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***Untersuchung neuer Targeting-Strategien
für intrazelluläre Tumorantigene und
für kombiniert-zielgerichtete Immuntherapie
der Akuten Myeloischen Leukämie***

Dissertation

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vorgelegt von

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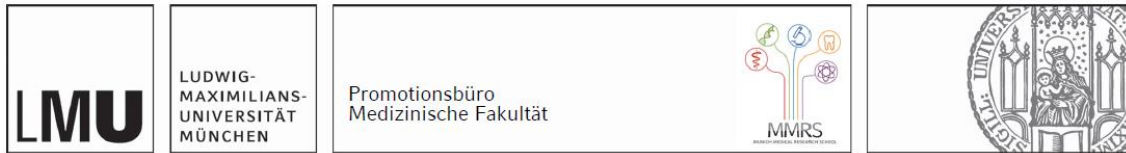
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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Untersuchung neuer Targeting-Strategien für intrazelluläre Tumorantigene und für kombiniert-zielgerichtete Immuntherapie der Akuten Myeloischen Leukämie

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Abkürzungsverzeichnis

ALL	Akute Lymphatische Leukämie
AML	Akute Myeloische Leukämie
CAR	chimärer Antigenrezeptor (engl. chimeric antigen receptor)
CD	engl. Cluster of differentiation
ELN	European LeukemiaNET
FAB	engl. French-American-British
HLA	Humanes Leukozyten-Antigen
IgG	Immunglobulin G
MHC	Haupthistokompatibilitätskomplex (engl. major histocompatibility complex)
PDX	engl. patient-derived xenograft
qPCR	quantitative Polymerasekettenreaktion (engl. polymerase chain reaction)
TCB	T-Cell-Bispecific
TCR	T-Zell-Rezeptor (engl. T cell receptor)
WHO	Weltgesundheitsorganisation (engl. World Health Organization)
WT1	Wilms-Tumor-Protein (alt. Wilms-Tumor 1)

Liste der Publikationen

Publikation 1:

Targeting intracellular WT1 in AML with a novel RMF-peptide-MHC specific T-cell bispecific antibody

Augsberger C, Hänel G, Xu W, Pulko V, Hanisch LJ, Augustin A, Challier J, Hunt K, Vick B, Rovatti PE, Krupka C, Rothe M, Schönle A, Sam J, Lezan E, Ducret A, Ortiz-Franyuti D, Walz AC, Benz J, Bujotzek A, Lichtenegger FS, Gassner C, Carpy A, Lyamichev V, Patel J, Konstandin NP, Tunger A, Schmitz M, von Bergwelt-Baildon M, Spiekermann K, Vago L, Jeremias I, Marrer-Berger E, Umaña P, Klein C, Subklewe M

Blood. 2021 Dec 23;138(25):2655-2669. doi: 10.1182/blood.2020010477. PMID: 34280257.

Publikation 2:

Coexpression profile of leukemic stem cell markers for combinatorial targeted therapy in AML

Haubner S, Perna F, Köhnke T, Schmidt C, Berman S, Augsberger C, Schnorfeil FM, Krupka C, Lichtenegger FS, Liu X, Kerbs P, Schneider S, Metzeler KH, Spiekermann K, Hiddemann W, Greif PA, Herold T, Sadelain M, Subklewe M

Leukemia. 2019 Jan;33(1):64-74. doi: 10.1038/s41375-018-0180-3. Epub 2018 Jun 26. PMID: 29946192; PMCID: PMC6326956.

Beitrag zu den Publikationen

1.1 Beitrag zu Publikation 1

Publikation 1 ist das Ergebnis einer präklinischen Forschungskollaboration mit dem *Roche Innovation Center Zürich* und wurde im Jahr 2021 unter dem Titel „*Targeting intracellular WT1 in AML with a novel RMF-peptide-MHC specific T-cell bispecific antibody*“ in der Fachzeitschrift *Blood* veröffentlicht. Das Forschungsprojekt, mit dem ich von 2015 bis 2019 als verantwortlicher Doktorand betraut war, beinhaltete die umfassende Charakterisierung eines neuartigen T-Zell-rekrutierenden Antikörpers, der gegen das intrazelluläre Tumorantigen Wilms-Tumor-Protein (WT1) gerichtet ist und zur Behandlung von akuter myeloischer Leukämie (AML) entwickelt wurde.

Als Erstautor des Fachartikels habe ich den Großteil des Manuskripts verfasst und alle Projektaktivitäten am Klinikum koordiniert. Ferner bestand mein Beitrag insbesondere in den folgenden von mir durchgeführten experimentellen Studien:

- Analyse und Interpretation von qPCR-Daten aus dem Labor für Leukämiediagnostik, um die Expression von WT1 in Knochenmarksproben von AML-Patientinnen/Patienten über den Verlauf der Krankheit hinweg zu verfolgen.
- Weiterentwicklung eines Feederzell-basierten Langzeitkultursystems für primäre AML-Zellen, um die Wirksamkeit des T-Zell-rekrutierenden Antikörpers in einem therapielevanten Zeitrahmen untersuchen zu können.
- Untersuchung der Wirksamkeit des T-Zell-rekrutierenden Antikörpers im Kurz- und Langzeitkultursystem: Hierbei wurden AML-Zelllinien und primäre AML-Zellen mit allogenen und/oder autologen T-Zellen in Anwesenheit von verschiedenen Antikörper-Konstrukten ko-kultiviert und schließlich die Antikörper-vermittelte Zytotoxizität mittels Durchflusszytometrie oder alternativen Methoden (T-Zell-Aktivierung, Zytokinfreisetzung und T-Zell-Rezeptor-Signaling) bestimmt. Die Durchführung erforderte die eigenständige Methodenetablierung, die Optimierung von Zellzahl, Effektor- zu Zielzellen-Verhältnis, Kulturdauer, Antikörperkonzentrationen sowie die Zusammenstellung und Messung umfangreicher *multi-color* FACS-Panels zur Bestimmung des Phänotyps der Effektor- und Zielzellen.
- Entwicklung und Durchführung von Assays, um die Zytotoxizität von WT1-spezifischen T-Zell-Klonen (zur Verfügung gestellt von Prof. Dr. Marc Schmitz, TU

- Dresden) gegen primäre AML-Zellen mit der Antikörper-vermittelten T-Zell-Zytotoxizität durch den WT1-TCB vergleichen zu können.
- Etablierung und Durchführung von CFU Assays zur Abschätzung der Toxizität des T-Zell-rekrutierenden Antikörpers gegen gesunde hämatopoetische Stammzellen: Hierzu wurden gesunde Knochenmarkszellen aus Femurköpfen von Hüftpatienten isoliert, denen eine Hüftgelenk-Endoprothese eingesetzt wurde.
 - Durchführung von Studien zur Kombination des T-Zell-rekrutierenden Antikörpers mit dem immunomodulatorischen Wirkstoff Lenalidomide und Charakterisierung des Einflusses von Lenalidomide auf die Zytokinfreisetzung und Effektorfunktion von T-Zellen.

Nach meinem Ausscheiden aus der Arbeitsgruppe führte Gerulf Hänel das Projekt als verantwortlicher Doktorand weiter. Es entstanden so zusätzliche Daten zur Abschätzung der Toxizität gegen gesunde hämatopoetische Stammzellen, zur Antikörper-vermittelten T-Zell-Aktivierung und Zytokinfreisetzung sowie zusätzliche Mausdaten mit AML „Patient-derived Xenograft (PDX)“ Mausmodellen. Ferner gelang es Gerulf Hänel, Zelllysate von primären AML-Zellen in ausreichender Menge und Qualität zu gewinnen, um eine Quantifizierung von WT1-Peptid-MHC-Komplexen auf den Zellen mittels Massenspektrometrie zu ermöglichen. Auch zur Finalisierung des Manuskripts und zum Einreichungsprozess bei der Fachzeitschrift trug Gerulf Hänel in der Zeit von 2019-2021 erheblich bei.

1.2 Beitrag zu Publikation 2

Für den Fachartikel „*Coexpression profile of leukemic stem cell markers for combinatorial targeted therapy in AML*“, der im Jahr 2019 in der Fachzeitschrift *Leukemia* publiziert wurde, musste ein umfangreicher Datensatz, bestehend aus Durchflusszytometriedaten von 356 AML- und 34 normalen Knochenmarksproben, analysiert werden. Im Fokus der Studie stand eine Co-Expressionsanalyse der Marker CD33, CD123, CLL1, TIM3, CD244 und CD7 auf leukämischen Stammzellen sowie übrigen AML-Zellen und auf gesunden hämatopoetischen Stammzellen.

Mein Beitrag zu diesem Forschungsvorhaben bestand insbesondere in der Validierung der Studiendaten und -ergebnisse. Im Rahmen dieser Validierung habe ich alle Daten und Analysen auf Vollständigkeit und Plausibilität geprüft und ferner bei deren Auswertung und Interpretation mitgewirkt. Außerdem habe ich zur Finalisierung und Überprüfung des Manuskripts beigetragen.

2. Einleitung

2.1 Akute Myeloische Leukämie

2.1.1 Entstehung und Klassifikation

Mit einer Inzidenz von 3-4 Fällen pro 100.000 Einwohnerinnen/Einwohnern pro Jahr in Deutschland stellt die Akute Myeloische Leukämie (AML) die häufigste akute Leukämie im Erwachsenenalter dar, ist aber dennoch eine insgesamt seltene Erkrankung (Nennecke et al., 2014). Das Risiko, an einer AML zu erkranken, steigt mit zunehmendem Alter, welches zum Zeitpunkt der Erstdiagnose im Mittel bei 68 Jahren liegt (Shallis et al., 2019).

Die AML ist eine maligne Erkrankung des blutbildenden Systems, die auf einer unkontrollierten Vermehrung von dysfunktionalen myeloischen Vorläuferzellen im Knochenmark beruht (Döhner et al., 2015). Im frühen Verlauf der Erkrankung, die genetisch und klinisch sehr heterogen ist, wird die normale Blutbildung durch die wachsende Zahl an malignen Zellen zunehmend verdrängt, wodurch Zytopenien entstehen, die zu Symptomen wie verminderter Leistungsfähigkeit und Infektanfälligkeit führen. Ohne Behandlung schreitet die Erkrankung schnell voran, führt im weiteren Verlauf zu lebensbedrohlichen Komplikationen und schließlich zu Organversagen und Tod (Döhner et al., 2015; E. Estey & Döhner, 2006; Grimwade & Mrózek, 2011).

Die Einteilung der AML erfolgte früher vor allem nach der French-American-British (FAB) Klassifikation, die auf Basis von morphologischen und zytochemischen Untersuchungen acht Subtypen der AML unterscheidet und für die Diagnosestellung einen Grenzwert von mindestens 30% myeloischen Vorläuferzellen (Blasten) im Knochenmark vorsieht (Bennett et al., 1976). Neben der FAB-Klassifikation ist heute auch eine modernere Klassifikation nach der *World Health Organisation* (WHO) von Bedeutung, die zuletzt im Jahr 2016 revidiert wurde. Das Ziel der WHO-Klassifikation ist es, basierend auf dem wachsenden Verständnis der Pathogenese der AML und anhand von klinischen Merkmalen, Morphologie, Immunphänotypisierung sowie Zyto- und Molekulargenetik, klinisch bedeutsame Krankheitsentitäten zu definieren. So lassen sich aktuell sechs Subgruppen definieren, die zum Teil noch weiter in Subtypen (insgesamt weitere 22 Subtypen) unterschieden werden. Nach der WHO-Klassifikation ist die Diagnose AML in

der Regel bereits ab einen Anteil von mindestens 20% myeloischen Vorläuferzellen (Blasten) im Knochenmark zu stellen (Arber et al., 2016; Vardiman et al., 2002, 2009).

Neben den FAB- und WHO-Klassifikationen haben auch die Empfehlungen des *European LeukemiaNETs* (ELN) zur Diagnose und Behandlung von AML sowie zur Risikostratifizierung breite Anerkennung in der Praxis, innerhalb klinischer Studien und bei regulatorischen Behörden erfahren. So sieht das in den Empfehlungen enthaltene ELN-Risikosystem die Kategorisierung einer AML-Erkrankung auf Basis der diagnostizierten zyto- und molekulargenetischen Anomalien in die drei Risikogruppen *favorable*, *intermediate* und *adverse* vor. Welcher dieser Kategorien die AML-Erkrankung einer Patientin/eines Patienten zugeordnet wird, spielt heute für Therapieentscheidungen, neben weiteren prognostischen Faktoren wie Alter, Performance Status und Komorbiditäten, ebenfalls eine große Rolle (Döhner et al., 2010b; Döhner et al., 2017; Herold et al., 2020).

2.1.2 Behandlungsstandards und Prognose

Der Behandlungsstandard der AML zum Zeitpunkt der Erstdiagnose basiert seit Jahrzehnten auf Chemotherapie und umfasst, sofern der gesundheitliche Zustand der Patientin/des Patienten es zulässt, zunächst eine Induktionstherapie mit einem Anthrazyklin (Daunorubicin, Idarubicin oder Mitoxantron) für 3 Tage, gefolgt von Zytarabin für 7 Tage. Mit diesem „3+7“ Schema gelingt es, bei 60-85% der Patientinnen/Patienten unter 60 Jahren und bei 40-60% der Patientinnen/Patienten über 60 Jahren eine komplette Remission zu erreichen. Jedoch besteht im weiteren Verlauf ein hohes Rezidiv-Risiko durch residuale chemo-resistente leukämische Zellen, weshalb der anschließenden Konsolidierungstherapie eine zentrale Rolle zukommt (Burnett et al., 2011; Döhner et al., 2015).

Abhängig vom individuellen Risikoprofil der Patientin/des Patienten finden hierzu als Postremissionsstrategien sowohl konventionelle Chemotherapien als auch die allogene hämatopoetische Stammzelltransplantation Anwendung. Bei Patientinnen/Patienten mit einem günstigen Risikoprofil kann eine Erhaltungstherapie mit Cytarabin in mittlerer Dosierung zu Langzeitremissionen in 60-70% der Fälle führen. Patientinnen/Patienten mit ungünstiger Prognose profitieren hingegen nicht von dieser Behandlung und auch bei mittlerem Risikoprofil werden lediglich Langzeitremissionen in 10-15% der Fälle erreicht. Insbesondere bei mittlerem oder ungünstigem Risikoprofil stellt eine allogene hämatopoetische Stammzelltransplantation die einzige kurative antineoplastische

Behandlungsoption dar, die das Gesamtüberleben nachweislich verlängert (Döhner et al., 2010a, 2015; E. Estey & Döhner, 2006; E. H. Estey, 2012).

Jedoch kommt für einen erheblichen Teil der Patientinnen/Patienten, insbesondere für ältere Patientinnen/Patienten, die unter Komorbiditäten leiden, aufgrund ihrer körperlichen Verfassung weder eine intensive Chemotherapie noch eine Stammzelltransplantation in Frage. In diesen Fällen verbleiben zur Behandlung nur neuartige Therapieansätze (z.B. Antikörper-basierte oder zelluläre Immuntherapien im Rahmen klinischer Studien) und Best Supportive Care. Häufig kommen zudem noch Hydroxyurea, Zytarabin in niedriger Dosierung und hypomethylierende Substanzen (Decitabine und Azacitidine) zum Einsatz (Döhner et al., 2015; Döhner et al., 2017; E. H. Estey, 2012; Ossenkoppele & Lowenberg, 2015).

Insgesamt können heute mit den zugelassenen Therapien Langzeitüberlebensraten von 35-40% bei Patientinnen/Patienten unter 60 Jahren und 5-15% bei Patientinnen/Patienten über 60 Jahren erreicht werden. Aufgrund der daraus abzuleitenden noch immer hohen Sterberaten, insbesondere bei älteren Patientinnen/Patienten und bei ungünstigem Risikoprofil, ist der medizinische Bedarf an neuartigen Therapien für AML nach wie vor sehr hoch (Döhner et al., 2015).

2.2 Antikörper-basierte Immuntherapie in AML

2.2.1 Überblick und Historie

Jede Krebsimmuntherapie zielt darauf ab, tumor-gerichtete Immunantworten auszulösen. In den letzten Jahrzehnten haben neue Krebsimmuntherapien, darunter insbesondere T-Zell- und Antikörper-basierte Ansätze die Onkologie regelrecht revolutioniert. Nachdem die allogene hämatopoetische Stammzelltransplantation über lange Zeit die einzige Immuntherapie zur Behandlung von AML darstellte, werden heute auf Basis des deutlich besseren Verständnisses von hämatologischen Krebserkrankungen auch zur Behandlung von AML eine Vielzahl von immuntherapeutischen Ansätzen entwickelt, darunter insbesondere T-Zell-basierte Strategien (Daver et al., 2021; Waldman et al., 2020).

Hierzu zählen zum einen adoptive T-Zell-Therapien, bei denen T-Zellen der Patientin/des Patienten entweder durch einen natürlichen T-Zell-Rezeptor (TCR), der gegen ein Epitop auf der Tumorzelle gerichtet ist, oder durch einen chimären

Antigenrezeptor (CAR) modifiziert werden, um dann nach Rückführung in die Patientin/den Patienten tumor-gerichtete Immunantworten auszulösen (Brenner, 2013; Fujiwara, 2014; June & Sadelain, 2018; Pizzitola et al., 2014; Prommersberger et al., 2018; Spranger et al., 2012; Stromnes et al., 2014; Xue et al., 2005).

Darüber hinaus kommt den bispezifischen T-Zell-rekrutierenden Antikörpern, die auch in dieser Dissertation im Mittelpunkt stehen, eine zentrale Rolle zu. Sie erkennen ein Zielantigen auf der Tumorzelle und binden simultan an CD3 im T-Zell-Rezeptorkomplex (TCR). Somit gelingt es, T-Zellen zur Tumorzelle zu rekrutieren, sie dort zu aktivieren und schließlich eine tumor-gerichtete T-Zell-Antwort auszulösen, die unabhängig von der eigentlichen TCR-Spezifität der T-Zellen ist (Daver et al., 2021; Laszlo et al., 2014; Sliwkowski & Mellman, 2013; Tian et al., 2021).

Neben weiteren Ansätzen ist schließlich vor allem die Immuncheckpoint-Blockade zu nennen, die darauf abzielt, durch den Einsatz von antagonistischen Antikörpern, die Rezeptor/Liganden-Interaktion von entzündungshemmenden Immuncheckpoints zu unterdrücken und somit entscheidende „*Tumor Escape*“-Mechanismen zu verhindern. Hierdurch lassen sich bereits vorhandene Immunantworten reaktivieren oder verstärken. Insbesondere kombinatorische Ansätze, bei denen Immuncheckpoint-Blockade mit weiteren Antikörper-basierten Immuntherapien kombiniert wird, stehen seit Jahren im Fokus des Interesses und wurden/werden in einer Vielzahl von Krebsindikationen evaluiert (Alatrash et al., 2016; Daver et al., 2021; Waldman et al., 2020).

2.2.2 Identifikation geeigneter Tumorantigene

Spätestens mit der Zulassung des ersten bispezifischen T-Zell-rekrutierenden Antikörpers Blinatumumab zur Behandlung von rezidivierender/refraktärer Akuter Lymphatischer Leukämie (ALL) im Jahr 2014 wurde die Wirksamkeit von T-Zell-rekrutierenden Antikörpern zur Behandlung von hämato-onkologischen Erkrankungen bestätigt. Blinatumumab richtet sich auf den Tumorzellen gegen CD19, ein Oberflächenprotein, das Bestandteil des erweiterten B-Zellrezeptor-Komplexes ist und auf malignen B-Zellen, wie ALL- oder Lymphomzellen, aber auch auf den gesunden Zellen der B-Linie exprimiert wird. Neben Blinatumumab haben sich auch weitere gegen CD19 gerichtete Ansätze, wie der monoklonale Immunglobulin G (IgG)-basierte Antikörper Tafasitamab oder die CD19 CAR-T-Zell-Therapie erfolgreich in diesem Feld etabliert. Eine Vielzahl weiterer Konstrukte, die meist ebenfalls gegen zellursprungsspezifische Oberflächenmarker (engl. Lineage Marker) gerichtet sind, werden derzeit in

präklinischen und klinischen Studien evaluiert (Abramson, 2020; Krishnamurthy & Jimeno, 2018; Salles et al., 2020; Suurs et al., 2019; Topp et al., 2015).

Da Lineage Marker stets auch auf den gesunden Zellen der entsprechenden Linie, wie den myeloischen Zellen, vorhanden sind, kommt es häufig zu unerwünschten Nebenwirkungen, wie „on-target off-tumor“ Toxizität, worunter die therapie-induzierte Depletion der gesunden Zellen, die den Lineage Marker ebenfalls exprimieren, zu verstehen ist. Während diese unerwünschten Nebenwirkungen im Falle von anti-CD19-Therapien klinisch kontrollierbar sind und somit dennoch eine effiziente Therapie ermöglichen, haben sich in der Vergangenheit, insbesondere bei Therapien gegen myeloische Ziel-Antigene erhebliche Komplikationen gezeigt. So wurden klinische Studien zu CD123-spezifischen Antikörpern vorzeitig gestoppt oder auch, im Falle von CD33, ein gegen CD33 gerichtetes Antikörper-Wirkstoff-Konjugat zur Behandlung von AML, aufgrund seines ungünstigen Nutzen/Risiko-Verhältnisses, über mehrere Jahre vom Markt genommen (Lichtenegger et al., 2015, 2017).

Eine der größten Herausforderungen ist und bleibt daher die Identifikation von geeigneten Tumorantigenen, die einerseits leukämie-spezifisch oder zumindest leukämie-assoziiert, also auf leukämischen Zellen und insbesondere auf leukämischen Stammzellen überexprimiert, aber andererseits nicht lineage-spezifisch und somit nicht auf gesunden hämatopoetischen Stammzellen exprimiert werden. Lösungsstrategien hierzu bestehen neben der Suche nach neuen Tumorantigenen, wie beispielsweise intrazellulären Tumorantigenen, auch in kombinatorischen Multi-Targeting-Strategien, die sich parallel oder sequenziell gegen eine Kombination von Tumorantigenen richten (Daver et al., 2021).

2.2.3 Ziel dieser Arbeit

Das Ziel dieser Arbeit ist es, den derzeitigen Herausforderungen bei der Suche nach geeigneten Zielstrukturen für Antikörper-basierte Immuntherapien der AML mit zwei unterschiedlichen Ansätzen zu begegnen.

Im Hauptteil der Arbeit wird ein neuartiger Ansatz vorgestellt, mit dem es gelingt, neben Zelloberflächenmarkern auch intrazelluläre Tumorantigene für Immuntherapien mit T-Zell-rekrutierenden Antikörpern zugänglich zu machen. Hierzu erkennt der in dieser Studie untersuchte T-Zell-rekrutierende Antikörper ein prozessiertes Peptid des intrazellulären Wilms-Tumor-Proteins (WT1), welches im Kontext des humanen Leukozyten-Antigen-Systems (HLA) auf der Zelloberfläche präsentiert wird. Bisher gibt

es nur wenige vergleichbare Ansätze (z.B. ImmTacs), von denen bislang in der Hämato-Onkologie aber keiner bis zur klinischen Entwicklung verfolgt wurde (Ataie et al., 2016; Dubrovsky et al., 2014; He et al., 2019; Oates et al., 2015; Veomett et al., 2014).

Der zweite Ansatz beschäftigt sich mit der Frage nach geeigneten Kombinationen von Tumorantigenen und insbesondere damit, welche Tumorantigen-Paare sich für paralleles oder sequenzielles Targeting von AML-Zellen und leukämischen Stammzellen auf Basis von Expressionsprofilen besonders eignen, um die Spezifität und Wirksamkeit der Behandlung zu erhöhen und Escape-Mechanismen zu verhindern, ohne dabei das Risiko für unerwünschte Toxizitäten zu steigern (Braciak et al., 2018; Huang et al., 2020; Kontermann, 2012).

3. Zusammenfassung

Die Akute Myeloische Leukämie (AML) ist eine maligne Erkrankung des blutbildenden Systems, für die nach wie vor großer medizinischer Bedarf an neuartigen Therapien besteht. Antikörper-basierte Immuntherapien, darunter T-Zell-rekrutierende Antikörper, stellen vielversprechende neue Strategien zur Behandlung von AML dar, aber die Identifikation von geeigneten Zielstrukturen bleibt eine zentrale Herausforderung bei deren Entwicklung. Die meisten der bisherigen Ansätze richten sich gegen Lineage Marker auf der Zelloberfläche, die auch auf gesunden hämatopoetischen Zellen exprimiert werden. Dies bringt ein Risiko für unerwünschte Nebenwirkungen wie Hämatotoxizität mit sich.

Diese Dissertation befasst sich mit zwei Strategien, um einerseits durch das Targeting von intrazellulären Tumorantigenen eine Vielzahl neuer geeigneter Zielstrukturen für Antikörper-basierte Immuntherapien zugänglich zu machen und andererseits bestmögliche Kombinationen von bekannten Tumorantigenen für neue kombinatorisch-zielgerichtete Ansätze zu identifizieren, die eine erhöhte Spezifität ermöglichen und Escape-Mechanismen verhindern.

Für die erste Strategie wurde ein bispezifischer T-Zell-rekrutierender Antikörper evaluiert, der auf Basis von „CrossMab“- und „Knobs-into-holes“-Technologie entwickelt wurde. Dieser *T-Cell-Bispecific* (TCB) erkennt ein prozessiertes Peptid des intrazellulären Tumorantigens Wilms-Tumor-Protein (WT1) im Kontext des Humanen Leukoyten-Antigens HLA-A2 auf der Zelloberfläche. Durch die simultane Bindung an CD3 werden T-Zellen zur Tumorzelle rekrutiert und schließlich tumor-gerichtete T-Zellantworten vermittelt, die unabhängig von der eigentlichen T-Zell-Rezeptorspezifität der T-Zelle sind. Im Rahmen der durchgeführten Studien wurde zunächst gezeigt, dass das WT1-Zielpeptid im Kontext von HLA-A2 auf primären AML-Zellen präsentiert wird. Darüber hinaus induzierte der WT1-TCB in Ko-Kulturen mit gesunden Spender-T-Zellen oder autologen Patienten-T-Zellen und primären AML-Zellen spezifische T-Zell-Antworten, die zu einer HLA-restringierten WT1-spezifischen Lyse der AML-Zellen führten. Ferner konnte die spezifische Lyse der AML-Zellen durch Kombination des WT1-TCB mit dem immunomodulatorischen Wirkstoff Lenalidomide weiter gesteigert werden. Auch in humanisierten Mausmodellen wurde die Vermittlung von spezifischer T-Zell-Zytotoxizität bestätigt. Auf Basis dieser Ergebnisse wird der WT1-TCB derzeit in einer klinischen Phase-I-Studie evaluiert.

Der zweite Ansatz befasst sich mit der Identifikation von geeigneten Kombinationen von bisher bekannten Tumorantigenen für das Targeting von AML-Zellen und leukämischen Stammzellen mit Multi-Targeting-Strategien. Das Ziel hierbei ist, durch paralleles oder sequenzielles Targeting der Tumorantigene die Spezifität und Wirksamkeit der Behandlung zu erhöhen und Escape-Mechanismen zu verhindern, ohne dabei das Risiko für unerwünschte Toxizitäten zu steigern. Hierzu wurde auf Basis von Proteinexpressionsdaten von über 300 AML-Patientinnen/Patienten und gesunden Knochenmarkspendern ein Proteinexpressionsprofil der bekanntesten Tumormarker erstellt. Die Expressionsdaten wurden außerdem mit einem Datensatz zur Proteinexpression in gesundem Gewebe verglichen, um schließlich die vielversprechendsten Kombinationsmöglichkeiten zu ermitteln. Auf Basis dieser Daten konnten die Kombinationen von CD33/TIM3 und CLL1/TIM3 als potenziell vielversprechendste Multi-Targeting-Strategien identifiziert werden.

Zusammenfassend handelt diese Dissertation von zwei neuartigen Strategien, einem T-Zell-rekrutierenden Antikörper zum Targeting von intrazellulären Tumorantigenen und der Identifikation von vielversprechenden Tumorantigen-Kombinationen für das Targeting von AML-Zellen und leukämischen Stammzellen, die beide darauf abzielen, die Herausforderungen bei der Suche nach geeigneten Zielstrukturen für die AML zu überwinden.

4. Abstract (English)

Acute myeloid leukemia (AML) is a malignancy of the hematopoietic system with a high medical need for novel therapeutic option. Antibody-based immunotherapy, including T-cell engaging antibodies, represent a strategy for immunotherapy of AML but the identification of suitable target antigens remains a key challenge for their development. Most current approaches are targeting lineage markers on the cell surface that are also expressed on healthy hematopoietic cells. This involves a risk for unwanted side effects like hematotoxicity.

This thesis addresses two strategies to make a variety of new suitable targets available for antibody-based immunotherapies by targeting intracellular tumor antigens on the one hand, and by identification of best possible tumor antigen combinations for new combinatorial-targeting approaches to enable increased specificity and prevent tumor-escape mechanisms on the other hand.

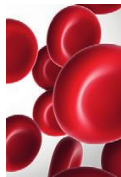
For the first strategy, a bispecific T-cell recruiting antibody was evaluated, which was developed based on "CrossMab" and "Knobs-into-holes" technology. This T-cell-bispecific (TCB) recognizes a processed peptide of the intracellular tumor antigen Wilms tumor protein (WT1) in the context of the human leukocyte antigen HLA-A2 on the cell surface. Simultaneous binding to CD3 recruits T-cells to the tumor cell and mediate tumor-directed T-cell responses that are independent of the T cell receptor specificity. It was shown that the WT1-derived peptide is presented in the context of HLA-A2 on primary AML cells. Moreover, WT1-TCB induced specific T cell responses in co-cultures with healthy donor T cells or autologous patient-derived T cells with primary AML cells, resulting in HLA-restricted WT1-specific lysis of AML cells. Furthermore, specific lysis of AML cells was further enhanced by combining WT1-TCB with the immunomodulatory agent lenalidomide. Induction of specific T cell cytotoxicity by the WT1-TCB was also confirmed in humanized mouse models. Based on these results, WT1-TCB is currently being evaluated in a phase I clinical trial.

In the second approach suitable combinations of previously known tumor antigens for targeting AML cells and leukemic stem cells with combinatorial strategies for parallel or sequential targeting were identified. The aim of this study was to increase the specificity and efficacy of the treatment and to prevent escape mechanisms by combining tumor antigens without increasing the risk of unwanted toxicities. Protein expression data from over 300 AML patients and healthy bone marrow donors were combined to generate a

protein expression profile of the most prominent tumor markers. The expression data were further compared with an integrated dataset of protein expression in healthy tissue to finally identify the most promising combination options. Based on these data, the combinations of CD33/TIM3 and CLL1/TIM3 were identified as the most promising multi-targeting strategies.

In summary, this thesis addresses two promising strategies, a T-cell recruiting antibody for targeting of intracellular tumor antigens on the one side and the identification of suitable tumor antigen combinations for targeting AML cells and leukemic stem cells on the other side, both of which aim to overcome the challenges in the identification of suitable target structures for AML.

5. Publikation I



IMMUNOBIOLOGY AND IMMUNOTHERAPY

Targeting intracellular WT1 in AML with a novel RMF-peptide-MHC-specific T-cell bispecific antibody

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KEY POINTS

- A T-cell bispecific antibody targeting intracellular WT1 presented on HLA-A2 for treatment of AML is characterized in vitro and in vivo.
- The novel, clinical-stage WT1-TCB mediated high-level killing of primary AML cells, which was enhanced by the addition of lenalidomide.

Antibody-based immunotherapy is a promising strategy for targeting chemoresistant leukemic cells. However, classical antibody-based approaches are restricted to targeting lineage-specific cell surface antigens. By targeting intracellular antigens, a large number of other leukemia-associated targets would become accessible. In this study, we evaluated a novel T-cell bispecific (TCB) antibody, generated by using CrossMAB and knob-into-holes technology, containing a bivalent T-cell receptor-like binding domain that recognizes the RMFPNAPYL peptide derived from the intracellular tumor antigen Wilms tumor protein (WT1) in the context of HLA-A*02. Binding to CD3 α recruits T cells irrespective of their T-cell receptor specificity. WT1-TCB elicited antibody-mediated T-cell cytotoxicity against AML cell lines in a WT1- and HLA-restricted manner. Specific lysis of primary acute myeloid leukemia (AML) cells was mediated in ex vivo long-term cocultures by using allogeneic (mean 6 standard error of the mean [SEM] specific lysis, 67.6% after 13–14 days; n = 18) or autologous, patient-derived T cells (mean 6 SEM specific lysis, 54.6% after 11–14 days; n = 8). WT1-TCB-treated T cells exhibited higher cytotoxicity against primary AML cells than

an HLA-A*02 RMF-specific T-cell clone. Combining WT1-TCB with the immunomodulatory drug lenalidomide further enhanced antibody-mediated T-cell cytotoxicity against primary AML cells (mean 6 SEM specific lysis on days 3–4, 45.4% vs 70.8% ± 8.3%; P = .015; n = 9–10). In vivo, WT1-TCB-treated humanized mice bearing SKM-1 tumors exhibited a significant and dose-dependent reduction in tumor growth. In summary, we show that WT1-TCB facilitates potent in vitro, ex vivo, and in vivo killing of AML cell lines and primary AML cells; these results led to the initiation of a phase 1 trial in patients with relapsed/refractory AML (#NCT04580121).

Introduction

Recruitment of T cells to tumor cells by T-cell-recruiting antibody constructs is a proven and effective way to treat cancer independently of preexisting tumor-specific T cells.^{1–5} Blinatumomab, a bispecific T-cell engager (BiTE, Amgen, Thousand Oaks, CA) consisting of 2 single-chain variable fragments directed against CD3 $\times$ CD19, has been approved for the treatment of Philadelphia chromosome-positive and -negative, relapsed or refractory B-cell

precursor acute lymphoblastic leukemia.^{6,7} Several other bispecific antibodies having different targets, mainly lineage antigens, are currently being evaluated in clinical trials.^{8–15} However, these targets are indiscriminate between healthy and tumor cells. Whereas a depleted healthy compartment is manageable in cases of B-cell malignancy, targeting lineage antigens in myeloid malignancies is associated with the risk of hematotoxicity and poses a potential safety concern.^{16,17}

In contrast, the intracellular antigen Wilms tumor protein (WT1) displays a rather restricted expression profile. It is a transcriptional and post-transcriptional regulator playing crucial roles in embryonic development and organ homeostasis.^{18,19} In adults, WT1 expression is restricted to a few tissues (kidney podocytes; Sertoli and granulosa cells in the testes and ovaries, respectively; a few mesothelial cells; and 1% of bone marrow cells).¹⁸⁻²¹ In addition to its physiological role in healthy cells, WT1 has reportedly contributed to the development of cancer, although its specific function in relation to this observation is not completely understood. WT1 is overexpressed in leukemias and solid cancers and is therefore widely regarded as a potential target antigen for immunotherapeutic approaches.^{22,23}

Targeting intracellular antigens with antibody constructs requires their binding to an HLA-restricted peptide epitope. For WT1, several epitopes have been identified. In particular, the non-peptide WT1₁₂₆₋₁₃₄ (RMFPNAPYL; "RMF"), which is presented on HLA-A*02, has been targeted in the context of several cancer entities, including acute myeloid leukemia (AML).²⁴⁻²⁶

Few approaches for targeting WT1 have reached clinical development. In a phase 1/2 clinical trial, adoptive transfer of donor-derived ex vivo-expanded, WT1-specific CD8⁺ T cells was shown to be safe and to result in antileukemic activity.²⁷ Adoptively transferred WT1_{RMF}-specific T-cell receptor (TCR)-transgenic T cells were also shown to be safe and to persist in patients with refractory AML or high-risk myelodysplastic syndrome taking part in a phase 1 clinical trial; they were able to prevent relapse if administered posttransplant, supporting further evaluation of this target.^{28,29} In a phase 2 clinical trial for postremission therapy of AML, vaccination with dendritic cells electroporated with WT1 messenger RNA (mRNA) was shown to reduce the risk of relapse after standard chemotherapy.^{30,31} Various WT1 peptide vaccines have been tested in phase 1 and phase 2 clinical trials.³² In preclinical studies, the therapeutic efficacy of WT1-specific chimeric antigen receptor T cells was shown in a xenograft mouse model.³³ Antibody-based immunotherapeutic approaches with TCR-mimicking T cell-recruiting antibodies have also been developed but could not be translated from mouse models into the clinic.³⁴⁻³⁷ WT1 has thus been validated as a safe and promising target antigen independently of the genetic subtype of AML.

In the current study, we evaluated a novel T-cell bispecific (TCB) antibody based on the TCR-like antibody 11D06 for the immunotherapy of AML. The TCB is directed against WT1 by recognition of the WT1_{RMF} peptide, which is processed and presented on HLA-A*02. In contrast to the aforementioned approaches, the construct relies on bivalent recognition of the tumor antigen, which is facilitated by an additional Fab domain fused to the N terminus of the VL domain of a CrossMAB^{VH-VL}.³⁸⁻⁴⁰ This results in a trivalent "2 + 1" immunoglobulin G (IgG) antibody for bivalent recognition of WT1-peptide major histocompatibility complexes (pMHC). Complementary monovalent binding to CD3ε in the TCR complex recruits T cells irrespective of their specificity. The TCB is based on human IgG1 and contains a mutated Fc region with a P329G LALA mutation; this confers extended half-life properties but excludes complement-dependent cellular cytotoxicity and antibody-dependent cellular cytotoxicity. Upon crosslinking, T cells are activated to form an immunologic synapse, which induces

apoptosis in tumor cells via the perforin/granzyme apoptosis pathway.⁴¹

Methods

Patients

Peripheral blood (PB) or bone marrow samples were collected from healthy donors (HDs) and patients with AML at primary diagnosis, relapse, or complete remission after written informed consent was received in accordance with the Declaration of Helsinki and approval was granted by the Institutional Review Board of the Ludwig-Maximilian-Universität (Munich, Germany).

WT1-TCB antibody generation and selection

Antibodies specific for the WT1-RMF peptide HLA-A*02 complex were generated by using phage display using standard protocols (further details are given in the supplemental Data, available on the *Blood* Web site).⁴² Of 34 complex-recognizing clones, the 11D06 antibody was selected due to its selectivity profile and characterization in cell assay, and reformatted into a trivalent 2 + 1 IgG TCB antibody for bivalent recognition of the WT1-pMHC complex and monovalency for the CD3ε chain of the TCR. This was facilitated by fusion of a second Fab domain to the N terminus of the VL domain of the anti-CD3ε Fab, resulting in a heterotrimeric CrossMAB^{VH-VL}.³⁸ Introduction of a P329G LALA mutation⁴³ renders it inactive for complement-dependent cytotoxicity. For the work presented here, two different CD3ε binders were incorporated, one based on the humanized antibody V9 derived from UCHT1, resulting in "WT1-TCB," and one related to the antibody Sp34 termed CH2527, yielding "WT1-TCB*." Both variants exhibited comparable potency in vitro and in vivo (supplemental Figure 1; Table 1). Accordingly, "WT1-TCB" is used throughout the article to refer to both constructs.

All antibodies were purified from transiently transfected HEK293-EBNA or CHO cells via protein A affinity chromatography and size exclusion chromatography.^{41,44,45} Affinities and avidities were characterized in surface plasmon resonance experiments by using a Biacore T200 (GE Healthcare, Chicago, IL).

Flow cytometry, cytokine quantification, and real-time polymerase chain reaction

Surface expression of CD33 (REA775), CD2 (REA972; both Miltenyi Biotec, Bergisch Gladbach, Germany), CD69 (FN50), PD1 (29F.1A12), TIM-3 (F38-2E2), CD45RA (HI100), and CCR7 (G43H7; all BioLegend, San Diego, CA) was assessed by using flow cytometry (CytoFLEX S, Beckman Coulter, Krefeld, Germany). Cytokine concentrations in cell culture supernatants were quantified by using the Human Th1/Th2 Cytokine Kit (BD Biosciences, Heidelberg, Germany). Alternatively, cytokines were measured on a Luminex 100 Analyzer (Bio-Rad, Hercules, CA) by using a custom ProcartaPlex panel (Invitrogen, Vienna, Austria). WT1 expression levels were quantified by using real-time quantitative polymerase chain reaction (qPCR), as previously described.⁴⁶

In vitro dose-response assays

T cells were isolated from PB of HDs and cocultured with tumor cell lines at an effector-to-target (E:T) ratio of 4:1 in the presence of various concentrations of WT1-TCB*. Mean specific lysis was assessed by flow cytometry and calculated relative to a control

Table 1. Affinities of WT1-TCB

pMHC affinity	pMHC avidity	CD3 affinity
$K_D = 50 \text{ nM}$ (25°C) $k_a = 7.7 \pm 0.0 \times 10^5 \text{ 1/M s}$ (37°C) $k_d = 3.7 \pm 0.0 \times 10^{-2} \text{ 1/s}$ (37°C)	0.45 nM	WT1-TCB $K_D = 3.7 \pm 0.8 \text{ nM}$ (25°C) $11.3 \pm 0.1 \text{ nM}$ (37°C) $k_a = 1.4 \pm 0.0 \times 10^5 \text{ 1/M s}$ (37°C) $k_d = 1.6 \pm 0.0 \times 10^{-3} \text{ 1/s}$ (37°C) WT1-TCB* $K_D = 3.0 \pm 0.1 \text{ nM}$ (25°C) $6.3 \pm 0.2 \text{ nM}$ (37°C) $k_a = 2.1 \pm 0.1 \times 10^5 \text{ 1/M s}$ (37°C) $k_d = 1.3 \pm 0.0 \times 10^{-2} \text{ 1/s}$ (37°C)

K_D , equilibrium dissociation constant; k_a , association rate constant; k_d , dissociation rate constant.

antibody construct (Ctrl-TCB) that recognizes a nontumor target derived from the human antibody germline repertoire.

Ex vivo short- and long-term cytotoxicity assays

Primary AML cells were thawed and pre-cultivated on a feeder layer of irradiated murine MS5 stromal cells in a 6-well plate for 3 to 4 days. For short-term cytotoxicity assays, cells were subsequently labeled with CellTrace Far Red (Life Technologies, Eugene, OR) to assess specific lysis. Cells were cocultured for 72 hours with autologous or HD peripheral blood mononuclear cells (PBMCs) at an E:T ratio of 5:1 and with various concentrations of WT1-TCB.

Ex vivo long-term cytotoxicity assays were performed as previously described.⁴⁷ After pre-cultivation on MS5 cells, primary AML cells were transferred onto a fresh feeder layer in a 96-well plate. WT1-TCB* was added at a concentration of 10 nM and HD T cells at an E:T ratio of 1:4 to 1:5 for 14 days.

Downstream TCR signaling

Downstream TCR signaling was measured by cocultivation of primary AML cells with NFAT Luciferase Reporter Jurkat cells (MilliporeSigma, Burlington, MA). Primary AML cells were precultured in the aforementioned culture system, followed by cocultivation with reporter cells in the presence of WT1-TCB* at an E:T ratio of 1:1 for 16 hours. Luminescence was induced by using the Bright-Glo Luciferase Assay System (Promega, Fitchburg, WI) and measured on an Infinite M1000 Plate Reader (Tecan, Männedorf, Switzerland).

In vivo studies with humanized mice

NSG mice (NOD.Cg-Prkdcscid-Il2rgtm1Wjl/SzJ) were humanized with human HLA-A*02⁺ CD34⁺ cord blood cells (details are provided in the supplemental Data). After successful engraftment and development of human T cells, 1×10^7 WT1-expressing HLA-A*02⁺ SKM-1 tumor cells were subcutaneously inoculated. After 7 days, when tumor size reached an average volume of 150 mm³, mice received weekly administrations of 1 mg/kg WT1-TCB or histidine buffer (vehicle) for 6 weeks. The experimental study protocol was reviewed and approved by local government authorities (2011-128).

In vivo study with AML patient-derived xenograft cells

NSG mice were engrafted with patient-derived xenograft (PDX) cells expressing enhanced firefly luciferase, and tumor burden was monitored by bioluminescence imaging as previously described.⁴⁸ After successful engraftment, HD T cells were transplanted, followed by weekly administration of 3 mg/kg WT1-TCB or Ctrl-TCB beginning on day 30. One WT1-TCB-treated mouse died during imaging on day 32. The experimental study protocol was reviewed and approved by local government authorities (ROB-55.2-2532.Vet_02-20-159).

Results

Quantitative PCR shows WT1 is expressed in >90% of AML samples at initial diagnosis and at relapse

We analyzed WT1 expression levels in PBMCs of 112 patients with AML by using quantitative real-time PCR at initial diagnosis, in remission, and at time of relapse or in refractory patients (Figure 1A). WT1 expression, defined as >10 WT1 copies per 10⁴ copies of ABL, was observed in 59 of 65 AML patient samples taken at the time of initial diagnosis.⁴⁹ Moreover, WT1 expression levels correlated with the percentage of AML blasts: expression was observed in 2 of 26 samples taken at the time of complete remission with a blast percentage <5%. Conversely, upon relapse or in refractory disease, WT1 was expressed in 17 of 21 cases. Furthermore, WT1 expression was observed in 79% (n = 38) of cancer cell lines (Figure 1B).

Similarly, WT1 expression in bone marrow samples correlated to the percentage of blasts (Figure 1C). WT1 expression was observed in 35 of 38 samples taken at primary diagnosis. In posttherapy samples, WT1 expression was detected in 1 of 8 cases in which the blast percentage was <5%. On the contrary, 10 of 16 samples were WT1 positive if the blast percentage exceeded 5% as a result of relapsed or refractory disease.

The WT1 expression level (mean $\pm$ standard error of the mean [SEM]) tended to be higher for NPM1 mutant samples compared with wild-type (1055 $\pm$ 251 vs 985 $\pm$ 429 WT1 copies/10⁴ ABL copies; $P = .1$) and was higher in samples with mutated FLT3-ITD (2155 $\pm$ 922 vs 582 $\pm$ 103 WT1 copies/10⁴ ABL copies; $P < .05$) and lower in CEBPA mutant samples (92 $\pm$ 43 vs 1299 $\pm$ 482 WT1 copies/10⁴ ABL copies; $P < .005$) (Figure 1D).

Specific binding to WT1_{RMF}-pMHC complexes

For the work presented here, trivalent 2 + 1 IgG TCB antibodies were generated with bivalent recognition of the WT1_{RMF}-pMHC complex and monovalency for the CD3 ϵ chain of the TCR (Figure 2A). The monovalent affinities of WT1-TCB for CD3 ϵ and WT1-pMHC as determined by surface plasmon resonance are shown in Table 1.

Alanine scanning revealed the key amino acids required for binding of the RMF peptide (Figure 2B; supplemental Figure 2A-B). These data were confirmed by using a novel peptide-MHC array in which the binding contribution of each amino acid can be investigated by replacement of the remaining amino acids (Figure 2C; supplemental Figure 2C). Both methods showed that apart from the anchor residues Met2 and Leu9, the residues Arg1, Phe3, Asn5, and Ala6 are particularly important for recognition of the

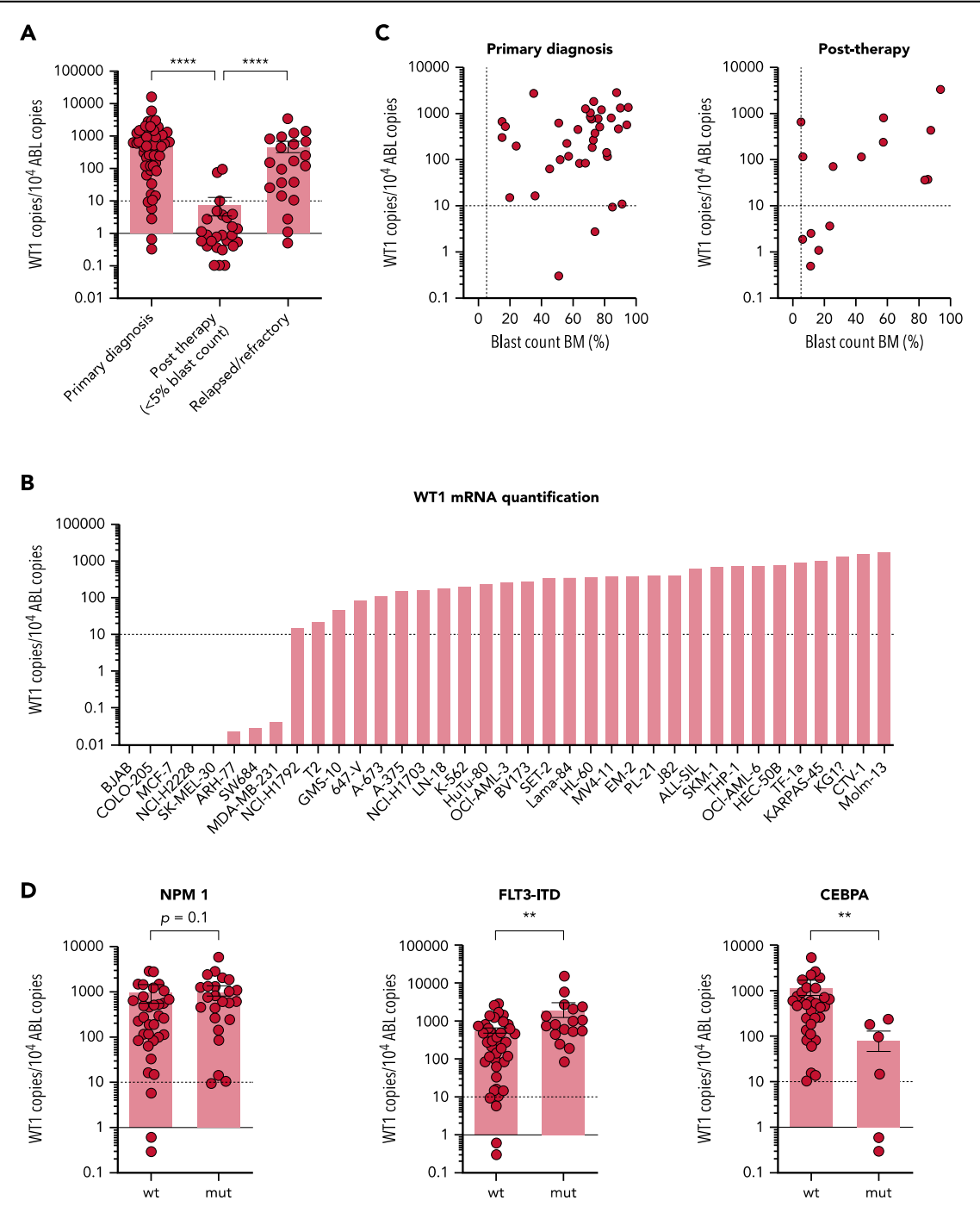


Figure 1. Analysis of WT1 expression in primary AML cells and tumor cell lines by real-time qPCR. (A) WT1 expression in primary AML cells at different time points during the course of the disease (primary diagnosis, $n = 65$; posttherapy, $n = 26$; relapsed/refractory, $n = 21$). (B) WT1 expression in tumor cell lines ($n = 37$). (C) Correlation of blast count and WT1 expression in bone marrow (BM) samples of patients with AML at primary diagnosis ($n = 38$) and posttherapy ($n = 24$). (D) Correlation of WT1 expression and NPM1 (wild-type [wt], $n = 37$; mutant [mut], $n = 25$), FLT3-ITD (wt, $n = 45$; mut, $n = 17$), and CEBPA (wt, $n = 34$; mut, $n = 6$) mutational status. Bars represent mean $\pm$ SEM. Statistical analysis: Mann-Whitney U test. ** $P < .01$, **** $P < .0001$.

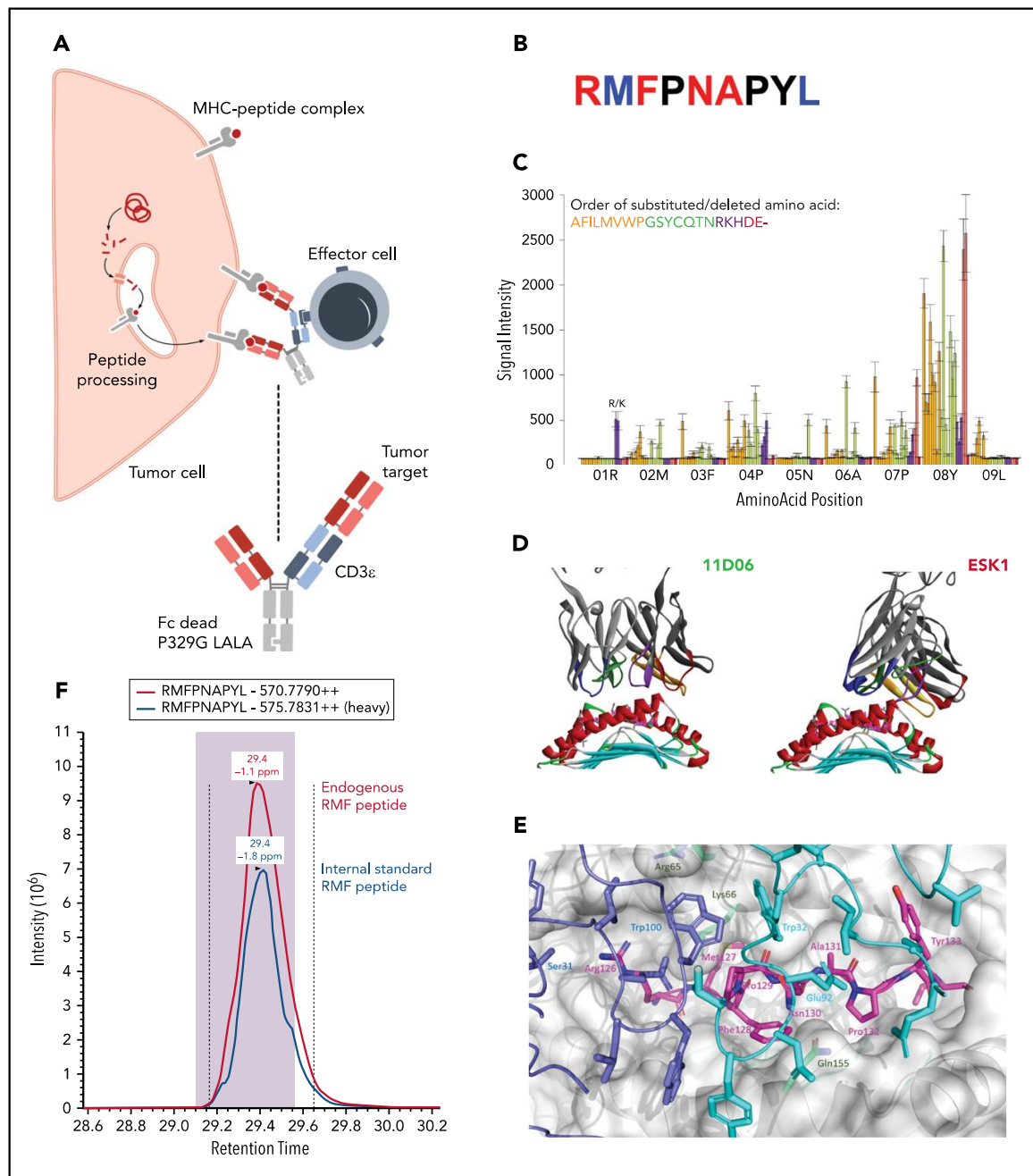


Figure 2. Structure of WT1-TCB, mode of action, binding motif, and crystallography. (A) Scheme of WT1-TCB-mediated T-cell engagement. (B) Binding motif as determined by alanine scanning and pMHC array. Anchor residues Met2 and Leu9 (marked in blue) can be replaced by few amino acids, whereas Arg1, Phe3, Asn5, and Ala6 (red) are important for recognition of the RMF-pMHC complex by 11D06. Residues Pro4, Pro7, and Tyr8 (black) are less critical for binding. (C) Single-substitution plot for RMF peptide in the context of HLA-A*02-β2m heterodimer bound by 11D06 antibody. Each bar corresponds to substitution with 1 of 20 amino acids, and a deletion is shown by the last bar. The height of each bar indicates the signal intensity. Bars are ordered as follows: the 8 nonpolar residues (AFILMVWP; orange); the 7 polar/neutral residues (GSYCQTN; green); the 3 positively charged residues (RKH; blue); and the 2 negatively charged residues (DE; pink). (D) Crystal structures of the 11D06-RMF-pMHC (pdb entry 4WUU) complexes. (E) View of the peptide-binding site of pMHC in the Fab 11D06-HLA-A*02 RMF-pMHC complex. Heavy and light chains of Fab 11D06 are colored in blue and cyan, respectively, and the RMF peptide is colored in magenta. The HLA-A*02 pMHC is shown as a transparent surface in white, with selected side chains highlighted in green. (F) Quantification of endogenous RMF peptide purified from a primary AML patient sample. Mass spectrometry chromatogram overlay of dioxidized endogenous RMF peptide precursor ion [m/z = 570.7790 (2+); red] and dioxidized internal standard [m/z = 575.7831 (2+); blue]. Endogenous RMF peptide was immunoprecipitated by using WT1-TCB. Figure 2A created with BioRender.com.

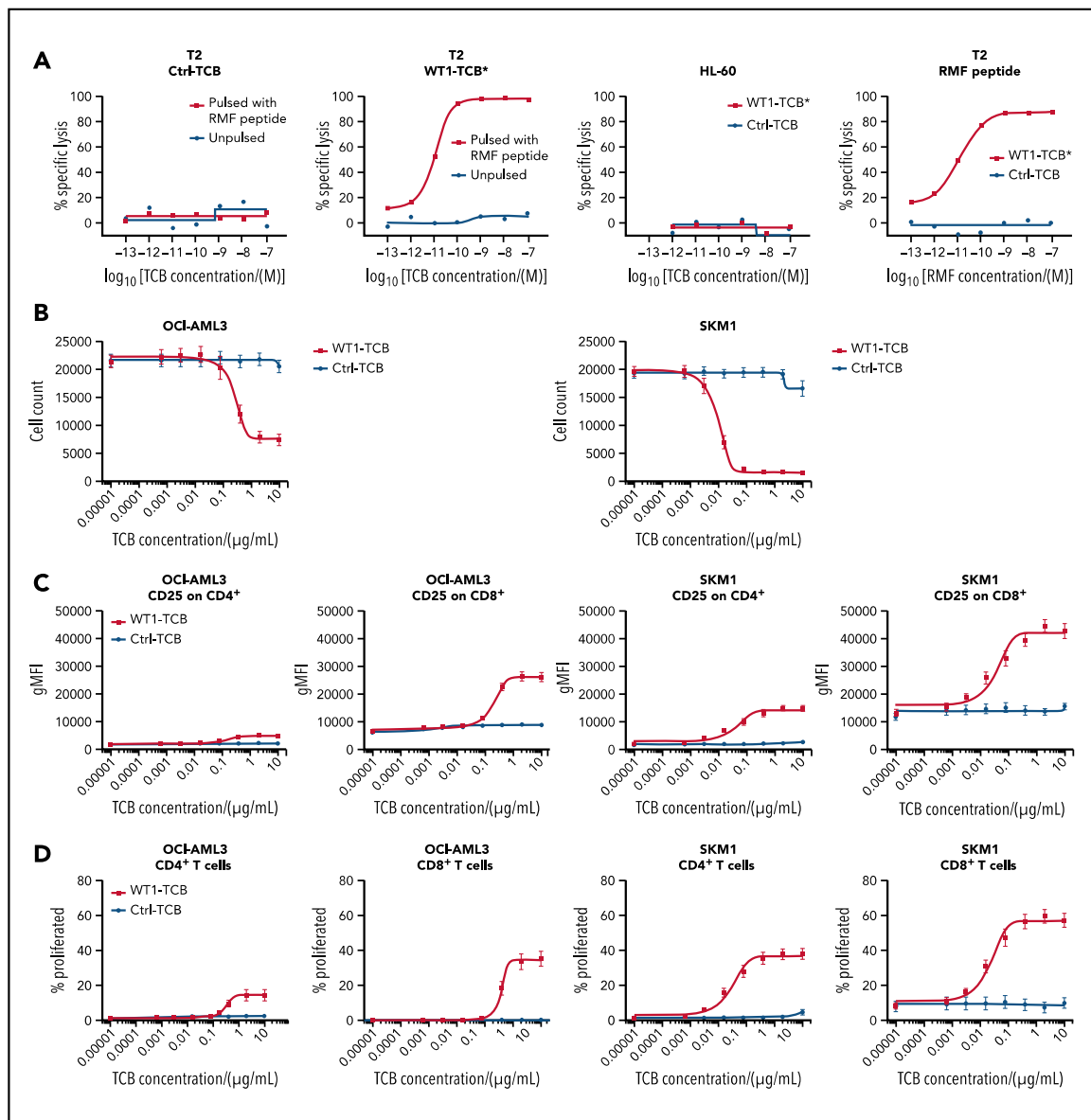


Figure 3. Dose-response relationship after treatment with WT1-TCB in cocultures of tumor cell lines with HD T cells. (A) Transporter associated with antigen processing-deficient HLA-A*02⁺ T2 cells, unpulsed (black) vs RMF peptide pulsed (red) after treatment with Ctrl-TCB (left) or WT1-TCB* (second from left). Second from right: control with HLA-A02⁺ HL-60 cells and Ctrl-TCB (black) or WT1-TCB* (red). Right: T2 cells pulsed with increasing doses of RMF peptide and treatment with 10⁻⁸ M Ctrl-TCB (black) or WT1-TCB* (red). (B) Killing assay performed on AML cell lines (OCI-AML3 and SKM-1) and treated with varying concentrations of WT1-TCB (red) or Ctrl-TCB (black). Tumor cell killing was evaluated after 44 hours (SKM-1) or 68 hours (OCI-AML3); n = 10. CD25 expression (C) and proliferation of CD4⁺ and CD8⁺ T cells (D) after 3 days in killing assays using AML cell lines (OCI-AML3 and SKM-1) at varying concentrations of WT1-TCB (red) or Ctrl-TCB (black); n = 6. gMFI, geometric mean fluorescence intensity.

RMF-pMHC complex by 11D06, whereas residues Pro4, Pro7, and Tyr8 are less critical for binding.

The X-ray structure of the HLA-A*02/RMF-pMHC complex bound by the 11D06 Fab fragment, resolved at 2.64 Å (pdb code 7BBG), allowed a closer examination of the binding motif compared with the known crystal structure of the ESK1 antibody (Figure 2D-E).³⁴ The structure shows that the Fab 11D06 bound to MHC by

contributions of all complementarity-determining regions. The RMF-pMHC-11D06 complex is characterized by a contact surface area of ~397 Å² in the center of the pMHC complex, which corresponds to a peptide contribution of ~107 Å² (27%); for the ESK1 antibody, the contact surface area is ~505 Å² with a peptide contribution of ~60 Å² (12%) that is shifted to the N terminus.³⁷ ESK1 binds HLA via Arg1 of the RMF peptide, whereas 11D06 interacts with the central bulge of the peptide, mainly through

Table 2. Summary of cell line killing mediated by WT1-TCB

Cell line	Disease of tissue origin	WT1 mRNA	HLA-A typing based on TRON database	Time of EC ₅₀ evaluation (h)	EC ₅₀ (nM) T-cell killing in vitro	EC ₅₀ (μg/mL) T-cell killing in vitro
SKM-1	AML	+	33:03, 02:07	44	0.16-1.25	0.032-0.25
OCI-M1	AML	+	03:01, 02:01	68	0.75-2.15	0.15-0.43
OCI-AML3	AML	+	23:01, 02:01	68	0.20-6.75	0.04-1.35
Set-2	AML	+	33:03, 02:01	48	0.8-7.6	0.16-1.52
COV318	Ovarian carcinoma	+	03:01, 02:01	48	4	0.8
Ovcar-3	Ovarian carcinoma	+	02:01, 29:02	48	10.8	2.16
T98G	Glioblastoma	+	02:01, 02:01	48	0.5	0.1
MDA-MB-231	Breast adenocarcinoma	+	02:01, 02:17	48	0.87	0.174
BV173	Blast phase chronic myelogenous leukemia	+	30:01, 02:01	48	0.97-13.75	0.19-2.75
SW480	Colorectal adenocarcinoma	+	02:01, 24:02	48	0.05-5.4	0.01-1.08
SW620	Colorectal adenocarcinoma	+	02:01, 24:02	48	5.8-8.5	1.16-1.7
HEC-50B	Endometrial adenocarcinoma	+	02:07, 02:07	48	5.95-6.2	1.19-1.24
ARH-77	Plasma cell leukemia	—	02:01, 03:01	NA	NA	NA
HL-60	Promyelocytic leukemia	+	01:01	NA	NA	NA

NA; not applicable.

Asn5. The peptide's central core is arranged similarly to the natural TCR-HLA assemblies, with the CDR3 loops of both chains arranged around the peptide amino acids 4-6. A similar pattern of binding is observed in the complex formed between the affinity-enhanced WT1- $\alpha_7\beta_2$ TCR and A2-RMF (supplemental Figure 3; supplemental Tables 1-3).⁵⁰

Targeted quantification of HLA-A*02 presented WT1_{RMF} peptide using immunoprecipitation combined with mass spectrometry

To provide evidence for RMF peptide presentation on MHC, we developed a novel mass spectrometry-based approach (details are given in the supplemental Data). To test the performance of the method, the RMF peptide was pulsed on T2 cells. The exogenously loaded peptide was recovered and detected with a concentration-dependent signal. Furthermore, the existence of the endogenous peptide on SKM-1 cells was confirmed by its detection, albeit at very low levels (supplemental Figure 4). The workflow has been further optimized for primary AML cells, including immunoprecipitation by WT1-TCB and sample upscaling. Thereby, the endogenous RMF peptide was quantified up to 17 fmol, which is equivalent to 152 copies per cell for AML patient no. 1 (Figure 2F). Patient characteristics are provided in supplemental Table 8.

Antibody binding is dependent on WT1 and HLA-A*02 expression and results in T-cell cytotoxicity against tumor cells

We evaluated the WT1-TCB*-mediated T-cell cytotoxicity by coculturing T cells from HDs with different tumor cell lines.

Flow cytometry measurements showed that WT1-TCB* elicited antibody-mediated T-cell cytotoxicity in cocultures with RMF peptide-pulsed T2 cells in a WT1-TCB and RMF peptide dose-dependent manner. No lysis was observed in cocultures with unpulsed T2 cells, HLA-A*02⁻ WT1⁺ HL-60 cells, or Ctrl-TCB (Figure 3A). Killing of HLA-A*02⁺ tumor cells expressing the RMF peptide from endogenous WT1 protein by WT1-TCB was assessed on a panel of cell lines by counting living target cells or analyzing distinct dead cell protease activity. The most potent killing was observed by using the HLA-A*02:07 cell line SKM-1; other tested HLA-A*02:01 AML cell lines, including OCI-AML3, were killed with lower potency, whereas non-HLA-A*02-expressing cells were not killed (Figure 3B; Table 2).

We also assessed the ability of WT1-TCB to activate and expand T cells by measuring upregulation of CD25 and proliferation of dye-labeled cells. Consistent with the results regarding tumor cell killing, T-cell activation and proliferation were strongest in the SKM-1 cell line and lower with OCI-AML3. In addition, T-cell activation and proliferation were consistently higher for CD8⁺ compared with CD4⁺ T cells (Figure 3C-D).

Finally, we assessed the killing of other HLA-A*02⁺ tumor cell lines that either express or do not express WT1 (based on mRNA values). The killing of non-AML cell lines was not as efficacious as the killing of SKM-1 cells, with 50% effective concentration (EC₅₀) values in most cases being >10-fold higher (Table 2).

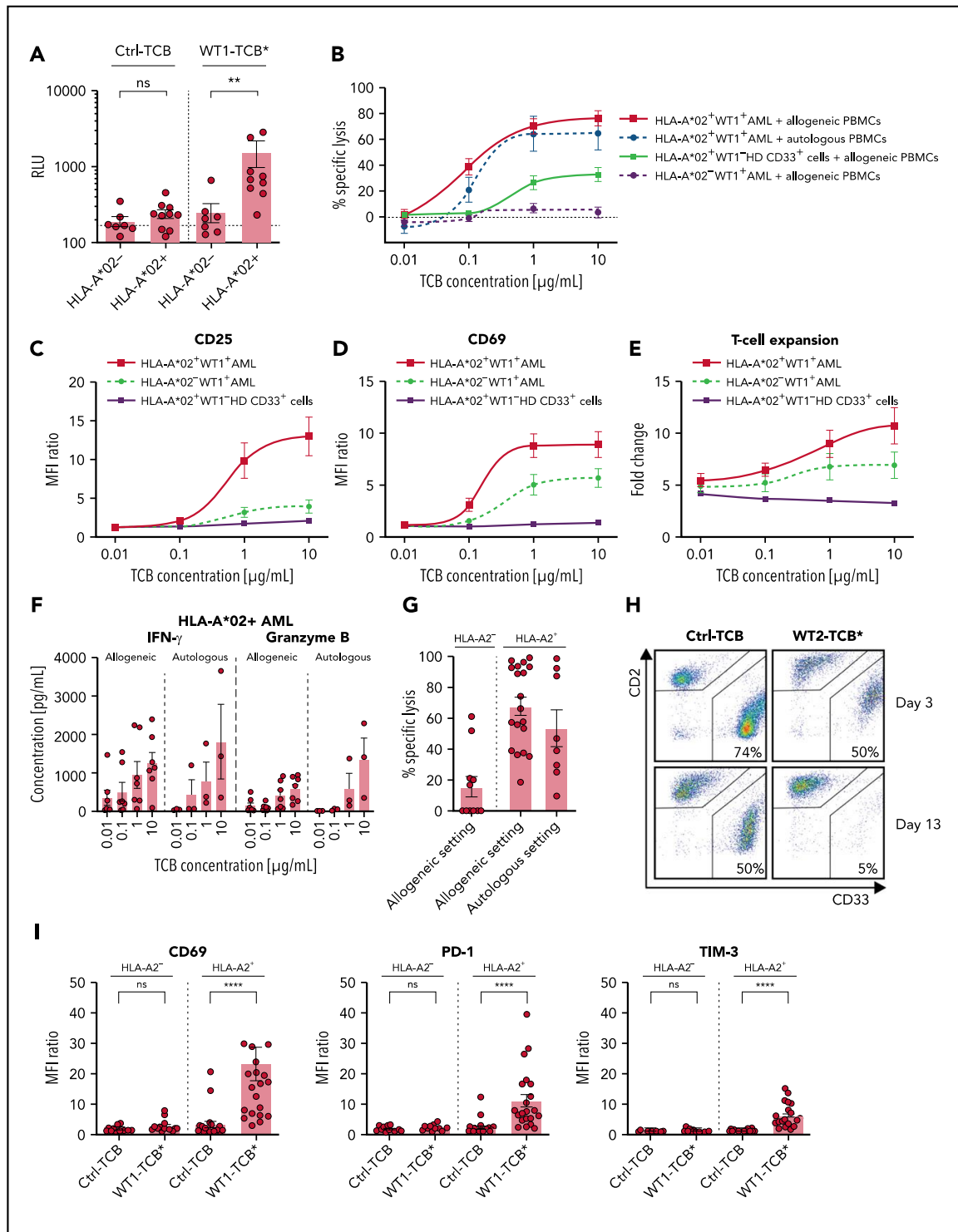


Figure 4. WT1-TCB-mediated T-cell cytotoxicity and T-cell activation. (A) Induction of downstream TCR signaling in NFAT Jurkat Reporter cells upon recognition of RMF peptide-MHC complexes on primary AML cells. Statistical analysis, Mann-Whitney U test ($n = 10$). (B) Dose-dependent specific lysis of primary HLA-A*02⁺ AML cells by allogeneic (black solid line; $n = 9$) or autologous (black dashed line; $n = 3$) PBMCs after 48 hours in short-term cytotoxicity assays with WT1-TCB. Controls: HLA-A*02⁺ WT1⁺ CD33⁺ HD cells (gray solid line; $n = 6$) and primary HLA-A*02⁺ AML cells (gray dashed line; $n = 10$). CD25 (C) and CD69 (D) expression on CD3⁺ T cells after 48 hours in

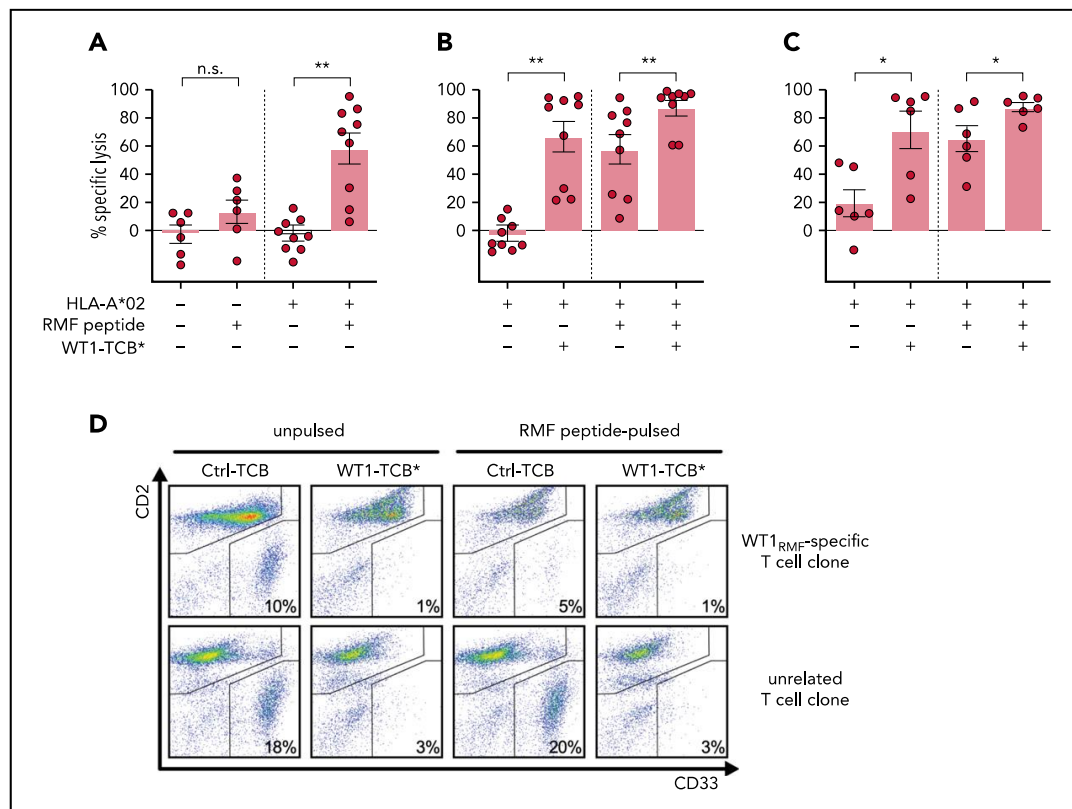


Figure 5. WT1-TCB* vs WT1-specific T cells. (A) Specific lysis of unpulsed and RMF peptide-pulsed primary AML cells from HLA-A*02⁻ and HLA-A*02⁺ patients by WT1_{RMF}-specific T-cell clone after 3 days in feeder layer-based long-term cocultures. Specific lysis was calculated by subtraction of unspecific lysis by HD T cells under the same conditions (n = 6-9). (B) Specific lysis of primary AML cells after 3 days in feeder layer-based long-term cocultures with WT1_{RMF}-specific T-cell clone and treatment with WT1-TCB* compared with HD T cells (n = 9). (C) Specific lysis of primary AML cells (all NPM1 wild-type) after 3 days in feeder layer-based long-term cocultures with WT1_{RMF}-specific T-cell clone and treatment with WT1-TCB*. Specific lysis was calculated by subtraction of unspecific lysis by a T-cell clone targeting an unrelated epitope of mutated NPM1 (n = 6). (D) Representative dot plots from flow cytometry analysis showing CD2 and CD33 expression in lysis experiments. Bars represent mean $\pm$ SEM. Statistical analysis: Mann-Whitney U test. *P < .05, **P < .01. n.s., not significant.

Induction of downstream TCR signaling in cocultures with primary AML cells

We monitored downstream TCR signaling by coculturing NFAT Luciferase Reporter Jurkat cells with primary AML cells in the presence of WT1-TCB* (10 nM). WT1-TCB* significantly induced downstream TCR signaling in cocultures with HLA-A*02⁺ primary AML cells but not with cells from HLA-A*02⁻ patients. No signal was observed if the nonspecific Ctrl-TCB was used (Figure 4A). Induction of TCR signaling was dose dependent, with an EC₅₀ of WT1-TCB* of 5.7 nM in HLA-A*02⁺ AML cells (supplemental Figure 5).

Lysis of primary AML cells is dose dependent

We assessed WT1-TCB-mediated cytotoxicity against primary AML cells by autologous or HD PBMCs in conjunction with various concentrations of WT1-TCB. WT1-TCB mediated the killing of

HLA-A*02⁺ AML cells in the allogeneic and autologous settings (mean $\pm$ SEM specific lysis after 48 hours with 10 μ g/mL TCB, 76.6 $\pm$ 5.9% and 64.7 $\pm$ 12.4%; n = 9 and 3, respectively). Lysis of AML cells was dose dependent, with an EC₅₀ value of $\sim$ 0.1 μ g/mL (0.5 nM) for both settings. Conversely, no lysis of HLA-A*02⁻ AML cells was observed, and only low levels of lysis in HD-derived HLA-A*02⁺ WT1⁻ myeloid cells were recorded (Figure 4B). Correspondingly, using HLA-A*02⁺ AML cells, we observed dose-dependent T-cell activation, expansion, interferon- γ secretion, and granzyme B secretion (Figure 4C-F).

T-cell cytotoxicity against primary AML cells ex vivo

To further support the potency of WT1-TCB* against primary AML cells, we cocultured primary AML cells with HD or autologous

Figure 4 (continued) killing assays with allogeneic PBMCs and primary HLA-A*02⁺ AML cells (black solid line; n = 9), primary HLA-A*02⁻ AML cells (gray dashed line; n = 10), or HLA-A*02⁺ WT1⁻ CD33⁺ HD cells (gray solid line; n = 6). (E) T-cell proliferation after 96 hours in killing assays with allogeneic PBMCs and primary HLA-A*02⁺ AML cells (black solid line; n = 8), primary HLA-A*02⁻ AML cells (gray dashed line; n = 7), or HLA-A*02⁺ WT1⁻ CD33⁺ HD cells (gray solid line; n = 3). (F) Secretion of interferon- γ (IFN- γ) and granzyme B in allogeneic and autologous settings after 72 hours in a short-term cytotoxicity assay with primary HLA-A*02 AML cells. (G) Specific lysis of primary AML cells by allogeneic T cells (left: HLA-A*02⁻ AML, n = 11; center: HLA-A*02⁺ AML, n = 19) or autologous T cells (right: HLA-A*02⁺ AML, n = 8) after 10 to 13 days in long-term cocultures and treatment with WT1-TCB*. (H) Representative dot plots from flow cytometry analysis showing CD2 and CD33 expression in long-term coculture samples. (I) Upregulation of activation and surrogate exhaustion markers on CD3⁺ T cells on days 3 to 4 in long-term cocultures with primary HLA-A*02⁺ and HLA-A*02⁻ AML cells (n = 14-22). Bars represent the mean $\pm$ SEM. Statistical analysis: Kruskal-Wallis test and subsequent Dunn's test. **P < .01, ****P < .0001. MFI, mean fluorescence intensity; n.s., not significant; RLU, relative luminescence units.

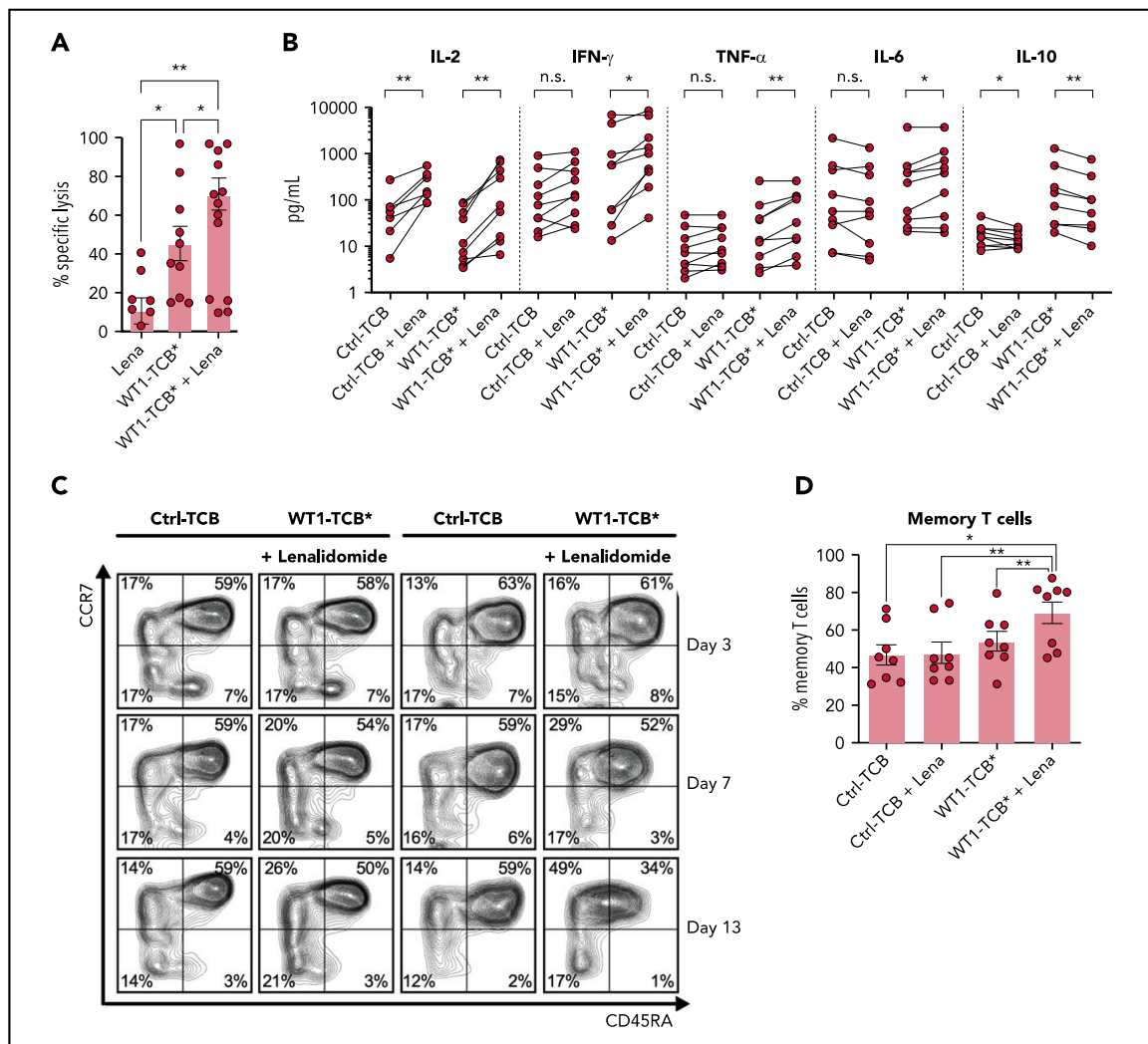


Figure 6. WT1-TCB*-mediated cytotoxicity in combination with lenalidomide (Lena). (A) WT1-TCB*-mediated specific lysis of primary AML cells significantly increased in combination with Lena after 4 days of coculture with HD T cells ($n = 9-10$). Lena was added at a concentration of $10 \mu\text{M}$ together with 10 nM WT1-TCB* in ex vivo cytotoxicity assays with primary AML cells and HD T cells at an E:T ratio of 1:2 for 4 days. (B) Levels of proinflammatory cytokines increase after combination of WT1-TCB* with Lena and levels of the anti-inflammatory interleukin 10 (IL-10) decrease ($n = 9$). (C) Representative example of CD45RA and CCR7 expression analysis. (D) Percentages of central memory T cells after 7 to 10 days of treatment ($n = 8$). Bars represent the mean $\pm$ SEM. Statistical analysis: Wilcoxon matched-pairs signed-rank test. * $P < .05$, ** $P < .01$. n.s., not significant; TNF- α , tumor necrosis factor α .

T cells in a feeder layer-based long-term culture system. This action enabled us to assess the specific lysis of AML cells over a therapeutically relevant time frame. WT1-TCB* induced specific cytotoxicity of primary HLA-A*02⁺ AML cells upon addition of allogeneic T cells from HDs (mean $\pm$ SEM specific lysis, $67 \pm 6\%$ after 13-14 days; $n = 19$). WT1-TCB* also mediated the killing of primary AML cells in an autologous setting (mean $\pm$ SEM specific lysis, $54 \pm 12\%$ after 11-14 days; $n = 8$). HLA-A*02⁻ AML cells were not significantly killed (Figure 4G-H). Corresponding upregulation of T-cell activation and checkpoint molecules was only observed with HLA-A*02⁺ AML cells (mean $\pm$ SEM fluorescence intensity fold change: CD69, 9.3 ± 1.5 ; PD-1, 5.1 ± 0.7 ; TIM-3, 4.7 ± 0.6 ; $n = 22$) (Figure 4I).

Lysis of healthy, bone marrow-derived CD34⁺ cells is low

To evaluate on-target off-leukemia WT1-TCB*-mediated cytotoxicity against healthy HLA-A*02⁺ bone marrow cells, we prepared cocultures with autologous T cells and varying concentrations of WT1-TCB. Specific lysis of CD34⁺ cells was observed in a dose-dependent manner at 48 hours, and the EC₅₀ value was $0.15 \mu\text{g/mL}$. The effect was minimal at $0.1 \mu\text{g/mL}$ (mean $\pm$ SEM specific lysis, $8.3 \pm 3.3\%$; $n = 8$) (supplemental Figure 6A) and increased to $34.2 \pm 9.0\%$ at $1 \mu\text{g/mL}$. Expression of the activation marker CD69 was low after 24 hours in the presence of WT1-TCB at concentrations equal to the EC₅₀ value or lower (mean $\pm$ SEM fluorescence

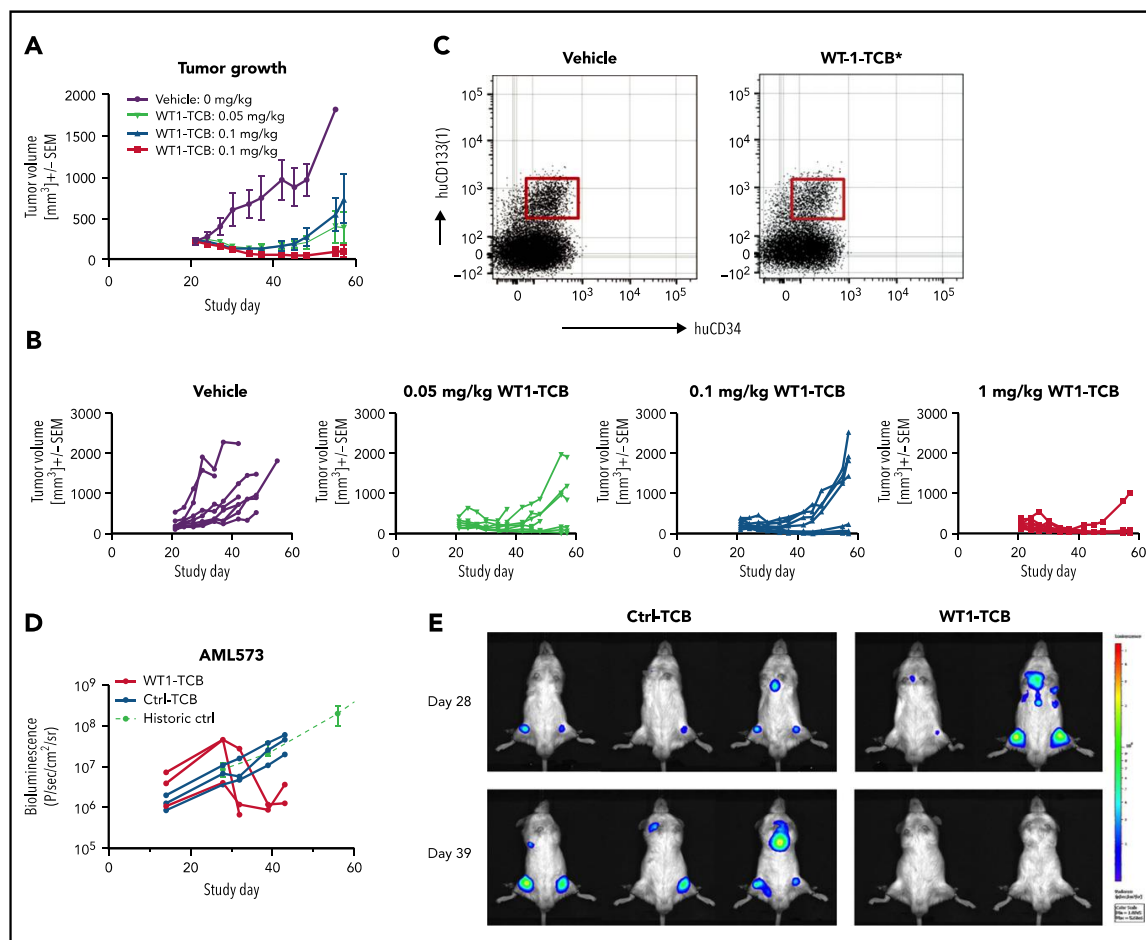


Figure 7. Antitumor efficacy of WT1-TCB in humanized mouse models. (A-B) Tumor growth curves of the SKM-1 model in hSC-NSG mice treated once weekly with vehicle control or 0.05, 0.1, or 1 mg/kg of WT1-TCB. (C) Cytometric analysis of the CD34⁺ CD133⁺ stem cell population in the bone marrow of SKM-1 tumor-bearing hSC-NSG mice engrafted with cord blood from HLA-A*02⁺ donors after 54 days of treatment with WT1-TCB*. (D-E) Tumor growth in AML PDX mice (AML573) treated once weekly with 3 mg/kg WT1-TCB or Ctrl-TCB beginning on day 30, assessed by in vivo imaging. One WT1-TCB-treated mouse died during imaging on day 32.

intensity ratio, 62.4 ± 39.0 ; $n = 8$; $0.1 \mu\text{g/mL}$ TCB). Pulsing with the RMF peptide exacerbated the lysis of CD34⁺ cells at all concentrations and increased T-cell activation (supplemental Figure 6B). To further validate the absence of negative effects on healthy hematopoiesis, we performed colony-forming unit assays with HD bone marrow cells. No difference in colony formation was observed for samples treated for 48 hours with either 0.1 or $2 \mu\text{g/mL}$ WT1-TCB compared with Ctrl-TCB ($n = 3$ and 1 , respectively) (supplemental Figure 6C).

The cytotoxicity against AML cells is higher compared with a WT1_{RMF}-specific T-cell clone

We compared the cytotoxicity of T cells mediated by WT1-TCB* vs that of a WT1_{RMF}-specific T-cell clone isolated from HDs by streptamer technology.⁵¹ A WT1_{RMF}-specific T-cell clone was cocultured with primary AML cells for 3 days in a feeder layer-based long-term culture system with or without

WT1-TCB*. To determine the extent of nonspecific background lysis, we prepared additional control samples with HD T cells or an unrelated T-cell clone without addition of WT1-TCB*. Specific lysis was calculated relative to these controls.

No specific lysis of HLA-A*02⁺ primary AML cells by WT1_{RMF}-specific T-cell clone was observed. Surprisingly, specific lysis against HLA-A*02⁺ primary AML cells was only observed after we loaded target cells with RMF peptide (mean $\pm$ SEM specific lysis, $-3 \pm 4\%$ [unpulsed]; $58 \pm 11\%$ [pulsed]; $n = 9$) (Figure 5A). Conversely, addition of WT1-TCB* induced specific lysis of HLA-A*02⁺ primary AML cells also without RMF peptide loading (mean $\pm$ SEM specific lysis, $67 \pm 11\%$ [unpulsed], $88 \pm 5\%$ [pulsed]; $n = 9$) (Figure 5B). Similar results were obtained by using an unrelated T-cell clone as a reference instead of HD T cells (mean $\pm$ SEM specific lysis, $71 \pm 13\%$ [unpulsed]; $88 \pm 3\%$ [pulsed]; $n = 6$) (Figure 5C-D).

Lenalidomide enhances cytotoxicity against primary AML cells

The addition of lenalidomide (AbMole BioScience, Brussels, Belgium) enhanced WT1-TCB*-mediated T-cell cytotoxicity in cocultures of primary AML cells and HD T cells (mean $\pm$ SEM specific lysis on days 3–4, $45.4 \pm 9.0\%$ vs $70.8 \pm 8.3\%$; $P = .015$; $n = 9$ –10) (Figure 6A). Similarly, addition of lenalidomide with WT1-TCB* increased the secretion of pro-inflammatory cytokines and reduced levels of IL-10 (Figure 6B). Furthermore, combination with lenalidomide promoted the differentiation of naive T cells toward a memory phenotype characterized by downregulation of CD45RA (Figure 6C–D).

Tumor growth inhibition in in vivo mouse models

We assessed the pharmacokinetic parameters of WT1-TCB in a human FcRn transgenic mouse model and observed a mean terminal elimination half-life of 10.7 days (supplemental Figure 7). Next, we evaluated the antitumor activity of WT1-TCB in a SKM-1 xenograft model in NSG mice engrafted with human stem cells (hSC-NSG). We tested doses of 0.05, 0.1, and 1 mg/kg WT1-TCB* administered once weekly and observed a dose-dependent efficacy (Figure 7A–B).

Notably, WT1-TCB* treatment did not result in adverse effects on CD34⁺ hSCs when tested in hSC-NSG mice engrafted with HLA-A*02⁺ stem cells (Figure 7C). Although WT1-TCB* mediated potent antitumor efficacy at concentrations of 1 and 10 mg/kg, it did not induce signs of weight loss or depletion of CD34⁺ stem cells in the bone marrow of hSC-NSG mice at day 57. This supports the in vivo specificity of WT1-TCB* (based on the 11D06 binder) in a system expressing HLA-A*02 on hSCs and in the derived cell lineages in blood, bone marrow, and lymphoid tissues.

In addition, antitumor activity of WT1-TCB was seen in an AML PDX model. We observed a reduction of the tumor burden after weekly administration of WT1-TCB compared with Ctrl-TCB, as assessed by in vivo imaging (Figure 7D–E). In a second experiment using a different PDX model, WT1-TCB-treated mice had a reduced number of human CD45⁺CD33⁺ cells in spleen and bone marrow compared with Ctrl-TCB-treated mice after 10 days of treatment (supplemental Figure 8).

Discussion

The current study evaluated a novel 2 + 1 TCB antibody construct that targets an epitope of the intracellular antigen WT1 in the context of HLA-A*02. WT1 is overexpressed in a number of tumor entities, including AML, and is thus a universal target antigen in immunotherapeutic strategies. It has been extensively described as a leukemia-associated antigen with a disease-restricted expression profile.^{22,23} Analysis of WT1 expression in bone marrow sampled from patients with AML during the course of the disease revealed a strong correlation between the percentage of leukemic blasts in the bone marrow and the number of copies of WT1 mRNA. Accordingly, WT1 has been used as a prognostic marker for assessing minimal residual disease.^{49,52–55} The recurrence of WT1 expression at time of relapse supports the hypothesis that WT1 plays a pathophysiological role in leukemic cell survival. Several in vitro studies have shown the modulation of leukemic

cell growth by upregulation and downregulation of WT1; however, the precise role of WT1 in leukemia remains elusive.^{56–58}

WT1-TCB was designed to specifically recognize the WT1-derived RMF peptide in the context of HLA-A*02. Alanine scanning and a novel peptide MHC array method showed that 6 of the 9 residues of the RMF peptide are important for binding WT1-TCB. A TCR-mimicking antibody targeting the same epitope, named ESK1, has been reported.^{34,35,59,60} However, this antibody has not entered clinical development, most likely owing to off-target binding, as epitope binding of ESK1 relies almost exclusively on peptide residue Arg1, allowing recognition of several other self peptides.³⁷ X-ray crystallography of the RMF-pMHC complex bound to the WT1-TCB Fab fragment further revealed a considerably higher contribution of the RMF peptide to the overall contact surface area compared with ESK1.^{37,50}

The confirmation that the RMF peptide is presented as part of a pMHC complex relies on cellular data, but so far the RMF peptide could not be extracted from cancer cells.^{61,62} By developing a novel mass spectrometry-based approach, we provide qualitative evidence for endogenous RMF peptide presentation using SKM-1 cells. Further optimization, including immunoprecipitation by WT1-TCB, allowed the first quantification of endogenous RMF peptide presentation on an AML patient sample. This provides evidence that HLA-A*02/RMF-pMHC complexes are presented on primary AML cells at a reasonable density and also shows the selectivity of WT1-TCB toward this complex.

In short-term cytotoxicity experiments, we observed the dose-dependent WT1-TCB-mediated lysis of primary AML cells, underlining that endogenous RMF peptide is presented. Using our pre-established ex vivo long-term culture system for AML cells,⁴⁷ we observed WT1-TCB-mediated cytotoxicity against primary AML cells by allogeneic and autologous T cells over a therapeutically relevant time frame. Interestingly, cytotoxicity was higher in the allogeneic setting, which supports previous findings on T-cell dysfunction in AML.^{63,64} Importantly, our in vitro findings were supported by our in vivo demonstration of tumor growth inhibition by WT1-TCB using different AML PDX models and a SKM-1-xenograft model. In the latter model, some animals exhibited tumor progression after initial tumor control. In an RNA-sequencing analysis of the possible escape mechanisms, we did not observe downregulation of WT1, in contrast to HLA-A*02; however, this was not the case in all samples.

Because low frequency of WT1 RNA-expressing hSCs has been reported,²¹ we evaluated WT1-TCB-mediated on-target, off-leukemia cytotoxicity against HD CD34⁺ bone marrow cells. We observed only low WT1-TCB-mediated target cell lysis and T-cell activation and did not observe killing of CD34⁺ hSCs in the humanized NSG mouse model. This indicates that the RMF peptide is not presented on the surface of HLA-A*02⁺ healthy CD34⁺ cells to an extent that WT1-TCB initiates T-cell activation. This observation is consistent with results from a clinical trial with WT1_{RMF}-TCR transgenic T cells that reported no corresponding adverse events.²⁹

Target antigen recognition by WT1-TCB mimics the physiological MHC-TCR interaction but differs by bivalent vs monovalent binding. WT1-TCB mediated the lysis of unpulsed and RMF peptide-pulsed primary AML cells, whereas a WT1_{RMF}-specific

T-cell clone mediated the killing of only peptide-pulsed target cells. One explanation for these findings is that the 2 + 1 molecular format confers higher avidity (double-digit nanomolar affinity for the pMHC complex as opposed to the micromolar affinity that is typical for TCRs), thus enabling target cell lysis even if only low numbers of pMHC complexes are presented. The TCR affinities for nonmutated self peptides such as WT1 have been shown to be lower than those for foreign antigens, which also impedes the generation of high-affinity TCR transgenic T cells for adoptive transfer. Nevertheless, the adoptive transfer of WT1-specific T cells or TCR transgenic T cells has exhibited antileukemic activity in clinical trials.^{27,29} Recently, WT1^{RMF}-TCR transgenic T cells were reported to prevent AML relapse in a small cohort of patients after hematopoietic cell transplantation, providing proof-of-concept for targeting WT1.²⁹

To further augment cytotoxicity, we evaluated the combination of WT1-TCB with lenalidomide, a drug that has been shown to have a stimulatory effect on T cells.^{65,66} We observed a significant increase of specific cytotoxicity and levels of pro-inflammatory cytokines, as well as a shift in the T-cell compartment toward the memory phenotype. The latter is likely a secondary effect due to increased secretion of interleukin-2, resulting in increased T-cell proliferation and activation. Our observations are consistent with results from other trials of lenalidomide combinations, underlining the rationale behind this combinatorial approach.⁶⁷⁻⁶⁹

In summary, we showed that WT1-TCB facilitates potent in vitro, ex vivo, and in vivo killing of AML cell lines and primary AML cells. These properties led to the initiation of an entry-into-human clinical trial (#NCT04580121) for the treatment of relapsed/refractory AML.

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Authorship

Contribution: C. Klein, W.X., P.U., and M. Subklewe designed the study and supervised the project; C.A., C. Klein, V.P., G.H., M. Subklewe, M.v.B.-B., and F.S.L. wrote the manuscript; C.A., G.H., V.P., L.J.H., A.A., J.C., K.H., P.E.R., A.S., J.S., E.L., A.D., D.O.-F., J.B., A.B., C.G., A.C., V.L., and J.P. performed the experiments and analyzed and/or interpreted the data; M. Subklewe, W.X., C. Krupka, M.R., F.S.L., L.V., B.V., I.J., E.M.-B., and A.-C.W. were involved in research design and data interpretation; N.K. and K.S. performed the real-time qPCR analyses; A.T. and M. Schmitz generated and provided the WT1-specific T-cell clones; and A.B. and J.B. determined and analyzed the crystal structure.

Conflict-of-interest disclosure: C. Klein, L.J.H., J.S., V.P., A.S., J.C., and P.U. are, and W.X. was, employed at the Roche Innovation Center Zurich. F.S.L., A.C., A.B., and C.G. are, and C. Krupka was, employed at the Roche Innovation Center Munich, and E.M.-B., A.-C.W., A.D., J.B. A.A., E.L., and D.O.-F. are employed at the Roche Innovation Center Basel. V.L. and J.P. were employed at Roche NimbleGen. These authors also declare patents and ownership of Roche stock. M. Subklewe has received industry research support from Amgen, Gilead, Miltenyi Biotec, MorphoSys, Roche, and Seattle Genetics; has served as a consultant/advisor to Amgen, BMS, Celgene, Gilead, Pfizer, Novartis, and Roche; sits on the advisory boards of Amgen, Celgene, Gilead, Janssen, Novartis, Pfizer, and Seattle Genetics; and serves on the speakers bureau at Amgen, Celgene, Gilead, Janssen, and Pfizer. The remaining authors declare no competing financial interests.

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Footnotes

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There is a *Blood* Commentary on this article in this issue.

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1 Supplemental Data

2 Supplemental Methods

3 Generation of antibodies specific for WT1_{RMF} peptide HLA-A*02 complex by phage display

4 Phage display was performed using a library displaying monomeric Fab fragments on M13
5 phage, consisting of six heavy chain (VH1_46, VH1_69, VH3_15, VH3_23, VH4_59, VH5_1) and
6 five light chain antibody frameworks (Vk1_5, Vk3_20, Vk2D_28, V13_19, Vk4_1,) each being
7 randomized in the CDR3 region. After four rounds of phage display, individual clones were
8 bacterially expressed and supernatants were screened for specific binding to HLA-
9 A*02/WT1_{RMF} by ELISA and in a kinetic screening experiment by surface plasmon resonance
10 analysis using Proteon XPR36 biosensor (BioRad, Hercules, CA, USA). Fabs with the highest
11 affinity constants and specific binding were identified and the corresponding phagemids were
12 sequenced. In total, 34 clones were found that bind specifically HLA-A*02/WT1_{RMF}. Fab coding
13 sequences were cloned into human IgG1-expressing DNA formats with PG LALA mutation on
14 Fc, rendering it inactive for complement-dependent cytotoxicity. IgGs were purified from
15 transiently transfected HEK 293 EBNA cells.

16 Comparison of the killing efficacy of WT1-TCB CD3 variants *in vitro*

17 HD T cells were isolated from PBMCs using human Pan T cell isolation kit (Miltenyi Biotec,
18 Bergisch Gladbach, Germany) according to the manufacturer's instructions. Two WT1-TCBs
19 with different CD3 clones (CH2527 and V9) were tested at various concentrations together
20 with enriched T cells and target cells at an E:T ratio of 5:1. Two different target cells were
21 used: WT1⁺ SKM1 cells and T2 cells, which were first pulsed with various concentrations of
22 RMF peptide [10^{-5} , 10^{-6} , 10^{-7} , and 10^{-9} M (GenScript, Piscataway, NJ, USA)] for 2 h at 37°C.

23 Target cell killing was evaluated after 20 h using a Cytotox Glo (Promega, Madison, WI, USA)
24 readout assay according to manufacturer's protocol. Luminescence was measured using a
25 Perkin Elmer plate reader (PerkinElmer, Waltham, MA, USA).

26 ***In vivo* comparison of the anti-tumor activity of WT1-TCB CD3 variants**

27 Mice were maintained under specific-pathogen-free condition with daily cycles of 12 h
28 light/12 h darkness according to committed guidelines (GV-Solas; Felasa; TierschG). The
29 experimental study protocol was reviewed and approved by local government authorities
30 (2011-128). Upon arrival, animals were maintained for one week to become accustomed to
31 their new environment and for observation. Continuous health monitoring was carried out on
32 a regular basis. For humanization, NSG female mice (purchased from Charles River
33 Laboratories), aged 4–5 weeks at start of the experiments, were injected with busulfan
34 (15mg/kg) followed 24 h later by injection of 100,000 human CD34⁺ T cells isolated from HLA-
35 A*02:01 cord blood (StemCell Technologies). Fourteen to 16 weeks after stem cell injection,
36 humanized mice were screened for human T-cell frequencies in blood by flow cytometry. Only
37 humanized mice that exhibited a humanization rate greater than 20% (i.e., 20% circulating
38 human immune cells within all leucocytes) were used for the experiment. Mice were injected
39 sub-cutaneously with 10x10⁶ SKM-1 tumor cells and after 7 days, when tumor size reached an
40 average volume of 150 mm³, they were randomized into three different groups and started
41 receiving weekly injections of WT1-TCB molecule variants with CH2527 or V9 CD3 at the dose
42 of 1 mg/kg or phosphate-buffered saline as a vehicle control for 6 weeks. Animals were
43 controlled daily for clinical symptoms and detection of adverse events. Tumor volume was
44 measured by caliper every second day. Study exclusion criteria for the animals were described
45 and approved in the corresponding license ZH193/2014. Termination criteria for animals were

46 visible sickness: scruffy fur, arched back, breathing problems, impaired locomotion, tumor
47 volume > 3000 mm³.

48 **Alanine scan**

49 To evaluate which amino acids of the RMF peptide directly interact with the HLA-
50 A*02/WT1_{RMF} binder (termed 11D06) of the *WT1-TCB*, 10 different single amino acid variants
51 of RMF peptide (R1Y, R1A, M2A, F3A, P4A, N5A, A6G, P7A, Y8A and L9A) were generated
52 (GenScript, Piscataway, NJ, USA). T2 cells pulsed for 2 h with 10⁻⁵ M RMF peptide variant were
53 incubated with Jurkat NFAT reporter cells at a ratio of 1:5 and various concentrations of WT1-
54 TCB. After 1 h, Jurkat NFAT activation was evaluated by ONE-Glo Luciferase Assay System
55 (Promega, Madison, WI, USA) according to the manufacturer's protocol. Luminescence was
56 measured using a Perkin Elmer plate reader.

57 **Peptide-MHC array**

58 Peptide synthesis was accomplished using light-directed array synthesis in a maskless array
59 synthesizer (MAS) using an amino-functionalized surface as previously reported.¹ Peptide
60 library included a complete substitution and deletion variants at each position of the RMF
61 nonamer peptide. Each peptide was synthesized in five copies. After synthesis, arrays were
62 stored without deprotection in a desiccated box at room temperature.

63 HLA-A*02:01 heavy chain was purified from insoluble inclusion bodies from *E. coli*. Soluble
64 HLA-A*02:01 heavy chain was prepared by dissolving the washed inclusion body pellet in
65 20 mM Tris-HCl, pH8, 8 M urea, 0.5 mM EDTA, 0.5 mM PMSF, 100 mM β-mercaptoethanol at
66 4°C overnight. Insoluble material was removed by centrifugation at 4,500 *g* for 10 min at 4°C.
67 Human β-2-microglobulin (β2m) was purchased from Scripps Laboratories (San Diego, CA,
68 USA).

69 To prepare HLA-A*02–b2m complex, b2m and HLA-A*02 were quickly diluted at a ratio of 6:1
70 (w/w) in 0.1X buffer pH 10 (di-sodium tetraborate/sodium hydroxide, Sigma) at room
71 temperature. The sample was immediately concentrated in an Amicon Ultra 0.5 ml 3K filter
72 (Millipore) at 14,000 *g* for 15 min. The retained solution was filtered through a 0.45 µm micro
73 spin filter (Fisher Scientific) at 4,000 *g* for 2 min. Samples with HLA-A*02–b2m complex and
74 antibody were prepared by mixing of assembled HLA-A*02–b2m complex and 11D06
75 antibody. The peptide array was deprotected and briefly washed in water. Samples were
76 loaded on separate sub-arrays and incubated at 4°C overnight. The array was subsequently
77 washed and stained with Cy3 goat anti-human IgG antibody (Sigma) for 1 h at 4°C, followed
78 by staining with Alexa 647 mouse HLA class I MEM-123 antibody (Novus) for 1 h at 4°C. The
79 array was scanned at 532 nm (IgG detection) and at 635 nm (HLA detection) at 2 µm resolution
80 using an InnoScan 1100 AL scanner (Innopsys, Chicago, IL, USA). Data pre-processing,
81 normalization, statistical tests and amino acid substitution plots were performed using the
82 language R.

83 **Crystallization, data collection and structure determination of the Fab 11D06 HLA-A02/RMF–**
84 **pMHCI complex**

85 The complex was prepared by mixing equimolar amounts of the Fab fragment of antibody
86 11D06 with HLA-A*02/WT1_{RMF}–pMHC. After incubation for 4 h at 21°C, the protein complex
87 was concentrated to 20 mg/mL. Initial crystallization trials were performed in sitting-drop
88 vapor diffusion setups at 21°C. Within four days, crystals materialized from 0.1 M Tris (pH 8.0)
89 containing 20% PEG 4000. Crystals were harvested directly from the screening plate without
90 any further optimization step.

91 For data collection, crystals were flash cooled at 100 K in crystallization solution supplemented
92 with 15% glycerol. X-ray diffraction data were collected at a wavelength of 1.0000 Å using a
93 PILATUS 6M detector at the beamline X10SA of the Swiss Light Source (Villigen, Switzerland).
94 Data were processed with XDS³ and scaled with SADABS (Bruker). The crystals of the complex
95 belong to space group P2₁ with cell axes of $a = 54.11$ Å, $b = 67.00$ Å, $c = 139.36$ Å with $\beta =$
96 90.57° and diffracted to a resolution of 2.64 Å. The structure was determined by molecular
97 replacement with PHASER 2.6.1⁴ using the coordinates of an in-house Fab and MHC complex
98 structure as a search model. Difference electron density was used to place the RMF peptide
99 and to change amino acids according to the sequence differences. Structures were refined
100 with programs from the CCP4 suite⁵ and BUSTER 2.11.7.⁶ Manual rebuilding was done with
101 COOT.⁷ Data collection and refinement statistics are summarized in supplemental Table 1. All
102 graphical presentations were prepared with PYMOL (The Pymol Molecular Graphics System,
103 Version 1.7.4. Schrödinger, LLC.).

104 **Mass spectrometry based quantification of WT1_{RMF} peptide**

105 An immune-enrichment-based, MHC-I-associated peptide proteomics (MAPPs) experiment
106 was conducted to quantify the WT1_{RMF} peptide (RMFPNAPYL) presented on MHC-I receptors
107 at the surface of cells. WT1 peptide-pulsed T2 cells (A*02:01, B*51:01, Cw*01:02) or SKM-1
108 cells (A*02:07, A*33:03; B*40:01, B*59:01, C*01:02, C*03:04) were lysed for 1 h at 4 °C in lysis
109 buffer (20 mM Tris-HCl pH 7.8, 5 mM MgCl₂, 1% Triton X-100, 1X protease inhibitor).
110 Solubilized peptide–MHC-I complexes were then captured with a biotinylated pan-MHC-I
111 antibody (clone W6/32) or a biotinylated pan-HLA-A*02 antibody (clone BB7.2; both
112 Biolegend). Immune complexes were enriched on an Agilent BRAVO automated liquid
113 handling platform (Agilent Technologies, Santa Clara, CA, USA) equipped with 5 µL

streptavidin cartridges (Agilent). After multiple washing, peptide–MHC-I complexes were eluted in 0.1% (v/v) trifluoroacetic acid supplemented with 25 fmol of the internal standard ($^{13}\text{C}_6$, $^{15}\text{N}_4$ -RMFPNAPYL; Cambridge Research Biochemicals). Oxidation of the MHC-I peptides was performed using 10% (v/v) H_2O_2 (final concentration) overnight at 4°C. The solutions were further purified using a 5 μL Reverse Phase S cartridge (Agilent) and MHC-I peptides were eluted in 40% (v/v) methanol, 20% (v/v) acetonitrile, 5% (v/v) formic acid solution, and dried in a speed-vac concentrator.

For analysis of primary AML cells, samples were selected for high viability, high expression of WT1 and HLA-A*02, and expanded for 3 days on MS5 feeder cells. The workflow was modified as follows: 6×10^7 AML cells were lysed for 1 h at 4°C in lysis buffer (50 mM HEPES pH 7, 1% NP-40, 1x EDTA-free protease inhibitor) and the solubilized peptide-MHC-I complexes were captured with the biotinylated WT1-TCB. Immune complexes were enriched on Pierce High Capacity Streptavidin Agarose beads and eluted in 5% (v/v) formic acid, supplemented with 12.5 fmol of the internal standard. Oxidation of the MHC-I peptides was performed using 20% (v/v) H_2O_2 (final concentration) overnight at 4°C. The solution was purified on Sep-Pak Vac 1cc (50 mg) C_{18} cartridges (Waters), MHC-I peptides were eluted in 50% (v/v) acetonitrile and lyophilized for 3 h.

Samples were analyzed on a Q Exactive HFX Orbitrap mass spectrometer equipped with an Ultimate 3000 RSLCnano UPLC (both Thermo Fisher Scientific) and a nano-electrospray interface using a standard operating procedure.

Peptide identifications were performed using the PEAKS Studio software version 8.5 (Bioinformatics Solutions) against the Uniprot database (<http://www.uniprot.org>, release 2018_02) filtered for the taxonomy “human” (71803 TrEMBL and SwissProt entries). Data were searched with a mass tolerance of ± 5 ppm for parent ions and 0.025 Da for fragment

ions considering no cleavage specificity and methionine oxidation, asparagine and glutamine de-amidation, and pyroglutamic acid as differential modifications. Peptide identifications were filtered at a false discovery rate (FDR) of $\leq 1\%$. MHC-I peptide quantification using PRM data was carried out using Skyline² version 4.1 using the heavy-labeled peptide standard to normalize for response.

Copy number of RMF peptide was calculated according to the following formula:

$$\text{Copy/cell} = \frac{c(\text{internal standard})}{N_A \times \text{cell number}} \times \text{ratio} \left(\frac{\text{endogeneous peptide}}{\text{internal standard}} \right)$$

The amount of internal standard analyzed on column was 10 fmol, N_A is the Avogadro number (6.022×10^{23}), cell number was 5×10^6 , and "ratio" represents the signal intensity ratio of endogenous RMFPNAPYL versus $^{13}\text{C}_6$, $^{15}\text{N}_4$ -labeled RMFPNAPYL internal standard. Based on the fragmentation of the peptide in the mass spectrometer, quantification was based on the most abundant transition (b_6), whereas specificity was estimated on the internal signal ratio (endogenous/internal standard) obtained from the four most-abundant transitions (a_6 , b_6 , b_5 , and y_3).

Off-leukemia cytotoxicity assay utilizing healthy bone marrow cells

CD34⁺ cells were isolated from HLA-A*02⁺ HD bone marrow using the human CD34 MicroBead Kit (Miltenyi Biotec) and co-cultured for 2 days with allogenic or autologous T cells isolated from HDs at an E:T ratio of 1:1 and various concentrations of WT1-TCB. As control, target cells were pulsed with 10 μM RMF peptide (JPT, Berlin, Germany). Mean specific lysis and expression of CD69 on T cells was measured by flow cytometry. T-cell functionality was validated in control experiments with SKM-1 cells.

159 Colony-forming unit assay with healthy bone marrow cells

160 HLA-A*02⁺ HD bone marrow cells were cultured for two days with 0.1 or 2 µg/mL WT1-TCB or
161 Ctrl-TCB. This was followed by isolation of CD34⁺ cells using the human CD34 MicroBead Kit
162 (Miltenyi Biotec). Cell numbers were determined only for Ctrl-TCB-treated samples. For
163 subsequent colony-forming unit assays, 500 CD34⁺ cells were cultured in Methocult H4434
164 Classic medium (Stemcell, Vancouver, Canada) for 14 days. Equal cell volumes were used for
165 WT1-TCB-treated samples, ignoring the potential reduction in CD34⁺ cell numbers.
166 Experiments were performed in technical duplicate.

167 Pharmacokinetic assessment of WT1-TCB

168 Pharmacokinetics (PK) assessment was conducted in a human FcRn (hFcRn) transgenic (Tg32
169 homozygous) mouse model treated with 1 mg/kg of WT1-TCB by *i.v.* bolus administration.
170 Blood, processed to serum, was serially sampled from three animals at 5 min, then 7, 24, 48,
171 72, 168, 240, and 504 h post-dose. Drug concentrations were measured in mouse serum
172 samples using an enzyme-linked immunosorbent assay (ELISA). Noncompartmental analysis
173 was performed on the individual PK profiles to derive the mean terminal elimination half-life.

174 Antitumor efficacy of WT1-TCB in an AML PDX mouse model

175 NSG mice were engrafted *i.v.* with 2x10⁶ cells of an HLA-A*02⁺ AML patient-derived xenograft
176 (AML-PDX), followed by *i.v.* injection of 1x10⁶ HD T cells after 45 days. Mice were randomized
177 into two groups and received injections of either WT1-TCB or Ctrl-TCB every 5 days at a dose
178 of 3 mg/kg. Mice were sacrificed after 10 days treatment and analyzed for frequency of human
179 CD45⁺CD33⁺ cells in spleen and bone marrow.

180

181 **Supplemental Results**

182 **Targeted quantification of HLA-A*02 presented WT1_{RMF} peptide using immunoprecipitation** 183 **combined with mass spectrometry**

184 Whereas cellular data imply the presentation of the WT1-derived RMF peptide as part of a
185 peptide–MHC complex, the RMF peptide itself could not be directly extracted from cancer
186 cells. To this end, we developed an analytical method capable of detecting and quantifying
187 the presence of the RMF peptide presented on MHC-I immune complexes.

188 In this method, the cells of interest were first lysed and the peptide–MHC-I complexes
189 immunoprecipitated using either an anti-pan-MHC-I or a selective anti-HLA-A*02. MHC-I
190 peptides were separated from their cognate receptors using reverse-phase material and
191 eluted using strongly acidic conditions. A pre-defined amount of an internal standard
192 (isotopically labeled RMF peptide) was spiked into the eluate, to establish the expected
193 chromatographic retention time window and to calibrate the signal corresponding to the
194 endogenous RMF peptide. Peptide analysis and quantification were performed using nano-
195 reverse-phase chromatography electrospray tandem mass spectrometry (supplemental
196 Figure 4A).

197 During method development, we realized that the Met2 of the RMF peptide was readily
198 oxidized and co-existed in two versions (oxidized and non-oxidized) in the analysis. The overall
199 sensitivity of the assay was therefore reduced and prevented an accurate quantification of the
200 peptide presented on the MHC-I receptor. Thus, we used an oxidation step (using H₂O₂) to
201 convert the amino acid to its stable sulfone analog, which also increased the overall stability
202 of the peptide for mass spectrometry analysis. To validate our approach for peptide
203 quantification, we spiked the isotopically labeled RMF peptide in a MHC-I receptor complex

204 preparation prepared from 5×10^6 SKM-1 cells immunoprecipitated using a pan-MHC-I
205 antibody. We observed a linear response for the spiked peptide in the range of 100 amol to
206 10 fmol loaded on column with a technical limit of detection arbitrarily set to 50 amol loaded
207 on column (supplemental Figure 4B). In addition, the lower limit of detection (LloD) and lower
208 limit of quantification (LloQ) of the methods with and without oxidation were calculated using
209 calibration curves generated from the spiking of labeled RMF peptide into HeLa tryptic
210 extracts. The LloDs of non-oxidized and oxidized peptides are 2 and 3.5 fmol, respectively. The
211 LloQs of non-oxidized and oxidized peptides are 6.1 and 10.6 fmol, respectively.

212 We applied our newly developed method first to T2 cells (TAP-deficient) pulsed with
213 increasing amounts of the RMF peptide (1 nM to 10 μ M) for 2 h prior to lysis. The linearity in
214 peptide load increase was reflected in the recovery of RMF peptide as measured by mass
215 spectrometry, post-immunoprecipitation with the pan-MHC-I or HLA-A*02 specific antibody
216 (supplemental Figure 4C). Notably, the consistently higher amounts of RMF peptide detected
217 using the pan-MHC-I antibody was due to the fact that HLA-C*01:02 is also predicted to bind
218 this peptide with high affinity and therefore increased the recovery of the endogenous
219 peptide. By contrast, the negative controls, using non-targeting isotype antibodies for the pan-
220 MHC-I and HLA-A*02 antibodies, did not immunoprecipitate meaningful amounts of MHC-I
221 complexes. The conservation of the linear increase between RMF peptide loading and peptide
222 recovery demonstrated the technical robustness of the method and provided the proof-of-
223 principle for detecting specifically and quantitatively the RMF peptide on the MHC-I receptor.

224 In a second experiment, we investigated whether the developed mass spectrometry-based
225 quantification technique could be used to analyze the SKM-1 cell line, which expresses a high
226 amount of the WT1 protein. Five million SKM-1 cells were lysed per measurement and the
227 MHC-I receptor complexes were immunoprecipitated using either an HLA-A*02-specific or a

pan-MHC-I antibody. The captured MHC-I peptides were then analyzed and quantified as described. The signal for endogenous RMFPNAPYL was always below the limit of detection when using the HLA-A*02 as immunoprecipitating reagent, in multiple experiments. However, when using the pan-MHC-I immune-precipitating antibody, weak signals for the endogenous RMF peptide were observed in 30–50% of all performed experiments (supplemental Figure 4D). A similar observation was also made in a related WT1-expressing cell line (data not shown).

For primary AML patient samples, we thoroughly optimized the workflow by 1) upscaling to 60 million cultured primary AML cells selected for their high viability/WT1/HLA-A*02 expression, and 2) using the clinical candidate for immunoprecipitation. Under these optimal conditions, we detected the endogenous RMF peptide on AML blasts from one of the three donors, and were able to quantify it up to 17 fmol in this sample, which is equivalent to 152 HLA-A*02/RMF-pMHC complexes per cell. For the first time, the RMF peptide was detected at amounts largely above LoD and LoQ (8.5 times the LoD and 2.8 times the LoQ) (supplemental Figure 4E).

Pharmacokinetics of WT1-TCB

A pharmacokinetics (PK) study was conducted in a human FcRn (hFcRn) transgenic (Tg32 homozygous) mouse model, which is characterized by a lack of murine FcRn, the transgenic expression of hFcRn and a homogenous genetic background C57BL/6J (B6).⁸ This model is suitable for predicting the PK of monoclonal antibody (mAb) in humans, as it reflects well the interaction of the drug with the human FcRn salvage receptor.^{9,10} The mean PK profiles are shown in supplemental Figure 7. As a result, the mean terminal elimination half-life in the hFcRn model was estimated to be 10.7 days.

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277

278 **Supplemental Tables**279 **Supplemental Table 1.** Data collection and refinement statistics for 11D06 HLA-A*02/RMF–

280 pMHC complex

11D06 HLA-A*02/RMF–pMHC complex	
Data collection	
Space group	P2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	54.11, 67.00, 139.36
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90.57, 90
Resolution (Å)	2.64
<i>R</i> _{sym} or <i>R</i> _{merge}	0.10
<i>I</i> / <i>S</i> / <i>I</i>	13.10 (0.82)
Completeness (%)	99.9 (99.8)
Redundancy	3.79 (3.85)
Refinement	
Resolution (Å)	48.3–2.64
No. reflections	29606
<i>R</i> _{work} / <i>R</i> _{free}	17.10/23.00
No. atoms	
Protein	6395
Water	250
<i>B</i> -factors	
Protein	67.52
Water	57.46
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.20

281 *Values in parentheses are for highest-resolution shell. pdb code 7BBG

282

283

284 **Supplemental Table 2.** Interfacing residues between heavy and light chains of Fab 11D06 and
 285 RMF peptide (residues within a contact distance of 4 Å). Distances and types of interactions
 286 (distance, D; ionic, I; hydrogen bond, H) were calculated in MOE 2020.09 (Chemical
 287 Computing Group).

Position in heavy chain	Position in peptide	Distance (Å)	Type of interaction
Ser31	Arg1	4.05	D
Ile100	Arg1	3.86	D
Glu101	Arg1	4.16	DI
Glu101	Met2	4.05	D
Glu101	Pro4	4.34	D
Trp104	Pro4	4.08	D

288

Position in light chain	Position in peptide	Distance (Å)	Type of interaction
Ser28	Tyr8	4.06	D
Ser28	Ala6	4.35	D
Ser30	Tyr8	4.14	D
Trp32	Pro4	3.95	D
Trp32	Asn5	3.81	DH
Glu92	Asn5	4.02	DH

289

290 **Supplemental Table 3.** Interfacing residues between heavy and light chains of Fab 11D06 and
 291 HLA-A*02 (residues within contact distance of 4 Å). Distances and types of interactions
 292 (distance, D; ionic, I; hydrogen bond, H) were calculated in MOE 2020.09 (Chemical
 293 Computing Group).

Position in heavy chain	Position in HLA-A02	Distance (Å)	Type of interaction
Ser30	Glu58	4.19	D
Ser31	Glu58	3.65	DH
Ser31	Trp167	4.16	D
Tyr32	Glu58	4.02	D
Ile52	Glu166	4.33	D
Ile54	Arg170	3.97	D
Phe55	Glu166	4.14	D
Phe55	Trp167	4.06	D
Phe55	Arg170	4.02	D
Thr57	Glu166	3.77	DH
Ile100	Glu58	4.13	D
Ile100	Asp61	4.45	D
Ile100	Gly62	4.05	D
Ile100	Arg65	3.86	D
Glu101	Lys66	3.36	DIH
Trp103	Ala158	4.10	D
Trp103	Thr163	3.84	D
Trp104	Gly62	3.90	D
Trp104	Arg65	3.86	D
Trp104	Lys66	4.27	D
Gly105	Arg65	3.76	DH

294

Position in light chain	Position in HLA-A02	Distance (Å)	Type of interaction
Gln27	Ala149	4.41	D
Gln27	Ala150	4.19	DH
Trp32	Arg65	4.10	D
Trp32	Lys66	4.34	D
Trp32	Ala69	3.91	D
Tyr49	Arg65	3.94	D
Asp50	Arg65	3.50	DIH
Tyr91	Arg65	4.07	D
Glu92	Gln155	4.19	D
Asp93	Gln155	4.15	D
Tyr94	Ala158	4.10	D

295

296

297 **Supplemental Table 4.** WT1 expression of primary AML and healthy donor samples used in

298 WT1-TCB dose-dependent cytotoxicity experiments as determined by qPCR

Sample	WT1 copies/ 10 ⁴ ABL1 copies	Sample type	Effector cells
AML 1	6147.27	HLA-A*02 ⁺ AML	Allogenic HD PBMCs
AML 2	29.87		
AML 3	653.70		
AML 4	1261.14		
AML 5	18.51		
AML 6	1125.70		
AML 7	4122.55		
AML 8	1670.84		
AML 9	344.10		
AML 3	653.70	HLA-A*02 ⁺ AML	Autologous PBMCs
AML 4	1261.14		
AML 7	4122.55		
AML 10	949.92	HLA-A*02 ⁻ AML	Allogenic HD PBMCs
AML 11	1909.38		
AML 12	1053.00		
AML 13	1702.55		
AML 14	1311.04		
AML 15	2802.25		
AML 16	117.65		
AML 17	1758.85		
AML 18	101.98		
AML 19	87.07		
HD 1	n.d.	HLA-A2 ⁺ WT1 ⁻ HD CD33 ⁺ cells	Allogenic HD PBMCs
HD 2	n.d.		
HD 3	n.d.		
HD 4	n.d.		
HD 5	n.d.		
HD 6	n.d.		

299 n.d.: not detected

Supplemental Table 5. WT1 expression of primary AML samples used in *ex vivo* long-term culture cytotoxicity experiments as determined by qPCR

Sample	WT1 copies/ 10 ⁴ ABL1 copies	Sample type	Effector cells
AML 1	6147.27	HLA-A*02 ⁺ AML	Allogenic HD T cells
AML 3	653.70		
AML 6	1125.70		
AML 7	4122.55		
AML 20	1199.94		
AML 21	459.53		
AML 22	636.56		
AML 23	1180.89		
AML 24	2934.4		
AML 25	n.a.		
AML 26	102.25		
AML 27	n.a.		
AML 28	n.a.		
AML 29	50.64		
AML 30	881.72		
AML 31	14.66		
AML 32	877.08		
AML 33	53.10		
AML 34	1145.06		
AML 3	653.70	HLA-A*02 ⁺ AML	Autologous T cells
AML 23	1180.89		
AML 28	n.a.		
AML 30	881.72		
AML 35	47.07		
AML 36	n.a.		
AML 37	456.56		
AML 38	153.05		
AML 11	1909.38	HLA-A*02 ⁻ AML	Allogenic HD T cells
AML 13	1702.55		
AML 15	2802.25		
AML 16	117.65		
AML 17	1758.85		
AML 18	101.98		
AML 19	87.07		
AML 43	n.a.		
AML 44	573.45		
AML 45	314.62		
AML 46	n.a.		

n.a.: data not available

304 **Supplemental Table 6.** WT1 expression of primary AML samples used in cytotoxicity
 305 experiments in combination with lenalidomide as determined by qPCR

Sample	WT1 copies/ 10 ⁴ ABL1 copies
AML 1	6147.27
AML 3	653.70
AML 4	1261.14
AML 23	1180.89
AML 29	50.64
AML 30	881.72
AML 31	14.66
AML 32	877.08
AML 33	53.1
AML 34	n.a.

306 n.a.: data not available

307 **Supplemental Table 7.** WT1 expression of samples used in CFU assays as determined by
 308 qPCR

Sample	WT1 copies/ 10 ⁴ ABL1 copies
Donor 1	26.51
Donor 2	145.46
Donor 3	52.21

309

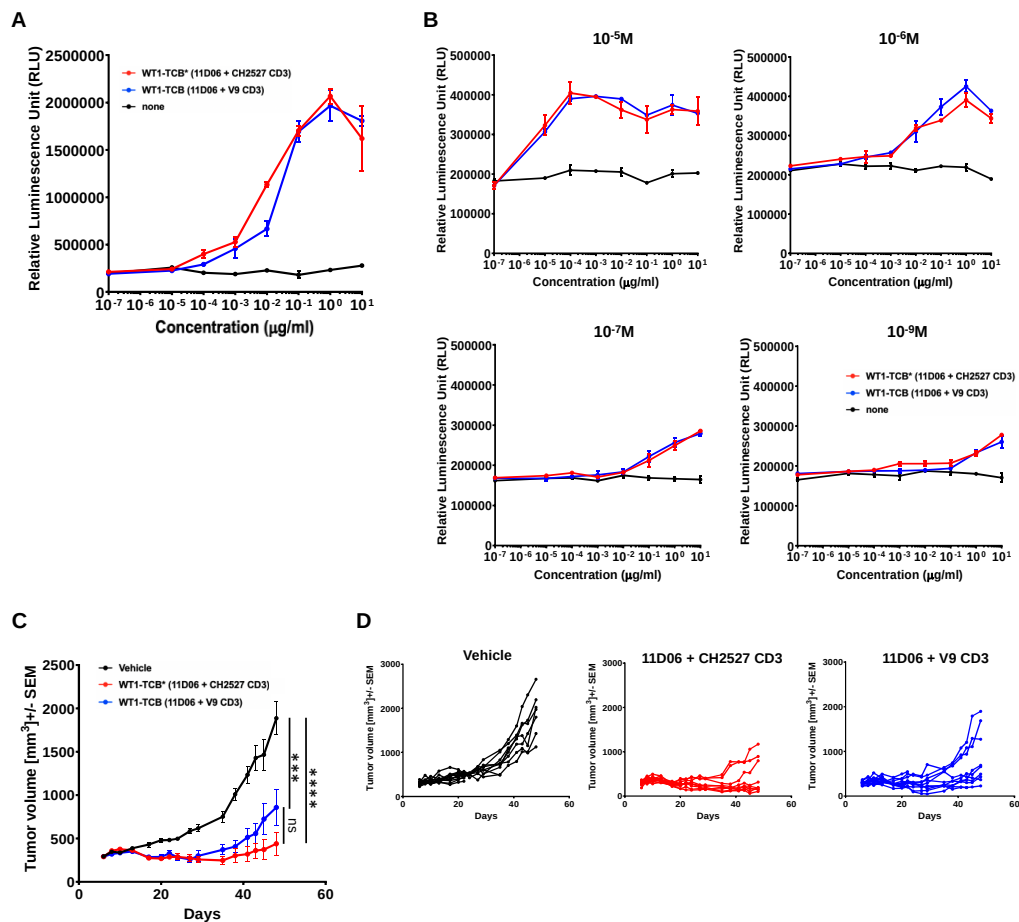
310 **Supplemental Table 8.** Characterization of AML patient No. 1

Gender	Male
Age	67 years
HLA-A type	02:01, 03:01
FAB classification	M1
ELN risk group	Favorable
Mutations	Result
ASXL1	negative
CEBPA	negative
FLT3-ITD	negative
FLT3-TKD	negative
IDH1	negative
IDH2	negative
KMT2A-PTD	negative
NPM1	Type A mutation
RUNX1	negative
TP53	negative
5q31 deletion	negative
7q31 deletion	negative
BCR-ABL1 rearrangement	negative
CBFB-MYH11 rearrangement	negative
ETV6 deletion	negative
KMT2A rearrangement	negative
MECOM rearrangement	negative
RUNX1-RUNX1T1 rearrangement	negative
TP53 deletion	negative

311

Target expression		determined by
WT1 copies/10 ⁴ ABL1 copies	6147.27	qRT-PCR
HLA-A*02 (MFI ratio)	166.37	flow cytometry
HLA-A*02/RMF pMHC complexes/cell	152	mass spectrometry
Cytotoxicity assays	Specific lysis	Antibody concentration
Specific lysis after 48h <i>in ex vivo</i> short-term cytotoxicity assay using allogenic HD PBMCs	-0.3%	0.01 µg/mL WT1-TCB
	33.6%	0.1 µg/mL WT1-TCB
	86.8%	1 µg/mL WT1-TCB
	93.4%	10 µg/mL WT1-TCB
Specific lysis after 14 days in <i>ex vivo</i> long-term cytotoxicity assay	88.9 %	10 ⁻⁸ M WT1-TCB*

312

313 **Supplemental Figures**314 **Supplemental Figure 1.**

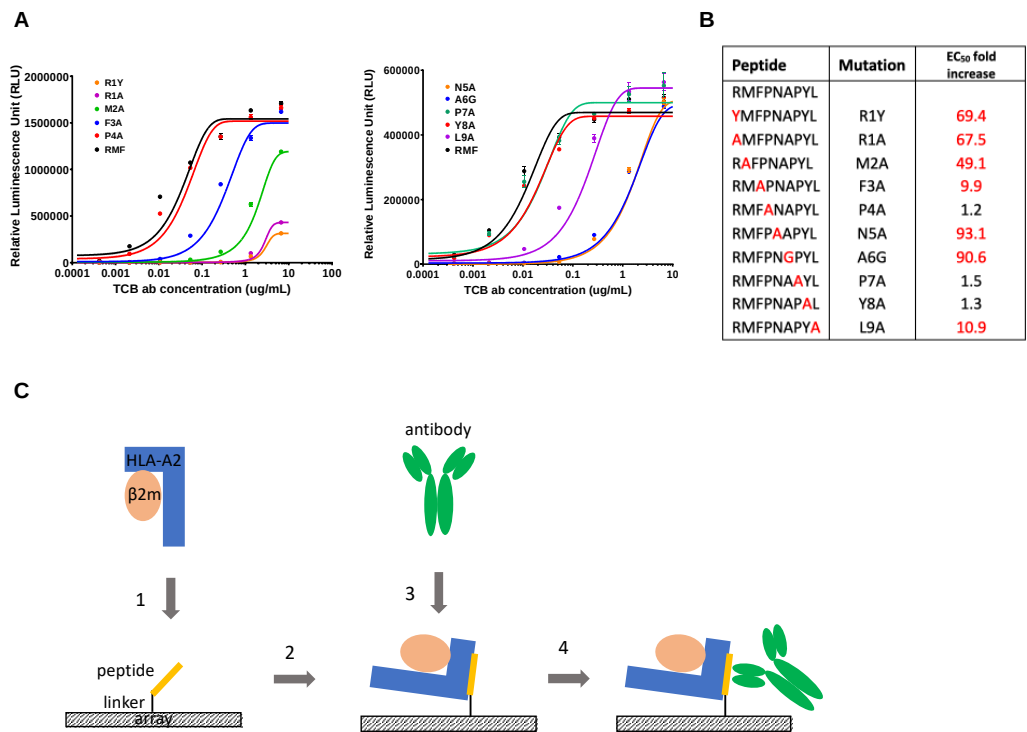
315

316 **Comparable efficacy of WT1-TCBs with CH2527 and V9 CD3 binders *in vitro* and *in vivo*.**

317 *In vitro* killing of WT1⁺ SKM-1 target cells (A) and T2 cells pulsed with four different
 318 concentrations of RMF peptide (B) induced by WT1-TCB with two different CD3 binders
 319 (CH2527 and V9), after 20 h co-incubation with enriched T cells from a representative healthy
 320 donor depicted as RLU over WT1-TCB concentration ($n=3$). (C) Average SKM-1 tumor volume
 321 and SEM over 48 days of treatment with two WT1-TCB molecules (1mg/kg) with different CD3
 322 binders (CH2527 and V9). Statistical analysis: One-way ANOVA multi comparison and Fisher's

323 LSD test, based on study day 48 (***p*<0.001; *****p*<0.0001; no significant differences
324 between *WT1-TCB* treatment groups). (D) Single mouse tumor volume plots depicted as SKM-
325 1 tumor volume over SEM.
326

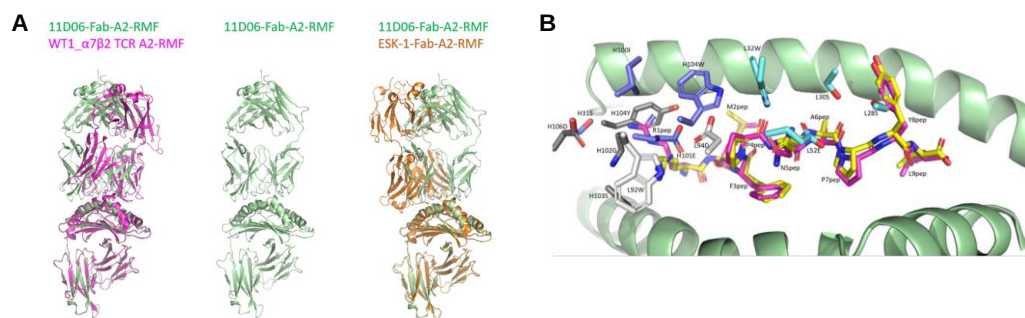
327 **Supplemental Figure 2.**



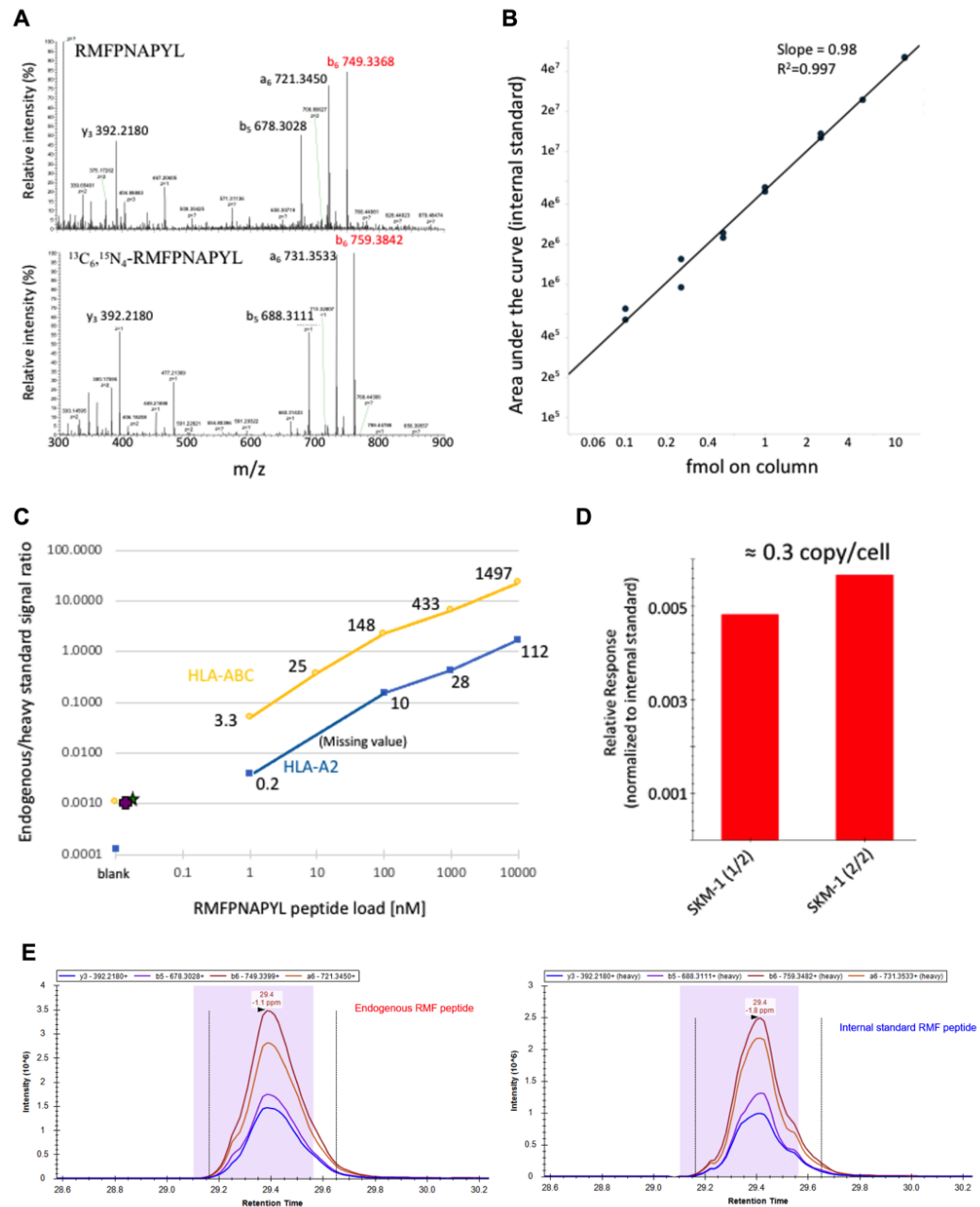
328 **Evaluation of the WT1-TCB RMF peptide-binding motif by alanine scan and peptide array.**
329 (A) Jurkat NFAT activation induced by WT1-TCB binding to different RMF peptide single amino
330 acid variants plotted as RLU versus WT1-TCB concentration. (B) EC₅₀ fold increase of Jurkat
331 NFAT activation induced by RMF peptide single amino acid variants. (C) Schematic
332 representation of HLA-A*02-b2m-peptide-antibody complex detection on peptide array.
333 Pre-formed HLA-A*02-b2m heterodimer binds to the peptide array (step 1) and forms HLA-
334 A*02-b2m-peptide complex (step 2). Antibody recognizes HLA-A*02-b2m-peptide (step 3)

336 and binds forming HLA-A*02-b2m-peptide-antibody complex (step 4). The complex is
337 detected by Cy3 anti-human IgG antibody (not shown).

338 **Supplemental Figure 3.**



347 Supplemental Figure 4.

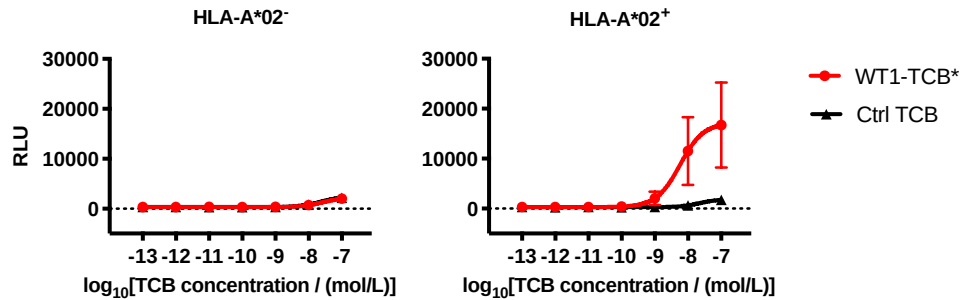


348

349 **Quantification of RMF peptide by targeted mass spectrometry.** (A) Representative tandem
350 mass spectra of the RMF peptide (upper panel: endogenous peptide; lower panel: $^{13}\text{C}_6, ^{15}\text{N}_4$ -
351 labeled RMFPNAPYL internal standard). Both species were subjected to Met oxidation to yield

the sulfonyl Met species. The qualifying transitions are labeled whereas the quantification transition is marked in red. (B) Reverse calibration curve of the internal standard (100 amol–10 fmol) spiked into an MHC-I receptor complex preparation prepared from 5 million SKM-1 cells immunoprecipitated using a pan-MHC-I antibody. All measurements were performed in duplicate. Based on this experiment, a technical limit of detection was arbitrarily set to 50 amol RMFPNAPYL loaded onto the column. (C) Detection of RMF peptide externally loaded on T2 cells. The immunoprecipitation conditions are represented as follows: HLA-A*02, blue line; HLA-ABC, yellow line. The results for the isotype controls of the HLA-A*02 and HLA-ABC antibodies are represented by “+” and “-” in the blank category for comparison. The signal of the captured peptide is ratioed against the spiked internal standard. The copy number per cell of RMFPNAPYL (as indicated on top of the curves for each conditions) is calculated assuming 100% recovery. (D) Detection of the MHC-I cognate peptide RMFPNAPYL in SKM-1 cells using the immunoprecipitating panMHC-I antibody. The signal of the captured peptide was ratioed against the spiked heavy internal standard. The copy number of RMFPNAPYL was calculated assuming 100% recovery and no experimental losses. (E) Quantification of endogenous RMF peptide versus internal standard purified from primary AML cells. Mass spectrometric transitions signals of dioxidized endogeneous [RMFPNAPYL] peptide purified from AML blasts (precursor $m/z=570.7790$ (2+); left) and dioxidized internal standard $^{13}\text{C}_6$, $^{15}\text{N}_4$ -labeled peptide (precursor $m/z=575.7831$ (2+); right). Total area under the curve (AUC) per peptide is the sum of the four most abundant [RMFPNAPYL] transitions (a_6 , b_6 , b_5 , and y_3). The estimated copy number of endogenous [RMFPNAPYL] per cell is calculated by comparison of total AUCs to the spiked-in isotopically labeled [RMFPNAPYL] peptide, assuming 100% recovery for both standard and endogenous peptides at the end of the immunoprecipitation and oxidation steps.

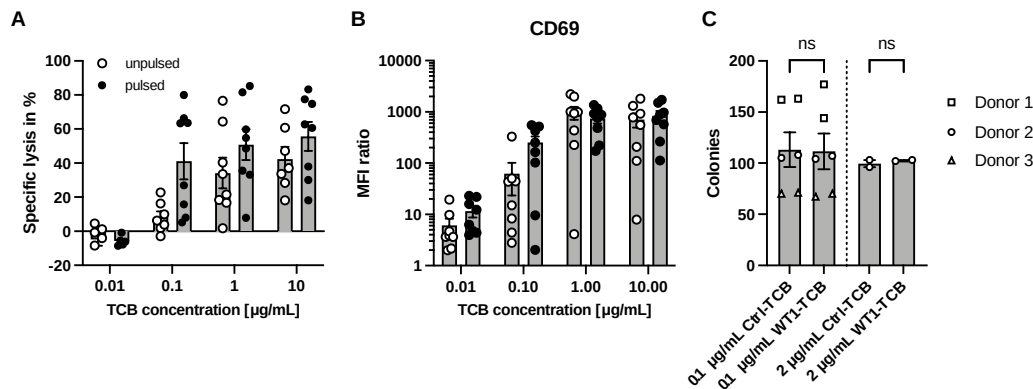
Supplemental Figure 5.



377

378 **Dose-dependent downstream TCR signaling induced by WT1-TCB*.** Induction of downstream
 379 TCR signaling in NFAT Jurkat Reporter cells in co-cultures with primary AML cells from HLA-
 380 A*02⁻ (left) or HLA-A*02⁺ (right) patients by various concentrations of WT1-TCB* (red) or Ctrl-
 381 TCB (black).

Supplemental Figure 6.

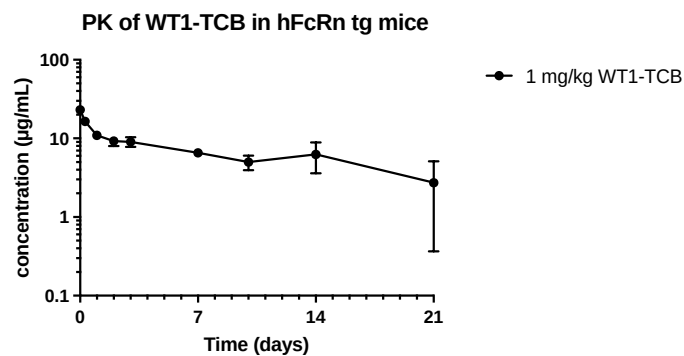


383

384 **WT1-TCB-mediated Cytotoxicity against healthy HLA-A*02⁺ Bone marrow cells.** (A) Specific
 385 lysis of healthy CD34⁺ bone marrow cells by HD T cells after and (B) expression of T-cell
 386 activation marker CD69 after 24 h in a cytotoxicity assay with healthy bone marrow. Bars
 387 represent mean $\pm$ SEM; $n=8$. Cells were either unpulsed (empty circles) or pulsed with RMF
 388 peptide (filled circles). (C) Colony-forming unit assay with CD34⁺ cells isolated from bone

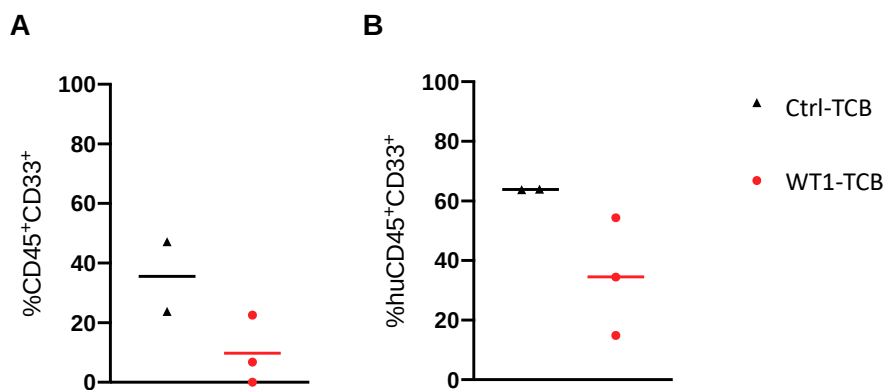
marrow after 48 h treatment with WT1-TCB. Bars represent mean $\pm$ SEM; $n=1-3$ with technical
 duplicates for every healthy donor.

Supplemental Figure 7.



Pharmacokinetics of WT1-TCB. Mean pharmacokinetics profiles of WT1-TCB for C57BL/6J (B6)
 mice expressing transgenic human FcRn and lacking murine FcRn ($n=3$).

Supplemental Figure 8.



Antitumor efficacy of WT1-TCB in an AML PDX mouse model. Reduction of human
 CD45⁺CD33⁺ cells in spleen (A) and bone marrow (B) in mice engrafted with a patient-derived
 xenograft sample after 10-day treatments with 3 mg/kg WT1-TCB or Ctrl-TCB.

6. Publikation II

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ARTICLE



Acute myeloid leukemia

Coexpression profile of leukemic stem cell markers for combinatorial targeted therapy in AML

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Abstract

Targeted immunotherapy in acute myeloid leukemia (AML) is challenged by the lack of AML-specific target antigens and clonal heterogeneity, leading to unwanted on-target off-leukemia toxicity and risk of relapse from minor clones. We hypothesize that combinatorial targeting of AML cells can enhance therapeutic efficacy without increasing toxicity. To identify target antigen combinations specific for AML and leukemic stem cells, we generated a detailed protein expression profile based on flow cytometry of primary AML ($n = 356$) and normal bone marrow samples ($n = 34$), and a recently reported integrated normal tissue proteomic data set. We analyzed antigen expression levels of CD33, CD123, CLL1, TIM3, CD244 and CD7 on AML bulk and leukemic stem cells at initial diagnosis ($n = 302$) and relapse ($n = 54$). CD33, CD123, CLL1, TIM3 and CD244 were ubiquitously expressed on AML bulk cells at initial diagnosis and relapse, irrespective of genetic characteristics. For each analyzed target, we found additional expression in different populations of normal hematopoiesis. Analyzing the coexpression of our six targets in all dual combinations ($n = 15$), we found CD33/TIM3 and CLL1/TIM3 to be highly positive in AML compared with normal hematopoiesis and non-hematopoietic tissues. Our findings indicate that combinatorial targeting of CD33/TIM3 or CLL1/TIM3 may enhance therapeutic efficacy without aggravating toxicity in immunotherapy of AML.

Introduction

Despite some advances in the treatment of acute myeloid leukemia (AML) in recent years, overall prognosis remains poor [1]. Allogeneic stem cell transplantation is still the

only curative option in high risk and relapsed AML, but morbidity and mortality are high owing to transplant-related side effects and refractory disease [2]. Targeted immunotherapy provides a potent option to specifically eliminate chemoresistant leukemic stem cells, which are reported to be the main cause of relapse [3]. In relapsed B-cell acute lymphoblastic leukemia (B-ALL), targeting of CD19 via bispecific T-cell-engaging antibody constructs and chimeric antigen receptor (CAR) T-cell products has shown remarkable antileukemic effects and a tolerable safety profile [4–10]. Given its success in B-ALL, the translation of T cell-based targeted immunotherapy to AML is of major interest and currently being evaluated in preclinical and clinical trials. However, choice of suitable target antigens in AML has proven to be challenging.

In B-ALL, CD19 can be considered an ideal target antigen owing to its high expression on leukemic cells and its restricted expression profile on normal cells: it is expressed on essentially all B-lineage cells, whereas negative on all other hematopoietic lineages or other normal tissues [11, 12]. In line with the CD19 expression pattern, the on-target

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Table 1 Patient characteristics of primary AML samples for flow cytometric analysis

	All cases	Initial Dx	Relapse
Samples, <i>n</i>	356	302	54
Age, median (range)	63 (16–92)	65 (16–92)	55 (23–80)
Gender, <i>n</i> (%)			
Male	201 (56%)	170 (56%)	31 (57%)
Female	155 (44%)	132 (44%)	23 (43%)
FAB, <i>n</i> (%)			
M0	18 (8%)	12 (7%)	6 (19%)
M1	68 (32%)	61 (34%)	7 (23%)
M2	45 (21%)	40 (22%)	5 (16%)
M3	9 (4%)	8 (4%)	1 (3%)
M4	41 (19%)	35 (19%)	6 (19%)
M5	30 (14%)	24 (13%)	6 (19%)
M6	2 (1%)	2 (1%)	0 (0%)
M7	0 (0%)	0 (0%)	0 (0%)
Unknown	143	120	23
Cytogenetics, <i>n</i> (%)			
Normal karyotype	139 (43%)	123 (44%)	16 (36%)
Complex karyotype	73 (23%)	60 (21%)	13 (30%)
t(8;21)	9 (3%)	9 (3%)	0 (0%)
t(9;11)(p21-22;q23) or t(11;19)(q23;p13)	10 (3%)	9 (3%)	1 (2%)
inv(16)/t(16;16)(p13;q22)	9 (3%)	9 (3%)	0 (0%)
Other adverse risk abnormalities	22 (7%)	17 (6%)	5 (11%)
Non-classified abnormalities	62 (19%)	53 (19%)	9 (20%)
Unknown	32	22	10
Mutations (normal karyotype), <i>n</i> (%)			
NPM1 mut/FLT3 wt	38 (28%)	37 (31%)	1 (6%)
NPM1 mut/FLT3-ITD	35 (26%)	30 (25%)	5 (31%)
NPM1 wt/FLT3-ITD	14 (10%)	14 (12%)	0 (0%)
NPM1 wt/FLT3 wt	50 (36%)	40 (33%)	10 (63%)
CEBPA mut	6 (10%)	6 (10%)	0 (0%)
KMT2A mut	16 (12%)	12 (10%)	4 (22%)
MRC, <i>n</i> (%)			
Favorable	46 (14%)	44 (16%)	2 (4%)
Intermediate	178 (55%)	154 (55%)	24 (53%)
Adverse	100 (31%)	81 (29%)	19 (42%)
Unknown	32	23	9
ELN2010, <i>n</i> (%)			
Favorable	64 (20%)	62 (23%)	2 (4%)
Intermediate-I	90 (28%)	74 (27%)	16 (36%)
Intermediate-II	64 (20%)	55 (20%)	9 (20%)
Adverse	100 (31%)	82 (30%)	18 (40%)
Unknown	38	29	9

off-leukemia toxicity of CD19 CAR T-cell therapy is tolerable and limited to B-cell aplasia [13]. In AML, however, the most suitable target antigen still needs to be defined. Several

surface-bound target antigens are known to be overexpressed on AML cells [14–32]. Some of these, including CD33, CD123, CLL1, CD47, CD96, CD157, CD244, TIM3 and CD7, have been reported to be expressed on leukemic stem cells (LSC) [20, 21, 24, 27, 32–36]. However, single-targeting approaches against these LSC-associated antigens is complicated as none of the antigens are exclusively expressed on AML cells, leading to severe on-target off-leukemia toxicity [37–40]. Furthermore, clonal heterogeneity or antigen escape mechanisms could lead to persistence of AML cells upon single-targeting therapy.

Perna et al. [12] recently provided the rationale that a combinatorial targeting approach with well-matched target antigens could have the potential to enhance therapeutic efficacy without increasing on-target off-leukemia toxicity. In our study, we analyzed the coexpression profile of the most commonly targeted and leukemic stem cell-associated antigens within a single cohort of >300 AML patients, comparing primary AML cells to normal hematopoietic cells and non-hematopoietic tissues. We sought to identify combinations of target antigens that show high coexpression in AML compared with normal cells and thereby provide options to reduce toxicity and overcome antigen escape mechanisms as well as clonal heterogeneity in AML.

Materials and methods

Patient samples and clinical data

For flow cytometric analysis, peripheral blood or bone marrow aspirate samples of AML patients at initial diagnosis (*n* = 302) and relapse (*n* = 54) as well as bone marrow aspirates from healthy donors (*n* = 34) were used. Sample size was chosen based on feasibility and experience with previous analysis [34]. All samples were collected after written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig Maximilian University Munich. Patient characteristics are summarized in Table 1. Diagnostic workup to establish diagnosis of AML included cytomorphology, cytogenetics, fluorescence in situ hybridization, molecular genetics and immunophenotyping. Combined cytogenetic and molecular risk stratification groups were assigned in accordance with the Medical Research Council (MRC) and European LeukemiaNet (ELN) recommendations [41, 42].

Flow cytometry

After collection, all samples were analyzed immediately, without prior cryoconservation. Samples were stained with the following fluorochrome-conjugated anti-human

monoclonal antibodies: CD7 (clone 8H8.1, Beckman Coulter, #A97050), CD33 (clone D3HL60.251, Beckman Coulter, #A54824), CD34 (clone 581, Beckman Coulter, #B49202), CD38 (clone LS198.4.3, Beckman Coulter, #B49200), CD45 (clone J.33, Beckman Coulter, #B36294), CD123 (clone 7G3, BD, #560087), CD244 (clone C1.7, Biolegend, #329508), TIM3 (clone 344823, R&D, #FAB2365A). Corresponding isotype controls were used for each sample. Surface antigen expression was assessed using a 10-color Navios flow cytometer (Beckman Coulter, Brea, CA, USA). Gating was performed as described in Supplemental Fig. 1A. As measure of antigen expression intensity, the median fluorescence intensity (MFI) ratio was used. MFI ratio was calculated by dividing the MFI value of the antigen-specific antibody by the MFI value of the respective isotype control (Supplemental Fig. 1B), as previously described [34]. We compared the isotype-based MFI ratio with an alternative MFI index, which is based on normalization to lymphocytes (Supplemental Fig. 2). The MFI ratio highly correlated with the MFI index for the myeloid-associated antigens CD33, CD123 and CLL1 (Spearman $r > 0.88$). In contrast, we observed no or lower correlation of the MFI ratio with the MFI index for the lymphoid-associated antigens TIM3, CD244 and CD7 (Spearman $r < 0.79$). The latter was owing to expression of these antigens on lymphocytes, which therefore did not serve as appropriate negative control. For this reason, we chose the isotype-based MFI ratio for our analysis. Positive expression in the majority of cells was defined as MFI ratio ≥ 1.5 . MFI values were determined using FlowJo software (Version 9.8.5) (Tree Star Inc., Ashland, Oregon).

Normal tissue proteomics

Protein expression data for normal tissues were retrieved from the integrated dataset generated by Perna et al. [12], including three independent protein expression data repositories: the Human Protein Atlas, the Human Proteome Map and the Proteomics Database.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA). The significance of differences was determined using the Mann–Whitney U test for unpaired samples and the Wilcoxon matched-pairs signed rank test for paired samples. Statistical significance was considered for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). Results are shown as medians $\pm$ 95% confidence interval or as indicated. Graphs were generated using GraphPad Prism 7, R Studio (R Studio, Boston, USA) and Adobe Illustrator CS6 (Adobe Systems, San José, USA).

Results

Antigen expression on AML cells at initial diagnosis and at relapse

To analyze the expression profile of AML-associated surface antigens, we performed multicolor flow cytometry on primary AML samples at initial diagnosis ($n = 302$) and at relapse ($n = 54$). We quantified the antigen expression levels of CD33, CD123, CLL1, TIM3, CD244 and CD7 on AML bulk cells (as described in Supplemental Fig. 1). Antigens were considered positive in the majority of cells if expression intensity exceeded an MFI ratio of 1.5. At initial diagnosis, AML bulk cells in most patients were positive for CD33 (96.4%), CD123 (97.0%), CLL1 (80.1%), TIM3 (87.3%) and CD244 (96.7%). Also at relapse, AML bulk cells in most patients were positive for CD33 (98.1%), CD123 (98.1%), CLL1 (71.4%), TIM3 (80.0%) and CD244 (97.1%). The aberrant antigen CD7 was positive in 35.6% of the patients at initial diagnosis and 48.1% of the patients at relapse (Fig. 1a, Supplemental Table 1). Both at initial diagnosis and at relapse, our analysis did not show any correlation of antigen expression levels with patient age (Supplemental Fig. 3). Subgroup analysis of de novo vs. secondary AML did not find any significant differences in expression levels of CD33, CD123, CLL1, TIM3 and CD7. In contrast, CD244 was found to be significantly higher in sAML after MDS compared with de novo AML ($p = 0.02$) (Supplemental Fig. 4). In addition, we analyzed the expression level of antigens in relapsed AML after intensive chemotherapy alone ($n = 33$) and after allogeneic stem cell transplantation ($n = 15$) (Supplemental Figure 5). In this small subgroup analysis, antigen expression levels of CD33, CD123, CD244 and CD7 were not significantly different after intensive chemotherapy alone compared with allogeneic stem cell transplantation. Statistical analysis of TIM3 and CLL1 expression levels was not performed owing to low sample numbers.

Antigen expression on AML bulk cells and leukemic stem cells

On AML bulk cells, we found varying degrees of surface antigen density both at initial diagnosis and at relapse, hereafter quantified as median MFI ratios (Fig. 1a, Supplemental Table 1; negative: < 1.5 ; low: 1.5–5; medium: 5–15; high: > 15): High expression was found for CD33 (Initial diagnosis: 27.1/Relapse: 34.1) and CD244 (18.9/35.8). Moderate expression was found for CD123 (8.5/9.2), CLL1 (13.5/3.6) and TIM3 (3.9/3.5). Median expression of CD7, which is known to be aberrantly expressed in AML, was negative per our definition. Comparing antigen expression levels at initial diagnosis and at relapse, no

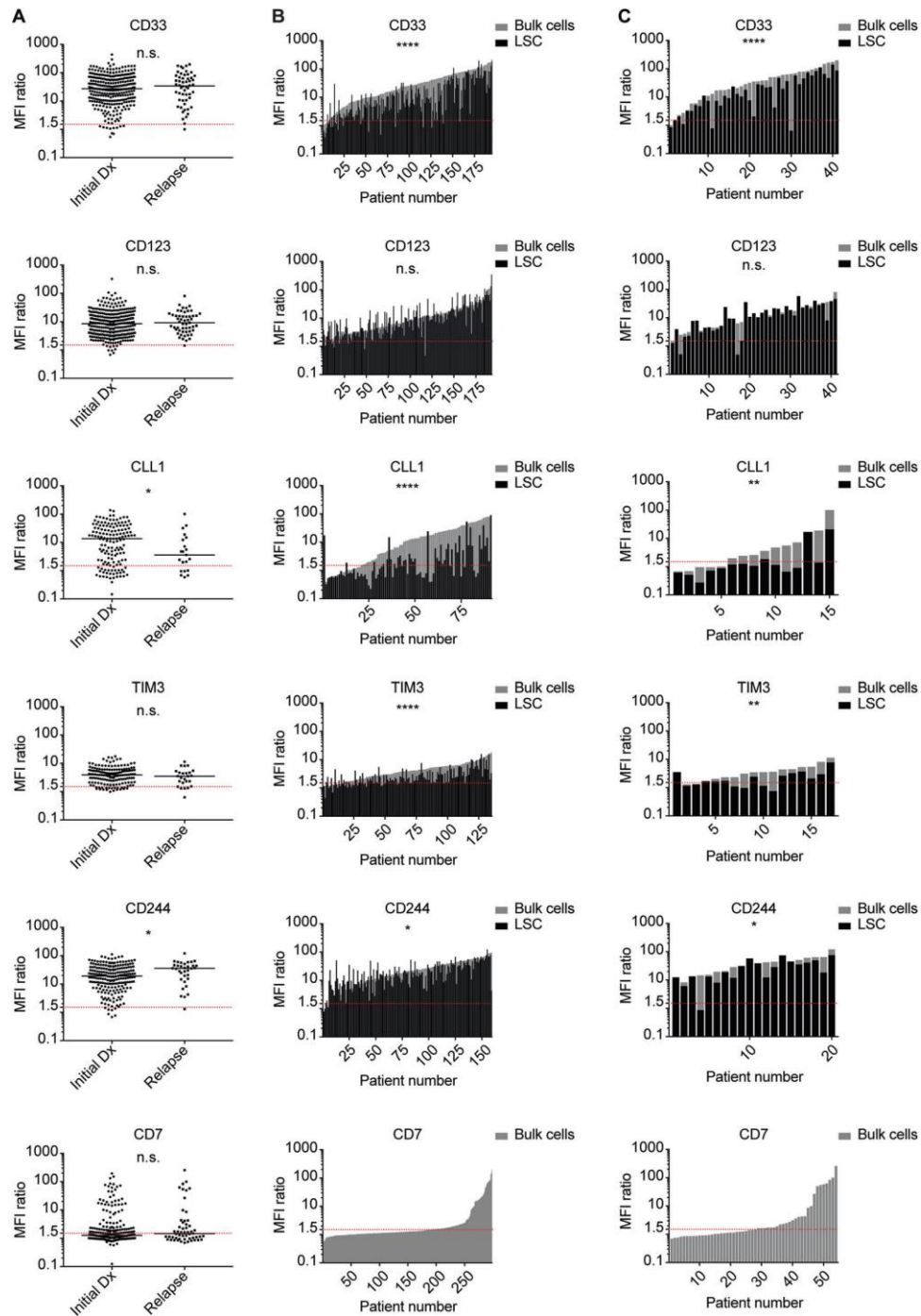


Fig. 1 Antigen Expression on AML Bulk Cells and LSC at Initial Diagnosis and Relapse. Antigen expression (MFI ratio) on primary AML samples at initial diagnosis and relapse was determined via flow cytometry. Each dot or bar represents one patient sample. Red dotted

line indicates MFI ratio of 1.5 as cutoff for positivity. **a** Initial diagnosis vs. relapse: unpaired analysis of AML bulk cells. **b** Initial diagnosis: paired analysis of AML bulk cells (gray) and LSC (black). **c** Relapse: paired analysis of AML bulk cells (gray) and LSC (black)

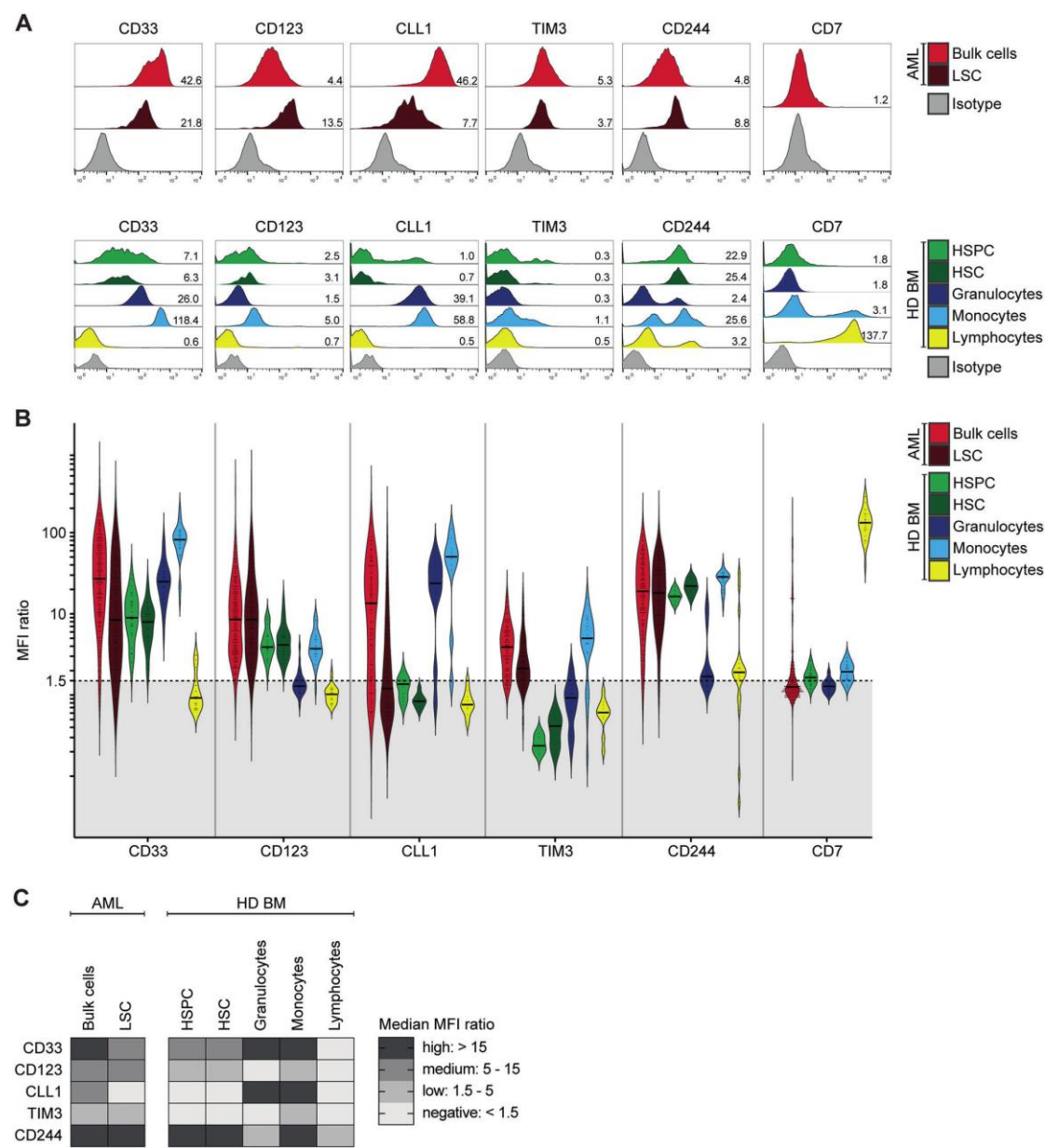


Fig. 2 Antigen Expression in AML and Normal Hematopoiesis. Antigen expression on primary AML samples at initial diagnosis and healthy donor-derived bone marrow cell populations (HD BM) as indicated in legend. **a** Representative primary AML sample and healthy donor-derived bone marrow sample. Histograms indicate fluorescence intensity. Numbers indicate MFI ratio. **b** Antigen

expression in AML and normal hematopoiesis, shown as MFI ratio. Dots indicate measured samples. Violin plots illustrate distribution of antigen expression for each analyzed cell population. Black dotted line indicates MFI ratio of 1.5 as cutoff for positivity. **c** Summary of antigen expression levels (median MFI ratio)

significant differences were found for CD33, CD123, TIM3 and CD7. CLL1 expression was significantly lower at relapse, whereas CD244 was significantly higher at relapse. The same trend could be observed in matched-pair analysis

at initial diagnosis and relapse, however, without statistical significance that may owe to low sample numbers (Supplemental Figure 6). We next evaluated antigen expression on LSC. At initial diagnosis, LSC in most patients were

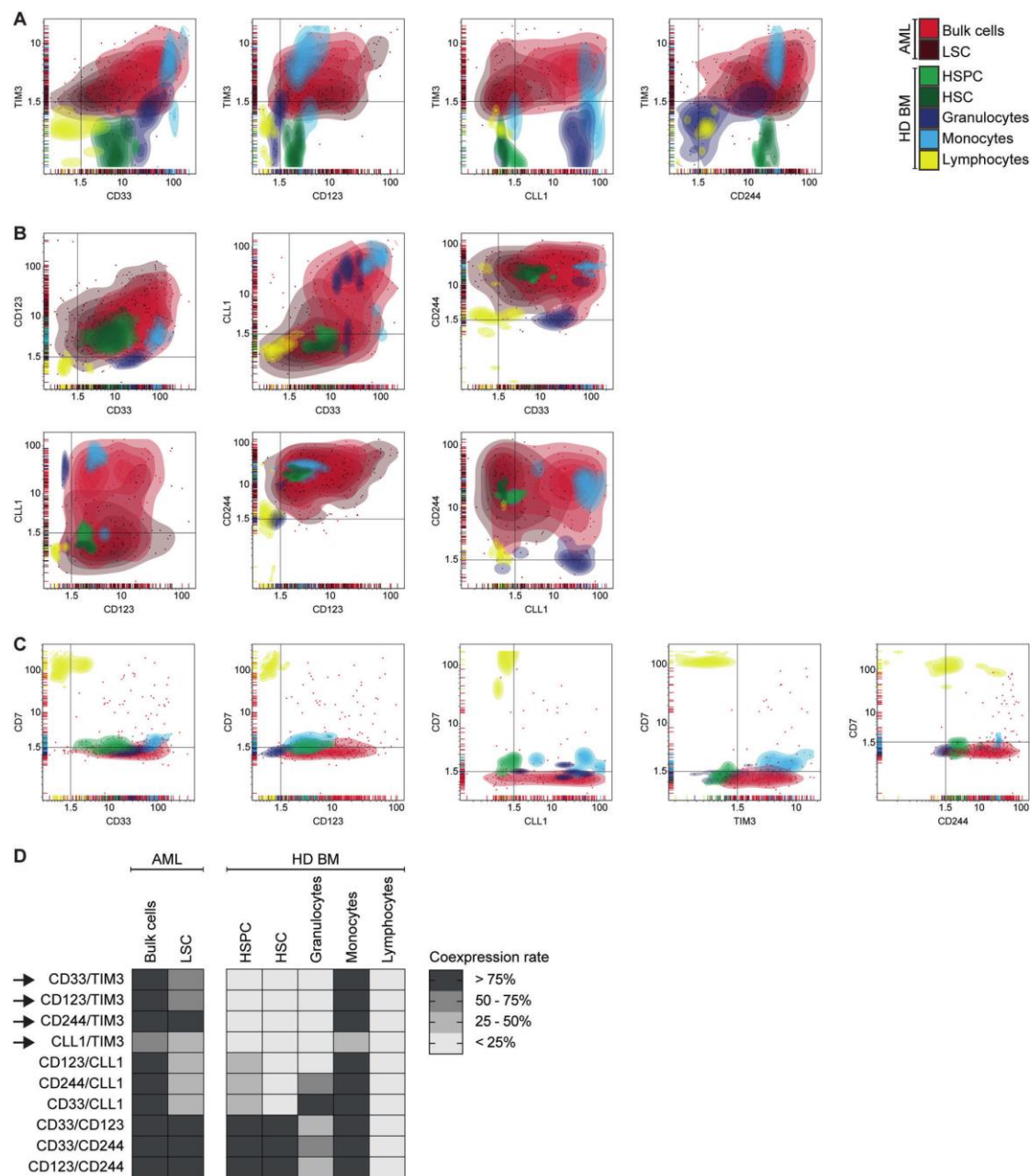


Fig. 3 Antigen Coexpression in AML and Normal Hematopoiesis. Antigen coexpression on primary AML cells and on normal hematopoietic cells of healthy donor-derived bone marrow (HD BM). Each dot indicates one independent sample measurement and the MFI ratios for the respective pair of antigens. Colors indicate different cell populations. Colored areas indicate density of dots. Black lines indicate MFI ratio of 1.5 as cutoff for positivity. Coexpression rate

indicates the proportion of samples with antigen coexpression (MFI ratio >1.5 for both antigens) in relation to all measured samples. **a** Suitable target antigen combinations. **b** Unsuitable target antigen combinations. **c** Target antigen combinations including CD7. **d** Summary of antigen coexpression data. Arrows indicate suitable target antigen combinations

positive for CD33 (88.7%), CD123 (95.3%), TIM3 (78.5%) and CD244 (98.1%) (Fig. 1b, Supplemental Table 1). Also at relapse, LSC in most patients were positive for CD33 (90.2%), CD123 (92.7%), TIM3 (64.7%) and CD244 (95.0%) (Fig. 1c, Supplemental Table 1). In contrast, CLL1 expression on LSC showed interindividual variability, with only a subgroup of patients being positive at initial diagnosis (45.1%) and at relapse (20.0%). Comparing antigen expression levels on AML bulk cells and LSC, we found CD33, CLL1, TIM3 and CD244 to be significantly less expressed on LSC, both at initial diagnosis and at relapse. In contrast, we found a trend toward higher CD123 expression on LSC compared with bulk cells, both at initial diagnosis and at relapse ($p = 0.05$ and $p = 0.08$, respectively) (Fig. 1b, c, Supplemental Table 1).

Antigen expression in genetically defined AML subgroups

To verify potential correlations of AML-associated antigens with genetic characteristics, we analyzed the level of antigen expression in different cytogenetically and molecularly defined AML risk groups according to the MRC and ELN2010 criteria (Supplemental Figure 7A). ELN2010 favorable and adverse risk AML samples did not have significantly different antigen expression levels of CD33, CD123, CLL1, TIM3, CD244 and CD7. We next analyzed antigen expression in different molecularly defined subtypes of AML. Normal karyotype AML samples were divided into sub-cohorts based on the mutational status of NPM1, FLT3, CEBPA and KMT2A (Supplemental Figure 7B). NPM1 mut/FLT3 wt ($n = 37$), NPM1 mut/FLT3-ITD ($n = 30$) and NPM1 wt/FLT3-ITD ($n = 14$) AML samples each had significantly higher expression of CD33 and CD123 compared with NPM1 wt/FLT3 wt ($n = 40$) AML samples, indicating that mutations of NPM1 and FLT3 are independently associated with higher expression of CD33 and CD123 compared with AML with non-mutated NPM1 or FLT3, respectively. Of note, in all evaluated molecularly defined sub-cohorts CD33, CD123, CLL1, TIM3 and CD244 were positive in most samples.

Antigen expression on AML cells and normal hematopoietic cells

As RNA expression of CD33, CD123, CLL1, TIM3, CD244 and CD7 was similar in bone marrow of AML patients at initial diagnosis and complete remission (Supplemental Figure 8, Supplemental Table 4), we next evaluated the differential protein expression on AML bulk cells and LSC in comparison with healthy donor-derived hematopoietic stem/progenitor cells (HSPC/HSC), granulocytes,

monocytes and lymphocytes (representative measurements in Fig. 2a; gating in Supplemental Fig. 1A). Overall, HSPC were positive for CD33 and CD123, but negative for CLL1 and TIM3. Granulocytes were positive for CD33, CLL1 and CD244, but negative for CD123 and TIM3. Monocytes were positive for all tested antigens. Lymphocytes were only positive for CD244 and CD7 (Fig. 2, Supplemental Table 2). This heterogeneous expression pattern both in the stem/progenitor and differentiated compartments implied different on-target off-leukemia hematotoxicity profiles for each of our tested target antigens.

Combinatorial antigen expression on AML cells and normal hematopoietic cells

We hypothesized that combinatorial targeting of antigens with non-overlapping expression on normal cells can enhance therapeutic efficacy without increasing toxicity. To identify the most AML-specific dual antigen combinations, we analyzed the antigen coexpression profile of CD33, CD123, CLL1, TIM3, CD244 and CD7 in primary AML compared with healthy donor-derived bone marrow samples, which were analyzed separately (Fig. 3). To systematically screen for suitable target antigen combinations, we calculated for each cell population the percentage of samples with dual antigen positivity (MFI ratio ≥ 1.5 for both antigens) and then compared antigen coexpression in AML vs. normal hematopoiesis (Supplemental Table 3). Suitable target antigen combinations were defined as having antigen coexpression on HSPC in 0% of samples and on granulocytes and/or lymphocytes in $< 25\%$ of samples. Combinations of the most commonly targeted antigens, CD33/CD123, CD33/CLL1 and CLL1/CD123, did not fulfill these criteria, owing to high coexpression on hematopoietic stem/progenitor cells (Fig. 3b). Remarkably, target antigen combinations that included TIM3 were found to have a more suitable coexpression profile with absent coexpression on HSPC and granulocytes (Fig. 3a). Thus, we identified CD33/TIM3, CD123/TIM3, CLL1/TIM3 and CD244/TIM3 as suitable target antigen combinations based on the expression profile in AML and normal hematopoiesis. Coexpression on single cell level could be confirmed both on AML bulk cells and LSC (Supplemental Figure 9).

Combinatorial antigen expression in normal non-hematopoietic tissue

To evaluate whether the selected target combinations were coexpressed on other, non-hematopoietic cells, we analyzed protein expression of CD33, CD123, CLL1, TIM3 and CD244 in normal tissues by using the integrated data set reported by Perna et al. [12] (Fig. 4). Based on these data, CD123 and CD244 were found to be expressed in a broad

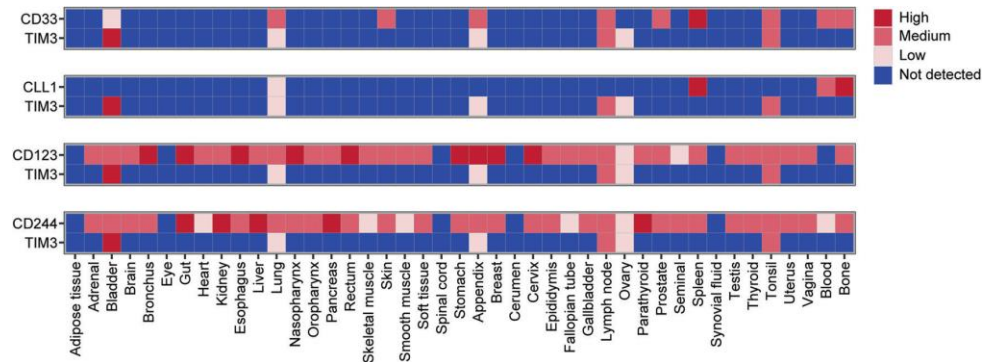


Fig. 4 Antigen coexpression in normal tissues. Protein expression levels in normal tissues were defined by using a previously reported integrated dataset. Paired expression is illustrated for possible target

antigen combinations CD33/TIM3, CLL1/TIM3, CD123/TIM3 and CD244/TIM3. Protein expression data range from 0 (not detected) to 3 (high)

range of non-hematopoietic tissues, including several vital organs: high positivity was detected for CD123 in lung and gut and for CD244 in gut, liver and kidney. In contrast, CD33, TIM3 and CLL1 showed an expression pattern that was largely restricted to hematopoietic tissue or organs with high immune cell infiltration. Combinatorial analysis of CD33/TIM3 and CLL1/TIM3 revealed non-overlapping expression patterns in normal tissues: Excluding organs with known immune infiltration, dual expression of CD33/TIM3 was only found in bladder. Remarkably, for CLL1/TIM3, there was no dual expression in any tissue except for low expression levels in lung.

Starting from our panel of six AML-associated antigens and all possible dual antigen combinations thereof ($n = 15$), our analysis revealed that CD33/TIM3 and CLL1/TIM3 were coexpressed in most AML samples, but largely spared in normal hematopoiesis and non-hematopoietic tissues, thus meeting our criteria of suitable target antigen combinations.

Discussion

In this study, we provide a detailed cell surface protein expression analysis of six commonly targeted and leukemic stem cell-associated antigens in AML. For the first time, we directly compare the antigen expression levels and coexpression of CD33, CD123, CLL1, TIM3, CD244 and CD7 on AML bulk cells, LSC and normal bone marrow cells, based on a cohort of > 300 AML patients and 34 healthy donors.

We show that CD33, CD123 and CLL1 are highly expressed on AML cells of most patients, which is consistent with previous reports [14, 21, 34, 43]. Notably, we are the first to compare the antigen expression levels on AML bulk cells and LSC at initial diagnosis and relapse. As multiple clinical trials are currently targeting CD33 and CD123 in relapsed and refractory (r/r) AML, our data support the target

suitability by showing homogeneous expression of CD33 and CD123 at relapse. Yet, consistent with independent data from our previous report, we show significantly lower expression of CD33 on LSC compared to bulk cells, underlining the difficulty to specifically target LSC [34]. In comparison, we find CD123 to be more specifically overexpressed on AML cells, but positivity on hematopoietic stem and progenitor cells as well as high expression in multiple normal tissues could lead to on-target toxicity, which may explain the pre-clinically observed hematotoxicity and the clinically reported grade 4 and 5 events upon CD123-targeting therapy [12, 37, 44, 45]. For CLL1, we find positivity on AML bulk cells in most cases, but lower and interindividually variable expression on LSC suggests that CLL1 is not a universal LSC marker. This is in agreement with a previous report stating CLL1 as a useful marker for LSC but also describing a heterogeneous expression pattern [35]. Given the limited CLL1 expression on normal HSPC and non-hematopoietic tissues, CLL1 may be a preferable target in a selected subgroup of patients. For CLL1-targeting therapy in AML, selection of patients based on the individual level of CLL1 expression may be necessary.

Beside the commonly targeted AML-associated antigens, CD244 and TIM3 have been reported to be overexpressed on LSC and to have a direct leukemia-promoting effect by maintaining the proliferative capability of LSC [22, 32, 46–50]. In our study, we show ubiquitous CD244 expression on AML bulk cells and LSC. However, high CD244 expression on HSPC and monocytes as well as in several vital non-hematopoietic tissues suggest that CD244 is a very unspecific AML-associated antigen. On the contrary, we describe a more suitable expression profile for TIM3, with positivity on AML bulk cells and LSC in most patients and negativity in the majority of normal HSPC, granulocytes, lymphocytes and most normal non-hematopoietic tissues. However, small subpopulations with variations in antigen

expression level, e.g., TIM3-positive T cells, might be underrepresented in our study. In the context of immune activation, upregulation of TIM3 on activated and exhausted T cells could result in on-target toxicity with depletion of TIM3-positive normal T cells [51]. We previously reported that TIM3 is expressed on < 5% of peripheral blood T cells in AML patients at initial diagnosis and relapse [52]. The relevance of TIM3-directed toxicity of CAR T cells against TIM3-positive normal T cells needs to be evaluated. However, TIM3-specific CAR T-cell fratricide or suicide may be limited in a CAR setting with optimized transgene delivery, thereby reducing exhaustion and TIM3 expression to a minor percentage of CAR T cells [53]. In addition, targeted deletion of the TIM3 gene may prevent CAR T-cell fratricide. Beside its relatively suitable expression profile on normal cells, TIM3 is part of an autocrine stimulatory loop that promotes self-renewal of LSC and thereby progression of AML [46]. Considering the pro-leukemic function of TIM3, targeting TIM3 may be less prone to escape via antigen loss and may enable specific elimination of LSC.

Overall, our protein expression data indicate that single-targeting of CD33, CD123, CLL1, TIM3 or CD244 may have antileukemic efficacy in most AML patients. However, on-target off-leukemia toxicity both in hematopoietic and non-hematopoietic tissues as well as high risk of relapse owing to antigen escape and clonal heterogeneity may limit therapeutic success. Among all tested antigens, we report a suitable expression pattern for TIM3, which may qualify for single-targeting or combinatorial targeting approaches in AML.

We hypothesize that combinatorial targeting strategies with well-matched target antigens enhance therapeutic efficacy without increasing on-target off-leukemia toxicity. Several preclinical reports of combinatorial targeting have been published so far and are either based on triplebodies or combinatorial CAR approaches [54–57]. In a combinatorial CAR setting with coexpression of two CARs (CAR + CAR), T cells eliminate any cells expressing at least one of the two targets, thereby reducing the chance of antigen escape. In a setting with coexpression of a CAR and a chimeric costimulatory receptor (CAR + CCR), T cells only eliminate cells that coexpress both targets, thereby limiting cytotoxicity to double-positive tumor cells and relatively sparing single-positive normal tissue [55]. For both approaches, our study defines suitable AML-associated target antigen combinations with non-overlapping expression patterns in normal cells. Out of $n = 15$ possible target antigen pairs, we identify CD33/TIM3 and CLL1/TIM3 as the most suitable antigen combinations, with high coexpression in most AML samples and largely absent coexpression in normal hematopoiesis and non-hematopoietic tissues, excluding tissues with known immune infiltration. Notably, targeting of CD33/TIM3 and CLL1/TIM3 may lead to monocyte depletion. Although the tolerability of monocyte

depletion needs to be further evaluated, there may be a rationale for targeting not only AML cells but also monocytes that are involved in disease pathogenesis and progression [58]. The target antigen combination CD33/TIM3 could be particularly suitable for combinatorial CAR + CCR approaches, thereby minimizing stem cell and myeloid hematotoxicity and prioritizing LSC-targeting. However, this setting might facilitate immune escape of single-positive AML cells. Targeting CLL1/TIM3 could be feasible not only in the CAR + CCR setting, but also in CAR + CAR approaches, thereby maximizing the number of targetable AML cells and minimizing chances of antigen escape.

In summary, our comprehensive analysis of > 300 primary AML samples demonstrates antigen positivity of CD33, CD123, CLL1, TIM3 and CD244 in most cases at initial diagnosis and relapse, irrespective of the genetic background. While none of these antigens are truly AML-specific, we describe a suitable expression profile of TIM3 with limited expression on normal cells. Our coexpression analysis of hematopoietic cells and non-hematopoietic tissues identifies CD33/TIM3 and CLL1/TIM3 as promising antigen combinations that should be validated in dual-targeting immunotherapeutic strategies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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(entfällt in der elektronischen Fassung)