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Die Rolle Natürlicher Killerzellen in der Immunkontrolle akuter Leukämien im Kindesalter

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1. Abkürzungsverzeichnis

ADCC	antidody-dependent cell-mediated cytotoxicity; antikörperabhängige zelluläre Toxizität
ALL	Akute lymphatische Leukämie
AML	Akute myeloische Leukämie
CIK	Zytokin-induzierte Killerzellen
CLL	Chronisch lymphatische Leukämie
CMV	Zytomegalievirus
CR	complete remission, komplette Remission
DCleu	Leukämieantigen präsentierende Zellen
FcyRIIIa	Fc-gamma-Rezeptor-III alpha
GMP	good manufacturing practice
GvHD	graft-versus-host-disease
GvL	graft-versus-leukemia effect
HCV	Hepatitis C Virus
HIV	Humanes Immundefizienzsyndrom
HLA	Humanes Leukozytenantigen
HSZT	Hämatopoetische Stammzelltransplantation
IgG	Immunglobulin G
IL-2, 12, 15, 18	Interleukin 2, 12, 15, 18
iNKT	invariante NKT-Zellen
KIR	Killerzell-Immunglobulin-ähnliche Rezeptoren
MDS	Myelodysplastisches Syndrom
MFI	mean flourescence intensity
MFSC	mean forward scatter
MHC	major histocompatibility complex; Haupthistokompatibilitätskomplex
MIC-A	MHC class I chain-related gene A
MIC-B	MHC class I chain-related gene B
MLC	Mischlymphozytenkultur
MNZ	Mononukleäre Zellen

mRNA	messenger ribonucleic acid; Botenribonukleinsäure
NCAM	neural cell adhesion molecule
NK-Zellen	Natürliche Killerzellen
PBMZ	Periphere mononukleäre Zellen
PCR	Polymerase chain reaction; Polymerasekettenreaktion
rt-PCR	real time polymerase chain reaction
ULBP1-4	UL16 bindendes Protein 1-4

2. Einleitung

a. Natürliche Killerzellen

Natürliche Killer (NK-) Zellen wurden erstmals 1975 als Immunzellen beschrieben, versehen mit der Fähigkeit schnell und ohne vorherige Induktion zytotoxisch aktiv sein zu können[1-3]. Sie stellen neben T- und B-Zellen die dritte eigenständige Lymphozytenpopulation der zellulären Immunabwehr im Blut, sowie im lymphatischen Gewebe dar und werden anhand ihrer Oberflächenmarker als CD3- (negative) Lymphozyten definiert, die CD56 (neural cell adhesion molecule, NCAM) und/oder CD16 (Fc-gamma-Rezeptor-IIIalpha, FcγRIIIa) tragen[4-6]. CD16 ist ein niedrig-affiner Rezeptor für Fc-Fragmente von IgG Antikörpern, der die sogenannte antikörperabhängige zelluläre Zytotoxizität (antibody-dependent cell-mediated cytotoxicity, ADCC) vermittelt. NK-Zellen spielen eine wichtige Rolle in der angeborenen Immunantwort, indem sie eine der ersten Verteidigungslinien gegen Virus-infizierte und entartete Zellen bilden[7, 8]. Im Zuge dieser Immunantwort ist es die Aufgabe der NK-Zellen zytotoxisch aktiv zu sein, Zytokine wie IL-5, IL-10, IL-13, den Wachstumsfaktor GM-CSF sowie die Chemokine MIP-1α, MIP-1β, IL-8, und RANTES zu sekretieren[9-15], um dadurch andere Immunzellen zu rekrutieren. Außerdem wird durch die NK-Zell-Antwort die Zeit überbrückt, die das adaptive Immunsystem benötigt, bis es spezifisch auf Pathogene reagieren kann.

NK-Zellen sind in der Lage zwischen körpereigenen („selbst“) und fremden Strukturen zu differenzieren und somit zu erkennen, gegen welche Zielstrukturen sie vorgehen müssen, und gegen welche nicht (sog. Selbsttoleranz). Karre et al konnten diese Selbsttoleranz erstmals belegen und es entstand die so genannte „missing-self“ – Hypothese (Abb. 1)[16, 17]. Diese „Selbst“-Strukturen konnten schließlich als Haupthistokompatibilitätskomplex (major histocompatibility complex, MHC) Klasse I-Moleküle identifiziert werden. Im Falle der Expression dieser Moleküle, bindet daran ein inhibitorischer NK-Zellrezeptor, die NK-Zelle wird inhibiert und eine zytotoxische Reaktion verhindert. Ohne oder bei verringerter Expression der Klasse I-Moleküle bleibt das inhibitorische Signal aus und bei gleichzeitigem stimulatorischem Signal kommt es zur Aktivierung der NK-Zelle (Abb. 1)[18].

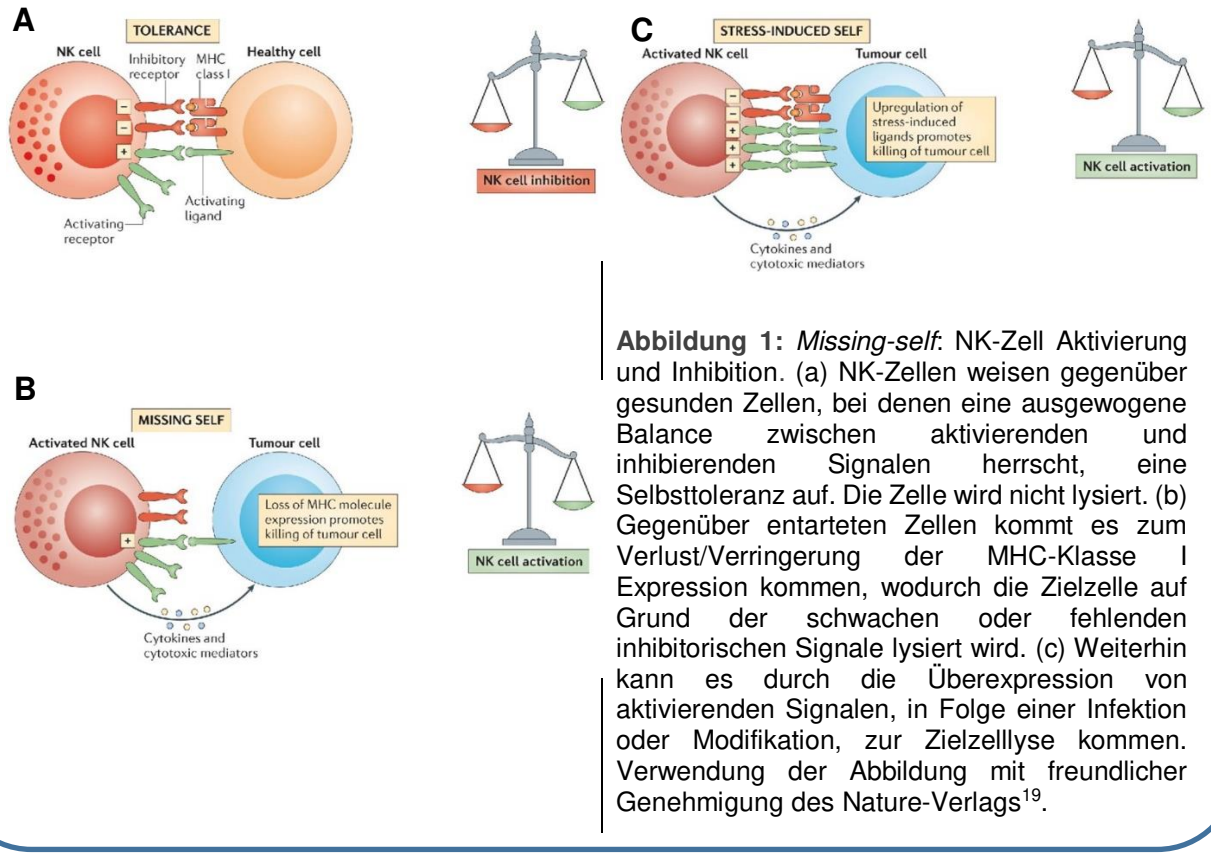


Abbildung 1: *Missing-self*: NK-Zell Aktivierung und Inhibition. (a) NK-Zellen weisen gegenüber gesunden Zellen, bei denen eine ausgewogene Balance zwischen aktivierenden und inhibierenden Signalen herrscht, eine Selbsttoleranz auf. Die Zelle wird nicht lysiert. (b) Gegenüber entarteten Zellen kommt es zum Verlust/Verringerung der MHC-Klasse I Expression kommen, wodurch die Zielzelle auf Grund der schwachen oder fehlenden inhibitorischen Signale lysiert wird. (c) Weiterhin kann es durch die Überexpression von aktivierenden Signalen, in Folge einer Infektion oder Modifikation, zur Zielzelllyse kommen. Verwendung der Abbildung mit freundlicher Genehmigung des Nature-Verlags[19].

Ausschlaggebend für die Reaktivität einer NK-Zelle ist die Balance zwischen verschiedenen aktivierenden und inhibierenden NK-Zell Rezeptoren[17, 20]. Hieraus entstand die *„at least one“* Hypothese[21, 22]. Demnach muss jede NK-Zelle mindestens einen, für das eigene HLA-Klasse I Molekül spezifischen Rezeptor exprimieren, um eine gesunde, eigene Körperzelle mit intakter HLA-Klasse I Expression zu verschonen. In der Folge erweiterten mehrere Studiengruppen diese Ansicht, wonach es entgegen der zuvor postulierten „*at least one*“ Theorie NK-Zellen gibt, die über keinen inhibitorischen Rezeptor für HLA Klasse I verfügen. Diese hyporesponsiven NK Zellen weisen eine verringerte Zytotoxizität auf[23-25]. Erst durch einen als Lizenzierung bezeichneten Prozess erlangt die NK-Zelle durch den Erwerb eines inhibitorischen Rezeptors für „selbst“ schließlich ihre funktionale Reife[26].

b. NK-Zellrezeptoren

Rezeptoren auf der Oberfläche von NK-Zellen regulieren diese inhibitorischen und stimulatorischen Signale. Zwei Subtypen von Rezeptoren, die beim Menschen HLA-Klasse-I-spezifisch sind, bilden hierfür die molekulare Basis. Die Familie der Killerzell-Immunglobulin-ähnlichen Rezeptoren (killer-cell immunoglobulin-like receptors, KIR) und die lektinartigen NKG2-Rezeptoren (Abb. 2).[27]

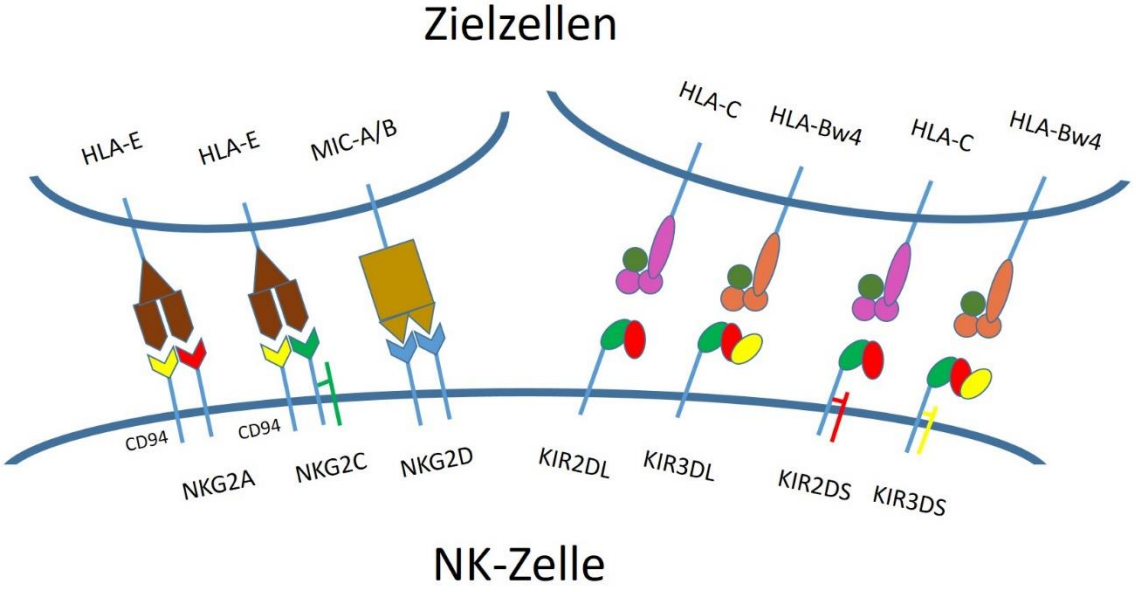


Abbildung 2: Exemplarische schematische Darstellung inhibitorischer (KIR2DL, KIR3DL) und stimulatorischer KIRs (KIR2DS, KIR3DS) sowie Mitgliedern der NKG2-Familie (NKG2A, -C und –D). Verwendung der Abbildung mit freundlicher Genehmigung des Wiley-Blackwell-Verlags[28].

Beide Rezeptorfamilien finden sich sowohl auf NK-Zellen als auch auf einer Subpopulation der T-Zellen. Die Rezeptoren sind nicht in allen Lebewesen zu finden. So besitzt die Maus statt der KIR-Rezeptoren NK-Rezeptoren der lektinähnlichen Ly49-Genfamilie, die jedoch im Menschen wiederum bis auf ein inaktives Genfragment verschwunden sind[27, 29].

Die NKG2 Rezeptoren werden im humanen System in den inhibitorischen NKG2A Rezeptor und die stimulatorischen NKG2C, -D und -E Rezeptoren unterteilt. Sie zählen zu den MHC abhängigen Rezeptoren (den C-Typ-Lektin-Antigen Rezeptoren) und werden auf Chromosom 12 kodiert[30, 31].

	Rezeptor (CD Nomenklatur)	Ligand
	KIR2DL1 (CD158a)	HLA-C2
inhibitorisch	KIR2DL2 (CD158b1)	HLA-C1, C2
inhibitorisch	KIR2DL3 (CD158b2)	HLA-C1
inhibitorisch	KIR3DL1 (CD158e1)	HLA-Bw4
inhibitorisch	KIR3DL2 (CD158k)	HLA-A*3,*11
inhibitorisch	NKG2A (CD159a)	HLA-E
inhibitorisch	LIR-1 (CD85j)	HLA-A, -B, -G
stimulatorisch	KIR2DS1 (CD158h)	HLA-C2
stimulatorisch	KIR2DS2 (CD158j)	unbekannt
stimulatorisch	KIR2DS3	unbekannt
stimulatorisch	KIR2DS4 (CD158i)	HLA-A*11, HLA-C
stimulatorisch	KIR2DS5	unbekannt
stimulatorisch	KIR3DS1 (CD158e2)	unbekannt
stimulatorisch	NKG2C (CD159c)	HLA-E
stimulatorisch	NKG2D (CD314)	ULBP 1-4, MIC-A, MIC-B

Tabelle 1: Liste wichtiger inhibitorischer und stimulatorischer NK-Zellrezeptoren und ihrer Liganden der KIR, LIR und NKG2 Genfamilien

Die Rezeptoren der NKG2-Familie werden nicht nur auf NK-Zellen, sondern auch einer Subpopulation der T-Zellen exprimiert. Die NKG2 Rezeptoren bilden mit Ausnahme von NKG2D mit CD94 Heterodimere[32]. Letzterer ist mit dem Adaptorprotein DAP10 assoziiert, bildet als einziger Rezeptor der NKG2 Familie Homodimere und bindet an stress-induzierte Liganden wie z.B. MICA und MICB (*MHC class I chain-related protein*

A/B) und den virusinduzierten ULBPs (*UL16 binding proteins)*[33-38]. Der mit CD94 ein Heterodimer bildende Sowohl der inhibitorische Rezeptor NKG2A als auch der aktivierende Rezeptor NKG2C bildet mit CD94 ein Heterodimer und bindet an das nicht klassische HLA-E Molekül, welches ubiquitär exprimiert wird[39-41]. HLA-E präsentiert die Signalpeptide der klassischen HLA-Moleküle A, B und C und vermittelt so den gesamten HLA- Klasse I Status der Zelle. Zwei ITIM Motive (*immunoreceptor tyrosine-based inhibitory motifs)* übernehmen die inhibitorische Signalkaskade im zytoplasmatischen Teil von NKG2A[31] (Houchins, *et al* 1991) während die ITAM Motive (*immunoreceptor tyrosine-based activation motifs)* von DAP12, das mit NKG2C assoziiert ist, ein stimulatorisches Signal erzeugen und mit einer geringeren Affinität an HLA-E binden [42].

Die KIR-Rezeptoren lassen sich ebenso wie die NKG2-Familie in stimulatorische und inhibitorische Rezeptoren unterscheiden. Der Unterschied zwischen diesen beiden Gruppen besteht aus einem inhibitorischen Motiv der zytoplasmatischen Domäne, das den stimulatorischen KIR fehlt. Die Spezifität der 6 stimulatorischen KIR-Rezeptoren ist weitestgehend unbekannt und könnte auch Nicht-HLA-Liganden umfassen[43].

Die Liganden der inhibitorischen KIR sind HLA-Klasse-I Moleküle, vor allem HLA-C-Allele. Sowohl der inhibitorische KIR2DL1 als auch der stimulatorische KIR2DS1 besitzen als einzige Liganden Moleküle, die zur HLA-C-Gruppe 2 (HLA-C2) gezählt werden, wobei die Affinität von HLA-C2 zu KIR2DL1 höher ist als zu KIR2DS1[44]. Für die inhibitorischen Rezeptoren KIR2DL2 und KIR2DL3 sowie für den stimulatorischen Rezeptor KIR2DS2 stellen in abnehmender Affinität HLA-C-Gruppe 1-Moleküle (HLA-C1) die Liganden[45-47]. Zwischen KIR und HLA-C Molekül interagiert und inhibiert die Bindung von KIR2DL1 und HLA-C2 am stärksten, gefolgt von KIR2DL2/HLA-C1 und KIR2DL3/HLA-C1[48]. KIR2DL2 sowie wenige KIR2DL3-Allele können im Gegensatz zu KIR2DL1 mit beiden HLA-C Molekülen interagieren. Die höchste Bindungsaffinität besitzen diese beiden Rezeptoren jedoch für HLA-C1[49-51]. KIR3DL1 besitzt als Liganden HLA-Bw4[52-54] während HLA-Bw6 keinen KIR-Liganden darstellt[54]. KIR3DS1 besitzt ähnliche Ligand-Spezifitäten wie KIR3DL1, die Interaktion mit HLA-Bw4 ist jedoch stark Peptid-abhängig[55, 56]. HLA-A3 repräsentiert den Liganden für KIR3DL2 und HLA-A11 für KIR3DL2 und KIR2DS2[57, 58]. Zusätzlich interagiert KIR3DL2 auch mit HLA-F und löslichen CpG-Oligodesoxynukleotiden [59, 60]. Eine breitere Spezifität besitzt

KIR2DS4 für die Liganden HLA-C1, HLA-C2, HLA-F und HLA-A1102[59, 61]. Für KIR2DS5 und KIR2DL5 konnten bisher keine Liganden identifiziert werden (Tab. 1).

c. KIR-Genetik

Es gibt 15 verschiedene KIR-Loci, die zu insgesamt 17 verschiedenen KIR-Genen führen. Diese sind dicht gruppiert im Leukozyten-Rezeptor-Komplex auf Chromosom 19q13.4 lokalisiert, weisen eine hohe genetische Diversität auf und treten interindividuell in unterschiedlicher Anzahl und in unterschiedlichen allelischen Varianten auf (Abb. 3)[21].

Von den definierten 17 verschiedenen KIR-Genen besitzen acht inhibitorische Eigenschaften (KIR2DL1-3, KIR2DL5A-B, KIR3DL1-3) während sechs KIR-Gene stimulatorische Eigenschaften aufweisen (KIR2DS1-5, KIR3DS1, Abb. 1). Man unterscheidet je nach Struktur 3 Gruppen von KIR-Genen: i) die KIR2D Gene (KIR2DP1, KIR2DL1-3 und KIR2DS1-5), die für zwei extrazelluläre Proteine kodieren und die 8 Exons sowie das inaktivierte Pseudoexon 3 besitzen. Innerhalb dieser Gruppe unterscheiden sich die Gene KIR2DL1-3 von KIR2DS1-5 lediglich durch die Länge des für den zytoplasmatischen Teil kodierenden Abschnitt in Exon 9. ii) die übrigen KIR2D Gene KIR2DL4 und KIR2DL5 mit einem produktiven Exon 3; iii) die KIR3D-Gene (KIR3DL1-3 und KIR3DS1), kodierend für extrazelluläre Proteine, die ebenfalls 9 Exons besitzen und sich untereinander vor allem durch die für den zytoplasmatischen Teil des Rezeptors kodierende Region unterscheiden[43].

Innerhalb des KIR-Lokus sind die KIR-Gene in Haplotypen organisiert, die in 2 Gruppen eingeteilt werden (Abb. 3): Haplotyp A setzt sich aus einem definierten Set von 6 Genen zusammen (KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2 und KIR2DS4). Die genetische Variabilität dieses Sets, in dem mit Ausnahme von KIR2DS4 alle KIRs inhibitorisch wirken, ist auf Allelebene beschränkt. Haplotypen der Gruppe B unterscheiden sich im Gegensatz dazu vor allem in Art und Anzahl der vorhandenen Gene. Letztere wirken überwiegend stimulatorisch (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 und KIR3DS1).

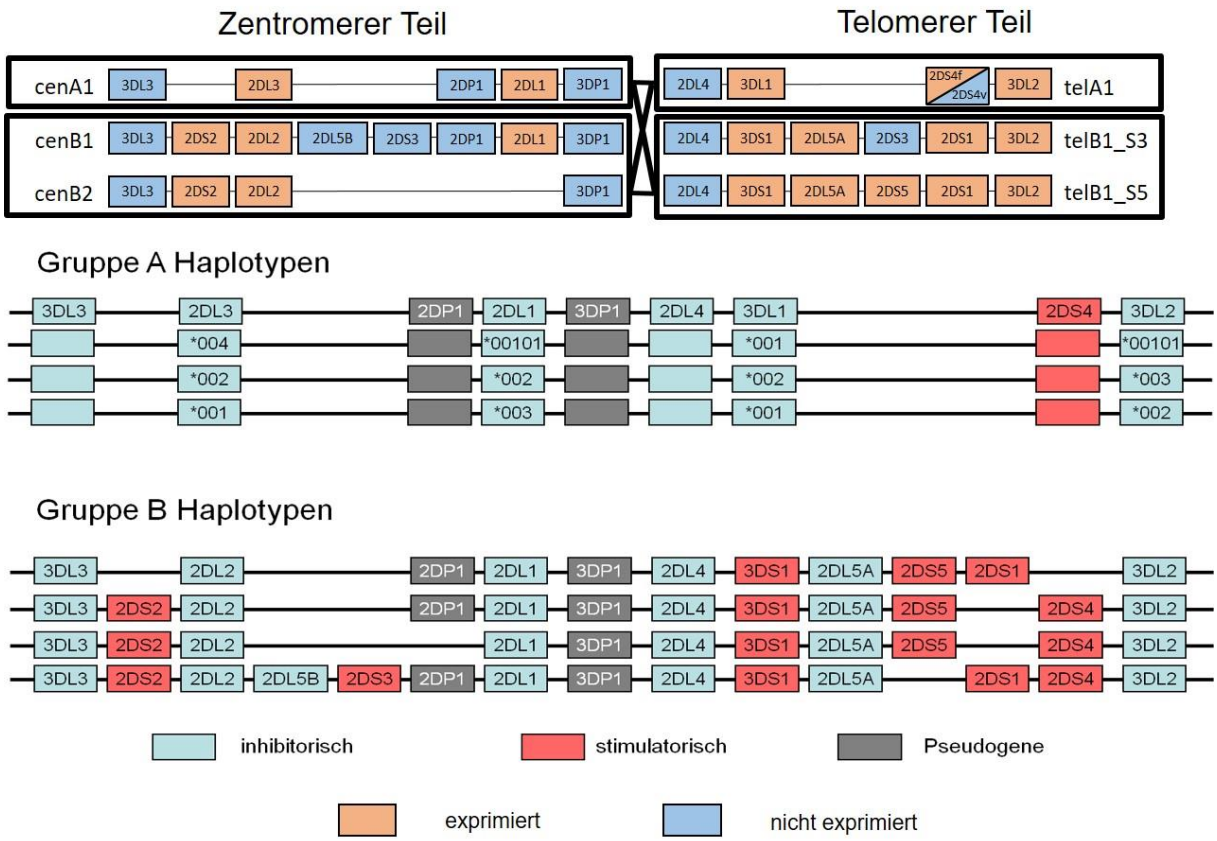


Abbildung 3: Genomische Organisation und schematische Darstellung der Gruppe A und B Haplotypen. Unterteilung des KIR-Locus in einen zentromeren und einen telomeren Bereich. Die häufigen Haplotypen setzen sich genetisch aus drei verschiedenen zentromeren und drei telomeren Teile zusammen. Es zeigt sich ein extensiver Polymorphismus der KIR-Gene. Gruppe A Haplotypen setzen sich immer aus dem gleichen Satz an KIR-Genen zusammen und variieren nur auf Ebene der Allele. Gruppe B Haplotypen zeichnen sich durch einen Anwesenheits-/Abwesenheits-Polymorphismus aus (Polygenie) und können 5 weitere stimulatorische KIR-Gene (KIR2DS2, KIR2DS3, KIR3DS1, KIR2DS5 und KIR2DS1) sowie das inhibitorische KIR2DL5 beinhalten. Verwendung der Abbildung mit freundlicher Genehmigung des Wiley-Blackwell-Verlags[62].

Jeder KIR-Haplotyp besteht aus 4 sogenannten „Framework“-Genen, die, bis auf wenige Ausnahmen, in jedem Individuum vorhanden sind. Die KIR Genanordnung wird zentromer von KIR3DL3 und telomer von KIR3DL2 flankiert. Zentral liegen KIR3DP1 und KIR2DL4. In der Vergangenheit konnten Assoziationen des KIR-Polymorphismus mit der Häufigkeit und dem Verlauf viraler Infektionen (HIV und HCV), mit Autoimmunkrankheiten (Psoriasis, rheumatoide Arthritis) sowie mit dem Auftreten solider Tumoren (Zervixkarzinom) gezeigt werden[63].

d. Lizensierung

Eine humane NK-Zelle kann jede beliebige Kombination der KIR-Rezeptoren, die im Genom der jeweiligen Person vorhanden ist, exprimieren[22]. Ihr volles funktionales Potenzial kann eine NK-Zelle jedoch nur dann ausschöpfen, wenn sie mindestens einen inhibitorischen Rezeptor für die eigene HLA-Klasse I besitzt. Dieser Prozess, der sich aus dem „missing-self“ Modell[16, 17] ergibt, wird Lizenzierung oder Erziehung genannt[26]. Hierbei wird die Effektivität der NK-Zellantwort maßgeblich von der Konstellation der inhibitorischen KIR und des NKG2A-Rezeptors auf der einzelnen Zelle beeinflusst. Das wird erkennbar, wenn NK-Zellen, die keinen inhibitorischen KIR für die HLA-Klasse-I-Liganden HLA-C1, -C2 oder -Bw4 exprimieren, trotzdem in vollem Umfang funktionsfähig sein können, solange sie durch die Expression von NKG2A effektiv inhibiert werden.

Spender	Lizensierung		Empfänger	Alloreaktive NK-Zellen	
KIR Ligand	Lizensiert	Unlizensiert	KIR Ligand	Lizensiert	Unlizensiert
C1/C1	KIR2DL3	KIR2DL1	C1/C1	-	KIR2DL1
C1/C2	KIR2DL1/KIR2DL3	-	C1/C1	KIR2DL1	-
C2/C2	KIR2DL1	KIR2DL3	C1/C1	KIR2DL1	-
C1/C1	KIR2DL3	KIR2DL1	C1/C2	-	-
C1/C2	KIR2DL1/KIR2DL3	-	C1/C2	-	-
C2/C2	KIR2DL1	KIR2DL3	C1/C2	-	-
C1/C1	KIR2DL3	KIR2DL1	C2/C2	KIR2DL3	-
C1/C2	KIR2DL1/KIR2DL3	-	C2/C2	KIR2DL3	-
C2/C2	KIR2DL1	KIR2DL3	C2/C2	-	KIR2DL3

Tabelle 2: NK-Zelllizensierung und Alloreaktivität durch HLA-C kodierte KIR-Liganden

Die funktionstüchtigsten NK-Zellen in Bezug auf die Detektion von HLA-Klasse-I Veränderungen sind jene, die nur einen einzigen inhibitorischen KIR für einen bestimmten HLA-Klasse-I-Liganden exprimieren (sogenannte „single-KIR“ NK-Zellen). Eine Verminderung der HLA-B-Expression wird somit von einer KIR3DL1

exprimierenden NK-Zelle nur dann erkannt, wenn sie nicht gleichzeitig KIR2DL1 (HLA-C2), KIR2DL2 (HLA-C1 und –C2), KIR2DL3 (HLA-C1) oder NKG2A exprimiert.
Die Lizenzierung ist auch der zugrundeliegende Mechanismus, der eine Autoreaktivität der NK-Zellen, welche dem eigenen Organismus gefährlich werden könnte, verhindert. Aufgrund der Lokalisation von KIR- und HLA-Klasse-I-Genen auf unterschiedlichen Chromosomen wird ein Toleranzmechanismus benötigt, der das Auftreten autoreaktiver NK-Zellen verhindert. Es lassen sich jedoch auch im NK-Zell-Repertoire gesunder Individuen NK-Zellen finden, die weder NKG2A noch einen passenden inhibitorischen KIR exprimieren[24]. Diese potenziell autoreaktiven NK-Zellen verbleiben in einem hyporesponsiven Status und erlangen im Gegensatz zu NK-Zellen, die inhibitorische KIR für körpereigene HLA-Klasse-I exprimieren, keine volle Funktionalität. Der Mechanismus der Lizenzierung spielt auch bei einer möglichen Alloreaktivität in der klinischen Transplantationssituation eine wichtige Rolle[24-26, 64-66].

3. Ziele der vorgelegten Arbeiten

Das Ziel der folgenden Studien lässt sich im Wesentlichen in drei Hauptfelder unterteilen.

1. Es sollten molekulare Suszeptibilitätsmarker im NK-Zellsystem identifiziert werden, die bei der Entstehung und der Behandlung von Leukämien im Kindesalter eine entscheidende Rolle spielen. Es wurden hierzu drei verschiedene Studien durchgeführt. Mittels PCR wurden die Gene der NK-Zellrezeptoren (KIR) in hoher Auflösung bestimmt, um so die Frage zu beantworten, ob das Vorhandensein spezifischer KIRs Risikofaktoren für die Entstehung einer akuten lymphatischen Leukämie im Kindesalter darstellt. Darüber hinaus sollte die Frage beantwortet werden, ob es einen Zusammenhang mit dem klinischen Verlauf der ALL in einer uniform behandelten Patientengruppe gibt (siehe Abschnitt 3. I). In einer zweiten, größeren Kohortenstudie wurden ausgewählte Liganden von NK-Zellrezeptoren im HLA-System untersucht. Es sollte eine mögliche Assoziation mit dem Auftreten von Leukämien untersucht und ein Zusammenhang zwischen Krankheitsrückfällen und einzelnen HLA-Genen hergestellt werden (siehe Abschnitt 3. III). In einer dritten Studie sollte ermittelt werden, durch welche Mechanismen leukämische Zellen der Erkennung durch NK-Zellen entkommen. Hierfür wurde die Expression HLA-Klasse I-kodierter NK-Zell-Liganden auf den gesunden und entarteten Lymphozyten pädiatrischer ALL-Patienten anhand von durchflusszytometrischen Analysen untersucht (siehe Abschnitt 3. IV).

2. Ein zweiter Schwerpunkt der vorgelegten Arbeit bestand in Untersuchungen zur zeitlichen Veränderung des NK-Zellrepertoires einschließlich der Subpopulationen bei Kindern mit ALL bzw. AML im Krankheitsverlauf. Es sollten dabei insbesondere „*Escape*“-Mechanismen von Leukämiezellen gegenüber der NK-vermittelten Immunabwehr untersucht werden. In wieweit beeinflusst der KIR Polymorphismus die NK-Zellfunktion? Welche Chancen und Risiken bestehen in einem adoptiven NK-Zelltransfer als neuem immuntherapeutischem Ansatz? (siehe Abschnitt 3. II). Weiters wurden hierzu

detaillierte Bestimmungen von Immunzellpopulationen bei ALL-, AML- und CLL-Patienten im Vergleich zu gesunden Kontrollpersonen durchgeführt (siehe Abschnitt 3. V).

3. Aus Vorstudien von Erwachsenen mit AML war bekannt, dass das Überleben von Patienten nach hämatopoetischer Stammzelltransplantation positiv beeinflusst wird, wenn der jeweilige Stammzellspender möglichst viele dem KIR-B Haplotyp assoziierte Gene besitzt. Es sollte im dritten Teil der vorgelegten Arbeit untersucht werden, ob dies auch im Kindesalter zutrifft.
   Hierzu wurden DNA-Proben von 317 im Rahmen der multizentrischen, prospektiven Therapieoptimierungsstudie ALL-SZT-BFM-2003 behandelten Hochrisiko-ALL-Patienten und deren Stammzellspender mittels PCR untersucht. Ziel war es, die Auswirkungen von KIR- und HLA-Genetik des Stammzellspenders auf die Prognose von Hochrisiko-ALL-Patienten nach hämatologischer Stammzelltransplantation zu analysieren und der Frage nachzugehen, ob das Auftreten von Transplantations-assoziierter Mortalität ebenfalls davon beeinflusst wird. Anhand der Ergebnisse sollte ein Auswahlsystem für Stammzellspender entwickelt werden, wofür es bis jetzt nur wenige aussagekräftige Studien im Kindesalter gibt. In Relation gesetzt und um ein wichtiges Charakteristikum bereichert sollen hierdurch potenziell die Überlebenschancen für Kinder mit Leukämien weiter verbessert werden können (siehe Abschnitt 3. VI).

4. Eigene Untersuchungen und Ergebnisse

I. KIR-Gene sind nicht mit dem Auftreten von Leukämien im Kindesalter assoziiert

Lack of association between KIR genes and acute lymphoblastic leukemia in children

Babor F, Manser A, Schönberg K, Enczmann J, Borkhardt A, Meisel R, and Uhrberg M, *Blood*, 2012. 120(13): 2770-2.

2011 veröffentlichten Almalte und Kollegen eine Studie, in der die KIR Genetik bei Patienten mit akuter lymphatischer Leukämie (ALL) untersucht wurde[67]. Es wurde die Frage gestellt, ob die individuelle genetische Ausstattung und Konfiguration der einzelnen KIR-Loci die Suszeptibilität für die Entwicklung einer ALL im Kindesalter beeinflusst. Der Großteil der 172 Kinder war franko-kanadischer Herkunft, zum Vergleich dienten 245 gesunde Kontrollen. Obwohl zu diesem Zeitpunkt bereits mehrere Assoziationsstudien zu einzelnen Entitäten (z.B. Morbus Crohn) existierten[68], war dies die bis dahin einzige Untersuchung an ALL-Patienten. Die Studie von Almalte et al lieferte bemerkenswerte Ergebnisse: das Auftreten von allen sechs untersuchten stimulatorischen KIR-Genen war bei ALL-Patienten im Vergleich zu Gesunden signifikant verringert.

Das Ziel der vorliegenden Studie war es, in einer unabhängigen Kohorte die stimulatorischen, aber auch inhibitorischen KIR-Gene zu analysieren und mit einer repräsentativen Kontrollgruppe zu vergleichen. Hierfür wurden 218 pädiatrische Patienten untersucht, die zu 92% deutschen Ursprungs waren und im Zeitraum von 1992-2012 an der Klinik für Kinder-Onkologie, -Hämatologie und Klin. Immunologie der Heinrich-Heine-Universität Düsseldorf aufgrund einer B- (n=185) oder T-ALL (n=33) behandelt wurden. Als Vergleich diente eine Kontrollkohorte aus 204 gesunden Spendern. Die durchgeführte Analyse beinhaltete sowohl die PCR-basierte Genotypisierung der 6 stimulatorischen (*KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5* und *KIR3DS1*), aber auch der 6 inhibitorischen KIR-Gene (*KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1* und *KIR3DL2*) nach Vilches et al[69]. Zur Qualitätskontrolle wurden 10% der Proben zufällig ausgewählt und mit einer unabhängigen Typisierungsmethode retypisiert[70]. Proben, die einen seltenen KIR-Genotyp ergaben (<0,5% Frequenz in der Normalbevölkerung) wurden ebenfalls auf

diese Weise retypisiert. Insgesamt 9 Proben wurden aufgrund widersprüchlicher Mehrfachtypisierungen von der Studie ausgeschlossen.

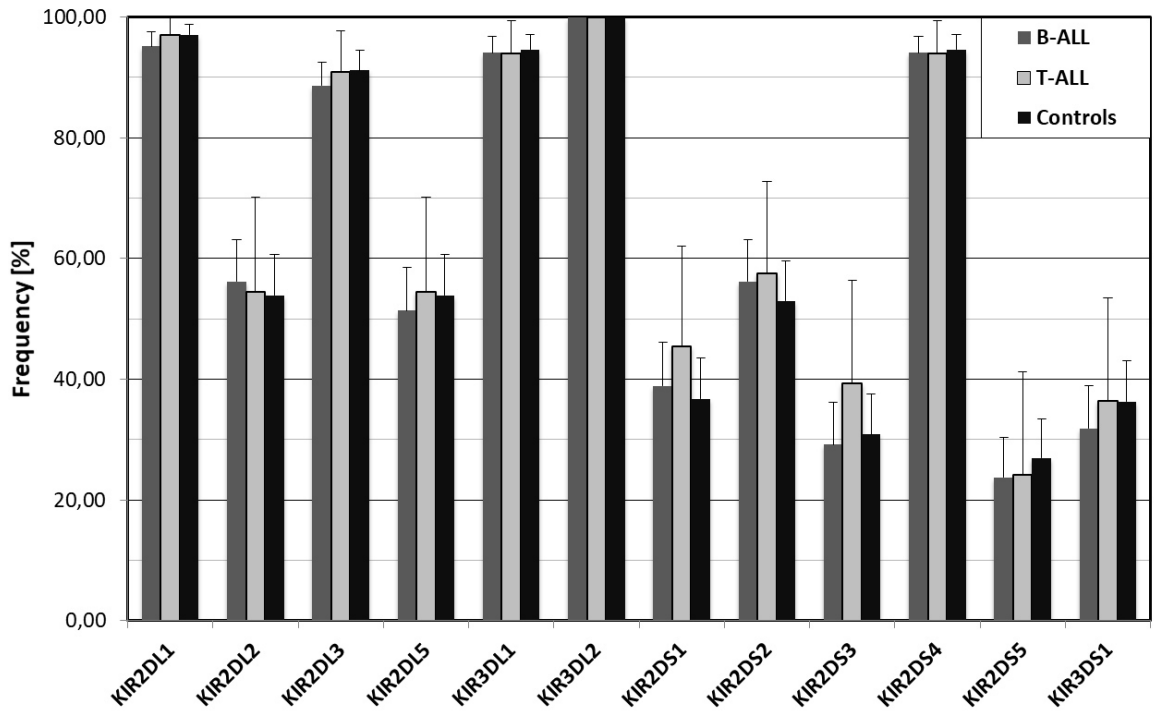


Abbildung 4: Keine Assoziation zwischen pädiatrischer ALL und der Häufigkeit von KIR-Genen. Analyse der Frequenz von 6 inhibitorischen (KIR2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 3DL2) und 6 stimulatorischen KIR Genen (KIR2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1) in pädiatrischen ALL Patienten. Verglichen wurden 185 Patienten mit B-ALL und 33 Patienten mit T-ALL mit 204 gesunden Spendern. Verwendung der Abbildung mit freundlicher Genehmigung des ASH-Verlags[71].

Es zeigte sich, dass im Patientenkollektiv die Häufigkeit der inhibitorischen und stimulatorischen KIR Gene vergleichbar war mit der Kontrollgruppe (Abb. 4). Auch in den Frequenzen der KIR-Genotypen sowie der Zugehörigkeit zu den KIR-Haplotypen A und B ergaben sich keine Unterschiede zur gesunden Population. In einem letzten Schritt folgten Analysen der zentromeren und telomeren Haplotypen sowie der kumulativen Anzahl der stimulatorischen KIR-Gene. Auch diese Untersuchung ergab keine signifikanten Unterschiede zwischen gesunden Kindern und Kindern, die eine ALL entwickelt haben.

Die Ergebnisse der vorgelegten Studie stehen in deutlichem Gegensatz zu der kurz zuvor veröffentlichten Analyse von Almalte et al[67].

Die Bestimmung sowohl stimulatorischer als auch inhibitorischer KIR-Gene stellt eine wichtige Plausibilitätskontrolle dar, da die Gene eine starke Ähnlichkeit zueinander aufweisen und in einer Vielzahl allelischer Variationen vorliegen. Zusätzlich stellt die Genotypisierung älterer Rückstellproben häufig eine technische Herausforderung dar, da es zu verminderter Amplifikationseffizienz kommen kann.

Durch das *linkage disequilibrium* zwischen stimulatorischen und inhibitorischen Genen wäre zu erwarten, dass in der Studie von Almalte et al auch inhibitorische Gene signifikante Abweichungen in der ALL-Kohorte aufweisen (z.B. KIR2DL2, das eng an KIR2DS2 gekoppelt ist). Diese Plausibilitätskontrolle wurde von den Autoren jedoch nicht durchgeführt. In einer Nachfolgestudie von Venstrom et al wurden ebenfalls keine erhöhten oder verringerten Frequenzen für stimulatorische oder inhibitorische KIR Gene in Erwachsenen mit AML gefunden[72].

Zusammenfassend konnte in der vorliegenden Studie eine Assoziation von stimulatorischen KIR-Genen mit dem Auftreten einer akuten lymphatischen Leukämie im Kindesalter nicht belegt werden.

Der Arbeitsgruppe von Almalte und Kollegen wurde zeitgleich die Möglichkeit gegeben auf die im Gegensatz zu ihren Arbeiten stehenden Ergebnisse zu antworten. Zwar gehen Almalte et al auf die Problematik der Typisierungsmethodik ein und erwähnen technische Schwierigkeiten ihrerseits bei der Typisierung älterer Proben. Es wird jedoch verabsäumt die Typisierungsmethode nach der die angegebenen KIR-Gene untersucht worden sind explizit zu nennen. Eine Retypisierung von Patienten- und Kontrollproben erbrachte laut den Autoren eine Bestätigung ihrer zuvor publizierten Ergebnisse. Die von uns für notwendig befundene Bestimmung der inhibitorischen KIR-Gene wurde von Almalte und Kollegen in Aussicht gestellt, Ergebnisse hierfür wurden jedoch nicht dargelegt. Für eine eindeutige Klärung der Diskrepanz der Ergebnisse der beiden Studien wäre eine einheitliche Methode zur Typisierung der Patienten- und Kontrollproben beider Kohorten erforderlich. Je nach DNA-Qualität kämen hierfür die Methoden nach Uhrberg et al[70] oder Vilches et al[69] in Frage.

II. Die Rolle von KIR-Genen und deren Liganden in der Leukämiekontrolle

The role of KIR genes and ligands in leukemia surveillance.

Babor F, Fischer JC, and Uhrberg M, *Frontiers in Immunology*, 2013. 4, 27.

Alloreaktive T-Zellen spielen eine wichtige Rolle bei der Eliminierung residualer Leukämiezellen nach hämatopoetischer Stammzelltransplantation (HSZT). Diese Lymphozytensubpopulation ist jedoch auch hauptverantwortlich für die Entstehung der akuten bzw. akuten und chronischen Reaktion der Spenderzellen gegen Gewebe des Empfängers (graft-versus-host-disease, GvHD). Die Alloreaktivität der transplantierten Immunzellen richtet sich gegen die fremden Histokompatibilitätsantigene des Empfängers, die jedoch nicht nur auf Leukämiezellen, sondern auch auf Zellen anderer Gewebearten zu finden sind. Im Falle einer GvHD werden Strukturen wie Leber-, Haut- oder Darmzellen von zytotoxischen Effektor-T-Zellen angegriffen. Es gibt unterschiedliche Strategien, um diese potenziell schwerwiegende Komplikation zu vermeiden, indem T-Zellsubgruppen aus dem Transplantat eliminiert werden, z.B. durch CD6+ T-Zelldepletion[73], CD3+/CD19+ T- und B-Zelldepletion[74] oder eine TCR-αβ/CD19 Depletion[75]. Es verbleiben großteils CD34+ Stamm- und NK-Zellen im Transplantat während potenziell GvHD-auslösende T-Zellen zum größten Teil eliminiert werden.

Der klinische Stellenwert von NK-Zellen bei der HSZT konnte von Ruggeri et al[76] eindrucksvoll gezeigt werden: AML Patienten, die eine haploidente HSZT von einem Spender mit KIR-Liganden-Mismatch erhielten, waren einem starken graft-versus-leukemia (GvL-) Effekt ausgesetzt. Die NK-Zellen reagierten somit gegen die fremden (Leukämie-)zellen und eliminierten diese. Weiters begünstigen alloreaktive NK-Zellen das Engraftment nach HSZT und reduzieren das Risiko einer GvHD. Andere Studien konnten diese Ergebnisse jedoch nicht konsistent bestätigen[77-79]. Die unterschiedlichen Transplantationsprotokolle, die in den spezialisierten Zentren zur Anwendung kommen, könnten hierfür eine mögliche Erklärung sein. Es entwickelten sich in der Folge unterschiedliche Spenderauswahlmodelle, die darauf beruhen, dass NK-Zellen von Spendern dann alloreaktiv (=lizensiert) sind, wenn sie einen inhibitorischen KIR-Rezeptor exprimieren und zusätzlich der jeweilige Ligand (HLA-C1, -C2 oder -Bw4) im Empfänger nicht vorhanden ist[80, 81].

Durch die Koadministration von Zytokinen wie Interleukin-(IL-)2, IL-15 und IL-21 oder von Kombinationen aus IL-12 und IL-18 werden NK-Zellen aktiviert und das Überleben, die Proliferation, die Regulation zytotoxischer Moleküle und von Adhäsionsstrukturen, die Zytokinproduktion sowie der GvL-Effekt gesteigert[82]. IL-2-aktivierte NK-Zellen sind in der Lage sowohl *in vitro* als auch *in vivo* bösartig veränderte Zellen zu eliminieren und so das Überleben von NOD/SCID Mäusen mit multiplem Myelom oder metastasiertem Neuroblastom zu verlängern[83]. Durch die kurze Halbwertszeit der Interleukine sind wiederholte Gaben notwendig, die akute Toxizitäten und die unerwünschte Aktivierung anderer Zellpopulationen hervorrufen können. IL-2 ist an der Erhaltung regulatorischer T-Zellen beteiligt. Diese kann die Tumorabwehr behindern, wohingegen IL-15 diese Eigenschaft nicht teilt und als GMP-Produkt zur *in vivo* Stimulation bereits Zulassung als Arzneimittel gefunden hat.

NK-Zell-vermittelte Therapiestrategien bewirken somit ähnlich wie T-Zelltherapien einen GvL-Effekt ohne dabei jedoch eine GvHD auszulösen. Dies konnte auch an Patienten unter konventioneller Chemotherapie gezeigt werden, die keiner Stammzelltransplantation unterzogen wurden. Bereits 2005 wurden adoptive haploidente NK-Zellen bei AML Patienten eingesetzt[84]. Nach einem chemotherapiebasierten Konditionierungsregime mittels Cyclophosphamid, Methylprednisolon oder Fludarabin, erhielten die Patienten haploidente NK-Zellen begleitet von subkutanen IL-2 Infusionen für 2 Wochen. Die NK-Zellen expandierten *in vivo* und ihre Funktionalität war auch nach 2 Wochen noch erhalten. Zu beobachten war jedoch auch, dass die intensive Konditionierung sowie die IL-2 Gaben zu erheblichen Toxizitäten und verlängerter Hospitalisierung führten.
Rubnitz et al[85] wählten in einer darauffolgenden Studie für pädiatrische AML-Patienten nach Erreichung einer kompletten Remission ein milderes Konditionierungsregime bevor ein adoptiver Transfer haploidenter NK-Zellen durchgeführt wurde. Auch im Rahmen dieser Studie erhielten die Patienten mehrere Gaben IL-2. Die Ergebnisse dieser Studie bildeten die Grundlage für eine Phase-II-Studie einer adoptiven NK-Zelltherapie als Konsolidierungstherapie für Kinder mit AML. Es folgten weitere Studien, die zeigen konnten, dass die Infusion aufgereinigter NK-Zellen sicher durchführbar ist und dass die alloreaktiven NK-Zellen in der Lage sind, die Leukämiezellen der Empfänger zu eliminieren ohne schwerwiegende Toxizität oder

GvHD[86, 87]. Die alleinige NK-Zelltherapie erscheint jedoch bei Patienten mit Leukämien zur Remissionsinduktion bzw. –erhaltung nicht in allen Fällen ausreichend.

III. HLA-C2: Ein unabhängiger Risikofaktor für die Entstehung einer ALL im Kindesalter und deren späten Rückfall

The KIR ligand C2 is associated with increased susceptibility to childhood acute lymphoblastic leukemia and confers an elevated risk for late relapse

Babor F, Manser AR, Fischer JC, Scherenschlich N, Enczmann J, Chazara O, Moffett A, Borkhardt A, Meisel R, and Uhrberg M, *Blood*, 2014, Oct 2;124(14):2248-51

Obwohl die Assoziationsstudie von Almalte et al[67] eine Rolle der stimulatorischen KIR-Gene bei der Entstehung pädiatrischer ALL nahelegte, konnte eine eigene Studie an einem größeren Kollektiv durch Typisierung der stimulatorischen aber auch der inhibitorischen KIR-Gene diese Ergebnisse nicht bestätigen[71]. In der vorliegenden Nachfolgestudie sollte Analyse um die Rolle der KIR-Gene und –Allele sowie deren Liganden (HLA-Klasse-I) erweitert werden. Hierfür wurde eine pädiatrische ALL-Kohorte (n=320) KIR-genotypisiert, HLA-typisiert und mit einer Kontrollkohorte von 1515 gesunden Spendern verglichen. Die KIR-Genotypisierung wurde mit Sequenz-spezifischen Primern per Polymerasekettenreaktion durchgeführt[69]. Zusätzlich wurden 10% der Proben zufällig ausgewählt und mit einer unabhängigen Methode typisiert[70]. Hierbei ergab eine vollständige Übereinstimmung der Ergebnisse. Die HLA-Typisierung erfolgte mittels Luminex-Verfahren (One Λ), zusätzlich wurden die HLA-C1/C2 Epitope mittels PCR-SSP typisiert[88].

Zunächst wurden die zu vergleichenden Kohorten anhand des HLA-C1/C2 Dimorphismus in 3 Gruppen eingeteilt: HLA-C1 homozygote Individuen, HLA-C1/C2 heterozygote Individuen und HLA-C2 homozygote Individuen. Überraschenderweise war der Anteil an HLA-C1/C1 homozygoten Individuen in der Patientenkohorte signifikant verringert (p=0,0051, Tab. 3), während eine HLA-C2/C2 Homozygotie im Vergleich zur Kontrollkohorte überrepräsentiert war (p=0,0764, Tab. 3). Auch die Anzahl an C2-Trägern (HLA-C1/C2 oder HLA-C2/C2) war in der ALL-Kohorte unabhängig vom Immunphänotyp signifikant erhöht (p=0,0037).

	Patienten		Gesunde			
	n	%	n	%	OR (95% CI)	P
ALL						
C1/C1	98	30,6	592	39,1	0,69 (0,53-0,89)	0,0051
C1/C2	161	50,3	695	45,9	1,19 (0,94-1,52)	0,1563
C2/C2	61	19,1	228	15,0	1,33 (0,97-1,82)	0,0764
C1 Allele	357	55,8	1879	62,0	0,77 (0,65-0,92)	0,0037
C2 Allele	283	44,2	1151	38,0	1,29 (1,09-1,54)	
B-ALL, kaukasischer Herkunft						
C1/C1	57	27,9	420	41,2	0,54 (0.39-0.75)	0,0004
C1/C2	110	53,9	463	45,4	1,44 (1.07-1.95)	0,0312
C2/C2	37	18,1	137	13,4	1,44 (0.97-2.15)	0,0987
C1 Allele	224	54,9	1303	63,8	0,69 (0,56-0,85)	0,0008
C2 Allele	184	45,1	737	36,2	1,45 (1,17-1,80)	
Bw4/Bw4	15	11,0	139	13,7	0,78 (0,44-1,37)	0,4243
Bw4/Bw6	68	50,0	481	47,6	1,10 (0,77-1,58)	0,6478
Bw6/Bw6	53	39,0	391	38,7	1,01(0,70-1,46)	1,0000

Tabelle 3: HLA Klasse I kodierte KIR Liganden und pädiatrische ALL

Für die weiteren Analysen wurde die Patientenkohorte auf Kinder kaukasischer Herkunft vereinheitlicht, da die Distribution von HLA-C1 und HLA-C2 zwischen verschiedenen Ethnizitäten sehr unterschiedlich ist. Auch in der ethnisch angeglichenen Kohorte von 204 Patienten waren HLA-C1-homozygote Individuen signifikant unterrepräsentiert. Bemerkenswerterweise gab es bezüglich einer HLA-C1-Trägerschaft keinen signifikanten Unterschied zur Kontrollkohorte, während der Anteil an HLA-C2-Trägern in der ALL-Kohorte signifikant erhöht und somit der Anteil an HLA-C1 homozygoten Individuen signifikant verringert war (p=0,0004, Tab. 3). Hieraus kann die Hypothese abgeleitet werden, dass HLA-C2 einen Risikofaktor für die

Entstehung einer pädiatrischen ALL darstellt und in gleichem Maße die Entstehung einer Leukämie fördert wie die homozygote Trägerschaft von HLA-C1 davor schützt.

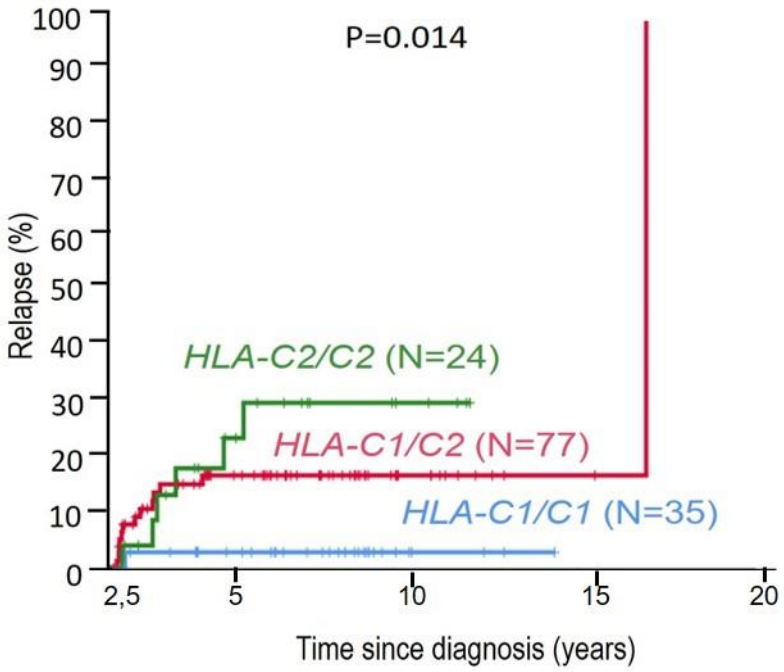


Abbildung 5: HLA-C2+ B-ALL Patienten haben ein erhöhtes Risiko für das Auftreten eines späten Rezidivs. Die Kohorte wurde in 3 Gruppen, basierend auf dem HLA-C Genotyp, aufgeteilt (C1/C1, C1/C2, and C2/C2). Eingeschlossen wurden Patienten, die a) eine komplette Remission erreichten, b) die Standardtherapie protokollgemäß beendeten und c) mind. 2,5 Jahre rezidivfrei blieben. Verwendung der Abbildung mit freundlicher Genehmigung des ASH-Verlags[89].

Im nächsten Schritt wurde die Frequenz der HLA-C2 spezifischen KIR-Gene *KIR2DL1* und *KIR2DS1* untersucht. Da mehr als 95% der Bevölkerung das *KIR2DL1* Gen besitzen, wurden sowohl die Allele von *KIR2DL1* als auch die Anzahl der vorhandenen Kopien untersucht. Beide Analysen ergaben für *KIR2DL1* und *KIR2DS1* keine signifikanten Unterschiede.

Es folgte eine Analyse des Einflusses der KIR-Liganden auf den klinischen Verlauf der Patienten. Hier zeigte sich, dass die Anzahl an *HLA-C2* Allelen signifikant mit dem Auftreten von späten Rezidiven der Erkrankung assoziiert war (Abb. 5). Späte Rezidive treten definitionsgemäß mindestens 30 Monate nach Diagnosestellung auf. Eine Standardbehandlung nach aktuellen Therapieoptimierungsstudienprotokollen wie AEIOP-BFM oder CoALL sieht eine Behandlungsdauer von 24 Monaten für Kinder mit

ALL vor. Danach ist das Immunsystem ohne zytostatische Medikamente erstmals seit Diagnosestellung wieder auf sich alleine gestellt und es kommt zu späten Rezidiven.
Die vorliegende Studie zeigt nicht nur, dass der HLA-C2, der Ligand des KIR-Rezeptors KIR2DL1, mit dem vermehrten Auftreten einer kindlichen ALL assoziiert ist, sondern dieser auch einen Risikofaktor für das Auftreten eines späten Rezidivs nach Therapieende darstellt. Dies legt eine Beeinträchtigung der NK-Zell-vermittelten Leukämiekontrolle mit daraus resultierender Begünstigung der Entstehung einer Leukämie nahe. Der inhibitorische Rezeptor KIR2DL1 bindet mit höherer Affinität HLA-C2 als KIR2DL2/3 an –C1 und KIR2DL2 an –C2. Somit kommt es aufgrund der stärkeren Bindung zu einer stärkeren Inhibition der NK-Zellen und folglich zu verringerter Effizienz der Leukämiekontrolle[90].
Die vorliegende Studie liefert erstmals Hinweise dafür, dass HLA-C-kodierte KIR-Liganden mit dem Auftreten akuter lymphatischer Leukämien assoziiert sind und auch die Wahrscheinlichkeit eines Rückfalls durch diese beeinflusst wird. Sollten sich diese Ergebnisse in unabhängigen Kohorten bestätigen, wäre aus den Ergebnissen ableitbar, dass HLA-C2 ALL Patienten (vor allem HLA-C2 homozygote Patienten) aufgrund eines gesteigerten Rezidivrisikos eine erhöhte Therapieintensität benötigen.

IV. Verminderte Oberflächenexpression von HLA-C und HLA-E bei der ALL im Kindesalter

Selective downregulation of HLA-C and HLA-E in childhood acute lymphoblastic leukaemia

Reusing S, Manser AR, Enczmann J, Mulder A, Claas FH, Carrington M, Fischer JC, Borkhardt A, Babor F*, and Uhrberg M*, *British Journal of Haematology*, 2015 Nov 3. doi: 10.1111/bjh.13777

In einer zuvor veröffentlichten eigenen Studie konnte gezeigt werden, dass der KIR-Ligand HLA-C2 das Risiko erhöht an einer pädiatrischen ALL zu erkranken. Dem gegenüber steht ein protektiver Effekt von HLA-C1/C1[89]. Darüber hinaus konnte gezeigt werden, dass HLA-C2 auch mit dem Auftreten später Rezidive (>30 Monate nach Diagnosestellung) assoziiert ist.

Das Ziel der vorliegenden Studie war es, die Rolle der HLA kodierten KIR-Liganden näher zu beleuchten, indem die Oberflächenexpression der Epitope HLA-Bw4, HLA-Bw6, HLA-C1, HLA-C2 und HLA-E auf Blasten und gesunden Zellen untersucht werden.

Zur durchflusszytometrischen Bestimmung der Oberflächenexpression wurden Antikörper gegen Bw4 (MUS4H4) and Bw6 (OUW4F11)[91], gegen HLA-C/E (DT9)[41, 92], und HLA-E (3D12, BioLegend, San Diego, CA, USA) eingesetzt. Die Antikörper gegen Bw4, Bw6 und C/E sind nicht kommerziell erhältlich und wurden von internationalen Kooperationspartner (A. Mulder, Leiden und M. Carrington, Cambridge) zur Verfügung gestellt.

Um durch die unterschiedlichen Bindungsstärken der Antikörper bedingte Fehlinterpretationen der duchflusszytometrischen Analysen zu verhindern, kamen ausschließlich unkonjugierte HLA-Klasse I Antikörper zum Einsatz. Diese wurden in einem zweiten Schritt mit sekundären anti-IgG Antikörpern gefärbt. Zur Ermittlung der Oberflächenexpression wurde die mittlere Fluoreszenzintensität (MFI) berechnet, wodurch das Signal mit der tatsächlichen Größe der Zelle in Relation gesetzt werden kann. Andernfalls wäre ein Vergleich der Expressionsstärke durch unterschiedliche Zellgrößen nicht zulässig. Die Messungen aller HLA-Klasse I spezifischen Antikörper wurden mit identen Geräteeinstellungen übernommen, die MFI wurde gegen den mittleren forward scatter normalisiert (MFI/MFSC).

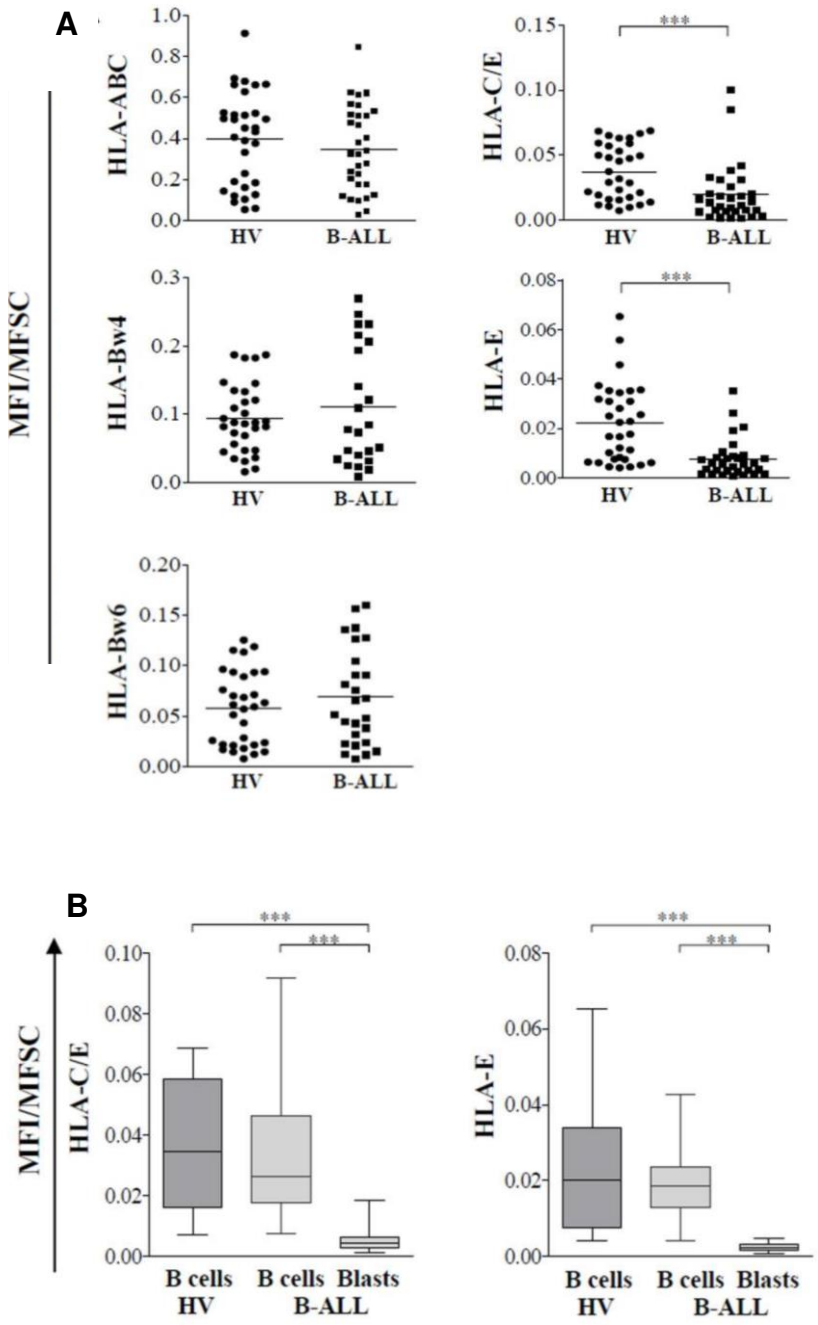


Abbildung 6: Selektive Verringerung der Oberflächenexpression von HLA-C and HLA-E in B-ALL Patienten. (A) Abgebildet ist die Oberflächenexpression der jeweiligen HLA Klasse-I Moleküle (HLA-ABC, HLA-Bw4, HLA-Bw6, HLA-C/E und HLA-E) auf CD19+ B-Zellen von gesunden Spendern (n=32) und ALL-Patienten (n=31). Jeder Punkt repräsentiert ein Individuum, horizontale Linien repräsentieren den Mittelwert. **(B)** In den Box-plots kommt die Oberflächenexpression von HLA-C and HLA-E auf leukämischen und nicht-leukämischen B-Zellen von Patienten sowie B-Zellen gesunder Spender zur Darstellung. Horizontale Linien repräsentieren Mittelwerte. Statistische Signifikanzen wurden mithilfe

des Mann-Whitney U Tests berechnet (***= p<0.001). Verwendung der Abbildung mit freundlicher Genehmigung des Wiley-Blackwell-Verlags[93].

Zunächst wurden CD34+/CD19+ (Blasten) und CD34-/CD19+ Zellen (gesunde B-Zellen) aus PBMCs von 31 Kindern mit neu diagnostizierter ALL isoliert und gesortet. Die Gesamtoberflächenexpression von HLA-Klasse-I wurde mittels des pan-HLA Klasse I Antikörpers W6/32 bestimmt. Diese zeigte sich auf Blasten und nicht-leukämischen CD19+ B-Zellen von Patienten sowie auf CD19+ B-Zellen gesunder Spender vergleichbar (Abb. 6A). Ein ähnliches Bild ergab sich für HLA-B: sowohl die Expression von HLA-Bw4 (Ligand des inhibitorischen Rezeptors KIR3DL1) als auch von HLA-Bw6 (Ligand unbekannt) war auf den drei untersuchten Zellpopulationen vergleichbar. Die Untersuchung der Oberflächenexpression von HLA-C (Ligand von KIR2DL1, KIR2DL2 und KIR2DL3) und HLA-E (Ligand von NKG2A) erbrachte hingegen signifikante Unterschiede mit einem deutlichen Verlust von –C und –E auf der Oberfläche von ALL-Blasten. Weiterhin zeigte sich ein hoch signifikanter Unterschied im Expressionsgrad von HLA-C und –E zwischen leukämischen (CD34+) und nicht-leukämischen (CD34-) B-Zellen der ALL-Patienten (Abb. 6B). Nach Erreichen einer kompletten Remission waren die pathologischen B-Zellpopulationen mit verringerter HLA-Expression wie auch die Blasten nicht mehr zu detektieren.

Im nächsten Untersuchungsschritt sollte überprüft werden, ob der Verlust an HLA-C und –E durch Unterdrückung der Transkription zustande kommt. Hierzu wurden leukämische und nicht-leukämische CD19+ B-Zellen der Patienten isoliert und anschließend mittels rt-PCR analysiert. Hier zeigte sich, dass das mRNA Niveau von HLA-C1, -C2 und –E in Blasten signifikant geringer war als in gesunden B-Zellen.

Das lediglich um das 2-4-fache verringerte mRNA Niveau könnte darauf hinweisen, dass die deutliche Verringerung der Oberflächenexpression auch eine Folge posttranskriptioneller Prozesse ist.

Bei der vorliegenden Studie handelt es sich um die erste tiefergehende Analyse von HLA-C und –E kodierenden NK-Zellliganden in einem Kollektiv pädiatrischer ALL Patienten. Der HLA-C spezifische Antikörper DT9 weist Kreuzreaktivitäten mit HLA-E auf, die für einen Teil des Verlusts des DT9 Signals verantwortlich sein könnten. Zusätzlich detektiert der HLA-E spezifische Antikörper 3D12 in schwacher Ausprägung auch HLA-B und –C Allele. Dennoch können diese Kreuzreaktivitäten die deutlich verringerten Niveaus nicht erklären, da die Expression von HLA-A und –B unverändert ist.

Verheyden et al[91] konnten in einer kleinen ALL-Kohorte ebenfalls eine verringerte HLA-C Expression beobachten, allerdings wurden für die Studie mehrere Antikörper für verschiedene HLA-C Allotypen verwendet. Der mRNA Nachweis von HLA-E bei fehlender Oberflächenexpression steht in Konflikt mit einer vorangegangenen Studie[94], die ein komplettes Fehlen von HLA-E auf mRNA Niveau postulierte.

Gemäß der „*missing-self*" Hypothese[16, 17] sollte der hier beschriebene Verlust von HLA-C und –E zu einer besseren Leukämiekontrolle durch NK-Zellen, die den jeweiligen inhibitorischen Rezeptor tragen, führen. Unabhängig davon, ob in einem Spender den inhibitorischen Rezeptor für HLA-C2 (KIR2DL1), HLA-C1 (KIR2DL3), HLA-Bw4 (KIR3DL1) oder HLA-E (NKG2A) vorhanden war, zeigte sich lediglich eine geringe CD107 Mobilisation von kurzzeit-stimulierten NK-Zellen gesunder Spender gegen primäre ALL Blasten. Ob das Fehlen entsprechender stimulatorischer Rezeptoren hierfür mitverantwortlich ist, bleibt zum aktuellen Zeitpunkt ungeklärt. Weiterhin sind die Mechanismen unklar, die zum Verlust von HLA-C und –E auf der Zelloberfläche führen.

NK-Zellen, die keinen inhibitorischen Rezeptor für HLA-Klasse I besitzen, weisen eine verringerte Zytotoxizität auf und sind hyporesponsiv[23-25]. Eventuell werden NK-Zellen jedoch auch durch das beinahe-Fehlen von HLA-Klasse I kodierten Liganden in einen derartigen hyposresponsiven Zustand versetzt.

Weitere Untersuchungen über den funktionalen Zustand von NK-Zellen von Leukämiepatienten sind notwendig, um die zugrundeliegenden Mechanismen hinter dem spezifischen Verlust an Liganden von NK-Zellrezeptoren besser zu verstehen.

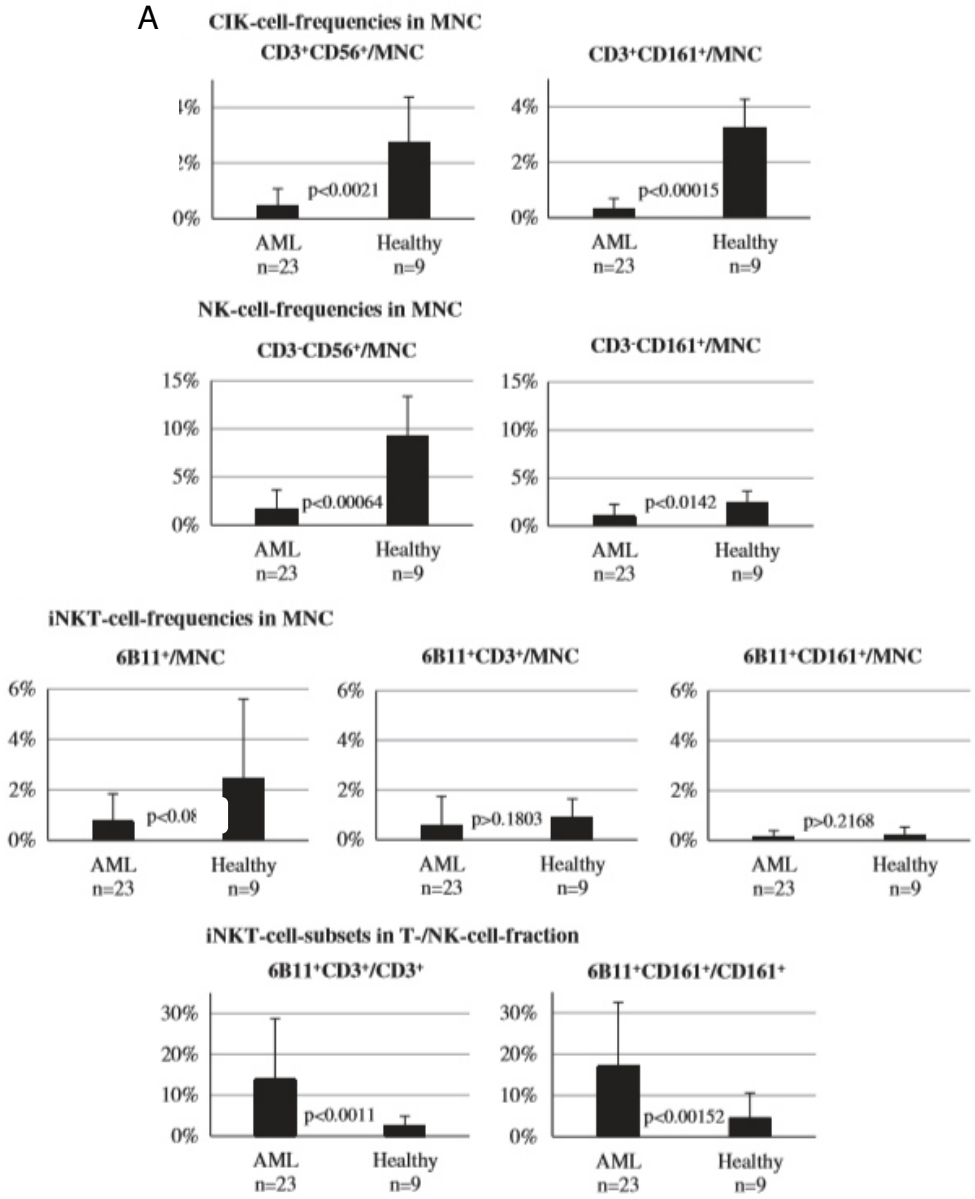
V. Die Frequenz, Zusammensetzung und/oder die antileukämische Aktivität (DC stimulierter) invarianter NKT-, NK- und CIK-Zellen beeinflussen die Prognose von Patienten mit AML, ALL und CLL

Significance of frequencies, compositions and/or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells on the outcome of patients with AML, ALL and CLL

Boeck CL, Amberger DC, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, Schuster F, Salih H, Babor F, Borkhardt A, and Schmetzer H, *Journal of Immunotherapy*, 2017 Jul/Aug;40(6):224-248.

Als wichtiger Teil des angeborenen Immunsystems übernehmen NK-Zellen, invariante NKT-Zellen (iNKT) und Zytokin-induzierte Killerzellen (CIK) eine wichtige Rolle bei der Entstehung und Bekämpfung bösartiger Erkrankungen. Anhand der Oberflächenproteine werden NK Zellen als CD3-CD56+CD161+ definiert[95]. CIK Zellen, die als Zwischenglied zwischen angeborenem und adaptivem Immunsystem phänotypisch und funktional Eigenschaften von NK- und T-Zellen aufweisen, sind CD3+CD56+CD161+[95]. iNKT-Zellen exprimieren einen semi-invarianten T-Zellrezeptor sowie die humanen NK-Zell Oberflächenmarker NKR-P1a (CD161), CD69 und CD94 jedoch ohne Expression der NK-typischen Marker CD16 und CD56[96, 97].
Kürzlich wurde der monoklonale Antikörper 6B11 entwickelt, mit dessen Hilfe iNKT-Zellen charakterisiert werden können Dieser bindet an den invarianten T-Zellrezeptor Vα24Jα18 in der CD3 Region von iNKT-Zellen.
Im ersten Teil der Arbeit wurden iNKT-, NK- and CIK-Zellen und deren Subpopulationen im peripheren Blut von gesunden Spendern (n=9), Patienten mit akuter myeloischer Leukämie (AML; n=23), Patienten mit akuter lymphatischer Leukämie (ALL; n=20) sowie Patienten mit chronischer lymphatischer Leukämie (CLL; n=21) analysiert. Die AML- und CLL-Kohorten setzten sich ausschließlich aus erwachsenen Patienten zusammen. Die ALL-Kohorte beinhaltete sieben pädiatrische Leukämiepatienten (35%).

A


CIK-cell-frequencies in MNC
CD3+CD56+/MNC
CD3+CD161+/MNC
p<0.0021
p<0.00015
AML n=23
Healthy n=9
NK-cell-frequencies in MNC
CD3-CD56+/MNC
CD3-CD161+/MNC
p<0.00064
p<0.0142
iNKT-cell-frequencies in MNC
6B11+/MNC
6B11+CD3+/MNC
6B11+CD161+/MNC
p<0.08
p>0.1803
p>0.2168
iNKT-cell-subsets in T-/NK-cell-fraction
6B11+CD3+/CD3+
6B11+CD161+/CD161+
p<0.0011
p<0.00152

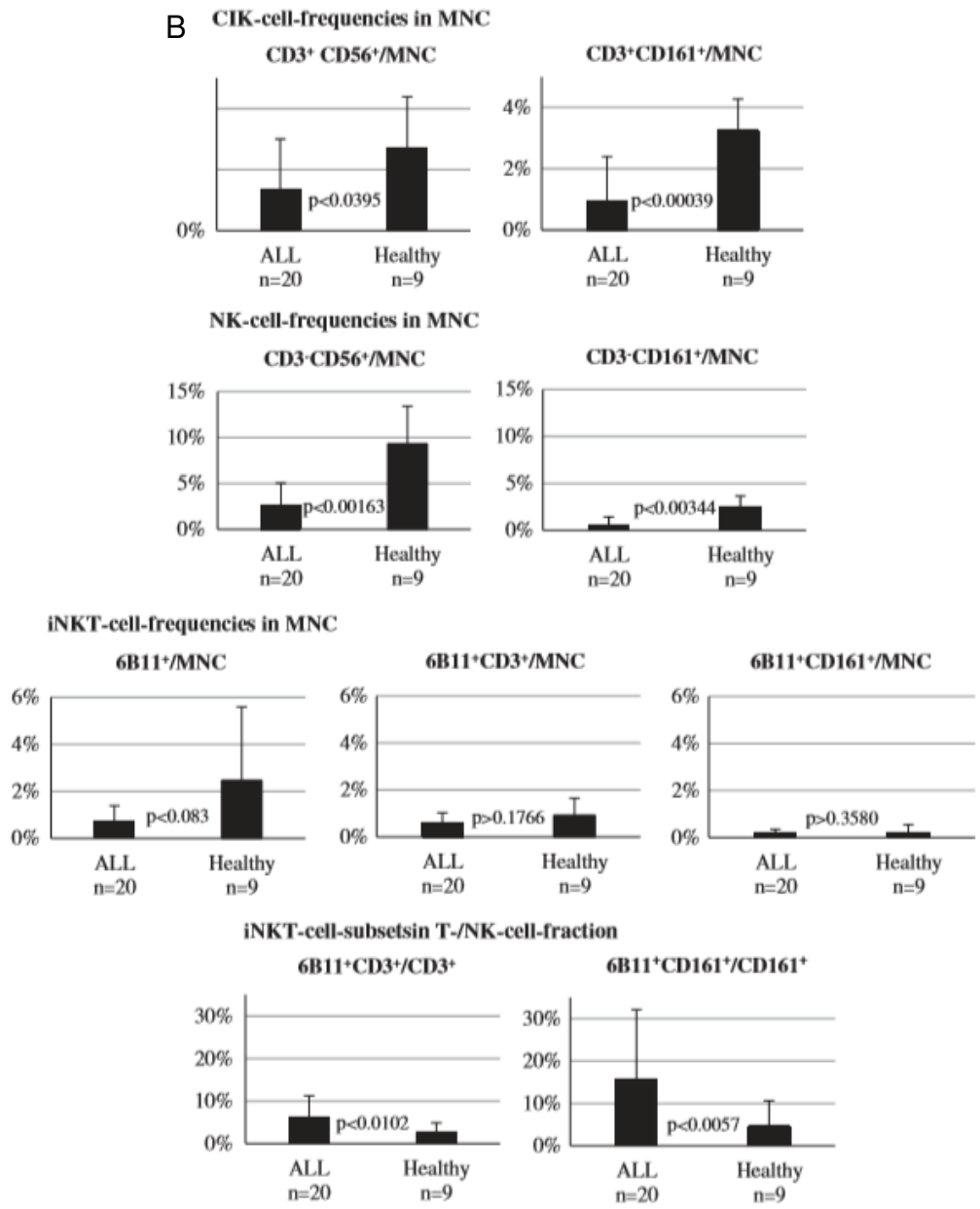


Abbildung 7: Frequenz von CIK-, NK- und iNKT-Zellen in AML und ALL Patienten verglichen mit gesunden Spendern. Anteile von CIK-Zellen (CD3+CD56+/MNC und CD3+CD161+/MNC), NK-Zellen (CD3-CD56+/MNC und CD3-CD161+/MNC) und iNKT-Zellen in MNC (6B11+, 6B11+CD3+/MNC, 6B11+CD161+/MNC) sowie innerhalb der T-Zellpopulation (6B11+CD3+/CD3+) und NK-Zellpopulation (6B11+CD161+/CD161+). Verwendung der Abbildung mit freundlicher Genehmigung des Lippincott Williams & Wilkins-Verlags[98].

Es konnte gezeigt werden, dass verglichen mit gesunden Kontrollen 6B11+/6B11+CD3+/ 6B11+CD161+/6B11+CD8+/6B11+Va24+ iNKT, CD3-CD56+/CD3-CD161+ NK, und CD3+CD56+/CD3+CD161+ CIK-Zellsubgruppen

signifikant seltener in AML Patienten zu finden sind. In diesem Patientenkollektiv war außerdem ein höherer Anteil an T- und NK-Zellen zu verzeichnen, die 6B11 koexprimierten (Abb. 7A).
Höhere Anteile von 6B11+ iNKT-Zellen waren mit niedrigerem Risikoscore, jüngerem Alter, dem Vorliegen einer primären AML, dem Fehlen extramedullärer Foci und einer kompletten Remission (CR) assoziiert. Höhere Frequenzen von NK- und CIK Zellen waren ebenfalls mit dem Fehlen extramedullärer Foci, stabiler CR sowie dem Erreichen einer CR nach Induktionschemotherapie assoziiert.
In der ALL-Kohorte wurde hingegen eine signifikant niedrigere Anzahl iNKT-, NK- sowie CIK-Zellen nachgewiesen (Abb. 7B). Analog zur AML-Kohorte wurde auch bei ALL-Patienten ein höherer Anteil von 6B11-exprimierenden T- und NK-Zellen nachgewiesen, gegensätzlich war jedoch ein kleinerer Anteil von CIK-Zellen im Vergleich zu Gesunden. Obwohl sich der Großteil des untersuchten Patientenkollektivs aus erwachsenen ALL-Patienten (65%) zusammensetzte, konnten Ergebnisse einer zuvor durchgeführten Studie von Bienemann et al[99], in der ebenfalls ein signifikant höherer Anteil an CD3+6B11+ iNKT-Zellen bei Kindern und Jugendlichen beschrieben wurde, bestätigt werden.
Auch bei ALL-Patienten korrelierte die Anzahl an iNKT- und NK-Zellen mit klinischen Verlaufsparametern. So waren höhere Werte eher mit dem Erreichen einer CR assoziiert, im Falle von iNKT auch mit seltenerem extramedullärem Befall.
Als dritte Entität wurden CLL-Patienten analysiert. Ähnlich wie bei ALL-Patienten lagen signifikant geringere Werte für iNKT-, NK- und CIK-Zellen vor. Wie im AML-Kollektiv war das Verhältnis zugunsten von 6B11 exprimierende T- und NK-Zellen verschoben. Dies traf nicht für CIK-Zellen zu.
Bei CLL-Patienten konnte gezeigt werden, dass höhere Anteile an 6B11+ iNKT-Zellen mit weniger Therapiebedarf, jüngerem Patientenalter und stabilerer Remission assoziiert waren. Letzteres zeigte sich auch für Patienten mit größeren Anteilen an NK- und CIK- Zellen.

Im zweiten Teil der Arbeit wurde heparinisiertes Vollblut und MNZ-Proben von gesunden Spendern (n=10), AML Patienten (n=5) und einem MDS Patienten analysiert.

Die Proben wurden mit unterschiedlichen „Cocktails“ vorbehandelt, die die Umwandlung von Leukämiezellen zu dendritischen Zellen leukämischen Ursprungs (DCleu, Leukämie-Antigen präsentierende Zellen) bewirken. Unbehandelte Proben dienten als Kontrolle,
iNKT-, NK- und CIK-Zellen wurden quantitativ und qualitativ vor und nach einer Koinkubation mit T-Zellen (MLC) unter physiologischen (21% O2) und hypoxischen Bedingungen (ca. 10% O2) untersucht.
Hier zeigte sich, dass iNKT-, NK- und CIK-Zellen nach MLC unabhängig vom verabreichten „Cocktail“ expandierten. Eine Vorbehandlung mit einem „Cocktail“ führte zu einem Anstieg der iNKT-Zellen. Nach MLC waren sämtliche Zellpopulationen sowohl unter Normoxie als auch unter Hypoxie vergleichbar und korrelierten mit der antileukämischen, lytischen Aktivität. Dies könnte ein Hinweis für die Beteiligung der Zellgruppen an der Leukämiebekämpfung sein.

Die Erkenntnis, dass ein höherer Anteil an iNKT-, NK- und CIK-Zellen mit einer verbesserten Prognose assoziiert ist und einer verbesserten antileukämischen Aktivität gegen AML, ALL und CLL, deutet auch darauf hin, dass die Größe dieser Zell-Subsets von prognostischem Wert bei Leukämiepatienten ist.
Bei der Entwicklung neuartiger immunotherapeutischer Protokolle sollte die Quantifizierung der drei Zellgruppen sowie deren Untergruppen berücksichtigt werden. Auch der sog. Cross-talk zwischen Immunzellen und löslichen Faktoren bei AML-Patienten sollte Gegenstand weiterer Untersuchungen sein, da ihm eine wichtige Rolle in der Immuntherapie bei AML zukommt.

VI. Einfluss des Spender KIR Status auf die Leukämiekontrolle nach Transplantation in pädiatrischer ALL

Donor KIR status impacts on leukemia control after transplant for childhood ALL.

Babor F, Peters C, Sauer M, Glogova E, Manser A, Pötschger U, Mytilineos J, Horn P, Ahlmann M, Oevermann L, Kremens B, Güngör T, Strahm B, Gruhn B, Siepermann M, Schuster F, Stachel D, Wössmann W, Cario G, Müller I, Scherenschlich N, Pichler H, Feuchtinger T, Mezger M, zur Stadt U, Zimmermann M, Schrappe M, Borkhardt A, Eckert C, Bader P, Uhrberg M, and Meisel R; *Bone Marrow Transplantation*, 2019 doi: 10.1038/s41409-019-0543-z

Kürzlich publizierte Studien legen nahe, dass unverwandte Stammzellspender, die einen *KIR B* Haplotyp aufweisen, einen positiven Einfluss auf das Überleben von AML Patienten haben[100, 101]. In einer ersten Studie untersuchten die Autoren den Effekt der KIR-Genotypen auf das Gesamt- und ereignisfreie Überleben bei 448 erwachsenen AML Patienten, die von HLA-identen Fremdspendern transplantiert wurden. Es zeigte sich, dass bei Transplantationen von Spendern mit KIR B Haplotypen das Gesamt- und ereignisfreie Überleben um jeweils 30% verbessert war[100]. In einer Nachfolgestudie analysierten die Autoren die Auswirkung von zentromeren und telomeren Genmotiven. Verglichen mit Gruppe A Haplotyp Spendern, bewirkten Spender mit zentromeren und/oder telomeren Gruppe B Motiven ein höheres Gesamtüberleben und eine verringerte Rezidivrate. Der stärkste Effekt war bei Spendern zu verzeichnen, die ein zentromeres B-Motiv trugen[101]. In einer weiteren Studie bewirkten die Gruppe B KIR-Gene KIR2DS1 und KIR3DS1 einen positiven Einfluss auf das Rezidivrisiko von AML Patienten. Allerdings befinden sich diese KIR-Gene auf dem telomeren Teils des KIR-Lokus[72]. Jüngste Studien zeigten, dass Stammzellspender mit B-Haplotypen nur dann einen Überlebensvorteil für den Patienten bewirken, wenn Ersterer mindestens ein HLA-C1 Allel aufweist[102].

Die zuletzt genannten Ergebnisse waren ausschließlich auf AML als Grunderkrankung beschränkt. In der 2010 publizierten Studie[101] befassten sich die Autoren auch mit ALL Patienten, konnten jedoch keinen Einfluss der KIR-Genetik auf das Überleben detektieren. Es ist anzunehmen, dass sich das Krankheitsbild einer ALL bei Erwachsenen von jenem bei Kindern unterscheidet und so stehen die Ergebnisse von

Cooley et al nur bedingt im Widerspruch zu jüngsten Ergebnissen von pädiatrischen ALL-Patienten, die von einem haploidenten Elternteil stammzelltransplantiert wurden[103]. Denn auch in diesem Patientenkollektiv von ausschließlich pädiatrischen ALL-Patienten ergab sich ein verringertes Rezidivrisiko nach Transplantationen von Spendern mit einem B-Haplotyp.

In der vorliegenden Studie sollte die Rolle der KIR-Gene im Rahmen der allogenen Fremdspender- und Geschwistertransplantation bei ALL-Patienten evaluiert werden. Hierfür wurden Patienten- und Spenderproben der prospektiven, multizentrischen Studie ALL-SZT 2003[104] analysiert. Von den 411 in die Studie eingeschlossenen Patienten konnten 317 Spender- und Empfängerrückstellproben retrospektiv mittels PCR analysiert werden. Die Kohorte der vorliegenden Studie unterschied sich nicht von den 94 nicht eingeschlossenen Patienten und Spendern der Primärstudie. Um zunächst die Rolle der B-Haplotypen nach Cooley et al zu evaluieren wurden die Stammzellspender in zwei Gruppen eingeteilt. A) Spender mit mindestens einem zentromeren KIR-B Gen und B) Spender ohne zentromeres KIR-B Gen. Analog zu den Ergebnissen der Literatur[101, 103, 105] waren Transplantationen von einem Spender mit KIR-B Gen mit einer signifikant geringeren Rezidivinzidenz assoziiert (Abb. 8A).

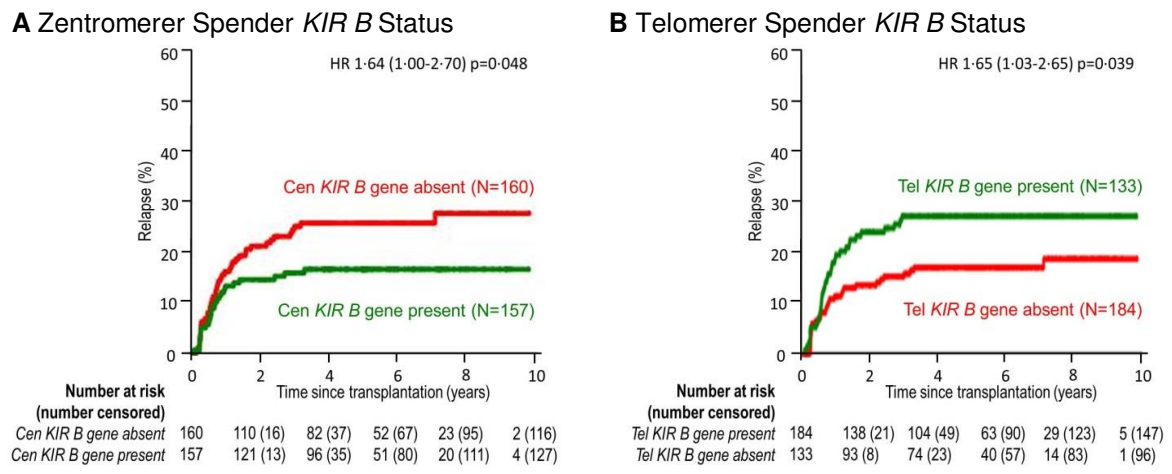


Abbildung 8. Kumulative Rezidivinzidenz stratifiiziert nach KIR B Gengehalt und chromosomaler Position. Verwendung der Abbildung mit freundlicher Genehmigung des Nature-Verlags[106].

In der gleichen Analyse des telomeren Abschnitts bot sich jedoch ein umgekehrtes Bild: eine signifikant niedrigere Rezidivinzidenz war für Patienten zu verzeichnen, die von einem Spender ohne telomeres KIR-B Gen transplantiert wurden (Abb.8B).
Um dieser dichotomen Rolle der KIR-B Gene Rechnung zu tragen, wurden die Spender anhand eines Scores (ct-KIR Score, Tab. 4) in 3 Gruppen unterteilt. Spender mit mindestens einem zentromeren aber ohne telomeres KIR-B Gen (cen B+/tel B-), Spender ohne zentromers aber mit mindestens einem telomeren KIR-B Gen (cen B-/tel B+) sowie die Gruppe der verbliebenen Spender (cen B+/tel B+ oder cen B-/tel B-).

Ct-KIR Score	**Spenderklassifikation**	***Zentromer***	***Telomer***
0	vorteilhaft	*CenB-*	*TelB+*
1	intermediär	*CenB+*	*TelB+*
		CenB-	*TelB-*
2	unvorteilhaft	*CenB+*	*TelB-*

Tabelle 4: Definition des *ct-KIR* Scores nach zentromeren und telomeren *KIR B* Motiven
Innerhalb des KIR-Locus sind die KIR-Gene in verschiedenen Haplotypen organisiert, die in 2 Gruppen eingeteilt werden: Haplotyp A setzt sich aus einem definierten Set von 6 Genen zusammen (KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2 und KIR2DS4). (als *CenB-* sind Spender definiert, die kein zentromere KIR B Gen aufweisen, CenB+ Spender besitzen mindestens ein KIR B Gen. Die gleiche Nomenklatur wird für die Telomerregion verwendet (*TelB-* and *TelB+)*.

Es stellte sich heraus, dass Transplantationen von cen B+/tel B- Spendern mit einer signifikant besseren Rezidivkontrolle (Rezidivinzidenz (RI) 12%) assoziiert waren als bei cen B-/tel B+ Spender (RI34%) und cen B+-/tel B+- Spender (RI 21%) (Abb. 9A).
Im nächsten Schritt sollte überprüft werden, ob die verbesserte Rezidivkontrolle auf Kosten der transplantationsassoziierten Mortalität zu verzeichnen war (Abb. 9B). Dies traf weder für die akute noch für die chronische GvHD zu. Besonders hervorzuheben ist, dass auch die extensive chronische GvHD, die zumeist die lebenslimitierende Form

der GvHD darstellt, in allen 3 Gruppen gleich häufig auftrat (Abb. 9C). Auch auf die kumulative Inzidenz des ereignisfreien Überlebens hatte der ct-KIR Score einen Einfluss, wenngleich mit grenzwertiger statistische Signifikanz (Abb. 9D).

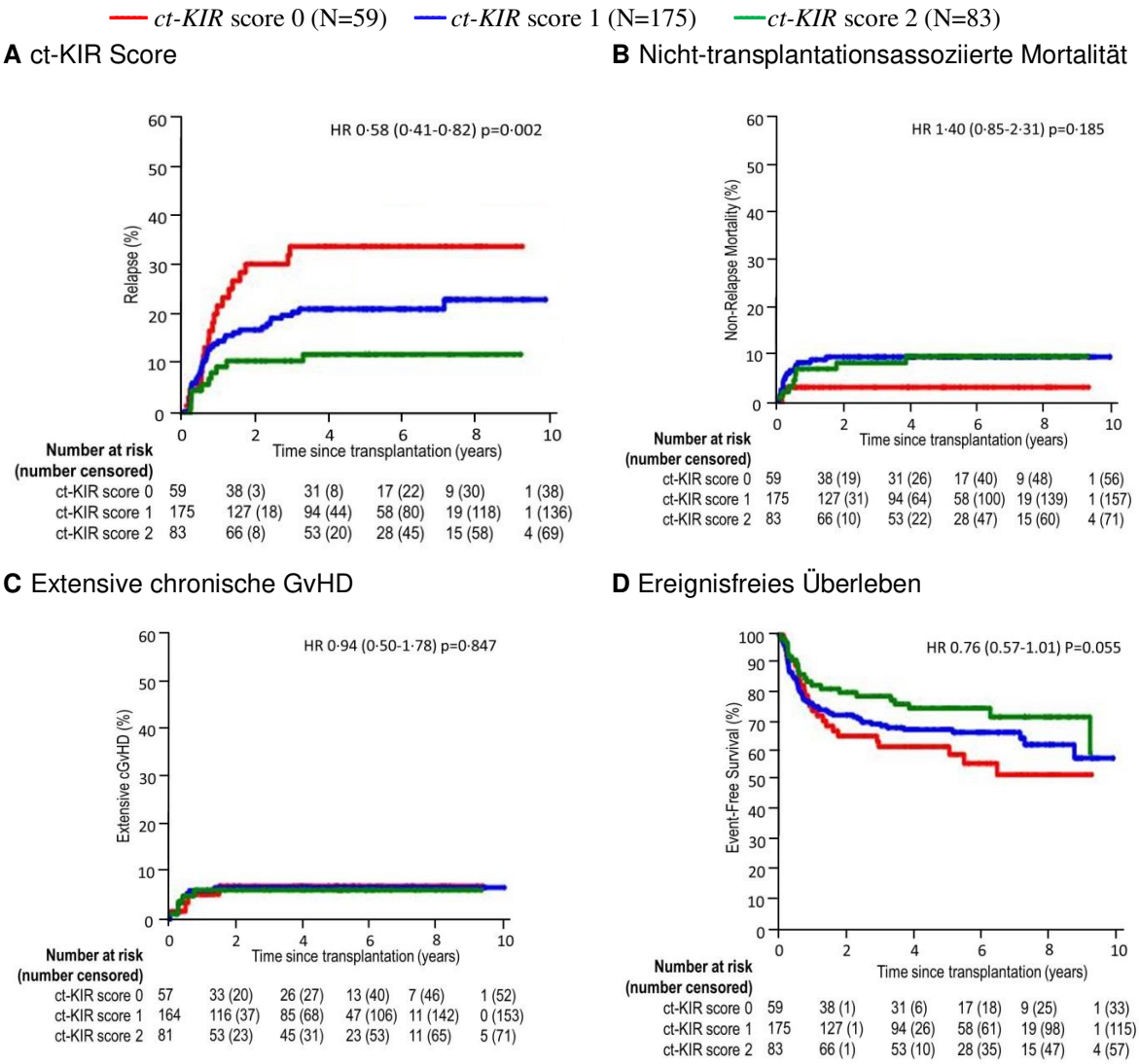


Abbildung 9. Kumulative Rezidivinzidenz nach ct-KIR Score, Inzidenz der nicht-transplantationsassoziierte Mortalität und extensiven chronischen GvHD sowie des ereignisfreien Überlebens nach *ct-KIR* Score. Verwendung der Abbildung mit freundlicher Genehmigung des Nature-Verlags[106].

Um den Stellenwert des ct-KIR Scores im Kontext anderer Spenderauswahlkriterien zu beleuchten, wurde dieser in einer Multivariatanalyse neben weiteren potenziell einflussreichen Patienten-, Spender- und Erkrankungsparameter getestet (ct-KIR Score, HLA-match, CMV-Status, Geschlecht, Blutgruppe, Immunphänotyp,

Rückfallrisiko). Es zeigte sich, dass der ct-KIR Score neben dem Rückfallrisiko der einzige Parameter mit signifikantem Einfluss auf die Rezidivinzidenz ist (p=0,0003). Für das ereignisfreie Überleben zeigte sich eine ähnliche Tendenz wie in der Univariatanalyse (p=0,059).

Die vorliegende Studie stellt die erste Analyse des Einflusses der KIR-Genetik auf das Überleben pädiatrischer ALL-Patienten nach HLA-gematchter allogener HSZT dar. Bisherige Studien haben sich mit Erwachsenen, AML-Patienten oder haploidenten Transplantationen bei Kindern beschäftigt.

Die multizentrische prospektive ALL-SZT 2003 Studie beinhaltet ein bezüglich Indikation, Spenderauswahl, Konditionierungstherapie und GvHD-Prophylaxe homogenes Patientenkollektiv, das zum Großteil (317/411 Patienten) in die vorliegende retrospektive Analyse eingeschlossen wurde. Die Erkenntnis, dass zentromere KIR-B Gene das Rezidivrisiko senken, deckt sich mit Ergebnissen von AML Patienten und haploidenter Transplantation bei Kindern[103]. Bisher unbeobachtet ist jedoch der negative Einfluss der telomeren KIR-B Gene auf das Überleben der Patienten. Der angewandte ct-KIR Score ermöglichte unter Berücksichtigung der dichotomen Rolle der KIR-Gene eine Einteilung in 3 Spendergruppen mit signifikant unterschiedlichen Rezidivraten. Dieser prognostische Stellenwert bestätigte sich in Multivariatanalysen mit weiteren Patienten-, Spender- und Erkrankungsparametern.

Ein negativer Einfluss des ct-KIR Scores auf andere Transplantationsparameter wie das Auftreten akuter oder chronischer GvHD oder der nicht-transplantationsassoziierten Mortalität war nicht zu verzeichnen.

Zusammenfassend liefert die vorliegende Studie Hinweise, dass eine Spenderauswahl nach dem in dieser Studie etablierten ct-KIR Score die Rezidivrate bei Kindern, die aufgrund ihrer ALL einer allogenen Stammzelltransplantation unterzogen werden, prospektiv verbessern kann ohne das Risiko für das Auftreten einer GvHD zu erhöhen. Besonders in Fällen, in denen mehr als ein passender Fremdspender zur Verfügung steht, ist die Identifikation eines optimalen Spenders nach dem ct-KIR Score ohne große Risiken durchführbar.

5. Zusammenfassung

Die hier präsentierte Arbeit bietet einen neuen Einblick in die Rolle der NK-Zell-vermittelten Immunantwort bei der Entstehung und Kontrolle akuter Leukämien im Kindesalter.

In einer Kohorte von pädiatrischen Patienten mit ALL konnte in Analysen von KIR- und HLA-Klasse I-Genen ein signifikant höheres Vorkommen des KIR-Liganden HLA-C2 beobachtet werden. Die Häufigkeit einzelner KIR-Gene oder –Haplotypen unterschieden sich hingegen nicht von der Kontrollgruppe gesunder Kinder. Korrelationen der genetischen Ergebnisse mit den Verläufen der Patienten zeigten, dass HLA-C2 ein Risikofaktor für die Entstehung einer akuten lymphatischen Leukämie im Kindesalter ist und das Risiko eines Rezidivs nach beendeter Therapie signifikant erhöht.

Die Identifizierung von HLA-C2 als neuen Risikofaktor für ein Rezidiv ist von besonderer klinischer Relevanz. Dies könnte rechtfertigen, dass HLA-C2 Patienten entsprechend länger therapiert oder engmaschiger kontrolliert werden, um das Auftreten eines Rezidivs zu verhindern und die Überlebensrate zu verbessern. Durchflusszytometrische Messungen der Oberflächenexpression auf den leukämischen Blasten ergaben ebenfalls in Bezug auf die Oberflächenexpression der zwei wichtigen NK-Zell-Liganden HLA-C und HLA-E in pädiatrischen ALL-Patienten wichtige Erkenntnisse. Im Gegensatz zu den anderen HLA-Klasse I Molekülen HLA-A und HLA-B (HLA-Bw4 und HLA-Bw6) war die Expression von HLA-E und HLA-C auf Blasten, verglichen mit B-Zellen von Patienten und gesunder Kontrollen, deutlich verringert. Interessanterweise waren die NK-Zellen der Patienten trotz der fehlenden inhibitorischen Liganden nicht in der Lage, die leukämischen Zellen zu eliminieren. Dies legt einen Defekt in der NK-Zell-Aktivierung nahe. Damit übereinstimmend war die Expression auf Transkriptionsebene in leukämischen CD34- CD19+ Blasten verringert.

Im Vergleich zu gesunden Individuen waren in AML, ALL und auch CLL Patienten signifikant höhere Anteile von iNKT-, NK- und CIK-Subpopulationen zu detektieren. Auch hier ergaben Korrelationen mit den klinischen Verläufen der Patienten eine prognostische Rolle von höheren Anteilen von iNKT-, NK- und CIK-Subpopulationen in AML, ALL und CLL Patienten.

Dass auch die KIR-Gene des Stammzellspenders von prognostischer Bedeutung für das rezidivfreie Überleben von ALL-Patienten sind, ergaben retrospektive Analysen einer multizentrischen internationalen Therapieoptimierungsstudie (ALL-SZT 2003). Durch Analysen des KIR Polymorphismus der Spender konnte gezeigt werden, dass zentromere, aber nicht telomere KIR B Haplotypen mit einer verbesserten Leukämiekontrolle einhergehen. Ein Spenderauswahl-Score, der dieser dichotomen Rolle der KIR B Gene Rechnung trägt, sollte in die aktuelle Spenderauswahl in dieser Krankheitsentität implementiert werden und so das Überleben von Kindern, die aufgrund einer ALL stammzelltransplantiert werden, maßgeblich zu verbessern.

Die in dieser Arbeit zusammengefassten Ergebnisse tragen zu einem besseren Verständnis der Rolle der NK-Zellen und der Interaktion von Rezeptor (KIR) und Ligand (HLA) bei der Entstehung und Kontrolle von Leukämien im Kindesalter bei. Die Identifizierung neuer Risikofaktoren (HLA-C2) und protektiver Faktoren (KIR B Gene), die die Rezidivwahrscheinlichkeit und somit unmittelbar das Überleben der Patienten beeinflussen, soll dazu beitragen, dass Therapieansätze und Spenderauswahlverfahren grundlegend verändert werden.

6. Abbildungs- und Tabellenverzeichnis

7. Referenzen

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8. Lebenslauf

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08/2007-01/2013	Facharztausbildung im Fach Kinderheilkunde Klinik für Kinderonkologie, -Hämatologie und –Klinische Immunologie (Direktor: Prof. Dr.med. Arndt Borkhardt), Zentrum für Kinder und Jugendmedizin, Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf
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28.01.2013	Erlangung der Gebietsbezeichnung „Facharzt für Kinder- und Jugendmedizin"
18.07.2013	Promotion an der Universitätskinderklinik Düsseldorf, unter der Leitung von Prof. Dr. med. Roland Meisel zum Thema „Infektionen in der Kinderonkologie"
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Gerätebeauftragter GMP-Labor
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Mitgliedschaften

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Österreichische Gesellschaft für Kinder- und Jugendmedizin (ÖGKJ)
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European Society of Paediatric Oncology (SIOPE)
Deutsche Gesellschaft für Pädiatrische Infektiologie (DGPI)
Society for Natural Immunity (SNI)

Förderung

Anschubförderung der Forschungskommission der Medizinischen Fakultät der Heinrich Heine Universität, Düsseldorf „Characterization and expansion of leukemia-specific NK cell subsets against pediatric ALL“ 2013-2015

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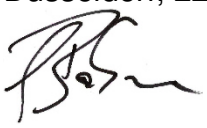
Clinical Research Training in Hematology – Scholarship der European Hematology Association, 2018-2019

Auszeichnungen/Preise

Düsseldorfer Forschungspreis für Kinder- und Jugendmedizin 2015
Posterpreis 3rd International Workshop on Biology, Prevention and Treatment of Relapse after Stem Cell Transplantation 2016, Hamburg

Düsseldorf, 22.08. 2020



Dr. med. Florian Babor

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Ebenso danke ich Professor Markus Uhrberg, ohne den es diese Habilitationsschrift nicht geben würde. Markus wurde von einem Kooperationspartner zu einem Mentor zu einem sehr lieben und engen Freund und bereichert mit seiner positiven Art jede wissenschaftliche Fragestellung und unsere Freundschaft. Ich habe von Markus sehr viel gelernt, tue das immer noch und er ist für mich als Wissenschaftler ein Vorbild.

Für mich einzigartig war auf diesem Weg auch die Zusammenarbeit mit seiner Arbeitsgruppe im ITZ. Hier vor allem mit Maryam Hejazi und Angela Manser. Vor allem Angela hat mit ihrer Expertise auf dem Gebiet der NK-Zellen wichtige Beiträge für diese Habilitationsschrift geleistet.

Danken möchte ich auch ganz besonders Sarah Reusing, die mich seit vielen Jahren in der Wissenschaft begleitet und die sich von einer jungen Masterstudentin zu einer eigenständigen Persönlichkeit und für mich zu einer unentbehrlichen FACS-Expertin entwickelt hat.

Nadine Scherenschlich, die in meinem wissenschaftlichen Werdegang eine sehr große Rolle eingenommen hat, danke ich besonders. Für mich herausragend ist ihre gewissenhafte und verlässliche Arbeit, die Bereitschaft über unseren Tellerrand hinauszublicken und auch mal etwas Verrücktes zu versuchen.

Danken möchte ich auch meiner Mutter, meinem jüngst verstorbenen Papa Lothar, sowie Georgi und Tanja.

Meinen Freunden Hemmen Sabir, Jan Grassmann, Meinolf Siepermann, Prasad Oommen und Sebastian Weinspach möchte ich auf diesem Weg ebenfalls danken. Ich hoffe wir stehen einander noch viele Jahre als enge Vertraute zur Seite.

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Und zuletzt gilt mein größter Dank meiner Frau Fabienne, die ich liebe, die mich glücklich macht und mich täglich unterstützt. Unsere Beziehung zueinander ist das, wonach ich mein Leben lang gesucht habe. Fabi, ohne Deine bedingungslose Liebe wäre das alles niemals möglich gewesen. Danke!

10. Eigene Veröffentlichungen

In dieser Habilitationsschrift sind folgende Originalarbeiten thematisch zusammengefasst:

1. Lack of association between KIR genes and acute lymphoblastic leukemia in children

Babor F, Manser A, Schönberg K, Enczmann J, Borkhardt A, Meisel R, and Uhrberg M

Blood, 2012. 120(13): 2770-2. **IF 9.06**

2. The role of KIR genes and ligands in leukemia surveillance.

Babor F, Fischer JC, and Uhrberg M

Frontiers in Immunology, 2013. 4, 27. Review

3. The KIR ligand C2 is associated with increased susceptibility to childhood acute lymphoblastic leukemia and confers an elevated risk for late relapse

Babor F, Manser AR, Fischer JC, Scherenschlich N, Enczmann J, Chazara O, Moffett A, Borkhardt A, Meisel R, and Uhrberg M

Blood, 2014, Oct 2;124(14):2248-51. **IF 10.452**

4. Selective downregulation of HLA-C and HLA-E in childhood acute lymphoblastic leukaemia

Reusing S, Manser AR, Enczmann J, Mulder A, Claas FH, Carrington M, Fischer JC, Borkhardt A, **Babor F** *, and Uhrberg M

British Journal of Haematology, 2015 Nov 3. doi: 10.1111/bjh.13777 **IF 5.401**

5. Significance of frequencies, compositions and/or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells on the outcome of patients with AML, ALL and CLL

Boeck CL, Amberger DC, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, Schuster F, Salih H, **Babor F**, Borkhardt A, and Schmetzer H

Journal of Immunotherapy, 2017 Jul/Aug;40(6):224-248 **IF 3,826**

6. Presence of centromeric but absence of telomeric group B KIR haplotypes in stem cell donors improve leukemia control after HSCT for childhood ALL

Babor F, Peters C, Sauer M, Glogova E, Manser A, Pötschger U, Mytilineos J, Horn P, Ahlmann M, Oevermann L, Kremens B, Güngör T, Strahm B, Gruhn B, Siepermann M, Schuster F, Stachel D, Wössmann W, Cario G, Müller I, Scherenschlich N, Pichler H, Feuchtinger T, Mezger M, zur Stadt U, Zimmermann M, Schrappe M, Borkhardt A, Eckert C, Bader P, Uhrberg M, and Meisel R

Bone Marrow Transplantation, 2019, doi: 10.1038/s41409-019-0543-z **IF 4,497**

11. Erklärungen und eidesstattliche Versicherung

Hiermit erkläre ich, dass bei den wissenschaftlichen Arbeiten, die Gegenstand meiner Habilitationsleistung sind, ethische Grundsätze und die jeweils gültigen Empfehlungen zur Sicherung guter wissenschaftlicher Praxis durch mich beachtet wurden.

Düsseldorf, 22.08.2020 

Dr. med. Florian Babor

Hiermit erkläre ich, dass keine weiteren Habilitationsverfahren eingeleitet oder erfolglos abgeschlossen worden sind.

Düsseldorf, 22.08.2020 

Dr. med. Florian Babor

Hiermit versichere ich an Eides statt, dass ich die Beiträge zu den Publikationen, die meiner Habilitationsschrift zugrunde liegen, eigenständig geleistet habe.

Düsseldorf, 22.08.2020 

Dr. med. Florian Babor

12. Zugrunde liegende Originalarbeiten

I–specific antibodies are not involved in the inhibitory effects reported here.

In conclusion, we demonstrated that antigen-specific CD8 T-cell activation after cross-presentation of immune complexes by BMDCs is strongly reduced in the presence of therapeutic doses of IVIg. This observation extends our previous observations showing that antigen-specific CD4 T-cell activation is inhibited by IVIg both in vitro and in vivo. Altogether, these results suggest that not only CD4 but also CD8 T-cell activation should be considered as therapeutic targets in the development of potent substitutes to IVIg.

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Contribution: P.T. designed and performed the experiments, analyzed the results, and wrote the paper; and R.B. designed the research, analyzed the results, and wrote the paper.

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10. Kaveri S, Vassilev T, Hurez V, et al. Antibodies to a conserved region of HLA class I molecules, capable of modulating CD8 T cell-mediated function, are present in pooled normal immunoglobulin for therapeutic use. *J Clin Invest.* 1996;97(3):865-869.

To the editor:

Lack of association between KIR genes and acute lymphoblastic leukemia in children

In a recent report, Almalte et al described novel associations between childhood acute lymphoblastic leukemia (ALL) and killer immunoglobulin-like receptor (KIR) genes in a case-control study including mostly French-Canadian patients.[1] The study was limited to the analysis of stimulatory *KIR* (*KIR-S*) and impressively, all of the 6 different *KIR-S* exhibited a strongly reduced frequency in the patient cohort. We performed a similar analysis in a cohort of childhood B-ALL (n = 185) and T-ALL (n = 33) patients of European origin (92% German, recruitment 1992-2012) from the pediatric oncology center in Düsseldorf, but also included inhibitory *KIR*, which enabled the identification of extended *KIR* genotypes. As shown in Figure 1A, none of the *KIR-S* genes exhibited a significant frequency deviation from our ethnically matched control cohort. Our control group exhibited comparable *KIR-S* frequencies to the French-Canadian control group from Almalte et al[1] except for *KIR2DS5*, which was unusually high in the Canadian study also when compared with other white cohorts from France, Germany, or the United Kingdom (data available at www.allelefrequencies.net). Because the strongest association in that study was seen for *KIR2DS2*, we looked for the frequency of the inhibitory *KIR2DL2*, which is in strong linkage disequilibrium with *KIR2DS2*. Again no decreased frequency of *KIR2DL2* was found in our ALL cohort. The data from Almalte et al also implicate that the frequency of group *A KIR* haplotypes, which are abundant in white populations and harbor only a single *KIR-S*, would be much higher in ALL patients. Again our analysis does not show any significant difference between patients and controls (Figure 1B). Further analysis of telomeric and centromeric *KIR* haplotypes[2] as well as the cumulative number of stimulatory *KIR* genes did not reveal any significant difference to the control cohort (data not shown).

Given the technical challenges associated with PCR-based *KIR* genotyping, which is due to the strong similarity between *KIR* genes and the increasing number of alleles, it is generally helpful to assess extended *KIR* genotypes when performing case-control studies. Because of the strong linkage disequilibrium between several pairs of *KIR*, the knowledge of *KIR* genotypes provides an important plausibility control for *KIR* typing results. Moreover, in our experience historic patient sample collections can be particularly challenging for *KIR* typing, leading to decreased amplification efficiency compared with high-quality control samples. Given the consistently decreased frequencies of all *KIR-S* genes in the Almalte et al study,[1] it would be highly desirable to know inhibitory *KIR* gene frequencies in this cohort, which would help to understand how the distribution of *KIR* genotypes is affected. Unfortunately, PCR primers and amplification conditions used for KIR

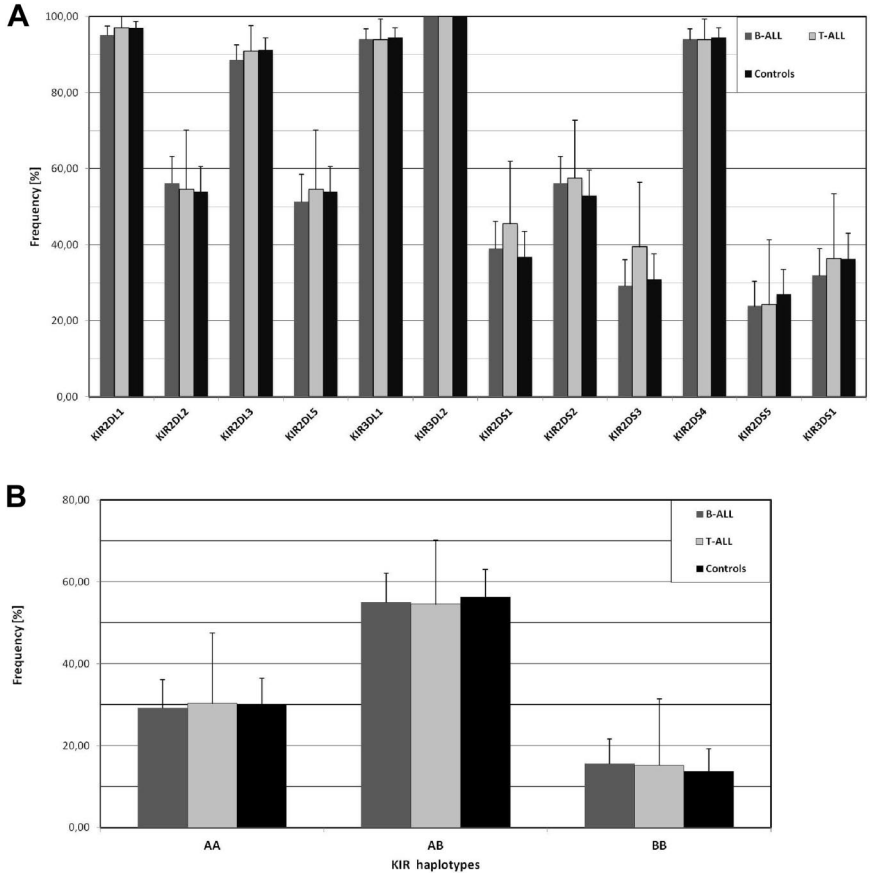

Figure 1. No association of childhood ALL with *KIR* gene frequencies. (A) The frequency of 6 inhibitory *KIR* genes (*KIR2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 3DL2*) and 6 stimulatory *KIR* genes (*KIR2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1*) was analyzed in ALL patients. Our study population (0-18 years) consisted of 185 children with B-ALL (dark gray columns) and 33 children with T-ALL (light gray columns) of European origin. The ethnically matched control group (black columns) consisted of 204 unrelated randomly selected healthy volunteers. PCR-based *KIR* genotyping was performed as described by Vilches et al.[3] As an additional quality control, 10% of samples were randomly selected and analysis repeated with an independent *KIR* typing protocol as described by Uhrberg et al.[4] Similarly, all samples exhibiting rare *KIR* genotypes (frequency $< 0.5\%$) were controlled in this way.[4] Samples with discordant typing results ($n = 9$) were excluded from the analysis. (B) Distribution of group *A* and *B KIR* haplotypes according to previous definitions.[5] Statistical significance was tested by 2-sided Student *t* test and 95% confidence intervals are indicated.

typing were not specified. The lack of these data in the study by Almalte et al makes it difficult to assess the observed discrepancies between the 2 studies.

In summary, we could not confirm the association of *KIR-S* genes with the risk of childhood ALL in our cohort and would generally recommend the assessment of extended *KIR* genotypes when performing case-control studies.

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Response

Associations between activating *KIR* genes and childhood leukemia

We are surprised to know that Babor et al did not detect any significant differences in the frequencies of activating *KIR* genes between acute lymphoblastic leukemia (ALL) patients and healthy controls.[1] Obviously, these results contradict those reported recently by us.[2] Interestingly, the frequencies of these genes in controls are more or less comparable in both studies. Babor and colleagues mentioned technical challenges associated with PCR-based *KIR* genotyping due to strong similarities between *KIR* genes and their many alleles. They further note that because of the archived nature of patient samples, the quality of the DNA may be compromised and hence it may be difficult to amplify *KIR* genes. Therefore, the authors imply that this could be one reason for decreased *KIR* gene frequencies in our patient samples. We have experienced such difficulties in amplifying *KIR* genes in both patient and control samples with longer amplifications. Such difficulties were alleviated when smaller segments of the genes were amplified. All of our PCR reactions for the *KIR* genes amplified $\leq$ 300 bp bands. Furthermore, we have used the same genotyping methods to determine gene frequencies of activating *KIR* genes in other diseases (eg, Crohn disease) and have found increased frequencies of several of the activating *KIR* genes in the patients compared with the controls (data not shown). We receive samples from both patients and controls for these studies from DNA banks. Furthermore, all of the patient and control DNA samples yielded bands in positive control reactions. Therefore, we do not believe that the decreased frequencies of activating *KIR* genes in leukemia patients are due to inherent unsuitability of our DNA samples for PCR-based amplifications. After receiving an invitation to respond to the letter by Babor et al, we regenotyped a subset of our patients and controls. Again, we found significantly decreased frequencies of all activating *KIR* genes in our cohorts. It is noteworthy that decreased frequencies of some activating *KIR* genes in ALL patients have been described.[3,4]

The reasons for the discordant results in the 2 studies are not immediately apparent. However, we speculate that these differences may result from the use of different PCR primer sets. We would encourage Babor and colleagues to verify their results with our primer pairs. We can provide them or send them their exact sequences. We would also be willing to genotype our DNA samples (a subset or all) in our laboratory. Furthermore, it could also be possible that these associations may not be present in the German population. Literature is replete with examples where genetic associations with a disease in one population were not replicated in another.

Concerning inhibitory *KIR* genes in our ALL patients, we are in the process of genotyping them. It is premature to draw definitive conclusions. However, we can say with certainty that they are showing a trend opposite to that of activating genes. This trend also verifies the suitability of our DNA samples for PCR-based genotyping. Concerning KIR haplotypes, our results do suggest that B haplotypes reduce risk for ALL. However, we have not directly determined them in our patient and control samples.

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The role of KIR genes and ligands in leukemia surveillance

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The antileukemic potential of natural killer (NK) cells has been of rising interest in recent years. Interactions between inhibitory killer cell immunoglobulin-like receptors (KIR) and HLA class I ligands seem to be critically involved in the immunosurveillance process. It is also well established that mismatching of HLA class I-encoded KIR ligands in the setting of hematopoietic stem cell transplantation leads to allorecognition of leukemic cells by NK cells, which is in line with the concept of *missing-self* recognition. Recent data now suggest that *KIR* gene polymorphism constitutes another important parameter that needs to be taken into account for selection of suitable stem cell donors. Moreover, the role of *KIR* gene polymorphism for predisposition to leukemia is a current matter of debate. Here, we would like to review the role of KIR function and genetic polymorphism for recognition of leukemia and discuss the impact of these findings for developing novel concepts for NK cell-based immunotherapy strategies.

Keywords: NK cells, KIR, leukemia, ALL, AML, oncology, HSCT

INTRODUCTION

Natural killer (NK) cells represent a subset of innate lymphoid cells that act as first line of defense against viral infections, early cellular transformation and tumor growth (Lanier, 2005; Spits and Di Santo, 2011). The "missing-self" concept, put forward by Kärre and colleagues in the 1980s, formed the basis for understanding NK-derived allorecognition by describing one simplistic mechanism by which NK cells target tumor cells deficient in MHC-I expression (Kärre et al., 1986; Lanier, 2005). According to this hypothesis NK cells expressing a cognate inhibitory receptor for the respective major histocompatibility complex (MHC) class I molecule can detect target cells via their reduced or "missing" expression of "self" MHC class I (Ljunggren and Karre, 1990; Algarra et al., 1997). In humans this is based on human leukocyte antigen (HLA) class I-specific receptors, which are mainly encoded by the killer cell immunoglobulin-like receptor (KIR) family and the lectin-like receptor family of NKG2 genes. Additional MHC class I receptors are encoded by the LIR gene family (Colonna et al., 1997; Cosman et al., 1997). KIRs are the genetically most polymorphic of these receptors, interacting with the equally polymorphic HLA class I system. In rodents, in the absence of *KIR* genes, a comparable MHC class I-specific system is in place encoded by the Ly49 family (Karlhofer et al., 1992; Raulet et al., 1997; Trowsdale et al., 2001).

Killer cell immunoglobulin-like receptors recognize specific motifs of HLA class I molecules, which are the products of highly polymorphic genes of the MHC located on chromosome 6 (Klein and Sato, 2000a,b; Vilches and Parham, 2002; Marsh et al., 2010). HLA-C plays a key role for KIR-mediated recognition of target cells and all allelic variants invariably provide ligands for inhibitory KIR. All HLA-C allotypes carry valine (V) at position 76, while position 80 displays a dimorphism of either asparagine or lysine. HLA-C group 1 with asparagine at position 80 provides the ligand for KIR2DL2 and KIR2DL3, whereas HLA-C group 2 with lysine at position 80 provides the ligand for KIR2DL1. However, it has been shown recently that KIR2DL2 and to a lesser extend KIR2DL3 bind to certain HLA-C2 group alleles as well, while KIR2DL1 exhibits exquisite specificity for HLA-C2 only (Moesta et al., 2008). KIR3DL1 binds the HLA-B motif Bw4, which is also present on some HLA-A molecules whereas KIR3DL2 has specificity for HLA-A alleles (A3 and A11) but also for CpG oligonucleotides (Marcenaro et al., 2009). The ligand specificity of stimulatory KIR is less well described with the exception of KIR2DS1. Activating KIR2DS1 and inhibitory KIR2DL1 share ligand specificity for the HLA-C2 group, which is consistent with their highly similar extracytoplasmic domain. In case of KIR2DS4, weak but significant interactions with subsets of HLA-C alleles as well as HLA-A*11 could be detected (Graef et al., 2009). The ligands of KIR3DS1 have yet to be identified but recent studies have suggested that the HLA-Bw4-T80 allotype HLA-B*2705 is a potential putative ligand for KIR3DS1 (Martin et al., 2002; Alter et al., 2007, 2009; Korner and Altfeld, 2012). Specificity of the stimulatory KIR KIR2DS2, KIR2DS3, and KIR2DS5 is not known and might comprise also non-HLA ligands (Kim et al., 1997; Vales-Gomez et al., 1998; Winter et al., 1998; Vilches and Parham, 2002; Carr et al., 2007; Della Chiesa et al., 2008; VandenBussche et al., 2009).

Killer cell immunoglobulin-like receptors are displayed on the surface of NK cells in diverse combinations. This clonally-distributed expression mode leads to the generation of complex NK cell repertoires, which basically comprise NK cells expressing all possible combinations of receptors (Valiante et al., 1997; Raulet et al., 2001; Yawata et al., 2006; Andersson et al., 2010; Schonberg et al., 2011a). The clonal expression mode and the cell type specificity of *KIR* genes are epigenetically regulated on the levels of DNA methylation and histone modifications in addition to promoter-derived transcriptional regulation (Uhrberg, 2005b).

NK CELL-MEDIATED TUMOR SURVEILLANCE

Natural killer cell function is determined by the net effect of signaling through several receptor families including activating, inhibiting, adhesion, and cytokine receptors. In this way, NK cells have demonstrated not only the ability of killing virally infected cells, but also of exerting antitumor cytotoxicity against lymphoblastic or myeloid hematologic malignancies and solid tumors like ovarian, breast, and colon cancer (Pende et al., 2005; Re et al., 2006; Stein et al., 2006). Notably, it has been shown that cytotoxicity of NK cells in the peripheral blood of leukemia patients is significantly reduced (Costello et al., 2002; Fauriat et al., 2007). Various mechanisms and characteristics could account for this reduced cytotoxicity: (a) an increased expression of MHC class I on leukemic blasts, (b) a reduced expression of ligands of various stimulatory NK cell receptors like NKG2D, NCR, and KIR on leukemic blasts, and (c) a reduced expression of these activating receptors on NK cells of patients with leukemia (Nowbakht et al., 2005; Szczepanski et al., 2010). Most of the investigations so far concerning the role of NK cells in leukemia dealt with adult patients suffering from acute myeloid leukemia (AML; Moretta et al., 2011). Little is known regarding the role of NK cells in the emergence and prevention of acute lymphoblastic leukemia (ALL), especially in the pediatric setting. Several studies suggest a less efficient interaction between NK cells and ALL blasts compared to the interaction between NK cells and AML blasts at the time of diagnosis (Nasrallah and Miale, 1983; Ruggeri et al., 2008). In this regard, surface density of HLA class I molecules seems to be higher on ALL compared to AML blasts (Pende et al., 2005). Other mechanisms of immune escape in ALL might be the absence or down-regulation of ligands for activating NK cell receptors and the expression of ligands for inhibitory receptors. Remarkably, information on the expression of KIR on NK cells of patients suffering from acute leukemia is scarce. Thus, the influence of an emerging acute or chronic leukemia on the functional NK cell repertoire as well as the question of adaptation to self-HLA class I and the licensing status in ALL patients remain to be analyzed in detail.

In HLA class I-matched hematopoietic stem cell transplantation (HSCT), NK cells were shown to contribute to leukemia surveillance based on the specific make-up of their KIR locus (Cooley et al., 2009, 2010). Interestingly, stimulatory *KIR* genes are emerging as important variables in this process (Venstrom et al., 2012). Since specificity of most of the stimulatory *KIR* genes is not well defined, the precise mechanism of their contribution constitutes a challenging but important question.

LICENSING AND ALLOREACTIVITY

Since KIR and HLA class I genes segregate on different chromosomes, a tolerance mechanism has to be in place to prohibit development of autoreactive NK cells, e.g., NK cells with an inhibitory KIR for HLA class I ligands not present in the genome. NK cells expressing orphan inhibitory KIR are indeed present in most NK cell repertoires and pose a potential threat (Anfossi et al., 2006). However, only NK cells expressing inhibitory receptors for self-HLA class I acquire full functional competence, whereas potential autoreactive NK cells remain in a hyporesponsive state, a process referred to as education or licensing (Fernandez et al., 2005; Kim et al., 2005, 2008; Anfossi et al., 2006; Yu et al., 2007; Yawata et al., 2008).

In clinical stem cell transplantation, licensed NK cells become potentially alloreactive when transferred in an HLA class I mismatched patient. Moreover, non-licensed NK cells might be reactivated in the cytokine-rich environment of the reconstituting hematopoietic system, providing an additional source of alloreactive NK cells (Hsu et al., 2005, 2006; Clausen et al., 2007; Miller et al., 2007). In this regard, it could be shown that NK cells can be aberrantly activated after transplantation and achieve effector function and functional competence in spite of lacking class I ligand for donor inhibitory KIR (Hsu et al., 2005, 2006; Clausen et al., 2007; Miller et al., 2007; Yu et al., 2009). Moreover, Yu et al. (2009) revealed that in HSCT settings "unlicensed" NK cells with inhibitory KIR for non-self-HLA are functional while "licensed" NK cells with inhibitory KIR for self-HLA appeared to be hyperresponsive. These findings were opposed by Bjorklund et al. (2010) who demonstrated that NK cells without self-HLA receptors remained hyporesponsive after HSCT thus remaining tolerant. In contrast, Vago et al. (2008) showed a general hyporesponsiveness of NK cells in the presence of leukemic blasts. Licensed and unlicensed NK cell subsets with alloreactive potential can be qualitatively predicted in the HSCT setting as outlined in **Table 1** and the size of the alloreactive populations estimated by flow cytometry.

Another interesting facet of KIR-mediated alloreactivity is given by the recently described education of stimulatory KIR. It was shown that NK cells expressing KIR2DS1, which is an activating receptor specific for the HLA-C2 allotype are functional only when derived from HLA-C1/C2 or HLA-C1/C1 donors but hyporesponsive in donors homozygous for HLA-C2. The data imply that expression of KIR2DS1 in the presence of HLA-C2 generally renders NK cells hyporesponsive independent of inhibitory KIR or

Table 1 | Pattern of NK cell licensing and alloreactivity for HLA-C-encoded KIR ligands.

Donor	Education		Recipient	Alloreactive NK cells	
KIR ligand	Licensed	Unlicensed	KIR ligand	Licensed	Unlicensed
C1/C1	KIR2DL3	KIR2DL1	C1/C1	–	KIR2DL1
C1/C2	KIR2DL1/ KIR2DL3	–	C1/C1	KIR2DL1	–
C2/C2	KIR2DL1	KIR2DL3	C1/C1	KIR2DL1	–
C1/C1	KIR2DL3	KIR2DL1	C1/C2	–	–
C1/C2	KIR2DL1/ KIR2DL3	–	C1/C2	–	–
C2/C2	KIR2DL1	KIR2DL3	C1/C2	–	–
C1/C1	KIR2DL3	KIR2DL1	C2/C2	KIR2DL3	–
C1/C2	KIR2DL1/ KIR2DL3	–	C2/C2	KIR2DL3	–
C2/C2	KIR2DL1	KIR2DL3	C2/C2	–	KIR2DL3

Of note, to keep the scheme simple the contribution of KIR2DL2 and KIR2DS1 were not considered here.

CD94/NKG2A expression (Fauriat et al., 2010). In line with this, in a recent evaluation of allogeneic HSCT for AML, grafts possessing KIR2DS1 on a C1/C1 background were associated with the lowest incidence of relapse (Venstrom et al., 2012). However, no significant effect of KIR2DS1 was seen in C2/C2 patients, which generally exhibited worse outcome compared to C1/C1. It was thus suggested that the lack of C2/C2 (or alternatively the presence of C1/C1) is another dominant genetic influence in AML patients that is independent of KIR2DS1 (Fischer et al., 2007; Fischer and Uhrberg, 2012). Remarkably, Pende et al. (2009) reported that KIR2DS1-expressing NK cell clones generated from C2/C2 recipients following haploidentical transplantation were highly cytolytic against patients' C2/C2 leukemic blasts and overriding inhibition by NKG2A or inhibitory KIR. Clearly, the clinical role of KIR2DS1 and other stimulatory KIR for NK cell alloreactivity should be clarified in further studies.

DEVELOPMENT OF ALLOGENEIC NK CELL REPERTOIRES

Natural killer cells develop from hematopoietic progenitor cells (HPC) in distinct stages, defined by a panel of surface markers (Yokoyama et al., 2004; Freud et al., 2006; Caligiuri, 2008). NK progenitor cells ($CD34^{dim}/CD117^{-}/NKG2A^{-}$) differentiate into immature $CD56^{+}$ NK cells, which still lack the CD94/NKG2A heterodimer ($CD34^{-}/CD117^{+}/CD56^{+/-}$). In a next step, these immature NK cells develop into $CD56^{bright}$ NK cells, which express CD94/NKG2A ($CD34^{-}/CD117^{+/-}/CD94^{+}$). Finally, mature NK cells are characterized by $CD56^{dim}$, $CD16^{+}$ and either NKG2A or KIR expression ($CD34^{-}/CD117^{-}/CD94^{+}/CD16^{+}/KIR^{+}$; Freud et al., 2006). In this context, it was already shown that $CD56^{bright}$ cells found in peripheral blood and secondary lymphoid organs can be directly differentiated into mature $CD56^{dim}CD16^{+}$ NK cells (Ferlazzo et al., 2004; Chan et al., 2007; Romagnani et al., 2007; Huntington et al., 2009).

After HSCT, NK cells are known to be the lymphocyte subset showing the fastest reconstitution *in vivo*, which is a prerequisite to efficiently exert antileukemic effects early after HSCT (Pfeiffer et al., 2010). They are $CD56^{+}$ and predominantly $CD16^{-}$ and KIR^{-}. It could be shown that $CD56^{dim}$ NK cells from patients who recently underwent hematopoietic transplantation transiently overexpress NKG2A, compared with cells from healthy donors (Dulphy et al., 2008; Vago et al., 2008; Beziat et al., 2009, 2010). In many respects, the early recovering NK cells closely resemble the $CD56^{bright}$ subset of peripheral blood NK cells, whereas the fraction of cells corresponding to the $CD56^{dim}CD16^{+}$ NK cell fraction increases and predominates at later times after transplantation (Shilling et al., 2003). This orderly appearance of $CD56^{bright}$ and $CD56^{dim}CD16^{+}$ subsets supports the model that $CD56^{bright}$ cells are the principal progenitors of mature $CD56^{dim}CD16^{+}$ NK cells.

Another factor that influences the composition of allogeneic NK cell repertoires following HSCT is the sequential mode of KIR acquisition during NK cell differentiation. *In vitro*, NK cell differentiation from hematopoietic progenitors leads to early expression of KIR2DL2/3 whereas KIR2DL1 is only expressed at later time points (Miller and McCullar, 2001; Schonberg et al., 2011b). The model of differentiation is given in **Figure 1**. Similar observations were made in studies following the reconstitution of NK cell repertoires following HSCT (Shilling et al., 2003). These patterns of expression influence the overall frequency of functionally competent NK cells including putative leukemia-reactive clones. In this context, it could be shown that the presence of HLA-C1, which is the ligand for the early-expressed KIR receptor KIR2DL3 was associated with increased survival if present in the recipient, whereas the presence of HLA-C2 ligands in the recipient constituted a risk factor (Fischer et al., 2007). These findings were in line with previous clinical observations (Giebel et al., 2003; Hsu et al., 2005). Notably, HLA-C allele matching (four-digit level) had differential effects in HLA-C1 (*C1/C1*) and HLA-C2 (*C1/C2* and C2/C2) subgroups of patients: in HLA-C1 patients with myeloid leukemia, HLA-C-matching was associated with improved survival as described previously (Morishima et al., 2002; Flomenberg et al., 2004; Petersdorf et al., 2004; Woolfrey et al., 2011). Surprisingly, the opposite was true for HLA-C2 patients, which profited from having an HLA-C allele mismatch on the allelic level (Fischer et al., 2012), i.e., two different HLA-C-alleles that both belong to the HLA-C2 ligand group. HLA-C2 patients with these allele-mismatched, but "KIR-neutral" donors generally showed superior survival with substantial improvement in treatment related mortality and relapse incidence compared to HLA-C1 patients with HLA-C mismatches. The data suggest that HLA-C mismatching should be avoided in HLA-C1 patients as reported previously but might be favorable for HLA-C2 patients. Furthermore, the study suggested a novel model in which matched HLA-C1 and mismatched HLA-C2 patients constitute a low-risk group, whereas mismatched HLA-C1 and matched HLA-C2 patients would represent a high-risk group with inferior overall survival, increased TRM and higher relapse rate [in myeloid leukemia and myelodysplasia syndrome (MDS); Fischer et al., 2012].

KIR GENETICS AND ALLOREACTIVITY

Two *KIR* haplotype groups, *A* and *B*, have been identified based on differential *KIR* gene content (Uhrberg et al., 1997). Group *A* and *B* haplotypes have four framework genes in common: the *KIR* gene cluster is flanked by the non-expressed *KIR3DL3* at the centromeric end, *KIR3DL2* at the telomeric end, with *KIR3DP1* and *KIR2DL4* in the middle. Deletion and duplication of genes have led to many different haplotypes (Vierra-Green et al., 2012). Group *A* haplotypes are present in all populations and consist of five productive inhibitory *KIR* (*KIR2DL3*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, *KIR3DL2*), and the stimulatory *KIR2DS4*, which is frequently present in an allelic variant that is not expressed on the cell surface (Maxwell et al., 2002). Group *B* comprises *KIR* haplotypes with diverse gene content including several genes (*KIR2DL2*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1*) that are not part of group *A* haplotypes. As a consequence, most group *B* haplotypes encode more activating KIRs compared to group *A* haplotypes. All individuals can be categorized as having one of two KIR genotypes: *A/A*, which is homozygous for group *A* KIR haplotypes, or *B/x*, which contains either one (*A/B* heterozygotes) or two (*B/B* homozygotes) group *B* haplotypes.

There have only been a few studies regarding the impact of genetic polymorphisms of activating and inhibitory KIR in humans on the susceptibility and resistance to leukemia.

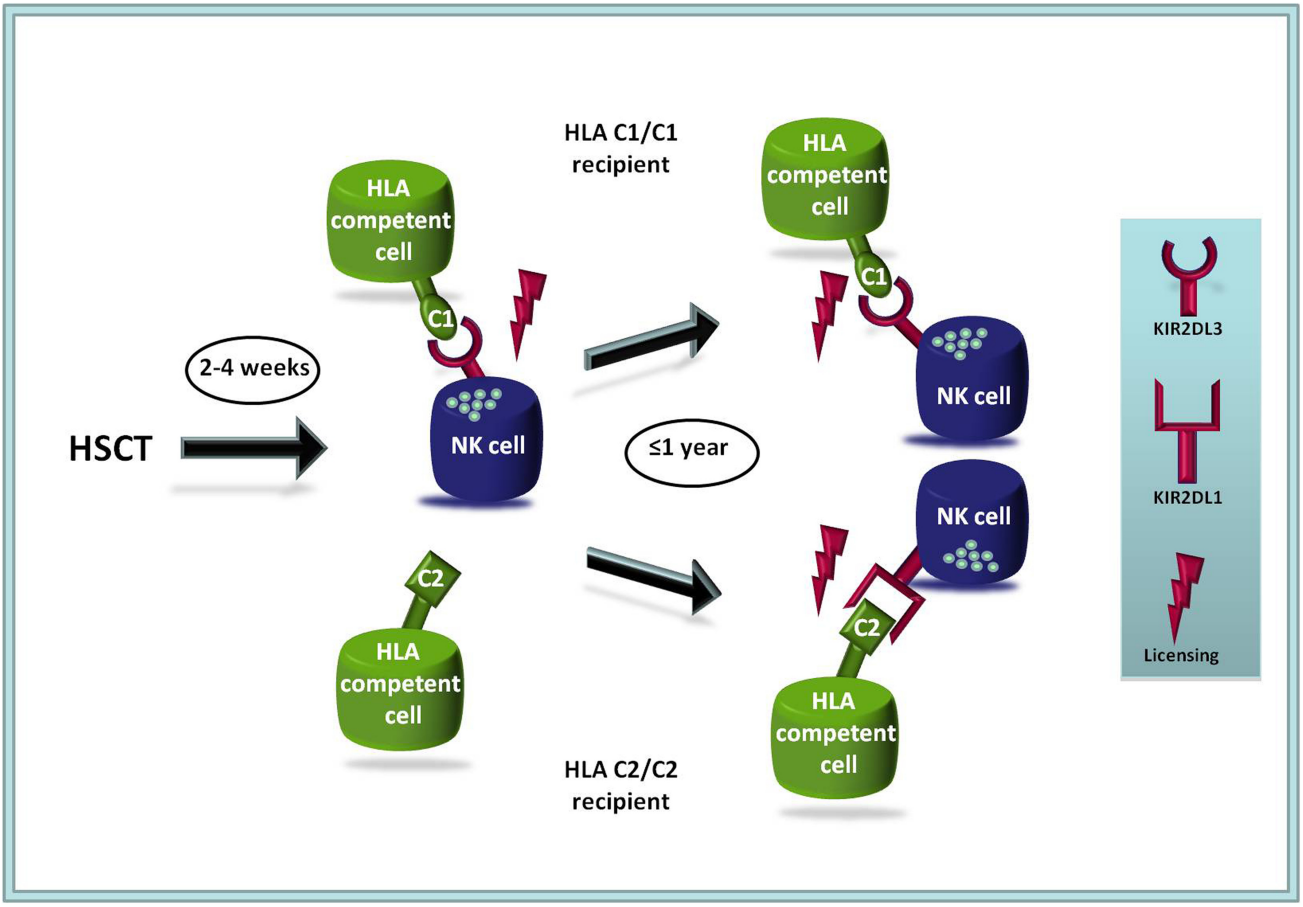

FIGURE 1 | Model of NK cell reconstitution and NK cell receptor expression following allogeneic HSCT. The HLA-C1-specific KIR2DL2/3 receptor is expressed at earlier time points on reconstituting NK cells than the HLA-C2-specific KIR2DL1 (Shilling et al., 2003; Nguyen et al., 2005; Fischer et al., 2007). Thus, in the clinical setting C2/C2 recipients possess a lower frequency of functionally competent NK cells (expressing KIR2DL1) than C1/C1 patients (expressing KIR2DL2/3), which may lead to a disadvantage in the early phase following HSCT. The model is compatible with decreased overall survival rates and increased relapse rates in C2/C2 individuals that were found in several different studies (Fischer et al., 2007, 2012; Venstrom et al., 2012).

Verheyden et al. (2004) revealed an association of the inhibitory *KIR2DL2* gene with leukemia. In a polymorphic cohort of AML, ALL, CML (chronic myeloid leukemia), and CLL (chronic lymphoblastic leukemia) patients they found a significantly increased frequency of the KIR haplotype combination *A/B* in leukemic patients compared to controls. This finding was related to the high prevalence of the inhibitory *KIR2DL2* gene. Another significant association with leukemia was found for two distinct KIR genotypes, which included all inhibitory KIRs (Uhrberg et al., 2002; Verheyden and Demanet, 2008). In a more recent study of pediatric ALL patients, Almalte et al. (2011) reported a highly significant association between the presence of stimulatory *KIR* genes and decreased risk for ALL. The risk for ALL progressively decreased with the cumulative number of stimulatory *KIR*. In a subsequent study, no significant association was found between childhood ALL and stimulatory *KIR* genes (Babor et al., 2012). In the latter study neither stimulatory nor inhibitory genes exhibited a significant deviation in frequency from an ethnically matched control group. Analyses of the cumulative number of stimulatory and inhibitory *KIR* genes as well as further analysis of telomeric and centromeric *KIR* haplotypes did not reveal any significant difference to the control cohort. Similarly, in a recent study analyzing a large cohort of AML patients, frequencies of stimulatory *KIR* genes were again not different to published healthy donor cohorts (Venstrom et al., 2012). More thorough analysis of *KIR* genes on the allelic level will be necessary to settle the question if *KIR* genes constitute a major disease susceptibility locus for acute leukemia in children and adults. Notably, the identification of single *KIR* genes as disease markers is generally complicated by the fact that groups of *KIR* genes are tightly linked in a small number of common centromeric and telomeric haplotypes (Uhrberg, 2005a). Moreover, centromeric KIR genes could be linked to the neighboring LIR gene family including inhibitory and stimulatory LIR genes that are known to be expressed on NK cells as well (Cosman et al., 1997; Young et al., 2001).

In the clinical transplant setting, Stringaris et al. (2010) revealed that three donor *B* haplotype *KIR* genes, *KIR2DL5A*, *KIR2DS1*, and *KIR3DS1*, were associated with significantly less relapse in

patients with AML undergoing HLA-identical sibling HSCT. The authors could not confirm these findings in patients with other myeloid or lymphoid malignancies. Recent data suggest evidence of beneficial effects of KIR *B* genotype donors in AML patients in an unrelated HLA-matched HSCT setting (Cooley et al., 2009, 2010). Cooley et al. (2009) analyzed the effect of KIR genotypes on outcome in a comparatively homogeneous study cohort of 448 AML patients who received T cell replete transplants from unrelated donors. They could show that the use of donors with KIR group *B* haplotypes was associated with significant improvement in relapse-free and overall survival, with 30% better relative risk in both endpoints. The same group subsequently performed analyses regarding gene content motifs, which further revealed that compared with group *A* haplotype motifs, centromeric and telomeric group *B* motifs both contributed to relapse protection and improved survival. Centromeric B homozygosity (*Cen-B/B*) had the strongest independent effect. Overall, significantly reduced relapse was achieved with donors having two or more *B* gene-content motifs in both HLA-matched and mismatched transplants (Cooley et al., 2010). In contrast, Venstrom et al. (2012) revealed in their study of AML transplant patients a beneficial effect of telomeric but not centromeric genes, namely *KIR2DS1* and *KIR3DS1*. Together, these findings highlight the need for further studies of KIR polymorphism in HSCT, possibly on the allelic level, to enable incorporation of *KIR* gene content status along with HLA-matching in future algorithms for donor selection in allogeneic/haploidentical HSCT settings.

EXPLOITING KIR AND KIR LIGAND MISMATCHING IN HSCT

In HSCT settings, donor-derived T cells are essential not only for early cellular engraftment following HSCT but also for eradication of remaining leukemic blasts. On the other hand, T cells are major effectors of rejection or acute or chronic graft versus host disease (GvHD). T cell-mediated alloreactivity is directed against histocompatibility antigens not only displayed on the stem cell recipient's leukemic cells but also on other tissues. Especially cytotoxic $CD8^+$ effector T cells may then attack tissues such as liver, intestines, and skin, which results in acute or chronic GvHD. Currently, in order to avoid GvHD various manipulations of HSCT grafts such as $CD6^+$ T cell depletion (Soiffer et al., 2001), $CD3^+/CD19^+$ T and B cell depletion (Federmann et al., 2012) or α/β T cell (Handgretinger, 2012) depletion are implemented in different transplant centers. With this approach, the numbers of potentially graft rejecting or GvHD causing T cells can be reduced considerably while HPC and NK cells remain in the graft and can be transferred into the patient. Such grafts, mainly consisting of mature NK cells, $CD34^+$ stem cells and only a few remaining T cells (max. 5×10^4/kg BW) of donor origin repopulate the recipient's hematopoietic system and rebuild the recipient's new immune system that quickly reconstitutes immunity to viral and bacterial infections and eliminates leukemic blasts that survived chemotherapy (graft versus leukemia effect – GvL). Especially in $CD3^+/CD19^+$ depleted grafts large numbers of mature donor NK cells are co-transfused.

Previous *in vitro* studies already demonstrated the alloreactive potential of NK cells (Ciccone et al., 1992; Colonna et al., 1992) and showed that the kind and frequency of alloreactive NK cells can be predicted if KIR expression in the donor and presence of KIR ligands in the recipient are known (Valiante et al., 1997). The clinical contribution of NK cells to alloreactivity was impressively shown by Ruggeri et al. (2002) who demonstrated a strong GvL effect for AML patients receiving a haploidentical allogeneic transplant mismatched for HLA-C KIR ligands. In this setting alloreactive donor-derived NK cells are thought to promote engraftment, reduce GVHD, and decrease leukemic relapse. Whereas some subsequent studies did confirm the KIR ligand mismatch model (Giebel et al., 2003) others could not find a beneficial effect of KIR ligand mismatching (Davies et al., 2002; Hsu et al., 2006; Miller et al., 2007), which might be explained by specific differences in HSCT protocols performed in the different transplant centers. Subsequently, several modified donor selection models were suggested based on the consideration that donor NK cells expressing a certain inhibitory KIR would be alloreactive if the respective ligands (C1, C2, or Bw4) in the patient are missing. This model was originally proposed by Leung et al. (2004) and demonstrated better clinical outcome in haploidentical HSCT for pediatric lymphoid leukemia thereby proofing that NK cell-mediated relapse control is not restricted to myeloid leukemia. Hsu et al. (2005) applied the same model to HLA-identical HSCT showing that patients with missing KIR ligands for which the donor possessed a specific KIR exhibited increased survival through decreased relapse incidence in AML and MDS patients. An overview of studies investigating the role of *KIR* genes in clinical stem cell transplantation is given in **Table 2**.

NK CELL IMMUNOTHERAPY: ONLY AT THE BEGINNING OF GREAT POSSIBILITIES?

Cytokines such as IL-2, IL-15, and IL-21, as well as the combination of IL-12 and IL-18 are known to activate NK cells (Carson et al., 1995; Singh et al., 2000; Trinchieri, 2003; Young and Ortaldo, 2006). Coadministration of such NK cell-activating cytokines have the potential to increase survival of adoptively transferred NK cells, enhance proliferation, up-regulation of cytotoxic and adhesion molecules, augment cytokine production and increase GvL effects (Becknell and Caligiuri, 2005). IL-2 activated NK cells kill tumor cells *in vitro* and *in vivo* by localizing to the tumor sites to elicit their cytotoxic action and they prolong the survival of tumor bearing mice. This was seen in mice with multiple myeloma (Alici et al., 2007) and in NOD/SCID mice with metastatic neuroblastoma (Castriconi et al., 2004). Unfortunately, administered cytokines such as IL-2, IL-15, or IL-18 are rapidly cleared, thus requiring repeated injections (subcutaneous) of large amounts. This in turn increases the potential for toxicity considerably and furthermore might elicit activation of unwanted cell populations or even cause activation-induced cell death of NK cells. IL-2 but not IL-15 is known to be involved in the maintenance of T regulatory cells (Treg), which could impair antitumor immune response (Waldmann, 2006). Thus, IL-15 administration holds promise to specifically boost NK cell alloreactivity without undesired stimulation of Tregs. Importantly, the use of IL-15 as GMP product has recently been approved by the U.S. Food and Drug Administration (FDA). However, since full biological activity of IL-15 is dependent on its trans-presentation on the cell surface, suitable application strategies have to be developed to increase biological stability and

Table 2 | An overview of studies analyzing the role of KIR for susceptibility and clinical HSCT for leukemia.

	KIR/model	Observation	Disease	Treatment	Reference
Genetic associations	*KIR2DL2*	Increased frequency	AML/ALL	–	(Verheyden et al., 2004)
	KIR-S	Decreased frequency	ped. ALL	–	(Almalte et al., 2011)
	KIR-L / KIR-S	No association	ped ALL	–	(Babor et al., 2012)
Inhibitory KIR	Missing KIR ligand	Relapse ↓	ped. AML/ALL	Haploidentical	(Leung et al., 2004)
	Missing KIR Ligand	Survival ↑, Relapse ↓	AML/ALL	MSD HSCT	(Hsu et al., 2005)
	KIR ligand mismatch	Relapse ↓, Survival ↑	AML	Haploidentical	(Ruggeri et al., 2007)
	KIR3DL1	Survival ↓	Acute leukemia	Unrelated HSCT	(Gagne et al., 2009)
Stimulatory KIR	KIR2DS2	Survival ↓	AML (ALL)	MSD HSCT	(Cook et al., 2004)
	KIR3DS1	Survival ↓	Acute leukemia	Unrelated HSCT	(Gagne et al., 2009)
	KIR2DS1	Relapse ↓	AML	Unrelated HSCT	(Venstrom et al., 2012)
Haplotype structure	Group B	Relapse ↓ chronic GvHD ↑	Adult AML	Unrelated HSCT	(Cooley et al., 2009)
	Group B haplotype score	Relapse ↓	AML (ALL)	Unrelated HSCT	(Cooley et al., 2010)

HSCT, hematopoietic stem cell transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; Cen, centromeric; MSD, matched sibling donor; ped, pediatric; indiv, individuals.

activity. These include administration of pre-associated complexes of IL-15 with soluble IL-15Rα-IgG1-Fc or stimulation of native IL-15Rα expression with, e.g., anti-CD40 (Zhang et al., 2009; Steel et al., 2012).

Advanced cell therapy trials with donor NK cells post-haploidentical stem cell transplantation provide a promising treatment option for patients with high risk leukemia and tumors (Leung et al., 2004, 2005). While the established adoptive T cell therapies are associated with the risk of GvHD, NK cells mediate GvL effects without substantial induction of acute GvHD. These findings encouraged scientists and clinicians to conduct clinical trials utilizing adoptive transfer of cytokine-activated NK cells, which could serve as an attractive cell therapy option not only in recipients of haploidentical stem cell transplantation but in other oncologic diseases as well (Miller et al., 2005; Passweg et al., 2006; Rubnitz et al., 2010). In 2005, Miller and colleagues were the first to infuse haploidentical NK cells into AML recipients in a non-transplant setting (Miller et al., 2005). Before NK cell infusion patients received an intensive immunosuppressive regimen of low-dose cyclophosphamide and methylprednisolone or fludarabine. Successful transfer of haploidentical KIR ligand-mismatched NK cells was followed by a subcutaneous administration of IL-2 for 2 weeks. NK cells expanded *in vivo* and were functional at day 14 following infusion (Miller et al., 2005). But still, the intensive conditioning regimen and high doses of IL-2 resulted in significant hematologic and non-hematologic toxicities as well as prolonged hospitalization. In order to further optimize this strategy of donor-derived NK cell transfer, Rubnitz et al. (2010) extended investigations concerning the safety and feasibility of low-dose immunosuppression followed by NK cell infusion. This study on transfer of alloreactive haploidentical KIR ligand-mismatched NK cells has been performed in children with AML after achievement of complete remission. In order to minimize toxicity while still allowing engraftment of haploidentical NK cells a low-intensity regimen was administered to patients followed by NK cell infusion and multiple doses of IL-2. The study gave encouraging results, and has allowed commencement of a phase II trial as consolidation therapy in children with AML. In multiple myeloma, infusion of haploidentical KIR ligand-mismatched NK cells after conditioning therapy with melphalan and fludarabine has been proposed in relapsed patients (Shi et al., 2008). Curti et al. (2011) demonstrated that infusion of purified NK cells is feasible and safe in elderly patients with high-risk AML and these donor alloreactive NK cells were shown to kill recipient leukemia cells. Again, no NK cell-related toxicity, including GVHD, was observed. Donor-versus-recipient alloreactive NK cells were shown *in vivo* by the detection of donor-derived NK clones that killed recipient's targets.

Together, these trials show that adoptive immunotherapy with haploidentical NK cells obtained from KIR ligand-mismatched donors is feasible and safe in adults as well as in children. Nevertheless, further studies are highly warranted to specifically assess the role of NK cell therapy to elucidate which patient collective (AML/ALL/adults/children) is best suited at which stage of disease (remission/relapse) and at which stage of therapy (after/prior to/without HSCT). Furthermore, these studies should address the question, which NK cell subset (single KIR^+ NK cells, licensed or unlicensed) mediates strongest reactivity against leukemia.

CONCLUSION

KIR genes have two unusual features that could be harnessed for improving current HSCT strategies: firstly, *KIR* genes are highly polymorphic leading to novel opportunities beyond classical HLA matching strategies for selection of optimal stem cell donors based on *KIR* genetic diversity. Secondly, KIR are expressed in a clonally distributed manner, which leads to the formation of NK cell repertoires encompassing tolerant as well as potentially alloreactive NK cell clones. Both features have a great potential to be exploited in the setting of clinical stem cell transplantation.

While recent studies could not detect a predisposition for leukemia based on presence or absence of particular inhibitory and stimulatory *KIR* genes or KIR haplotype classification, there is growing evidence for beneficial effects of KIR B genotype donors in HSCT for AML. In general, NK cells and their variable capability of killing residual leukemic blasts become a criterion of growing importance as clinicians are offered another important factor concerning donor selection in unrelated, sibling, and haploidentical transplant settings along with HLA matching, CMV status, blood group, age, and gender. Furthermore, recent investigations suggest that homozygous HLA-C2 stem cell transplant recipients constitute a high-risk group that needs special attention. In this regard, recent data suggest that survival of C2/C2 patients might be improved by allele mismatching of donor/recipient pairs for HLA-C2, a novel concept that has to be confirmed in retrospective studies of larger cohorts. On the basis of optimally defined matching/mismatching strategies an important next step for successful NK cell-based intervention in HSCT will be to qualitatively and quantitatively define patient-specific alloreactive subsets among donor NK cell repertoires opening the possibility to stimulate, enrich, and expand those subsets *in vitro* or *in vivo* for immunotherapy.

Antileukemic NK cells, either allogeneic or unlicensed autologous NK cells, emerge as a feasible therapy option and might improve clinical outcome in myloid leukemia. Further integration into established HSCT protocols may indeed improve survival rates in adults and children. It will be an important task to define, which patients are most suitable for NK cell-mediated antileukemic therapy based on incorporation of multiple factors like genetic predisposition, HLA, KIR and the nature of leukemic disease. It would be desirable to expand the possible range of applications to ALL and lymphoma as well as toward NK cell-based therapy of solid tumors. Toward this goal, our biological understanding of NK cell function and interaction with leukemic target cells has to be further improved.

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Brief Report

LYMPHOID NEOPLASIA

KIR ligand C2 is associated with increased susceptibility to childhood ALL and confers an elevated risk for late relapse

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Key Points

- C2 confers increased susceptibility to childhood B-ALL.
- C2 is associated with increased risk of late relapse in childhood B-ALL.

A role for *HLA class I* polymorphism in childhood acute lymphoblastic leukemia (ALL) has been suggested for many years, but unambiguous associations have not been found. Here, we show that the *HLA-C*–encoded supertypic epitope C2, which constitutes a high-affinity ligand for the inhibitory natural killer (NK) cell receptor KIR2DL1, is significantly increased in ALL patients (n = 320; *P* = .005). Stratification for ethnicity and disease subtype revealed a strong association of *C2* with B-ALL in German cases (*P* = .0004). The effect was independent of *KIR2DS1* and *KIR2DL1* allelic polymorphism and copy number. Analysis of clinical outcome revealed a higher incidence of late relapse (>2.5 years) with increasing number of *C2* alleles (*P* = .014). Our data establish C2 as novel risk factor and homozygosity for C1 as protective for childhood B-ALL supporting a model in which NK cells are involved in immunosurveillance of pediatric B-ALL via interaction of KIR with HLA-C ligands. (*Blood*. 2014;124(14):2248-2251)

Introduction

Natural killer (NK) cells serve important functions in immunosurveillance of hematologic malignancies. Inhibitory killer cell immunoglobulin-like receptors (KIR) recognizing the *HLA-C*–encoded ligands C1 and C2 are central to this process. C2 is recognized by the inhibitory KIR2DL1 with high affinity and specificity as well as by the stimulatory KIR2DS1 receptor. KIR2DL2/3 interact with C1 ligands with less affinity and specificity, ie, KIR2DL2 and to a lesser extent KIR2DL3 show some cross-reactivity with C2 ligands.[1] In the setting of hematologic stem cell transplantation, the presence of C2 ligands was already identified as risk factor in patients with myeloid leukemia.[2-4] In lymphoblastic leukemia, recent studies suggest that KIR or KIR ligands may play a role.[5,6] Here, we asked the question if the *C1/C2* dimorphism constitutes a susceptibility locus for childhood acute lymphoblastic leukemia (ALL).

Study design

Patients and controls

We analyzed a cohort of 320 pediatric ALL cases treated at the University Hospital Düsseldorf between 1988 and 2013. Patient characteristics and inclusion and exclusion criteria are given in supplemental Table 1 and supplemental Figure 1, available on the *Blood* Web site. The healthy control cohort was taken from the Düsseldorf Bone Marrow Registry as part of a prospective *HLA* class I typing project involving randomly selected donors (n = 1515). Donors did not undergo any specific medical examination or selection according to health status, were not related to any of the patients, and were not previously selected for stem cell donation for an unrelated leukemic patient. German ethnicity was assigned to donors with first and last name of the German-speaking area and residency in Germany.

KIR and *HLA* genotyping

KIR genotyping was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) as previously described.[7] A total of 10% of samples were randomly retyped by an independent *KIR* typing method with full concordance.[8] Subtyping for *KIR2DL1* alleles (*KIR2DL1*001-004*) was done as previously described.[9] Additional SSPs were used for *KIR2DL1*004* (forward-CATCTTCTTCCAGGTAACCCCC, reverse-TTTTGTTGGAGCACCAGCA). *KIR2DL1* copy-number variation was analyzed by SYBR green–based real-time PCR (StepOnePlus PCR system; Applied Biosystems). Results were normalized to *KIR3DL2*, which served as internal 2-copy control gene (gene frequency in whites of 99.7%).[10] *HLA* class I typing was performed by Luminex technology (One Λ). Confirmatory typing for *C1/C2* epitopes was performed by PCR-SSP as previously described.[11]

Statistics

Two-tailed Fisher's exact test was used to compare categorized groups. Cumulative incidences of relapse and late relapse (defined as relapse occurring ≥30 months from remission[12]) were analyzed after adjusting for the competing risk of nonrelapse mortality. Interactions of different covariates on the analytical end point relapse were evaluated by stepwise proportional hazards general

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Table 1. Association of *KIR* and *HLA* class I ligands with incidence of childhood ALL

	Cases		Controls			
	n	%	n	%	OR (95% CI)	*P*†
ALL						
C1/C1	98	30.6	592	39.1	0.69 (0.53-0.89)	**.0051**
C1/C2	161	50.3	695	45.9	1.19 (0.94-1.52)	.1563
C2/C2	61	19.1	228	15.0	1.33 (0.97-1.82)	.0764
C1 alleles‡	357	55.8	1879	62.0	0.77 (0.65-0.92)	**.0037**
C2 alleles	283	44.2	1151	38.0	1.29 (1.09-1.54)	
B-ALL, German						
C1/C1	57	27.9	420	41.2	0.54 (0.39-0.75)	**.0004**
C1/C2	110	53.9	463	45.4	1.44 (1.07-1.95)	.0312
C2/C2	37	18.1	137	13.4	1.44 (0.97-2.15)	.0987
C1 alleles‡	224	54.9	1303	63.8	0.69 (0.56-0.85)	**.0008**
C2 alleles	184	45.1	737	36.2	1.45 (1.17-1.80)	
Bw4/Bw4	15	11.0	139	13.7	0.78 (0.44-1.37)	.4243
Bw4/Bw6	68	50.0	481	47.6	1.10 (0.77-1.58)	.6478
Bw6/Bw6	53	39.0	391	38.7	1.01 (0.70-1.46)	1.0000
***KIR2DL1*§**						
0	9	6.3	16	2.7	2.45 (1.06-5.67)	.0393
1	42	29.4	172	28.7	1.04 (0.69-1.55)	.9182
2	91	63.6	409	68.2	0.82 (0.56-1.20)	.3216
3	1	0.7	3	0.5	1.40 (0.15-13.5)	.5756
**001/*002*	85	41.1	357	35.7	1.25 (0.92-1.70)	.1542
**003*	89	43.0	452	45.2	0.91 (0.68-1.23)	.5912
**004*	32	15.5	186	18.6	0.80 (0.53-1.20)	.3212
Other	1	0.5	4	0.4	1.21 (0.13-10.86)	1.0000
***KIR2DS1*‖**						
0	91	61.9	376	62.7	0.97 (0.67-1.40)	.9243
1	50	34.0	197	32.8	1.05 (0.72-1.54)	.8450
2	6	4.1	27	4.5	0.90 (0.37-2.23)	1.0000

†*P* values were calculated by Fisher's exact test, ORs are denoted with 95% CI, and significant *P* values (after correction for multiple testing according to the Bonferroni method) are highlighted in bold.

‡Allele frequency = (x/n)/2 (where x is the number of alleles and n the number of individuals).

§*KIR2DL1* copy number (from 0-3 gene copies) and subtyping (indicated alleles) was performed for all cases and controls having *C2*. *KIR2DL1*001* and **002* encode identical mature proteins and were not differentiated by our typing method.

‖*KIR2DS1* copy number (from 0-2 gene copies) was performed for all cases and controls having *C2*.

linear model (PHGLM) analysis using Cox regression models analyzing time to relapse with nonrelapse mortality as competing risk. Differences between time-to-relapse distributions were compared by a log-rank test.

Results and discussion

In order to examine the influence of *HLA-C*–encoded KIR ligands, a cohort of pediatric ALL patients (n = 320) was stratified into 3 groups according to the *C1/C2* dimorphism. As shown in Table 1, the frequency of patients with 2 *C1* alleles was significantly decreased, whereas the corresponding carrier frequency for *C2* was significantly increased in the ALL cohort ($P = .0051$). Furthermore, allele frequencies were significantly biased toward *C2* in ALL patients ($P = .0037$). This observation was true for B-cell ALL (B-ALL) (n = 273), whereas in T-cell ALL (T-ALL) (n = 43), the distribution was similar to controls (data not shown). Because *C1* and *C2* frequencies vary greatly between different ethnicities even within Europe, further analysis was restricted to B-ALL patients of German origin, which constituted the largest disease- and ethnically matched subgroup in our patient cohort (n = 204). *C2* allele frequencies of the German control cohort (n = 1020, 36.2%) were comparable to those from populations of the same area retrieved from a public database,[13] confirming that the control cohort indeed is representative for German ethnicity (supplemental Figure 2). Similar to the previous analysis comprising all cases, homozygosity for *C1* was significantly decreased in B-ALL patients of German ethnicity (Table 1). Of note, no significant difference was seen for *C1* carrier frequency (*C1/C1* + *C1/C2*) between cases and controls (81.9% vs 86.6%). In contrast, *C2* carrier frequency (*C1/C2* + *C2/C2*) was significantly increased in the German B-ALL cohort (72.1% vs 58.8%, $P = .0004$). Together with the fact that the odds ratios (ORs) are similar for *C1/C2* and *C2/C2* individuals (OR = 1.44) in B-ALL German cases (Table 1), this clearly suggests that presence of *C2* constitutes a risk factor in B-ALL, whereas homozygosity for *C1* confers an equally strong protective effect (Table 1).

It was next examined if frequencies of C2-specific *KIR* genes *KIR2DL1* and *KIR2DS1* were also affected. In our previous study of B-ALL patients (cases included in the present larger cohort), no significant deviations from control values were seen for any *KIR* gene.[14] However, epistatic interaction of the C2-specific *KIR2DL1* gene with C2 might be difficult to detect given the high carrier frequency of *KIR2DL1* in whites (>95%).[10,15] We thus attempted to detect a possible epistatic interaction by analyzing allelic and copy-number variation (CNV). *KIR2DL1* CNV of German B-ALL cases was comparable to German controls except cases with 0 copies, which were found more frequently in B-ALL patients (Table 1 and supplemental Table 2). However, following adjustment for multiple testing, significance was lost. We also considered allelic variation of *KIR2DL1* as possible contributing factor. Analysis of allele-specific expression in NK cells of healthy controls by flow cytometry revealed a significantly decreased expression of KIR2DL1*004, an allele that was previously shown to have impaired signal-transduction capacity (supplemental Table 3).[16] However, PCR-SSP subtyping for the most common alleles (covering >98% of *KIR2DL1* allotypes in whites)[15] including *KIR2DL1*004* did not reveal significant differences (Table 1 and supplemental Table 2). Similarly, no significant changes in frequency and CNV were found for the C2-specific stimulatory *KIR2DS1* (Table 1 and supplemental Table 2). Of note, *KIR2DS1* exhibits only minor allelic polymorphism (*2DS1*002* present in >97% of whites).[15] Thus, our thorough analysis of KIR2DL1 polymorphism did not reveal any overt epistatic interaction with C2 in B-ALL.

We next asked if *HLA-C*–encoded KIR ligands would also impact clinical outcome. Indeed, B-ALL patients homozygous for *C2* exhibited a nonsignificant increase in relapse frequency (Figure 1A). Importantly, time-dependent analysis revealed that *C1/C1* patients exhibited a very low frequency of late relapse and that the number of *C2* alleles was significantly associated with increasing risk for late relapse (Figure 1B). Distribution of known confounding factors (age, sex, initial leukocyte count, and immunophenotype) was comparable among the 3 groups (supplemental Table 1). Multivariate analysis including the above-mentioned confounding factors and considering the number of C2 ligands as covariate confirmed a significantly increased risk for relapse in B-ALL with C2 ligands ($P = .013$; OR = 2.32; 95% confidence interval [CI], 1.18-4.57). A significant association of C2 ligands with relapse was also seen when National Cancer Institute criteria for pediatric high-risk leukemia (white blood cell count ≥50 000/μL and/or age <1 year or >10 years) were applied for multivariate analysis ($P = .046$; OR = 1.21; 95% CI, 1.00-1.46). We next evaluated if the C2 effect was sensitive to the changes in

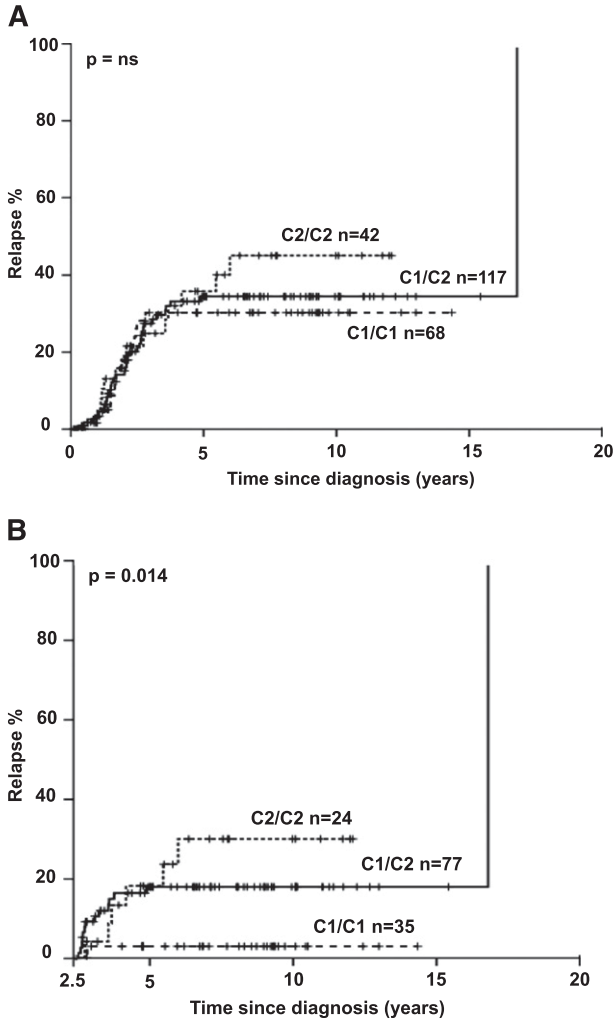

Figure 1. Increased frequency of late relapse in B-ALL patients with *HLA-C2*. Kaplan Meier analysis was performed for all B-ALL patients with available clinical and follow-up information. Patients not achieving remission during induction chemotherapy and patients undergoing hematopoietic stem cell transplantation without prior relapse were excluded from this analysis. Cases were divided into 3 groups based on *C1/C1*, *C1/C2*, and *C2/C2* genotypes. (A) Kaplan-Meier estimates are shown for all patients for the full observation time starting at the time of diagnosis. (B) Only patients who achieved complete remission, finished standard treatment, and remained event free for at least 2.5 years were analyzed (qualifying for late relapse ≥30 months after diagnosis). *P* values were calculated by 2-sided log-rank test; A: $P = .391$).

treatment protocols that were implemented in the last 2 decades. Splitting the cases into an "early" (1988-2003) and a "late" cohort (2004-2010) of similar size revealed a stable C2 effect, suggesting that evolution of treatment regimen did not confound analyses (supplemental Figure 3). Notably, no significant association with leukemia-free survival and overall survival was found (supplemental Figure 4).

The present study shows that HLA-C2 ligands not only are associated with increased susceptibility to B-ALL but also constitute a risk factor for late relapse. These observations are compatible with a model in which C2 ligands impair NK cell–mediated surveillance for leukemic cells at both the stage of leukemia initiation and in remission following completion of chemotherapy. Mechanistically, the inhibitory KIR2DL1 binds to C2 with higher affinity than KIR2DL2/3 to C1 and KIR2DL2 to C2. The exceptional specificity and affinity of the KIR2DL1-C2 interaction might lead to stronger inhibition of NK cells, which could compromise the efficiency of leukemia surveillance. The fact that we could not detect a major influence of *KIR2DL1* allelic variation or CNV is not necessarily at odds with this affinity model. Firstly, *KIR* CNV is associated with increased expression frequency but not with increased mean expression on the cell surface and does not majorly influence target cell recognition.[17] Thus, CNV effects on leukemia recognition might be quite subtle. Secondly, significant allele-specific expression differences were restricted to KIR2DL1*004, which is a relatively rare variant in whites. Our study might thus be underpowered to detect a significant allele-specific effect.

Notably, we did not find any association of the KIR ligand Bw4 with B-ALL (Table 1). It is currently unclear how these results compare with a recent study describing an association with Bw4 in non-Hispanic white ALL patients.[6] Notably, in both studies, high-affinity inhibitory KIR ligands seem to be associated with susceptibility to pediatric ALL.

This is the first study of childhood B-ALL reporting an association of KIR ligands with clinical outcome in a nontransplant setting. If confirmed in independent studies, these observations might have relevance for adjustment of therapy (eg, intensity, duration) to the increased risk of late relapse in B-ALL patients with C2 ligands, particularly those with homozygous C2 ligands.

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Authorship

Contribution: F.B. and A.R.M. contributed equally to this work; F.B., A.R.M., and M.U. designed the project, performed the experiments, and wrote the paper; N.S. performed the experiments; J.C.F. and J.E. contributed samples and performed statistical analysis; O.C. and A.M. performed analyses and revised the manuscript; A.B. and R.M. contributed clinical samples, treated patients according to the current therapy protocol, and critically revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Supplement

Supplemental material and methods

Flow Cytometry: Analysis of KIR2DL1 surface expression was performed using PBMC from buffy coats (kindly provided by Dr. G. Spohn, German Red Cross Blood Service Baden-Württemberg/Hessen) using the following antibodies: CD56-ECD (Beckman Coulter, clone N901), CD3-PECy7 (Biolegend, clone UCHT1) and KIR2DL1-FITC (R&D systems, clone 143211) on a CyAn® ADP flow cytometer (Beckman Coulter). Frequency and geometric mean fluorescence intensity of KIR2DL1 was analyzed following gating on $CD56^{dim}CD3^{-}$ NK cells.

Table S1: Clinical data and characteristics of 320 pediatric ALL patients according to *HLA-C* ligand status.

	total	*C1/C1*	*C1/C2*	*C2/C2*	*P*†
median age (range)	5 y 7 m (3 m – 20 y 10 m)	6 y 10 m (4 y – 17 y 11 m)	5 y 8 m (4 m – 20 y 10 m)	4 y 5 m (3 m – 19 y)	0.300
age < 1y or ≥ 10 y	34%	37%	34%	30%	0.814
males/cases	195/320 (60.9%)	63/98 (64.3%)	94/161 (58.4%)	38/61 (62.3%)	0.622
median leucocyte count x 10*3/µl (range)*	12.3 (0.3-600)	10.6 (0.5-300)	12.5 (0.7-493)	22.6 (0.3-600)	0.705
	n (%)	n (%)	n (%)	n (%)	
low risk subtype	266 (83.1%)	76 (77.5%)	141 (87.6%)	49 (80.3%)	0.091
pre-ALL	88 (27.5%)	24 (24.5%)	48 (29.8%)	16 (26.2%)	0.629
pre-pre-ALL	11 (3.4%)	3 (3.1%)	5 (3.1%)	3 (4.9%)	0.780
c-ALL	144 (45.0%)	45 (45.9%)	76 (47.2%)	23 (37.7%)	0.436
B-ALL unclassified	23 (7.2%)	4 (4.1%)	12 (7.5%)	7 (11.5%)	0.199
high risk subtype	54 (16.9%)	22 (22.5%)	20 (12.4%)	12 (19.7%)	0.091
pro	5 (1.6%)	1 (1.0%)	3 (1.9%)	1 (1.6%)	0.867
T-ALL	43 (13.4%)	18 (18.4%)	15 (9.3%)	10 (16.4%)	0.088
bcr/abl	2 (0.6%)	1 (1.0%)	0	1 (1.6%)	0.321
biphenotypic	4 (1.3%)	2 (2.0%)	2 (1.2%)	0	0.530

*In 130/320 patients initial leucocyte counts could not be obtained from medical records.

†p values were calculated by Fisher's Exact Test (for sex, age < 1y or ≥10y and subtype) and Kruskal Wallis test (for age and initial leucocyte count).

Table S2: *KIR2DL1* and *KIR2DS1* copy number and allelic variation in *C1C2* and *C2C2* patients

C1C2		**B-ALL cases, German**		**controls, German**			
		n	%	n	%	OR (95% CI)	*P**
KIR2DL1†	0	6	5.6	11	2.4	2.44 (0.88-6.76)	0.1068
	1	31	29.0	125	27.0	1.10 (0.69-1.76)	0.7184
	2	69	64.5	324	70.0	0.78 (0.50-1.21)	0.2970
	3	1	0.9	3	0.6	1.45 (0.15-14.04)	0.5657
	**001/*002*	60	39.0	282	36.1	1.13 (0.79-1.62)	0.5220
	**003*	73	47.4	355	45.4	1.08 (0.77-1.53)	0.6590
	**004*	20	13.0	142	18.2	0.67 (0.41-1.11)	0.1307
	other	1	0.6	3	0.4	1.70 (0.18-16.42)	0.5134
KIR2DS1‡	0	69	62.7	289	62.4	1.01 (0.66-1.56)	1.0000
	1	36	32.7	152	32.8	1.00 (0.64-1.55)	1.0000
	2	5	4.5	22	4.8	0.95 (0.35-2.58)	1.0000

C2C2		**B-ALL cases, German**		**controls, German**			
		n	%	n	%	OR (95% CI)	*P**
KIR2DL1†	0	3	8.3	5	3.6	2.40 (0.55-10.56)	0.3655
	1	11	30.6	47	34.3	0.84 (0.38-1.86)	0.8430
	2	22	61.1	85	62.0	0.96 (0.45-2.04)	1.0000
	**001/*002*	25	47.2	75	34.6	1.69 (0.92-3.10)	0.1122
	**003*	16	30.2	97	44.7	0.54 (0.28-1.02)	0.0629
	**004*	12	22.6	44	20.3	1.15 (0.56-2.37)	0.7075
	other	0	0.0	1	0.5	-	1.0000
KIR2DS1‡	0	22	59.5	87	63.5	0.84 (0.40-1.77)	0.7034
	1	14	37.8	45	32.8	1.24 (0.59-2.65)	0.5638
	2	1	2.7	5	3.6	0.73 (0.08-6.48)	1.0000

*p values were calculated by Fisher's exact test, odds ratios (OR) are denoted with 95% confidence intervals (95% CI).

†*KIR2DL1* copy number (from zero to three gene copies) and subtyping (indicated alleles) was performed for all cases and controls having *C2*. *KIR2DL1*001* and **002* encode identical mature proteins and were not differentiated by our typing method.

‡*KIR2DS1* copy number (from zero to two gene copies) was performed for all cases and controls having *C2*.

Table S3. Allele-specific expression of KIR2DL1 on NK cells

KIR	MFI†	KIR %*	2DL1*002	2DL1*003/*002	2DL1*003	2DL1*003/*004	2DL1*004	2DL1*004/*002
			21.47 % (6.95)	25.10 % (8.71)	26.99 % (12.85)	28.05 % (9.47)	16.59 % (12.50)	19.11 % (6.24)
2DL1*002	6.42 (2.11)			0.3157‡	0.2474	0.0935	0.2898	0.4348
2DL1*003/*002	5.62 (1.73)		0.3639		0.7054	0.4793	0.089	0.0937
2DL1*003	5.42 (0.99)		0.1914	0.7587		0.837	0.0756	0.098
2DL1*003/*004	4.54 (1.82)		0.0467	0.1914	0.1952		0.0299	0.0227
2DL1*004	3.17 (1.25)		**0.0004**	**0.0014**	**0.0002**	0.0584		0.5722
2DL1*004/*002	5.27 (1.03)		0.1404	0.598	0.7513	0.281	**0.0005**	

*Frequency (%) of cell surface expression on NK cells for *KIR2DL1* alleles in homozygous or, if separated by a slash, heterozygous state. Standard deviation is shown in brackets and was calculated from 10 samples for each allelic combination (total n=60).

†Geometric mean fluorescence intensity (MFI) with standard deviation shown in brackets.

‡Statistical significance was calculated for expression frequencies (upper right half) and MFI (lower left half) by two-sided t-test. Significant p values (after correction for multiple testing according to the Bonferroni method) are highlighted in bold.

Figure S1. Flow diagram of pediatric ALL patients identified, included and excluded in analyses. FUP indicates follow up; CR, complete remission; HSCT, hematopoietic stem cell transplantation

Figure S2. European *HLA-C2* allele frequencies for 59 populations from the Allele Frequency Net database (www.allelefrequencies.net). Only non-migrant populations are included. The city of Düsseldorf is indicated.

Figure S3. Analyses of relapse risk according to date of diagnosis. Kaplan Meier analysis was performed for all B-ALL patients with available clinical and follow-up information. The cohort was split into two similar-sized groups, one “early” group of patients being diagnosed from 1988-2003 (A), and one “late” group being diagnosed from 2004-2010 (B). Only patients who achieved complete remission, finished standard treatment and remained event free for at least 2.5 years were analyzed (qualifying for late relapse ≥ 30 months after diagnosis). p values calculated by two-sided logrank test were p=0.084 in (A) and p=0.103 in (B). Patients not achieving remission during induction chemotherapy and patients undergoing HSCT without prior relapse were excluded from this analysis.

Figure S4. Analysis of leukemia free survival and overall survival in 242 pediatric B-ALL patients. The pediatric B-ALL cohort (242 patients with available clinical and follow-up information, 73 *C1C1* patients (blue line), 125 *C1C2* patients (green line) and 44 *C2C2* patients (red line)) was evaluated for leukemia free survival (A), overall survival (B), and relapse probability (C) on the basis of *HLA-C* status. p values calculated by two-sided log rank test were p=0.273 in (A), 0.920 in (B) and 0.115 in (C).

Figure S1

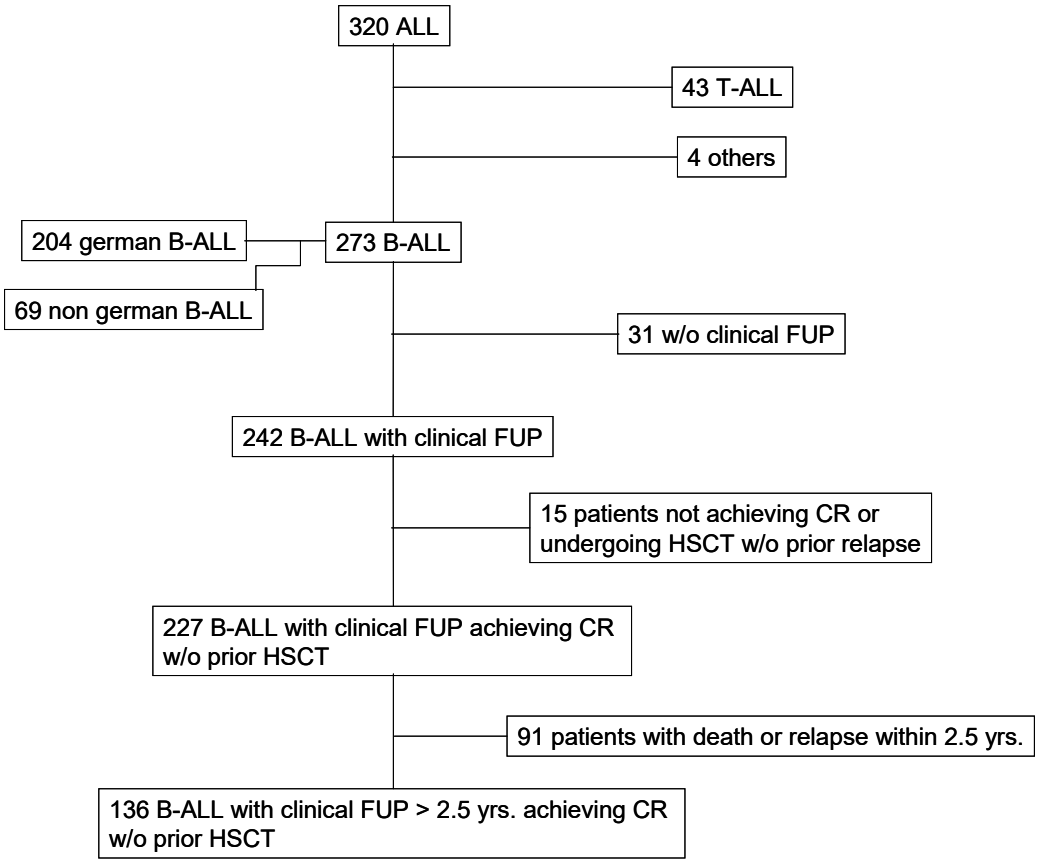
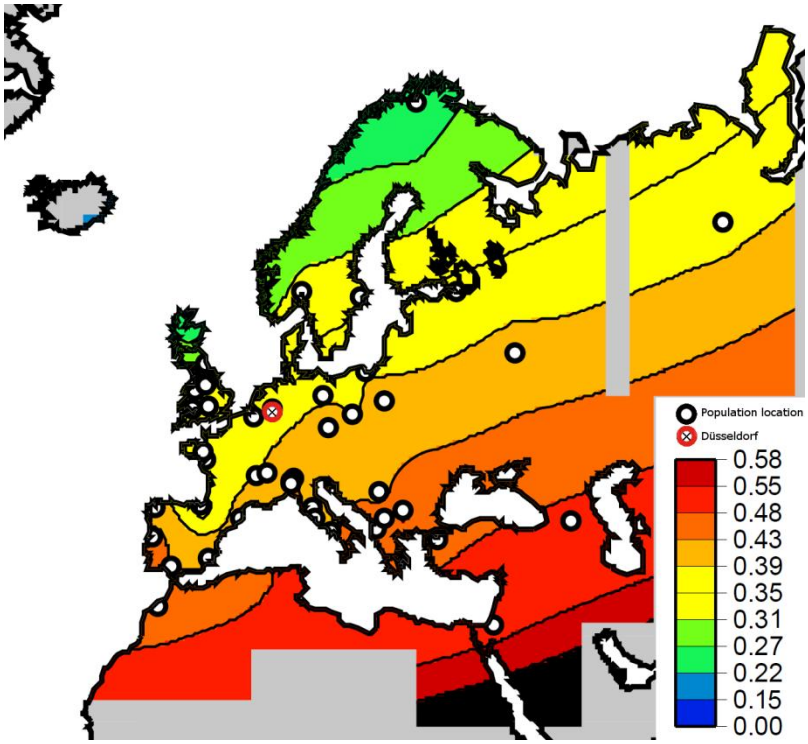

320 ALL
43 T-ALL
4 others
204 german B-ALL
273 B-ALL
69 non german B-ALL
31 w/o clinical FUP
242 B-ALL with clinical FUP
15 patients not achieving CR or undergoing HSCT w/o prior relapse
227 B-ALL with clinical FUP achieving CR w/o prior HSCT
91 patients with death or relapse within 2.5 yrs.
136 B-ALL with clinical FUP > 2.5 yrs. achieving CR w/o prior HSCT

Figure S2


Population location
Düsseldorf
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0.48
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0.39
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0.31
0.27
0.22
0.15
0.00

Figure S3

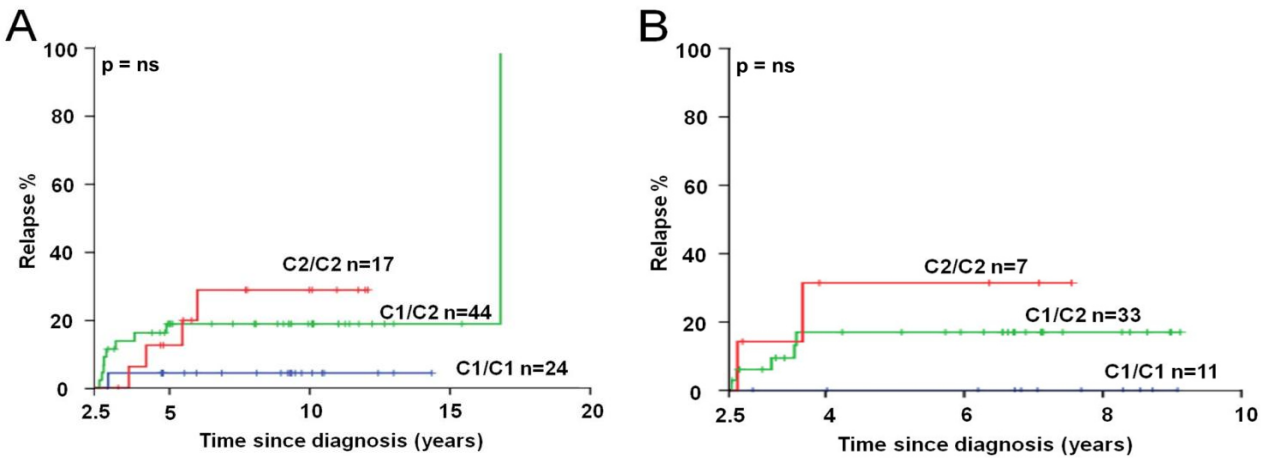
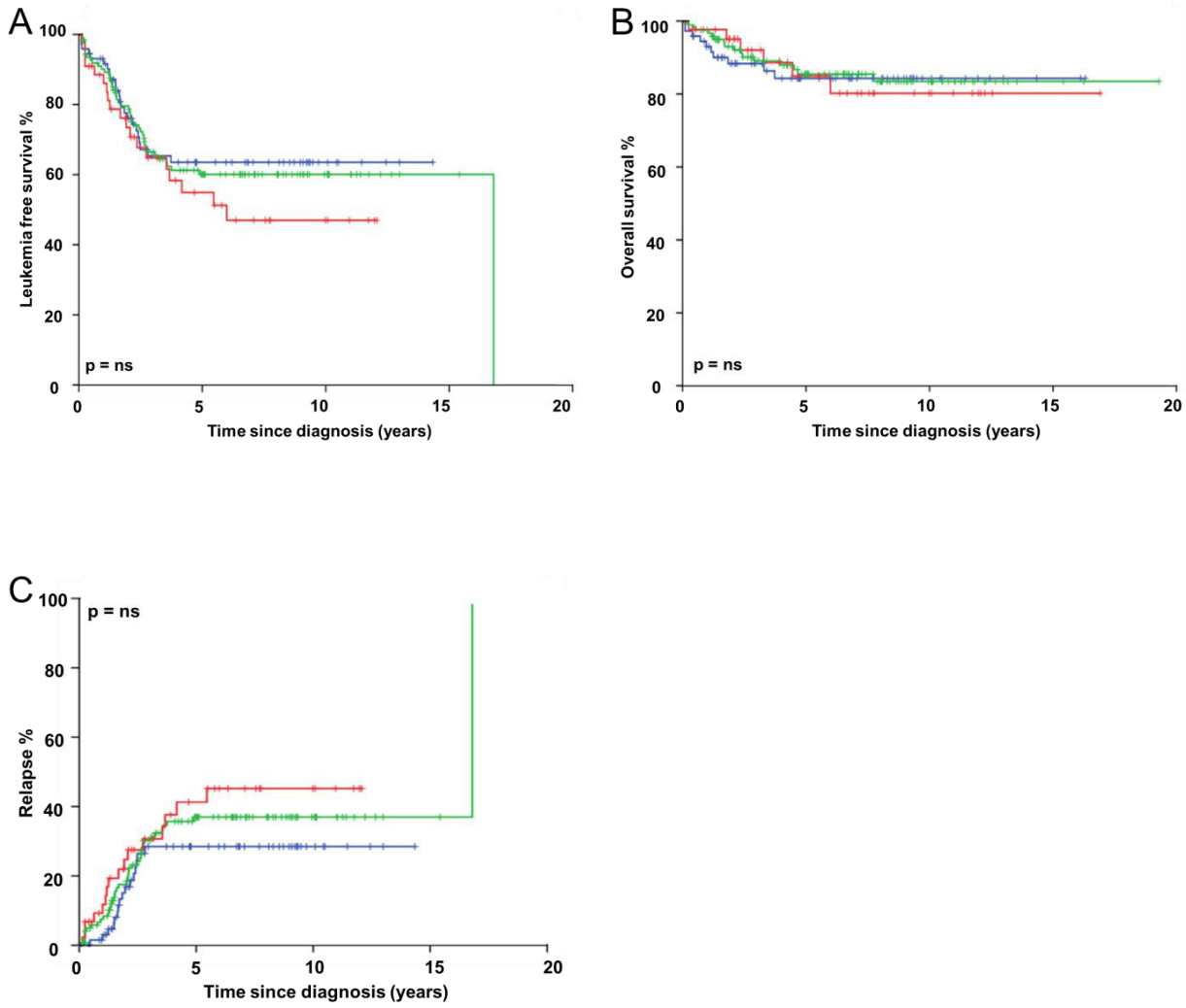

A
p = ns
Relapse %
100
80
60
40
20
0
C2/C2 n=17
C1/C2 n=44
C1/C1 n=24
2.5
5
10
15
20
Time since diagnosis (years)
B
p = ns
Relapse %
100
80
60
40
20
0
C2/C2 n=7
C1/C2 n=33
C1/C1 n=11
2.5
4
6
8
10
Time since diagnosis (years)

Figure S4


A
Leukemia free survival %
100
80
60
40
20
0
p = ns
0
5
10
15
20
Time since diagnosis (years)
B
Overall survival %
100
80
60
40
20
0
p = ns
0
5
10
15
20
Time since diagnosis (years)
C
Relapse %
100
80
60
40
20
0
p = ns
0
5
10
15
20
Time since diagnosis (years)

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Selective downregulation of HLA-C and HLA-E in childhood acute lymphoblastic leukaemia

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Keywords

NK cells; HLA class I; KIR; childhood leukaemia; ALL

We have recently shown that susceptibility to childhood B-cell acute lymphoblastic leukaemia (B-ALL) is influenced by the presence of *HLA-C* encoded ligands C1 and C2 for killer cell immunoglobulin-like receptors (KIR), (Babor *et al,* 2014). B-ALL patients exhibited an increased frequency of the C2 ligand and, moreover, C2 was associated with increased risk of late relapse. The study suggested that the expression of *HLA* class I-encoded KIR ligands on tumour cells influences disease susceptibility and Natural Killer (NK) cell-mediated control of B-ALL. In order to extend our knowledge about expression of the major inhibitory NK cell receptor ligands in childhood B-ALL, Bw4, *HLA-C*-encoded C1 and C2 epitopes, and HLA-E were assessed at the surface and mRNA level in leukaemic cells. To this end, we employed antibodies for specific detection of Bw4 (MUS4H4) and Bw6 (OUW4F11) as previously described (Verheyden *et al,* 2009), for HLA-C/E (DT9), (Braud *et al,* 1998, Thomas *et al,* 2009) and HLA-E (3D12, BioLegend, San Diego, CA, USA) in peripheral blood samples in 31 children (age 0.3–19.3 years, mean 7.2) with newly diagnosed B-ALL (common ALL: n=18; preB-ALL: n= 11; proB-ALL: n=2). In order to

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Author contributions

F.B. and M.U. contributed equally to this work. S.B.R. and A.R.M. designed the project, performed the experiments and wrote the manuscript; J.E. and J.C.F performed experiments and reviewed the manuscript; A.M., F.H.C., and M.C. contributed essential reagents and reviewed the manuscript, A.B. contributed clinical samples and reviewed the manuscript; F.B. and M.U. designed the project and wrote the paper.

Conflict of interest

The authors state that they have no conflict of interest.

accommodate for possible differences in cell size between leukaemic blasts and unaffected B cells, mean fluorescence intensity was normalized to mean forward scatter levels of the respective cell population. All HLA class I-specific antibodies were unconjugated and subsequently stained with secondary fluorescein isothiocyanate-coupled anti-IgG antibodies to avoid a systematic bias of directly conjugated antibodies due to differences in coupling efficiencies and flow cytometric compensation.

Overall levels of HLA class I surface expression, which were measured with a panHLA class I antibody (W6/32, BioLegend), were comparable and within the same range in $CD19^+$ B cells of patients and healthy controls (Fig 1A). Similarly, we could not detect B-ALL-specific changes in overall expression levels of HLA-B allotypes carrying the Bw4 epitope, which constitutes the ligand for the inhibitory KIR3DL1 receptor, nor the Bw6 epitope, which is not recognized by inhibitory KIR. In all cases, the flow cytometry results were consistent with the HLA class I typing status, confirming the specificity of the Bw4 and Bw6 reagents (data not shown). Whereas panHLA class I and Bw4/Bw6 expression remained unchanged, strong and consistent downregulation of HLA-C and HLA-E surface expression was observed in patients (Fig 1A). At initial diagnosis, leukaemic patients but not healthy controls exhibited B cell populations with very low HLA-C and -E expression levels (Fig 1B) that could be consistently allocated to the $CD19^+CD45^-$ leukaemic subset (Fig 1C). The differences in HLA-C and -E expression levels between non-leukaemic and leukaemic B cells were highly significant (Fig 1D). Upon complete remission, B cell populations with low HLA-C and HLA-E levels were no longer detectable, consistent with the disappearance of the leukaemic blast population (Fig 1B, C). In contrast, Bw4- and Bw6-bearing allotypes led to homogenous staining in leukaemic and control samples (Fig 1B).

To determine whether downregulation of HLA-C and –E expression on the cell surface was due to repression of transcription, B cells were separated into leukaemic and non-leukaemic subsets by flow cytometric cell sorting and subsequently subjected to reverse transcription polymerase chain reaction analysis. Indeed, the mRNA levels for C1 and C2-bearing *HLA-C* alleles as well as for *HLA-E* were clearly lower in $CD34^+$ leukaemic blasts than in the residual non-leukaemic B cells (Fig 2A). Nonetheless, except one case where no *HLA-E* message could be detected (data not shown), the reduction of mRNA levels was moderate (2- to 4-fold compared to normal B cells) pointing to additional post-transcriptional mechanisms that might explain the many cases exhibiting an almost complete lack of surface expression. Notably, the distribution of the well-characterized *HLA-E* dimorphism at position 107 (Geraghty *et al,* 1992) was not different from the control cohort (data not shown).

This is the first comprehensive analysis of *HLA-C* and *HLA–E*-encoded NK cell ligands in childhood ALL. It is important to mention that the HLA-C-specific reagent (DT9) cross-reacts with HLA-E, which might partially account for the decrease in DT9 signals. Moreover, the HLA-E-specific antibody (3D12) weakly recognizes certain HLA-B and HLA-C alleles (Corrah *et al,* 2011). However, these cross-reactions would not explain our observation that both reagents gave vastly diminished signals on leukaemic blasts whereas the same leukaemic cells showed normal levels of HLA-A and -B expression. Our observations are in line with a previous study showing downregulation of HLA-C in a small

ALL cohort, employing a panel of antibodies for single HLA-C allotypes (Verheyden *et al,* 2009). Furthermore, our data show that HLA-E is expressed at the mRNA but not cell surface level on leukaemic blasts, which is in contrast to a previous study, which reported complete absence of *HLA-E* mRNA level (Majumder *et al,* 2006).

The downregulation of HLA-C and HLA-E as reported here should theoretically lead to 'missing-self' recognition of leukaemic cells by NK cells expressing the respective cognate inhibitory receptor. However, CD107 mobilization of short-term stimulated NK cells from healthy donors remained low, even on NK cell subsets expressing a single inhibitory KIR for C2 (KIR2DL1), C1 (KIR2DL1), Bw4 (KIR3DL1) or HLA-E (NKG2A) (Fig 2B). The observation that B-ALL blasts are not efficiently recognized by NK cells despite their consistent downregulation of NK cell receptor ligands might be explained by the lack of appropriate ligands for stimulatory receptors, such as NCR, NKG2D, and DNAM1 (Pende *et al,* 2005). However, the driving force behind the downregulation of HLA-C and HLA-E is yet unknown. Possible explanations involve the escape from T cells that recognize peptides presented in the context of HLA-C or HLA-E. A thought-provoking but speculative explanation would be that the presence of HLA-C and HLA–E-negative leukaemic cells in haematopoietic stem cell niches, such as bone marrow, might drive NK cells into a hyporesponsive state. In this regard, it was shown that TAP-deficient patients that express almost no HLA class I (Sleiman *et al,* 2014) have NK cells that are not licensed and exhibit decreased cytotoxicity and cytokine production. Further experiments analysing the functional state of patient NK cells will hopefully help to clarify the underlying mechanism behind the specific downregulation of NK cell receptor ligands in childhood B-ALL.

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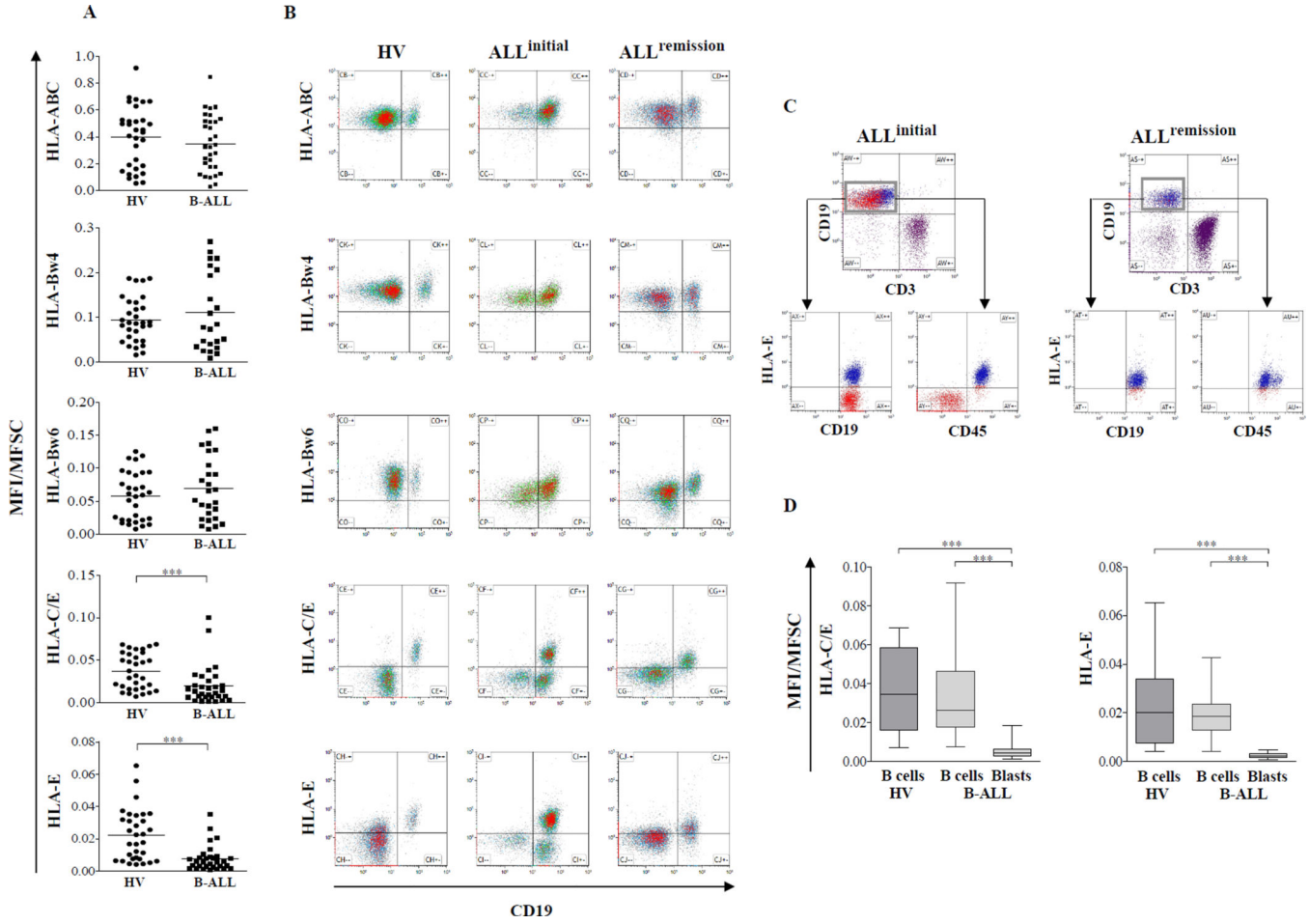

Figure 1. Selective downregulation of HLA-C and HLA-E in paediatric B-ALL patients

Peripheral blood mononuclear cells (PBMC) from 31 newly diagnosed B-ALL patients and 32 healthy volunteers (HV) were stained with HLA class I-specific human monoclonal antibodies (mAbs) for HLA-ABC, HLA-Bw4, HLA-Bw6, HLA-C/E and HLA-E. Measurements of all HLA class I-specific antibodies were made with the same instrument settings. The mean fluorescence intensity (MFI) was normalized to mean forward scatter (MFI/MFSC) to adjust for possible differences in cell size. **(A)** Plots representing the surface expression of the respective HLA class I molecules on B cells ($CD19^+$ subset) of healthy volunteers and leukaemic patients. Each dot represents one individual and horizontal bars represent mean values. **(B)** Flow cytometric data are shown for a representative leukaemic patient (middle panel) at initial diagnosis and complete remission (right panel) following chemotherapy in comparison to a healthy volunteer (left panel). **(C)** Representative flow cytometric analysis illustrating the presence of two $CD19^+$ subsets with either high ($CD45^+$ non-leukaemic B cells) or low ($CD45^-$ leukaemic blasts) HLA-E expression at initial diagnosis (upper panel) and the subsequent disappearance of the leukaemic HLA-E-negative B cell subset following chemotherapy and complete remission (lower panel). **(D)** Box plots representing surface expression of HLA-C and HLA-E for non-leukaemic and leukaemic B cells in comparison to B cells from healthy volunteers. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (***= $p<0.001$).

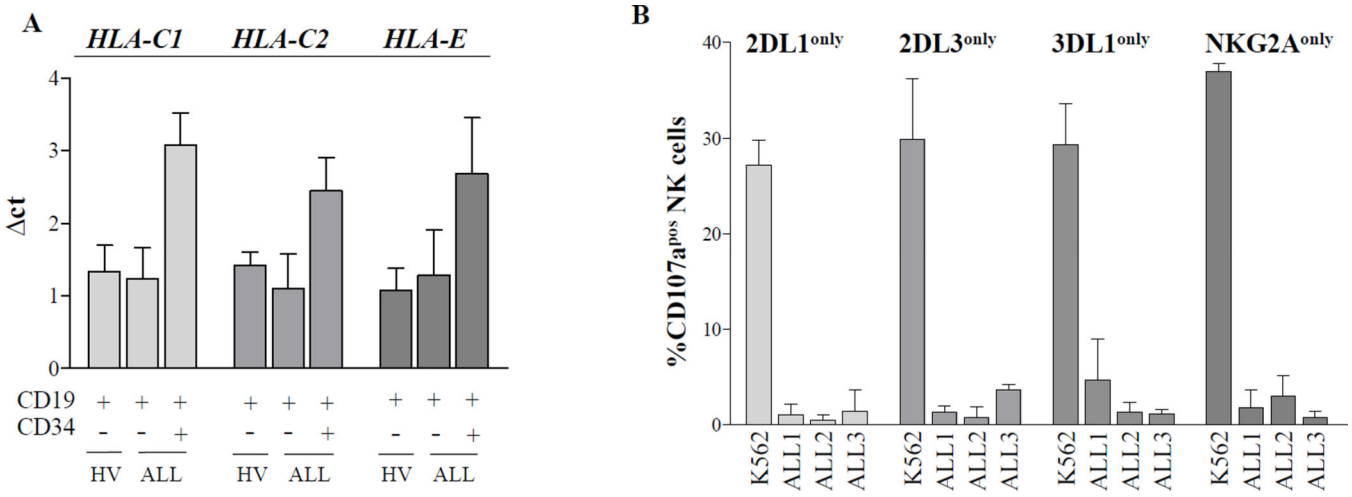

Figure 2. Reduced *HLA-C* and *HLA-E* mRNA expression and lack of NK cell activation by leukaemic cells

(A) Semiquantitative reverse transcription (RT) PCR analysis of *HLA-C1, HLA-C2* and *HLA-E* mRNA expression. Leukaemic blasts ($CD34^+CD19^+$) and B cells ($CD34^-$ $CD19^+$) from acute lymphoblastic leukaemia (ALL) patients as well as B cells from healthy volunteers were separated by flow cytometric cell sorting. mRNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by cDNA synthesis (Moloney murine leukaemia virus, Promega, Madison, WI, USA). Transcriptional levels were analysed by SYBR green-based real-time PCR (StepOnePlus PCR-System, Applied Biosystems, Foster City, CA, USA) and normalized to expression levels of *ACTB*. Bars represent the mean expression level of three healthy (far left bar in each grouping) and three leukaemic individuals (middle and right bars in each grouping), each heterozygous for *HLA-C1* and *HLA–C2*, respectively; error bars represent standard error of the mean (SEM). Primers for *HLA-C*-encoded ligands were from (Babor *et al,* 2014). Primers for *HLA-E* were as follows: forward: 5'-GCACATGGCACGTGTATCTC-3', reverse: 5'-CCTTCTGGAGAGGAGCAGAG-3'. **(B)** CD107a mobilization assay of healthy NK cells stimulated overnight with interleukin 2 (1000 u/ml) against paediatric B-ALL blasts (frequency >80% of peripheral blood mononuclear cells) indicated as ALL1-3 or against K562 (erythroleukaemic HLA-class I deficient cell line) as a positive control (effector/target ratio of 10:1). CD107a monoclonal antibody (mAb) (H4A3, BioLegend) was added prior to incubation. Subsequent flow cytometric staining included mAbs for CD56 (HCD56, Biolegend), CD3 (UCHT1, BioLegend), CD159a (NKG2A, Z199, Beckman Coulter, Brea, CA, USA), CD158a/h (KIR2DL1/S1, 11PB6, Miltenyi Biotec, Bergisch Gladbach, Germany), CD158b1/b2/j (KIR2DL2/L3/S2, GL183, Beckman Coulter) and CD158e1 (KIR3DL1, DX9, BioLegend). CD107a expression was determined on $CD56^{dim}CD3^-$ NK cells expressing the indicated inhibitory receptor. Bars represent the mean expression of three healthy individuals; error bars represent standard error of the mean (SEM).

Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients With AML, ALL and CLL

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Summary: Invariant natural killer T (iNKT)/natural killer (NK)/cytokine-induced killer (CIK) cells are important for immune surveillance. (I) Novel combinations of antibody 6B11 (targeting the Vα24-Jα18-invariant T-cell receptor) with CD4/CD8/CD1d/Vα24 for iNKT subset detection and "T/NK cell-like"-iNKT subsets were defined. Compared with healthy peripheral blood mononuclear cells (MNC) (significantly) lower proportions of iNKT cells ($6B11^+$/$6B11^+CD3^+$/$6B11^+CD161^+$), NK cells ($CD3^-CD56^+$/$CD3^-CD161^+$), and CIK cells ($CD3^+CD56^+$/$CD3^+CD161^+$) were found in peripheral blood MNC from acute myeloid (AML)/acute myeloid, lymphoid (ALL)/chronic lymphoid leukemia (CLL) patients in acute disease stages. Subtyping of iNKT cells revealed (significantly) higher proportions of $CD3^+$ T cells and $CD161^+$ NK cells in AML/ALL/CLL expressing 6B11 compared with healthy MNC. Prognostic evaluations showed higher proportions of iNKT/NK/CIK cells in favorable AML subgroups (younger age, primary, no extramedullary disease, achievement/maintenance of complete remission) or adult ALL and CLL patients. (II) iNKT/NK/CIK cell frequencies increased after (vs. before) mixed lymphocyte cultures of T-cell–enriched immune reactive cells stimulated with MNC/whole blood with or without pretreatment with "cocktails" (dendritic cells generating methods/kits inducing blasts' conversion to leukemia-derived dendritic cells from AML patients). Individual "cocktails" leading to "highest" iNKT cell frequencies could be defined. Antileukemic blast lytic activity correlated significantly with frequencies of iNKT/NK/CIK cells. In summary healthy MNC show significantly more iNKT/NK/CIK cells compared with AML/ALL/CLL MNC, a shift in the iNKT cell composition is seen in healthy versus leukemic samples and iNKT/NK/CIK cell-proportions in AML/ALL/CLL MNC samples correlate with prognosis. "Cocktail"-treated AML blasts lead to higher iNKT/NK/CIK cell frequencies and samples with antileukemic activity show significantly higher frequencies of iNKT/NK/CIK cells. Proportions of iNKT/NK/CIK cells should regularly be evaluated in AML/ALL/CLL diagnosis panels for quantitative/prognostic estimation of individual patients' antileukemic potential and their role in dendritic cells/leukemia-derived dendritic cells triggered immune surveillance.

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Key Words: iNKT, NK, CIK, DC_{leu}, AML, ALL, CLL

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Acute myeloid (AML), lymphoid (ALL) or chronic lymphoid leukemia (CLL) are clonal diseases with uncontrolled proliferation of myeloid or lymphoid blasts. Prognostic rates of complete remission (CR) and survival depend on grade of anemia, thrombocytopenia, white blood cell expansion and karyotypes, resulting in different therapeutic strategies. Therapy for AML, ALL, and CLL patients with advanced stages consists of chemotherapy and stem cell transplantation (SCT), but the rate of early failures and relapses is still unsatisfying. AML patients can be categorized into 3 risk groups.[1] ALL patients are primarily children with survival rates of about 90% and higher incidences for an assignment to standard risk-groups compared with adult patients with higher tendencies for high risk leukemia.[2] CLL usually occurs in elderly patients and these are classified in Binet-staging groups: Binet A (early), B (intermediate), and C (advanced stage).[3]

Effective immune surveillance of patients with hematologic malignancies such as leukemia is mediated by arms of the innate and adaptive immune system. The innate immune system includes macrophages, dendritic cells (DC), and natural killer (NK) cells, which respond quickly to an immunologic threat.[4] NK cells have the ability to kill tumor cells without activation, arise from $CD34^+$ bone marrow (BM) cells and are defined as $CD3^-CD56^+CD161^+$.[5] The adaptive immune system includes T and B cells, which mediate tumor immunity by antigen-specific responses and provide long-lasting protection by effector-memory responses. T cells ($CD3^+$) express T-cell receptors (TCR) that recognize (peptide)-antigens on blasts, which have to be presented by majorhistocompatibility complex (MHC)-I or MHC-II molecules via antigen-presenting cells.[4] A specialty of myeloid blasts is, that they can differentiate to leukemia-derived dendritic cells (DC_{leu}), presenting the complete leukemic antigen repertoire, thereby specifically and efficiently activating an antileukemic T-cell response.[6–8] T cells occur in various differentiation and functional subsets [eg, naive (T_{naive}, $CD45RO^-$), non-naive ($T_{non\text{-}naive}$, $CD45RO^+$), central-memory (T_{cm}, $CD45RO^+CCR7^+$), effector/memory ($T_{eff\text{-}em}$, $CD45RO^+CCR7^-$), and regulatory (T_{reg}, $CD25^{++}CD127^{+low}$)].[9,10] Furthermore, other cells at the interface of the innate and the adaptive immune system are important mediators in antitumor,

autoimmune, and antimicrobial responses and tumor surveillance[4]: cytokine-induced killer (CIK) cells have phenotypic and functional features of T and NK cells as they are $CD3^+CD56^+CD161^+$, they are expandable in culture like T cells; however, they do not recognize cells via TCR or MHC molecules as T cells.[5] NKT cells are a heterogenous lymphoid population, that bridge innate and adaptive immunity and, in general, share properties of T and NK cells. Similar to NK cells, NKT cells have the ability to react rapidly to antigenic stimulation by quickly secreting large amounts of cytokines and chemokines within minutes to hours.[4] In addition, they activate DC, NK, and $CD4^+$/$CD8^+$ T cells, thereby triggering innate and adaptive immune responses.[11] Similar to T cells, NKT cells respond via the TCR, recognizing glycolipid antigens presented by the MHC-like molecule CD1d. TCR from NKT cells react with many self and foreign antigens, while the TCR from T cells only reacts with one epitope.[4] It was shown, that the positive selection of NKT cells is strictly dependent on CD1d during ontogeny in the thymus and therefore CD1d restriction has become the defining characteristic of NKT cells (NK markers are only used to define subpopulations).[12] The NKT cell population consists of many phenotypically and functionally diverse subsets, which are subdivided either by surface markers, TCR, tissue location, antigen recognition or by effector functions. Concerning their heterogenous TCR rearrangements, NKT cells are divided into 2 main groups: type-I NKT cells are referred to as invariant NKT cells (iNKT cells) as they express a semi-invariant TCR, characterized in humans by Vα24-Jα18 and Vβ11, while β-chains have a limited variety. In contrast, NKT cells that do not express this semi-invariant TCR are referred to as type-II NKT cells, which are less well studied than iNKT cells.[11] iNKT cells are known for enhancement of tumor immunity, while type-II NKT cells are known for suppression of it, resulting in opposed roles in tumor immunity and crossregulate of each other.[4]

NKT cells originate from a precursor pool of $CD4^+$ $CD8^+$ double positive thymocytes, that have to undergo diverse *TCR* gene rearrangements, are then positively selected by $CD1d^+$ double positive thymocytes in the thymic cortex, undergo 4 differentiation and maturation steps and then differentiate into mature NKT cells. Expression of CD4 and CD8 allow a subdivision of NKT cells in different subpopulations: a majority is $CD4^+$ (90% in mice), the remainder is $CD4^-CD8^-$ double negative and a small population is $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$, but $CD8^+$ subsets only exist in humans (not in mice), predominantly in healthy persons with latent Epstein-Barr virus infection.[12]

The evaluation of human iNKT cells is challenging, as their frequency in peripheral blood (PB) is very low. Classically, iNKT cells have been identified using CD1d tetramers loaded with α-galactosylceramide or monoclonal antibodies (moAbs) against the Vα24 and Vβ11 chains, what however, can lead to an overestimation of iNKT cells, as noninvariant, non-CD1d restricted $V\alpha24^+$ T cells can also pair with Vβ11. Recently iNKT cells were shown to be identified using the moAb 6B11, which recognizes the invariant CDR3 loop of their Vα24Jα18 TCR-rearrangement with high specificity and sensitivity.[13] CD161 (NKR-P1A) is a C-type lectin receptor and is an important marker for NK and iNKT cell identification as all human NK cells, high proportions of iNKT cells, and $T_{eff/em}$ and T_{cm} express it. Recently it was shown, that $CD161^+$ T cell subsets are highly functional during infections: low frequencies correlate with higher incidence of (viral) infections.[14–18]

Considering that the antileukemic function of T cells can be stimulated by DC_{leu},[9,10] our supposition is that the iNKT/NK and CIK cell activity might also be enhanced under DC/DC_{leu} stimulation. DC_{leu} can be generated in vitro by converting myeloid leukemia cells in mononuclear cells (MNC) or whole blood (WB) using DC-generating methods/kits ("cocktails," containing immune response modifiers in combination with cytokines) and represent as well leukemic (eg, CD13, CD33, CD117) and DC antigens (eg, CD80, CD83, CD86).[19,20]

Physiological conditions in the stem cell niche of the BM as well as in the PB are hypoxic with Oxygen (O_2) concentrations between 0.1% and 0.6 % in the BM, 12% in arterial blood and 4%–15% in PB.[21,22] Previous studies suggest an influence of the partial pressure of oxygen (pO_2) on several pathophysiological mechanisms.[23,24] However, hematopoiesis takes place under physiologically low pO_2/O_2-concentration and hematopoietic cells are continually exposed to dynamic pO_2-values.[25] Moreover, a recent in vitro study showed that hypoxia might have an enhancing effect on NK cells.[26]

The aim of this study was (1) to test the suitability of different markers and combinations (Table 1) to characterize and quantify iNKT, NK, and CIK cells/subsets; (2) to quantify iNKT, NK, and CIK subsets in MNC from patients with AML, ALL, and CLL compared with healthy controls; (3) to correlate findings with disease entities and prognostic subgroups; (4) to quantify iNKT, NK, and CIK subsets under stimulation with DC/DC_{leu}, that are generated from WB/MNC with various "cocktails".

MATERIALS AND METHODS

Sample Collection

After obtaining informed consent, heparinized peripheral WB samples were taken from patients in acute phases of AML, ALL, CLL, and from healthy controls. MNC were prepared from WB samples by density gradient centrifugation using the Ficoll-Hypaque technique (Biocoll separating solution; Biochrom, Berlin, Germany) with a density gradient of 1.077 g/mL. MNC were washed and suspended in phosphate-buffered saline (PBS; Biochrom). $CD3^+$ T cells were enriched using the MACS technology (Milteney Biotech, Bergisch Gladbach, Germany). The purity of $CD3^+$ T cells was on average (Ø) 89% (range: 69%–98%). Cells were quantified using Neubauer counting chambers, used directly or frozen and thawed according to standardized protocols.

Patients' Characteristics and Diagnostics

Patients and Samples Included for Surface Marker Expression Analyses on Thawed Cells

Quantitative and qualitative characterizations of several immune reactive cells were performed with thawed MNC from patients with blast-rich phases of AML (n = 23), ALL (n = 19), and CLL (n = 21). Samples were provided by the University-Hospitals of Tubingen, Dusseldorf, Munich, Oldenburg and Augsburg. Cells were obtained by aspirates of PB or BM, which were anticoagulated with heparin after patients' written informed consent in accordance with the Helsinki protocol and the local Ethic Committee (Pettenkoferstr. 8a, 80336 München,

TABLE 1. Subtypes of T/iNKT/NK/CIK Cells, Blasts and DC as Evaluated by Flow Cytometry

	Names of Subgroups	Referred To	Surface Marker	Abbreviation	Explanatory Note/Permise	References
T cells	$CD3^+$ pan-T cells	MNC(WB) or MLC1*	$CD3^+$	$CD3^+$/MNC(WB) or MLC		Schick et al[10]
	$CD4^+$-coexpressing T cells	MNC(WB) or MLC	$CD3^+CD4^+$	$CD4^+$/MNC(WB) or MLC	$CD4^+$ T cells	Schick et al[10]
	$CD4^+$-coexpressing T cells	$CD3^+$	$CD3^+CD4^+$	$CD4^+/CD3^+$	$CD4^+$ T cells	Schick et al[10]
	$CD8^+$-coexpressing T cells	MNC(WB) or MLC	$CD3^+CD8^+$	$CD8^+$/MNC(WB) or MLC	$CD8^+$ T cells	Schick et al[10]
	$CD8^+$-coexpressing T cells	$CD3^+$	$CD3^+CD8^+$	$CD8^+/CD3^+$	$CD8^+$ T cells	Schick et al[10]
	Naive T cells	MNC(WB) or MLC	$CD3^+CD45RO^-$	T_{naive}/MNC(WB) or MLC	Unprimed T cells	Vogt et al[9]
	Naive T cells	$CD3^+$	$CD3^+CD45RO^-$	$T_{naive}/CD3^+$	Unprimed T cells	Vogt et al[9]
	Non-naive T cells	MNC(WB) or MLC	$CD3^+CD45RO^+$	$T_{non\text{-}naive}$ /MNC(WB) or MLC	Memory + effector T cells	Vogt et al[9]
	Non-naive T cells	$CD3^+$	$CD3^+CD45RO^+$	$T_{non\text{-}naive}$ /$CD3^+$	Memory + effector T cells	Vogt et al[9]
	Central (memory) T cells	MNC(WB) or MLC	$CD3^+CCR7^+CD45RO^+$	T_{cm}/MNC(WB) or MLC	Long-term immunity	Vogt et al[9]
	Central (memory) T cells	$CD3^+$	$CD3^+CCR7^+CD45RO^+$	$T_{cm}/CD3^+$	Long-term immunity	Vogt et al[9]
	Effector (memory) T cells	MNC(WB) or MLC	$CD3^+CCR7^-CD45RO^+$	T_{ems}-T_{effs}/MNC(WB) or MLC		Vogt et al[9]
	Effector (memory) T cells	$CD3^+$	$CD3^+CCR7^-CD45RO^+$	T_{ems}-$T_{effs}/CD3^+$		Vogt et al[9]
	Proliferating T cells	MNC(WB) or MLC	$CD3^+CD69^+$	T_{prol}/MNC(WB) or MLC	proliferating T cells	Schick et al[10]
	Proliferating T cells	$CD3^+$	$CD3^+CD69^+$	$T_{prol}/CD3^+$	proliferating T cells	Schick et al[10]
	Viable T cells	MNC(WB) or MLC	$CD3^+7AAD^-$	T_{via}/MNC(WB) or MLC		Schick et al[10]
	Viable T cells	$CD3^+$	$CD3^+7AAD^-$	$T_{via}/CD3^+$		Schick et al[10]
	TCRVα24$^+$-coexpressing T cells	MNC(WB) or MLC	$CD3^+V\alpha24^+$	Vα24$^+$/MNC(WB) or MLC	Vα24 segment joined with Jα18	Montoya et al[13]
	TCRVα24$^+$-coexpressing T cells	$CD3^+$	$CD3^+V\alpha24^+$	Vα24$^+$/$CD3^+$	Vα24 segment joined with Jα18	Montoya et al[13]
	$CD1d^+$-coexpressing T cells	MNC(WB) or MLC	$CD3^+CD1d^+$	$CD1d^+$/MNC(WB) or MLC	$CD1d^+$ is a MHC class I-like molecule	Matsuda et al[51]
	$CD1d^+$-coexpressing T cells	$CD3^+$	$CD3^+CD1d^+$	$CD1d^+/CD3^+$	$CD1d^+$ is a MHC class I-like molecule	Matsuda et al[51]
CIK cells	$CD3^+CD56^+$ CIK cells	MNC(WB) or MLC	$CD3^+CD56^+$	$CD3^+CD56^+$/MNC(WB) or MLC	Cells expressing T cell (CD3) and	Pittari et al[5]
	$CD3^+CD161^+$ CIK cells	MNC(WB) or MLC	$CD3^+CD161^+$	$CD3^+CD161^+$/MNC(WB) or MLC	NK cell (CD56,CD161) markers	
	$CD3^+$ $CD161^+$ CIK cells	$CD3^+$	$CD3^+CD161^+$	$CD3^+CD161^+/CD3^+$		
NK cells	$CD3^-CD56^+$ NK cells	MNC(WB) or MLC	$CD3^-CD56^+$	$CD3^-CD56^+$/MNC(WB) or MLC	Cells expressing NK markers (CD56,	Pittari et al[5]
	$CD3^-CD161^+$ NK cells	MNC(WB) or MLC	$CD3^-CD161^+$	$CD3^-CD161^+$/MNC(WB) or MLC	CD161), but not expressing T cell	Montoya et al[13]
	$CD3^-CD161^+$ NK cells	$CD161^+$	$CD3^-CD161^+$	$CD3^-CD161^+/CD161^+$	markers (CD3)	Montoya et al[13]
iNKT cells	6B11$^+$(PE) iNKT cells	MNC(WB) or MLC	6B11$^+$	6B11$^+$(PE)/MNC(WB) or MLC	6B11 recognizes the invariant CDR3	Bienemann et al[11]
	6B11$^+$(FITC)iNKT cells	MNC(WB) or MLC	6B11$^+$	6B11$^+$(FITC)/MNC(WB) or MLC	loop of the TCR α-chain of iNKT cells	Bienemann et al[11]
	$CD3^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD3^+$	6B11$^+CD3^+$/MNC(WB) or MLC	6B11$^+CD3^+$ iNKT cells	Montoya et al[13]
	$CD3^+$ coexpressing 6B11$^+$ iNKT cells	$CD3^+$	6B11$^+CD3^+$	6B11$^+CD3^+/CD3^+$	6B11$^+CD3^+$ iNKT cells	Bienemann et al[11]
	$CD4^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD4^+$	6B11$^+CD4^+$/MNC(WB) or MLC	6B11$^+CD4^+$ iNKT cells	Montoya et al[13]
	$CD4^+$ coexpressing 6B11$^+$ iNKT cells	$CD4^+$	6B11$^+CD4^+$	6B11$^+$CD4+/CD4+	6B11$^+CD4^+$ iNKT cells	Montoya et al[13]
	$CD8^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD8^+$	6B11$^+CD8^+$/MNC(WB) or MLC	6B11$^+CD8^+$ iNKT cells	Montoya et al[13]
	$CD8^+$ coexpressing 6B11$^+$ iNKT cells	$CD8^+$	6B11$^+CD8^+$	6B11$^+CD8^+/CD8^+$	6B11$^+CD8^+$ iNKT cells	Montoya et al[13]
	$CD1d^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD1d^+$	6B11$^+CD1d^+$/MNC(WB) or MLC	6B11$^+CD1d^+$ iNKT cells	Montoya et al[13]
	$CD1d^+$ coexpressing 6B11$^+$ iNKT cells	6B11$^+$	6B11$^+CD1d^+$	6B11$^+CD1d^+$/6B11$^+$	6B11$^+CD1d^+$ iNKT cells	Montoya et al[13]
	$CD161^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD161^+$	6B11$^+CD161^+$/MNC(WB) or MLC	6B11$^+CD161^+$ iNKT cells	Montoya et al[13]
	$CD161^+$ coexpressing 6B11$^+$ iNKT cells	6B11$^+$	6B11$^+CD161^+$	6B11$^+CD161^+$/6B11$^+$	6B11$^+CD161^+$ iNKT cells	Montoya et al[13]
	$CD161^+$ coexpressing 6B11$^+$ iNKT cells	$CD161^+$	6B11$^+CD161^+$	6B11$^+CD161^+/CD161^+$	6B11$^+CD161^+$ iNKT cells	Montoya et al[13]
	TCRVα24$^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+V\alpha24^+$	6B11$^+V\alpha24^+$/MNC(WB) or MLC	6B11$^+V\alpha24^+$ iNKT cells	Montoya et al[13]
	TCRVα24$^+$ coexpressing 6B11$^+$ iNKT cells	6B11$^+$	6B11$^+V\alpha24^+$	6B11$^+V\alpha24^+$/6B11$^+$	6B11$^+V\alpha24^+$ iNKT cells	Montoya et al[13]
	$CD45RO^+$ coexpressing 6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD45RO^+$	6B11$^+CD45RO^+$/MNC(WB) or MLC	6B11$^+CD45RO^+$ iNKT cells	Montoya et al[13]
	$CD45RO^+$ coexpressing 6B11$^+$ iNKT cells	6B11$^+$	6B11$^+CD45RO^+$	6B11$^+CD45RO^+$/6B11$^+$	6B11$^+CD45RO^+$ iNKT cells	Montoya et al[13]
	$CD45RO^-$6B11$^+$ iNKT cells	MNC(WB) or MLC	6B11$^+CD45RO^-$	6B11$^+CD45RO^-$/MNC(WB) or MLC	6B11$^+CD45RO^-$ iNKT cells	
	$CD45RO^-$6B11$^+$ iNKT cells	6B11$^+$	6B11$^+CD45RO^-$	6B11$^+CD45RO^-$/6B11$^+$	6B11$^+CD45RO^-$ iNKT cells	
Blast cells	Blast cells†	MNC(WB) or MLC	bla$^+$	bla$^+$		
	Blasts coexpressing CD161	MNC(WB) or MLC	bla$^+CD161^+$	bla$^+CD161^+$/MNC(WB) or MLC		
	Blasts coexpressing CD161	bla$^+$	bla$^+CD161^+$	bla$^+CD161^+$/bla$^+$		
	Blasts coexpressing 6B11	MNC(WB) or MLC	bla$^+$6B11$^+$	bla$^+$6B11$^+$/MNC(WB) or MLC		
	Blasts coexpressing 6B11	bla$^+$	bla$^+$6B11$^+$	bla$^+$6B11$^+$/bla$^+$		
DC	DC	Cells	DC$^+$ (CD80, CD86, CD206)	DC/cells	$\geq$ 10 % DC in cells	Schmetzer et al 2007
	Leukemia derived DC	Cells	DC$^+$bla$^+$	DC_{leu}/cells	$\geq$ 5% DC_{leu} in cells, $\geq$ 10% DC, $\geq$ 10% bla	Schmetzer et al 2007
	Blasts converted to DC_{leu} in blast fraction	bla$^+$	DC$^+$bla$^+$	DC_{leu}/bla$^+$	$\geq$ 5% DC_{leu} in cells, $\geq$ 10% DC, $\geq$ 10% bla	Schmetzer et al 2007
	DC_{leu} in DC fraction	DC	DC$^+$bla$^+$	DC_{leu}/DC	$\geq$ 5% DC_{leu} in cells, $\geq$ 10% DC, $\geq$ 10% bla	Schmetzer et al 2007
	Migratory mature DC in DC fraction	Cells	DC$^+$CCR7$^+$	DC_{mig}/DC	$\geq$ 10% DC in cells	Grabrucker et al 2010
	Viable DC in DC-fraction	Cells	DC$^+$7AAD$^-$	DC_{via}/DC	$\geq$ 10% DC in cells	Grabrucker et al 2010

Surface marker combinations as well as T/iNKT/NK/CIK cell and DC subtypes after flow cytometric staining with fluorochrome-labeled antibodies are given.

*Total proportions of cell subsets in uncultured MNC/WB or in MLC (after culture with T cells and IL-2).

†Cells expressing blast markers: patients' individually selected blast-markers with the highest expression (Table 2: blast phenotype).

CIK indicates cytokine-induced killer; DC, dendritic cells; iNKT, invariant natural killer T; NK, natural killer; MLC, mixed lymphocyte cultures; MNC, mononuclear cells; WB, whole blood.

Ludwigs-Maximilians-University Hospital in Munich; Vote-No 339-05). The mean age of AML patients was 50 (range: 23–81) years, of ALL patients 22 (range: 3–50) years and of CLL patients 57 (range: 33–72) years. The female to male ratio of AML patients was 1:1.9, of ALL patients was 1:1.7, and of CLL patients was 1:0.9 (Table 2A).

Diagnosis and classification of AML patients was based on the "French American British" classification: minimally differentiated AML (M0: n = 1), AML without maturation (M1: n = 6), AML with granulocytic maturation (M2: n = 6), acute myelomonocytic leukemia (M4: n = 4), acute myelomonocytic leukemia together with BM eosinophilia (M4eo: n = 2), acute monocytic leukemia (M5: n = 3). Patients presented with primary AML (n = 18) or with secondary AML (n = 5). Patients stages were: first diagnosis (n = 20), relapse before (n = 1) or after SCT (n = 2). Patients were classified in cytogenetic risk groups based on the National Comprehensive Cancer Network (NCCN) guidelines as "favorable" (n = 3), "intermediate" (n = 11), or "adverse risk" (n = 4); for 5 patients no further data were available.

The subtypes of the 19 ALL patients were immune cytologically classified according to the European Group of Immunophenotyping of Leukemias classification: pro B-ALL (BI: n = 1), c ALL (BII: n = 5), pre B-ALL (BIII: n = 2), My$^+$c ALL (BII + My: n = 3), My$^+$pre B-ALL (BIII + My: n = 1), pro T-ALL (TI: n = 1), pre T-ALL (TII: n = 1), cortical T ALL (TIII: n = 4), mature T ALL (TIV:n = 1). Patients presented with primary ALL (n = 5) or with secondary ALL (n = 14). Stages of samples were: diagnosis before SCT (n = 17) or at relapse after SCT (n = 2). Risk stratification of adult ALL was based on the Study Group for Adult Acute Lymphoblastic Leukemia (GMALL) as "standard" (n = 3), "high" (n = 1) or "highest risk" (n = 3); for 12 patients no further data were available.

All CLL patients were classified as pB-CLL (n = 21) at diagnosis (n = 2) or with persisting disease (n = 19). Risk stratification was based on the Binet-classification: Binet A (n = 14), Binet B (n = 6) or Binet C (n = 1).

Cell Lines Included for Surface Marker Analyses

The following cell lines were studied for surface marker profiles: HL-60 (AML-M2), OCI-AML2 (AML-M4), Mono-Mac 6 (AML-M5), THP-1 (AML-M5), MOLM (AML-M5a), RAMOS (B-ALL–L3), RAJI (B-ALL–L3) and JURKAT (T-ALL). Cell lines were purchased from the American Type Culture Collection and were cultured according to the manufacturer's instructions (Table 2A).

AML Patients and Samples Included for Culture Experiments

Cellular composition of immune reactive cells in MNC or WB samples from 5 AML patients, 1 myelodysplastic syndrome (MDS) patient and from 10 healthy volunteers were studied before or after culture with/without DC/DC_{leu}-generating strategies or mixed lymphocyte culture (MLC) with (T) cells from the patients. Details about patient and sample characteristics are given in Table 2B.

Cell cultures were either performed under "normoxic conditions" (37°C, 5% CO_2 and 21% O_2). Further we studied the influence of hypoxia on the composition and function of different immune reactive cells and cultured samples in parallel under "hypoxic conditions" (37°C, 5% CO_2 and with either varying O_2-concentrations between 0% and 17% during the incubation time in some cases or with a defined O_2-concentration of 6% or 10%) using an InVivo400 working station (Ruskinn Technology, Bridgend, United Kingdom).

DC Generation From Isolated MNC or WB

DC/DC_{leu} were generated from 4 to 5×10^6 isolated MNC from healthy volunteers or AML/MDS patients in blast-rich stages of the disease as described previously by others or us[19,27] using Kit-D, Picibanil 1 "(Pici1)" or "Pici2" (Table 3) (D.C. Amberger, personal written communication). Therefore, cells were pipetted into 12-multiwell tissue culture plates (ThermoFisher Scientific, Darmstadt, Germany) and were diluted in 2 mL serum-free X-Vivo15-medium (Lonza, Basel, Swiss).

Moreover, DC/DC_{leu} were generated from WB (presenting the physiological cellular and soluble composition of the individual samples) obtained from AML/MDS patients in blast-rich stages of the disease or from healthy volunteers.[20,29] A total of 500 µL WB was pipetted in 12-multiwell plates and diluted 1:2 in X-Vivo15-medium to imitate the physiological conditions. DC were generated from WB using 6 different DC-generating methods: "Pici1," "Pici2," Kit-D, Kit-I, Kit-K, and Kit-M (Table 3). A patent was written to save the idea of Kit-compositions (102014014993.5, German Patent Office), but no financial conflicts of interest have to be declared. In the subsequent chapters we summarize all DC-generating methods and Kits under the term "cocktails." WB/MNC cultures without added response modifiers severed as a control. All substances used for the DC generation are approved for human treatment.

DC-subtypes were quantified as described in the chapter "Cell-characterization by flow cytometry".[19,20,29]

"Pici1"

DC were generated from MNC or WB with the DC-generating protocol "Pici1" containing 500 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 250 U/mL interleukin 4 (IL-4) (PeproTech, Berlin, Germany). After 6–7 days, 10 µg/mL Picibanil (OK 432)—a lysis product from *Streptococcus pyogenes* which has unspecific immune modulatory effects (Chugai Pharmaceutical Co., Kajiwara, Japan) and 1 µg/mL prostaglandin E_2 (PGE_2) (PeproTech) were added.[19,28] After 7–10 days of incubation cells were harvested and used for further experiments.

"Pici2"

DC were generated from MNC or WB with the "Pici2"-DC-generating protocol—a protocol developed by our group, with the same composition as given above for "Pici1," however substituting PGE_2 by PGE_1 (PeproTech; D.C. Amberger, personal communication).

Kit-D

The generation of DC from MNC or WB with Kit-D was performed using 800 U/mL GM-CSF, 10 µg/mL Picibanil and 1 µg/mL PGE_2.[20] After 2–3 days the same amounts of cytokines were added and after 7–10 days of incubation cells were harvested and used for subsequent experiments.

TABLE 2. Patients' and Cell-lines' Characteristics are Presented

(A) Uncultured AML, ALL, CLL Samples and Celllines Studied for Proportions and Coexpression of iNKT, NK, and CIK cells

	Pat.#	Age at Dgn./Sex	Subtype	Stage	Cell Source	Blast Phenotype (CD)	Blasts %	Cytogenetic Marker at Dgn.	Risk score (NCCN)	Resp. to SCT1	Resp. to Induction Chemotherapy (CLL: need for initial therapy)
	P1050	**32**/F	s/M1	Dgn.	PB	**117,33,**13	78	46,XX	ND	**CR**	ND
	P1053	**25**/M	p/M1	Rez.	PB	**117,34,**33,56	65	ND	ND	**NCR**	ND
	P1056	**27**/M	p/M4	Dgn.	PB	**117,34,**33,13,15	53	46,XY	ND	ND	**CR**
	P1057	**32**/M	p/M5	Dgn.	PB	**117,34,**33,13,14,15,38,2	39	inv(16)pq, +22	**Favorable**	ND	ND
	P1058	**71**/M	p/M1	Dgn.	PB	**117,34,**33,13,15,38,45,71,w65	91	r+11	**Intermediate**	ND	ND
	P1059	**66**/M	p/M4	Dgn.	PB	**117,34,**33,13,7	84	del(5)qq, add(17)p, add(21)q, der(9)	**Adverse**	ND	**CR**
	P1060	**81**/F	s/n.d.	Dgn.	PB	**117,34,**33,13	97	ND	ND	ND	ND
	P1061	**62**/F	p/M0	Rel. a. SCT	PB	**117,34,**33,13	96	46,XX	**Intermediate**	**NCR**	ND
	P1062	**41**/F	p/M4	Dgn.	PB	**117,34,**33,13,14,15,71	61	t(6;9)pq, add(21), -7, del(8), del(15), +mar	**Adverse**	ND	**NCR**
	P1063	**26**/M	p/M5	Rel. a. SCT	PB	**117,34,**2	86	ND	ND	ND	ND
	P1066	**60**/M	s/M2	Dgn.	PB	**117,34,**33,65	74	46,XY	**Adverse**	ND	ND
AML	P1067	**27**/M	p/M4eo	Dgn.	PB	**117,34,**33,13,15,65	41	inv(16)pq, +8	**Favorable**	ND	**CR**
	P1068	**76**/M	p/M2	Dgn.	PB	**117,34,**33,13	39	ND	**Intermediate**	ND	ND
	P1069	**33**/M	p/M4eo	Dgn.	PB	**117,34,**33,13,15	61	inv(16)pq, t(12;17)pq	**Favorable**	ND	**CR**
	P1071	**71**/F	s/M1	Dgn.	PB	**117,34,**33,13,15	60	r+4	**Intermediate**	ND	**CR**
	P1072	**58**/M	p/M2	Pers.	PB	**117,34,**33,13	70	-4,-5,-7,-11,-12,-14,-16,-21,+mar	**Adverse**	ND	**NCR**
	P1073	**23**/M	p/M5	Dgn.	PB	**117,34,**33,13,15,7,65	18	+8,+13	**Intermediate**	ND	ND
	P1076	**47**/F	s/M1	Dgn.	PB	**117,34,**33,13,14	99	r+8	**Intermediate**	**CR**	**CR**
	P1077	**41**/F	p/M1	Dgn.	PB	**117,34,**33,13,15,7	71	del(12)p	**Intermediate**	**CR**	**CR**
	P1078	**73**/M	p/M2	Dgn.	PB	**117.34**	84	46,XY	**Intermediate**	ND	ND
	P1083	**41**/M	p/M2	Dgn.	PB	**117,133,**33,13,65	43	46,XY	**Intermediate**	**CR**	**CR**
	P1084	**55**/M	p/M4	Dgn.	PB	**117,33,**13,71	84	46,XY	**Intermediate**	ND	**CR**
	P1085	**71**/F	p/M2	Dgn.	PB	**117,33,**56	96	46,XX	**Intermediate**	ND	ND
	P1106	**24**/F	p/BII+My	Dgn.	PB	**34,19,**33,133,13	81	46,XX	**High risk**	ND	**CR**
	P1107	**31**/M	s/BII+My	Dgn.	PB	**34,19,**33,10,24	36	t(9;11)qq	**Highest risk**	ND	**CR**
	P1108	**32**/M	s/TIII	Dgn.	PB	**5,2,**1a,15,cy3	98	46,XY	**Standard**	ND	**CR**
	P1109	**21**/M	p/BII+My	Dgn.	PB	**34,19,**33,13,10,38	85	46,XY	**Standard**	ND	**CR**
	P1110	**50**/F	p/BIII+My	Dgn.	PB	**34,133,**33	59	t(9;22)qq, der(22)	**highest risk**	ND	**CR**
	P1111	**26**/M	s/BII	Rel. a. SCT	PB	**34,19,**10	64	del(11q)	ND	ND	ND
	P1112	**37**/M	s/BII	Rel. a. SCT	PB	**34,19,**10	17	ND	ND	ND	ND
	P1113	**64**/F	/B	Rez./pers.	PB	**34,19,**15		ND	ND	ND	ND
	P1114	**22**/M	p/BIII	Dgn.	PB	**19,20,**34,38	97	46,XY	**Highest risk**	ND	**CR**
ALL	P1115	**45**/M	p/TIII	Dgn.	PB	**5,7,**38,71	99	46,XY	**Standard**	ND	**CR**
	P1120	**20**/M	s/TII	Dgn.	PB	**19,5,**34,20,3	28	46,XY	ND	**CR**	ND
	P1121	**25**/F	s/BI	Dgn.	PB	**34,19,** 33	32	ND	ND	ND	ND
	P1122	**23**/M	s/TIII	Dgn.	PB	**4,8,**1,2,5,7	86	46,XY			
	P1129	**11**/M	s/BII	Dgn.	BM	**34,19,**10,22	85	46,XY	ND	ND	**CR**
	P1132	**12**/M	s/BIII	Dgn.	BM	**19,10,**22	84	ND	ND	ND	**CR**
	P1133	**3**/F	s/BII	Dgn.	BM	**34,19,**10,22	55	46,XX	ND	ND	**CR**
	P1135	**17**/M	s/TI	Dgn.	BM	**34,7,**4,5,10,13,33	98	46,XY	ND	ND	**CR**
	P1136	**5**/F	s/TIII	Dgn.	PB	**34,7,**1a,2,3,5,10	82	46,XX	ND	ND	**CR**
	P1137	**3**/F	s/BII	Dgn.	BM	**34,10,**19,22	71	46,XX	ND	ND	**CR**
	P1146	**8**/F	s/TIV	Dgn.	PB	**7,3,**1,34	98	46,XX	ND	ND	**CR**
	P1088	**44**/F	p/B-CLL	Pers.	PB	**5,19,**20	98	ND	**A**	ND	**Yes**
	P1089	**54**/M	p/B-CLL	Pers.	PB	**5.19**	95	ND	**B**	ND	**Yes**
	P1090	**43**/M	p/B-CLL	Dgn.	PB	**5,19,**kappa	95	add(1)q, del(9)q,del(11)qq	**A**	ND	**Yes**
	P1091	**68**/M	p/B-CLL	Pers.	PB	**5,19,**20	96	del(13)q	**A**	ND	**No**
	P1092	**66**/M	p/B-CLL	Pers.	PB	**5,19,**20	71	ND	**B**	ND	**No**
	P1093	**51**/F	p/B-CLL	Pers.	PB	**5,23,**kappa	94	ND	**A**	ND	**No**
	P1094	**67**/M	p/B-CLL	Pers.	PB	**5,19,**kappa	91	ND	**A**	ND	**No**
	P1095	**65**/F	p/B-CLL	Pers.	PB	**5.19**	88	del(13)q	**A**	ND	**Yes**
	P1096	**64**/M	p/B-CLL	Pers.	PB	**5,19,**lambda	95	ND	**A**	ND	**No**
	P1097	**72**/F	p/B-CLL	Pers.	PB	**5,19,**20,22,kappa	97	ND	**A**	ND	**Yes**
CLL	P1098	**60**/F	p/B-CLL	Pers.	PB	**5,19,**kappa	93	ND	**B**	ND	**No**
	P1099	**67**/M	p/B-CLL	Dgn.	PB	**5,19,**20,23,lambda	89	ND	**A**	ND	**No**
	P1100	**36**/M	p/B-CLL	Pers.	PB	**5,19,**lambda	91	del(17)p, der(11)q	**B**	ND	**Yes**
	P1101	**52**/M	p/B-CLL	Pers.	PB	**5,19,**20	96	ND	**A**	ND	**Yes**
	P1102	**45**/F	p/B-CLL	Pers.	PB	**5.19**	91	del(13)q	**A**	ND	**Yes**
	P1103	**67**/F	p/B-CLL	Pers.	PB	**5,19,**20	87	del(17)q	**A**	ND	**Yes**
	P1104	**66**/F	p/B-CLL	Pers.	PB	**5,19,**kappa	94	ND	**A**	ND	**Yes**
	P1116	**66**/F	p/B-CLL	Pers.	PB	**5,19,**kappa	96	t(8;13)qq	**A**	ND	**Yes**
	P1117	**33**/M	p/B-CLL	Pers.	PB	**5,19,**23,kappa	40	del(13)q	**B**	ND	**Yes**
	P1118	**60**/F	p/B-CLL	Pers.	PB	**5,19,**20,22,23,kappa	32	del(11)q	**C**	ND	**Yes**
	P1119	**55**/F	p/B-CLL	Pers.	PB	**5,19,**20,22,23,38,kappa	57	ND	**B**	**CR**	**Yes**

Celllines and their origin

Name	Age /Sex	Subtype	Cell type	Cell Source	Blast phenotype (CD)
HL-60	**35**/F	FAB M2	**AML**	PB	**33,**13,15
OCI-AML2	**65**/M	FAB M4	**AML**	PB	**13,**14,15,33,4
THP-1	**1**/M	FAB M5	**AML**	PB	**33,**13,14,15
Mono-Mac-6	**64**/M	FAB M5	**AML**	PB	**13,**14,15,33,68
MOLM-13	**20**/M	FAB M5a	**AML**	PB	**33,**13,15,4
RAMOS	**3**/M	B-lymphoblastic	**Burkitt lymphoma**	Ascitic fluid	**19,**10,20,37,38,80
RAJI	**11**/M	B-lymphoblastic	**Burkitt lymphoma**	Left maxilla	**20,**10,13,19,37
JURKAT	**14**/M	my^2 T-linear	**T-ALL**	ND	**33,**13,2,3,4,5,6,7,34

(B) AML-samples used for culture experiments in hypoxia vs. normoxia

	Pat.#	Age at dgn./sex	Subtype (FAB)	Stage	Cell Source	Blast phenotype	Blasts %	Cytogenetic marker at dgn.
	P1424	**37**/F	p/M4	Rez.	PB	**117,**13,33,45	30	46,XX
	P1426	**61**/F	p/M5	Dgn.	PB	**34,117,**13,33,64	40	**ND**
AML	P1430	**79**/M	p/M5/M6	Dgn.	PB	**34,**13,33,117	70	46,XY
	P1433	**59**/M	p/MDS (RAEB-II)	Dgn.	PB	**34,**13,15	18	ND
	P1434	**61**/F	s/ND	Dgn.	PB	**34,117,**64,56,33,13,7	61	46,XX, t(3;8)
	P1439	**61**/F	s/M5	Dgn.	PB	**34,117,**13,33	9	inv(16)

AML/ALL/CLL subtypes, age, sex, stages of the disease, proportions of blasts, blast phenotypes evaluated by flow cytometry are given.
[1]Complete remission (CR) achieved or not achieved (NCR) after treatment with SCT.
Bold letters antibodies used for (co)expression analyses.
ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; BM, bone marrow; CIK, cytokine-induced killer; CLL, chronic lymphoid leukemia; Dgn., first diagnosis; F, female; FAB, French American British classification; iNKT, invariant natural killer T; M, male; NCCN, National Comprehensive Cancer Network; ND, no data; NK, natural killer; Pat.#, patient's number; PB, peripheral blood; Pers., persisting disease; Rel., relapse; Rel.a.SCT, relapse after stem cell transplantation.

TABLE 3. Overview of the Different DC-generating Methods/Kits ("Cocktails")[19,20,28]

"Cocktails"	Composition	Mode of Action	Culture Time (d)
"Picibanil 1" (Pici1)	GM-CSF	GM-CSF: induction of myeloid (DC) differentiation	7–10
	IL-4		
	OK-432		
	PGE_2		
"Picibanil 2" (Pici2)*	GM-CSF	IL-4: induction of DC-differentiation	7–10
	IL-4		
	OK-432		
	PGE_1		
		Picibanil (OK-432): lysis product from *Streptococcus pyogenes*; stimulates DC-differentiation	
Kit-D	GM-CSF		7–10
	OK-432		
	PGE_2		
Kit-I	GM-CSF	PGE_2: increases CCR7-expression and enhances migration	7–10
	OK-432		
Kit-K	GM-CSF		7–10
	PGE_2		
Kit-M	GM-CSF	PGE_1: effects are comparable with PGE_2	7–10
	PGE_1		

*Personal communication D.C. Amberger.

DC indicates dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-4, interleukin 4; OK-432, Picibanil; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2.

Kit-I

DC were generated with Kit-I using 800 U/mL GM-CSF and 10 μg/mL Picibanil.[20] Incubations were performed in analogy to Kit-D.

Kit-K

Kit-K consisted of 800 U/mL GM-CSF and 1 μg/mL PGE_2.[20] Incubations were performed in analogy to Kit-D.

Kit-M

For the generation of DC with Kit-M, 800 U/mL GM-CSF and 1 μg/mL PGE_1 were added to the culture.[20] Incubations were performed in analogy to Kit-D.

MLC of T-Cell–Enriched Immune Reactive Cells With "Cocktails" Pretreated or Not Pretreated Stimulator Cell Suspensions From MNC or WB

Immune reactive cells were enriched with 1×10^6 positively selected $CD3^+$ T cells (effector cells) from AML patients or healthy controls and cocultured in 24-multiwell tissue culture plates (ThermoFisher Scientific) with a stimulator cell suspension containing 2.5×10^5 DC/DC_{leu} ($MLC^{*"MNC\text{-}DC"}$ or $MLC^{*"WB\text{-}DC"}$ which were generated with different "cocktails." The same setting, but with a stimulatorcell suspension without pretreatment with "cocktails" ($MLC^{*"MNC"}$ or $MLC^{*"WB"}$) severed as a control. In 1 parallel tested case the stimulator cell suspensions were irradiated with 33 Gy to inactivate residual immune reactive cells (eg, iNKT/NK and CIK cells). The total volume of the cell culture was adjusted to 1 mL with RPMI-1640 medium (Biochrom) containing 1% Penicillin (Biochrom) and 50 U/mL IL-2 (PeproTech). The $MLC^{*"MNC"}$ or $MLC^{*"MNC\text{-}DC"}$ further contained 15% human serum (Healthcare Europe GmbH, Vienna, Austria). After 2–3 days 50 U/mL IL-2 were added to the WB and MNC cultures. Half medium exchange for MNC cultures was carried out every 2–3 days. Cells were harvested after 6–9 days and were used for the cytotoxicity-fluorolysis assay as described below.

Before and after culture different cell subsets in the MNC-fraction and WB-fraction were quantified by flow cytometry (Table 1).

Enzyme-linked Immunosorbent Assay (ELISA)

Cell culture supernatants after AML-WB-DC culture stimulated with different "cocktails" ("Pici1," "Pici2," Kit-D, Kit-I, Kit-K, and Kit-M) and after $MLC^{*"WB\text{-}DC"}$ ($n = 4$) were analyzed for IL-10, IL-17, Monocyte Chemoattractant Protein-1 (MCP-1) and interferon gamma (IFN-γ) secretion using the human IL-10, IL-17A, MCP-1, and IFN-γ immunoassay kits (DRG Instruments GmbH, Marburg, Germany). Moreover, WB-DC cultures without added response modifiers and $MLC^{*"WB"}$ severed as a control. The samples were evaluated with a Tristar LB941 ELISA reader (Berthold company, Bad Wildbach, Germany) and the concentration of the 4 different cytokines evaluated using the corresponding standard curve.

Cell Characterization by Flow Cytometry

Flow cytometric analyses were carried out to evaluate and quantify amounts, subsets and phenotypes of leukemic cells, B, T, iNKT, NK, and CIK cells, monocytes and DC in the MNC and WB fractions before and after different cultures. Panels with several moAbs labeled with fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE), or allophycocyanin (APC) were used. Antibodies were provided by Beckman Coulter, Krefeld, Germany ([a]); Becton Dickinson, Heidelberg, Germany ([b]); Biozol, Eching, Germany ([c]); Caltag, Darmstadt, Germany ([d]); Bioscience, Heidelberg, Germany ([e]); Miltenyi Biotech, Bergisch Gladbach, Germany ([f]); and ThermoFisher Scientific ([g]). FITC-conjugated moAbs against $CD3^a$, $CD8^b$, $CD33^a$, $CD34^a$, $CD45RO^a$, $CD83^a$, $CD161^b$, $6B11^c$, and $V\alpha24^a$ were used. We used PE-conjugated moAbs for $CD1d^e$, $CD3^a$, $CD4^b$, $CD34^a$, $CD80^a$, $CD83^a$, $CD117^a$, $CD206^a$, and $6B11^b$. MoAbs against $CD3^a$, $CD4^a$, $CD5^a$, $CD14^b$, $CD15^b$, $CD19^a$, $CD34^a$, $CD80^b$, $CD117^a$, and $CD197^b$ were labeled with Cy7-PE. As APC-labeled moAbs we used $CD1a^a$, $CD3^a$, $CD4^{a,b}$, $CD5^a$, $CD7^e$, $CD8^b$, $CD10^a$, $CD14^a$, $CD15^b$, $CD19^a$, $CD20^a$, $CD33^a$, $CD34^{a,d}$, $CD45RO^d$, $CD56^a$, $CD69^b$, $CD86^g$, $CD117^a$, $CD133^f$, $CD206^b$, and $CD209^b$. To detect dead cells $7AAD^b$ was used.

Erythrocytes in WB samples were lysed using Lysing-Buffer (BD, Heidelberg, Germany) according to the manufacturer's instructions. To stain cells (MNC or WB) with moAbs they were resuspended in PBS (Biochrom), containing 5%–10% fetal calf-serum (Biochrome) to avoid unspecific bindings and were incubated for 15 minutes in the dark at room temperature. Afterwards cells were washed, centrifuged, and resuspended in 100–200 μL PBS. At least 5000 events were evaluated with a fluorescence-activated cell-sorting Flow Cytometer (FACSCalibur) and

Cell-Quest data acquisition and analysis software (Becton Dickson, Heidelberg, Germany). Isotype controls were conducted according to the manufacturer's instructions.

For the analysis and quantification of T, iNKT, NK, and CIK cells/subtypes were quantified in the total cell-fraction (eg, $CD3^+$ cells) or in the subpopulations (eg, $6B11^+CD3^+$). According to their expression profile we quantified proportions of immune reactive cells as given in Table 1: T cells: $CD3^+$, $CD8^+$, $CD4^+$, $V\alpha24^+$, $CD1d^+$ T-cells, T_{naive}, $T_{non\text{-}naive}$, T_{cm} or $T_{eff\text{-}em}$.[9,10,13] CIK cells: $CD3^+CD56^+$ or $CD3^+CD161^+$ cells. NK cells: $CD3^-CD56^+$ or $CD3^-CD161^+$ cells.[5,13] ($6B11^+$) iNKT cells: $6B11^+CD3^+$, $6B11^+CD161^+$, $6B11^+V\alpha24^+$, $6B11^+CD4^+$, $6B11^+CD8^+$, $6B11^+CD1d^+$, $6B11^+CD45RO^+$ or $6B11^+CD45RO^-$ iNKT cells. Exemplarily, dot plots iNKT, NK, and CIK cells and the gating strategy of iNKT cells/subsets are given in Figure 1. Moreover, aberrant expressions of CD161 and 6B11 on blasts were studied (Table 1).[13]

For the analysis and quantification of DC/DC_{leu} subtypes in the total cell fraction or in subtype cell fraction after DC culture in the MNC or WB fraction (DC_{leu}/cells) we used a refined gating strategy[6,8]: subgroup analyses were conducted only in cases with $\geq 10\%$ DC. DC_{leu} were quantified in the total fraction (DC_{leu}/cells) in the DC fraction (DC_{leu}/DC) or in the blast fraction, to quantify the amount of blasts converted to DC_{leu} (DC_{leu}/bla), mature DC (coexpressing the migration marker CCR7) and viable DC (negative for 7AAD) were quantified in the DC fraction after culture (DC_{mig}/DC; DC_{via}/DC, Table 1,

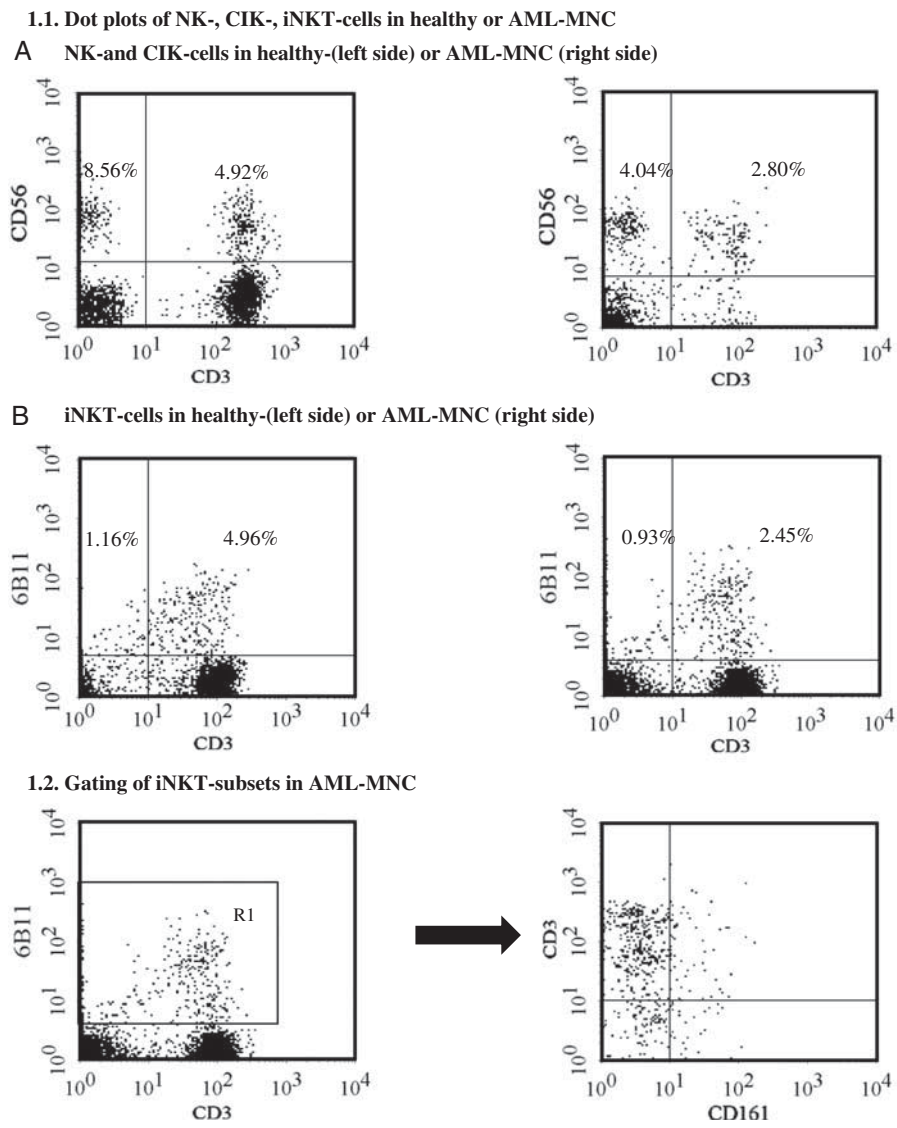

FIGURE 1. Gating strategy and corresponding dot plots of CIK, NK and iNKT cells in healthy or AML MNC are given. 1.1. Various frequencies of NK ($CD3^-CD56^+$) and CIK cells ($CD3^+CD56^+$) in healthy or AML MNC are given (A). Various frequencies of iNKT cells ($6B11^+CD3^+$ and $6B11^+CD3^-$) in healthy or AML MNC are given (B). 1.2. Gating of $6B11^+$ cells and characterization of corresponding subsets ($6B11^+CD3^+CD161^+$, $6B11^+CD3^-CD161^+$) are given. ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T. R1 = gate surrounding all 6B11 + cells in the AML-MNC fraction.

lower part). For this purpose, cells were stained with patient-specific "blast"-staining antibodies (eg, CD15, CD34, CD65, and CD117) according to diagnostic reports before culture in combination with "DC"-staining antibodies (eg, CD80, CD83, CD86, CD206, and CD209), which were not expressed before culture.

Cytotoxicity (Fluorolysis) Assay

To analyze the blast lytic activity of T-cell–enriched immune reactive cells after MLC with "cocktails"-pretreated or not pretreated stimulator cell suspensions from MNC or WB a fluorolysis assay was performed.[19] Therefore, effector cells (E) were cocultured 1:1 with thawed blast-containing target cells (T) for 3 and 24 hours at 37°C, with 21% O_2 and 5% CO_2. As a control effector cells and target cells were cultured for the same time separately and mingled on ice shortly before the flow cytometric analyses were carried out. Before culture, target cells were stained for 15 minutes with FITC, PE or APC-conjugated blast, monocyte or T cell-specific target cell antibodies. To evaluate viable cells and the lytic activity of effector cells, the cultures were harvested after 3 and 24 hours and resuspended in PBS containing 7AAD (Becton Dickson) and a defined number of Fluorospheres beads (Beckman Coulter). For analyses, a refined gating was used.[8] Therefore, viable target cells were gated in a FSC/7AAD$^-$ gate. With a FACS Calibur Flow Cytometer and a Cell-Quest software (Becton Dickson) cells were analyzed. The lytic activity was calculated and defined as the difference in the percentage of viable target blasts before and after the effector cell contact.

Statistical Methods

Data were presented as mean ± SD. Statistic comparisons for 2 groups were performed using the *t* test and Mann-Whitney-Wilcoxon test. The statistical analysis was done with Microsoft Excel 2010 or 2013 (Microsoft, Redmond, WA) and JMP10.0 statistical software (SAS Institute, Cary, NY). Differences were considered as "not significant" (ns) with *P*-values >0.1, as "borderline significant" (significant*) with *P*-values between 0.1 and 0.05, as "significant" (significant**) with *P*-values between 0.05 and 0.005 and as "highly significant" (significant***) with *P*-values <0.005.

RESULTS

Prolog

During the development of acute and chronic leukemia B, T lymphocytes and monocytes are displaced by increasing blast cell-populations. In our samples between 8% and 99% of blast cells and varying proportions of the remaining hematopoietic cells were detectable. Details of the cellular composition of AML/ALL/CLL samples are shown in Table 4. Samples with blasts, that aberrantly expressed CD3, CD4, CD8, CD14 or CD19, were excluded from quantification analyses for the respective lineage markers.

In our first approach, we compared frequencies and compositions of iNKT, NK, CIK cells as well as T cell subtypes in (unstimulated) MNC from healthy donors with AML, ALL and CLL patients and correlated findings with prognosis of these patients. Moreover, we studied potential (aberrant) coexpressions of iNKT markers on leukemic cell lines. In our second approach we analyzed proportions of AML blasts and immune reactive cells (with a special focus on iNKT, NK, and CIK cells) and their expression profiles after MLC.

TABLE 4. Cellular Composition of AML/ALL/CLL and Healthy Samples

	Cell Type	Ø (MNC) (%)	Range (MNC) (%)
4.1. AML/ALL/CLL and healthy samples used for surface marker expression analyses			
AML	Blasts (myeloid blasts)	69	18–99
	T cells (CD3$^+$)	6	1–33
	B cells (CD19$^+$)	6	1–33
	Monocytes (CD14$^+$)	8	2–18
	Blasts (B or T lineage blasts)	71	17–99
ALL	T cells (CD3$^+$)*	14	5–33
	B cells (CD19$^+$)†	8	3–11
	Monocytes (CD14$^+$)	5	1–29
	Blasts (B lineage blasts)	85	32–98
CLL	T cells (CD3$^+$)	11	1–60
	Monocytes (CD14$^+$)	4	1–25
	T cells (CD3$^+$)	41	24–64
Healthy	B cells (CD19$^+$)	24	14–32
	Monocytes (CD14$^+$)	3	1–6
	Cell type	Ø (WB/MNC) (%)	Range (WB/MNC) (%)
4.2. AML and healthy samples used for culture experiments			
AML	Blasts	28/17	8–60/15–18
	T cells (CD3$^+$)	21/11	2–54/5–17
	B cells (CD19$^+$)	1/5	0–2/2–8
	Monocytes (CD14$^+$)	11/7	0–32/3–13
	T cells (CD3$^+$)	18/35	14–21/9–48
Healthy	B cells (CD19$^+$)	3/7	1–4/3–12
	Monocytes (CD14$^+$)	6/7	5–8/3–12

*Only quantified in B lineage ALL.
†Only quantified in T lineage ALL.
ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; MNC, mononuclear cells; WB, whole blood; Ø, mean.

The 6B11-antibody, targeting the invariant CDR3 loop of the Vα24Jα18 TCR, is regarded as a specific antibody to detect iNKT cells. In our experiments, we used 2 different antibodies: 6B11 (clone 6B11) labeled with PE (6B11-PE, delivered by BD) and 6B11 (clone 6B11) labeled with FITC (6B11-FITC, delivered by Biozol). First comparative analyses with these markers in MNC from AML, ALL, CLL patients revealed significantly* higher proportions of iNKT cells detected with the 6B11-FITC compared with 6B11-PE (AML: 1.52% ± 1.74% vs. 0.75% ± 1.10%, $P < 0.0652$; ALL: 1.24% ± 1.52% vs. 0.74% ± 0.64%, $P < 0.103$; CLL: 0.92% ± 0.87% vs. 0.78% ± 0.47%, $P < 0.299$). However, proportions of iNKT cells detected with 6B11-FITC were significantly** lower in healthy samples compared with leukemic samples (AML: 0.40% ± 0.26% vs. 1.52% ± 1.74%, $P < 0.01073$; ALL: 0.40% ± 0.26% vs. 1.24% ± 1.52%, $P < 0.01622$; CLL: 0.40% ± 0.26% vs. 0.92% ± 0.87%, $P < 0.02267$),

while iNKT cells detected with 6B11-PE were significantly* higher in healthy samples compared with leukemic samples (AML: 2.47% ± 3.12% vs. 0.75%% ± 1.10%, $P < 0.081$; ALL: 2.47% ± 3.12% vs. 0.74% ± 0.65%, $P < 0.083$; CLL: 2.47% ± 3.12% vs. 0.78% ± 0.47%, $P < 0.0827$). We decided for better comparibility to present only data obtained with 6B11-PE.

We evaluated 8 different leukemic cell lines (HL-60, OCI-AML2, THP-1, Mono-Mac-6, MOLM-13, RAMOS, RAJI, JURKAT) to analyze if 6B11 or CD161 is aberrantly expressed on blasts. We could demonstrate that neither CD161 nor 6B11 were (aberrantly) expressed on blasts of leukemic myeloid and B lineage cell lines with a mean coexpression on blasts of 2.59% ± 0.02% or 2.67% ± 0.03%. However, 8.38% $6B11^+$ bla^+ and 10.03% $CD161^+$ bla^+ cells were found in the T-linear Jurkat cell line. Moreover, we could demonstrate that neither CD161 nor 6B11 were aberrantly expressed on leukemic blasts obtained from patients with AML, ALL or CLL. On average, coexpression of CD161 and 6B11 on blasts was: AML: 0.23% ± 0.01% or 0.35% ± 0.01%; ALL: 0.55% ± 0.01% or 0.66% ± 0.01%; CLL: 0.17% ± 0.00% or 1.60% ± 0.01%.

Therefore, analyzing iNKT cells with 6B11-PE and NK/CIK cells with CD161 represent specific results as 6B11 and CD161 antibodies do not show unspecific bindings on blasts, although combinations of (fluorochrome)-labeled antibodies have to be tested thoroughly.

moAbs and their Combinations for iNKT, NK, and CIK Cell/Subtype Analyses

For iNKT cell detection in healthy and AML, ALL, and CLL samples, we used moAb 6B11 alone or in combination with CD3, CD161, Vα24, CD1d, CD45RO, CD4, and CD8. We defined iNKT cells detected with 6B11 in combination with T cell markers (CD3, Vα24, CD1d, CD45RO, CD4, and CD8) as "T cell-like" iNKT cells and iNKT cells detected with 6B11 in combination with NK cell markers (CD161) as "NK cell-like" iNKT cells. The frequencies of iNKT cells detected with 6B11 alone were similar in AML, ALL, and CLL patients (0.75% ± 1.10% vs. 0.74% ± 0.65% vs. 0.78% ± 0.47%). Frequencies of iNKT cells detected with 6B11 in combination with CD3 ($6B11^+$ $CD3^+$/MNC) were slightly lower in AML, ALL, and CLL patients (0.58% ± 1.15% vs. 0.61% ± 0.42% vs. 0.46% ± 0.47%), but the percentages were comparable to results with 6B11 in combination with CD1d ($6B11^+$ $CD1d^+$/MNC, 0.66% ± 1.16% vs. 0.66% ± 0.96% vs. 0.48% ± 0.64%). Proportions of $6B11^+$ $CD161^+$/MNC iNKT cells were even smaller in AML, ALL, and CLL patients (0.14% ± 0.25% vs. 0.18% ± 0.16% vs. 0.22% ± 0.26%), while $6B11^+$ $V\alpha24^+$/MNC could only detect very few frequencies of iNKT cells (0.09% ± 0.10% vs. 0.08% ± 0.18% vs. 0.03% ± 0.03%). We could show higher frequencies of 6B11 + CD4 + /MNC versus $6B11^+$ $CD8^+$/MNC iNKT cells detected in AML, ALL, and CLL patients ($CD4^+$ iNKT cells: 0.18% ± 0.20% vs. 0.32% ± 0.33% vs. 0.25% ± 0.12%; $CD8^+$ iNKT cells: 0.03% ± 0.04% vs. 0.07% ± 0.09% vs. 0.06% ± 0.05%). Moreover, we could show that the majority of $6B11^+$ iNKT cells express CD45RO in AML, ALL, and CLL patients (88.58% ± 14,43% vs. 79.75% ± 30.00% vs. 64.58% ± 29.53%).

In conclusion, frequencies of iNKT cells and their subsets can be detected with 6B11 alone (PE-labeled clone preferred) or in combination with CD3, CD161, CD1d or Vα24. The combination of 6B11 with CD4, CD8, and CD45RO can be used for further subset-analyses of iNKT cells.

Frequencies and Compositions of iNKT, NK, and CIK Cells From AML Patients Compared With Healthy Controls and their Correlation With Prognosis

AML Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly*** lower frequencies of $CD3^+$ $CD56^+$ CIK cells in MNC of AML patients compared with healthy MNC (0.46% ± 0.62% vs. 2.74% ± 1.64%, $P < 0.0021$) and significantly*** lower percentages of $CD3^+CD161^+$ CIK cells (0.30% ± 0.40% vs. 3.25% ± 1.02%, $P < 0.000152$, Fig. 2A). In addition, we could show significantly*** lower proportions of $CD3^-CD56^+$ NK cells (1.68% ± 1.97% vs. 9.33% ± 4.06%, $P < 0.00064$) and significantly** lower proportions of $CD3^-CD161^+$ NK cells in MNC of AML patients compared with healthy MNC (1.01% ± 1.26% vs. 2.44% ± 1.20%, $P < 0.0142$, Fig. 2B). We could demonstrate significantly* lower percentages of $6B11^+$ iNKT cells (0.75% ± 1.10% vs. 2.47% ± 3.12%, $P < 0.081$) and (ns) lower proportions of $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cells in MNC of AML patients compared with healthy MNC (Fig. 2C). Moreover, $6B11^+CD8^+$ and $6B11^+V\alpha24^+$ iNKT cells were found in lower proportions in MNC from AML patients versus healthy MNC [data not shown (dns)].

In summary frequencies of $6B11^+$/$6B11^+CD3^+$/$6B11^+CD161^+$/$6B11^+CD8^+$/$6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+$/$CD3^-CD161^+$ NK, and $CD3^+CD56^+$/$CD3^+CD161^+$ CIK cells/subsets were (significantly) lower in MNC from AML patients than in healthy MNC.

(Significantly) Higher Proportions of T and NK Cells Express 6B11 in AML Patients Compared With Healthy Controls

We found significantly*** increased proportions of $CD3^+$ T cells expressing 6B11 in AML patients compared with healthy controls (14.03% ± 14.66% vs. 2.69% ± 2.17%, $P < 0.00111$, Fig. 2D). Moreover, we found significantly** higher frequencies of $CD4^+$ (8.36% ± 15.22% vs. 0.31% ± 0.38%, $P < 0.0123$) and $CD161^+$ cells expressing 6B11 (17.19% ± 15.36% vs. 4.62% ± 5.97%, $P < 0.0152$, Fig. 2D) and significantly* increased frequencies of $CD8^+$ cells expressing 6B11 in MNC of AML patients versus healthy controls (1.89% ± 3.55% vs. 0.59% ± 1.12%, $P < 0.0723$). We did not find significant differences of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T cell fraction of AML patients compared with $CD3^+CD161^+$ CIK cells in the healthy T cell fraction (dns).

In summary a shift to higher proportions of ($CD3^+$/$CD4^+$/$CD8^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in AML patients compared with healthy samples. No differences of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T-cell fraction were observed.

AML Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT, NK, and CIK Cells

AML patients who achieved CR after induction chemotherapy were characterized by significantly** higher frequencies of $CD3^+CD56^+$ CIK cells in MNC-fractions than AML patients with no complete remission (NCR; 0.4% ± 0.33% vs. 0.05% ± 0.05%, $P < 0.0094$, Fig. 5.1A, left side), whereas proportions of NK or iNKT cells were not different in MNC fractions of patients who achieved versus not achieved a CR (dns). AML-patients with

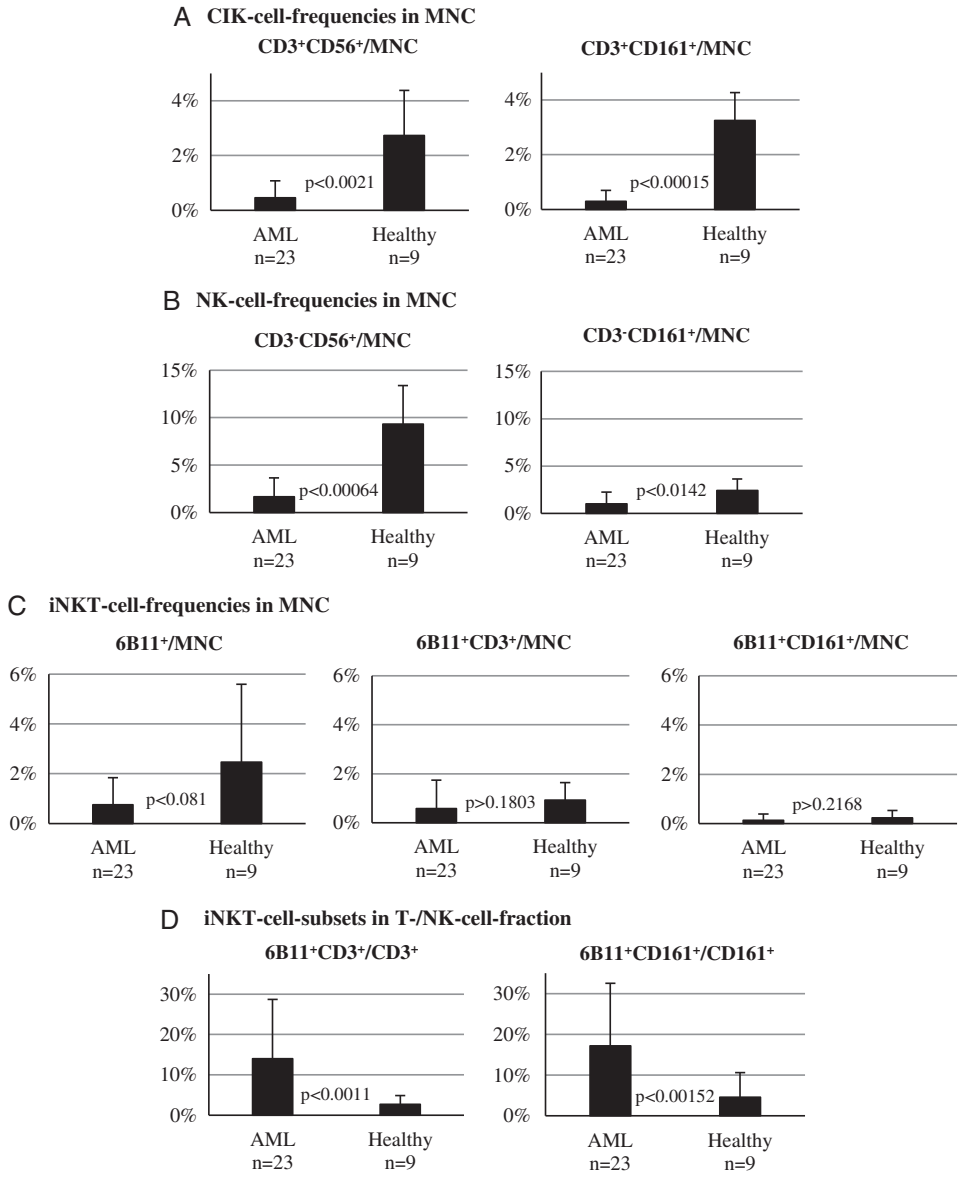

FIGURE 2. Frequencies of CIK, NK and iNKT cells and their subsets in healthy versus AML MNC are given. Percentages of CIK cells ($CD3^+CD56^+$/MNC and $CD3^+CD161^+$/MNC) (A), NK cells ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) (B), iNKT cells in MNC ($6B11^+$, $6B11^+CD3^+$/MNC, $6B11^+CD161^+$/MNC) (C), and iNKT cell subsets in the T cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) (D) are given. ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

favorable versus adverse NCCN risk-score presented with (ns) higher values of $6B11^+$-iNKT cells in the MNC-fraction (0.64% ± 0.11% vs. 0.56% ± 0.09%; Fig. 5.1A, middle row). Comparable results were found for $6B11^+$ $Va24^+$ and $6B11^+CD161^+$ iNKT cell-proportions in the MNC-fraction of AML-patients with favorable versus adverse NCCN risk-score (dns).

AML-patients younger than 60 years presented with (ns) higher frequencies of $6B11^+$ iNKT cells in the MNC-fraction compared with AML-patients older than 60 years (0.89% ± 1.35% vs. 0.53% ± 0.40%, Fig. 5.1A, right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell proportions in the MNC-fraction of AML-patients younger than 60 years versus older than 60 years (dns). No differences were found for proportions of NK or CIK cells in the groups compared (dns).

AML-patients with primary versus secondary AML presented with (ns) higher frequencies of $6B11^+$ and $6B11^+$ $CD3^+$ iNKT cells in the MNC-fraction (0.78% ± 1.21% vs. 0.62% ± 0.46%; 0.66% ± 1.29% vs. 0.32% ± 0.23%). No differences were found for proportions of NK or CIK cells in the groups compared (dns).

AML-patients without extramedullary foci (n = 20) compared with those with extramedullary foci (n = 3) presented with significantly*** increased proportions of $CD3^+CD56^+$ CIK cells in the MNC-fraction (0.53% ± 0.63% vs. 0.00% ± 0%,

$P < 0.0023$; Fig. 5.1B left side). Moreover, the percentages of $CD3^-CD56^+$ NK cells was significantly*** higher in cases without compared with patients with extramedullary foci (1.97% ± 0.21% vs. 0.08% ± 0.04%, $P < 0.0017$, Fig. 5.1B, middle row) Furthermore we found (ns) higher frequencies of $6B11^+$ iNKT cells in the MNC fraction of AML-patients without versus with extramedullary foci (0.81% ± 1.16% vs. 0.37% ± 0.24%; Fig. 5.1B right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell-proportions in the MNC-fraction in the groups compared (dns).

AML-patients who stayed in stable CR versus no stable CR (NCR) showed (ns) higher frequencies of $CD3^+CD161^+$ CIK cells and $CD3^-CD161^+$ NK cells in the MNC-fraction (0.47% ± 0.58% vs. 0.16% ± 0.08%; 1.21% ± 1.73% vs. 0.90% ± 072%; Fig. 5.1C left side, middle row). Moreover, we found (ns) higher percentages of $6B11^+$ iNKT cells in the MNC-fraction of AML-patients who stayed in stable CR versus NCR (1.04% ± 1.63% vs. 0.37% ± 0.10%; Fig. 5.1C, right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell proportions in the MNC fraction in the groups compared (dns).

In summary in AML-patients (significantly) higher proportions of $6B11^+$ iNKT cells correlated with favorable NCCN risk-score, younger age, primary AML, no extramedullary foci and stable CR. Similar correlations were found for $6B11^+CD3^+/6B11^+CD161^+$ and $6B11^+V\alpha24^+$ iNKT-subsets. High frequencies of $CD3^-CD56$ NK cells and $CD3^+CD56^+$ CIK cells NK and CIK cells correlated significantly with no extramedullary foci. Moreover, higher proportions of $CD3^-CD161^+$ NK cells and $CD3^+CD161^+$ CIK cells correlated with stable CR in AML-patients and $CD3^+CD56^+$ CIK cells correlated with the achievement of CR after induction chemotherapy in AML-patients.

Frequencies and Subtypes of iNKT, NK, and CIK Cells From ALL-Patients Compared With Healthy Controls and their Correlation With Prognosis

ALL-Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly** lower frequencies of $CD3^+CD56^+$ CIK cells in MNC of ALL-patients compared with healthy MNC (1.36% ± 1.63% vs. 2.74% ± 1.64%, $P < 0.0395$) and significantly*** lower percentages of $CD3^+CD161^+$ CIK cells (0.94% ± 1.45% vs. 3.25% ± 1.02%, $P < 0.00039$, Fig. 3A). Moreover, we found significantly*** lower proportions of $CD3^-CD56^+$ NK cells (2.68% ± 2.35% vs. 9.33% ± 4.06%, $P < 0.00163$) and significantly*** lower proportions of $CD3^-CD161^+$ NK cells in MNC of ALL-patients versus healthy MNC (0.52% ± 0.92% vs. 2.44% ± 1.20%, $P < 0.00344$, Fig. 3B). We could show significantly* lower frequencies of $6B11^+$ iNKT cells in MNC of ALL-patients versus healthy MNC (0.74% ± 0.65% vs. 2.47% ± 3.12%, $P < 0.083$, Fig. 3C). Moreover, $6B11^+CD3^+/6B11^+CD8^+/6B11^+CD161^+$ and $6B11^+V\alpha24^+$ iNKT cells were found in (ns) lower proportions in MNC from ALL-patients versus healthy MNC (dns).

In summary frequencies of $6B11^+/6B11^+CD3^+/6B11^+CD161^+/6B11^+CD8^+/6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+/CD3^-CD161^+$ NK, and $CD3^+CD56^+/CD3^+CD161^+$ CIK cells/subsets were (significantly) lower in ALL-MNC than in healthy MNC.

(Significantly) Higher Proportions of T and NK Cells Express 6B11 in ALL-Patients Compared With Healthy Controls

We found significantly* lower percentages of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T-cell fraction of ALL-patients compared with $CD3^+CD161^+$ CIK cells in the healthy $CD3^+$ T-cell fraction (5.25% ± 7.17% vs. 8.92% ± 2.82%, $P < 0.053$), but significantly** higher proportions of $CD3^+$ T cells expressing 6B11 in ALL-patients compared with healthy controls (6.03% ± 5.25% vs. 2.69% ± 2.17%, $P < 0.0102$, Fig. 3D). Furthermore, we found significantly** higher frequencies of $CD4^+$ (7.27% ± 11.04% vs. 0.31% ± 0.38%, $P < 0.00745$) and $CD161^+$ cells expressing 6B11 (15.63% ± 16.46% vs. 4.62% ± 5.97%, $P < 0.0057$, Fig. 3D) and significantly* higher percentages of $CD8^+$ cells expressing 6B11 in ALL patients versus healthy controls (1.28% ± 1.44% vs. 0.59% ± 1.12%, $P < 0.081$).

In summary a shift to higher proportions of ($CD3^+/CD4^+/CD8^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in ALL-patients compared with healthy samples. However, unlike in AML-patients, lower frequencies of $CD3^+CD161^+$ CIK cells were found in the $CD3^+$ T-cell fraction of ALL-patients versus in the healthy $CD3^+$ T-cell fraction.

Adult ALL-Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT and NK Cells

The ALL-patients' cohort included children and adults. As only 1 child relapsed only adult ALL-patients (treated with GMALL-therapy) were included in prognostic analyses.

Adult ALL-patients who achieved a CR after GMALL-induction chemotherapy were characterized by (ns) higher frequencies of $CD3^-CD161^+$ NK cells in MNC fractions than ALL-patients who achieved without CR (1.09% ± 1.48% vs. 0.18% ± 0.11%, dns). Moreover, we found (ns) higher percentages of $6B11^+$, $6B11^+CD3^+$, and $6B11^+V\alpha24^+$ iNKT cells in the MNC-fraction of ALL-patients who achieved CR versus NCR (0.95% ± 1.02% vs. 0.65% ± 0.37%; 0.61% ± 0.34% vs. 0.58% ± 0.26%; 0.16% ± 0.30% vs. 0.03% ± 0.05%, dns). No differences were found for CIK cells.

Adult ALL-patients without extramedullary foci compared with those with extramedullary foci presented with (ns) higher proportions of $6B11^+$, $6B11^+CD3^+$, and $6B11^+V\alpha24^+$ iNKT cells in the MNC fraction (1.12% ± 1.07% vs. 0.54% ± 0.30%; 0.63% ± 0.38% vs. 0.56% ± 0.21%; 0.20 ± 0.32% vs. 0.02% ± 0.02%, dns). No differences were found for NK and CIK cells.

Although only few data were available we can demonstrate, that higher proportions of $6B11^+/6B11^+CD3^+/6B11^+V\alpha24^+$ iNKT cells correlate with adult ALL-patients who achieved CR and without extramedullary foci. Moreover, $CD3^-CD161^+$ NK cells correlated with adult ALL-patients who achieved CR. no correlations were found for CIK cells.

Frequencies and Subtypes of iNKT, NK, and CIK Cells From CLL Patients Compared With Healthy Controls and Correlations With Prognosis

CLL-Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly*** lower frequencies of $CD3^+CD56^+$ CIK cells in MNC of CLL-patients compared with healthy MNC (0.67% ± 0.73% vs. 2.74% ± 1.64%,

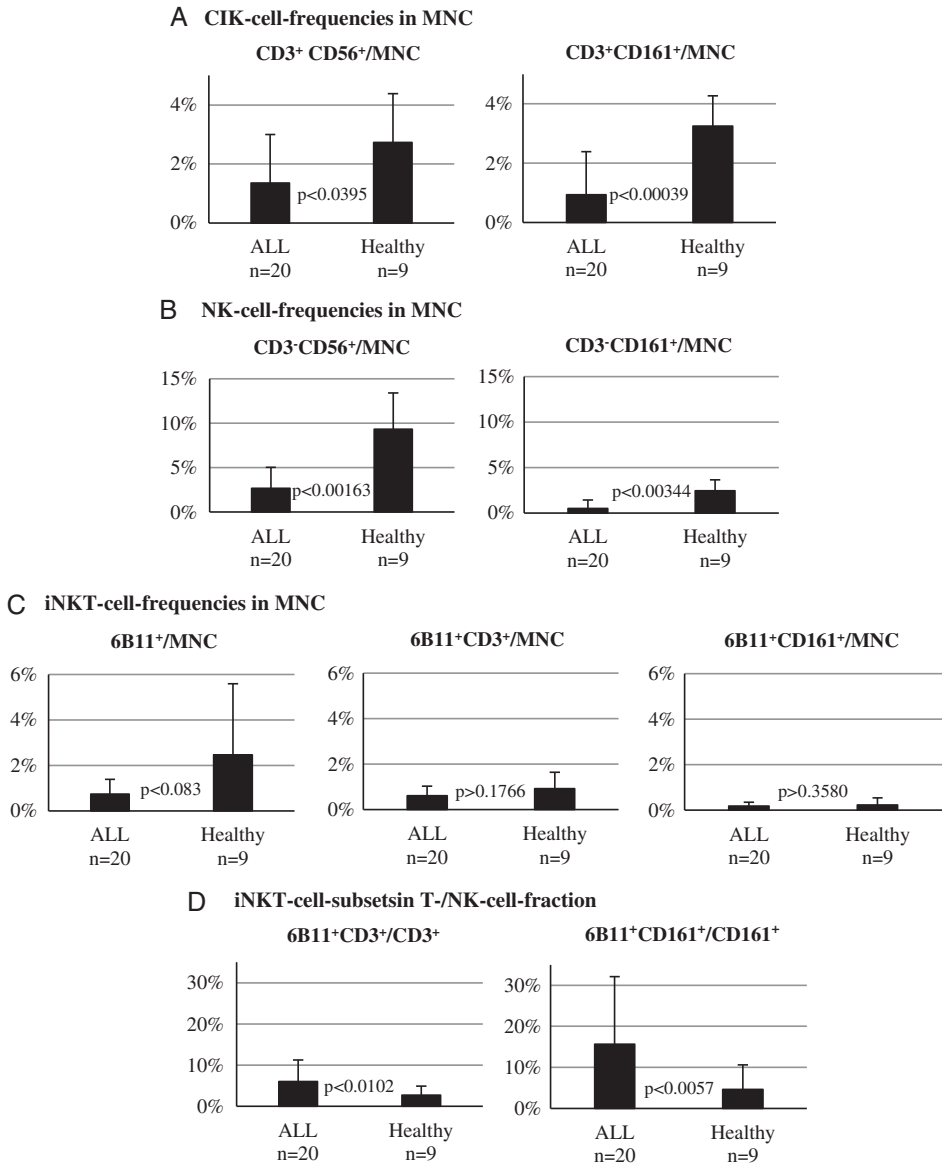

FIGURE 3. Frequencies of CIK, NK and iNKT cells and their subsets in healthy versus ALL MNC are given. Percentages of CIK cells ($CD3^+CD56^+$/MNC and $CD3^+CD161^+$/MNC) (A), NK cells ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) (B), iNKT cells in MNC ($6B11^+$, $6B11^+CD3^+$/MNC, $6B11^+CD161^+$/MNC) (C), and iNKT cell subsets in the T cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) (D) are given. ALL indicates acute lymphoid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

$P < 0.0036$) and significantly*** lower frequencies of $CD3^+$ $CD161^+$ CIK cells ($0.67\% \pm 0.84\%$ vs. $3.25\% \pm 1.02\%$, $P < 0.000168$, Fig. 4A). Moreover, we found significantly*** lower proportions of $CD3^-CD56^+$ NK cells ($3.27\% \pm 3.26\%$ vs. $9.33\% \pm 4.06\%$, $P < 0.00243$) and significantly*** lower proportions of $CD3^-CD161^+$ NK cells in MNC of CLL-patients versus healthy MNC ($0.45\% \pm 0.48\%$ vs. $2.44\% \pm 1.20\%$, $P < 0.00302$, Fig. 4B). Moreover, we found significant* lower frequencies of $6B11^+$ ($0.78\% \pm 0.47\%$ vs. $2.47\% \pm 3.12\%$, $P < 0.0828$), $6B11^+CD3^+$ ($0.46\% \pm 0.47\%$ vs. $0.93\% \pm 0.72\%$, $P < 0.072$, Fig. 4C) and $6B11^+V\alpha24^+$ iNKT cells in MNC of CLL patients versus healthy MNC ($0.03\% \pm 0.03\%$ vs. $0.16\% \pm 0.27\%$, $P < 0.09522$). Moreover, lower proportions of $6B11^+CD8^+$ and $6B11^+CD161^+$ iNKT cells were found in MNC from CLL patients versus healthy MNC (dns).

In summary frequencies of $6B11^+/6B11^+CD3^+/6B11^+CD161^+/6B11^+CD8^+/6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+/CD3^-CD161^+$ NK and $CD3^+CD56^+/CD3^+CD161^+$ CIK cells/subsets were (significantly) lower in CLL MNC than in healthy MNC.

Significantly Higher Proportions of T and NK Cells Express 6B11 in CLL-Patients Compared With Healthy Controls

We found significantly*** higher frequencies of $CD3^+$ T cells expressing 6B11 in CLL-patients compared with

healthy controls (11.27% ± 11.83% vs. 2.69% ± 2.17%, $P < 0.00384$, Fig. 4D). Furthermore, we found significantly** higher frequencies of for $CD4^+$ cells expressing 6B11 (7.14% ± 5.90% vs. 0.31% ± 0.38%, $P < 0.00908$) and significantly*** higher frequencies of $CD161^+$ cells expressing 6B11 in CLL-patients versus healthy controls (23.55% ± 20.47% vs. 4.62% ± 5.97%, $P < 0.00043$, Fig. 4D). We did not find significant differences in the numbers of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T cell fraction and $CD8^+$ T cells expressing 6B11 (dns).

In summary a shift to higher proportions of ($CD3^+$/$CD4^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in CLL-patients compared with healthy samples. Like in AML patients, but unlike in ALL patients, no differences were found for CIK cells in the $CD3^+$ T-cell fraction. Unlike AML and ALL patients no differences were found for $CD8^+$ T cells expressing 6B11.

CLL-Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT, NK, and CIK Cells

CLL-patients with no need versus need for therapy presented with significantly* higher proportions of $CD3^+CD56^+$ CIK cells in the MNC fraction (1.07% ± 1.03% vs. 0.43% ± 0.28%, $P < 0.093$; Fig. 5.2A, left side). We found (ns) higher frequencies of $6B11^+$ iNKT cells in the MNC fraction of CLL patients with no need versus need for therapy (0.81% ± 0.50% vs. 0.73% ± 0.40%; Fig. 5.2A, middle row). Comparable results were found for $6B11^+CD3^+$ and $6B11^+V\alpha24^+$ iNKT cell proportions in the MNC fraction

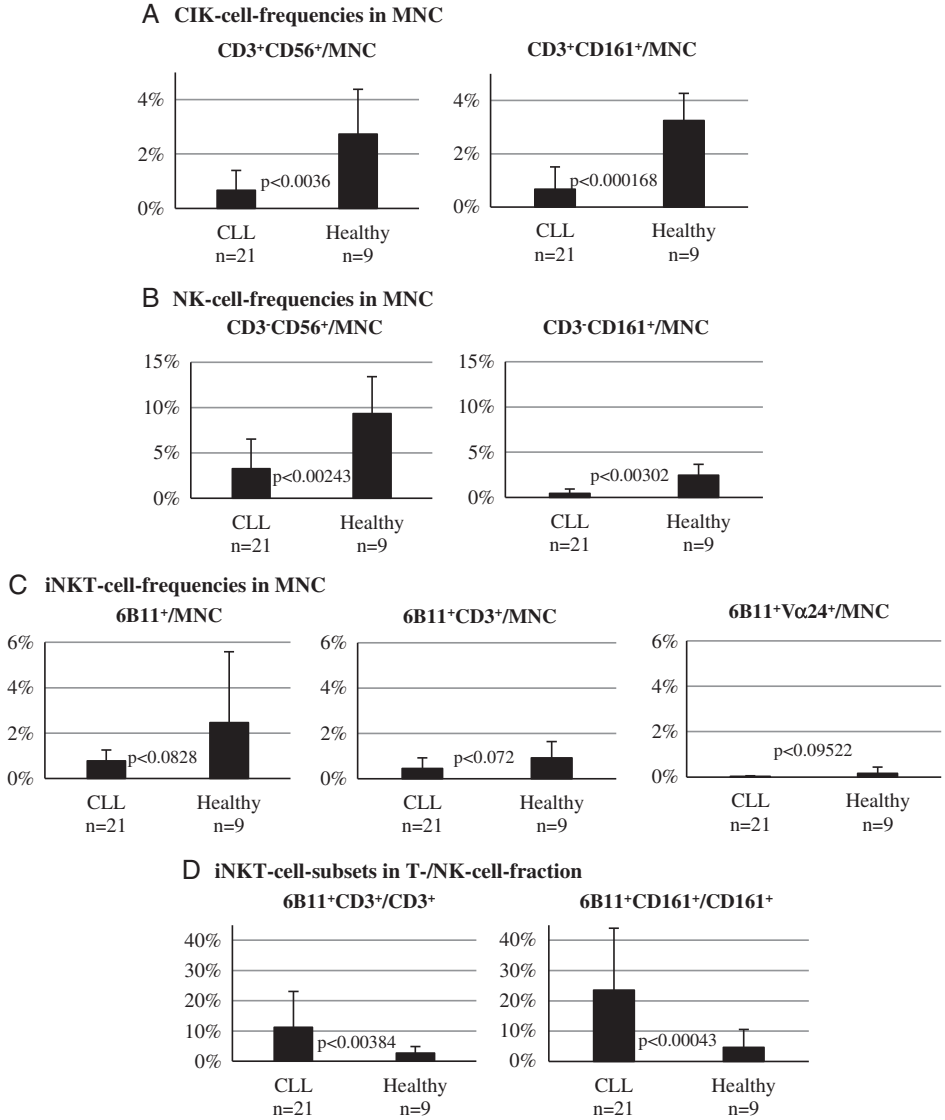

FIGURE 4. Frequencies of CIK, NK, and iNKT cells and their subsets in healthy versus CLL MNC are given. Percentages of CIK cells ($CD3^+CD56^+$/MNC and $CD3^+CD161^+$/MNC) (A), NK cells ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) (B), iNKT cells in MNC ($6B11^+$, $6B11^+CD3^+$/MNC, $6B11^+V\alpha24^+$/MNC) (C), and iNKT cell subsets in the T-cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) (D) are given. CIK indicates cytokine-induced killer; CLL, chronic lymphoid leukemia; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

of CLL patients with no need versus need for therapy (dns). No differences were found for NK cells.

CLL-patients younger versus older than 60 years presented with significantly* higher frequencies of $6B11^+$ and $6B11^+V\alpha24^+$ iNKT cells in the MNC fraction (0.87% ± 0.55% vs. 072% ± 0.40%; 0.04% ± 0.03% vs. 0.01% ± 0.01%; $P < 0.0711$, Fig. 5.2A, right side).

CLL-patients with stable versus no stable disease (NCR, relapse or death with disease) presented with (ns) higher percentages of $CD3^+CD56^+$ CIK cells and $CD3^-CD56^+$ NK cells in MNC fraction (0.70% ± 0.79% vs. 0.47% ± 0.23%; 3.40% ± 3.33% vs. 3.50% ± 2.64%, Fig. 5.2B, left side; middle row). Moreover, we could demonstrate significantly** higher frequencies of $6B11^+$ (0.82% ± 0.49%

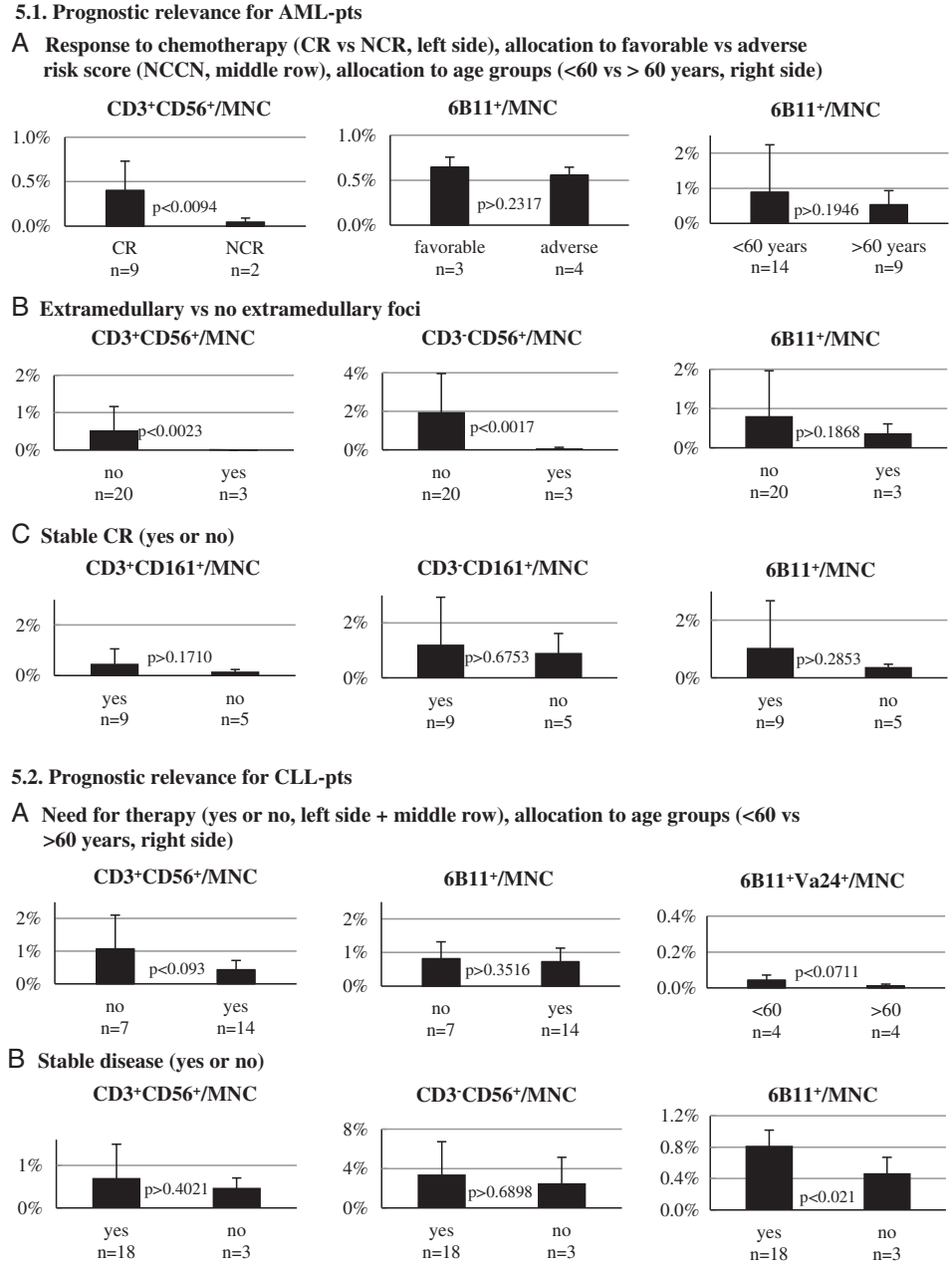

FIGURE 5. Relevance of CIK, NK, and iNKT cells and their subsets for prognosis of AML and CLL patients. 5.1. AML patients: Frequencies of CIK cells in patients with CR versus NCR after induction chemotherapy, iNKT cells in patients with favorable versus adverse risk score (NCCN) and younger than 60 versus older than 60 years (A), CIK, NK, and iNKT cells in patients with extramedullary versus no extramedullary foci (B), and CIK, NK, and iNKT cells in patients with stable versus no stable disease are given (C). 5.2. CLL patients: frequencies of CIK and iNKT cells in patients with no need versus need for therapy, iNKT cells in patients younger than 60 versus older than 60 years (A) and CIK, NK, and iNKT cells in patients with stable versus no stable disease are given (B). AML indicates acute myeloid leukemia; CIK, cytokine-induced killer; CLL, chronic lymphoid leukemia; CR, complete remission; iNKT, invariant natural killer T; MNC, mononuclear cells; NCCN, National Comprehensive Cancer Network; NCR, no complete remission; NK, natural killer; pts, patients.

vs. 0.47% ± 0.06%, $P < 0.021$, Fig. 5.2B) and significantly* higher frequencies of $6B11^+CD3^+$ iNKT cells in the MNC fraction of CLL patients with stable CR versus NCR (0.49% ± 0.48% vs. 0.17% ± 0.09%, $P < 0.0597$ right side).

In summary (significantly) higher proportions of $6B11^+$ iNKT cells correlated with no need for therapy, CLL patients younger than 60 years and stable CR. Comparable correlations were found for $6B11^+CD3^+$/$6B11^+V\alpha24^+$ iNKT subsets. $CD3^-CD56^+$ NK and $CD3^+CD56^+$ CIK cells regularly correlated with stable CR, while only $CD3^-CD56^+$ NK cells correlated with no need for therapy.

iNKT, NK, and CIK Cells and their Subtypes Increase Under the Influence of IL-2 and After Prestimulation With DC/DC_{leu}

It is well known, that DC/DC_{leu} are professional stimulators of T cells, thereby giving rise to antileukemic active effector cells after MLC (containing IL-2).

We generated DC/DC_{leu} from healthy or leukemic MNC or WB (DC_{leu} were only analyzed in cases where the amount of DC were $\geq 10\%$) and achieved the following results.

"MNC-Healthy"

With "Kit-D" we generated Ø 10.79% ± 2.07%, with "Pici 1" 15.42% ± 4.97% and with "Pici 2" 15.33% ± 6.09% DC in MNC.

"WB-Healthy"

With "Kit-D" we generated Ø 7.44% ± 1.28%, with "Kit-I" 11.05% ± 6.82%, with "Kit-K" 8.62% ± 2.00%, with "Kit-M" 8.08% ± 3.38%, with "Pici 1" 10.50% ± 2.62% and with "Pici 2" 9.48% ± 1.85% DC in WB.

"MNC-AML"

With "Kit-D" we generated Ø 36.51% ± 7.33% DC and 6.70% ± 0.58% DC_{leu}, with "Pici 1" 23.53% ± 0.00% DC and 12.63% ± 0.00% DC_{leu} and with "Pici 2" 19.39% ± 7.39% DC and 10.20% ± 0.00% DC_{leu} in MNC.

"WB-AML"

With "Kit-D" we generated Ø 6.85% ± 1.10% DC, with "Kit-I" 13.18% ± 7.43% DC and 19.95% ± 0.00% DC_{leu}, with "Kit-K" 10.48% ± 3.77% DC and 8.11% ± 2.38% DC_{leu}, with "Kit-M" 9.92% ± 3.93% DC and 13.62% ± 0.00% DC_{leu}, with "Pici 1" 10.16% ± 0.00% DC and 8.98% ± 0.00% DC_{leu} and with "Pici 2" 12.88% ± 0.00% DC and 11.39% ± 0.00% DC_{leu} in WB.

Pooling all results from the controls without added response modifiers ("MNC-healthy," "WB-healthy," "MNC-AML," "WB-AML") we found Ø 6.28% ± 2.09% DC.

Here we studied, whether iNKT/NK/CIK cells (in MNC or WB samples) could contribute to improve the overall antileukemic activity after stimulation with DC/DC_{leu}. Therefore, we stimulated in MLC (prepared with MNC or WB) T-cell–enriched immune reactive cells with a stimulator cell suspension containing DC/DC_{leu} (MLC*"MNC-DC" or MLC*"WB-DC") which were generated with different "cocktails." The same setting, but with a stimulator cell suspension without pretreatment with "cocktails" (MLC*"MNC" or MLC*"WB") served as a control. First, we quantified these cells before and after MLC with added T cells and IL-2. In a second step, we analyzed the effect of different "cocktail"-generated DC/DC_{leu} on the composition of immune reactive cells after stimulation. Further, we correlated our findings with antileukemic reactivity in a context with iNKT, NK, CIK cells, and T-cell subsets.

In MLC of Healthy and AML-Patients (MLC*"WB-DC" or only MLC*"WB") Proportions of iNKT/NK and CIK Cells Increase in the Presence of IL-2

At start of MLC we found lower total frequencies of iNKT cells and a shift to higher proportions of T/NK cells coexpressing 6B11 in AML patients (n = 6) versus healthy WB samples (n = 5; Fig. 6A). In a next step we quantified these cells after MLC and found that iNKT cells significantly(*)* increased in both AML and healthy WB-samples after MLC: at start of MLC versus after MLC*"WB"(AML $6B11^+$/MLC: 4.18% ± 1.32% vs. 8.63% ± 4.40%, $P < 0.0745$*; healthy $6B11^+$/MLC: 4.68% ± 3.92% vs. 12.34% ± 8.10%, $P < 0.0305$**; healthy $6B11^+CD3^+$/MLC: 4.60% ± 3.90% vs. 12.11% ± 8.10%, $P < 0.0385$**; healthy $6B11^+CD3^+$/$CD3^+$: 5.17% ± 4.63% vs. 31.77% ± 22.85%, $P < 0.0732$*; Figs. 6A, B) and start of MLC versus after MLC*"WB-DC" (healthy $6B11^+$/MLC: 4.68% ± 3.92% vs. 13.67% ± 5.01%, $P < 0.0323$**; healthy $6B11^+CD3^+$/MLC: 4.60% ± 3.90% vs. 13.03% ± 4.91%, $P < 0.0393$**; $6B11^+CD3^+$/$CD3^+$: 5.17% ± 4.63% vs. 21.51% ± 7.38%, $P < 0.0093$**, Fig.s 6A, C).

Pooling all results after MLC*"WB-DC" compared with MLC*"WB", an astonishing finding was, that cellular compositions in healthy samples were comparable in both settings (Figs. 6B, C). This could be due to the general influence of IL-2 in MLC. However, in AML-cases the proportions of iNKT cells increased after MLC*"WB-DC" compared with MLC*"WB" (Figs. 6B, C), although differences were not significant. Comparable results were found with MNC as cell sources (dns).

At start of MLC we found lower total frequencies of CIK and NK cells in AML (n = 6) versus healthy WB-samples (n = 5) (Fig. 7A). In a next step we quantified these cells after MLC and found that CIK and NK cells (significantly) increased in both AML and healthy WB-samples after MLC: at start of MLC versus after MLC*"WB"(AML $CD3^+CD161^+$/MLC: 1.41% ± 0.74% vs. 3.28% ± 1.59%, $P < 0.0487$**; healthy $CD3^+CD161^+$/MLC: 2.35% ± 2.59% vs. 3.30% ± 2.04%; AML $CD3^-CD161^+$/MLC: 0.15% ± 0.15% vs. 1.62% ± 1.81%; healthy $CD3^-CD161^+$/MLC: 1.41% ± 2.48% vs. 0.80% ± 0.86%; Figs. 7A, B) and start of MLC versus after MLC*"WB-DC" (AML $CD3^+CD161^+$/MLC: 1.41% ± 0.74% vs. 3.15% ± 2.13%; healthy $CD3^+CD161^+$/MLC: 2.35% ± 2.59% vs. 4.20% ± 2.02%; AML $CD3^-CD161^+$/MLC: 0.15% ± 0.15% vs. 1.73% ± 1.98%; healthy $CD3^-CD161^+$/MLC: AML 1.41% ± 2.48% vs. 0.57% ± 0.37%; Figs. 7A, C).

Pooling all results after MLC*"WB-DC" compared with MLC*"WB", an astonishing finding was, that cellular compositions in healthy samples were comparable in both settings (Figs. 7B, C). This could be due to the general influence of IL-2 in MLC.

Moreover, we determined values of IL-10, IL-17A, IFN-γ and MCP-1 after MLC*"WB-DC", MLC*"WB", WB-DC culture and WB-DC control in the supernatant of samples from AML patients (n = 4) using ELISA. An increased cytokine release was shown for IL-17A, MCP-1

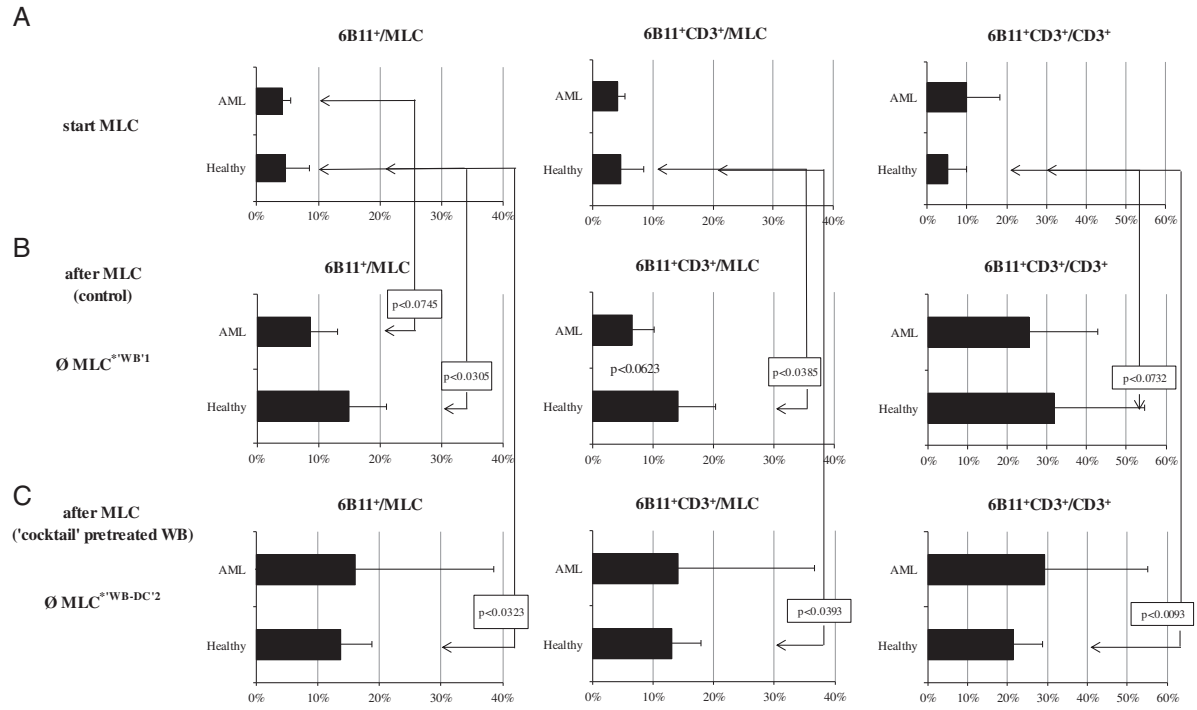

FIGURE 6. Frequencies of iNKT cells before (A) or after (B, C) MLC (WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB (MLC*"WB"1 (control)) (B) or with a stimulator cell suspension pretreated with "cocktails" (MLC*"WB-DC"2) (C). 1MLC*"WB" T-cell enriched immune-reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB with "cocktails". 2MLC*"WB-DC" T-cell-enriched immune-reactive cells were stimulated with a stimulator cell suspension pretreated with "cocktails," "cocktails" = all DC-generating methods/Kits. DC indicates dendritic cells; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; WB, whole blood.

and IL-10 in WB-DC supernatants compared with WB-DC control—what correlated with higher DC counts compared with control. An increased release of the chemoattractant MCP-1 was found after MLC*"WB-DC" with all DC-generating methods/Kits compared with MLC*"WB". An increased antitumor and anti-inflammatory cytokine profile (IL-17A and IL-10) was found after MLC*"WB-DC" especially with Kit-M pretreated WB (dns) compared with MLC*"WB".

In summary iNKT as well as CIK and NK cells increased after MLC independent of the stimulator cell suspension, what might be explained by a general iNKT, NK, and CIK cells inducing effect by IL-2. AML samples treated with "cocktails" and used as stimulator cells seemed to increase iNKT but not CIK and NK cell counts compared with not pretreated controls, pointing to an "iNKT-inducing influence" of these different "cocktails."

Highest iNKT Frequencies After MLC Could be Found in Cases With DC Generation Conducted With Prostaglandin-containing "Cocktails"

Studying the influence of different DC/DC_{leu}, generated with "cocktails," on the frequencies of 6B11 $^+$ iNKT cells after MLC showed, that in MNC/WB samples iNKT cell proportions increased in general during the MLC. For each individual patient we defined the best stimulator cell source resulting in the "highest" (+ + +) 6B11 $^+$ iNKT cell frequencies after MLC (Ø6B11 $^+$ /WB: 24.91% ± 19.61%, Ø6B11 $^+$ /MNC: 10.98% ± 7.05%); in "high" (+ +) 6B11 $^+$ iNKT cell frequencies (only defined for WB, Ø6B11 $^+$ /WB: 13.56% ± 6.59); with a "good" (+) frequency of 6B11 $^+$ iNKT cells (Ø6B11 $^+$ /WB: 8.06% ± 2.77%; Ø6B11 $^+$ /MNC: 5.88% ± 4.42%) and with "no increase" (–) or even in a decrease of iNKT cells (Ø6B11 $^+$ /WB: 3.94% ± 2.45%, Ø6B11 $^+$ /MNC: 0.93% ± 0.21%). Frequencies were applied for healthy and AML samples.

Similar distributions were found for 6B11 $^+$ CD161 $^+$ and 6B11 $^+$ CD3 $^+$ iNKT cells. We could show that especially in cases in that the DC generation was performed with prostaglandin-containing (PGE_1, PGE_2) "cocktails" the percentages of iNKT cells increased the most (Kit-D, Kit-K, Kit-M, "Pici1," "Pici2," Fig. 8.1) Moreover, we found that in almost every given patient (except P1433) we could select at least one of several "cocktails," that increased iNKT proportions— pointing to an effect of a certain pretreatment with "cocktails" on the frequencies of iNKT cells in healthy as well as of AML samples. Effects seen in MNC and WB samples from healthy and AML samples were comparable (Figs. 8.1A, B). Comparable effects were found for CIK cells (dns).

Moreover, we correlated percentages of 6B11 $^+$ / 6B11 $^+$ CD3 $^+$ /6B11 $^+$ CD161 $^+$ iNKT cells (defined as "highest," "high," "good," and "no increase") after MLC in MNC (Fig. 7.1A, healthy "MNC") for each individual sample with the frequencies of DC after culture of healthy MNC (n = 8) with different "cocktails." We found that samples with the "highest" (Ø6B11 + /MNC: 10.98% ± 7.05%) frequencies of iNKT cells showed (although not significant) higher percentages of DC after the DC-generating process compared with MLC with "no increase" (Ø6B11 $^+$ /MNC: 0.93% ± 0.21%) or even a

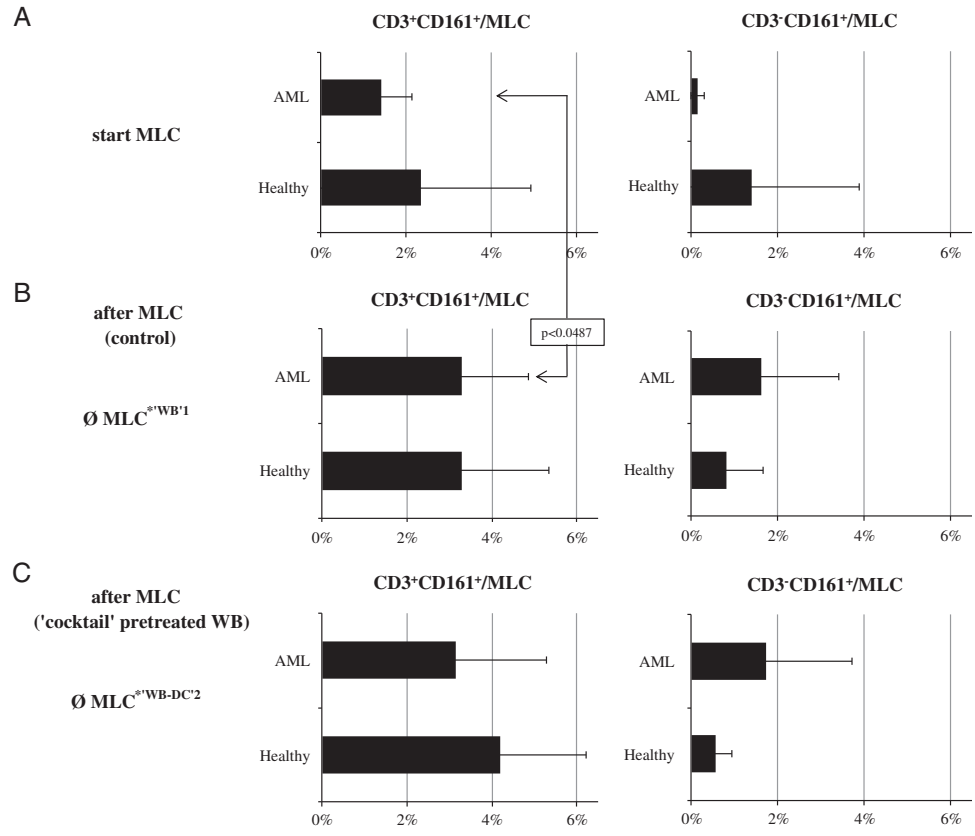

FIGURE 7. Frequencies of CIK/NK cells before (A) or after (B, C) MLC (WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB (MLC*"WB"1 (control)) (B) or with a stimulator cell suspension pretreated with "cocktails" (MLC*"WB-DC"2) (C). 1MLC*"WB" T-cell enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB with "cocktails." 2MLC* "WB-DC" T-cell enriched immune reactive cells were stimulated with a stimulator cell suspension pretreated with "cocktails." "Cocktails" = all DC-generating methods/Kits. CIK indicates cytokine-induced killer; DC, dendritic cells; MLC, mixed lymphocyte cultures; WB, whole blood.

decrease of iNKT cells (**Ø** DC-counts: 12.87% ± 5.44% vs. 9.11% ± 8.81%; Fig. 8.2). Those correlations were not found for MNC of the 2 AML-patients we have analyzed (dns). In a next step, we correlated these values with DC/DC-subtype values obtained from 5 healthy and 6 AML-WB samples and did not find correlations between DC/DC subtype counts and iNKT cells after MLC (dns).

In summary, differences in iNKT cell compositions after MLC could be found in individual samples (healthy or AML) after pretreatment with different "cocktails." Highest percentages were found with prostaglandin-containing methods. Together, high frequencies of DC correlated with "highest" frequencies of iNKT cells after MLC in MNC of healthy controls, while lower percentages of DC correlated with "no increase" or even a decrease of iNKT cells after MLC in MNC of healthy controls—however, these correlations were not found for the 2 AML patients analyzed.

Physiological Hypoxia Does Not Influence Frequencies and Compositions of iNKT, NK, and CIK Cells Compared With Normoxic Conditions

Under physiological conditions the O_2-concentration in PB is lower than the normoxic 21%. With some preliminary experiments, we wanted to work out results under physiologically most adapted conditions in order to draw first conclusions for the functional relevance on immune-reactive cells: physiological low O_2 concentrations could possibly influence the conversion of blasts to DC_{leu} and in consequence the composition and reactivity of immune-reactive cells. Here we studied whether hypoxic (compared with normoxic) conditions influence the composition of iNKT/NK/CIK cells after MLC (in WB samples). Results included in this chapter were cultured in 6% or 10% O_2 or with varying O_2-concentrations between 0% and 17%. For our evaluations, all results were pooled. In a first step, we quantified DC-(subtype) proportions in healthy WB samples (n = 4) with or without stimulation with 3 "cocktails" (Kit-I, Kit-K or Kit-M). We could show that DC proportions [overall and mature DC (DC_{mig})] were not significantly different in cases cultured under normoxic versus hypoxic conditions (dns).

In a next step, we quantified iNKT/NK/CIK cell proportions after MLC*"WB-DC" and MLC*"WB" under hypoxic versus normoxic conditions. Pooling all results after MLC*"WB-DC" we found comparable proportions of iNKT/NK/CIK cells after MLC under normoxic versus hypoxic conditions (dns).

In summary low O_2 culture concentrations do not seem to have neither a significant effect on the generation of DC nor on the amount and composition of iNKT, NK, and CIK cells after MLC.

8.1. Frequencies of iNKT-cells (subsets) after MLC

A 6B11+/6B11+CD3+/6B11+CD161+-iNKT-cells after MLC*'MNC-DC' and MLC*'MNC'				
Healthy 'MNC'	**Kit-D**	**'Pici1'**	**'Pici2'**	**control**
P1418	+	+++	-	+
P1421	+	+	+++	+
P1422	+	+++	+	+
P1425	+++			+
P1428	+		+++	+
P1429		+++	-	-
P1436		+++	+	+++
P1438	+++	+	+	+
AML 'MNC'	**Kit-D**	**'Pici1'**	**'Pici2'**	**control**
P1424	-		-	+++
P1426	+++	+	-	+

B 6B11+/6B11+CD3+/6B11+CD161+-iNKT-cells after MLC*'WB-DC' and MLC*'WB'							
Healthy 'WB'	**Kit-D**	**Kit-I**	**Kit-K**	**Kit-M**	**'Pici1'**	**'Pici2'**	**control**
P1420	+++	+	+	++	+	+	++
P1428	+	+	+++	++	+	++	++
P1429	++	+	+	+	++	+	+++
P1436				+	+++	+	+
P1438	-	-	-	+++	++	-	+
AML 'WB'	**Kit-D**	**Kit-I**	**Kit-K**	**Kit-M**	**'Pici1'**	**'Pici2'**	**control**
P1424	+++	-	-	-	-	-	-
P1426	++		+	+	+	+	+++
P1430	+	+	+++				++
P1433		-					-
P1434		+++	+++	++			+
P1439				+++			++

8.2. iNKT-cells after MLC correlate with DC-values from healthy MNC

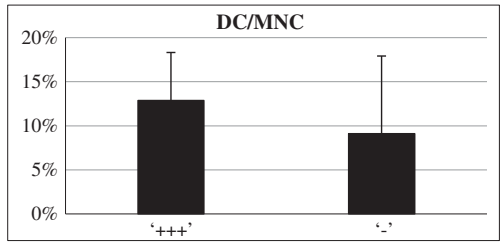

FIGURE 8. Frequencies of iNKT cells (6B11+ or 6B11+CD3+ or 6B11+CD161+ iNKT cells) after MLC (MNC and WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of MNC/WB [MLC*"MNC/WB"1 (control)] (B) or with a stimulator cell suspension pretreated with "cocktails" (MLC*"MNC/WB-DC"2) (C). AML indicates acute myeloid leukemia; DC, dendritic cells; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; MNC, mononuclear cells; WB, whole blood. MNC/WB: "+++" "highest" counts of iNKT cells; "++" "high" counts of iNKT cells, "+" "good" counts of iNKT cells, "-" "no" increase or decrease of iNKT cells.

iNKT, NK, CIK Cells Contribute to Antileukemic Activity

As shown above iNKT cell proportions increase after MLC*"WB-DC" or MLC*"MNC-DC". Here we studied, whether iNKT/NK/CIK cells could contribute to improve the overall-antileukemic activity after MLC. In a first step, we quantified these cells and their subtypes in individual AML-WB samples (precultured with various "cocktails") after MLC (with added T cells and IL-2). In the next step, we correlated frequencies of iNKT, NK, CIK cells with the antileukemic activity [blast-lysis evaluated with a cytotoxicity (fluorolysis) assay] of the individual WB samples from AML patients.

We found that cases with versus without an antileukemic activity ("lysis" vs. "nonlysis") were characterized by significantly*** increased frequencies of 6B11+ iNKT cells in the total MLC (7.25% ± 4.21% vs. 2.60% ± 0.96%, $P < 0.0022$) and significantly** higher frequencies of 6B11+CD161+/6B11+CD3+ iNKT cells in MLC (3.67% ± 3.55% vs. 1.06% ± 0.29%, $P < 0.0083$; 5.10% ± 2.57% vs. 2.12% ± 1.04%, $P < 0.0232$, Fig. 9A). However, the subtype-compositions of iNKT and CIK cell subsets were comparable in the 2 groups compared (6B11+CD161+/CD161+: 52.63% ± 24.14% vs. 51.34% ± 9.10%; 6B11+CD3+/CD3+: 18.45% ± 15.34% vs. 17.25% ± 9.79%; CD3+CD161+/CD3+: 15.08% ± 14.03% vs. 14.88% ± 5.34%,

Fig. 9B). Moreover, significantly* higher proportions of $CD3^+$ $CD161^+$ CIK cells in MLC (3.26% ± 2.35% vs. 1.85% ± 0.48%, $P < 0.0509$) and significantly*** increased frequencies of $CD3^-CD161^+$ NK cells in MLC were found in cases with versus without an antileukemic activity (2.07% ± 2.13% vs. 0.21% ± 0.13%, $P < 0.0023$, Fig. 9C). However, significantly* higher proportions of proliferating T cells (T_{prol}: $CD3^+$ $CD69^+$/MLC) and significantly** higher frequencies of central memory T cells (T_{cm}: $CD3^+CD45RO^+CCR7^+$/MLC) were found in cases with versus without an antileukemic activity (T_{prol}: 17.56% ± 7.86 vs. 15.29% ± 2.61%, $P < 0.0825$, T_{cm}: 9.35% ± 10.47% vs. 2.32% ± 1.5%, $P < 0.01451$, Fig. 9D). No significant differences were found in the composition of naive/non-naive T cells in the groups compared (dns).

Moreover, we assorted our samples according to their antileukemic activity ("lysis" vs. "nonlysis") and evaluated predictive cutoff values for NK/CIK/iNKT cells after MLC*"WB-DC" and MLC*"WB". A total of 100% of samples with >4% $6B11^+$ iNKT cells, with > 4% $6B11^+CD3^+$ iNKT cells and with >1.5% $6B11^+CD161^+$ iNKT cells after MLC*"WB-DC" and MLC*"WB" showed antileukemic activity (lysis). Furthermore, 100% of samples with >2.3% $CD3^+CD161^+$ CIK cells and with >1.9% $CD3^+CD56^+$ CIK cells after MLC*"WB-DC" and MLC*"WB" showed antileukemic activity. Moreover, 89%–100% of samples with >0.4% $CD3^-CD161^+$ NK cells and with >1.3% $CD3^-CD56^+$ NK cells after MLC*"WB-DC" and MLC*"WB" showed antileukemic activity.

Further, we analyzed whether the addition of "cocktails" to cultures improves the antileukemic activity after MLC compared with controls. Samples with more lysis compared with controls, were defined as improved blast-lysis, samples with less lysis compared with controls as "not improved" blast-lysis. Samples with improved blast-lysis showed significantly* higher $6B11^+$ iNKT cell frequencies compared with samples with not improved

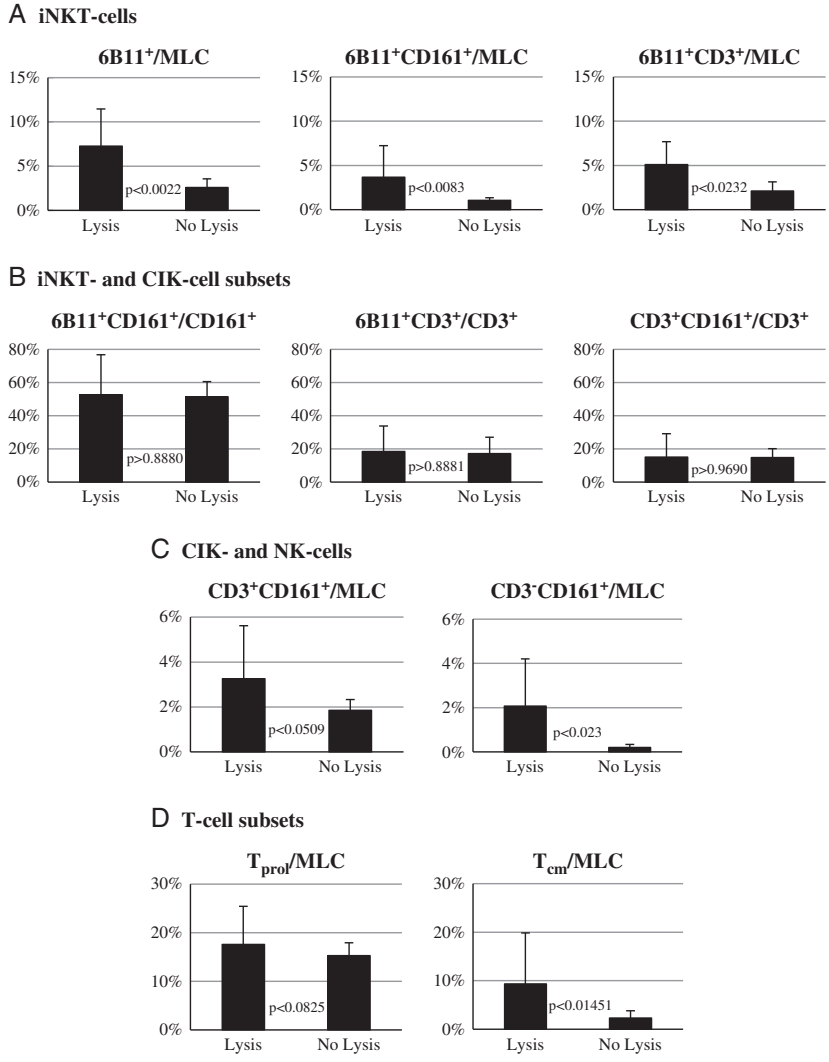

FIGURE 9. Frequencies of iNKT cells (A), CIK cells (B), NK cells (C), and T cell subsets (D) after MLC (MNC/WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of MNC/WB [MLC*"MNC/WB"1 (control)] or with a stimulator cell suspension pretreated with "cocktails" (MLC*"MNC/WB-DC"2) were compared in cases with/without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay. CIK indicates cytokine-induced killer; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; NK, natural killer; Tprol, proliferating T cells ($CD69^+CD3^+$), Tcm, central-memory T cells ($CD45RO^+CCR7^+$).

blast-lysis (7.94% ± 4.12% vs. 4.39% ± 3.12%, $P < 0.0921$). Moreover, samples with improved blast-lysis showed (ns) higher frequencies of $6B11^+CD161^+$ and $6B11^+CD3^+$ iNKT cells compared with samples with not improved blast-lysis (4.82% ± 3.49% vs. 1.80% ± 2.90%; 5.25% ± 2.13% vs. 3.50% ± 1.84%). Furthermore, samples with "improved" blast-lysis showed (ns) higher frequencies of $CD3^+CD161^+$ CIK cells and $CD3^-CD161^+$ NK cells compared with samples with not improved blast-lysis (3.99% ± 2.51% vs. 2.10% ± 1.83%; 2.73% ± 1.95% vs. 0.95% ± 1.87%).

Furthermore, we correlated findings of ELISA of supernatants of MLC*"WB-DC" and MLC*"WB" of samples from AML patients (n = 4) with antileukemic activity ("lysis" vs. "no lysis"). We could show that cases with achieved "lysis" compared with "no lysis" after MLC*"WB-DC"/MLC*"WB" were characterized by higher release of antitumor and anti-inflammatory cytokines (IL-17A and IL-10), what correlated with higher iNKT, NK, and CIK cell frequencies.

With one exemplary experiment, we could show that a radiation of stimulator-cells did not reduce proportions of T/iNKT/NK and CIK cells; however, reduced the overall blast-lysis compared with unirradiated settings. This could point to a role of iNKT/NK and CIK cells beside T cells in antileukemic reactions (dns).

In summary in cases with antileukemic blast-lytic activity after MLC not only a T cell stimulation was induced, followed by a creation of memory T cells, but also that iNKT, NK, CIK cell proportions are significantly increased—pointing to an involvement of these cells in antileukemic reactions. Moreover, the presence of increased proportions (above cutoff values) of iNKT as well as of CIK and NK cells after MLC might correlate with successful blast-lysis.

DISCUSSION

Role of T, iNKT, NK, and CIK Cells in Tumor-immune Surveillance

T, iNKT, NK, and CIK cells and their subsets are important mediators of immune responses: T cells are known to be activated by tumor or leukemia antigen-presenting DC and—as shown exemplarily by our DC/DC_{leu} ex vivo strategies—their antitumor functionality can be improved by DC_{leu}.[9] $T_{eff\text{-}em}$ enable—in case of a secondary challenge—a very quick and immediate secondary immune response, while T_{reg} are able to downregulate immune responses—important to inhibit autoaggressive immune reactions—but also in a context to downregulate antitumor responses.[9,10] In contrast to T cells, iNKT, NK, and CIK cells react quickly to an immunologic threat by secreting cytokines and chemokines[4,30] and kill tumor cells without prior activation and, unlike T and iNKT cells, NK and CIK cells do not recognize target cells by a TCR.[5] iNKT cells can produce a variety of chemokines and cytokines like IFN-γ, IL-4/IL-2/IL-12, tumor necrosis factor (TNF)-α, transforming growth factor-β, and GM-CSF that in consequence activate different cells like DC, NK cells, $CD4^+/CD8^+$ T cells, but also T_{reg}.[31,32] A previous study could show that all subsets of iNKT cells produce almost the same amounts of IFN-γ and TNF-α, while IL-4 is mainly produced by PB-$CD4^+$ iNKT cells.[13] Moreover, iNKT cells show a strong cytolytic potential and can directly kill tumor cells as they express granzyme-B, Perforin, and FasL.[4,31] Therefore, it might be promising to use or even to increase the antileukemic potential of NK, CIK cells and especially iNKT cells to enhance the patients' anti-tumor immune response.

Methods to Detect iNKT, NK, and CIK Cells

The identification and quantification of iNKT cells (in healthy blood samples) is known to be difficult due to their low frequency as well as missing marker (combinations). Previous methods using CD1d tetramers or the combination of Vα24-/Vβ11-antibodies did not yield the necessary specific results.[13] Therefore, the first aim of our study was to develop a highly specific iNKT cell detection panel for the quantification of iNKT cells/subsets in healthy, but also to be used for leukemia patients. Furthermore, we compared strategies and markers to detect and quantify NK and CIK cells and their subsets in healthy, but especially in leukemic patients. A previous study performed with healthy samples could show that a new moAb (clone 6B11-PE) can recognize the invariant CDR3 loop of the Vα24Jα18-TCR rearrangement on the cell surface and can be used to specifically characterize iNKT cells in combination with anti-CD3.[13] In our current study, we also used 6B11-PE, but also 6B11-FITC (purchased from different companies) to characterize and define iNKT cells. In previous studies,[11,13] only 6B11-PE was used for analysis, but not 6B11-FITC. We found (significantly) higher proportions of iNKT cells detected with 6B11-FITC compared with 6B11-PE in AML, ALL, CLL patients. The proportions of iNKT cells detected with 6B11-FITC compared with 6B11-PE were (significantly) lower in healthy samples. Moreover, 6B11-FITC-stainings seemed to yield varying results in several stainings, whereas 6B11-PE-stainings showed stable and precise results for iNKT detection. It is well known, that moAbs can react and bind differently to their targets—depending not only on the selected clones, but also on the purchasing companies, the fluorochromes used or the combination with partner moAbs in a panel.[33] In order to exclude these variations, we focused mainly on 6B11-PE results in our setting.

Coexpression analyses of 6B11 with myeloid (eg, CD33, CD34, CD117) or lymphoid (eg, CD1a, CD5, CD7, CD10, CD19, CD20) markers on blasts from malignant myeloid and lymphoid cell lines showed, that 6B11 was not expressed on myeloid or B-lymphoid blasts. However, we found a low expression of 6B11 on T-linear cells of the Jurkat cell line. As we do not know, whether this cell line has a Vα24-/Vβ11-rearrangement, typical for iNKT cells we cannot decide whether this expression is "aberrant" on this cell line or specific. In general, our results show, that the 6B11-PE-marker, but not 6B11-FITC, can be regarded as a very specific marker to detect iNKT cells without cross-reactivity or aberrant expression on leukemic cells. Previous studies did not analyze whether 6B11 is aberrantly expressed on leukemic blasts and if 6B11-FITC is specific for iNKT cells analyses. Thereby, we can confirm preliminary findings of other groups, that the detection of iNKT cells with 6B11 alone or in combination with subtype markers is very precise, as 6B11 specifically recognizes the CDR3 loop of the invariant TCR of CD1d-restricted iNKT cells.[13]

It is known, that the iNKT cell population consists of different subsets with diverse phenotypic and functional characteristics that can be subdivided according to their expression of surface molecules, receptors, effector functionality or tissue localization.[4] In a former study it was shown that 6B11 could be used in combination with further

T (CD27, CD28, CD45RA, CD45RO) and NK cell markers (CD16, CD56, CD161) to analyze iNKT subsets.[13] Moreover, iNKT cells were characterized by the combination of CD1d and CD3 or the combination of Vα24 and Vβ11. The percentage of iNKT cells expressing CD4 or CD8 was evaluated in a region comprising $6B11^+CD3^+$ iNKT cells. Like the previous study, we used 6B11 in combination with CD3, CD45R0, and CD161. Moreover we included the combinations of 6B11 with CD1d, Vα24, CD4 or with CD8. This means that we evaluated the expression of T cell (CD1d, CD3, CD45RO, Vα24) and NK cell markers (CD161) on $6B11^+$ iNKT cells and divided them into "T cell-like" and "NK cell-like" iNKT subsets. We are the first group, that defined "T cell-like" iNKT subsets as $CD3^+6B11^+$, $CD4^+6B11^+$, $CD8^+6B11^+$, $V\alpha24^+6B11^+$, $CD1d^+6B11^+$, $CD45RO^+6B11^+$, and $CD45RO^-6B11^+$ iNKT cells.

Classically, iNKT cells have been identified with CD1d-tetramers loaded with α-galactosylceramide, but this method could lead to an overestimation of iNKT cells as some T cell subsets also express CD1d.[13] This study did not find significant differences in $6B11^+$ iNKT cells compared with $CD3^+6B11^+$ iNKT cells or $CD3^+CD1d^+$ iNKT cells in healthy samples. Although we did not combine CD1d with CD3, but with 6B11, we could also show that frequencies of $6B11^+CD3^+$ and $6B11^+CD1d^+$ iNKT cells were comparable in healthy samples. We could show in addition that frequencies of $6B11^+CD3^+$ and $6B11^+CD1d^+$ iNKT cells were similar in AML, ALL, and CLL samples. Unlike the previous study,[13] we detected higher numbers of $6B11^+$ iNKT cells compared with $6B11^+CD3^+$ and $6B11^+CD1d^+$ iNKT cells in healthy samples as well as in AML, ALL, and CLL samples.

Using CD3-/Vα24-antibodies, we could demonstrate specific results and detected lower frequencies of $V\alpha24^+6B11^+$ iNKT cells compared with $CD3^+6B11^+$ iNKT subsets in healthy as well as in AML, ALL, and CLL patients. These findings are consistent with a previous study, which found 3 times more $CD3^+6B11^+$ iNKT cells compared with $CD3^+V\alpha24^+V\beta11^+$ iNKT cells in healthy adult and children's samples.[11] However, this group combined Vα24 with CD3 and Vβ11, while we combined Vα24 with 6B11. Unlike these results, another group found similar proportions of $CD3^+6B11^+$ iNKT cells and $V\alpha24^+V\beta11^+$ iNKT cells in healthy controls.[13] However, this group used the combination of Vα24 and Vβ11 without combining it with CD3. These differences may be explained by the fact, that different combinations of Vα24 with CD3, Vβ11 or 6B11 were used for iNKT cell detection. Furthermore, the results or our group and Bienemann may be explained by the fact that the invariant α-chain of the TCR sometimes binds with other β-chains than Vβ11, which could lead to an underestimation of iNKT cells.[11]

Both type I iNKT cells and type II iNKT cells, are divided into $CD4^+$, $CD4^-CD8^-$, $CD8\alpha\alpha^+$, and $CD8\alpha\beta^+$ iNKT cells, with higher proportions being $CD4^+$, lower proportions being $CD4^-CD8^-$ and only a few cells $CD8\alpha\alpha^+$ or $CD8\alpha\beta^+$, which could only be found in humans but not in mice.[4] Unlike previous studies, we combined 6B11 with CD4 and CD8 for subtype analyses. We can confirm that the majority of $6B11^+$ iNKT cells coexpresses $CD4^+$ and the minority $CD8^+$ in healthy samples. We can add as a new finding in addition, that higher proportions of $6B11^+CD4^+$ iNKT cells and lower proportions of $6B11^+CD8^+$ iNKT cells were also found in AML, ALL and CLL patients. A previous study suggested that $CD4^+$ iNKT cells produce both Th1 and Th2 cytokines, whereas $CD4^-$ iNKT cells tend to produce mainly Th1 cytokines.[31] However, another study found that $CD4^+$, $CD4^-$, and $CD8^+$ iNKT cells produced similar amounts of IFN-γ and TNF-α, but $CD4^+$ iNKT cells produced significantly more IL-4.[13] In our patients' cohort, we had not further analyzed the functional role of CD4 and CD8 expressions on iNKT cells. However, we suggest to combine 6B11 with CD4 and CD8 to analyze the diffences between $6B11^+CD4^+$, $6B11^+CD4^-$, $6B11^+CD8^+$, and $6B11^+CD8^-$ iNKT cells.

Consistent to a previous study we found that the majority of iNKT cells is $CD45RO^+6B11^+$, while the minority is $CD45RO^-6B11^+$, what could be interpreted as iNKT cells with a memory-effector phenotype.[13]

We defined "NK-like" iNKT cells as $CD161^+6B11^+$ iNKT cells and found lower proportions of $CD161^+6B11^+$ iNKT cells compared with $6B11^+$ iNKT cells and $6B11^+CD3^+$ iNKT cells ("T-like" iNKT cells). A previous study showed different NK cell markers expressed by iNKT cells and demonstrated that the majority of iNKT cells expressed CD161, while only a minority expressed CD56 and CD16. Furthermore, this study showed that CD161 was expressed on significantly higher frequencies of $CD8^+$ or double negative iNKT cells compared with $CD4^+$ iNKT cells. CD161 (KLRB1) is a C-type lectin receptor expressed on human NK cells, but also on iNKT and T cell subsets—especially on subgroups found in liver and gut.[13–15,18] In humans, the CD161 receptor interacts with its ligand LLT1 and suppresses the cytotoxic potential of NK cells. Moreover, it was shown that an infection with cytomegalovirus leads to decreased expression of CD161 on NK cells.[34]

In conclusion, we suggest that the most precise detection of iNKT cells should be based on 6B11-PE alone or in combination with CD3 and CD161-moAbs. Moreover, we found new detection methods for iNKT cells/subsets using 6B11 in combination with CD1d, Va24, CD4 or CD8. Furthermore, we could demonstrate that 6B11 can be used to analyze iNKT cells of leukemia patients, knowing that there is no aberrant expression of 6B11 on blasts. Our results confirm the heterogeneity of iNKT cells, as we could show that there are various "T cell-like" and "NK cell-like" iNKT subsets. This highlights that the iNKT cell population consists of several phenotypically and potentially functionally different subsets with diverse surface markers. A previous study could demonstrate that neonatal $CD4^+$, adult $CD4^+$, and adult $CD4^-$ iNKT cells are phenotypically diverse and show different effector/memory T-cell markers and NK cell markers. Neonatal $CD4^+$ iNKT cells show a more naive phenotype and can be modified more easily in their capacity to acquire Th1-like or Th2-like functions compared with adult iNKT cells, which are mainly resistant to functional reprogramming, but show higher cytotoxic functions.[35]

To detect NK and CIK cells, we used recommended combinations of CD3 with CD161 or CD56 moAbs. NK cells were defined as $CD3^-CD56^+$ or $CD3^-CD161^+$ cells and CIK cells were defined as $CD3^+CD56^+$ or $CD3^+CD161^+$ cells. Both combinations detect similar amounts of NK and CIK cells in healthy controls. However, CD56 is known to be aberrantly expressed on blasts of certain AML-subtypes and correlates with a worse prognosis.[36] In these cases, NK cells cannot be evaluated with a CD56-

marker. We recommend using the CD161-moAb to quantify NK cells in cases with aberrant expression of CD56. Therefore, CD161 can be regarded as a better NK and CIK cell marker for leukemic patients with aberrant CD56 expression.

In conclusion, we suggest that NK and CIK cells can always be detected with the combination of CD3 with CD161-moAbs and with CD3 and CD56-moAbs in cases without aberrant expression of CD56. The potential functional differences in these subsets, however, should be evaluated.

iNKT, NK, and CIK Cells in AML, ALL, and CLL Patients Compared With Healthy Samples

The present study shows that AML, ALL as well as CLL patients' MNC are characterized by (significantly) lower frequencies of CIK cells ($CD3^+CD56^+$ or $CD161^+$), NK cells ($CD3^-CD56^+$ or $CD161^+$) and iNKT cells ($6B11^+$, $6B11^+CD3^+$ or $CD8^+$ or $CD161^+$ or $V\alpha24^+$) compared with healthy donors. This could be confirmed by another group that demonstrated significantly lower median values of NK cells, NKT, and iNKT cells in AML patients compared with healthy donors' PB (cells/μL: 303.47 vs. 101.54; 55.86 vs. 21.85; 0.515 vs. 0.0814).[7] However, this group did not compare values of CIK cells in AML patients versus healthy and did not consider different NK and iNKT subsets: NK and NKT cells were detected with CD3 and CD16/56 and iNKT cells with CD3 in combination with Vα24 and Vβ11.[7] In general, these findings can be expected due to the displacement of immune-reactive cells by uncontrolled proliferating and expanding blasts.

In summary, we could show that a high blast-load in AML, ALL or CLL patients directly correlated with low frequencies of iNKT, NK, and CIK cells. Therefore, the majority of iNKT, NK, and CIK cells is displaced by blasts in AML, ALL, and CLL patients.

An interesting finding was, that—although proportions of iNKT, NK, and CIK cells were comparable in leukemic patients, their subtype composition was different in leukemic patients compared with healthy donors showing a shift to (significantly) higher frequencies of T and NK cells coexpressing 6B11 in AML, ALL, and CLL patients compared with healthy donors. This might suggest that certain T/NK-derived iNKT cells in leukemic patients might either be subtypes with downregulated antileukemic functionality or could be special subsets that could be triggered in antileukemic reactivity.

In summary, we present the new finding that proportions of iNKT, NK, and CIK cells were significantly lower in AML, ALL, and CLL patients and compared with healthy donors. Their subset compositions as well as iNKT subsets were comparable in AML, ALL, and CLL patients. We can demonstrate in addition to findings in the literature a shift to higher proportions of T and NK cells expressing 6B11 in AML, ALL, and CLL patients compared with healthy donors, what might point to comparable mechanisms in leukemic diseases, that favor the extension of subsets in these diseases and what might help to develop new iNKT, NK, and CIK cell–based immune therapeutic strategies for leukemia patients.

Prognostic Relevance of iNKT, NK, and CIK Cells in AML, ALL, and CLL Patients

A previous study could show, that the overall number of iNKT cells in AML patients is an important prognostic factor as iNKT cell proportions >0.2 cells/μL are associated with favorable survival.[7] Furthermore, this study could demonstrate, that a lower number of iNKT cells in PB in combination with higher proportions of $CD4^+$ and $CD8^+$ T cells in BM at diagnosis is characterized by a lower overall survival (OS) in AML patients. Another study could demonstrate that a high expression of NKp30/NKp46 on $CD16^+$ NK cells in elderly AML patients correlated with leukemia-free and better OS.[37] Moreover, previous studies pointed out that CIK cells (especially $CD3^+CD56^+$) show strong cytotoxic potential, for example against leukemia,[4,30] but did not correlate the frequencies of CIK cells with survival or favorable prognosis for leukemia patients. With our results we confirm these findings and can add in addition, that higher percentages of iNKT, NK, and CIK cells correlate with the allocation of patients to diverse prognostically more favorable subgroups not only in AML, but also in ALL and CLL.

We could show, that higher frequencies of iNKT cells in leukemic patients correlate with prognostically better subsets in AML, adult ALL, and CLL patients. AML patients with favorable (vs. adverse) NCCN risk score showed higher percentages of $6B11^+$, $6B11^+CD161^+$, and $6B11^+Va24^+$ iNKT cells. Moreover, AML and CLL patients younger (vs. older) than 60 years showed higher numbers of $6B11^+$, $6B11^+CD161^+$, $6B11^+CD3^+$, and $6B11^+Va24^+$ iNKT cells. AML patients with primary (vs. secondary) AML showed higher frequencies of $6B11^+$ and $6B11^+CD3^+$ iNKT cells. Furthermore, AML and adult ALL patients without (vs. with) extramedullary foci showed higher frequencies of $6B11^+$, $6B11^+CD3^+$, $6B11^+CD161^+$, and $6B11^+Va24^+$ iNKT cells. Moreover, adult ALL patients achieving CR after GMALL induction chemotherapy (vs. NCR) and CLL patients with no need (vs. need) for therapy showed higher frequencies of $6B11^+$, $6B11^+CD3^+$, and $6B11^+Va24^+$ iNKT cells. AML and CLL patients with stable CR (vs. no stable CR: relapse or death by disease) showed (significantly) higher percentages of $6B11^+$, $6B11^+CD3^+$, and $6B11^+CD161^+$ iNKT cells, however, 1 patient had died from pneumonia. This data can confirm previous findings[7,37,38] that low frequencies of iNKT cells can be correlated with worse prognosis and worse OS. In contrast to previous studies and our results, another study found that lower frequencies of NK and NK like T cells correlated with higher survival in AML patients, while higher frequencies were correlated with poor survival.[7,37,38]

Moreover, we could show, that higher frequencies of NK cells in leukemia patients correlate with prognostically better subsets in AML, adult ALL, and CLL patients. AML patients without (vs. with) extramedullary foci showed (significantly) higher frequencies of $CD3^-CD56^+$ NK cells. Moreover, adult ALL patients, who had achieved CR after GMALL induction chemotherapy, showed higher numbers of $CD3^+CD161^+$ NK cells. Furthermore, AML and CLL patients, who stayed in stable CR (vs. no stable CR) showed higher amounts of $CD3^-CD161^+$ and $CD3^+CD56^+$ NK cells. Consistent to previous studies,[7,37,38] our data show that higher percentages of NK cells

can be correlated with better prognosis and stabilization of disease. In contrast to a previous study, we could not correlate low frequencies of NK cells with better survival.[7,37,38]

We can add in addition, that higher frequencies of CIK cells in leukemia patients correlate with prognostically better subgroups in AML and CLL patients. AML patients who achieved CR (vs. NCR) and CLL patients with no need for initial therapy (vs. need for initial therapy) showed (significantly) more CIK cells. Moreover, AML as well as ALL patients without extramedullary foci presented with significantly more CIK cells. Furthermore, AML and CLL patients, who stayed in stable CR (vs. no stable CR), showed higher numbers of $CD3^+CD161^+$ and $CD3^+CD56^+$ CIK cells. Consistent to previous studies,[4,30] these data might point to an antileukemic reactivity of CIK cells—leading to stabilized disease.

We conclude that higher frequencies of iNKT, NK as well as CIK cells can be regarded as a favorable prognostic factor for AML, adult ALL, and CLL patients. For the future, we recommend to perform subtype-analyses of iNKT, NK as well as CIK cells in more detail to be able to allocate defined subtypes to prognostic groups. Moreover, we recommend multifactorial statistical analyses to work out the role of cellular partners involved in the mediation of anti-leukemic reactions and better prognosis for individual pts in more detail.

iNKT, NK, CIK Cells Contribute to Antitumor and Antileukemic Activity

A previous study has shown, that iNKT cells are important mediators in tumor-protection, as reduced frequencies of iNKT cells were correlated with a variety of cancers and increased frequencies of iNKT cells were related with favorable response to therapy.[39] Another study showed, that NK and CIK cells have cytotoxic potential against leukemia and other cancers.[30,40–42]

In the current study, we could show in general, that higher proportions of iNKT, NK, and CIK cells and higher frequencies of proliferating T cells and T_{cm} correlated with antileukemic activity (blast-lysis). Moreover, we could show (although only with one exemplary experiment up to now), that radiation of stimulator-cells did not reduce proportions of T/iNKT/CIK and NK cells, but reduce the overall blast lysis of (stimulator cell activated) effector cell-mediated antileukemic activity compared with unradiated settings—what could be explained by a functional knock-out of these T/iNKT/CIK and NK cells and therefore point to their antileukemic contribution. Consistent, a previous study has shown that in knockout experiments in mice absence of iNKT cells was correlated with tumor-growth and poor survival, while a transfer of iNKT cells into mice could reduce tumor growth.[39] All of these results emphasize the relevance of iNKT/CIK and NK cells in antileukemic reactions. More studies have to be performed to work out the specific role of these cell fractions in the mediation of antileukemic reactions. To further analyze the function of different iNKT/CIK or NK cells and their subtypes we recommend performing, for example blocking experiments knocking out certain cellular subtypes and correlating results with antileukemic function.

With an attempt to evaluate the predictivity of iNKT/CIK and NK cell frequencies we defined cutoff values: we could show that cases presenting with higher values of iNKT (subtypes) or CIK or NK cells were characterized by a higher chance to belong to the group with "antileukemic activity."

In summary, we suggest to define and applicate cut-off values for iNKT/CIK and NK cells and their subtypes for leukemia-pts in the future to enable a refined estimation of prognosis.

Induction of iNKT, NK, and CIK Cells After Stimulation With DC/DC_{leu}

It was already shown, that a crosstalk between NK cells and DC improves antitumor reactions. Recently, details about the mechanisms behind the increase of antitumor-reactivity (eg, pathogen-associated molecular patterns or cytokines) have been detected.[39,43] Moreover cytokines, cellular factors, antibodies promote NK cell activations, and DC-NK crosstalk establishing a micro-environment which enables antitumor reactions.[44,45] Moreover, a crosstalk between CIK cells and DC has been reported recently, demonstrating an influence of a DC stimulation on the phenotype as well as antileukemic cytotoxicity of CIK cells.[46] Recently a crosstalk between DC and iNKT cells has been shown (mediated, eg, by chemokine receptor-expression), leading to an amplification of antitumor-immune reactions.[4,47]

With our data, we contribute that iNKT cells not only respond to a stimulus with IL-2, but in addition are significantly expanded in cases with previous treatment of AML-or healthy MNC/WB with "cocktails." Comparable phenomena were found for CIK cells. These finding might point to an induction of these cell-populations by DC/DC_{leu} stimulation.

Moreover, we can add important new data to a previous study that highlighted 3 possible mechanisms of antitumor-reactivity mediated by iNKT cells: indirect cytotoxicity, direct cytotoxicity and modulation of the tumor microenvironment. "Indirect cytotoxicity" means that iNKT cells and DC stimulate each other by TCR/CD1d and CD40/CD154 interactions leading to a release of cytokines (e.g. IL-10, IL-17A, IFN-γ, and IL-12) and increased antitumor activity of other effector cells.[39,43] Mature DCs are known to produce IL-12 stimulating iNKT cells to increase IFN-γ release coactivating NK cells and their production of IFN-γ,[39] what could in return lead to a release of pro-inflammatory cytokines by DC.[43] Our preliminary data obtained with some cases in part confirm these findings: increased release of cytokines (IL-17A, IL-10) correlated with achieved "lysis"/antitumor activity and with higher iNKT, NK and CIK cell frequencies—pointing to a cytokine-mediated antitumor activity.[39] Moreover, we could show that "highest" iNKT cell frequencies were correlated with higher DC—pointing to a coactivation between iNKT cells and DC.

Recently it was shown, that soluble as well as cellular components of the immune-system are involved in a "crosstalk" with NK/iNKT cells leading to a "cross-priming" in infectious diseases or cancer: for example, IL-21 improved cytotoxicity and increased IFN-γ production of NK cells against breast cancer cells,[48] TH17 frequencies directly correlated with iNKT frequencies and good prognosis in CLL[49] and iNKT cells were shown to be involved in cross-talk between DC and $CD8^+$ cells—at least in infectious diseases.[50] These findings emphasize the importance to study these kinds of cross-talks in detail in future studies.

We can add in addition that the highest 6B11$^+$ iNKT and CIK values were found after culture in prostaglandin-containing "cocktails." This could point to a special effect of these "cocktails" on the allocation and recruitment of iNKT and CIK cells—resulting in an improved antileukemic activity—up to now an effect of Prostaglandin E_2 on the maturation of DC has been shown.[19,28] Alternatively, it might be discussed, that "only" the 6B11 or CD56-antigens are upregulated on T/NK cells, however without expansion of functionally reactive iNKT/CIK cell populations.

Physiologic Culture-conditions (Hypoxia, WB) Do Not Have an Effect on the Generation of DC Nor on the Frequencies and Composition of iNKT, NK or CIK Cells

Preliminary experiments with MNC/WB cultured under hypoxic versus normoxic conditions did not yield an effect on the generation of DC nor on the frequencies and composition of iNKT, NK, and CIK cells after MLC. Although more experiments have to be performed we can assume, that working under normoxic conditions yields "physiological" data.

CONCLUSIONS

In conclusion, we recommend to regularly evaluate proportions of iNKT/NK/CIK cells and include specific markers in diagnosis panels (based on 6B11/CD161/CD56/CD3 antibodies) in AML/ALL/CLL for quantitative, qualitative, and prognostically relevant estimation of individual patients antileukemic potential. Detection of iNKT cells should be based on 6B11-(PE)-staining alone or in combination with (especially) CD3 and CD161; NK and CIK cell detection should be based on the combination of CD3 with CD161 or with CD56 (in cases without aberrant expression of CD56). As we could demonstrate a shift to higher proportions of T and NK cells expressing 6B11 in AML, ALL, and CLL patients compared with healthy donors, we suggest that mechanisms behind these subtype extensions have to be studied in detail. These findings might be considered in the development of iNKT-based immunotherapies.

Considering that higher frequencies of iNKT, NK, and CIK cells correlate with prognostically better subgroups and with antileukemic activity (blast lysis) in AML, adult ALL, and CLL patients, high amounts of iNKT, NK, and CIK cells can be regarded as a favorable prognostic factor in leukemic patients.

Moreover, proportions of iNKT and CIK cells increase under stimulation of MLC with IL-2 and particularly after culture with (prostaglandin-containing) DC/DC_{leu}-inducing "cocktails". Cytokine profiling revealed a correlation of release of antitumor and anti-inflammatory cytokines with higher frequencies of iNKT, NK, and CIK cells after MLC, what correlated with improved antileukemic cytotoxicity. These findings not only point to a cross-talk between soluble factors and these immune reactive cells and DC, but in addition a correlation with an improved antileukemic reactivity. In conclusion, "cocktails" not only activate antileukemic T cells, but also iNKT and CIK cells and improve the antileukemic activity. Inclusion of prostaglandin-containing DC/DC_{leu}-generating methods might be especially promising in the recruitment of antileukemic active immune reactive cells.

Although the quantification of these small cell populations (using selected markers and special gating-strategies in flow cytometric settings) has to be performed from experienced groups and the contribution of these cell populations to antileukemic reactions should not be underestimated.

Novel immunotherapeutic protocols in the treatment of pts with leukemia should be designed, that include the quantification of iNKT, NK, and CIK cells and their subtypes in diagnostic panels. Moreover, the role of iNKT, NK, and CIK cells as well as soluble factors in a cross-talk in DC/DC_{leu}-triggered antileukemic reactions in AML patients should be studied in detail and probably be included in immunotherapeutic protocols against AML.

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ARTICLE

Presence of centromeric but absence of telomeric group *B KIR* haplotypes in stem cell donors improve leukaemia control after HSCT for childhood ALL

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Abstract

Although allogeneic hematopoietic stem-cell transplantation (HSCT) provides high cure rates for children with high-risk acute lymphoblastic leukaemia (ALL), relapses remain the main cause of treatment failure. Whereas donor killer cell immunoglobulin-like receptor (*KIR*) genotype was shown to impact on relapse incidence in adult myeloid leukaemia similar studies in paediatric ALL are largely missing. Effect of donor *KIR* genotype on transplant outcome was evaluated in 317 children receiving a first myeloablative HSCT from an *HLA*-matched unrelated donor or sibling within the prospective ALL-SCT-BFM-2003 trial. Analysis of donor *KIR* gene polymorphism revealed that centromeric presence and telomeric absence of KIR *B* haplotypes was associated with reduced relapse risk. A centromeric/telomeric *KIR* score (*ct-KIR* score) integrating these observations correlated with relapse risk (hazard ratio (HR) 0.58; $P = 0.002$) while it had no impact on graft-versus-host disease or non-relapse mortality. In multivariable analyses *ct-KIR* score was associated with reduced relapse risk (HR 0.58; $P = 0.003$) and a trend towards improved event-free survival (HR 0.76; $P = 0.059$). This effect proved independent of MRD level prior to HSCT. Our data suggest that in children with ALL undergoing HSCT after myeloablative conditioning, donor selection based on *KIR* genotyping holds promise to improve clinical outcome by decreasing relapse risk and prolonged event-free survival.

Introduction

Allogeneic hematopoietic stem-cell transplantation (HSCT) provides high cure rates in children suffering from very high-risk primary or relapsed acute lymphoblastic leukaemia (ALL) who display poor prognosis with conventional chemotherapy. Recent improvements in donor selection relying on high-resolution human leucocyte antigen (*HLA*) typing as well as optimisation of supportive care, including effective graft-versus-host-disease (GvHD) prophylaxis and early diagnosis and pre-emptive treatment of infections, have substantially reduced non-relapse morbidity and mortality, in particular in the setting of transplantation from *HLA*-matched unrelated donors (MUD). Consequently, current results of HSCT from MUDs are comparable to those from *HLA*-identical sibling donors (ISD) and approach 4-year event-free survival rates of 65–70% [1].

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Relapses of leukaemia, however, represent the main cause of treatment failure, thus warranting investigation of novel strategies to improve leukaemia control in paediatric HSCT.

As therapeutic efficacy of HSCT in addition to high dose chemo-/radiotherapy critically relies on immune effects provided by allogeneic donor cells various strategies to identify the immunologically optimal stem-cell donor have been promoted. Among those, the employment of leukaemia-directed effects provided by donor natural killer (NK) cells play a prominent role [2]. The function of NK cells is regulated by killer cell immunoglobulin-like receptors (*KIR*) that comprise of 14 polymorphic inhibitory or stimulatory receptors whose genes vary in number and content between individuals. The specific set of *KIR* genes present in an individual at the independently segregating centromeric and telomeric *KIR* gene loci defines two common haplotypes: Group *A* haplotypes provide high-affinity recognition of all major *HLA* class I-encoded ligands to secure self-tolerance, whereas group *B* haplotypes have variable gene content contributing to the diversification of NK cell repertoires [3].

Recent large registry-based analyses primarily focusing on adult patients suffering from acute myeloblastic leukaemia (AML) have identified donor selection based on presence and chromosomal position of the *KIR B* haplotype, with a predominant role of the centromeric *KIR* genes KIR*2DL2/2DS2*, to provide superior leukaemia control [4–6]. Subsequent studies, however, have revealed that the effect of donor *KIR* haplotypes is highly context-dependent, i.e., depending on the specific transplant scenario with regard to underlying malignancy, conditioning intensity, donor type, and in vivo and/or in vitro T cell depletion. [5, 7–9].

As data in the paediatric *HLA*-matched setting are largely missing, we here evaluated the impact of donor *KIR* haplotypes and their respective chromosomal position on the clinical outcome of 317 children with ALL homogeneously treated within the ALL-SCT-BFM-2003 trial.

Methods

Patients and donors

This study retrospectively investigated the impact of donor *KIR* genotype on outcome in paediatric patients who received a first myeloablative HSCT from an *HLA*-identical sibling or an *HLA*-matched unrelated donor within the prospective ALL-SCT-BFM-2003 trial. Between September 2003 and September 2011, 411 children with high-risk ALL, who received allogeneic SCT, were enroled in the ALL-BFM-SCT 2003 trial [1]. From 317 of these 411 patients and their stem-cell donors DNA samples could be collected and *KIR*-typed (Table S1). Ninety-four patients were not included in this study as DNA samples from donors were not available. Informed consent was obtained in accordance with the Declaration of Helsinki.

Kir typing and Kir haplotype assignment

KIR genotyping of 12 *KIR* genes (*KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5*, and *KIR3DS1*) was performed on genomic DNA from related and unrelated donors of hematopoietic stem-cells by polymerase chain reaction with sequence specific primers (PCR-SSP), as previously described [10]. A total of 10% randomly selected samples were retyped by an independent *KIR* typing method with full concordance [11]. Based on the presence/absence polymorphism and the strong negative linkage disequilibrium of some *KIR* genes, the centromeric and telomeric modules that constitute a *KIR* haplotype can be defined as follows: presence of *KIR2DL1* and *KIR2DL3* denotes a *centromeric A* (*Cen-A*) module whereas presence of *KIR2DL2* and/or *KIR2DS2* denotes a *centromeric B* (*Cen-B*) module. On the telomeric part of the KIR locus the presence of *KIR3DL1* and *KIR2DS4* indicates the *telomeric A* (*Tel-A*) module while presence of *KIR3DS1* and/or *KIR2DS1* indicates the *telomeric B* (*Tel-B*) module.

Statistical analyses

For non-time to event variables, χ^2 test was used to compare groups for categorical variables. Wilcoxon rank-sum test (Kruskal–Wallis test for more than two populations) was used for continuous variables. The overall and the event-free survival probabilities were evaluated using the Kaplan–Meier method and the log-rank test. Cumulative incidences of events were calculated by the method of Kalbfleisch and Prentice [12], and compared using the Gray test [13]. Competing events were defined as follows: for relapse incidence: deaths from any cause and secondary malignancies; for chronic GVHD: death from any cause; and for non-relapse mortality: relapses and secondary malignancies. The impact of donor *KIR* haplotypes and the donor *ct-KIR* score on time-dependent outcome variables (cumulative incidence of relapse, chronic GvHD and non-relapse mortality; probability of event-free and overall survival) was further analysed in univariable und multivariate Cox regression models.

The Cox proportional hazards model was used to estimate survival, and the proportional subdistribution hazards model of Fine and Gray [14] for censored data subject to competing risks was applied for analyses of relapse incidence and non-relapse mortality. In order to perform a trend test the *ct-KIR* score was included into the models as a

numerical variable. More information on statistical methods is provided in the online supplement.

Results

Patient and donor characteristics

Characteristics of the study patients and their donors are listed in Table 1. After conditioning, all patients received stem-cell transplantations during first or later remission standardised with regard to donor selection, myeloablative conditioning, donor type-stratified GvHD prophylaxis and supportive care. Depending on donor availability, grafts originated from *HLA*-identical siblings ($n = 86$) or from ≥9/10 *HLA*-matched unrelated donors ($n = 231$). As shown in Table S1, a lower proportion of patients transplanted in >CR2 was the sole patient, disease and donor characteristic with significant difference between our study cohort and the 94 patients not included in the ALL-BFM-SCT 2003 trial.

Effect of presence and absence of donor centromeric and telomeric KIR B motifs

As previously reported by Cooley et al. and Bachanova et al., a relapse protective effect of donor *KIR B* haplotype motifs was identified in adult AML and non-Hodgkin lymphoma patients [4–6, 15]. In particular, centromeric group *B* motifs were found to be associated with improved disease control in these studies. To investigate the role of *KIR B* motifs in children with ALL in a candidate approach, patients were grouped according to the presence of donor *B* motifs in the centromeric (*CenB+* vs. *CenB−*) and the telomeric region (*TelB+* vs. *TelB−*). In accordance with the studies by Cooley and Bachanova, donors lacking a centromeric *KIR B* motif (*CenB−*) were associated with significantly increased relapse incidence (RI) (26% 95%-CI 23–29%) compared to donors with at least one centromeric *KIR B* motif (*CenB+*; 17% 95%-CI 14–20%; hazard ratio, 1.64; 95%-CI, 1.00–2.70; $P = 0.048$; Fig. 1a). In addition, we found that patients transplanted from donors with at least one telomeric *KIR B* motif (*TelB+*) experienced a higher relapse incidence (RI 27% 95%-CI 23–31%) compared to donors without telomeric *KIR B* motifs (*TelB-*; RI 17% 95%-CI 14–20%; hazard ratio, 1.65; 95%-CI, 1.03–2.65; $P = 0.039$; Fig. 1b). Together, these findings suggest that the absence of centromeric and presence of telomeric group *B* haplotypes are independent risk factors for relapse in childhood ALL.

To evaluate an independent effect of centromeric *KIR B* motifs vs telomeric *KIR B* motifs donors were divided into *CenB−TelB−*, *CenB+TelB−*, *CenB−TelB+* and *CenB+ TelB+* groups. *CenB + TelB-* donors were associated with the lowest RI (12% 95%-CI 8–16%) while patients transplanted from *CenB-TelB+*donors fared significantly worse (34% 95%-CI 28–40%). Patients who were transplanted from either *CenB + TelB +* or *CenB−TelB−* donors experienced intermediate relapse incidences (22% 95%-CI 17–27% and 21% 95%-CI 17–25%, respectively; Fig. 1c). These data confirm an independent effect of centromeric and telomeric *KIR B* motifs on relapse incidence.

To harness the dichotomous role of *KIR B* motifs in the centromeric vs. telomeric gene locus we integrated the individual donor *KIR* genotypes at these two regions into a composite centromeric/telomeric *KIR (ct-KIR)* score: The *ct-KIR* score was assigned the value 0 in donors with *KIR* haplotypes associated with higher relapse risk at both centromeric and telomeric position (i.e. *CenB-* and *TelB+*), the value 1 in donors with the presence of one *KIR* haplotype associated with higher relapse risk at either centromeric or telomeric position (*CenB+* and *TelB+* or *CenB−* and *TelB−*), and the value 2 in donors lacking any centromeric and telomeric KIR haplotype associated with higher relapse risk (*CenB+* and *TelB−*; Table 2). As shown in Fig. 1d, the donor *ct-KIR* score clearly discriminated paediatric ALL transplant recipients with high (*ct-KIR* score 0, $n = 59$), moderate (*ct-KIR* score 1, $n = 175$) and low (*ct-KIR* score 2, $n = 83$) cumulative incidences of relapse of 34% 95%-CI 28–40% vs. 21% 95%-CI 18–24% vs. 12% 95%-CI 8–16% (hazard ratio, 0.58; 95%-CI, 0.41–0.82; $P = 0.002$). Thus, with regard to relapse risk the *ct-KIR* score identifies unfavourable (*ct-KIR* score 0), intermediate (*ct-KIR* score 1) and favourable *(ct-KIR* score 2) donors (Fig. 1d) and this holds true for transplantations from both HLA-identical sibling donors and HLA-matched unrelated donors (Fig. S1 and S2).

Accounting for the current unavailability of an external validation cohort for this potentially practice-changing finding, we employed the method of bootstrapping to internally validate our *ct-KIR* score definition procedure. As described above, the *ct-KIR* score was defined according to the presence or absence of donor *B* motifs in the way to ensure an optimal association between the donor *KIR* status and the cumulative relapse incidence within the cohort of 317 patients. A potential statistical problem (model overfitting) related to the circumstance that we used the complete dataset to build the score, is that the model performance ability may shrink if measured on a new data set.

Our validation approach allowed for calculating the effect of model overfitting on the cumulative incidence of relapse for the subgroups of patients identified by the ct-KIR score: 33% (20-44%) for ct-KIR score 0, 22% (17-29%) for ct-KIR score 1, and 14% (8-22%) for ct-KIR score 0 (hazard ratio, 0.63; 95%-CI, 0.46-0.87; $P = 0.005$). These results validate the robust predictive value of the donor *ct-KIR* score on the cumulative incidence of relapse (Fig. 1e).

Table 1 Demographic and clinical characteristics of the patients

Characteristics	Donor with *ct-KIR* score							
	Total KIR study cohort ($N = 317$)		2 (favourable) ($N = 83$)		1 (intermediate) ($N = 175$)		0 (unfavourable) ($N = 59$)	
Patient age - year								
Median (range)	9.9 (0.6–18.5)		9.4 (0.9–18.2)		10.4 (0.6–18.5)		7.5 (1.0–18.3)	
Patient male sex - no. (%)	194	61%	49	59%	104	59%	41	69%
Remission status - no. (%)								
First complete remission	163	51%	41	49%	92	53%	30	51%
Second complete remission	138	44%	36	43%	75	43%	27	46%
>Second complete remission	16	5%	6	7%	8	5%	2	3%
Immunophenotype - no. (%)								
B lineage	240	76%	68	82%	129	74%	43	73%
Others	77	24%	15	18%	46	26%	16	27%
Donor age – year (median – range)								
Matched sibling donor	11.6 (0.8–27.2)		13.5 (2.3–27.2)		12.2 (0.8–23.9)		13.5 (2.3–27.2)	
Matched unrelated donor	35.6 (18.0–64.6)		36.6 (19.0–64.6)		34.9 (18.0–56.0)		36.6 (19.0–64.6)	
Donor type - no. (%)								
Matched sibling donor§	86	27%	22	27%	39	22%	25	42%
9/10 matched unrelated donor	108	34%	32	39%	63	36%	13	22%
10/10 matched unrelated donor	123	39%	29	35%	73	42%	21	36%
Cytomegalovirus match (donor-patient) - no. (%)								
Negative–negative	153	49%	42	51%	81	47%	30	51%
Negative–positive	53	17%	10	12%	35	20%	8	14%
Positive–negative	43	14%	14	17%	19	11%	10	17%
Positive–positive	65	21%	16	20%	38	22%	11	19%
Incomplete data	3		1		2		0	
Sex match (donor–patient) - no. (%)								
Female–male	72	23%	15	18%	42	24%	15	26%
Others	243	77%	68	82%	132	76%	43	74%
Incomplete data	2		0		1		1	
AB0 blood group match (donor–recipient) - no. (%)								
AB0 match	134	43%	33	40%	73	42%	28	47%
Minor mismatch	75	24%	18	22%	43	25%	14	24%
Major mismatch	106	34%	31	38%	58	33%	17	29%
Incomplete data	2		1		1		0	
Conditioning regimen - no. (%)								
Total body irradiation and etoposide	286	90%	76	92%	156	89%	54	92%
Chemotherapy conditioning	31	10%	7	8%	19	11%	5	8%
Graft type - no. (%)								
Bone marrow	239	75%	65	78%	127	73%	47	80%
Peripheral blood stem cells	65	21%	15	18%	41	23%	9	15%
Cord blood stem cells	4	1%	1	1%	1	1%	2	3%
Other	9	3%	2	2%	6	3%	1	2%
Disease recurrence risk[a]								
Low risk	232	73%	42	71%	132	75%	58	70%
High risk	85	27%	17	29%	43	25%	25	30%
Minimal residual disease (MRD) level prior to stem-cell transplantation - no. (%)								
$\geq 10^{-3}$ (high-risk)	17	10%	4	9%	12	13%	1	3%

Table 1 (continued)

Characteristics	Donor with *ct-KIR* score							
	Total KIR study cohort ($N = 317$)		2 (favourable) ($N = 83$)		1 (intermediate) ($N = 175$)		0 (unfavourable) ($N = 59$)	
$<10^{-3}$ (standard risk)	154	90%	42	91%	78	87%	34	97%
No minimal residual disease data	146		37		85		24	
CR1: time from diagnosis to SCT (months)								
Median (range)	6.6 (4.1–39.3)		6.4 (4.7–12.4)		6.7 (4.5–39.3)		6.6 (4.1–17.2)	
>CR1: time from relapse to SCT (months)								
Median (range)	5.2 (2.4–9.0)		5.0 (3.4–8.3)		5.2 (3.4–9.0)		5.2 (2.4–7.5)	
>CR1: time from diagnosis to SCT (months)								
Median (range)	30.1 (3.0–128)		27.2 (13.3–77)		32.0 (3.0–128)		28.9 (5.2–99.4)	

The χ^2 test was used for categorical variables, and the Wilcoxon rank-sum test (Kruskal–Wallis test for more than two populations) for continuous variables. Analyses compare 3 patient cohorts grouped according to *ct-KIR* score (values 0, 1, 2 representing groups of unfavourable, intermediate, and favourable donors). At the time of analysis in June 2016, the median follow-up time of survivors, defined as the time from SCT to last observation, was 5.9 years (range 1.1–10.64)

[a]Disease recurrence low-risk: transplantation in CR1 or in CR2 after late (>6 months after end of front-line therapy) relapse; high risk: transplantation in CR2 after early (<6 months after end of front-line therapy) relapse or in CR3

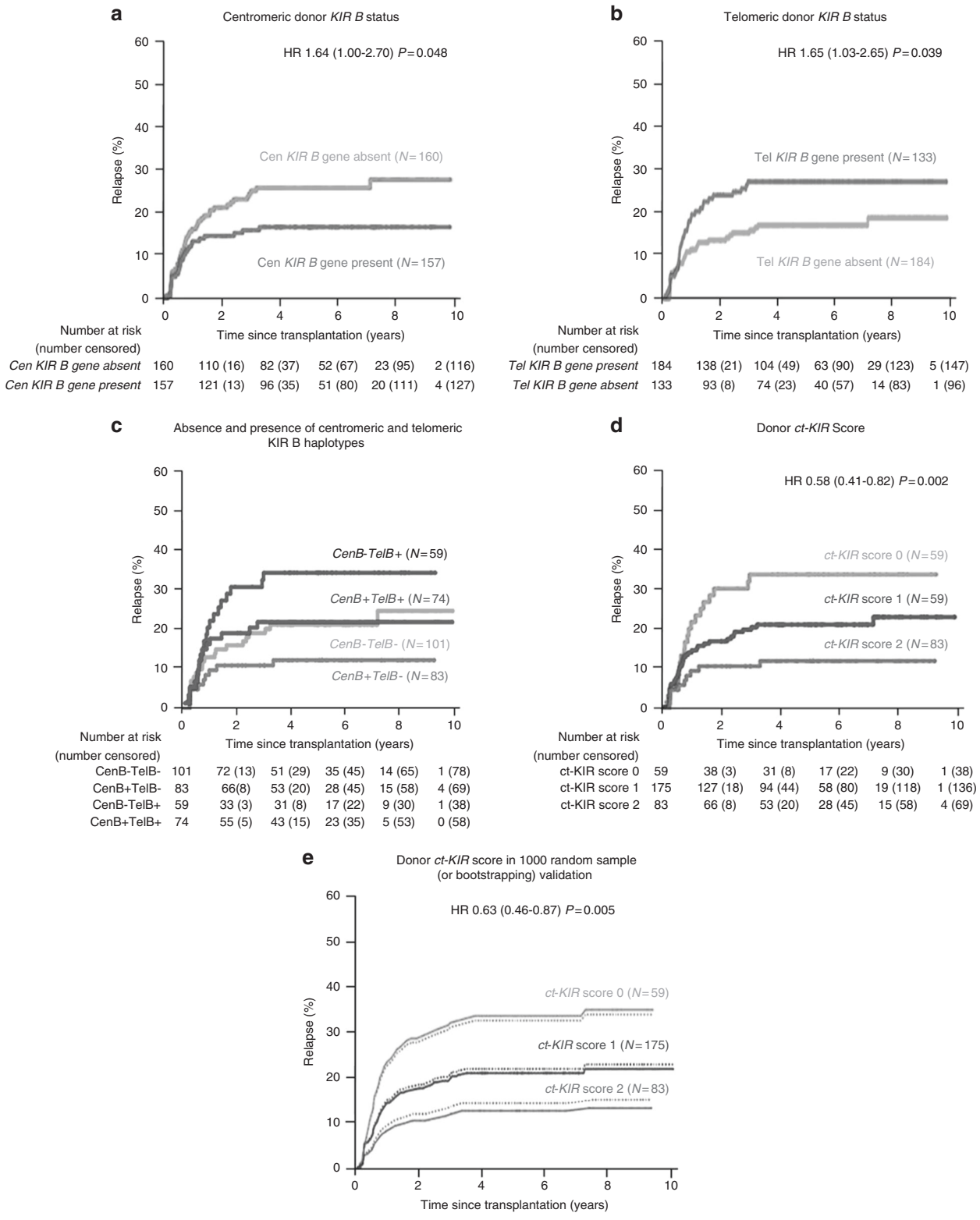
§ $P = 0.04$ for the difference in the distribution of characteristics among the three groups

Having identified this significant association of donor *KIR* status with relapse incidence we strived for carefully evaluating potential biases due to unequally distributed confounders between the patient groups discriminated by donor *ct-KIR* score. Donor *KIR* genotype and thus *ct-KIR* score status is segregating independently from other donor characteristics like *HLA* genotype and AB0 blood group based on their genomic positioning on different chromosomes. As shown in Table 1 donor *ct-KIR* score did not correlate with any patient, disease or donor characteristic except for a slightly higher prevalence of *HLA*-identical sibling donors in the unfavourable donor group. Importantly, patients with high minimal residual disease (MRD) burden prior to HSCT, representing one major risk factor for post-transplant relapses (Fig. S3), were equally distributed among the donor groups (Table 1). Taken together, these observations illustrate random distribution of the *ct-KIR* score in donors of our study cohort.

In order to confirm or reject an independent prognostic impact of donor *ct-KIR* score on relapse incidence, we performed multivariable analysis adjusting for patient, disease and donor selection criteria such as immunophenotype, disease recurrence risk (Fig. S4 and S5), donor type [16–19], CMV serostatus [20], sex match and AB0 blood group [19]. As shown in Table 3 donor *ct-KIR* score was independently associated with reduced relapse incidence (hazard ratio for one-point increment in *ct-KIR* score, 0.58; 95%-CI, 0.40–0.83; $P = 0.003$). The other factor reaching statistical significance in multivariable analysis was disease recurrence risk (hazard ratio, 2.84; 95%-CI, 1.64–4.92; $P < 0.001$) whereas no other patient, disease or donor characteristic including donor KIR – recipient KIR ligand mismatch had any effect on relapse incidence (Table 3 and Fig. S6). Given the fact that MRD level prior to HSCT has recently emerged as a prognostic factor for relapse risk [21], we performed additional multivariable analyses within all patients with available MRD data revealing that *ct-KIR* score and MRD level were the only independent risk factors for relapse thus validating that the effect of the donor *ct-KIR* score acts independently from MRD level prior to transplant (Table S3). Finally, donor *ct-KIR* score proved of prognostic significance within the subgroup of patients with low risk MRD level, while the subgroup of patient with high risk MRD was too small to study (Fig. S7).

Effect of donor *Ct-KIR* score on acute GvHD, chronic GvHD and non-relapse mortality

We next explored whether the relapse protective impact of donor *ct-KIR* status was achieved at the expense of increased risk of graft-versus-host-disease (GvHD). As shown in Table 4 for acute GvHD and Fig. 2a, b for any grade and extensive chronic GvHD we could not identify any detectable impact of donor *ct-KIR* status on GvHD risk. Most importantly, the incidence of extensive chronic GvHD representing the major cause of morbidity and non-relapse mortality in paediatric allogeneic HSCT [22] proved low and identical in all three donor *ct-KIR* score groups (unfavourable vs intermediate vs favourable, 7% 95%-CI 4–10% vs 7% 95%-CI 5–9% vs 6% 95%-CI 3–9%; hazard ratio, 0.94; 95%-CI, 0.50–1.78; $P = 0.847$; Fig. 2b).



Similar results were obtained when donor *ct-KIR* score groups were compared with regard to incidence of non-relapse mortality (NRM). In univariable analysis NRM incidence proved lowest in the unfavourable donor group (Fig. 2c). However, this difference between the donor *ct-KIR* score groups was not statistically significant, an observation that was confirmed in multivariable analysis of NRM (hazard ratio, 1.41; 95%-CI, 0.81–2.45; $P = 0.222$; Table 3).

◀ **Fig. 1** Cumulative Incidence of Relapse According to Presence and Chromosomal Position of Donor *KIR B* motifs and Donor *ct-KIR* score. The cumulative incidence of relapse is shown for patients with donors stratified according to the presence vs. absence of centromeric (**a**) and telomeric (**b**) *KIR B* motifs, and for patients with donors stratified according to the combination of presence and absence of centromeric and telomeric *KIR B* motifs (**c**). **d** The cumulative incidence of relapse is shown for patients with donors stratified according to the *ct-KIR* score. Donor *ct-KIR* scoring was performed as outlined in Table 2. Transplants from favourable donors (*ct-KIR* score 2) led to significantly better relapse control compared to those from unfavourable donors (*ct-KIR* score 0; $P = 0.003$). Transplants from intermediate donors (*ct-KIR* score 1) were associated with higher relapse incidence compared to those from favourable donors and lower relapse rates compared to those from unfavourable donors ($P = 0.07$ for both comparisons). The impact of model overfitting is shown by visualisation of validation results (**e**). Solid lines: cumulative incidence of relapse according *ct-KIR* score. Dashed lines show the cumulative incidence of relapse for the *ct-KIR* score, taking into account the estimated effect of model overfitting: *ct-KIR* score 0 ($N = 59$), 5-y CI of relapse 33% (20-44%); *ct-KIR* score 1 ($N = 175$), 5-y CI of relapse 22% (17–29%); *ct-KIR* score 2 ($N = 83$), 5-y CI of relapse 14% (8–22%); HR 0.63 (0.46–0.87), $p = 0.005$

Impact of donor *Ct-KIR* score on event-free survival

Finally, we investigated the effect of donor *ct-KIR* status on event-free survival (EFS). In univariable analysis we observed a beneficial effect of donor *ct-KIR* status with 5-year EFS of 63% 95%-CI 57–69% vs. 68% 95%-CI 64–72% vs. 75% 95%-CI 70–80% for the unfavourable vs. intermediated vs. favourable *ct-KIR* score donor group (hazard ratio, 0.76; 95%-CI, 0.57–1.01; $P = 0.055$; Fig. 2d). This was verified in multivariable analysis where donor *ct-KIR* status proved associated with improved EFS (hazard ratio for one-point increment in *ct-KIR* score, 0.76; 95%-CI, 0.57–1.01; $P = 0.059$; Table 3). Although no other donor factor had an effect on EFS, disease recurrence risk was affirmed as an adverse disease characteristic (hazard ratio, 1.91; 95%-CI, 1.26–2.89; $P = 0.002$).

Discussion

The importance of mismatching between KIR on donor NK cells and their HLA-encoded ligands in the recipient for control of myeloid leukaemia, also referred to as NK cell alloreactivity, was initially described for haploidentical HSCT [23, 24]. In the more prevailing setting of T cell-replete transplantation from HLA-matched donors, however, numerous studies yielded conflicting results on the impact of NK cell alloreactivity mainly attributed to widely varying degree of T cell depletion [25]. Notably, NK cell alloreactivity, defined by KIR – KIR ligand mismatch [26, 27], had no impact on relapse incidence in our analysis (Fig. S6). More recent analyses in adult AML suggested a critical role for conserved *KIR* haplotype structures consisting of centromeric and telomeric *KIR* linkage groups, the inhibitory *KIR2DL1* and the stimulatory *KIR2DS1* in the donor [4–6, 28, 29]. In lymphoid malignancy, similar reports in *HLA*-matched HSCT yielded rather inconclusive results in adults [5, 15, 28–31] or in the case of paediatric ALL, are entirely missing. The only existing data in the paediatric setting comes from a dual centre study of 85 paediatric ALL patients undergoing haploidentical transplantation showing a beneficial impact of *KIR B* haplotypes as defined by Cooley [5] on relapse incidence [7]. As ALL represents the most prominent indication for paediatric allogeneic HSCT the present study focussed on the role of donor *KIR* haplotype polymorphism in this scenario in a candidate approach. Aforementioned studies from Cooley et al. [5, 6, 32] were conducted in adult patients whereas our patient cohort consisted of a pure paediatric cohort. While ALL occurs in adults and children, there is evidence for different susceptibilities of childhood ALL and adult ALL towards NK cell-mediated leukaemia control that have been attributed to specific HLA class I downregulation in paediatric ALL [33]. These differences in the role of *KIR B* genes between adults and children, and their underlying disease indicate that investigations in other diseases are highly warranted.

The setup of the ALL-SCT-BFM-2003 trial [1] enabled retrospective analysis of *KIR* polymorphism in a cohort of 317 children, homogeneously treated with regard to transplant indication, donor selection, pre-transplant conditioning and GvHD prophylaxis within a prospective, multicentric clinical trial. In full concordance with previous observations in adult AML [4–6, 30, 34, 35] presence of

Table 2 Definition of *ct-KIR* score according to centromeric and telomeric *KIR B* motifs

Ct-KIR score	Donor assignment	Centromeric region	Telomeric region
0	Unfavourable	*CenB-*	*TelB+*
1	Intermediate	*CenB + CenB-*	*TelB + TelB-*
2	Favourable	*CenB +*	*TelB-*

The specific set of *KIR* genes defines two common *KIR* haplotypes A and B at the independently segregating centromeric and telomeric *KIR* gene loci (group *A* genes: *KIR2DL1, KIR2DL3, KIR3DL1*, and *KIR2DS4*; group *B* genes: *KIR2DL2*, *KIR2DL5, KIR2DS1, KIR2DS2*, and *KIR3DS1*). In the diploid genome donors lacking a centromeric *KIR B* gene are termed *CenB-* whereas those with at least one centromeric *KIR B* gene present are termed *CenB+*. The same nomenclature is used for the telomeric region resulting in *TelB−* and *TelB+*

Table 3 Proportional subdistribution hazards model for analyses of relapse incidence and non-relapse mortality and cox proportional hazard models for analyses of survival[a]

		Relapse incidence		Non-relapse mortality		Event-free survival	
		HR (95% HR CI)	*P*-value	HR (95% HR CI)	*P*-value	HR (95% HR CI)	*P*-value
ct-KIR score	1-point increment	0.58 (0.40–0.83)	0.003	1.41 (0.81–2.45)	0.222	0.76 (0.57–1.01)	0.059
	0 (unfavourable)[b]	1.00	–	1.00	–	1.00	–
	1 (intermediate)	0.65 (0.36–1.18)	0.158	2.42 (0.55–10.76)	0.245	0.79 (0.49–1.28)	0.341
	2 (favourable)	0.32 (0.15–0.69)	0.004	2.66 (0.55–12.78)	0.222	0.57 (0.32–1.03)	0.061
Donor type	Matched sibling	1.00	–	1.00	–	1.00	–
	10/10 matched unrelated	0.80 (0.45–1.43)	0.460	1.94 (0.65–5.76)	0.234	0.99 (0.60–1.64)	0.962
	9/10 matched unrelated	0.89 (0.48–1.65)	0.707	2.13 (0.72–6.34)	0.174	1.26 (0.76–2.09)	0.366
CMV serostatus (donor/recipient)	Positive/positive	1.00	–	1.00	–	1.00	–
	Negative/negative	0.82 (0.45–1.50)	0.517	0.48 (0.17–1.36)	0.168	0.75 (0.45–1.25)	0.269
	Negative/positive	0.57 (0.21–1.13)	0.094	1.25 (0.43–3.63)	0.680	0.73 (0.38–1.38)	0.327
	Positive/negative	0.61 (0.18–1.28)	0.142	0.49 (0.12–2.10)	0.337	0.81 (0.41–1.59)	0.539
Sex match (donor/recipient)	Other	1.00	–	1.00	–	1.00	–
	Female/male	0.73 (0.38–1.39)	0.340	1.45 (0.62–3.38)	0.389	0.98 (0.62–1.57)	0.944
AB0 blood group match	Match	1.00	–	1.00	–	1.00	–
	Minor mismatch	0.78 (0.43–1.42)	0.412	1.79 (0.71–4.48)	0.215	1.01 (0.61–1.66)	0.978
	Major mismatch	0.78 (0.38–1.42)	0.359	1.54 (0.65–3.62)	0.324	0.97 (0.61–1.53)	0.899
Immunophenotype	Other	1.00	–	1.00	–	1.00	–
	B-ALL	1.05 (0.62–1.78)	0.869	1.23 (0.47–3.27)	0.674	0.96 (0.62–1.49)	0.845
Disease recurrence risk[c]	Low risk	1.00	–	1.00	–	1.00	–
	High risk	2.84 (1.64–4.92)	0.000	0.78 (0.31–1.97)	0.601	1.91 (1.26–2.89)	0.002

Analyses were adjusted for donor type, CMV serostatus, sex match, AB0 match, immunophenotype of leukaemia and remission status at time of HSCT. In order to perform a trend test (1-point increment) the *ct-KIR* score was included into the models as a numerical variable. Data on AB0 blood group were missing for 2 donors, on sex for 2 donors, and on CMV serostatus for 2 patients and 1 donor

[a]Competing risks were accounted for according to Fine and Gray [14]

[b]For pairwise comparison of groups according to donor *ct-KIR* score this variable was included into the models as a categorical variable.

[c]Disease recurrence low risk: transplantation in CR1 or in CR2 after late (>6 months after end of front-line therapy) relapse; high-risk: transplantation in CR2 after early (<6 months after end of front-line therapy) relapse or in CR3

Table 4 Incidence and severity of acute graft versus host disease according to donor *Ct-KIR* score[a]

Characteristics	*KIR* study ($N = 317$)		Donor with *ct-KIR* score						*P*-value[a]
			0 (unfavourable) ($N = 59$)		1 (intermediate) ($N = 175$)		2 (favourable)		
Acute GvHD (grade)									0.942[a]
0	80	25%	19	32%	48	27%	13	16%	
1	130	41%	23	39%	66	38%	41	50%	
2	68	22%	10	17%	41	23%	17	21%	
3	25	8%	5	8%	13	7%	7	9%	
4	9	3%	2	3%	5	3%	2	2%	
Death without acute GvHD	4	1%	0	0%	2	1%	2	2%	
Unknown	1		0		0		1		

GvHD graft-versus-host disease

[a]The χ^2 test was used for categorical variables

centromeric group *B* motifs was significantly associated with relapse protection and was thus validated in our paediatric patient cohort. We extend these findings by showing that the presence of telomeric group *B* motifs is detrimental for leukaemia control. The *ct-KIR* score, an algorithm that accommodates this dichotomous role of centromeric versus

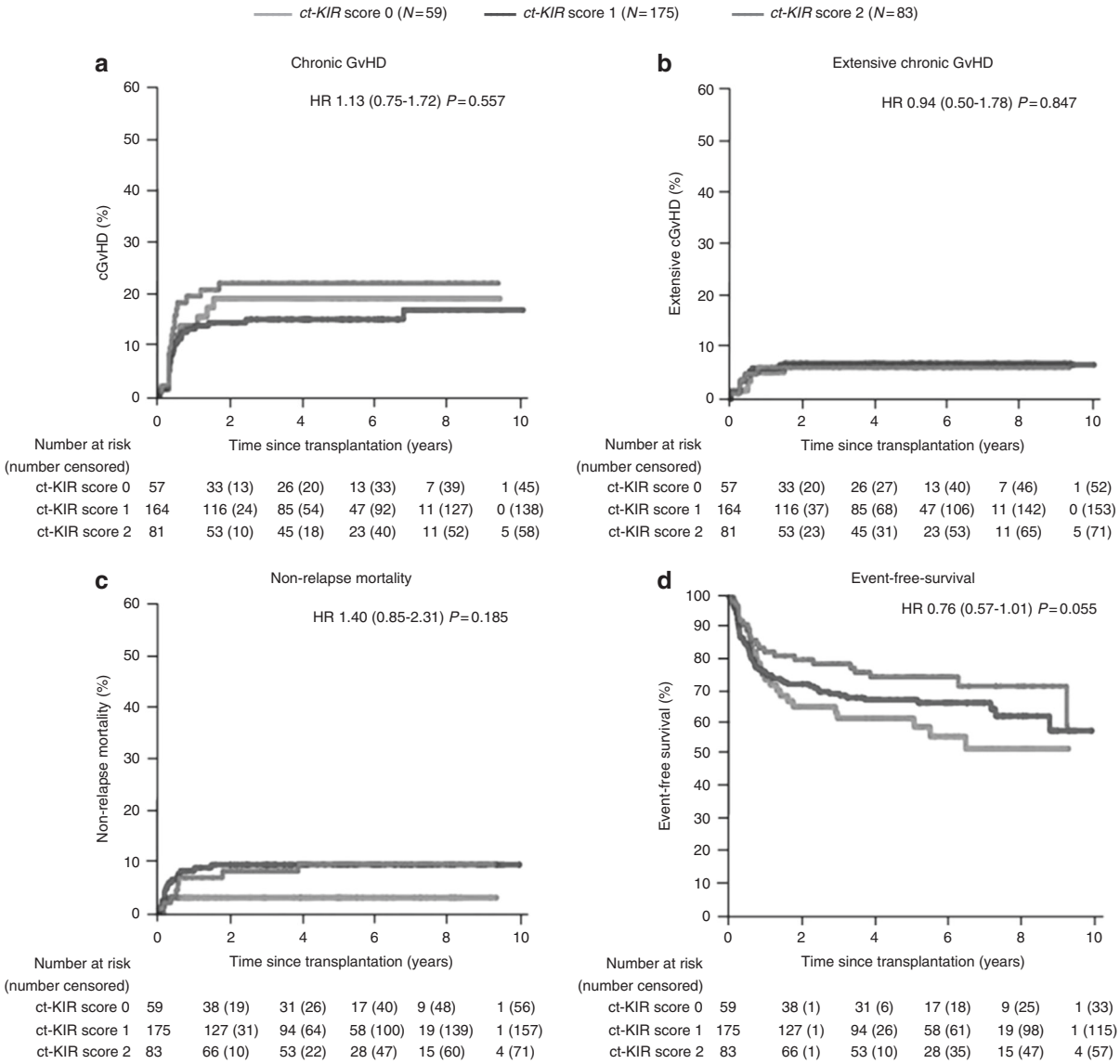


Fig. 2 Cumulative Incidence of chronic GvHD, extensive chronic GvHD, non-relapse mortality and estimate of event-free survival among patients stratified by donor *ct-KIR* score. The cumulative incidence of chronic GvHD (**a**), extensive chronic GvHD (**b**), non-relapse mortality (**c**) and estimate of event-free survival (**d**) are shown for patients transplanted from donors stratified according to the *ct-KIR* score

telomeric *KIR B* motifs enabled the distinction between three different groups including a favourable donor group that exhibited a substantially decreased relapse rate and improved event-free survival. This protective effect was not associated with any detrimental impact on other transplant outcomes including acute and chronic GvHD as well as non-relapse mortality. In multivariable analyses adjusting for disease, transplant and donor characteristics disease recurrence risk, reflected by remission status at transplant and time to first relapse, and donor *ct-KIR* score proved to represent the only prognostic factors for relapse incidence and event-free survival. Moreover, one thousand randomly selected bootstrap resamples of the >300 individuals in our analysis were utilised to re-assess the cumulative incidence of relapse for the subgroups of patients identified by donor *ct-KIR* score and validated the robust predictive value of the donor *ct-KIR* score on disease control (Fig. 1e).

As our analysis was retrospective in nature it may be subject to inherent biases. The retrospective design at the same time ascertains that transplant physicians were unaware of the donor *KIR* status at donor selection. Moreover, *ct-KIR* status, segregating independently from other donor characteristics including *HLA* genotype, did not correlate with patient, disease, and transplant characteristics, hence approving its random distribution in our study cohort.

Based on the paucity of paediatric studies donor selection criteria and their hierarchy in HSCT for childhood malignancy largely rely on registry-based analyses comprising a wide variety of underlying diseases, transplant modalities and patient ages [1, 18–20, 36, 37]. In multivariable analyses incorporating those characteristics that are currently employed for donor selection donor *ct-KIR* score proved the only donor factor associated with improved leukaemia control and event-free survival (Table 3). The question whether a patient would benefit from a favourable 9/10 MUD compared to an unfavourable 10/10 donor is of high clinical relevance. However, in order to be able to analyse more donor characteristics in a multivariable model, a higher number of patients would be needed.

The detrimental role of telomeric *KIR B* genes is difficult to interpret as far more genes are involved in telomeric haplotypes compared to centromeric haplotypes [38, 39]. Moreover, in contrast to the centromeric part, various ligands of receptors encoded by telomeric *KIR* genes are completely unknown [40]. Thus, any biologic rationale behind the telomeric *KIR B* effects would remain speculative. Clearly, more research is warranted in this area.

Recently, MRD level prior to HSCT has emerged as an important prognostic factor for relapse risk [21]. As MRD level prior to HSCT was not routinely investigated within the ALL-SCT-BFM-2003 trial, data were not available for the entire study population. To nevertheless account for the potential prognostic impact of MRD on our observations we evaluated the effect of *ct-KIR* score within 171 patients with available MRD data. Both univariate and multivariate analyses confirmed an MRD-independent prognostic impact of donor *ct-KIR* score on relapse incidence (Table S2/ Fig. S7).

In conclusion, within the attendant limitations of a retrospective analysis lacking formal validation in an independent patient cohort our data indicate that in children with ALL undergoing HLA-matched allogeneic stem-cell transplantation after myeloablative conditioning donor selection with a particular focus on the *ct-KIR* score holds promise to prospectively reduce relapse rates without detrimental effects on GvHD and non-relapse mortality. This may prove of particular clinical relevance if more than one appropriate donor with regard to conventional donor selection criteria is identified. Given the major hurdles to perform a randomised trial on *KIR* status-based donor selection in the paediatric setting studies such as ours are one means of providing informed guidance regarding donor selection. As post-transplant leukaemia relapses represent the major obstacle to treatment success and are associated with dismal prognosis our results may stimulate critical review of currently applied donor selection criteria for this group of patients. With more than 25 million registered stem-cell donors worldwide and the growing proportion of instantly available high resolution *HLA*- and *KIR* genotyping results in many registries the identification of an optimal donor based on donor *ct-KIR* score – representing 26% of all donors in our study and more than 31% of the Caucasoid population [3] seems a clinically feasible strategy which could readily be implemented into clinical care without any discernable risk.

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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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Supplement

ALL-BFM-SCT-2003 study trial

Within the ALL-BFM-SCT 2003 trial (Clinical trial information www.clinicaltrials.gov: NC01423747) patients with a matched sibling donor (MSD) or matched unrelated donor (MUD) received a conditioning regimen containing total-body irradiation and etoposide. GvHD prophylaxis consisted of cyclosporine (CSA) 3 mg/kg starting on day -1, in patients who received their grafts from MSDs or from MUDs. For those with MUDs, all patients received antithymocyte globulin (ATG), and methotrexate (10 mg/m2) on days +1, +3 and +6. Patients transplanted from MSD received no in vivo T cell depletion with ATG. This study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice and was approved by the local institutional review board at each participating site.

STATISTICAL ANALYSES

The enhanced bootstrapping method and the .632 estimator[1] were used for the purpose of internal validation of the score definition procedure. This method allowed us to quantify the amount of model overfitting (optimism) and respectively adjust performance measures. A model-based framework [2] has been applied to calculate the following performance measures: the calibration-in-the-large coefficient assessing the ability of the model to make unbiased estimates of the outcome, and the calibration slope coefficient additionally measuring the overfitting in the model's ability to separate subjects according their outcome. The effect of model overfitting on the cumulative incidence of relapse has been calculated as well.

Our score definition procedure [3] was validated by the method of enhanced bootstrapping and the .632 estimator[1].

This method allowed us to quantify the amount of model overfitting (optimism) and respectively adjust performance measures.

A model-based framework[2] has been applied to calculate the following performance measures: the calibration-in-the-large coefficient assessing the ability of the model to make unbiased estimates of the outcome, and the calibration slope coefficient additionally measuring the overfitting in the model's ability to separate subjects according their outcome. The effect of model overfitting on the cumulative incidence of relapse has been calculated as well.

To adjust for potential differences in disease recurrence risk prior to stem cell transplantation we analysed the impact of remission status and time to first relapse in our study cohort. We found that patients transplanted in either CR1 or CR2 with late relapse (i.e. later than 6 months after completion of front-line treatment) displayed a largely comparable and substantially lower relapse risk than patients transplanted in either CR2 with early relapse (i.e. within 6 months after completion of front-line treatment) or in CR3 (Figure S4). Based on these findings we stratified patients with regard to disease recurrence risk as low risk (CR1 and CR2 with late relapse) and high risk (CR2 with early relapse and CR3; Supplemental Figure S5).

In multivariable analyses the impact of the ct-KIR score was adjusted for covariates potentially associated with the major outcome parameters of this study, i.e. relapse incidence, non-relapse mortality and event-free survival. Patient disease and donor characteristics that either proved correlated to *donor ct-KIR* score (i.e. donor type; Table 1) or based on current knowledge are associated with the defined outcomes (i.e. disease recurrence risk and immunophenotype as characteristics reflecting disease biology; stem cell donor type, cytomegalovirus (CMV) serostatus, sex match and AB0 blood group match as impactful characteristics currently employed for donor selection) were entered into the final model.

P-values below 0.05 were considered significant. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).

Table S1: Comparison of Patients Enrolled in the *KIR* study and Patients not enrolled in the *KIR* study.*						
Characteristics	KIR study (N=317)		Non-KIR study (N=94)		Total ALL-SCT-BFM 2003 study	
Patient male sex - no. (%)	194	61%	62	66%	256	62%
Patient age - yr						
Median (range)	9.9 (0.6 - 18.5)		10.3 (0.5 - 18.4)		10.1 (0.5 - 18.5)	
Remission status - no. (%)						
First complete remission	163	51%	46	49%	209	51%
Second complete remission	138	44%	35	37%	173	42%
> Second complete remission§	16	5%	13	14%	29	7%
Immunophenotype - no. (%)						
B lineage	240	76%	69	73%	309	75%
Others	77	24%	25	27%	102	25%
Donor age – yr (median – range)						
Matched sibling donor	11.6 (0.8-27.2)		12.9 (1.7 - 38.1)		11.9 (0.8-38.1)	
Matched unrelated donor	35.6 (18.0-64.6)		34.7 (7.1 - 59.0)		35.3 (7.1-64.6)	
Donor type - no. (%)						
matched sibling donor	86	27%	19	20%	105	26%
9/10 matched unrelated donor	108	34%	38	40%	146	36%
10/10 matched unrelated donor	123	39%	37	39%	160	39%
Cytomegalovirus match (donor-patient) - no.						
Negative-negative	153	49%	39	41%	192	48%
Negative-positive	53	17%	10	11%	63	16%
Positive-negative	43	14%	13	14%	56	14%
Positive-positive	65	21%	23	24%	88	22%
Incomplete data	3		9		12	
Sex match (donor-patient) - no. (%)						
Female - male	72	23%	20	21%	92	23%
Others	243	77%	70	74%	313	77%
Incomplete data	2		4		6	
AB0 blood group match (donor-recipient) -						
AB0 match	134	43%	38	40%	172	44%
Minor mismatch	75	24%	22	23%	97	25%
Major mismatch	106	34%	19	20%	125	32%
Incomplete data	2		15		17	
Conditioning regimen - no. (%)						
Total body irradiation and etoposide	286	90%	84	89%	370	90%
Chemotherapy conditioning	31	10%	10	11%	41	10%
Graft type - no. (%)						
Bone marrow	239	75%	70	74%	309	75%
Peripheral blood stem cells	65	21%	23	24%	88	21%
Cord blood stem cells	4	1%	0	0	4	1%
Other	9	3%	1	1%	10	2%
Disease reccurence risk						
Low risk	232	73%	63	67%	295	72%
High risk	85	27%	31	33%	116	28%

*The chi-square test was used for categorical variables, and the Wilcoxon rank-sum test (Kruskal-Wallis test for more than two populations) for continuous variables. The P-value compares the ALL-SCT-BFM 2003 study patients not included into the *KIR* study vs. *KIR* study patients.

§ P=0.02 for the difference in the distribution of characteristics among *KIR* study and non-*KIR* study patients

Table S2. Proportional Subdistribution Hazards Model for Analyses of Relapse Incidence in 171 patients with available MRD data.*

		Relapse Incidence	
		HR (95% HR CI)	P
ct-KIR Score	1-point increment	0.55 (0.32-0.93)	0.026
	0 (unfavourable)§	1.00	-
	1 (intermediate)	0.43 (0.20-0.95)	0.037
	2 (favourable)	0.32 (0.12-0.84)	0.021
Donor type	Matched sibling	1.00	-
	10/10 matched unrelated	0.86 (0.40-1.86)	0.703
	9/10 matched unrelated	0.80 (0.34-1.86)	0.597
CMV serostatus (donor/recipient)	Positive/positive	1.00	-
	negative/negative	0.77 (0.34-1.86)	0.513
	negative/positive	0.62 (0.22-1.71)	0.363
	positive/negative	0.35 (0.12-1.75)	0.071
Sex match (donor/recipient)	Other	1.00	-
	female/male	0.67 (0.25-1.77)	0.419
AB0 blood group match	Match	1.00	-
	Minor mismatch	0.69 (0.26-1.79)	0.443
	Major mismatch	0.79 (0.37-1.67)	0.531
Immunophenotype	Other	1.00	-
	B-ALL	0.92 (0.40-2.12)	0.845
Disease recurrence risk $	Low risk	1.00	-
	High risk	1.78 (0.76-4.18)	0.188
MRD	Low risk	1.00	-
	High risk	3.34 (1.25-8.88)	0.016

* Competing risks were accounted for according to Fine and Gray[4]

Analyses were adjusted for donor type, CMV serostatus, sex match, AB0 match, immunophenotype of leukemia and remission status at time of HSCT. In order to perform a trend test (1-point increment) the *ct-KIR* score was included into the models as a numerical variable. Data on AB0 blood group were missing for 2 donors, on sex for 2 donors, and on CMV serostatus for 2 patients and 1 donor.

§ For pairwise comparison of groups according to donor *ct-KIR* score this variable was included into the models as a categorical variable.

$ Disease recurrence low risk: transplantation in CR1 or in CR2 after late (>6 months after end of front-line therapy) relapse; high risk: transplantation in CR2 after early (<6 months after end of front-line therapy) relapse or in CR3.

Patient, disease and donor characteristics of the patients with available MRD data vs without MRD data are provided in Table S3.

Table S3: Comparison of Patients With MRD Level Prior to HSCT Data Available and Patients With No MRD Data Available.*

Characteristics	MRD data (N=171)		No MRD data (N=146)		KIR study (N=317)	
Patient male sex - no. (%)§	97	57%	97	66%	194	61%
Patient age – yr						
Median (range)	8.7 (2.0 - 18.3)		10.8 (0.6 - 18.5)		9.9 (0.6 - 18.5)	
Remission status - no. (%)$						
First complete remission	74	43%	89	61%	163	51%
Second complete remission	93	54%	45	31%	138	44%
> Second complete remission	4	2%	12	8%	16	5%
Immunophenotype - no. (%)╪						
B lineage	140	82%	100	68%	240	76%
Others	31	18%	46	32%	77	24%
Donor age – yr (median – range)						
Matched sibling donor	9.2 (0.8-23.9)		13.5 (2.0 – 27.2)		11.6 (0.8-27.2)	
Matched unrelated donor	37.0 (18.0-64.6)		34.0 (19.0 - 57.0)		35.6 (18.0-64.6)	
Donor type - no. (%)						
matched sibling donor	44	26%	42	29%	86	27%
9/10 matched unrelated donor	57	33%	51	35%	108	34%
10/10 matched unrelated donor	70	41%	53	36%	123	39%
Cytomegalovirus match (donor-patient) - no.						
Negative-negative	80	48%	73	50%	153	49%
Negative-positive	27	16%	26	18%	53	17%
Positive-negative	28	17%	15	10%	43	14%
Positive-positive	33	20%	32	22%	65	21%
Incomplete data	3		0		3	
Sex match (donor-patient) - no. (%)∩						
Female – male	30	18%	42	29%	72	23%
Others	140	82%	103	71%	243	77%
Incomplete data	1		1		2	
AB0 blood group match (donor-recipient) -						
AB0 match	77	46%	57	39%	134	43%
Minor mismatch	40	24%	35	24%	75	24%
Major mismatch	52	31%	54	37%	106	34%
Incomplete data	2		0		2	
Conditioning regimen - no. (%)						
Total body irradiation and etoposide	159	93%	127	87%	286	90%
Chemotherapy conditioning	12	7%	19	13%	31	10%
Graft type - no. (%)						
Bone marrow	128	77%	111	78%	239	75%
Peripheral blood stem cells	36	22%	29	20%	65	21%
Cord blood stem cells	2	1%	2	1%	4	1%
Other	5		4		9	3%
Disease reccurence risk						
Low risk	124	73%	108	74%	232	73%
High risk	47	27%	38	26%	85	27%

*The chi-square test was used for categorical variables, and the Wilcoxon rank-sum test (Kruskal-Wallis test for more than two populations) for continuous variables. The P-value compares the KIR study patients with data on MRD level prior to HSCT available vs. *KIR* study patients with no data on MRD level prior to HSCT available.

§ P=0.02 for the difference in the distribution of patient age among MRD and non-MRD patients

$ P<0.001 for the difference in the distribution of complete remission among MRD and non-MRD patients

⋕ P=0.006 for the difference in the distribution of immunophenotype among MRD and non-MRD patients

∩ P=0.02 for the difference in the distribution of sex match among MRD and non-MRD patients

Figure legends

Figure S1. Cumulative Incidence of Relapse According to Donor ct-KIR score in Patients Undergoing HLA-identical Sibling Donor Transplantation.

Figure S2. Cumulative Incidence of Relapse According to Donor ct-KIR score in Patients Undergoing HLA-Matched Unrelated Donor Transplantation.

Figure S3. Cumulative Incidence of Relapse in Patients with MRD less than 10^{-3} leukemic cells (MRD standard risk) or MRD $\geq 10^{-3}$ leukemic cells (MRD high risk).

Figure S4. Cumulative Incidence of Relapse According to Number of Complete Remission and Time Point of Relapse before Second Remission. Early relapse defined as relapse within 6 months after completion of front-line treatment = fewer than 30 months after initial diagnosis; late relapse defined as relapse more than 6 months after completion of front-line treatment = later than 30 months after initial diagnosis.

Figure S5. Cumulative Incidence of Relapse in Patients undergoing Transplantation in CR1 or in CR2 after Late Relapse (low disease recurrence risk) vs. Transplantation in CR2 after Early Relapse or in CR3 (high disease recurrence risk).

Figure S6. Cumulative Incidence of Relapse in Patients and Donors with a KIR – KIR Ligand mismatch vs. KIR – KIR Ligand Match.

Figure S7. Cumulative Incidence of Relapse According to Donor ct-KIR score in Patients with Low Risk MRD Level Prior to HSCT (<10-3).

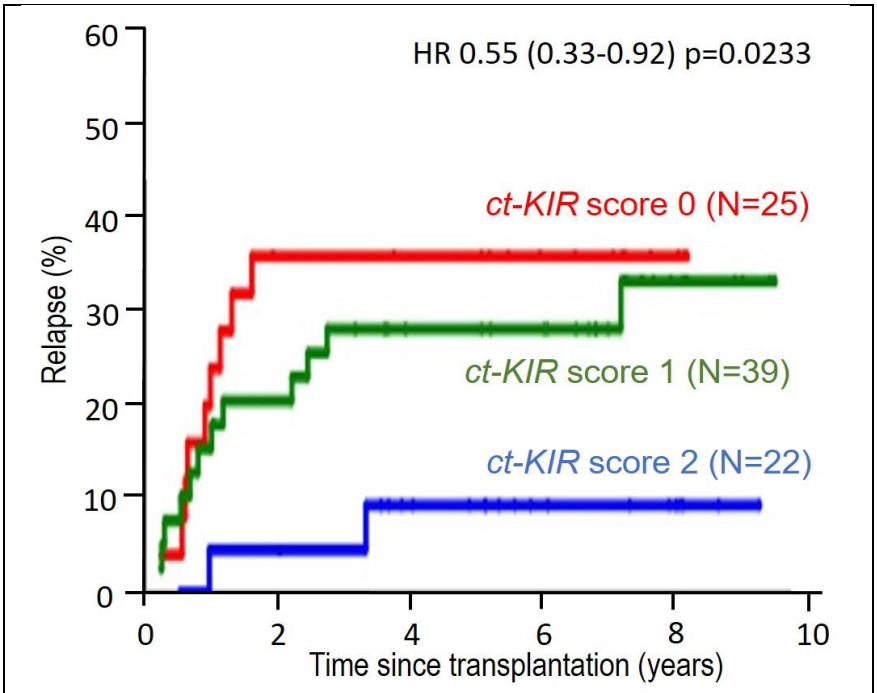

Figure S1. Cumulative Incidence of Relapse According to Donor *ct-KIR* score in Patients Undergoing HLA-identical Sibling Donor Transplantation.

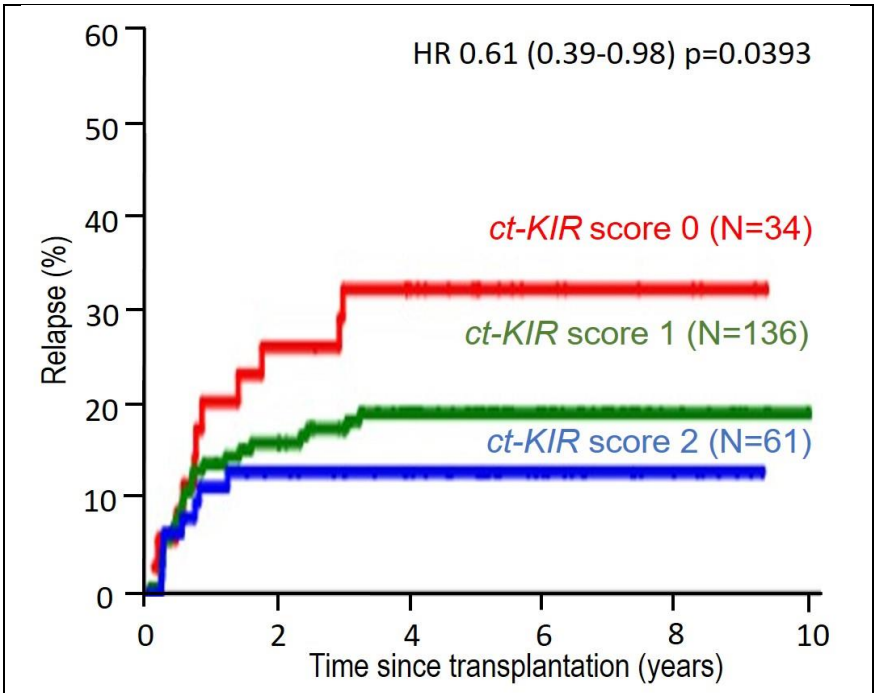

Figure S2. Cumulative Incidence of Relapse According to Donor *ct-KIR* score in Patients Undergoing HLA-Matched Unrelated Donor Transplantation.

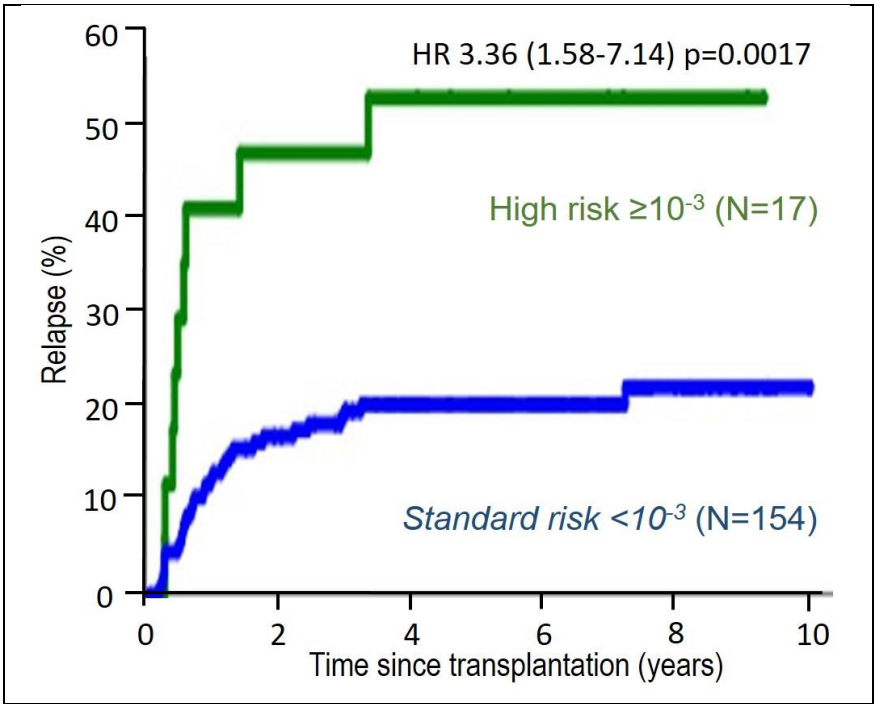

Figure S3. Cumulative Incidence of Relapse in Patients with MRD less than 10^{-3} leukemic cells (MRD standard risk) or MRD ≥10^{-3} leukemic cells (MRD high risk).

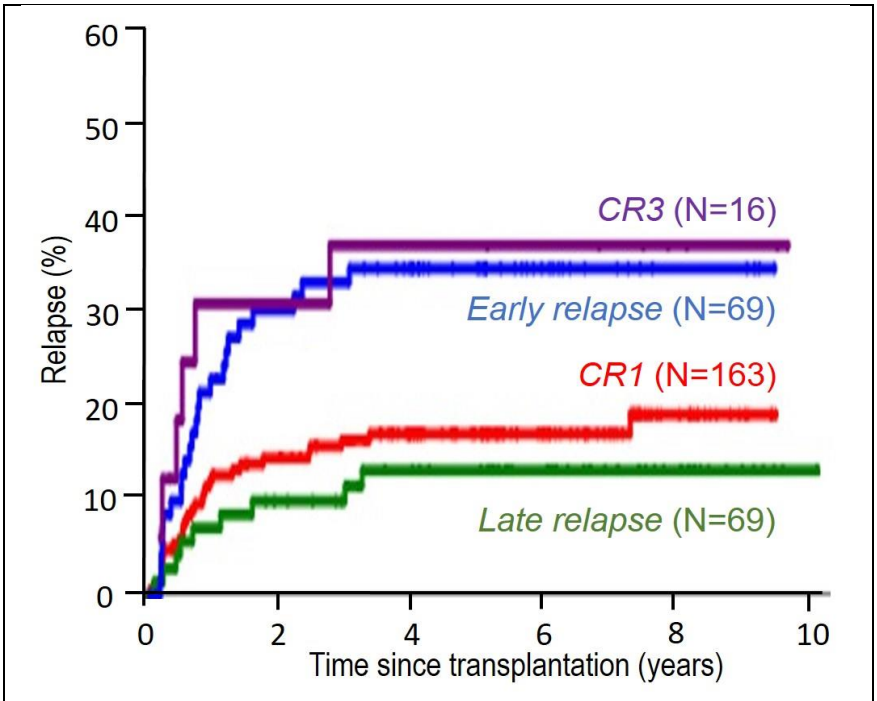

Figure S4. Cumulative Incidence of Relapse According to Number of Complete Remission and Time Point of Relapse before Second Remission.

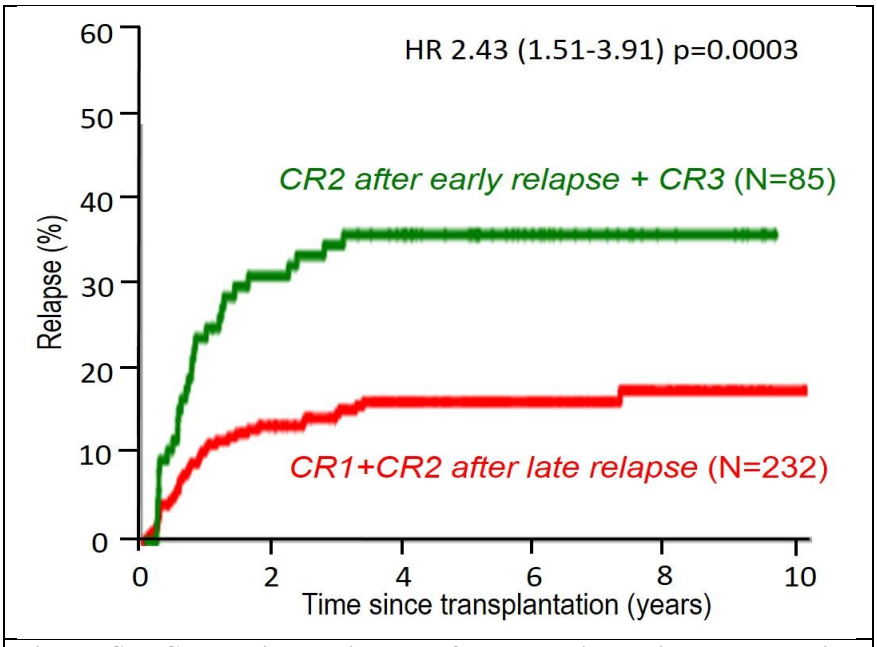

Figure S5. Cumulative Incidence of Relapse in Patients undergoing Transplantation in CR1 or in CR2 after Late Relapse (low disease recurrence risk) vs. Transplantation in CR2 after Early Relapse or in CR3 (high disease recurrence risk)

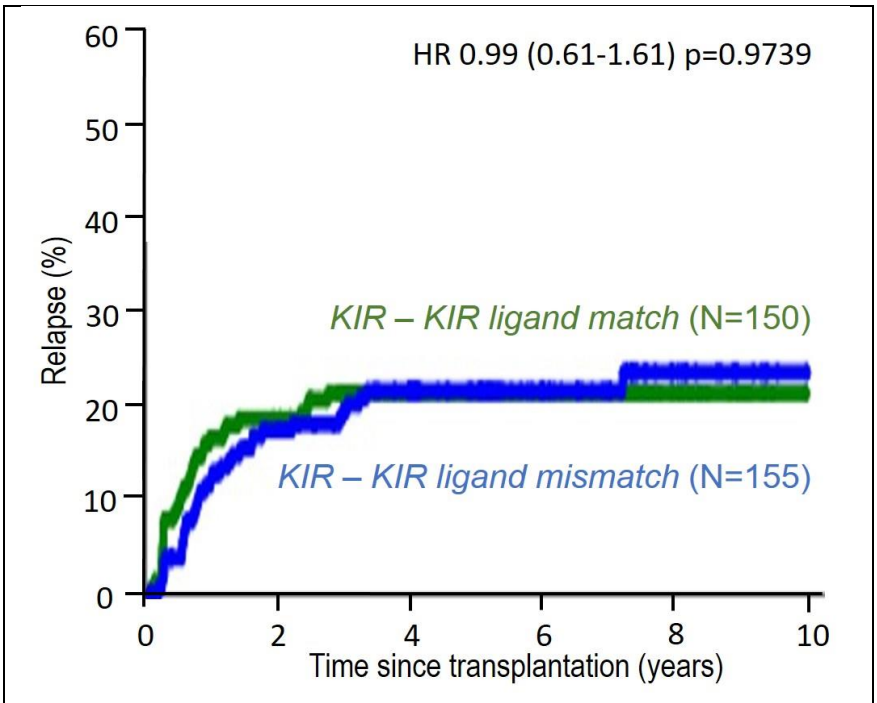

Figure S6. Cumulative Incidence of Relapse in Patients and Donors with a KIR – KIR Ligand mismatch vs. KIR – KIR Ligand Match.

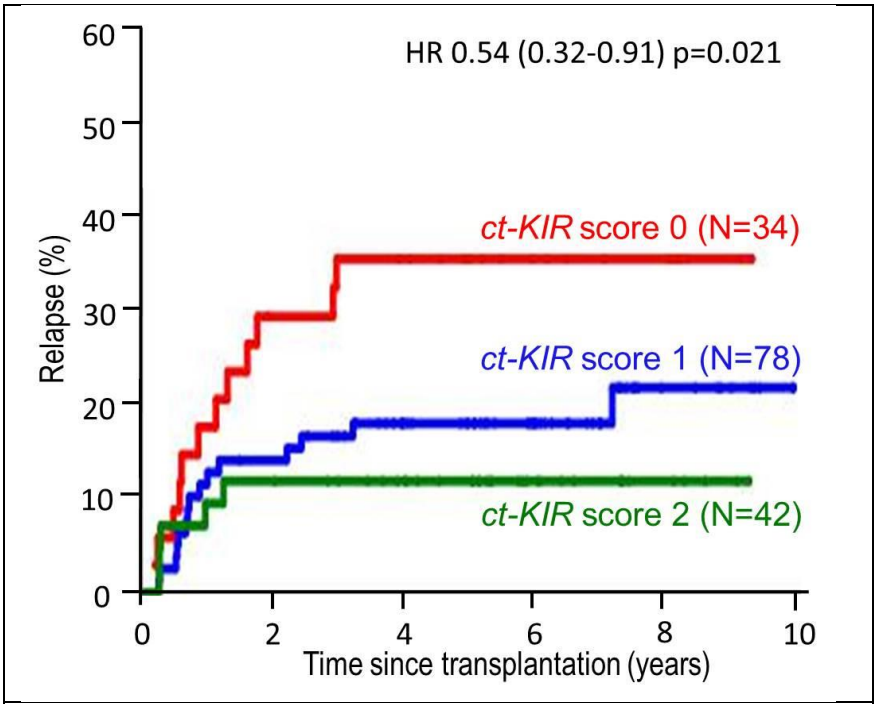

Figure S7. Cumulative Incidence of Relapse According to Donor *ct-KIR* score in Patients with Low Risk MRD Level Prior to HSCT ($<10^{-3}$).

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