischen leichten Ketten. Sowohl schwere als auch leichte Ketten besitzen eine variable sowie eine konstante Domäne. Die variable Domäne trägt sechs *complementarity determining regions* (CDR), die als Paratop spezifisch an ein Epitop eines Antigens binden. Die enzymatische Spaltung mit Papain teilt einen Antikörper in drei Fragmente: zwei antigenbindende Domänen (Fab) und eine Fc-Domäne, die die Effektorfunktion des Antikörpers vermittelt. Die molekulare Struktur der Fc-Domäne beeinflusst das Bindeverhalten an die verschiedenen Fc-Rezeptoren der Effektoren und bestimmt so indirekt die Gestalt der Effektormechanismen (Murphy et al. 2022). Aufgrund der potenten Aktivierung von ADCC, CDC und Phagozytose werden in der Tumortherapie vor allem Antikörper des Isotyps IgG1 verwendet (Delidakis et al. 2022).

Durch Veränderung der Aminosäuresequenz im Fc-Teil der Antikörper kann die Affinität zu Fc-Rezeptoren und somit die Aktivierung zytotoxischer Mechanismen optimiert werden (Delidakis et al. 2022). Auch Strukturalterationen im Fc-Teil, die eine Bindung an Fc-Rezeptoren verhindern, können therapeutisch genutzt werden. Dadurch können bei Antikörpern, deren therapeutisches Ziel lediglich die Interaktion mit Signalwegen darstellt, Nebenwirkungen durch unspezifische Effektoraktivierung vermieden werden (Schlothauer et al. 2016, Wilkinson et al. 2021).

Des Weiteren kann die Glykosylierungsstruktur der Fc-Domäne einen erheblichen Einfluss auf das Bindungsverhalten an Fc-Rezeptoren haben. Bei gezielter Veränderung der Glykosylierung spricht man von „*glycoengineering*“ (GE). Obinutuzumab ist der erste Antikörper, der auf diese Weise entwickelt wurde und nun zugelassen ist. Bei dessen Entwicklung konnte gezeigt werden, dass in Abwesenheit eines zentralen Fukoserestes an Asn297 im Fc-Teil von IgG die Bindungsaffinität zu FcyRIII, dem wesentlichen Rezeptor für die Aktivierung der ADCC sowie der Phagozytose, erhöht ist. Wird zusätzlich ein komplexer, verzweigter Oligosaccharid-Rest an dieser Stelle angehängt, kann die Affinität weiter gesteigert werden. Bei Obinutuzumab, der als anti-CD20 Antikörper durch diese beiden Modifikationen

verändert wurde, konnte eine klinisch relevante Verstärkung der ADCC nachgewiesen werden (Ferrara et al. 2011, Goede et al. 2015, Mössner et al. 2010).
Auch einzelne Antikörperfragmente können allein, zusammengesetzt als multispezifische Antikörper oder gekoppelt mit weiteren Effektormolekülen therapeutisch eingesetzt werden. (Jin et al. 2022)

5.3 T-Zelltherapie

5.3.1 Arten der T-Zelltherapie

Während klassischen Antikörpern lediglich Tumorantigene zugänglich sind, die auf der Zelloberfläche exprimiert werden, können T-Zellen auch veränderte intrazelluläre Peptidstrukturen detektieren, die über den MHC präsentiert werden. Über ihre effektive zytotoxische Funktion sowie die Ausschüttung von Zytokinen können sie eine körpereigene anti-tumorale Antwort generieren (Coulie et al. 1995, Wölfel et al. 1995). Vor diesem Hintergrund wurde die adoptive T-Zell-Therapie entwickelt, welche die günstigen Eigenschaften von T-Zellen ausnützt und durch gentechnische Methoden oder pharmakologische Stimulation verbessert. Dabei können im Wesentlichen drei Strategien unterschieden werden, die sich in Bezug auf die Quelle der verwendeten Zellen sowie der Art der Modifikation unterscheiden (Rosenberg et al. 2015). Tumorinfiltrierende Lymphozyten (TIL) können aus einem resezierten Tumor isoliert und dem Patienten wieder therapeutisch verabreicht werden, nachdem sie *in vitro* expandiert und stimuliert wurden. Mit dieser Strategie konnten bereits sehr früh erste Erfolge der adoptiven Zelltherapie bei Patienten mit malignem Melanom erzielt werden (Rosenberg et al. 1988). Die transferierten T-Zellen können noch Monate später als oligoklonale Populationen im Blut der Patienten nachgewiesen werden (Dudley et al. 2002, Shen et al. 2007).

Auch peripher isolierte T-Zellen können nach entsprechender Modifikation therapeutisch verwendet werden. Zum einen können T-Zellen mit T-Zellrezeptoren (*T-cell receptor*, TCR) transduziert werden, die gegen MHC-präsentierte Tumorantigene gerichtet sind (Morgan et al. 2003, Morgan et al. 2006). Die Anwendbarkeit dieser Therapie ist jedoch nicht nur von der Expression des gewählten Antigens durch die Tumorzellen des Patienten, sondern auch vom passenden Typ des humanen Leukozytenantigens abhängig (Shafer et al. 2022). Zum anderen können die T-Zellen mit einem chimären Antigenrezeptor (CAR) versehen werden, der Antigene auf der Zelloberfläche der Zielzellen durch eine Einzelkette des variablen Teils eines monoklonalen Antikörpers bindet (Gross et al. 1989). Über eine transmembrane

Domäne ist dieser mit einer intrazellulären stimulatorischen Einheit verbunden, die ähnlich dem Signalweg des T-Zellrezeptors zu einer Aktivierung der T-Zelle führt. Durch diese Fusionsrezeptoren können MHC-unabhängig Tumorantigene der Zelloberfläche detektiert und die Effektormechanismen der T-Zelle aktiviert werden (Hiltensperger et al. 2023, Schmidts et al. 2018).

5.3.2 Limitationen der CAR-T-Zelltherapie

Während sich autologe CAR-T-Zelltherapien im Bereich einiger maligner hämatologischer Erkrankungen bereits klinisch etablieren konnten, bestehen bei der Therapie von soliden Tumoren noch erhebliche Schwierigkeiten, die auf folgende Aspekte zurückzuführen sind. Nach der Applikation des T-Zellprodukts müssen die T-Zellen zunächst in den Tumor gelangen und dort expandieren. Durch chaotische Vaskularisierung des Tumors, verminderte Expression von Adhäsionsmolekülen an der Gefäßwand, eine unzureichende Chemotaxis sowie erhöhten interstitiellen Druck wird die Infiltration des Tumors jedoch erschwert (Slaney et al. 2014). Eine nachhaltige Persistenz und Aktivität von T-Zellen im Tumor wird schließlich durch das immunsuppressive TME sowie die Erschöpfung der T-Zellen eingeschränkt (Park et al. 2018). Auch die Mutation, verminderte Expression oder der vollständige Expressionsverlust des adressierten Antigens stellen eine Möglichkeit des Tumors zur Immunevasion dar (Hiltensperger et al. 2023). Bei TIL oder TCR-T-Zelltherapie können Tumorzellen der Therapie auch durch verminderte Expression von MHC-I entkommen (Garrido et al. 2016, Tran et al. 2016).

Neben den beschriebenen Problemen einer eingeschränkten anti-tumoralen Effizienz schränken Nebenwirkungen die klinische Anwendbarkeit ein. Die Aktivierung von Effektormechanismen durch Detektion von Antigenen im gesunden Gewebe abseits des Tumors kann als „*on target, off tumor*“-Toxizität teils zu fatalen klinischen Verläufen führen. Der Auswahl geeigneter Antigene, der Entwicklung von Detektionsstrategien mit hoher Spezifität sowie Mechanismen zur Unterbrechung bzw. Beendigung der Therapie kommt daher eine hohe Bedeutung zu (Morgan et al. 2010). Zudem tritt häufig ein Zytokinfreisetzungssyndrom (*cytokine release syndrome*, CRS) auf, bei dem es zu einer systemischen inflammatorischen Reaktion kommt, die sich als Grippe-ähnliche Symptomatik, Fieber oder sogar lebensbedrohliche Organtoxizität äußern kann. Auch neurologische Nebenwirkungen sind als Immuneffektorzell-assoziiertes Neurotoxizitätssyndrom (*immune effector cell-associated neurotoxicity syndrome*, ICANS) von klinischer Bedeutung (Morris et al. 2022). Zuletzt stellen zeitaufwändige

Expansionsprotokolle und hohe Kosten Nachteile der T-Zelltherapie dar (Santomasso et al. 2019).

5.3.3 Modulare CAR-T-Zelltherapie

Die Strategie der so genannten modularen (mod) oder universalen CAR-T-Zelltherapie versucht dem Antigenverlust der Tumorzelle zu begegnen, indem Antigenerkennung und CAR-Aktivierung voneinander getrennt werden. Dabei werden lösliche Antigen-bindende Adaptermoleküle, meist Antikörper, eingesetzt, die an die Tumorzelle binden. Die verabreichten modCAR-T-Zellen binden dann spezifisch an eine Domäne dieses Adaptermoleküls und werden so aktiviert. Dadurch können bei Verwendung eines einzigen T-Zellproduktes durch Variation des verwendeten Adaptermoleküls verschiedene Antigene als Zielstrukturen gewählt werden. Als Adaptermoleküle können bispezifische Antikörper, Antikörper mit besonderer Bindedomäne („*Tag*“, z. B. Biotin) sowie klassische Antikörper eingesetzt werden. Der Vorteil letzterer Strategie in Kombination mit chimären Fc-Rezeptoren ist die Anwendbarkeit bereits zugelassener therapeutischer Antikörper ohne notwendige Modifikation (Arndt et al. 2020).
Im Hinblick auf die Tumorevasion kann die modCAR-Strategie vorteilhaft sein, da simultan oder sequenziell verschiedene Antigene durch Kombination oder Wechsel der verwendeten Adaptermoleküle adressiert werden können. So kann eine hohe Spezifität der Therapie erreicht und auf einen Antigenverlust reagiert werden. Zudem könnte theoretisch ein einziges T-Zell-Produkt bei allen malignen Erkrankungen eingesetzt werden, sofern dafür geeignete Adaptermoleküle zur Verfügung stehen. Über das Applikationsschema und die Pharmakokinetik des Adaptermoleküls kann die Antigen-spezifische Wirkung der Zelltherapie gesteuert und begrenzt werden. Gerade bei Verwendung kleiner Adaptermoleküle mit kurzer Serumhalbwertszeit könnte Nebenwirkungen wie CRS, ICANS oder „*on-target, off-tumor*“ Toxizität durch Pausierung der Applikation rasch begegnet werden, ohne die Persistenz des T-Zellproduktes zu beeinträchtigen (Arndt et al. 2020, Darowski et al. 2019).

5.4 NK-Zellen

5.4.1 Funktion von NK-Zellen

Als ein wichtiger Bestandteil des angeborenen Immunsystems können Natürliche Killerzellen (NK-Zellen) rasch auf ein breites Feld von Stimuli reagieren, während sich die adaptive Immunantwort formiert. Zudem können sie die adaptive Immunantwort auslösen und verstärken. Entdeckt wurden sie als lymphoide Zellen mit zytotoxischer Eigenschaft gegen virusinfizierte sowie maligne veränderte Zellen (Herberman et al.

1975, Kiessling et al. 1975a, Kiessling et al. 1975b). Im Gegensatz zu B- und T-Zellen, die Antigen-spezifische Rezeptoren basierend auf somatischer Rekombination exprimieren, weisen sie ein großes Repertoire an Keimbahn-kodierten stimulatorischen und inhibitorischen Oberflächenrezeptoren auf. Ihre Aktivität wird durch ein komplexes Zusammenspiel dieser Signale gesteuert (Long et al. 2013). Beispielsweise exprimieren sie Rezeptoren, die durch Bindung an körpereigene MHC-I-Moleküle inhibitorische Signalwege initiieren und bei Kontakt mit gesunden Zellen eine Toleranz vermitteln. Bei erkrankten Zellen ohne MHC-I Präsentation fehlt diese Hemmung, so dass es zu einer Aktivierung der NK-Zellen kommen kann (Karre et al. 1986, Ljunggren et al. 1990). Dadurch können sie viral infizierte oder maligne veränderte Zielzellen angreifen, die durch eine verminderte Expression von MHC-I der adaptiven Immunantwort durch CD8+ T-Zellen entgehen. Pathogen-assoziierte Muster virusinfizierter Zellen oder die Expression von speziellen Liganden durch gestresste Zellen können durch Bindung an entsprechende Rezeptoren der NK-Zellen aktivierende Signale vermitteln. Auch Antikörper, die an die Antigene der Zielzelle gebunden sind, führen über Fc-Rezeptoren zur Aktivierung und können somit eine zytotoxische Reaktion auslösen (ADCC). Die Interaktion mit den Zielzellen wird zusätzlich durch den Kontakt mit anderen umgebenden Immunzellen und Zytokinen moduliert (Mujal et al. 2021).

Aktivierte NK-Zellen können über die Ausschüttung von Granzymen und Perforinen, über die Expression von Todesrezeptor-Liganden wie *tumor necrosis factor-related apoptosis inducing ligand* (TRAIL) oder den Fas-Liganden eine Apoptose der Zielzelle auslösen (Fehniger et al. 2007, Prager et al. 2019). Die zweite wichtige Funktion der NK-Zellen ist die Ausschüttung proinflammatorischer Zytokine, vor allem von Interferon-γ (IFN-γ) und Tumornekrosefaktor-α (TNF-α) (Martín-Fontecha et al. 2004).

Trotz ihrer Zuordnung zum angeborenen Immunsystem können in viralen Modellen, wie z. B. bei Infektion mit dem Zytomegalievirus, auch adaptive Eigenschaften von NK-Zellen nachgewiesen werden. Nach einer initialen Infektion verbleibt eine lang lebende Population an „Gedächtniszellen“, die bei erneuter Infektion zu einer raschen, robusten und antigenspezifischen Immunantwort fähig ist. Diese kann im murinen Modell noch Monate nach initialem Antigenkontakt fortbestehen und auf naive Tiere übertragen werden (Sun et al. 2009). Eine Gedächtnisfunktion der anti-viralen Immunität konnte auch für humane NK-Zellen gezeigt werden. Ähnliche Eigenschaften wurden bei wiederholter Stimulation von NK-Zellen durch Zytokine sowie bei Applikation

chemischer Haptene gezeigt (Cooper et al. 2009, Mujal et al. 2021, Reeves et al. 2015).

5.4.2 NK-Zellen in der Krebstherapie

Für einige solide Tumorentitäten wie Lungenkarzinome oder kolorektale Karzinome konnte eine Korrelation zwischen Infiltration durch NK-Zellen und einem besseren Gesamtüberleben gezeigt werden (Nersesian et al. 2021). Tumore können dennoch einer NK-Zellantwort entgehen, indem sie Bindungspartner aktivierender NK-Zellrezeptoren vermindert exprimieren und die Funktion der NK-Zellen durch ein immunsuppressives TME hemmen (Li et al. 2021). Dabei kann sich ein überwiegend Toleranz-vermittelndes Rezeptorprofil der NK-Zellen entwickeln. Zudem wurde ein Zustand der Anergie von NK-Zell bei Kontakt zu Tumoren ohne MHC-I Expression beschrieben, die durch Zytokinapplikation reversibel ist (Ardolino et al. 2014, Chauvin et al. 2020).

Zunehmend werden immuntherapeutische Strategien entwickelt, um die hervorragenden Effektoreigenschaften, die vielschichtige Regulierung ihrer Aktivität und die Langlebigkeit bestimmter Gedächtnispopulationen der NK-Zellen klinisch nutzbar zu machen (Laskowski et al. 2022). Ansätze sind der adoptive Transfer von NK-Zellen, die mit Zytokinen stimuliert oder mit einem CAR transduziert wurden, der Einsatz bi- oder multispezifischer NK-Zell-bindender Antikörper, die Therapie mit Zytokinen oder Zytokin-Superagonisten und die Applikation von Checkpoint-Inhibitoren (Liu et al. 2020, Miller et al. 2019).

5.5 Toll-*like*-Rezeptor-7

5.5.1 Eigenschaften von Toll-*like*-Rezeptor-7

Toll-*like*-Rezeptoren (TLR) stellen als mustererkennende Rezeptoren (*Pattern-recognition receptors*; PRR) einen weiteren wichtigen Teil der angeborenen Immunität dar. Durch Bindung von Pathogen-assoziierten molekularen Mustern kann eine Immunantwort hervorgerufen werden (Fitzgerald et al. 2020). Während membranständige TLR (TLR 1, 2, 4, 5, 6 und 10) Protein- und Lipidstrukturen erkennen, sind Rezeptoren der Erkennung von DNA und RNA endosomal lokalisiert (TLR 3, 7, 8 und 9) (West et al. 2006).

Als endosomaler Rezeptor wird TLR7 durch Bindung von Einzelstrang-RNA mit geringer nukleosidischer Modifikation, wie sie bei Bakterien oder Viren vorliegt, aktiviert

(Diebold et al. 2004, Hornung et al. 2005). Dabei dimerisiert der Rezeptor und führt über den *myeloid differentiation factor 88* (MyD88) zu einer Aktivierung der Transkriptionsfaktoren *nuclear factor kappa B* (NF-κB) sowie der Interferonregulationsfaktoren (IRF) 3 und 7 (Hiroaki Hemmi et al. 2002). Funktionell scheint die Expression von TLR7 durch Antigen-präsentierende Zellen (*antigen-presenting cells*, APC), vorrangig plasmazytoide dendritische Zellen, von zentraler Bedeutung zu sein (Kobold et al. 2014). Die Wirkung wird dabei über die Aktivierung weiterer myeloider und lymphoider Zellen durch Ausschüttung proinflammatorischer Zytokine, z. B. IFN-α, Interleukin 6 und 12, multipliziert. Im murinen Modell konnte bereits gezeigt werden, dass eine Stimulation von TLR7 durch Oligonukleotide vermittelt durch dendritische Zellen zu einer Aktivierung der NK-Zellpopulation führt. Dabei ist die Erhöhung deren Zytotoxizität gegenüber MHC-I-negativen Tumorzellen abhängig von IFN-α, während die Induktion der IFN-γ Ausschüttung der NK-Zellen abhängig von IL-12 ist (Bourquin et al. 2009). Auch eine direkte Wirkung auf dendritische Zellen im Sinne einer Reifung, erhöhten Zytotoxizität und verstärkten Antigenpräsentation konnte nachgewiesen werden (Drobits et al. 2012).

5.5.2 TLR7-Agonisten in der Krebstherapie

Um die günstigen, proinflammatorischen Eigenschaften von TLR7 zu nutzen, wurden diverse *small-molecule*-Agonisten für den therapeutischen Einsatz in der Tumortherapie entwickelt (Kobold et al. 2014). Im Vergleich zu RNA als natürlichem Liganden bieten diese den Vorteil einer besseren Stabilität sowie einer höheren Rezeptorspezifität. Imiquimod, ein Imidazoquinolin, ist aktuell der einzige TLR7-Agonist, der in Europa therapeutisch zugelassen ist. Er wird in der topischen Behandlung von humanem-Papillomavirus-assoziierten Warzen, aktinischer Keratose und superfiziellem Basalzellkarzinom eingesetzt (Leśniak et al. 2023).

In präklinischen Modellen solider Tumore konnte bereits früh ein anti-tumoraler Effekt bei systemischer Applikation von Imiquimod festgestellt werden (Sidky et al. 1992). Der mehrfache Versuch einer systemischen, tumortherapeutischen Anwendung beim Menschen scheiterte jedoch aufgrund dosisbegrenzender Nebenwirkungen wie Fieber, Kopfschmerzen und Fatigue in der klinischen Erprobung (Dudek et al. 2007, Patricia L. Witt et al. 1993, Savage et al. 1996). Um die Nebenwirkungen zu reduzieren, wurden weitere *small-molecule*-Agonisten mit dem Ziel einer verbesserten Pharmakokinetik und einem günstigerem Zytokinprofil entwickelt. Etliche befinden sich als Einzeltherapie, in Kombination mit anderen Immuntherapien, als Antiköperkonjugate sowie als Adjuvanz bei Vakzinierungsstrategien in der klinischen Erprobung (Rolfo et

al. 2023). Zudem werden biomaterial-basierte Systeme, zum Beispiel Nanopartikel, zur zielgerichteten Freisetzung der Agonisten im Tumor entwickelt, um systemische Nebenwirkungen zu reduzieren (Leśniak et al. 2023, Varshney et al. 2021).

5.6 Fragestellung der Arbeit

Der klinische Einsatz immuntherapeutischer Strategien zeigt bei vielen malignen Entitäten bereits große Erfolge. Dennoch profitiert nur eine Minderheit der Patienten im Sinne einer vollständigen Tumorelimination mit nachhaltigem Überleben. Die komplexen und vielschichtigen Mechanismen der Immunevasion machen eine Optimierung und Kombination bestehender Therapien sowie die Entwicklung neuer Strategien notwendig. Eine Herausforderung der Immuntherapie stellt gerade bei Antikörper- sowie CAR-T-Zelltherapie der Antigenverlust der Tumorzelle dar. Auch die verminderte MHC-I-Expression des Tumors trägt zum Versagen verschiedener immuntherapeutischer Strategien wie Checkpoint-Inhibition oder TIL-Therapie bei.

Die CAR-T-Zelltherapie mit chimären CD16-Rezeptoren, die mit bereits etablierten Antikörpern kombiniert werden kann, stellt einen Lösungsansatz für das Problem des Antigenverlustes dar. Antikörper, deren Affinität zu Fc-Rezeptoren durch eine veränderte Glykosylierung erhöht wurde, werden bereits klinisch eingesetzt und stellen auch in der modCAR-T-Zelltherapie eine Kombinationsmöglichkeit dar, die bislang noch nicht evaluiert wurde. Zudem ist unklar, wie sich Polymorphismen im extrazellulären Teil von CD16-CAR-Konstrukten auf die Effektivität dieser Therapie auswirken. Um diese Therapiestrategie weiterzuentwickeln, soll daher der Einfluss beider Modalitäten auf Aktivierung, Proliferation und Zytotoxizität der CD16-CAR-T-Zellen in verschiedenen humanen, präklinischen Modellen untersucht werden.

Tumore mit verringerter MHC-I-Expression stellen ein potenzielles Ziel von NK-Zellen dar. Dennoch können die tumor-induzierte Immunsuppression sowie die Anergie der NK-Zellen eine effektive Immunantwort verhindern. Durch den Einsatz von TLR-Agonisten könnte die NK-Zellpopulation aktiviert und dieser Weg der Immunevasion verhindert werden. Für den neuen TLR7-spezifischen Agonisten SC-1 konnte bereits eine Aktivierung von NK-Zellen im murinen Modell gezeigt werden. Vor einem klinischen Einsatz müssen jedoch Wirkungsmechanismus und therapeutischer Effekt genauer untersucht werden. Dabei soll die Rezeptorspezifität und das Zytokinprofil dieses Agonisten analysiert werden, da dies einen erheblichen Einfluss auf das zu erwartende Nebenwirkungsprofil hat (Geller et al. 2010, Holldack 2014,

Patricia L. Witt et al. 1993). Zudem ist der therapeutische Effekt dieses Agonisten in einem Tumormodell mit geringer MHC-Expression *in vivo* zu testen. Das Zytokinprofil und der therapeutische Effekt soll dabei mit dem etablierten TLR7/8-Agonisten Resiquimod (R848) verglichen werden.

6 Zusammenfassung

In dieser kumulativen Dissertation werden Ergebnisse zweier Originalpublikationen zusammengefasst. Dabei werden zwei unterschiedliche Ansatzpunkte der onkologischen Immuntherapie präklinisch evaluiert.

In der ersten Publikation, Rataj et al., evaluierten wir die therapeutische Relevanz von Polymorphismen in der extrazellulären Domäne eines modCAR-Konstruktes und verglichen die Effektivität von glykomodizierten (*glycoengineered*, GE) Antikörpern mit unmodifizierten Antikörpern. Dazu wurden Panc-1-Pankreaskarzinomzellen, Raji-Lymphomzellen und A375-Melanomzellen als präklinische Modelle verwendet und mit anti-EGFR-Antikörpern für Panc-1, anti-CD20-Antikörpern für Raji und anti-MCSP-Antikörpern für A375 kombiniert. Als Effektoren setzten wir humane T-Zellen ein, die retroviral mit FcγRIII-CD28-CD3ζ-Konstrukten transduziert wurden. Dabei wurden Konstrukte mit 158F/V und 48H/L Mutationen in der FcγRIII-Domäne verglichen. Wir konnten einen Vorteil der 158V- gegenüber der 158F-Variante im Hinblick auf IFN-γ Ausschüttung und Zytotoxizität nachweisen. Zudem konnte eine überlegene Aktivierung und Zytotoxizität der CD16-CAR-T-Zellen durch GE-Antikörper im Vergleich zu WT-Antikörpern für Raji und A375 demonstriert werden. Für eine hohe Effektivität in der CD16-CAR-T-Zelltherapie sollten daher GE-Antikörper und Rezeptorkonstrukte mit 158V Polymorphismus eingesetzt werden. Bezüglich einer unspezifischen Aktivierung der CAR-T-Zellen durch patienteneigene Antikörper bleibt die klinische Evaluation abzuwarten.

In der zweiten Publikation, Wiedemann et al., beschrieben wir die Wirkung des neuen TLR7-Agonisten SC1 auf die murine NK-Zellpopulation. Nach Verabreichung von SC1 *in vivo* konnten wir eine TLR7-abhängige Induktion eines proinflammatorischen Zytokinprofils zeigen, das ein geringeres Niveau als nach Applikation des TLR7/8-Agonisten R848 aufwies. *In vitro* und *in vivo* beobachteten wir eine TLR7-abhängige Aktivierung von NK-Zellen durch SC1 mit resultierender Zytotoxizität gegenüber MHC-I-negativen Lymphomzellen. Die therapeutische Anwendung von SC1 konnte in RMA-S-Tumor-tragenden Mäusen den Tumor TLR7- und IFN-α-abhängig zur

Regression bringen. Die Effektstärke war dabei mit der von R848 vergleichbar. In diesem Tumormodell konnte für die NK-Zellpopulation nach Behandlung mit SC1 eine Induktion aktivierender Oberflächenrezeptoren, eine verringerte Expression inhibitorischer Rezeptoren und eine Beseitigung der Anergie gezeigt werden.

7 Abstract

In this cumulative dissertation, the results of two scientific publications are summarized. Two different approaches of oncological immunotherapy are evaluated in preclinical studies.

In the first publication, Rataj et al., we evaluated the therapeutic relevance of polymorphisms in the extracellular domain of a modCAR construct and compared the effectiveness of glycoengineered (GE) antibodies with unmodified antibodies. Panc-1 pancreatic carcinoma cells, Raji lymphoma cells, and A375 melanoma cells were used as preclinical models. We combined anti-EGFR-antibodies for Panc-1, anti-CD20-antibodies for Raji, and anti-MCSP-antibodies for A375 with human T cells retrovirally transduced with FcγRIII-CD28-CD3ζ-chimeric receptors. In this setting, we compared constructs with 158F/V and 48H/L polymorphisms in the FcγRIII domain. We demonstrated an advantage of the 158V variant over the 158F variant in terms of IFN-γ secretion and cytotoxicity. Additionally, superior activation and cytotoxicity of CD16-CAR T cells through GE antibodies compared to wild-type antibodies were demonstrated for Raji and A375. Therefore, for high efficacy in CD16-CAR T cell therapy, GE antibodies and receptor constructs with the 158V polymorphism should be used. Clinical evaluation regarding unspecific activation of CAR-T cells by patient-specific antibodies is still warranted.

In the second publication, Wiedemann et al., we focused on the effect of the novel TLR7 agonist SC1 on the murine NK cell population. Upon *in vivo* administration of SC1, we observed a TLR7-dependent induction of a pro-inflammatory cytokine profile, which was lower in magnitude compared to the TLR7/8 agonist R848 except for levels of IFN-α. *In vitro* and *in vivo*, we observed TLR7-dependent activation of NK cells by SC1, resulting in cytotoxicity against MHC-I-negative lymphoma cells. Therapeutic application of SC1 was able to heal RMA-S tumor-bearing mice in a TLR7- and IFN-α-dependent manner. The therapeutic effect was comparable to that of R848. In this tumor model, treatment with SC1 showed induction of activating surface receptors, reduced expression of inhibitory receptors, and reversal of anergy in the NK cell population.

8 Publikationen

8.1 High-affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy.

8.1.1 Eigenanteil an der Publikation

Um für Figure 2c die Zytotoxizität von CAR-T-Zellen im Raji-Lymphommodell zu untersuchen, etablierte ich einen Durchflusszytometrie-basierten Assay. Dafür isolierte ich zunächst humane T-Zellen gesunder Spender, expandierte diese und transduzierte sie mit retroviralen Konstrukten. Die Viren wurden dabei von transfizierten Platinum A Packungszelllinien gewonnen. Mittels Durchflusszytometrie bestimmte ich die Effizienz der Transduktion und setzte eine Kokultur mit Raji-Zellen in der Kombination mit verschiedenen Antikörpern an. Zur Auswertung wurden zu Ende des Assays anti-CD3, anti-CD16, anti-CD19 Antikörper sowie ein *Live/Dead-Stain* hinzugegeben. Die absolute Zahl der verbliebenen Zellen der einzelnen Populationen wurde schließlich durchflusszytometrisch unter Verwendung von *Counting Beads* bestimmt. Als Antikörper verglich ich dabei den GE-Antikörper Obinutuzumab (GA101GE), für den bereits eine höhere Affinität zu CD16 durch Veränderung der Glykosylierung nachgewiesen werden konnte, sowie den korrespondierenden WT Antikörper (GA101) (Mössner et al. 2010). Zudem setzte ich als etablierte und zugelassene Positivkontrolle Rituximab und als Negativkontrolle den anti-EGFR-Antikörper Cetuximab ein.

Ich konnte mit diesem Versuch für alle anti-CD20-Antikörper in Kombination mit CD16-CAR-T-Zellen eine Lyse der Raji-Zellen zeigen. Für Rituximab konnte ich einen Vorteil der 158V Variante gegenüber der 158F Variante beobachten. Für die GE Variante des GA101 Antikörpers zeigte sich dabei keine erhöhte Zytotoxizität im Vergleich zum WT Antikörper. Dies könnte sich durch die bereits fast vollständige Lyse der Zielzellen durch die WT Variante erklären lassen. Der Einsatz eines modifizierten GA101-Antikörpers (GA101 LALA) mit aufgehobener Fc-Bindung in Kombination mit den CD16-CAR-T-Zellen resultierte in einer deutlich verminderten Zytotoxizität. Damit konnte ich in diesem Lymphommodell die Abhängigkeit des zytotoxischen Effektes von einer spezifischen, Antikörper-vermittelten Aktivierung der CAR-T-Zellen darstellen. Die Wiederholungen dieses Experiments erfolgten in Zusammenarbeit mit den Ko-Autoren.

Als nächstes evaluierte ich den Effekt von CD16-CAR-T-Zellen auf A375 Melanomzellen in einem Impedanz-basierten Zytotoxizitätsassay (Fig. 3c). Dabei

wurden der anti-MCSP-Antikörper LC007 und seine GE Variante mit erhöhter CD16-Affinität sowie als etablierte Positivkontrolle Cetuximab verglichen. Hier konnte ich für die GE Variante des Antikörpers in Kombination mit T-Zellen mit 158V Polymorphismus eine vollständige, nachhaltige Lyse der Zielzellen feststellen. Bei Einsatz der WT Variante des Antikörpers zeigte sich nur eine unzureichende zytotoxische Kontrolle der Tumorzellpopulation. Bei Einsatz der 158F Rezeptorvariante mit geringerer Fc-Affinität trat in Kombination mit keinem der Antikörper eine vollständige Lyse auf. Mit diesem Experiment konnte ich die Relevanz des 158V Polymorphismus in der Konstruktion von CD16-CARs und der Verwendung von GE-Antikörpern für eine effektive Tumorzelllyse in einem humanen Melanommodell aufzeigen. Zusätzlich konnte ich in diesem Modell durchflusszytometrisch eine Proliferation der transduzierten T-Zellen bei Einsatz der GE-Antikörper zeigen (Supplementary Fig. 1b). Für die korrespondierenden WT Antikörper zeigte sich kein Effekt auf die T-Zellproliferation.

Die Generierung der verwendeten Konstrukte mittels *overlap-PCR*, die Experimente zur Zytokinausschüttung und die Zytotoxizitätsanalysen unter Verwendung von PBMC und polyklonalen Antikörpern erfolgten durch die Ko-Autoren.

Meine Beiträge wurden mit einer Zweit-Autorenschaft an dieser Publikation honoriert.

8.1.2 Originalartikel

Der Originalartikel „High-affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy" (doi: 10.1038/s41416-018-0341-1) ist unter folgendem Link zu finden: https://www.nature.com/articles/s41416-018-0341-1

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ARTICLE
Immunotherapy

High-affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy

Felicitas Rataj[1], Severin J. Jacobi[1], Stefan Stoiber[1], Florian Asang[1], Justyna Ogonek[1], Nicholas Tokarew[1], Bruno L. Cadilha[1], Erwin van Puijenbroek[2], Constanze Heise[1], Peter Duewell[1], Stefan Endres[1], Christian Klein[2] and Sebastian Kobold[1]

BACKGROUND: CD16-chimeric antigen receptors (CAR) T cells recognise the Fc-portion of therapeutic antibodies, which can enable the selective targeting of different antigens. Limited evidence exists as to which CD16-CAR design and antibody partner might be most effective. We have hypothesised that the use of high-affinity CD16 variants, with increased Fc-terminus antibody affinity, combined with Fc-engineered antibodies, would provide superior CD16-CAR T cell efficacy.
METHODS: CD16-CAR T (wild-type or variants) cells were co-cultured with Panc-1 pancreatic cancer, Raji lymphoma or A375 melanoma cells in the presence or absence of anti-CD20, anti-MCSP, wild-type or the glycoengineered antibody variants. The endpoints were proliferation, activation, and cytotoxicity in vitro.
RESULTS: The CD16 158 V variant of CD16-CAR T cells showed increased cytotoxic activity against all the tested cancer cells in the presence of the wild-type antibody directed against MCSP or CD20. Glycoengineered antibodies enhanced CD16-CAR T cell activity irrespective of CD16 polymorphisms as compared with the wild-type antibody. The combination of the glycoengineered antibodies with the CD16-CAR 158 V variant synergised as seen by the increase in all endpoints.
CONCLUSION: These results indicate that CD16-CAR with the high-affinity CD16 variant 158 V, combined with Fc-engineered antibodies, have high anti-tumour efficacy.

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BACKGROUND

Adoptive T cell therapy (ACT) employs autologous T cells, which are expanded in vitro and therapeutically reinfuse into patients for the treatment of cancer. Clinically, three main avenues have been investigated: (1) tumour infiltrating lymphocytes (TIL) isolated from the patient´s tumour; (2) peripheral blood T cells transduced with T cell receptors (TCR) specific for cancer-associated antigens; and (3) peripheral blood T cells transduced with synthetic receptors, the so called chimeric antigen receptors (CARs).[1,2] All three strategies have and are being investigated in clinical trials with varying success rates.[3] The most advanced clinical strategies are CAR T cells specific for the B-cell differentiation antigen CD19. Various anti-CD19 CAR T cells have been evaluated in refractory B-cell malignancies, including acute lymphatic leukaemia (ALL) and diffuse large B-cell lymphoma (DLBCL). Based on previously unseen response rates, the prolongation of disease-free and overall survival, anti-CD19 CAR T cell therapies have been approved by the Food and Drug Administration (FDA) in 2017.[4,5]

In spite of these achievements, recent data also demonstrate that most patients are unlikely to benefit from these treatments in the long run and will relapse. Only a fraction of treated patients have a long-term benefit from CAR T cells or can be considered cured.[6] Causes for primary or secondary resistance and treatment failure to CAR T cell therapy include: (1) loss or downregulation of the target antigen, resulting in insufficient cancer cell recognition;[7] (2) insufficient access of the CAR T cells to the vicinity of the malignant cell—an aspect of particular relevance in the context of solid cancer treatment,[8] and (3) local and systemic immune suppression, which impedes CAR T cells activity.[9] These limitations will have to be overcome to broaden the applications of CAR T cell technology across several clinical indications. Access of T cells and circumvention of immune suppression will be of utmost importance when applying CAR-T cells to patients with solid cancers.

The first of these hurdles—loss, downregulation, or mutation of tumour-associated antigen[7,9,10]—could be addressed by the simultaneous or sequential targeting of different antigens. Given the manufacturing and regulatory burden to develop and produce a specific CAR T cell product, targeting different antigens with one CAR T cell product would bear considerable advantage.

A promising avenue is the use of CARs not based on a single-chain antibody variable fragment (for direct antigen recognition) but on the activating Fc-receptor CD16.[11] This platform requires co-administration of monoclonal antibodies and CD16 CAR T cells for cancer recognition. This has two major advantages: it allows the use of already approved antibodies for tumour targeting, and

[1]Center of Integrated Protein Science Munich (CIPS-M) and Division of Clinical Pharmacology, Department of Medicine IV, Klinikum der Universität München, LMU Munich (Member of the German Center for Lung Research (DZL), LMU Munich, Germany and [2]Roche Innovation Center Zurich, Schlieren, Switzerland
Correspondence: Sebastian Kobold (Sebastian.kobold@med.uni-muenchen.de)

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it enables a target-switch (in the same patient, with the same T cell product) under therapeutic pressure.

Currently, CD16-CAR T cells are under pre-preclinical and clinical testing, in combination with monoclonal antibodies such as rituximab. First clinical trials have been initiated combining the CD20-targeting antibody rituximab with a CD16-CAR T cell product (ClinicalTrials.gov Identifier: NCT03189836).

CD16 has polymorphisms distributed within the normal population, which could affect the affinity for the Fc-part of antibodies. We hypothesised that altering these sequences in a CD16-CAR would also modulate CAR T cell activity. We also applied antibody engineering, to promote activity of CD16-CAR T cell by increasing Fc-binding affinity via CD16. We postulated that both strategies might enhance CD16-CAR T cell activity, and their combination might further benefit tumour cell recognition and lysis (Supplementary Figure 1A).

We have demonstrated that the high-affinity CD16 158 V variant strongly promotes CD16-CAR T cell activity in combination with both wild-type and Fc-engineered antibodies. Glycoengineered antibodies with enhanced FcγRIIIa affinity further increase tumour cell recognition and CD16-CAR T cell activation. Furthermore, we have demonstrated the value of the strategy by using the approved Fc-glycoengineered clinical-grade anti-CD20 antibody GA101 (obinutuzumab),[12] the anti-MCSP antibody LC007 and their non-glycoengineered counterparts, in human B-cell lymphoma and melanoma cell lines, respectively.

MATERIALS AND METHODS

Cell lines

Raji, a human Burkitt lymphoma cell line, was purchased from the American Type Culture Collection (ATCC). Raji cells were cultured in RPMI (RPMI 1640; Lonza, Switzerland) supplemented with 10% foetal bovine serum (FBS, Life Technologies, USA), 100 U/ml penicillin and streptomycin (PS), and 2 mM L-glutamine, 1 mM sodium pyruvate (all from PAA, Germany), and 1 mM HEPES (Sigma-Aldrich, Germany). Panc-1, a human pancreatic cancer cell line, was purchased from the European collection of cell cultures (ECACC). A375, a human melanoma cell line, was previously described.[13] Panc-1 and A375 cells were cultured in DMEM3 + (DMEM with 10% FBS, 100 U/ml PS, and 2 mM L-glutamine). The retroviral amphitropic packaging cell line Platinum A was purchased from Cell Biolabs (USA). DMEM medium for Platinum A cells additionally contained 10 µg/ml puromycin and 1 µg/ml blasticidin (both from Sigma-Aldrich, Germany). The cell lines used in experiments were regularly checked for mycoplasma with the MycoAlert™ kit (Lonza, Basel, Switzerland). Primary human T cells were cultured in VLE-RPMI 1640 (Biochrom, Germany) supplemented with 2.5% human serum (Sigma-Aldrich, Germany), 100 U/ml PS and 2 mM L-glutamine, 1 mM sodium pyruvate, 10 µM NEAA (Sigma-Aldrich, Germany), and 50 µM β-mercaptoethanol (human TCM).

Antibody generation

Antibodies cetuximab (Merck, Darmstadt, Germany), panitumumab (Amgen, Munich, Germany), and rituximab (Roche, Basel, Switzerland) were commercially purchased. Obinutuzumab (GA101) is a glycoengineered Type II anti-CD20 antibody that mediates enhanced cell death induction as compared with rituximab.[12] Glycoengineered GA101 is produced in CHO cells co-expressing the glycosylating enzymes GnT3 and Man2.[14] For comparative analysis, non-glycoengineered wild-type GA101 expressed in conventional CHO cells was used. As a negative control, we used a mutant version of GA101 which was produced by transient expression in HEK293 cells. The mutant version of GA101 contains P329G LALA mutations that abolished FcγR binding.[15]

For MCSP targeting, the humanised antibody LC007 M4-3 ML2 and the respective glycoengineered MCSP antibody LC007 M4-3 ML2-g2 were used. Both antibodies were produced by transient expression in HEK293 cells, either in the presence or absence of GnT3 and Man2 co-expression, respectively.[16] All antibodies were purified via protein A chromatography and an ion exchange or size exclusion polishing steps. Purity and monomeric state were confirmed by CE-SDS and analytical size exclusion chromatography, respectively, and identity was confirmed by mass spectrometry.

Generation of T cell activating CD16-CAR

The constructs were generated by overlap extension PCR and recombinant expression cloning into the retroviral pMP71 vector (kindly provided by C. Baum, Hannover). The CD16 fusion receptor constructs consist of the signal peptide (accession number NM_000569.6 aa 1–53) and extracellular domain of human CD16 (Uniprot Entry P 08637 aa 17–208), the transmembrane and intracellular domain of human CD28 (Uniprot Entry P10747 aa 153–220) and the intracellular domain of human CD3ζ (Uniprot Entry P20963 aa 52–164). The CD16 fusion receptor variants were generated by point mutation PCR in the CD16 extracellular domain as follows: aa 48 L → H and aa 158 F → V. The CD16del constructs are composed of the aforementioned signal peptide fused to CD16 molecule without the intracellular CD28 and CD3ζ signalling domain (Uniprot Entry P 08637 aa 17–229).

Ethical approval and consent to participate

Use of PBMC samples from irreversibly anonymised healthy blood donors was approved by the local ethical committee.

Human T cell transduction

Human T cell transduction was performed as previously described.[8,13] In brief, the packaging cell line Platinum A (Cell Biolabs, USA) was seeded into six-well plates (Corning, Kaiserslautern, Germany) at varying cell numbers ($0.8 - 1.2 \times 10^6$) and cultured overnight until they reached confluency of ~70–90%. Subsequently, Platinum A cells were transfected with 18 µg of DNA, using the calcium phosphate precipitation method. Cell culture medium was changed 6 h post transfection, to retain cell viability, and virus was harvested both 48 h and 72 h after transfection, respectively.

In parallel, human PBMC were isolated by density gradient separation (Biocoll Separating Solution, Biochrom, Germany), from heparinised whole blood of healthy donors. $CD3^+$ cells were positively selected by MACS® Technology (Miltenyi Biotec, Germany) and activated for 48 h on anti-CD3- and anti-CD28-coated beads (eBioScience, Frankfurt, Germany, clones HIT3a and CD28.2) in six-well plates, in human TCM supplemented with 200 U/ml IL-2 (Peprotech, Hamburg, Germany). For subsequent cultures, 5 ng/ml IL-15 (Peprotech, Hamburg, Germany) and Dynabeads® Human T-Activator anti-CD3 anti-CD28 (Life Technologies, Darmstadt, Germany) were added. Twenty-four-well plates (Corning, Kaiserslautern, Germany) were coated for 24 h with 12.5 µg/ml of RetroNectin (TaKaRa Biotech, Japan) in PBS. Retrovirus from Platinum A cells was concentrated on the RetroNectin coated 24-well plates by centrifugation at 3000x g for 90 min at 32 °C, and activated T cells were added to virus-loaded plates at a concentration of 10^6 T cells per ml human TCM per well. In total, two transduction hits were performed at 24 h intervals. T cells were then cultured in human TCM supplemented with IL-2, IL-15, and β-mercaptoethanol at a concentration of 10^6 T cells/ml, with medium changes every 48 h. Transduction efficiency was assessed by staining for human CD16 (clone 3G8, BioLegend) (Supplementary Figure 2).

Co-cultures of tumour cells and T cells

T cells were co-cultured with either Panc-1, Raji, or A375 tumour cells in a 10:1 effector to target cell ratio using 96-well flat bottom plates (Corning, Kaiserslautern, Germany). Medium was

supplemented with different antibody concentrations, peripheral blood mononuclear cell (PBMCs) or polyclonal human immunoglobulins (IVIgs) (1 or 10 mg/ml, Privigen, CSL Behring, USA), as indicated in the respective figure. After 48 h of co-culture, supernatants were collected and release of human IFN-γ was assessed by ELISA (Becton Dickinson, Heidelberg, Germany).

To assess T cell proliferation, T cells were collected after 72 h of co-culture, an Fc-block with Human TruStain FcX™ (BioLegend) was applied and cells were stained with anti-human CD16, anti-human CD3 (clone HIT3a, BioLegend, USA), in addition to a live-dead stain (Zombie Aqua™ Fixable Viability Kit, BioLegend, USA). Equal amounts of Count Bright absolute counting beads (Life Technologies, USA) were added to each sample for absolute number quantification.

Flow cytometry-based lysis assay

Raji cells were co-cultured for 24 h with T cells in a 96-well flat bottom plate (Corning, Kaiserslautern, Germany) in DMEM supplemented with 0.1 mM NEAA, 1 mM sodium pyruvate (Life Technologies, Darmstadt, Germany), and different antibodies (antibody concentration 1 µg/ml) in a ratio of 10:1. Prior to surface staining, Fc-block was performed using Human TruStain FcX™ (BioLegend, USA). Subsequently, cells were stained with anti-human CD19 (clone HIB19, BioLegend, USA), anti-human CD3 (clone HIT3a, BioLegend, USA), and anti-human CD16 (clone 3G8, BioLegend, USA) in PBS. A live-dead stain (Zombie Aqua™ Fixable Viability Kit, BioLegend, USA) was used to limit analysis to viable cells. Cell numbers were determined by Count Bright absolute counting beads (Life Technologies, USA).

Real-time cytotoxicity assays

In total, 10.000 A375 cells were plated in 150 µl DMEM (Lonza, Basel, Switzerland) supplemented with 0.1 mM NEAA, 10% FBS, 100 µg/ml streptomycin, 1 IU/ml penicillin, 2 mM L-glutamine, and 1 mM sodium pyruvate (Life Technologies, Darmstadt, Germany) in an xCELLigence 96-well flat bottom plates (ACEA Biosciences, San Diego, USA). After overnight incubation T cells, in a 10:1 effector to target ratio, PBMCs in a 10:1 PBMC to T cell ratio and corresponding antibodies (10 µg/ml) as indicated in the figures, were added. T cell killing was determined as change in impedance every 6 min for 20 h and afterwards for 45 h every 15 min the followed by an additional 45 h every 15 min, by using the xCELLigence® RTCA SP device.

Statistics

The FACS data were analysed with FlowJo V9.2 or V10.3 software. Statistical analysis was performed by using GraphPad Prism software 5.0 or 7.0. Differences between experimental conditions were analysed using the unpaired two-tailed Student's *t* test, with *p*-values < 0.05 were considered significant. In the case of repeated testing of the same samples (longitudinal analysis over time), two-way ANOVA with Bonferroni-correction was used. Data are shown as mean values ± SEM of a minimum of three biological replicates or independent experiments, as indicated. Supplementary Table 1 contains all *p*-values associated with the figures.

RESULTS

CD16 158 V/F, but not 48 H/L variants, enhance activity of CD16-CAR T cells

To test the influence of CD16 polymorphisms on the activity of CD16-CAR T cells, we started by generating variants bearing sequential modifications that have been reported to enhance affinity of CD16 to the Fc-terminus of antibodies. We cloned and transduced the CD16-CARs 158 V 48 H, 158 V 48 L, and 158 F 48 L, as well as one construct without the signalling moieties (CD16-del, Fig. 1a). To test the impact of these receptors on T cell function, we took advantage of two approved anti-EGFR antibodies; the IgG1-based antibody cetuximab and the IgG2-based antibody panitumumab. The first would be expected to bind to CD16 and the latter not.

Primary human T cells bearing CD16-CAR, with the aforementioned structural changes were co-cultured with EGFR + Panc1 pancreatic cancer cells in the presence of either panitumumab or increasing concentrations of cetuximab. Cetuximab dose-dependently increased the activity of both the CD16 158 V 48H-

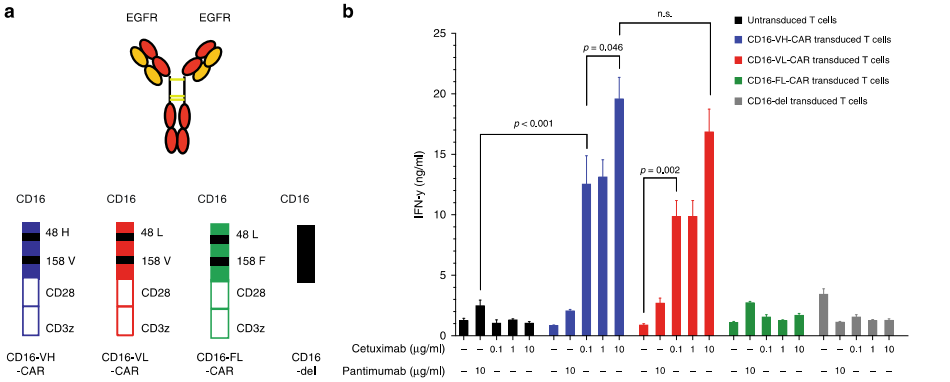


Fig. 1 CD16 158 V but not 158 F or 48 H/L polymorphisms enable activity of CD16-CAR transduced T cells. **a** Schematic overview of used constructs; anti-EGFR monoclonal antibodies cetuximab (IgG1) and panitumumab (IgG2), CD16-CAR with 48 H and 158 V (referred to as VH), 48 L and 158 F (referred to as FL), 48 L and 158 V (referred to as VL) variations and CD16-del, which is devoid of the intracellular moieties. **b** In total, 300.000 CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced or untransduced T cells were co-cultured for 48 h with 30.000 EGFR$^+$ Panc-1 pancreatic cancer cells in the presence or absence of 10 µg/ml of the anti-EGFR antibody panitumumab (IgG2) or increasing doses of the anti-EGFR antibody cetuximab (0.1, 1, and 10 µg/ml; IgG1), as indicated in the figure. IFN-γ production was measured by ELISA. All graphs show mean values of at least three technical replicate and each experiment shown is a representative figure of at least three independent experiments. A two-sided unpaired Student´s *t* test was used to determine the p-values and a p-value < 0.05 was considered statistically significant

and CD16 158 V 48L-CAR, and modestly CD16 158 F 48L-CAR (Fig. 1b). The CD16 48 H or 48 L variants had no influence on CD16-CAR function (Fig. 1b). As expected, co-culture in the presence of panitumumab did not induce IFN-γ. These results demonstrate that the CD16 158 V construct enhances CD16-CAR T cell activity.

Glycoengineered anti-CD20 antibodies enhance the activity of CD16-CAR T cells against CD20 + lymphoma cells, irrespective of CD16 variants

Next, we investigated the influence of the Fc-glycoengineered antibodies on the activity of CD16-CAR and its dependences on the CD16-CAR variants described above. We took advantage of the clinically approved anti-CD20 antibody GA101 (obinutuzumab), which is glycoengineered for about 10-fold enhanced CD16-binding (referred to as GE[12]). We generated a non-glycoengineered wild-type counterpart, which binds to the same epitope. We used another approved anti-CD20 antibody (rituximab) as a positive control as well as cetuximab as a negative control (Fig. 2a).

In T cell co-cultures with $CD20^+$ Raji lymphoma cells, GA101GE enhanced T cell activation (as seen by the increase in IFN-γ release) via CD16-CAR more efficiently than the identical concentrations of wild-type GA101 or rituximab, regardless of the CD16-CAR variant (Fig. 2b). For both GA101 and GA101GE, activation of CD16-CAR was correlated positively with the dose of antibody used (Fig. 2b). Activation of the high-affinity CD16 158V-CAR T cells was, however, higher in the presence of all anti-CD20 antibodies, as compared with the low-affinity CD16 158F-CAR T cells (Fig. 2b), with the same antibody concentration. Furthermore, the combination of GA101GE with the CD16 48 L variant enhanced CD16-CAR T cell activation more efficiently than the CD16 48 H variant (Fig. 2b). In all cases, activation correlated with the dose of antibody used, and to a lesser extent depending on whether the CD16 158 V or CD16 158 F variant of the CAR was used.

All anti-CD20 antibodies induced lysis of lymphoma cells in a CD16-CAR T cell-dependent manner. CD16 158V-CAR-T cells were more effective at lysing CD20 + cells than CD16 158F-CAR-T cells in combination with rituximab (Fig. 2c), congruent to the findings for T cell activation in Fig. 2b. Vice versa, the use of an Fc-incompetent GA101 (GA101 LALA) prevented CD16-CAR-mediated lysis confirming that CD16 engagement is the mode of action of CD16-CAR T cells. Importantly the known non-Fc-mediated activity of GA101 on B-cell lymphoma viability was conserved in GA101 LALA conditions.[17] For a given polymorphism, the activity of GA101 was higher than that seen with rituximab but was not further enhanced by glycoengineering, presumably because cell lysis was already maximal (Fig. 2c). We hereby provide evidence that co-administration of Fc-engineered antibodies may enhance the activity of CD16-CAR to target T cells against lymphoma cells.

Glycoengineered anti-MCSP antibody synergises with the CD16 158 V variant for increased activation of CD16-CAR T cells and enhanced melanoma cell lysis

To extend our findings on the relationship of CD16-CAR activity and affinity-driven binding of the Fc-portion of therapeutic antibodies, we next used the glycoengineered anti-MCSP antibody LC007 (LC007GE) and its non-engineered counterpart (LC007) (Fig. 3a). The aforementioned antibodies were tested in combination with the different CD16-CAR variants. Cetuximab was used as a positive control, on $MCSP^+EGFR^+CD20^-$ A375 melanoma cells. Co-culturing CD16-CAR T cells with A375 cells in the presence of antibodies, resulted in a dose-dependent increase in activation for both anti-MCSP antibodies (Fig. 3b). Again, activation was highest for T cells transduced with CD16 158V-CAR variants, as compared with CD16 158F-CAR-T cells. For the anti-MCSP antibodies, we did not detect major differences in the activation capacity between co-incubated CD16 48H- and CD16 48L-CAR-transduced T cells (Fig. 3b). Activation was paralleled by induction of T cell proliferation by the LC007 GE antibody but not the wild-type LC007 antibody (Supplementary Figure 1B).

Quantifying the cytotoxic potential of CD16-CAR T cells in combination with the different antibodies, we found that all variants of CD16-CAR T cells induced cell lysis to a certain degree. However, only the CD16 158V-CAR-T cells (CD16 VH or CD16 VL) together with the glycoengineered LC007 mediated complete lysis of the melanoma cells, while the wild-type LC007 only led to transient growth control of tumour cells (Fig. 3c). These results indicate that glycoengineered antibodies with enhanced CD16 affinity and high-affinity CD16-CAR T cells synergise for maximal T cell efficacy against A375 melanoma cells.

Presence of an excess of polyclonal human immunoglobulins and PBMCs modulates CD16-CAR T cell activation and tumour cell lysis

As a patient is a complex organism as compared with an in vitro culture system composed of two or three components, several factors can affect CD16 CAR T cell activity, targeting, and safety. The components that are most likely to affect the efficacy and safety are immunoglobulins and other immune cells, which are able to engage the CD16 CAR T cells. To address these potential issues, we probed the impact of an excess of immunoglobulin or PBMCs on the activity of CAR T cells in the presence or absence of anti-MSCP antibody (LC007 or LC007GE, Fig. 4a) against A375 melanoma cells. In the context of wild-type LC007, the addition of PBMCs to the culture system has a modulating effect on T cell activation, as seen by interferon-γ release (Fig. 4b), and limits the cytotoxic activity of VL, FL, and VH CD16-CAR T cells (Fig. 4c). However, in the context of LC007GE, the addition of whole blood PBMCs to the culture system reduces target tumour cell lysis of VL, FL, and VH CD16-CAR T cells (Fig. 4c). This reduction in tumour cell killing in the presence of PBMCs is attributed to a reduction in T cell activation (Fig. 4b).

We further extended our exploration by adding polyclonal human antibodies to the aforementioned culture system. The addition of polyclonal human antibodies to the culture system dose-dependently enhanced T cell activation as compared with antibody alone, (Fig. 4b) and mediated a complete lysis of melanoma cells in the context of the high-affinity receptors CD16 VH- and CD16 VL-CAR-T cells (Fig. 4c). Concentrations of polyclonal human antibodies comparable with physiological serum concentrations (10 mg/ml) but not sub-physiological concentrations (1 mg/ml) induced on-tumour activation (Fig. 4b). Activation was paralleled by on tumour cell lysis by the CD16- CAR T cells (Fig. 4c). Addition of polyclonal human immunoglobulins did, however, not impede the efficacy of the different CD16-CAR variants (Fig. 4b, c).

DISCUSSION

We demonstrate that affinity modulating CD16 sequence variants play a major role in binding of antibodies by CD16-CAR T cells and can enhance their activity. The high-affinity 158 V variant renders CD16-CAR T cells superior to the more prevalent 158 F low-affinity variant of CD16-CAR T cells. Glycoengineering of the Fc-portion of antibodies for enhanced CD16 binding promotes both the activation and lytic activity of CD16-CAR T cells.

Our results provide evidence that both strategies enhance CD16 function, namely CD16s' own affinity for the Fc portion and engineering the Fc portion itself can be employed to promote T cell activity against cancer cells.

Several groups have previously reported the use of CD16-CAR T cells in preclinical models, including B- and T cell non-Hodgkin lymphoma, neuroblastoma, and breast cancer.[11] Different designs have been used to trigger antibody-redirected cytotoxicity: in analogy to CAR, first (CD3ζ only) and second-generation (CD28 or

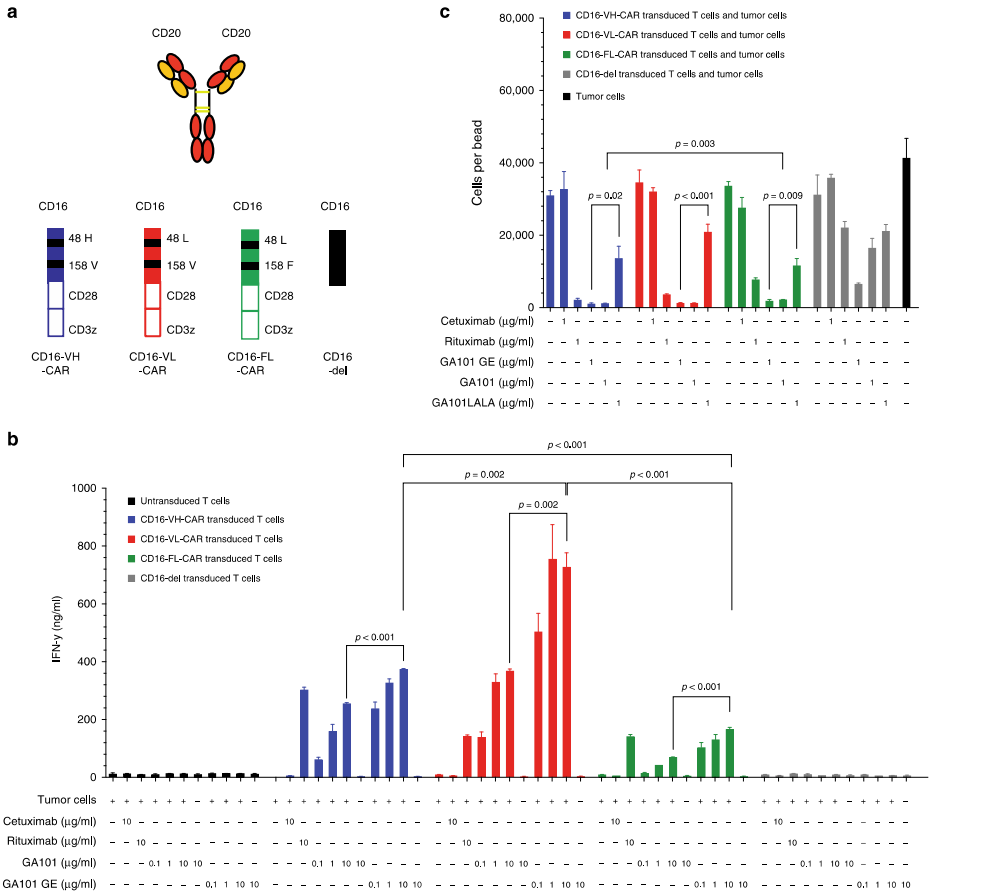


Fig. 2 Glycoengineered anti-CD20 antibody GA101 enhances the activity of CD16-CARs against B-cell lymphoma cells. **a** Schematic overview of investigated constructs; anti-CD20 monoclonal antibodies (rituximab, GA101 or glycoengineered (GE) GA101), CD16-CAR with VH, FL, VL variants and Vdel. **b** 300.000 CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced or untransduced T cells were co-cultured for 48 h with 30.000 $CD20^+$ Raji B-cell lymphoma cells in the presence or absence of either 10 µg/ml of the anti-EGFR antibody cetuximab (negative control), 10 µg/ml of the anti-CD20 antibody rituximab (positive control), increasing doses of the anti-CD20 antibodies GA101 or glycoengineered GA101GE (0.1, 1, and 10 µg/ml), as indicated in the figure. IFN-γ production was measured by ELISA. **c** CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced, or untransduced T cells were co-cultured for 24 h with 50.000 CD20 + Raji B-cell lymphoma cells in the presence or absence of either 1 µg/ml of the anti-EGFR antibody cetuximab, 1 µg/ml of the Fc-mutated anti-CD20 antibody GA101LALA (negative control), 1 µg/ml of the anti-CD20 antibody rituximab (positive control), 1 µg/ml of the anti-CD20 antibody GA101 or glycoengineered GA101 GE, as indicated in the figure. Cytotoxicity was assessed by flow cytometry by quantifying the absolute number of live Raji cells at the end of the assay. All graphs show mean values of at least three technical replicate and each experiment shown is a representative figure of at least three independent experiments. A two-sided unpaired Student´s *t* test was used to determine the *p*-values and a *p*-value < 0.05 was considered statistically significant

CD137 and CD3ζ) designs have been utilised for the CD16-CAR structure.[18–20] These CD16-CAR T cells represent a further development in respect to previously used CD16-transduced T cells.[21] Importantly, no study has yet compared these designs with one another, and it is unclear if the chosen architectures are suited for optimal CD16-CAR T cell activity. The CD16-CAR reported in the present study represent, according to the above-referenced nomenclature, a second-generation CD16-CAR (i.e., containing domains CD28 and CD3ζ). Unlike previously published receptors, however, our transmembrane domain is a sequence of CD28 rather than that of CD3ζ or CD8.

By default, previous studies have employed the CD16 extracellular domain containing a 158 V mutation, yet, all authors postulate that any advantage seen with natural CD16 would also

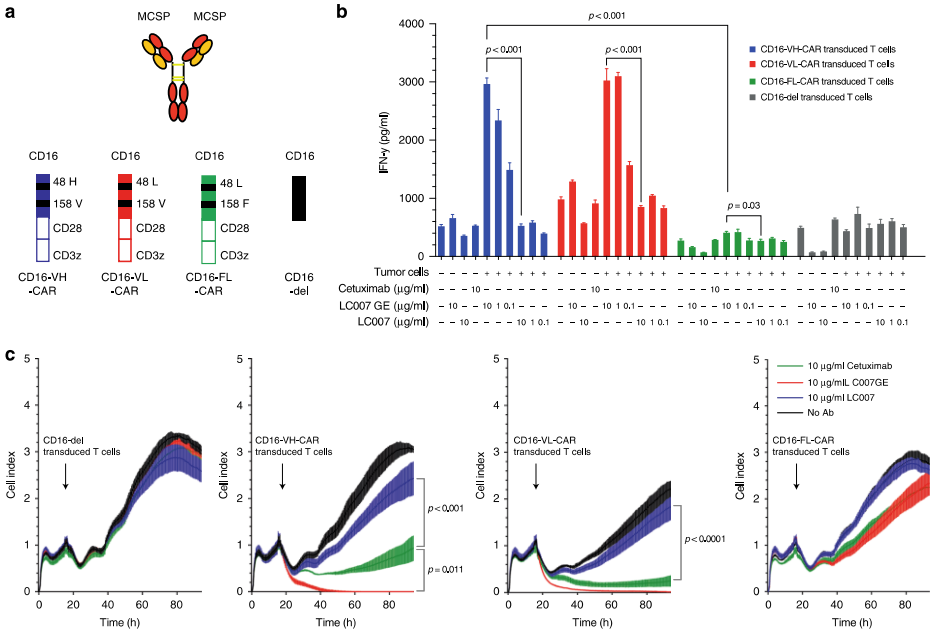


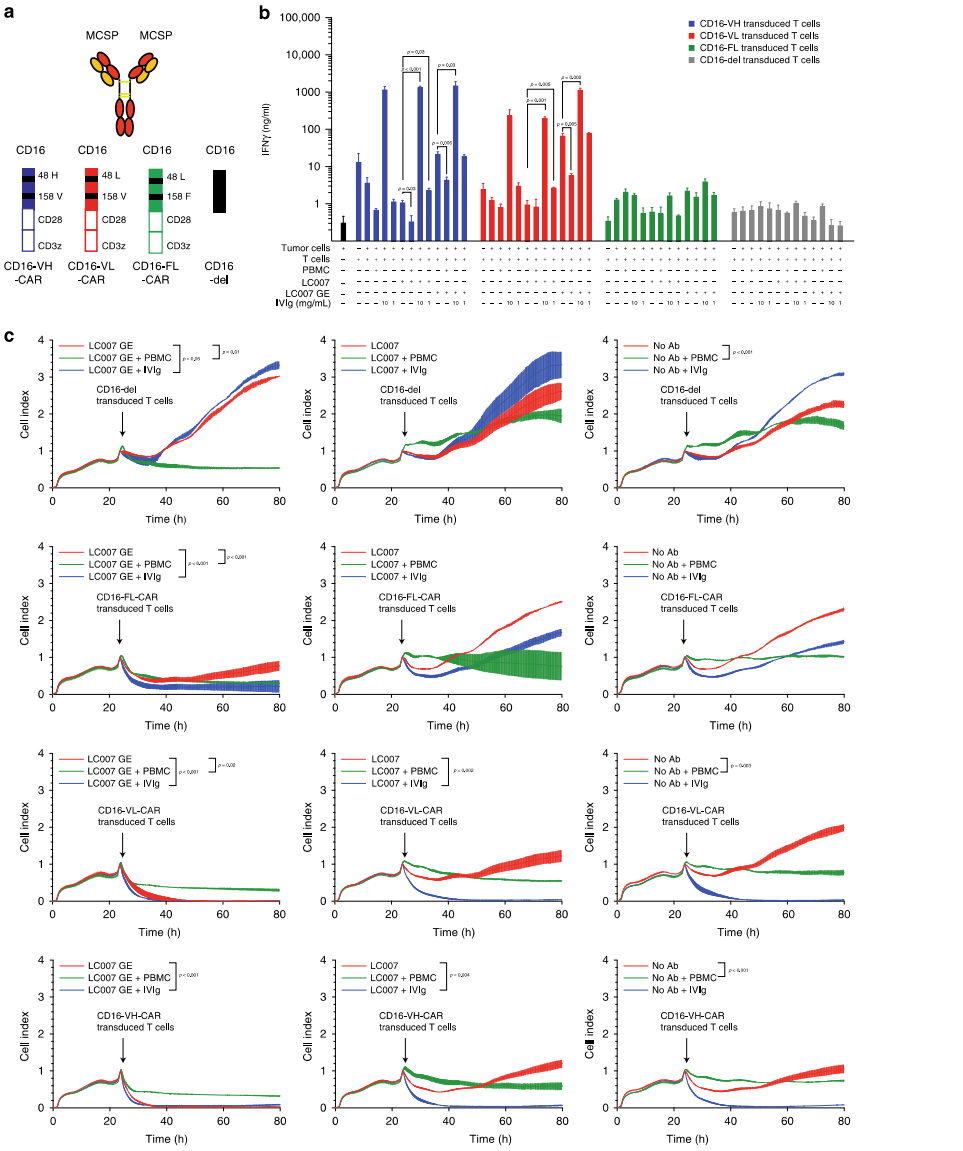
Fig. 3 Glycoengineered anti-MCSP antibody LC007 synergises with CD16-158V-CAR for effective melanoma cell recognition and lysis. **a** Schematic overview of constructs used in this figure; the anti-MCSP antibody (LC007 or glycoengineered LC007GE), CD16-CAR with VH, FL, VL variants and Vdel. **b** CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced T cells were co-cultured for 48 h with 20.000 $MCSP^+$ A375 melanoma cells in the presence or absence of either 10 µg/ml of the anti-EGFR antibody cetuximab (positive control), increasing doses of the anti-MCSP antibodies LC007 or increasing doses of the glycoengineered LC007GE (10, 1, and 0.1 µg/ml) as indicated in the figure. IFN-γ production was measured by ELISA. **c** 10^5 CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR- or CD16-del-transduced T cells were added after overnight incubation to 10^4 $MCSP^+$ A375 melanoma cells, in the presence or absence of either 10 µg/ml of the anti-EGFR antibody cetuximab, 10 µg/ml of the anti-MCSP antibody LC007 or glycoengineered LC007GE, as indicated in the figure. The time point when T cells and antibodies were added to the system are indicated by the black arrows. Cytotoxicity was measured in real-time, as a mean of cell viability by xCELLigence technology. All graphs show mean values of at least three technical replicate and each experiment shown is a representative figure of at least three independent experiments. For figure B a two-sided unpaired Student´s *t* test was used and for C a two-way ANOVA with Bonferroni-correction was used to determine the *p*-values. A *p*-value < 0.05 was considered statistically significant

be transferrable to CD16-CAR. We now demonstrate that the 158 V variant indeed improves CD16-CAR T cell activity, as compared with the 158 F variant. Owing to the controversial relevance of CD16 variants for the activity of therapeutically applied antibodies, we argue that this comparison is of particular importance.[22] The 48 H variant, although reported to enhance Fc affinity of CD16,[23] did not influence CD16-CAR activity in our study.

Glycoengineering or affinity enhancing mutations of the Fc-portion have been applied to enhance binding of therapeutic antibodies to Fc-receptors and thereby to confer enhanced anticancer activity.[24,25] Based on superior efficacy in comparison with rituximab, one such antibody—obinutuzumab—has been approved for the treatment of follicular lymphoma and chronic lymphatic leukaemia,[26] indicating that CD20-targeting can be improved through Fc-engineering. As the activity of CD16 is subjected to Fc-binding and affinity, enhancement of CD16-CAR activity by Fc-glycoengineering of co-administered antibodies appeared probable. We demonstrate that antibodies against MCSP and CD20, when glycoengineered, conferred higher CD16-CAR T cell activity regardless of CD16 variants, this increase was more pronounced in the CD16 158 V variant.

Our data indicate that Fc-engineered antibodies can enhance CD16-CAR T cell activity. Approved or advanced clinical phase glycoengineered antibodies would be the first choice when designing clinical trials with CD16-CAR T cells in combination with antitumour antibodies. As exemplified in the present study, the glycoengineered anti-CD20 antibody obinutuzumab, enhances CD16-CAR T cell activity. Other candidates include the glycoengineered anti-CD19 antibody MOR208, which is under investigation in refractory B-cell non-Hodgkin lymphoma, and the glycoengineered anti-CCR4 antibody KW-0761, mogamulizumab, tested for T cell lymphoma.[27,28] It is however important to bear in mind that enhanced activity, especially in the context of CAR T cells which can come with a severe side effect profile,[4] carries the risk of more dismal side effects too. However, the clear advantage of CD16-CAR might come in handy here, as any CD16-CAR activity will vanish with antibody half-life and decay, this providing an inherent safety switch to this strategy.

In patients, endogenous polyclonal IgG molecules will compete with co-administered therapeutic antibodies for Fc-binding by CD16. Here, glycoengineered antibodies come with a clear advantage: They will be preferentially bound to CD16 compared with non-engineered antibodies. This has been shown for



example, for rituximab versus obinutuzumab.[12] In autoimmune diseases, where autoantibody titres occur, such discriminatory capacity can be of the particular importance:[29] On the other hand, higher affinity autoantibodies might be able to trigger CD16-CAR T cells on their own right and this consideration might call for autoimmunity as an exclusion criteria for the use of CD16-CAR

Fig. 4 CD16-CAR T cell activation and cytoxicity in complex co-culture systems. **a** Schematic overview of constructs used in this figure; the anti-MCSP antibody (LC007 or glycoengineered LC007GE), CD16-CAR with VH, FL, VL variants and Vdel. **b** In total, 20×10^4 CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced T cells were co-cultured for 48 h with 2×10^4 $MCSP^+$ A375 melanoma cells in presence of either 10 µg/ml of the anti-MCSP antibody LC007, 10 µg/ml of the anti-MCSP glycoengineered antibody LC007GE or without antibody. Each condition was additionally tested in the presence or absence of 10 mg/ml or 1 mg/ml polyclonal immunoglobulin or 2×10^6 PBMC. IFN-γ production was measured by ELISA. **c** IN total, 10^5 CD16 VH-CAR-, CD16 VL-CAR-, CD16 FL-CAR-, CD16-del-transduced T cells were added after overnight incubation to 10^4 $MCSP^+$ A375 melanoma cells, in presence of either 10 µg/ml of the anti-MCSP antibody LC007, 10 µg/ml anti-MCSP glycoengineered antibody LC007GE or without antibody. Each condition was additionally tested in the presence or absence of 10 mg/ml or 1 mg/ml polyclonal human immunoglobulins (IVIgs) or 10^6 PBMC. The time point when T cells and antibodies were added to the system are indicated by arrows. Cytotoxicity was measured in real-time, as a mean of cell viability by xCELLigence technology. All graphs show mean values of at least three technical replicate and each experiment shown is a representative figure of at least three independent experiments. For figure B a two-sided unpaired Student's *t* test was used and for C a two-way ANOVA with Bonferroni-correction was used to determine the *p*-values. A p-value < 0.05 was considered statistically significant

T cells. As a matter of fact, current studies with CD16-CAR specifically exclude these patients from enrolment. Glycoengineered antibodies can help CD16-CAR T cells to better discriminate between endogenous and therapeutically applied antibodies for enhanced efficacy and safety.

In order to further investigate safety of the proposed therapy, we probed the impact of high-dose polyclonal immunoglobulins and PBMCs. In this system when we cultured CD16 CAR T cells with 10 mg/ml of polyclonal human immunoglobulins, a concentrations found in the serum of healthy donors,[30] we observed an increase in cytotoxicity and activation of enhanced affinity CD16 CAR T cells (VH and VL). Enhanced activity mediated by CD16-CAR in the presence of high doses of polyclonal immunoglobulins was due to the unspecific deposition of IVIgs on tumour cells, which subsequent activated the CAR T cells (data not shown). However, it is important to note that doses required for polyclonal triggering of CD16-CAR T cells were several log-concentrations higher than for monoclonal antibodies (microgram vs. milligram range). This question will be elucidated in ongoing clinical trials. In an in vivo context, this level of T cell activation could lead to autoimmunity or a cytokine storm,[31] an event which may be enhanced in a "leaky" tumours, where high molecular weight serum components can extravasate and deposit in the tumours milieu.[32] While in healthy tissues, the amount of deposited antibodies and higher molecular weight serum components should be negligible.[33] These data would argue for the exclusion of patients with IgG-associated pathologies where antibodies can be deposited in tissues.[34,35] Furthermore, our data would also argue for cautious T cell updosing to allow proper safety assessment in patients. In contrast, we observed that the addition of PBMC to our co-culture system inhibits cytotoxicity and activation of CD16 CAR T cells, what may counteract effect of polyclonal immunoglobulins in the peripheral blood and even prevent aforementioned pathologies. The observed dampening effect of PBMCs on the T cell response might have been caused by different subsets of PBMCs (e.g., regulatory T cells, myeloid derived suppressor cells) or alternatively, through competition over trophic factors. Moreover, aggregation of a high number of PBMC on the surface of cancer cells might have prevented the direct contact of effector T cells resulting in a decrease in the overall T cell activation level.

Different modular strategies have been employed to conditionally control T cell activity. For example, the "Uni-CAR"-platform employs a CAR recognising nanobody.[36] Such approaches come with the advantage of being modular and bearing an inherent safety switch based on the antibodies' half-life. A similar concept has been utilised when monoclonal antibodies were tagged with FITC and targeted by an anti-FITC CAR T cell.[37] However, all these strategies require both further antibody engineering of otherwise approved antibodies or modification and the development of cellular products. The major advantage of CD16-CAR T cells is the use of a single cellular product, irrespective of tumour-associated antigen to be targeted in combination with an approved monoclonal antibody. This combination has major regulatory advantages for development. The findings of this study may help to prioritise CD16-CAR design. It provides the rationale for the use of glycoengineered antibodies for optimal targeting of different types of cancer cells by CD16-CAR T cells.

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AUTHOR CONTRIBUTIONS

F.R., S.J.J., S.S., F.A., J.O., N.T., B.L.C., E.v.P., C.H. conducted experiments, analysed data and wrote the manuscript. P.D., S.E., C.K., S.K. supervised research, interpreted data and wrote the manuscript. S.K. designed the study.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41416-018-0341-1.

Competing interests: Parts of this work have been performed for the doctoral theses of S.J.J., S.S. and F.A. at the Ludwig-Maximilians-Universität München. The authors have declared that no other conflict of interest exists.

Data availability: Materials and data available upon reasonable request.

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8.2 A novel TLR7 agonist reverses NK cell anergy and cures RMA-S lymphoma-bearing mice

8.2.1 Eigenanteil an der Publikation

Nachdem durch Ko-Autoren die TLR7-abhängige Aktivierung von NF-κB und die Induktion proinflammatorischer Zytokine durch den TLR7-Agonisten SC1 im murinen Modell demonstriert werden konnte, untersuchte ich zunächst den Effekt von SC1 auf die Zytotoxizität muriner NK-Zellen. Nach subkutaner Applikation von SC1 im murinen Modell konnte ich eine erhöhte Zytotoxizität von isolierten NK-Zellen gegenüber NK-Zell-sensitiven YAC-1-Lymphomzellen nachweisen (Fig. 1d). Diesen Effekt konnte ich auch für die Kokultur von NK-Zellen mit RMA-S Zellen zeigen (Fig. S1A).

Um den Einfluss auf die zytotoxische Funktion von NK-Zellen auch *in vivo* zu untersuchen, wurden die Splenozyten β_2-Mikroglobulin-defizienter Spendermäuse in Mäuse transferiert, die zuvor mit SC1 oder Kontrolle behandelt worden waren. Dabei konnten wir durchflusszytometrisch einen zytotoxischen Effekt nach SC1 Applikation feststellen, der in TLR7 -/- Mäusen ausblieb (Fig. 2d). Weiter konnten wir die Relevanz des beobachteten Effektes in einem NK-Zell-sensitiven Tumormodell nachweisen. Dazu wurden Mäuse mit induzierten RMA-S Tumoren regelmäßig mit SC1, dem etablierten TLR7/8 Agonisten R848 oder Vehikel therapiert. Für die beiden Agonisten zeigte sich dabei ein vergleichbarer Effekt auf das Tumorwachstum und das Überleben der behandelten Mäuse. Eine Mehrheit der behandelten Mäuse konnte dabei geheilt werden. Diesen anti-tumoralen Effekt konnten wir weder in TLR7 -/- noch in IFN-α-Rezeptor -/- Mäusen nachweisen (Fig. 3).

Durchflusszytometrisch analysierte ich die NK-Zellpopulation der tumor-tragenden Mäuse. Dabei bestand keine Auswirkung der Therapie auf die Quantität der NK-Zellen in Tumor und Tumor-drainierenden Lymphknoten (TDL) RMA-S tragender Mäuse (Fig. 4a). Innerhalb der NK-Zellpopulation zeigte sich in den TDL jedoch eine gesteigerte Expression des aktivierenden Rezeptors *natural killer group 2D* (NKG2D). Zudem wiesen die NK-Zellen therapierter Mäuse sowohl im Tumor als auch im TDL eine erhöhte Expression von CD69 auf, der gemeinhin als Aktivierungsmarker lymphoider Zellen angesehen wird (Fig. 4b-c). Bei dieser aktivierten Zellpopulation konnte ich zudem eine geringere Expression der inhibitorischen Rezeptoren Ly49A und CD96 nach Therapie mit SC1 darstellen (Fig 4d-e). Für R848 zeigte sich ein ähnlicher Effekt auf die NK-Zellpopulation (Fig. S4a-c) in diesem Tumormodell. Für das RMA-S Tumormodell wurde bereits ein anerger Zustand der NK-Zellen, definiert als verringerte

Zytokinausschüttung und Degranulation zytotoxischer Vesikel auf Antikörper-vermittelte Stimulation hin, beschrieben (Ardolino et al. 2014). Ich konnte zeigen, dass die NK-Zellen der drainierenden Lymphknoten von RMA-S Tumoren eine verminderte Degranulation zytotoxischer Vesikel nach Stimulation mit anti-Nkp46 Antikörpern im Vergleich zur NK-Zellpopulation der Milz dieser Tiere aufwiesen ((Fig. 4G). Durch die Therapie mit SC1 konnte die Degranulation zytotoxischer Vesikel durch NK-Zellen aus TDL verstärkt werden. Dieser Effekt zeigte sich auch bei Applikation von R848 (Fig. S4D).

Für sämtliche beschriebene *in vivo* und *ex vivo* Experimente wählten wir einen subkutanen Applikationsweg für SC1. Im Rahmen der Revision konnte ich die Effekte des *in vivo* Zytotoxizitätsassays mit β_2-Mikroglobulin-defizienten Zellen sowie den Effekt auf das Tumorwachstum von RMA-S Tumoren auch bei intravenöser Applikation von SC1 nachweisen (Fig. S2). Ich konnte somit zeigen, dass der therapeutische Effekt von SC1 unabhängig von der gewählten parenteralen Applikationsvariante ist. Aufgrund der Pharmakokinetik könnte gerade im Hinblick auf Zytokin-induzierte Nebenwirkungen eine subkutane Anwendung in der Klinik jedoch vorteilhafter sein.

Mein Beitrag zu diesem Manuskript wurde mit einer *equally contributing* Zweitautorschaft honoriert. Aufgrund der langen Dauer der *in vivo* Tumorexperimente sowie der aufwändigen Zellaufarbeitung im Rahmen der *ex vivo* Experimente wurden diese in enger Zusammenarbeit mit der Erstautorin durchgeführt.

8.2.2 Originalartikel

Der Originalartikel „A novel TLR7 agonist reverses NK cell anergy and cures RMA-S lymphoma-bearing mice” (doi: 10.1080/2162402X.2016.1189051) ist unter folgendem Link zu finden:
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ORIGINAL RESEARCH

A novel TLR7 agonist reverses NK cell anergy and cures RMA-S lymphoma-bearing mice

Gabriela Maria Wiedemann[a,*,#], Severin Johannes Jacobi[a,*,#], Michael Chaloupka[a,*,#], Angelina Krächan[a,#], Svetlana Hamm[b], Stefan Strobl[b], Roland Baumgartner[c], Simon Rothenfusser[a,#], Peter Duewell[a,#], Stefan Endres[a,#], and Sebastian Kobold[a,#]

[a]Center of Integrated Protein Science Munich (CIPSM) and Division of Clinical Pharmacology, Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany; [b]4SC Discovery GmbH, Martinsried, Germany; [c]4SC AG, Martinsried, Germany

ABSTRACT
Toll-like receptor 7 (TLR7) agonists are potent immune stimulants able to overcome cancer-associated immune suppression. Due to dose-limiting systemic toxicities, only the topically applied TLR7 agonist (imiquimod) has been approved for therapy of skin tumors. There is a need for TLR7-activating compounds with equivalent efficacy but less toxicity. SC1, a novel small molecule agonist for TLR7, is a potent type-1 interferon inducer, comparable to the reference TLR7 agonist resiquimod, yet with lower induction of proinflammatory cytokines. *In vivo*, SC1 activates NK cells in a TLR7-dependent manner. Mice bearing the NK cell-sensitive lymphoma RMA-S are cured by repeated s. c. administrations of SC1 as efficiently as by the administration of resiquimod. No relevant toxicities were observed. Mechanistically, SC1 reverses NK cell anergy and restores NK cell-mediated tumor cell killing in an IFN-α-dependent manner. TLR7 targeting by SC1-based compounds may form an attractive strategy to activate NK cell responses for cancer therapy.

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Introduction

Toll-like receptors (TLR) are pattern-recognition receptors which recognize conserved molecular structures of viruses, bacteria and fungi.[1] Upon binding of their respective ligand, TLR initiate an intracellular signaling cascade, which culminates in the rapid induction of an inflammatory cytokine response and in the activation of cells of the innate and adaptive immune system.

TLR7 is located on the endosomal membrane and senses viral single-stranded RNA.[2,3] Synthetic ligands for TLR7 including RNA oligonucleotides, guanosine analogs and imidazoquinoline compounds such as imiquimod and resiquimod (R848) have been described and used in a number of applications.[4,5] Engagement of these agonists to TLR7 leads to the recruitment of MyD88, interferon regulatory factors and NF-κB and thereafter to rapid induction of a type-1 interferon response together with the secretion of proinflammatory cytokines such as IL-1β, IL-6 and IL-12.[6]

A hallmark of cancer is tumor-associated immunosuppression, defined by an anti-inflammatory cytokine profile and the accumulation of suppressive cells such as regulatory T cells or myeloid-derived suppressor cells.[7] TLR7 agonists have the ability to revert this suppressive milieu and to enable tumor cell killing by the innate and adaptive immune system.[8] The potency of TLR7 agonists for cancer immunotherapy is illustrated by imiquimod, the only TLR-agonist approved for clinical use. It induces remissions of basal cell carcinoma, of early stage melanoma or even of breast cancer skin metastases when applied topically.[9-12] A major limitation of TLR7 agonists applied systemically is side effects such as fever, fatigue and cardiac toxicity. This has prevented their systemic use at an effective dosage.[13] Based on local potency, a compound or a formulation of TLR7 agonist that achieves antitumoral efficacy without limiting systemic cytokine release may form a potent immunotherapeutic approach.

NK cells are innate immune effector cells which exhibit target cell killing in an antigen-unspecific manner and contribute to immunosurveillance and tumor control.[14,15] NK cel-mediated lysis of tumor cells is driven by stress-induced surface molecules or the lack of MHC class I expression on cancer cells.[16] However, a number of cancer types have included NK cell inhibition as a part of their immunsuppressive network.[17,18] A number of strategies aim at harnessing NK cells for cancer therapy, including *in vivo* activation of NK cells with cytokines such as IL-2, adoptive transfer of NK cells and agonistic antibodies to exploit NK cell antitumoral properties.[19-23] However, NK cell-based therapeutic protocols are not yet clinically established, as *in vivo* activation of NK cells by cytokine

CONTACT Sebastian Kobold, M.D. Sebastian.kobold@med.uni-muenchen.de Division of Clinical Pharmacology Klinikum der Universität München Lindwurmstraße 2a, 80337 München, Germany.
Supplemental data for this article can be accessed on the publisher's website.
*GMW, SJJ and MC contributed equally to this work.
#Member of the German Center for Lung Research.

administration is limited by systemic cytokine toxicity.[24] On the other hand, *ex vivo* generation, expansion and activation of NK cells need to be established experimentally for optimal *in vivo* efficacy.[25,26] An effective use of NK cells for cancer therapy would need an approach not only mediating enhanced tumor cell killing, but also reversing tumor-associated NK cell anergy.

The small molecule TLR7 agonist SC1 has been hypothesized to activate NK cells and to thereby mediate antitumor efficacy *in vivo*.[27] However, SC1s detailed mode of action has remained unaddressed. We have now performed a detailed analysis of the *in vivo* mode of action of SC1 with focus on NK cells. We demonstrate that SC1 treatment activates NK cells in a TLR7 and IFN-α dependent manner. SC1 thereby reverses NK cell anergy leading to efficient tumor cell lysis. We provide evidence that SC1 has equal therapeutic efficacy as the prototypical TLR7 agonist resiquimod while exhibiting a more favorable cytokine profile. This lays the basis for further therapeutic development.

Material and methods

Mice and cell lines

C57BL/6 mice were purchased from Janvier (St Berthevin, France). TLR7-deficient mice (C57BL/6 background) were provided by S. Akira (Osaka University, Osaka, Japan) and bred in the animal facility of the Ludwig-Maximilians Universität München. Female β_2-microglobulin-deficient mice (B6.129P2-*B2mtm1Unc*/J) were purchased from Jackson (Bar Harbor, USA). Female IFNAR-deficient mice (B6.129S2-*Ifnar1tm1Agt*/Mmjax) were a kind gift from T. Brocker (Institute of Immunology, Ludwig-Maximilians Universität München, Munich, Germany) and were later purchased at The Mutant Mouse Resource and Research Center (MMRRC) at Jackson Laboratories (Bar Harbor, USA). Mice were 5 to 12 weeks of age at the onset of experiments. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The TAP-deficient T cell lymphoma cell line RMA-S was provided by Dr J. Charo (Berlin, Germany) and was cultured in complete RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% FBS, 100 μg/mL streptomycin, 1 IU/mL penicillin and 2 mM L-glutamine (Life Technologies, Darmstadt, Germany). The human TLR7 reporter embryonic kidney cell line HEK-293T containing an NFκB luciferase reporter and transfected with TLR7 was kindly provided by Marc Lamphier (Eisai, Inc., Andover, Massachusetts, USA)(28). SC1 (patent WO 09/118296) was provided by 4SC AG (Martinsried, Germany), R848 was purchased from Enzo Life Sciences (Lörrach, Germany).

Luciferase assay

10^4 HEK-293T TLR7 reporter cells were stimulated with 10 μM R848 or SC1 for 24 h. The TLR4 ligand LPS (Sigma-Aldrich, Saint Louis, USA) and the TLR9 ligand CpG (ODN 1585, Invivogen, Toulouse, France) served as negative controls. After lysis of cells and addition of substrate, luminescence was measured using a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). To analyze TLR specificity of SC-1, 2×10^4 HEK^{TM}hTLR7, HEK^{TM}hTLR8, HEK^{TM}hTLR9 or HEK^{TM}Null cells bearing *SEAP* as reporter gene (Invivogen, Toulouse, France) were stimulated with SC1 and appropriate controls for 16 h. Expression of target gene was assessed using HEK-Blue^{TM} Detection reagent (Invivogen, Toulouse, France) according to manufacturer's instructions.

In vitro activation assays and cytokine analysis

Murine splenocytes were passed through a 40 μm cell strainer and red blood cell lysis was performed using Bio-Plex Cell Lysis Buffer (Bio-Rad, Hercules, USA). 2×10^5 splenocytes per well were cultured in 96-well plates in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% FCS, 100 μg/mL streptomycin, 1 IU/mL penicillin and 2 mM Lglutamine (Life Technologies). Cells were treated with 1 to 10 μM of SC1, vehicle or 1 ng/mL LPS. Cytokine levels were analyzed 36 h after treatment. IL-6 cytokine ELISA (BD Biosciences, Heidelberg, Germany) of culture supernatant was performed according to the manufacturer's protocol. Premixed 25-Plex Cytokine Array (Merck Millipore, Darmstadt, Germany) was performed according to manufacturer's protocols. IFNγ levels in cytotoxicity assay supernatants were measured by IFNγ ELISA (Biolegend, Fell, Germany) according to the manufacturer's protocol.

In vivo activation assays and flow cytometry

For *in vivo* activation assays, mice were injected with SC1 (2.6 mg/kg or 10 mg/kg), R848 (2 mg/kg) or vehicle solutions s. c. into the flank. For cytokine ELISA, plasma was collected by centrifugation of whole blood at 400 g for 7 min, 1 to 3 h after treatment. Spleens were harvested 1 to 12 h after treatment and were passed through a 40 μm cell strainer. Erythrocytes were removed and remaining lymphocytes were stained for flow cytometry analysis. Therefore, cells were incubated with fluorochrome-linked antibodies and ZombieAqua^{TM} Fixable Viability Dye (Biolegend) for 30 min at 4°C. After incubation, cells were washed and analyzed on a BD FACSCanto II (BD, Heidelberg, Germany). Where indicated, counting beads (CountBright Absolute Counting Beads, Life Technologies) were added for determination of absolute cell numbers. Fluorochrome-conjugated antibodies against CD3 (clone 145-2C11), CD69 (clone H1.2F3), B220 (clone RA3-6B2), NK1.1 (clone PK136), MHCI (clone KH95), NKG2D (clone CX5), Ly49A (clone YE1/48.10.06), DNAM-1 (clone 10E5), CD96 (clone 3.3) and CD107b (clone M3/84) for flow cytometry were purchased from Biolegend.

Cytotoxicity assays

For *in vitro* cytotoxicity assays, mice were injected s.c. with 10 mg/kg μg SC1, 2 mg/kg R848 or vehicle solution. 12 h after injection, mice were sacrificed and NK cells were isolated from the spleen by negative selection using magnetic-activated cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany). NK cells were coincubated with 2×10^4 YAC-1 or B16F10 target cells at different ratios for 4.5 or 6 h. For assessment of direct effects of SC1 on RMA-S cells, RMA-S cells were incubated for 4 h with 4 μg/mL SC1 or vehicle solution prior to coculture

with NK cells. Cytotoxicity was assessed by LDH release (CytoTox 96® NonRadioactive Cytotoxicity Assay, Promega, Madison, USA) and calculated using the following formula: %cytotoxicity = [(Compound-treated LDH activity – spontaneous LDH activity)/(Maximum LDH activity – spontaneous LDH activity)] × 100.

For *in vivo* cytotoxicity assays, mice were injected with 10 mg/kg SC1 or vehicle solution s. c. or in the flank or i. v. Splenocytes from β_2-microglobulin-deficient mice were stained with 1 μM of Cell Proliferation Dye eFluor 450 (eBioscience, Frankfurt am Main, Germany). Splenocytes from wild-type mice were stained with 0.1 μM of the same dye. Stained splenocytes were mixed at a 1:1 ratio and 10^7 splenocytes were injected i. v. into mice 2 h after treatment with SC1. Mice were sacrificed 12 h after i. v. injection and Cell Dye eFluor 450 positive cells in the spleens were analyzed by flow cytometry. Specific lysis was calculated as follows: %specific lysis = 100 – [100 × (eFlour 450_{high}/eFluor 450_{low})^{Treated}/(eFluor 450_{high}/eFluor 450_{low})^{Control}].

Mouse tumor models

Mice were injected with 10^6 RMA-S lymphoma cells subcutaneously into the right flank. Treatment with 10 mg/kg SC1, 2.6 mg/kg SC1 or 2 mg/kg R848 or vehicle solution was carried out s.c. into the left flank on days 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 after tumor induction. For i. v. treatment, 10 mg/kg SC1 or vehicle were injected i. v. on days 0, 5, 10, 15 and 20 after tumor induction. Tumor size was measured three times a week and mice were assessed for body condition every week. Criteria for early termination of the experiment were weight loss of >10 %, hunched posture with piloerection or dull fur and severely reduced activity. Mice were sacrificed when tumors had reached a size of 225 mm^2 or when tumors were seriously ulcerated. For analysis of tumor-infiltrating lymphocytes, tumors were harvested at a size of 100 mm^2, tumor tissue was homogenized with a scalpel and incubated with 1 mg/mL collagenase and DNAse 1 (Sigma-Aldrich) at 37°C for 30 min. Tumors were first passed through a 100 μm cell strainer followed by passing through a 40 μm cell strainer. Tumor tissue-derived lymphocytes were enriched using a gradient of 44% and 67% of EasyColl (d = 1.124, Biochrom Merck Millipore, Darmstadt, Germany) before centrifugation at 800 g for 30 min. Enriched lymphocytes were washed and analyzed by flow cytometry.

NK cell activation assays in tumor bearing mice

For analysis of NK cell activation, RMA-S tumor-bearing mice were injected with 10 mg/kg SC1, 2 mg/kg R848 or vehicle solution on days 15, 20 and 25 after tumor induction. Mice were sacrificed on day 26 and single cell suspensions of spleen and lymph nodes were created by passing organs through a 40 μm cell strainer. Wells of flat-bottom high protein-binding plates (Thermo Scientific) were coated with 0.5 μg of NKp46 (clone 29A1.4, Biolegend, Fell, Germany) mAb or rat IgG2a as isotype control. 2.5 × 10^5 cells per well were incubated for 5 h in the presence of 100 U recombinant human IL-2 (Peprotec, Hamburg, Germany) and CD107b mAb (clone M3/84, AbD Serotec, Martinsried, Germany) in RPMI medium supplemented with 10% FCS, 1% P/S, 1% L-glutamine, 1% NEAA and 1% sodium pyruvate. CD107b expression was analyzed by flow cytometry.

Statistics

Statistics were calculated with GraphPad Prism software 5.0. Differences between experimental conditions were analyzed using the unpaired, two-tailed Student's t test. p values < 0.05 were considered significant. For *in vivo* tumor models, differences between groups were calculated using two-way ANOVA with correction for multiple testing.

Results

The novel small molecule SC1 is a TLR7-specific agonist and activates NK cells for tumor cell lysis in vitro

In order to determine the specificity of SC1 for TLR7, we used an HEK-293T cell line stably transfected with TLR7 and a reporter plasmid leading to luciferase activity upon activation of the NFκB promoter region through TLR7. SC1 and the TLR7/8-agonist R848 but not LPS (TLR4 ligand) induced luciferase activity (Fig. 1A). Additionally, SC1 did not induce reporter gene expression in cells bearing human TLR8 or TLR9, indicating specificity for TLR7 (data not shown). SC1 dose dependently increased cytokine secretion from wild-type splenocytes (Fig. 1B). Vice versa, when splenocytes from TLR7-KO mice were incubated with SC1, cytokine secretion, as exemplified by IL-6, was abolished (Fig. 1C). This indicates TLR7-specific activity of SC1 *in vitro*. To assess the impact of SC1 on *in vitro* NK cell cytotoxicity, we injected mice with 200 μg SC1 or vehicle solution. 12 h after injection, NK cell cytotoxicity to YAC-1 target cells was measured *in vitro*. SC1-treatment significantly increased NK cell-mediated target cell lysis at all effector-to-target cell ratios tested, indicating potent effector cell activation (Fig. 1D).

SC1 induces type-1 interferon and mediates increased NK cell cytotoxicity in vivo

Systemic treatment with TLR7 agonists results in cytokine induction, leading to dose-limiting systemic adverse effects.[13,29] Resiquimod (R848) is a prototypical TLR7 agonist. It has so far failed clinical application in spite of potent antitumoral efficacy in preclinical models.[30] We used R848 as a benchmark compound in terms of antitumor efficacy and of systemic cytokine induction. We compared the plasma cytokine profiles 1 to 8 h after s. c. treatment with R848 or SC1. Mice were injected with equimolar amounts of R848 (2 mg/kg) and SC1 (2.6 mg/kg), based on the established therapeutic dosage of R848 in murine tumor therapy.[31] Plasma cytokine levels were determined 1, 2, 3, 6 and 12 h after injection. Peak cytokine levels were reached 1 h (IFN-α, IFNγ, IL-1β, IL-6, IL-10, KC, CXCL2, TNF-α), 2 h (G-CSF, GM-CSF, IL-12p40, IL-13, IL-17, CCL3, CCL4, CCL5) or 3 h (IP-10) after injection. Both compounds lead to a rapid systemic cytokine induction, but SC1 consistently induced lower levels of all cytokines as compared to R848 (Fig. 2A). We next injected mice s. c. with 10 mg/kg of SC1, an efficacious

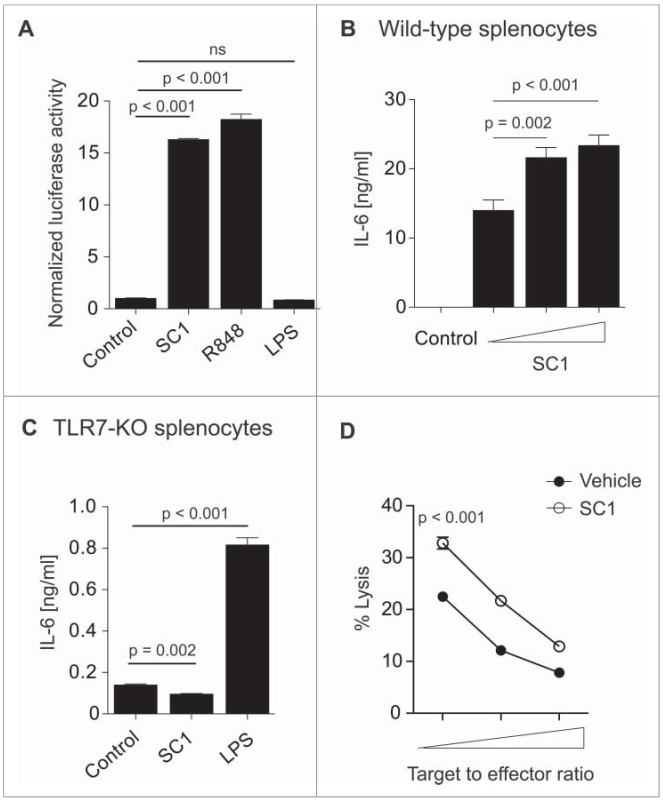

Figure 1. SC1 is a TLR7-specific agonist which enhances NK cell-mediated cytotoxicity *in vitro*. (A) 10^4 HEK293T cells, stably transfected with TLR7 and an NFkB reporter plasmid were stimulated over night with 10 μM SC1, R848 or LPS and luciferase activity was measured. (B) Splenocytes from C57Bl/6 mice were stimulated with 1, 5 or 10 μM of SC1 for 36 h. Supernatants were analyzed for IL-6 by ELISA. (C) TLR7-deficient splenocytes were stimulated with 4 μg/mL SC1, 3.1 μg/mL R848 or 1 ng/mL LPS for 36 h. IL-6 in supernatants was detected by ELISA. (D) Mice were treated s. c. with 10 mg/kg SC1 or vehicle solution. 12 h after treatment, NK cells were isolated from spleen and cultured with 2×10^4 YAC-1 target cells at target to effector cell ratios of 1 to 1.25, 1 to 2.5 and 1 to 5. Cytotoxicity was assessed by LDH release assay after 4.5 h of coculture. One representative experiment of three independent experiments is shown. Statistical significance was analyzed by two-tailed unpaired Student's t test. *p* values < 0.05 were considered significant. Error bars indicate SEM.

dose in antitumoral treatment determined in animal models and compared plasma cytokine levels to injection of a therapeutic dose of R848 (2 mg/kg). Induction of proinflammatory IP-10, TNF- α, CCL4, IL-6 and IL-10 was significantly lower after SC1 treatment compared to R848 treatment, in spite of a 4 times molar excess of SC1 compared to R848. However, IFN-α was the cytokine induced at the highest level by SC1 and did not differ significantly from the levels observed in R848-treated mice (Fig. 2B). To further dissect the functional consequences of the *in vivo* cytokine induction, we analyzed splenic $CD8^+$ T cells, B and NK cells after SC1 injection. Treatment with SC1 upregulated CD69 on all three cell types (Fig. 2C), indicating early activation of these cells. As a direct consequence of NK cell activation, we could observe a significant increase in NK cell-mediated lysis of β_2-microglobulin-deficient splenocytes in SC1-treated mice compared to mice treated with vehicle solution. The observed boost in NK cell cytotoxicity was entirely dependent on the presence of TLR7 in the treated mice, as no selective cytotoxicity was seen in TLR7-KO mice (Fig. 2D). In order to define, if NK cell cytotoxicity upon SC1 treatment was restricted to the rather NK cell-sensitive cell lines YAC-1 and RMA-S, we performed an *in vitro* cytotoxicity assay on the less immunogenic B16F10 melanoma cell line. We could show that NK cells from mice treated with either SC1 (10 mg/kg) or R848 (2 mg/kg) equally induced specific lysis of B16F10 melanoma cells which was not seen with untreated NK cells (Fig. 2E). Similarly, IFNγ levels measured under the same conditions were equally induced in the SC1 and R848 group (Fig. 2F). These results indicate that NK cell-derived IFNγ induction is comparable in SC1 and R848 treatment, even if systemically IFNγ induction was lower in the SC1 group (Fig. 2A).

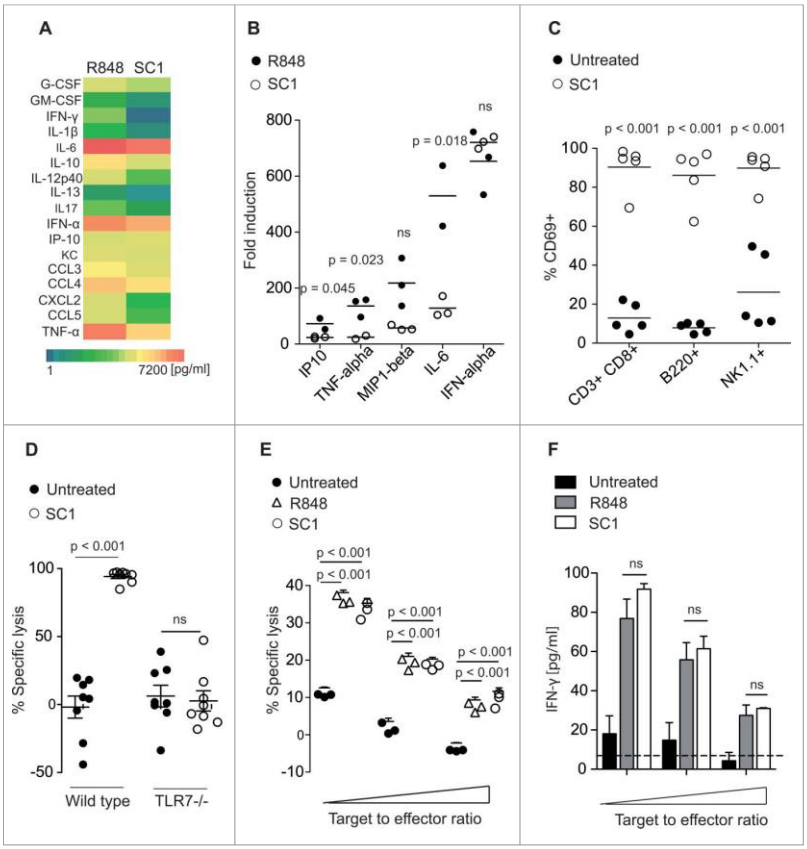

Figure 2. *In vivo* cytokine profile and immune cell activation after SC1 administration. (A) Mice (n = 4 per group) were injected s.c. with 2 mg/kg R848 or 2.6 mg/kg SC1. Cytokines in blood plasma were measured by ELISA before injection (0), 1, 2, 3 and 8 h after injection. Peak cytokine plasma levels are shown. (B) Mice (n = 3 per group) were injected s.c. with 2 mg/kg R848 or 10 mg/kg SC1. Plasma cytokine levels were analyzed 1, 2 or 3 h after injection by ELISA. Fold induction at the time point of peak cytokine levels is depicted. (C) Mice were injected with 10 mg/kg SC1 s. c. CD69 expression on splenic immune cells was measured by flow cytometry after 6 h and compared to expression in untreated animals. Pooled data from two independent experiments is shown and is representative of a total of five independent experiments. (D) Mice treated s. c. with 10 mg/kg SC1 or vehicle were injected with a 1:1 ratio of wild-type and β_2-microglobulin-deficient splenocytes labeled with 0.1 or 1 μM of Cell Dye eFluor450. After 12 h, the ratio of labeled cells in the spleen was determined by flow cytometry. Specific lysis was calculated as follows: specific lysis (%) = 100 − [100 × (β_2-microglobulin/wild-type) treated/(β_2-microglobulin/wild-type) untreated]. Results of three independent experiments are shown. (E, F) Mice were treated s. c. with 10 mg/kg SC1 or 2 mg/kg R848. 12 h after treatment, NK cells were isolated from spleen and cultured with 2 × 10^4 B16F10 target cells at target to effector cell ratios of 1 to 1.25, 1 to 5 and 1 to 10. Cytotoxicity was assessed by LDH release assay after 4.5 h of coculture. One representative experiment of two independent experiments is shown (E). IFNγ levels in coculture supernatants were determined by cytokine ELISA (F). Statistical significance was analyzed by two-tailed unpaired Student's t test. *p* values < 0.05 were considered significant. Error bars indicate SEM.

SC1 cures mice from NK cell-sensitive RMA-S lymphomas in a TLR7- and interferon-α-dependent manner

Efficacy of SC1 in antitumor treatment was assessed in mice bearing s. c. NK cell-sensitive RMA-S lymphomas.[32] Mice injected s. c. with 10^6 RMA-S cells were treated with 10 mg/kg SC1, 2 mg/kg R848 or vehicle solution s. c. every 5th day starting at day 0 (Fig. 3A). SC1 and R848 reduced growth of RMA-S tumors with similar efficacy (Fig. 3B). We could exclude a direct effect of SC1 on RMA-S tumor cells, as pretreatment of tumor cells with SC1 did not alter specific lysis of RMA-S (Fig. S1A). Most mice treated with SC1 or R848 were cured by the treatment (4 out of 6 versus 5 out of 6, respectively). Remarkably, all mice that rejected the tumor upon treatment remained tumor free during at least 150 d, when observation was ended (Fig. 3B). To compare the effect of SC1 in equimolar dosage to R848 (2.6 mg/kg) to the above used four times molar excess (10 mg/kg SC1), we next compared both dosages of SC1.

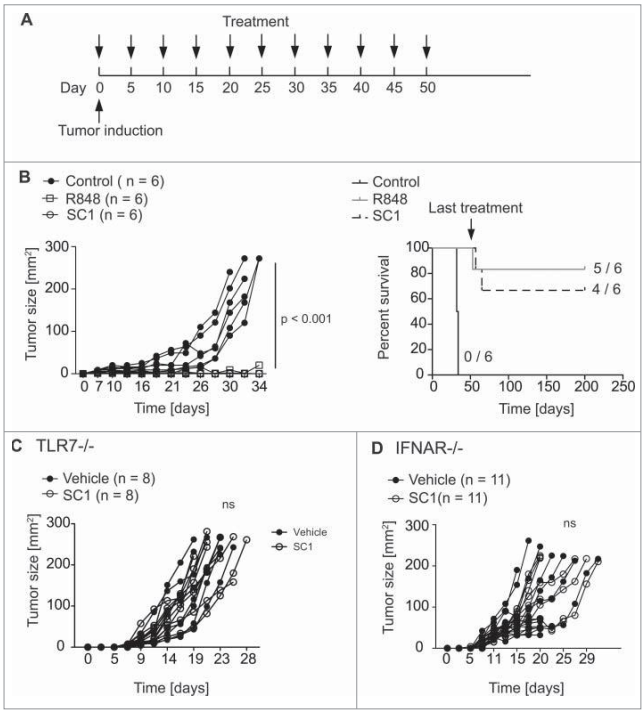

Figure 3. SC1 cures mice bearing RMA-S lymphoma. (A) C57BL/6 mice (n = 6 per group) were injected with 10^6 RMA-S cells s. c. into the flank. Mice were treated with 2 mg/kg R848, 10 mg/kg SC1 or vehicle s. c. into the contralateral flank (each n = 5) every 5th day beginning on day 0 through day 50. (B) Tumor growth and survival of C57BL/6 wild-type mice bearing s. c. RMA-S tumors. Results are representative of three independent experiments. (C) TLR7-deficient mice (n = 8 per group) were injected with 10^6 RMA-S cells s. c. in the flank. Contralateral s. c. treatment with 10 mg/kg SC1 or vehicle was administered every 5th day from day 0. Tumor growth over time is shown. (D) IFNAR-deficient mice (n = 11 per group) were injected with 10^6 RMA-S tumor cells s. c. into the flank. Contralateral s.c. treatment with 10 mg/kg SC1 or vehicle was administered every 5th day from day 0. Tumor growth over time is displayed. C and D show results of two independent experiments. Statistical significance was analyzed by two-way Anova with correction for multiple testing. p values < 0.05 were considered significant.

SC1 in the 2.6 mg/kg dosage resulted in a significant reduction of tumor growth, but growth inhibition and cure of mice was less pronounced than in the 10 mg/kg group (Fig. S1B). We thus used the 10 mg/kg dosage for all subsequent tumor experiments. To rule out that the chosen s. c. application route may have deleterious impact on treatment efficacy, we also performed a similar experiment with SC1 (10 mg/kg) using i.v. treatment with the same schedule. SC1 retained similar efficacy against RMA-S lymphoma both in *in vivo* cytotoxicity assays and in a therapeutic experiment (Fig. S2). We chose to keep the s. c. route for further experiments. Since some TLR7 agonists are known to function through TLR7-independent routes,[33] we sought to investigate the role of TLR7 expression on host cells in SC1-based therapy. TLR7-deficient mice bearing RMA-S tumors did not respond to SC1 treatment, supporting the notion that TLR7 is required for SC1-based antitumor efficacy (Fig. 3C). Because type-1 interferon is known to be crucial for the induction of NK cell cytotoxicity upon TLR7 stimulation,[5] we hypothesized that IFN-α was the key cytokine for the antitumoral effect of SC1. IFNAR-KO mice bearing RMA-S tumors failed to reject tumors upon SC1 treatment, indicating the dependence on IFN-α effects on host cells (Fig. 3D).

SC1 induces intratumoral NK cell activation and reverses tumor-induced NK cell anergy

To further explore the mode of action of SC1, we analyzed how NK cells are altered by SC1 to enhance antitumor efficacy. SC1 treatment of RMA-S tumor-bearing mice did not result in an increase of NK cell numbers in tumors or in tumor-draining lymph nodes (TDL) as compared to vehicle solution treated mice (Fig. 4A). In contrast, SC1 treatment significantly induced NK cell activation, as illustrated by enhanced expression of CD69 and NKG2D on NK cells (Fig. 4B,C and Fig. S3). In line with these observations, the NK inhibitory receptors, Ly49A and CD96, were both found to be downregulated on $CD69^+$ activated NK cells after treatment with SC1 (Fig. 4D and E). Similar results were observed for R848 (2 mg/kg) with

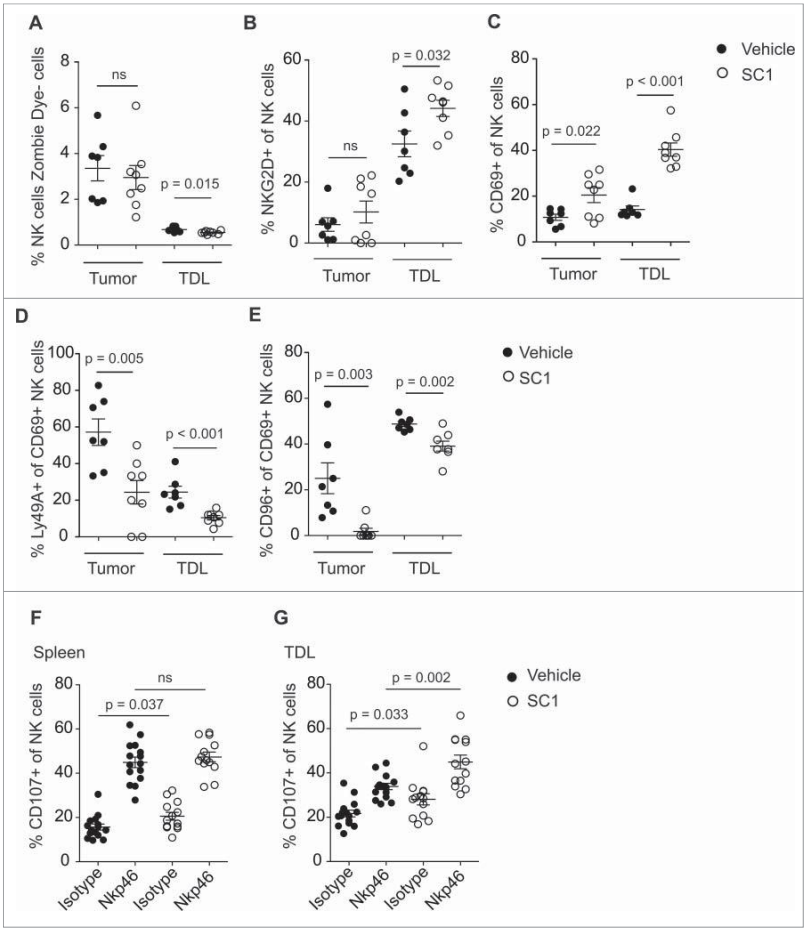

Figure 4. SC1 activates NK cells and reverts tumor-induced NK cell anergy. (A–E) C57BL/6 mice (n = 7 (vehicle) and n = 8 (SC1) per group) were injected s. c. with 10^6 RMA-S cells. 10 mg/kg SC1 was injected s. c. in the contralateral flank on days 15, 20 and 25 after tumor induction. Intratumoral and tumor-draining lymph node (TDL) NK cells, defined as $MHCI^+$, Zombie Dye^-, $CD3^-$, $NK1.1^+$, were quantified by flow cytometry on day 26. Data is representative of two independent experiments. (F, G) C57BL/6 mice (n = 6 per group) were injected s. c. with 10^6 RMA-S cells. 10 mg/kg SC1 were injected s. c. in the contralateral flank on days 15, 20 and 25 after tumor induction. NK cells from spleen and TDL were isolated on day 26 and 0.25 × 10^6 cells were stimulated for 5 h on plates coated with NKp46 antibody. NK cell expression of CD107b was measured by flow cytometry. Results of three independent experiments are shown. Statistical significance was analyzed by two-tailed unpaired Student's t test. *p* values < 0.05 were considered significant. Error bars indicate SEM.

comparable upregulation of NK cell activation markers in tumors and TDL (Fig. S4A–C), indicating that the observed impact on NK cells is pathway and not compound specific. It has been described that NK cells in a tumor-bearing organism have an exhausted or anergic phenotype, defined by the reduced capacity of intratumoral or TDL NK cells to degranulate upon adequate stimulation, as measured by the surface expression of CD107.[17] We investigated whether the ability of NK cells from tumor-bearing mice to degranulate after stimulation with agonistic NKp46 antibodies could be restored by treatment with SC1. As expected, splenic NK cells displayed no anergic phenotype due to the lack of direct tumor cell contact (Fig. 4F). NK cells derived from TDL of RMA-S tumor-bearing mice, however, showed a reduced ability to degranulate upon stimulation, which was restored by treatment with SC1 or R848 (Fig. 4G and Fig. S4D). These results suggest that reversal of anergy by SC1 contributes to the antitumor activity of the compound.

Discussion

Since the clinical approval of the TLR7 agonist imiquimod for topical treatment of certain cutaneous tumors, a number of clinical trials have investigated TLR7 agonists for local or systemic cancer therapy or as adjuvants in therapeutic vaccination strategies.[9] These trials have led to the preliminary conclusion that systemic toxicity limits the use of TLR7 agonists to topical treatment. Other TLR7 agonists such as loxoribin, 852A or TMX-101[34-36] have been clinically tested, but showed inadequate efficacy and/or dose-limiting toxicity. It was concluded that clinically feasible systemic TLR7 agonist treatment requires an equally potent immune activation with less systemic cytokine release. IFN-α is the central cytokine responsible for TLR7 agonist antitumor treatment efficacy[37] while proinflammatory cytokines, such as IL-1 or IL-6, might predominantly cause their side effects.[38] The novel TLR7 agonist SC1 has been designed to more selectively induce IFN-α. Previous work could demonstrate induction of proinflammatory cytokines in human PBMC and the ability to reduce lung metastases in the murine Renca model.[27] The mode of action of SC1 and especially the hypothesized more selective IFN-α induction remained unaddressed. Our results corroborate the concept that SC1 activates NFκB signaling and subsequently leads to dose-dependent secretion of IFN-α and of proinflammatory cytokines in immune cells after TLR7 engagement. *In vivo*, SC1 leads to rapid systemic induction of IFN-α with peak levels 1 h after injection accompanied by early activation of B, T and NK cells as measured by expression of CD69, comparable to the reference compound R848.

We observed similar effects on NK cell activation for i. v. injection compared to s. c. injection in cytotoxicity and *in vivo* tumor models (Fig. S2), which has already been shown for 852A.[39] The s. c. route may be preferable clinically to the i. v. route, in some circumstances, both for safety and applicability purposes, without loss of activity, as suggested for other therapeutic strategies.[40] We next compared the plasma cytokine profile induced by SC1 to the cytokine profile of R848. Our results indicate that concentrations of proinflammatory cytokines like IL-1β, IL-6 and TNF-α were significantly lower after treatment with SC1 than after treatment with equimolar amounts of R848. At a dose of SC1 four times higher than the equimolar dose of SC1 to R848, lower levels of IL-6, TNF-α, CCL4 and IP-10 in SC1-treated mice were found. A major obstacle in cancer therapy with systemic administration of TLR7 agonists is the occurrence of dose-limiting systemic adverse effects like fever, fatigue, nausea, dehydration, hepatic and cardiac toxicity—all potentially linked to cytokine release.

The therapeutically critical cytokine after TLR7 engagement is IFN-α.[41] Remarkably, SC1 was an equally effective inducer of IFN-α as R848, a finding supported by a comparable therapeutic efficacy in wild-type mice that was absent in IFNAR-KO mice. It has been reported, that IFNγ production by NK cells is critical for efficacy of TLR7/8 agonists.[42] In contrast, our *in vivo* data in IFNAR-deficient mice suggests that systemic type-1 interferon is critical for the antitumoral efficacy of TLR7 agonists. Here, we could, however, demonstrate that NK cell-derived IFNγ levels, but not systemic IFNγ levels, are comparable in R848- and SC1-treated mice, the latter being administered with the four times equimolar doses. A potent systemic IFN-α release may be preferable to induction of other cytokines not only in terms of efficacy, but also of diminished systemic side effects.

The reason of the observed differences in the cytokine profiles induced by SC1 and by R848 are surprising, since both agonists have long been considered to be selective TLR7 agonists in mice.[4,27] These differences may be due to discordances in cytokine secretion of TLR7 over TLR8 agonists. Agonists specific for TLR7 preferentially lead to a type-1 interferon response, whereas activation of TLR8 activation culminates in a proinflammatory cytokine response including cytokines like IL-12 and TNF-α.[41] SC predominantly activates TLR7 and has no effect on human TLR8 as shown in HEK293 reporter cells,[27] whereas R848 is known to bind to both TLR7 and TLR8. The functionality of TLR8 in mice is controversial. Mouse TLR8 is thought to be non-functional due to the lack of a five amino acid motif and missing recognition of ssRNA and other small molecule TLR8 agonists.[2,4,43] In contrast, MyD88-dependent cytokine response to TLR8 agonists in murine immune cells has been reported upon addition of polyT ODN to a TLR8 agonist, suggesting at least partial functionality of mouse TLR8.[44] In our hands, cytokine induction, NK cell activation as well as antitumoral efficacy of SC1 were completely dependent on TLR7, as they were absent in TLR7-deficient mice. It is thus likely that SC1 does not exert any TLR7-independent effects.

We have shown in previous studies that TLR7 agonist treatment in RMA-S tumor-bearing mice leads to an NK cell-mediated antitumor immune response but the mechanism of increased NK cell killing upon TLR7 activation was not studied.[5] NK cells are equipped with a large repertoire of inhibiting and activating receptors. Inhibitory receptors of the Ly49 family bind to the MHC-I complex and inhibit NK cell cytotoxicity upon engagement. This mechanism enables self-tolerance as well as lysis of MHC-I-negative cancer cells.[45] Another recently described inhibitory receptor on NK cells is CD96, which binds to CD155, a molecule expressed on dendritic cells and various epithelial cancer cells.[46] CD96 seems to be instrumental in cancer cell escape from NK cell killing, as CD96 neutralization mediates cancer regression.[46] NKG2D, in contrast, is a NK cell-activating receptor, which recognizes stress-induced ligands on tumor cells and virus-infected cells.[47] For efficient target cell killing, a combination of several of these receptors is required.[48] Although increased NK cell-mediated tumor cell lysis upon TLR7 agonist treatment has been previously described,[27,49] little is known on the effects of TLR7 agonists on NK cell receptor expression and phenotype. In the present study, we could show that SC1 efficiently controls tumor burden of s.c. RMA-S tumors and induces phenotypic changes on NK cells. SC1 also leads to an increased *in vitro* and *in vivo* NK cell cytotoxicity toward YAC-1 cells and β_2-microglobulin-deficient splenocytes. YAC-1 cell lysis by NK cells is dependent on expression of NKG2D ligands, whereas killing of β_2-microglobulin-deficient splenocytes is triggered by lack of MHC-I expression on these cells.[50] These results suggest that SC1 leads to a broad activation of NK cells, which is confirmed by our analysis of intratumoral and tumor-draining lymph node NK cells. NK cells did not accumulate in tumor and tumor-draining lymph node of SC1-treated mice, but showed an activated phenotype

characterized by the upregulation of activating receptors NKG2D and CD69 and downregulation of inhibitory receptors Ly49A and CD96. Recently, it could be shown that direct contact of NK cells to RMA-S cells induces NK cell anergy.[51] In addition, proliferation of NK cells in the presence of tumor cells contributes to NK cell exhaustion.[18] We could demonstrate that SC1 treatment reverses NK cell anergy in RMA-S tumor-bearing mice. Treatment with SC1 also upregulated NKG2D expression in NK cells from tumor-draining lymph node, further underlining reversal of NK cell exhaustion.

In summary, we identified SC1 as a small molecule TLR7 agonist with potent immunostimulatory and especially NK cell-activating properties. We describe for the first time a mechanism for TLR7-mediated NK cell activation and the associated phenotypic changes. This may form the basis for the development of TLR7 agonists to harness the therapeutic potential of NK cells in cancer therapy. Because of potent antitumor efficacy and lower inflammatory cytokine induction, SC1 or related compounds may be promising candidates for systemic therapy of NK cell sensitive tumors.

Disclosure of potential conflicts of interest

The authors from CIPSM and Division of Clinical Pharmacology have no conflict of interest to declare. The authors SH, SS and RB are employees of 4SC Discovery GmbH and 4SC AG and may have financial interest in the development of novel TLR7/8 agonists for cancer therapies.

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