Untersuchungen zu HLA-Klasse I-spezifischen Rezeptoren auf zytotoxischen Lymphozyten

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1. Einleitung

Eine Hauptaufgabe des Immunsystems ist die Bekämpfung von Infektionen. Ein Grundproblem, das hierbei gelöst werden muss, ist die Unterscheidung von Selbst- im Gegensatz zu Fremdstrukturen. Das Immunsystem hat im Verlauf der Evolution zwei unterschiedliche, sich ergänzende Strategien zur Lösung dieses Problems entwickelt. Das eine System beruht auf der Erkennung von Fremdantigenen mithilfe von antigenspezifischen Rezeptoren. Diese werden zum einen in Form von Antikörpern von B-Zellen gebildet und zum anderen in Form von T-Zell-Rezeptoren von T-Zellen. B- und T-Zellen begegnen der extremen Vielfalt der potentiellen Fremdantigene durch eine ebenso große Vielfalt ihrer Antigenrezeptoren, was durch die somatisches Rekombination vieler variabler Genelemente und die anschließende Selektion geeigneter rearrangierter Rezeptoren im Rahmen der B- und T-Zellreifung erreicht wird (1, 2). Jeder einzelne reife T- oder B-Zell-Klon trägt dabei jeweils einen klonotypischen Antigenrezeptor mit Spezifität für ein bestimmtes antigenes Epitop. Während B-Zellen Fremdantigene im Rahmen der humoralen Immunantwort direkt erkennen und ihre Antigenrezeptoren in Form von Antikörpern sezernieren können, ist die spezifische T-Zellantwort MHC- (Major Histocompatibility Complex) restringiert, d. h. das Fremdantigen wird nur im Kontext von autologen MHC-Molekülen erkannt (3).

Die zweite Strategie zur Unterscheidung von Selbst und Fremdstrukturen schlägt den umgekehrten Weg ein. Anstatt ein Fremdantigen aufzuspüren wird überprüft, ob eine spezifische Struktur auf der Oberfläche der eigenen Körperzellen vorhanden ist. Die Präsenz dieser Struktur führt zur Inhibition der Immunantwort. Fehlt die Struktur, wird die Zelle als fremd erkannt und eliminiert. Die Effektorzelle dieser, erstmals von Kärre 1981 postulierten, Strategie der "Missing-Self"-Erkennung ist die Natürliche Killer (NK)-Zelle (4). Die erwähnten Erkennungsstrukturen, eine Art molekularer Mitgliedsausweis, sind MHC-Klasse-I-Moleküle (5), die gleichen Moleküle, die auch die antigenspezifische, T-Zell-Rezeptor-vermittelte Erkennung durch zytotoxische T-Zellen ermöglichen. Im Gegensatz zur T-Zelle, die durch die Erkennung des Fremdpeptids im Kontext des autologen MHC-Moleküls aktiviert wird, führt die Erkennung der autologen MHC-Klasse-I-Strukturen zur Inhibition der NK-Zelle.

Das Konzept der "Missing-Self"-Erkennung ist der Schlüssel zum Verständnis zweier klinisch relevanter Beobachtungen, die sich durch das klassische Konzept der MHC-restringierten

Immunantwort nicht erklären ließen. Zum einen ist schon seit den 1950er Jahren das Phänomen der Hybrid-Resistenz bekannt (6, 7), bei dem die (A x B)F1-Generation homozygote Transplantate sowohl des A als auch des B-Typs abstößt (Wobei A und B den MHC-Typ bezeichnet). Nach den bekannten Gesetzen der Transplantation müsste die F1-Generation die parentalen Transplantate akzeptieren, da keine fremden MHC-Antigene übertragen werden. Der zweiten Beobachtung, die sich ebenfalls nicht mit den klassischen immunologischen Konzepten erklären ließ, verdanken die Natürlichen Killerzellen ihren Namen: Das Phänomen des "natural killing" bezeichnete eine frühe, nicht-MHC-restringierte Immunantwort gegen Tumorzellen (8, 9). Natürliche Killeraktivität wurde verstärkt bei Tumorzellen mit fremden oder fehlenden MHC-Antigenen festgestellt. In beiden Fällen liefert die "Missing-Self"-Theorie eine schlüssige Erklärung: Durch das Fehlen passender MHC-Antigene wird die Inhibition der NK-Zelle aufgehoben und der Prozess der natürlichen Zytotoxizität in Gang gesetzt.

Die molekulare Basis für die MHC-abhängige natürliche Killeraktivität sind MHC-Klasse-I-spezifische Rezeptoren, die sich auf der Oberfläche aller NK-Zellen und einer Subpopulation von T-Zellen befinden. Überraschenderweise besitzen Nagetiere und Primaten zwei strukturell unterschiedliche Familien von NK-Rezeptoren. Während die Maus NK-Rezeptoren der Lektin-ähnlichen Ly49-Genfamilie besitzt (10-12), sind diese im Menschen bis auf ein inaktives Genfragment verschwunden (13). Dafür besitzt der Mensch (sowie andere Primaten) NK-Rezeptoren der KIR- (Killer Cell Immunoglobulin-like Receptor) Familie (14-18), die in der Maus nicht vorhanden ist (19). Die Existenz zweier strukturell unverwandter Rezeptorfamilien mit sehr ähnlicher Funktion ist ein Paradebeispiel für konvergente Evolution, wobei die Gründe für diese erstaunliche Entwicklung nach wie vor unbekannt sind.

KIR-Rezeptoren gehören aufgrund ihrer Struktur zur Immunglobulin-Superfamilie und besitzen entweder zwei (Nomenklatur: KIR2D) oder drei (KIR3D) extrazelluläre Immunglobulin-ähnliche Domänen. Die Signaltransduktion der inhibitorischen KIR erfolgt über ITIM- (Immunoreceptor tyrosine-based inhibition motif) Motive in der zytoplasmatischen Kette, die phosphoryliert werden, wenn der entsprechende Ligand gebunden wird und anschließend eine inhibitorische Signalkaskade initiieren (20-22). Daneben existieren auch stimulatorische KIR, die eine kurze zytoplasmatische Kette ohne ITIM-Motive besitzen (17, 23-25). In der offiziellen Nomenklatur sind inhibitorische KIR

durch ein L (long = lange zytoplasmatische Kette) und stimulatorische KIR durch ein S (short) gekennzeichnet (26, 27).

KIR-Rezeptoren sind spezifisch für HLA-Klasse-I-Moleküle, die humane Variante der MHC-Antigene. Die HLA-Klasse-I-Gene sind hochvariabel und werden von den drei Genen HLA-A, -B, und -C kodiert. Für die verschiedenen HLA-Klasse-I-Gene existieren spezifische KIR-Rezeptoren, die bestimmte Varianten erkennen. Zwei verschiedene, inhibitorische KIR (KIR2DL1 und KIR2DL3) erkennen die beiden Varianten eines Dimorphismus des HLA-C-Moleküls (14, 28, 29), ein weiterer KIR (KIR3DL1) bindet spezifisch an das Bw4-Epitop des HLA-B-Moleküls (15, 30), und für HLA-A wurde ein weiterer KIR (KIR3DL2) beschrieben (31), dessen spezifische Bindungsstelle nicht bekannt ist. Für einige, vor allem stimulatorische, KIR ist der Ligand noch unbekannt. Neben der KIR-Familie existiert ein weiterer inhibitorischer, MHC-Klasse-I-spezifischer Rezeptor, das Dimer CD94:NKG2A, der sowohl in Maus als auch Mensch vorkommt (32-35). Während KIR-Rezeptoren bestimmte Untergruppen von HLA-Genen erkennen, besitzt CD94:NKG2A im Menschen eine breite Spezifität für die Produkte aller drei HLA-Klasse-I-Gene. Der eigentliche Bindungspartner von CD94:NKG2A ist HLA-E, ein nicht-klassisches Mitglied der HLA-Genfamilie, das auf die Präsentation von Signalpeptiden der drei klassischen HLA-Klasse-I-Gene A, B, und C spezialisiert ist (36-40). Die Erkennung des Komplexes aus HLA-E und Signalpeptid durch CD94:NKG2A führt zur Inhibition der NK-Zelle(41).

2. Eigene Untersuchungen

Die Entdeckung von MHC-Klasse-I-spezifischen NK-Rezeptoren in Maus und Mensch eröffnete die Möglichkeit, die wichtige Rolle der NK-Zellen im Rahmen der Bekämpfung von Virusinfektionen und der Eliminierung von Tumorzellen besser zu verstehen. Unser Ziel war es zunächst, experimentell die Funktion und Spezifität der NK-Zelle mit der Expression dieser Rezeptoren zu korrelieren und festzustellen, in welchem Maß die Erkennung von Zielzellen tatsächlich durch dieses Rezeptorsystem kontrolliert wird. Zu diesem Zweck wurden Methoden zur genetischen Analyse der KIR-Genfamilie entwickelt. Mithilfe der neuen Typisierungstechniken wurden dann die genetischen Grundlagen der humanen KIR-Genfamilie untersucht und das NK-Rezeptor-Repertoire auf NK- und T-Zellen im Detail beschrieben. Die immungenetischen Untersuchungen bildeten die Grundlage für die Analyse des funktionalen NK-Zell-Repertoires. Unter anderem wurde die Vorhersage der Missing-

Self-Hypothese getestet, dass jede NK-Zelle einen inhibitorischen Rezeptor für ein eigenes MHC-Antigen exprimieren muss um tolerant zu sein. Des Weiteren wurde die Expression von KIR auf T-Zellen untersucht und die Ergebnisse zu einer Hypothese zusammengefasst, die eine Rolle der KIR bei der Entwicklung der zytotoxischen Gedächtnis T-Zellen postuliert.

2.1 Genetische Analyse der KIR-Rezeptoren

2.1.1 Charakterisierung der KIR-Gene als polygene und polymorphe Genfamilie

Um die Expression der verschiedenen KIR-Gene genauer untersuchen zu können, wurde eine Methode entwickelt, die eine spezifische Typisierung der KIR mRNAs mittels Reverser Transkription (RT) und anschließender Polymerase-Kettenreaktion (PCR) erlaubt. Zu diesem Zweck wurde für jedes der 12 verschiedenen, bekannten KIR-Gene ein spezifisches Primerpaar ausgewählt, welches alle Varianten des jeweiligen Gens amplifizierte. Die Spezifität der Typisierungsmethode wurde an einer Serie von Zelllinien getestet, die mit jeweils einer KIR cDNA stabil transfiziert worden waren. Mithilfe dieser verifizierten Methode konnte gezeigt werden, dass eine große Variabilität in der Expression von KIR existiert. In 52 untersuchten Individuen wurden 18 verschiedene KIR-Expressionsmuster gefunden. Drei der zwölf KIR-Gruppen wurden in allen Individuen exprimiert, nämlich KIR2DL1, KIR2DL4 und KIR3DL2. Die anderen neun KIR-Gruppen wurden in verschiedenen Frequenzen von 0% (KIR2DS5) bis zu 91% (KIR3DL1) exprimiert. Um die Variabilität der KIR auch auf genetischer Ebene untersuchen zu können, wurde ein weiteres, auf DNA basierendes Typisierungssystem entwickelt. Es stellte sich heraus, dass die Anzahl und Art der KIR-Gene variabel ist und genau mit den Expressionsdaten korrelierte. Die Ergebnisse zeigen, dass die beobachteten Expressionsunterschiede nicht etwa durch eine differentielle Regulation der vorhandenen KIR-Gene zustande kommen, sondern durch die individuell unterschiedliche Zusammensetzung der KIR-Genfamilie auf genomischer Ebene. Grundsätzlich gilt, dass alle KIR-Gene, die vorhanden sind auch exprimiert werden (wobei die einzelne NK-Zelle im Normalfall nur einen Teil der vorhandenen KIR-Gene exprimiert, siehe 2.2). Es wurde ein bestimmter KIR-Haplotyp, bestehend aus fünf KIR-Genen der inhibitorischen Gruppe sowie einem KIR-Gen der stimulatorischen Gruppe, definiert, der mit einer Frequenz von 33% in der kaukasoiden Bevölkerung weitaus häufiger als alle anderen KIR-Haplotypen auftrat. Die weitere Untersuchung des KIR-Polymorphismus mithilfe von Hybridisierungen mit KIR-spezifischen Sonden sowie DNA-Typisierung ergab, dass dieser

als Haplotyp A bezeichnete häufige KIR-Typ auch in acht anderen ethnischen Bevölkerungsgruppen häufig vorkommt. Neben Haplotyp A wurde eine Vielzahl anderer KIR-Genkombinationen mit sechs bis zehn verschiedenen KIR-Genen gefunden, die als Gruppe B-Haplotypen zusammengefasst werden.

2.1.2 Identifikation der häufigsten KIR-Haplotypen durch Segregationsstudien

Um die Organisation der KIR-Gene genauer untersuchen zu können wurde eine Familienstudie durchgeführt. In 21 kaukasoiden Familien wurden 16 verschiedene Haplotypen gefunden. Für die 8 häufigsten KIR-Haplotypen konnte die Segregation vollständig aufgeklärt werden. Neben dem bekannten Haplotyp A besitzen die sieben häufigsten Gruppe B Haplotypen zwischen 7 und 11 KIR-Gene. Vor allem die Anzahl der stimulatorischen KIR-Gene variiert stark zwischen einem und bis zu fünf verschiedenen. Daneben treten auch Deletionen von inhibitorischen KIR-Genen in Gruppe B-Haplotypen auf, und zwar können das HLA-C-spezifische KIR2DL1 sowie das HLABw4-spezifische KIR3DL1 fehlen. Insgesamt scheint die Diversifizierung der KIR-Gene zweigeteilt zu sein: Auf der einen Seite erscheint die Genkonstellation des häufigen Haplotyps A konserviert und definiert damit eine minimale Grundausstattung an KIR-Genen und auf der anderen Seite entwickeln sich sehr schnell, wahrscheinlich durch Genduplikationen, neue Gruppe B-Haplotypen, die über ein überwiegend komplementäres Spektrum an KIR-Genen verfügen. Die ebenfalls durchgeführte HLA-Klasse-I-Subtypisierung der Familienmitglieder zeigt, dass in 99% aller Fälle mindestens ein HLA-Klasse-I-kodierter Ligand für ein inhibitorisches KIR-Gen vorhanden ist. Dies ist insofern interessant, da diese beiden hochpolymorphen Genfamilien auf verschiedenen Chromosomen liegen und damit unabhängig voneinander segregieren. In einem Fall kam es allerdings zu einer KIR/HLA Konstellation, bei der die betreffende Person über keine HLA-Liganden für eines der vererbten KIR-Gene verfügt. Es ist anzunehmen, dass in diesem Fall die notwendige Toleranzentwicklung der NK-Zellen durch die Expression des alternativ zur Verfügung stehenden, inhibitorischen Lektins CD94:NKG2A sichergestellt wird.

2.2 Expression und Funktion von KIR-Rezeptoren auf NK-Zellen

Die Interaktion verschiedener polymorpher inhibitorischer und stimulatorischer Rezeptoren auf NK-Zellen mit ebenfalls hochpolymorphen HLA-Klasse-I-Liganden auf Zielzellen stellt

ein komplexes System dar, welches sich auf polyklonaler Ebene, also in einer gemischten Population von NK-Zellen, nur schwer analysieren lässt. Es wurde deshalb ein experimentelles System entwickelt, dass eine Reduzierung der Untersuchungsparameter auf wenige Variablen ermöglicht: Zunächst wurden aus primären, polyklonalen NK-Zellen eines bestimmten Spenders durch Einzelzellsortierung und anschließende Kultivierung NK-Zellklone etabliert. Die Spezifität der NK-Zellklone für jedes der eigenen HLA-Klasse-I-Antigene wurde dann einzeln untersucht. Zu diesem Zweck wurden die sechs verschiedenen HLA-Klasse-I-Gene dieses Spenders sequenziert und jeweils einzeln in eine HLA-Klasse-I-defiziente Zelllinie (die EBV-transformierte Zelllinie 721.221) transfiziert. Die sechs so generierten, stabilen Transfektanten wurden dann separat als Zielzellen für jeweils einen der eigenen NK-Zellklone eingesetzt. Es zeigte sich, dass alle NK-Zellklone durch eine oder mehrere der autologen HLA Klasse-I-Antigene inhibiert wurden, wohingegen die parentale Zelllinie, die kein HLA-Antigen exprimierte, ausnahmslos von allen NK-Zellen lysiert wurde. Ein paralleles Experiment mit einem zweiten Spender führte zum gleichen Resultat: Alle NK-Zellklone waren tolerant gegen Zelllinien, die mit autologen HLA-Klasse-I-Antigenen transfiziert worden waren.

Um das funktionale Repertoire der verschiedenen NK-Zellen mit der Expression von NK-Zell-Rezeptoren korrelieren zu können, wurde das KIR- und CD94:NKG2A-Expressionsmuster jedes NK-Zellklons molekularbiologisch untersucht. Zu diesem Zweck wurden die KIR-Gene der beiden Spender sequenziert und die Expression der Rezeptoren auf den NK-Zellklonen mittels der in 2.1.1 dargestellten Typisierungsmethode untersucht. Diese Untersuchung ergab, dass die NK-Zellklone unterschiedliche Kombinationen der genomisch vorhandenen KIR-Rezeptoren exprimierten. Die Variationsbreite reichte dabei von der Expression eines einzigen bis zur Expression von neun verschiedenen KIR-Genen, wobei die meisten NK-Zellen in beiden Spendern 3 bis 5 KIR-Gene exprimierten. Der Vergleich der HLA-Spezifität der verschiedenen Klone mit ihrem KIR-Expressionsmuster ergab, dass die funktionale Inhibition einer NK-Zelle immer mit der Expression eines entsprechenden inhibitorischen KIR für ein autologes HLA-Klasse-I-Antigen korrelierte. Die Wirkung eines inhibitorischen KIR konnte dabei nicht durch die gleichzeitige Expression eines stimulatorischen KIR aufgehoben werden. Es stellte sich weiterhin heraus, dass jede NK-Zelle mindestens einen inhibitorischen KIR für ein autologes HLA-Klasse-I-Antigen oder alternativ den CD94:NKG2A Rezeptor exprimierte. Die Ergebnisse zeigen, dass sich die Zielzell-Spezifität einer NK-Zelle nahezu vollständig durch das Repertoire der beiden

komplementären Rezeptorsysteme KIR und CD94:NKG2A erklären lässt. Die Ergebnisse implizieren weiterhin, dass die Toleranzentwicklung von NK-Zellen wesentlich von der Expression geeigneter HLA-Klasse-I-spezifischer Rezeptoren abhängt.

Während NK-Zellen tolerant gegen eigene Körperzellen sind, kann es im Falle einer Stammzelltransplantation zu NK-Zell-vermittelten Abstoßungsreaktionen kommen (42). Um den Einfluss des NK-Zell-Repertoires auf die Alloreaktivität von NK-Zellen näher zu untersuchen, wurden gemischte Lymphozytenkulturen mit NK-Zellen eines Spenders und Zielzellen eines anderen Spenders durchgeführt. Es stellte sich heraus, dass manche NK-Klone zytotoxisch gegen fremde Zielzellen waren, während andere NK-Klone keine Reaktivität aufwiesen. Die Untersuchung der NKR-Expression zeigte, dass die alloreaktiven NK-Zellklone keinen inhibitorischen Rezeptor für eines der HLA-Klasse-I-Antigene besaßen, während tolerante Zellen immer einen inhibitorischen KIR für eines der HLA-Klasse-I-Antigene des Fremdspenders oder alternativ den CD94:NKG2A Rezeptor exprimierten. Damit konnte gezeigt werden, dass NK-Zell-vermittelte Alloreaktivität im wesentlichen von zwei molekularen Komponenten abhängt: Zum einen dem Polymorphismus der HLA-Klasse-I-Gene und zum anderen vom Repertoire der NK-Zell-Rezeptoren.

2.3 Expression und Funktion von KIR-Rezeptoren auf T-Zellen

2.3.1 Analyse der KIR- und CD94:NKG2A-Expression auf T-Zellen

KIR und CD94:NKG2 Rezeptoren werden nicht nur auf NK-Zellen, sondern auch auf Subpopulationen von αβ- und δγ-T-Zellen exprimiert (43, 44). Um das Repertoire dieser Rezeptoren auf T-Zellen genauer zu charakterisieren, wurden T-Zellen nach dem gleichen Protokoll, dass auch bei der NK-Zellklonierung angewendet wurde, kloniert (siehe 2.2). Um die Analyse des T-Zell-Repertoires mit den vorangegangenen Untersuchungen des NK-Zell-Repertoires vergleichen zu können, wurde wiederum peripheres Blut der gleichen Spender für die Klonierung verwendet. Die durchflusszytometrische Analyse ergab, dass die KIR-exprimierenden αβ-T-Zellklone beider Spender zytotoxische $CD8^+$ T-Zellen waren, während die meisten der δγ-T-Zellen $CD4^-CD8^-$ waren. Die Expression der verschiedenen KIR-Gene wurde mit dem gleichen RT-PCR-Typisierungsprotokoll untersucht, das auch für die Analyse von NK-Zellen verwendet worden war. Es stellte sich heraus, dass alle KIR-Gene, die auf genomischer Ebene vorhanden sind, vom jeweiligen Spender auf seinen T-Zellen exprimiert

werden können. Das KIR-Repertoire der T-Zellen war dabei dem NK-Zell-Repertoire sehr ähnlich. In beiden Spendern wurden sowohl inhibitorische als auch stimulatorische KIR in vergleichbaren Frequenzen auf T- und NK-Zellen exprimiert. Eine Ausnahme bildete dabei das HLA-C spezifische inhibitorische KIR2DL1, dass in beiden Spendern auf T-Zellen geringer exprimiert wurde. Größere Unterschiede waren hingegen bei der Expression des CD94:NKG2A Rezeptors zu beobachten: Während die Expression von CD94:NKG2A auf αβ-T-Zellen nur sehr selten zu beobachten war, war sie auf δγ-T-Zellen, ebenso wie auf NK-Zellen, auf über 50% aller Klone festzustellen. Insgesamt war das Repertoire der δγ-T-Zellen in beiden Spendern dem der NK-Zellen ähnlicher als dem der αβ-T-Zellen. Der Vergleich des HLA-Klasse-I-Typs der beiden Spender mit der Expression der entsprechenden inhibitorischen Liganden ergab, dass in einem Spender alle T-Zellen einen spezifischen KIR oder CD94:NKG2A-Rezeptor exprimierten. Im zweiten Spender ergaben sich jedoch gravierende Unterschiede zwischen den verschiedenen Lymphozyten-Populationen. Während alle NK-Zellen und über 80% der δγ T-Zellen dieses Spenders einen inhibitorischen Rezeptor für ein eigenes HLA-Klasse-I-Antigen exprimierten, war dies nur in 27% der αβ T-Zellklone der Fall. Damit konnte gezeigt werden, dass die Expression von NK-Rezeptoren auf T-Zellen nicht gemäß der "Missing-Self"-Hypothese erfolgt, nach der jede NK-Zelle einen inhibitorischen Rezeptor für ein autologes HLA-Klasse-I-Antigen exprimieren muss, um tolerant zu sein. Diese Beobachtung legte nahe, dass die Expression von inhibitorischen NK-Rezeptoren auf T-Zellen nicht der Toleranzentwicklung dient.

Des weiteren wurde untersucht, welche T-Zell-Rezeptor-Rearrangements in NK-Rezeptor-exprimierenden T-Zellen vorliegen. Zu diesem Zweck wurde eine bereits zu einem früheren Zeitpunkt entwickelte, familienspezifische RT-PCR-Methode eingesetzt (45, 46). Überraschenderweise exprimierten alle αβ-T-Zellklone des einen Spenders die Vβ16 Familie, die in den meisten Spendern nur in geringer Frequenz vorkommt (46). Insgesamt 55 verschiedenen T-Zellklone zeigten identische Rearrangements der hypervariablen Bereiche sowohl der TCRα- als auch der TCRβ-Kette und müssen somit nach der klassischen Definition als identische T-Zellklone angesehen werden, die durch klonale Expansion einer einzelnen aktivierten T-Zelle entstanden sind. Die Analyse der KIR-Expression dieser Klone zeigt jedoch ein heterogenes Muster mit 18 verschiedenen KIR-Kombinationen. Auch in dem anderen Spender konnten mehrere T-Zellklone nachgewiesen werden, die den gleichen T-Zell-Rezeptor aber ein unterschiedliches KIR-Repertoire aufwiesen. Diese Ergebnisse zeigen

eindeutig, dass die Expression und Diversifizierung von KIR erst nach Abschluss der TCR-Rearrangements auf dem Niveau der reifen T-Zelle initiiert wird.

2.3.2 Untersuchungen zur Funktion von KIR-Rezeptoren auf LIR1-exprimierenden T-Zellen

Neben der HLA-Klasse-I-spezifischen NK-Rezeptorfamilie KIR existiert mit LIR1 ein weiterer inhibitorischer Rezeptor, der auf NK- und T-Zellen vorkommt und mit geringer Affinität an einen nicht-polymorphen Bereich des HLA-Klasse I Moleküls bindet (47-49). LIR1 ist strukturell mit den KIR-Genen verwandt und befindet sich auf Chromosom 19 direkt neben den KIR-Genen (50). Die durchflusszytometrische Analyse des LIR1-Rezeptors in peripherem Blut von 20 verschiedenen Spendern zeigt, dass LIR1 und KIR auf überlappenden Populationen von zytotoxischen T-Zellen exprimiert werden, wobei die KIR-Expression auf NK-Zellen überwiegt, während die LIR1-Expression auf T-Zellen überwiegt. Um den funktionalen Zusammenhang von LIR1- und KIR-Expression genauer analysieren zu können, wurden wiederum von den gleichen Spendern, deren NK- und KIR^+T-Zellen bereits analysiert worden waren, $LIR1^+$T-Zellklone generiert. Überraschenderweise exprimierten fast alle in Zellkultur etablierten $LIR1^+$T-Zellklone auch KIR, während das *in vivo* bei nur ungefähr 30% der LIR^+T-Zellen der Fall war. Eine mögliche Erklärung hierfür wäre ein positiver Einfluss der KIR-Expression auf das Überleben der Zellen, was zu einer Anreicherung der KIR-exprimierenden T-Zellen im Laufe der mehrwöchigen Kultivierung führen würde. Um diese Hypothese zu überprüfen, wurden die verschiedenen T-Zellklone aktiviert, um ein Phänomen zu induzieren, das als aktivierungsinduzierter Zelltod bezeichnet wird (AICD) und für Effektor T-Zellen das normale Schicksal darstellt (51). Wurden die KIR^--T-Zellklone durch Kreuzvernetzung des CD3-Komplexes aktiviert, führte dies wie erwartet zum raschen apoptotischen Tod dieser Zellen. Im Gegensatz dazu zeigten KIR^+T-Zellklone eine starke Resistenz gegen AICD. Auch bei der antigenspezifischen Aktivierung durch HLA-Peptid-Komplexe zeigte sich, dass nur die KIR^+T-Zellklone resistent gegen AICD waren, während KIR^-T-Zellklone apoptotisch wurden. Schließlich zeigten durchflusszytometrische Analysen von $CD8^+$T-Zellen aus peripherem Blut, dass die KIR^+T-Zellen im Vergleich zu KIR^-T-Zellen wesentlich höhere Mengen an Bcl-2 exprimierten, einem Molekül, das eine wichtige Rolle bei der Resistenz gegen AICD spielt (52).

2.3.3 Entwicklung einer Hypothese zur Rolle von KIR bei der Entwicklung von $CD8^+$Gedächtnis-T-Zellen

Die oben dargestellten Ergebnisse zeigen erstmals einen Zusammenhang zwischen der Expression von NK-Rezeptoren und der Funktion von Gedächtnis T-Zellen auf. Auf der Basis dieser und der in Abschnitt 2.3.1 dargestellten Daten wurde die Hypothese entwickelt, dass die sequentielle Expression von NK-Rezeptoren eine wesentliche Rolle bei der Entwicklung des Repertoires zytotoxischer T-Zellen spielt: Im Rahmen einer Virusinfektion kommt es zur antigenspezifischen Aktivierung von zytotoxischen T-Zellen. Dies führt zunächst zur Expression von LIR1-Rezeptoren. Diese wird durch Experimente mit fluoreszenzmarkierten CMV- und EBV-spezifischen HLA-Peptidkomplexen belegt, sogenannten HLA-Tetrameren (53). Mehr als die Hälfte aller CMV und EBV-spezifischen cytotoxischen T-Zellen exprimierten LIR1-Rezeptoren, während die KIR-Expression nur auf weniger als 10% dieser Zellen zu finden war. Nach dieser Expansionsphase sterben die meisten der cytotoxischen Effektorzellen durch Apoptose im Rahmen von AICD ab. Eine kleine Population von antigenspezifischen $CD8^+$T-Zellen wird durch die Expression von inhibitorischen KIR-Rezeptoren und die damit verbundene Aktivierung der Bcl-2 Expression vor AICD geschützt und expandiert weiter. Die sequentielle Expression von KIR-Rezeptoren startet mit KIR2DL4. Dies ist der einzige KIR-Rezeptor, der auf den untersuchten T-Zellklonen alleine, also ohne Koexpression anderer KIR-Rezeptoren, gefunden wurde. Die immungenetischen Untersuchungen zeigten weiterhin, das KIR2DL4 im Gegensatz zu den meisten KIR-Genen auf allen KIR-Haplotypen vorkommt, was eine essentielle Funktion dieses Rezeptors unterstreicht. In einem nachfolgenden Schritt kommt es zur Expression weiterer KIR-Rezeptoren im Rahmen der klonalen Expansion dieser T-Zellen, wodurch ein KIR-Repertoire auf klonaler Basis entsteht, wie es auf den T-Zellklonen beider Spender gefunden wurde. Die Expression von KIR auf T-Zellen könnte damit im Rahmen der virusspezifischen Immunantwort zwei Funktionen erfüllen: Sie ermöglicht zum einen die Etablierung langlebiger zytotoxischer Gedächtnis-T-Zellen und führt zum anderen zu einer Diversifizierung des Repertoires durch die differentielle Expression verschiedener KIR-Rezeptoren. Durch die Expression verschiedener inhibitorischer und stimulatorischer KIR auf T-Zellen des gleichen Klons werden die spezifischen Aktivierungsbedingungen dieser T-Zellen individualisiert und ermöglichen die Diversifizierung einer virusspezifischen Immunantwort, die andernfalls monoklonal bleiben würde.

3. Schlussfolgerung

Die Ergebnisse der hier dargelegten detaillierten Studie zur Genetik und Funktion der humanen NK-Rezeptoren sind von erheblicher klinischer Relevanz. Kurz zusammengefasst konnte durch die vergleichende Analyse von NK-Rezeptor-Expression und NK-Zellfunktion gezeigt werden, dass die Spezifität der NK-Zelle vorwiegend durch das Repertoire an KIR-Rezeptoren bestimmt wird. Dieses Repertoire ist hochvariabel und hängt im wesentlichen von zwei Faktoren ab: Zum einen existiert ein ausgeprägter genetischer Polymorphismus sowohl in der Anzahl als auch der Art der KIR-Gene. Zum anderen exprimiert jede NK-Zelle ein individuelles Repertoire dieser polymorphen KIR-Rezeptoren. Das KIR-Repertoire spielt auch bei der Fremderkennung eine wichtige Rolle, wie die *in vitro* Stimulation von NK-Zellen durch allogene Zielzellen zeigt. Da die Fremdreaktivität der NK-Zelle direkt von der Expression bestimmter NK-Rezeptoren abhängt, drängt sich die Frage auf, ob der ausgeprägte Polymorphismus der KIR-Gene auch für die klinische Stammzelltransplantation relevant ist. Zur Zeit wird im Rahmen mehrerer Projekte der KIR-Polymorphismus von Spender/Empfänger-Paaren mit den klinischen Daten von Nabelschnurblut- und Knochenmarktransplantationen verglichen, um einen möglichen Zusammenhang aufzudecken und entsprechende Spenderauswahlkriterien zu definieren. Dabei müssen mehrere Möglichkeiten in Betracht gezogen werden. Auf der einen Seite garantiert die Auswahl von gleichen KIR-Typen in Spender und Empfänger, das sogenannte "Matching", dass die passenden inhibitorischen Liganden für die ebenfalls "gematchten" HLA-Klasse-I-Gene vorhanden sind. Auf der anderen Seite könnte ein intelligentes "Mismatching" der KIR-Rezeptoren, also die Übertragung eines Transplantats mit einer anderen Ausstattung an KIR-Genen, einen sogenannten "Graft-versus-Leukemia" (GvL)-Effekt verursachen, bei dem die übertragenen NK-Zellen residuale Tumorzellen aufgrund eines fehlenden KIR-Liganden erkennen und eliminieren. Ein NK-Zell-abhängiger GvL-Effekt konnte vor kurzem in einer klinischen Studie gezeigt werden, bei der eine transplantierte Patientengruppe, die einen Mismatch in einem KIR-Liganden aufwies, ein deutlich geringeres Risiko für das Wiederauftreten der Leukämie aufwies als eine vergleichbare Kohorte von Patienten mit übereinstimmenden KIR-Liganden (54).

Nicht zuletzt könnten die neuen Erkenntnisse zur Funktion der NK-Zelle dazu beitragen, neue Konzepte zur Therapie solider Tumoren zu entwickeln. Die gängigen Ansätze der Immuntherapie basieren größtenteils auf der Stimulation des adaptiven Immunsystems, z. B

die Stimulation von antigenpräsentierenden dendritischen Zellen mit Tumorantigenen. Eine Vielzahl von Studien belegt allerdings, dass der Tumor im Verlauf der Metastasierung die Expression von MHC-Klasse-I-Antigenen reduziert oder sogar vollständig einstellt, um der Erkennung durch MHC-restringierte, tumorspezifische T-Zellen zu entgehen (55). Die Erkennung dieser Tumor-Escape-Varianten durch aktivierte NK-Zellen ist vielfach beschrieben und beruht höchst wahrscheinlich auf der Erkennung durch spezifische NK-Rezeptoren (5). Die genaue Kenntnis von NK-Rezeptor Struktur und Funktion könnte es nun ermöglichen, tumorreaktive NK-Zellen spezifisch anzureichern und zu vermehren. Erste Ansätze einer NK-Zell-basierten Tumortherapie wurden vor kurzem publiziert (56, 57). Die Kombination von aktivierten dendritischen Zellen und aktivierten NK-Zellen könnte aufgrund der fundamental unterschiedlichen Prinzipien der Tumorerkennung einen synergistischen Anti-Tumor-Effekt bewirken.

Das Verständnis der Funktion von NK-Rezeptoren hat nicht nur Relevanz für die Entwicklung einer NK-Zell-basierten Immuntherapie, sondern spielt auch bei der Entstehung zytotoxischer Gedächtnis-T-Zellen eine Rolle. Die hier dargestellten Untersuchungen von KIR-exprimierenden T-Zellen deutet darauf hin, dass die sequentielle Expression von KIR die Selektion langlebiger Gedächtnis-T-Zellen ermöglicht, die über ein komplexes KIR-Repertoire verfügen. Die Induktion der KIR-Expression auf T-Zellen zeigt, dass NK-Rezeptoren auch eine Komponente der adaptiven Immunantwort sind. Vor kurzem konnte gezeigt werden, dass KIR auch auf einer Population von regulatorischen $CD4^+$ T-Zellen zu finden sind, die bei einer Untergruppe von Patienten mit rheumatoider Arthritis vermehrt auftreten (58, 59). Eine wichtige Voraussetzung für ein besseres Verständnis der Rolle der NK-Rezeptoren im Rahmen der T-Zellantwort ist die Kenntnis der Regulation der NK-Rezeptorgene. Leider sind die molekularen Vorgänge, die zur Expression von KIR führen, weitgehend unbekannt. Ein wichtiges Ziel zukünftiger Forschung sollte es deshalb sein, den oder die Faktoren zu isolieren, die zur Induktion, aber auch zur Abschaltung der KIR-Expression notwendig sind. Die gezielte Induktion der KIR-Expression wäre ein immuntherapeutisch hochinteressanter Weg, um die Reaktivität pathogener T-Zellen im Rahmen von Autoimmunerkrankungen, z. B. der rheumatoiden Arthritis, lokal zu inhibieren.

4. Literaturverzeichnis

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5. Anlagen

Sonderdrucke der folgenden Arbeiten

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Human Diversity in Killer Cell Inhibitory Receptor Genes

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Summary

The presence and expression of killer inhibitory receptor (KIR) and CD94:NKG2 genes from 68 donors were analyzed using molecular typing techniques. The genes encoding CD94:NKG2 receptors were present in each person, but KIR gene possession varied. Most individuals expressed inhibitory KIR for the three well-defined HLA-B and -C ligands, but noninhibitory KIR genes were more variable. Twenty different KIR phenotypes were defined. Two groups of KIR haplotypes were distinguished and occurred at relatively even frequency. Group A KIR haplotypes consist of six genes: the main inhibitory KIR, one noninhibitory KIR, and a structurally divergent KIR. Allelic polymorphism within five KIR genes was detected. Group B comprises more noninhibitory KIR genes and contains at least one additional gene not represented in group A. The KIR locus therefore appears to be polygenic and polymorphic within the human population.

Introduction

Natural killer (NK) cell function is regulated by the interaction of major histocompatibility (MHC) class I molecules with inhibitory cell surface receptors (Yokoyama, 1993; Lanier and Phillips, 1996). This mechanism is believed to protect healthy cells from lysis by autologous NK cells, while rendering cells for which class I expression is compromised by infection or transformation susceptible to NK cell-mediated lysis (Ljunggren and Kärre, 1990). Unlike cytotoxic $CD8^+$ T cells, which require recognition of a specific MHC class I allotype to lyse a target, NK cells are prevented from lysing a target by recognition of MHC class I (Kärre et al., 1986; Storkus et al., 1987; Ciccone et al., 1992; Karlhofer et al., 1992; Moretta et al., 1993).

Human NK cells use two types of structure as their HLA class I receptors: molecules of the immunoglobulin superfamily (IgSF) called killer cell inhibitory receptors (KIR) are specific for determinants shared by subsets of HLA-B or -C allotypes (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Long et al., 1996), whereas the CD94:NKG2-A heterodimer, which is related to C-type lectins, is specific for a determinant shared by most HLA-A, -B, and -C allotypes (Moretta et al., 1994; Lazetic et al., 1996; Phillips et al., 1996; Brooks et al., 1997; Carretero et al., 1997). Forms of the NKG2 polypeptide determine whether the lectin-like heterodimer mediates inhibition (NKG2-A) or stimulation (NKG2-C) (Houchins et al., 1997). For both kinds of receptor, transduction of an inhibitory signal requires the presence in the cytoplasmic tail of two immune receptor tyrosine-based inhibitory motifs (ITIM) (Burshtyn et al., 1996; Lazetic et al., 1996; Houchins et al., 1997). Several members of the KIR family have truncated cytoplasmic tails and lack such inhibitory motifs (Colonna and Samaridis, 1995; Moretta et al., 1995; Wagtmann et al., 1995; Biassoni et al., 1996); they are designated here as "noninhibitory KIR."

Every person can be expected to express HLA class I molecules that engage the CD94:NKG2-A receptor, whereas the number of KIR ligands depends on a person's HLA type. Three kinds of inhibitory KIR have well-defined HLA ligands, and an individual can have one, two, or three of these: inhibitory KIR with two IgSF domains recognize subsets of HLA-C allotypes determined by alternative amino acid sequence motifs at positions 77 and 80 of the α1 helix (Colonna et al., 1993). A KIR with three IgSF domains recognizes the subset of HLA-B allotypes that share the Bw4 sequence motif at positions 77–83 of the α1 helix (Litwin et al., 1994; Gumperz et al., 1995).

The genes for KIR, CD94:NKG2, and HLA are on different chromosomes (Yabe et al., 1993; Baker et al., 1995; Chang et al., 1995; Colonna and Samaridis, 1995; Wagtmann et al., 1995), so that ligands and receptors segregate independently in human pedigrees. As a consequence, a substantial proportion of the population has genes for KIR for which they have no HLA class I ligand (Gumperz et al., 1996). In the present investigation, we used novel molecular typing techniques for KIR and NKG2 genes to analyze a sample population, revealing an extensive polymorphism in KIR genotypes.

Results

A System for Typing Patterns of KIR and NKG2 mRNA Expression

To assess variation in the repertoires of KIR and NKG2 HLA class I receptors expressed by individual humans, typing assays based on the reverse transcription polymerase chain reaction (RT-PCR) were developed. The design of the KIR typing system was guided by the

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Table 1. PCR Primers

PCR Group	Template[a]	Sense Primer (5′-3′)	Antisense Primer (5′-3′)	Location[b]	Length[c]
KIR2DL1	RNA	GCA GCA CCA TGT CGC TCT	GTC ACT GGG AGC TGA CAC	−8–348	356
KIR2DL1	DNA	ACT CAC TCC CCC TAT CAG G	AGG GCC CAG AGG AAA GTC A	315–567	∼1750
KIR2DL2	RNA	CCA CTG CTT GTT TCT GTC AT	CAG CAT TTG GAA GTT CCG C	645–1015	370
KIR2DL2	DNA	CCA TGA TGG GGT CTC CAA A	GCC CTG CAG AGA ACC TAC A	228–523	∼1800
KIR2DL3	RNA	CCA CTG AAC CAA GCT CCG	CAG GAG ACA ACT TTG GAT CA	692–1044	352
KIR2DL3	DNA	CCT TCA TCG CTG GTG CTG	CAG GAG ACA ACT TTG GAT CA	792–1043	798
KIR2DL4	RNA	CTG TCC CTG AGC TCT ACA A	CAC TGA GTA CCT AAT CAC AG	206–747	541
KIR3DL1	RNA	ACA TCG TGG TCA CAG GTC C	TGC GTA TGT CAC CTC CTC	641–1197	556
KIR3DL1	DNA	CCA TCG GTC CCA TGA TGC T	AGA GAG AAG GTT TCT CAT ATG	542–679	∼1600
KIR3DL2	RNA;DNA	CGG TCC CTT GAT GCC TGT	GAC CAC ACG CAG GGC AG	546–914	368; ∼1900
KIR2DS1	RNA;DNA	TCT CCA TCA GTC GCA TGA A/G	AGG GCC CAG AGG AAA GTT	254–567	313; ∼1800
KIR2DS2	RNA;DNA	TGC ACA GAG AGG GGA AGT A	CAC GCT CTC TCC TGC CAA	179–435	256; ∼1750
KIR2DS3	RNA;DNA	TCA CTC CCC CTA TCA GTT T	GCA TCT GTA GGT TCC TCC T	315–594	279; ∼1800
KIR2DS4	RNA;DNA	CTG GCC CTC CCA GGT CA	GGA ATG TTC CGT TGA TGC	94–544	450; ∼2000
KIR2DS5	RNA;DNA	AGA GAG GGG ACG TTT AAC C	GCC GAA GCA TCT GTA GGC	184–600	416; ∼1950
KIR3DS1	RNA	GGC ACC CAG CAA CCC CA	AAG GGC ACG CAT CAT GGA	321–567	246; ∼1750
KIR3DS1	DNA	GGC AGA ATA TTC CAG GAG G	AGG GGT CCT TAG AGA TCC A	217–493	∼1800
CD94	RNA	GCA GTG TTT AAG ACC ACT CT	CTG TTG CTT ACA GAT ATA ACG	4–531	527
NKG2-A	RNA	CCA GAG AAG CTC ATT GTT GG	CCA ATC CAT GAG GAT GGT G	202–527	325
NKG2-A	DNA	AGG AGT AAT CTA CTC AGA CC	AGG GAA TAA CAA CTA TCG TTA C	12–283	661
NKG2-C	RNA	GGA AAT ATT CCA AGT AGA ATT AAA T	CTG ATG CAC TGT AAA CGC AAA T	108–727	619
NKG2-C	DNA	TTT CTG GCC AGC ATT TTA CCT	CTG ATG CAC TGT AAA CGC AAA T	477–727	∼1100
NKG2-D	RNA	CTG GGA GAT GAG TGA ATT TCA TA	GAC TTC ACC AGT TTA AGT AAA TC	35–451	416
NKG2-E	RNA	CTG TGC TTC AAA GAA CTC TTC T	CTG GTC TGA TAT AAG TCC ACG T	432–657	225

[a] Primer pairs were used for RNA-based typing (RNA), genomic typing (DNA), or both (RNA; DNA).
[b] Nucleotide positions of amplified fragments were calculated from the start codon according to the sequences previously reported (Houchins et al., 1991; Adamkiewicz et al., 1994; Biassoni et al., 1995; Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Döhring et al., 1996a; and Selvakumar et al., 1996).
[c] In cases where primer pairs were used for RNA- and DNA-based typing, the length (in base pairs) of the RT-PCR fragment is given first.

topology of a tree of KIR sequences. The 12 groups of KIR chosen as the types to be defined in the assay represent the smallest clades of the tree (Valiante et al., 1997a). Oligonucleotide primer pairs (Table 1) were designed to match polymorphic positions unique to each KIR group, as determined from an alignment of 36 different KIR sequences. The resulting assay accounts for all reported KIR sequences (except certain alternative splice variants) and permits comparison of the five groups of inhibitory KIR (KIR2DL1–3 and KIR3DL1–2), the six groups of noninhibitory KIR (KIR2DS1–5 and KIR3DS1), and the unusual KIR2DL4, for which a function has yet to be assigned (Table 2). Each KIR group comprises one to six members, which differ by 1–9 nucleotide substitutions and may represent alleles. By contrast, members of different groups differ by 20 nucleotides or more. Expression of the four NKG2 genes was monitored with specific amplifications covering the inhibitory NKG2-A gene (NKG2-B is a mRNA splice variant of NKG2-A) (Plougastel et al., 1996); the noninhibitory NKG2-C; and the NKG2-D and NKG2-E genes, of unknown function.

The validity of the PCR typing system was demonstrated in several ways. First, it was demonstrated by direct sequencing of the PCR products obtained from two "control" individuals (donors PP and NV) for whom the expressed KIR and NKG2 genes had been determined by cloning and sequencing of cDNA (Valiante et al., 1997b [this issue of *Immunity*]). Second, its validity was demonstrated by application of the typing system to a panel of seven transfected cell lines, each expressing single KIR or the lectin-like NKG2-A. When the PCR typing system was applied to RNA samples from donors PP and NV, the types obtained correlated precisely with those determined by cDNA cloning (data not shown). Similar analysis of individual NK cell clones obtained from PP and NV showed that the KIR and NKG2 type obtained with the PCR assay corresponded to that determined using a panel of KIR- and NKG2-specific antibodies and flow cytometry (Figure 1).

The Combination of KIR Genes Expressed Is Highly Polymorphic in the Human Population

Using the RT-PCR–based typing system, peripheral blood mononuclear cells (PBMC) from 52 blood donors were compared for their expression of KIR and NKG2. Considerable variability in KIR expression was observed. Within this panel, which consisted mostly of caucasoid donors, 18 different phenotypes were detected (Table 3). The most common phenotype was present in 33% of the donors and was composed of four major inhibitory KIR (KIR2DL1, KIR2DL3, and KIR3DL1–2), the noninhibitory KIR2DS4, and the divergent KIR2DL4. The other KIR phenotypes were less common; they were found in 2%–8% of the panel. Whereas the common phenotype consisted of KIR from six different amplification groups, the less common phenotypes could include KIR from as many as ten of the amplification groups (Figure 2A).

Three KIR groups were represented in all the individuals typed: KIR2DL1 and KIR3DL2 of the inhibitory type and KIR2DL4. The majority of individuals expressed four different inhibitory KIR (Figure 2B). KIR with the two different HLA-C inhibitory specificities were present in

Table 2. PCR-Defined Groups of KIR Sequences

Group[a]	Structure	Specificity[b]	cDNA Name[c]	Amino Acid Substitutions[d]
KIR2DL1	2Ig, inhibitory	C2	P58cl47-11	5V, 132R, 230L
			NKAT1	5V, 132P, 230P
			P58cl42	5F, 132P, 230P
KIR2DL2	2Ig, inhibitory	C1	NKAT6	385R, 386Q
			p58cl43	385S, 386E
KIR2DL3	2Ig, inhibitory	C1	NKAT2/p58cl6	9V, 127L, 151Q, 166H, 324P, 338H 399A, 413R
			NKAT2A	9V, 127L, 151Q, 166H, 324L, 338H, 399A, 413H
			NKAT2B	9V, 127L, 151Q, 166H, 324P, 338H, 399T, 413R
			KIR-023GB	9A, 127R, 151E, 166R, 324P, 338I, 399A, 413R
KIR2DL4	2Ig		KIR-103AS	138T, 209A, 271D
			KIR-103LP	138A, 209P, 271N
KIR3DL1	3Ig, inhibitory	Bw4	NKAT3/AMB11/cl11	2S, 13L, 23M, 68I, 75I, 259G
			NKB1/cl2	2L, 13F, 23V, 68V, 75L, 259R
KIR3DL2	3Ig, inhibitory	A?	cl5/AMC5	40P, 113L, 158D, 166R, 228A, 252I
			NKAT4	40P, 113L, 158E, 166R, 228A, 252I
			NKAT4A/cl1-1	40P, 113V, 158E, 166R, 228A, 252I
			NKAT4B	40P, 113V, 158E, 166H, 228A, 252I
			17-1c	40A, 113V, 158E, 166H, 228P, 252T
			8-11c	40A, 113V, 158E, 166H, 228A, 252I
KIR2DS1	2Ig, noninhibitory	C2	EB6act1	4T, 186R
			EB6act2	4M, 186K
KIR2DS2	2Ig, noninhibitory	C1	NKAT5/p58cl49	20G
			GL183act1	20W
KIR2DS3	2Ig, noninhibitory		NKAT7	
KIR2DS4	2Ig, noninhibitory		NKAT8/cl39	
KIR2DS5	2Ig, noninhibitory		NKAT9	
KIR3DS1	3Ig, noninhibitory		NKAT10	166R
			123FM	166H

[a] KIR genes were designated according to nomenclature agreed upon by E. Long (National Institutes of Health, Bethesda, MD) L. Lanier (DNAX, Palo Alto, CA), and M. Colonna (Basel Institute, Basel, Switzerland). KIR2D and KIR3D refer to receptors with two or three IgSF domains, respectively. L stands for receptors having long and S for those having short cytoplasmic tails, consistent with the presence or absence of ITIM motifs, respectively. Each KIR subfamily is designated by an individual number, for example KIR2DS1. In tables and figures the receptor subfamilies are sometimes abbreviated (e.g., 2DS1).

[b] The C1 and C2 groups of HLA-C alleles are distinguished by the dimorphic positions Ser 77–Asn 80 (C1) and Asn 77–Lys 80 (C2) (Colonna et al., 1993).

[c] Sequences with identical coding regions are listed together and separated by a slash. No alternative splicing forms are listed.

[d] Amino acid substitutions between members of each group and their positions as calculated from the start codon are shown.

all individuals; KIR2DL1 represented one specificity and either KIR2DL2 or KIR2DL3 represented the other (Table 3). By contrast, the HLA-B–specific inhibitory receptor, KIR3DL1, was not expressed by four members of the panel. These appear to be true negative results, since antibodies specific for KIR3DL1 failed to bind these individuals' NK cells and since amplification with alternative sets of primers specific for KIR3DL1 gave similarly negative results (data not shown).

The majority of the polymorphism in the expressed KIR phenotypes is due to the noninhibitory receptors. Every individual expresses between one and five noninhibitory KIR (Figure 2B). None of these receptor groups is shared by all individuals, the most common, KIR2DS4, being represented in 80% of the panel (Figure 3). With one exception, the other noninhibitory KIR were represented in 27% or more of the panel. KIR2DS5 was not represented in the donor panel, although it could be amplified from the original cDNA clone, suggesting that this KIR is relatively uncommon.

The frequency with which certain combinations of KIR are expressed by individuals exceeded that predicted by random association, whereas one combination (KIR2DS3/2DS4) was found at less than the expected value (Table 4). The former phenomenon could be due to linkage disequilibrium, indicating that these combinations of KIR represent haplotypes. One putative haplotype comprises the inhibitory KIR2DL2 and the noninhibitory KIR2DS2 and KIR2DS3. Linkage was also detected between the two noninhibitory KIR2DS1 and KIR3DS1, which are expressed together in 19 of the 21 samples expressing either KIR2DS1 or KIR3DS1. Segregation of these two linkage groups was observed in a family, where donor NV inherited KIR2DL2, KIR2DS2, and KIR2DS3 from his mother and KIR2DS1 and KIR3DS1 from his father. KIR2DL2 was present in all samples negative for KIR2DL3, a finding suggesting that these two functionally similar inhibitory KIR segregate on different haplotypes. The four samples, which were negative for the inhibitory KIR3DL1, were positive for the noninhibitory KIR3DS1, which is the most similar KIR. Again, this finding argues that these two receptors segregate independently and that they are related to each other as alleles.

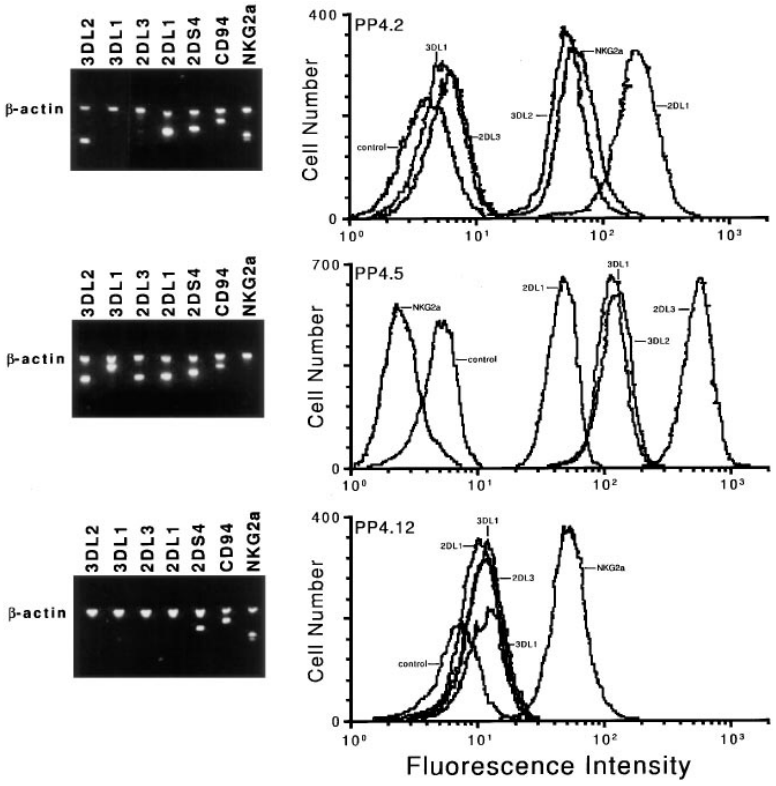

Figure 1. Detection of KIR and NKG2-A Transcripts in NK Clones by RT-PCR Correlates with Surface Expression

Three representative NK clones from donor PP were analyzed by RT-PCR for expression of KIR2DL1, KIR2DL3, KIR3DL1–2, and KIR2DS4 as well as the lectin-like receptors CD94 and NKG2-A (left). Detection of KIR or NKG2-A transcripts by RT-PCR correlated with receptor surface expression as determined by flow cytometry analysis using MAbs against KIR2DL1 (HP-3E4), KIR2DL3 (DX27), KIR3DL1 (DX9), and KIR3DL2 (DX31) as well as an CD94:NKG2-A–specific antiserum (right). Donor PP does not express the noninhibitory KIR2DS1 and KIR2DS2, which are also recognized by MAbs HP-3E4 and DX27, respectively (Lanier et al., 1997). Second-step antibodies alone served as negative controls.

Variability in the expression of the NKG2 receptor family was also observed. The inhibitory receptor NKG2-A was found to be expressed by all individuals, whereas in six samples no expression of the noninhibitory NKG2-C receptor was found (data not shown). The presence or absence of NKG2-C expression was not correlated with the expression of particular KIR haplotypes. The NKG2-D and NKG2-E genes, for which functions are unknown, are expressed by all the individuals we analyzed. Although the variation in NKG2 gene expression is more limited than that for KIR, it is similarly focused on a noninhibitory receptor, NKG2-C.

Distinct Patterns of KIR Expression Are Due to Differences in KIR Genes

The variation of KIR gene expression in the human population raised the question of whether it is due primarily to polymorphism of the genes or to differential regulation of gene expression. To address this issue, we developed a method for PCR typing of KIR and NKG2 genes using

Table 3. KIR Expression Patterns in the Population Survey

	KIR												
Phenotype	2DL1	2DL3	3DL1	3DL2	2DL2	2DS1	2DS2	2DS3	2DS4	3DS1	2DL4	Number[a]	Frequency (%)
1 (PP)	+	+	+	+	−	−	−	−	+	−	+	17	33
2	+	+	+	+	−	−	+	−	+	−	+	4	7.70
3	+	+	+	+	+	−	+	+	+	−	+	4	7.70
4	+	+	+	+	−	+	−	−	+	+	+	4	7.70
5 (NV)	+	−	+	+	+	+	+	+	+	+	+	3	5.80
6	+	+	+	+	+	+	+	+	−	+	+	3	5.80
7	+	+	+	+	+	−	+	−	+	−	+	3	5.80
8	+	+	+	+	−	+	+	−	+	+	+	2	3.80
9	+	+	+	+	+	+	+	−	+	+	+	2	3.80
10	+	+	+	+	+	−	+	+	−	−	+	2	3.80
11	+	+	+	+	+	−	−	−	+	−	+	1	1.90
12	+	−	−	+	+	+	+	+	−	+	+	1	1.90
13	+	+	+	+	+	−	+	−	+	+	+	1	1.90
14	+	+	−	+	−	+	−	−	−	+	+	1	1.90
15	+	+	−	+	+	+	+	+	−	+	+	1	1.90
16	+	+	−	+	−	+	−	−	+	+	+	1	1.90
17	+	+	+	+	−	+	−	−	−	+	+	1	1.90
18	+	+	+	+	−	+	+	−	−	−	+	1	1.90

[a] A total of 52 individuals were analyzed.

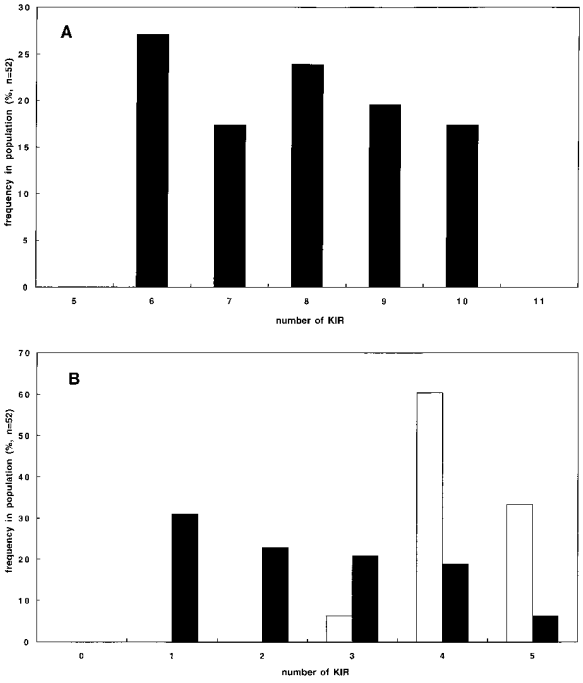

Figure 2. Variation in the Number and Type of KIR Genes Expressed by Different Individuals

KIR expression by PBMC was analyzed by KIR-specific RT-PCR.
(A) The total number of KIR expressed by individuals is plotted as a frequency distribution within the panel (n = 52).
(B) The number of inhibitory (open bars) and noninhibitory (filled bars) KIR is compared. The divergent KIR2DL4 of unknown function that is expressed by all individuals is included in the analysis shown in (A) but not that in (B).

genomic DNA as the template. We modified the primers used in the RT-PCR assay in order to limit the length of the amplification products to no more than 2 kb (Table 1). The DNA-based typing system was applied to samples of genomic DNA obtained from PBMC and the results compared to those obtained by RT-PCR typing of the same preparations of PBMC. For donors PP and NV, for whom the expressed KIR are known, identical

Table 4. Association of KIR in a Survey of 52 Individuals

KIR Combination	Observed[a]	Expected[b]	Factor[c]
2DL2/2DS2/2DS3	26.9	5.64	4.77
2DL2/2DS3	26.9	10.9	2.47
2DS1/3DS1	36.5	14.9	2.44
2DS2/2DS3	26.9	13.9	1.94
2DL2/2DS2	38.5	20.9	1.84
2DS1/2DS3	15.4	10.4	1.48
2DS3/3DS1	15.4	10.4	1.48
2DL2/3DS1	21.2	15.6	1.36
2DS1/2DS2	25	19.9	1.26
2DS2/3DS1	25	19.9	1.26
2DL2/2DS1	19.2	15.6	1.23
2DL2/2DS4	32.6	26.9	1.21
2DS2/2DS4	36.5	41.9	0.87
2DS4/3DS1	25	30.9	0.81
2DS1/2DS4	23.1	31.1	0.74
2DS3/2DS4	13.5	21.7	0.62

[a] Observed frequencies of KIR combinations in a survey of 52 samples. KIR combinations shown underlined are associated more frequently ($P < 0.0001$) than expected by random association, as determined by a test of statistical independence (G test). The results for KIR2DL1, KIR2DL3–4, and KIR3DL1–2 are not shown because they are expressed at frequencies greater than 0.9.
[b] Expected frequencies for KIR combinations are the product of each receptor's individual frequency.
[c] The fold increase of the observed frequencies over the expected frequencies.

KIR types were obtained in the two assays, a result that extended to all of ten other individuals analyzed (Figure 4). In no instance did an individual possess a KIR gene that was not found to be expressed by some cells within the population of PBMC. This correlation demonstrates that differences in the KIR expressed by individuals within the human population result from structural polymorphisms of the KIR gene family rather than from polymorphisms in the regulation of their expression.

DNA typing of KIR genes was next performed for 18 individuals of different ethnicity (Table 5). Within this panel the commonest genotype corresponded to the common phenotype seen at the RNA level in the panel of 52 mostly caucasoid individuals. This genotype, which carries a single noninhibitory KIR gene (KIR2DS4), was

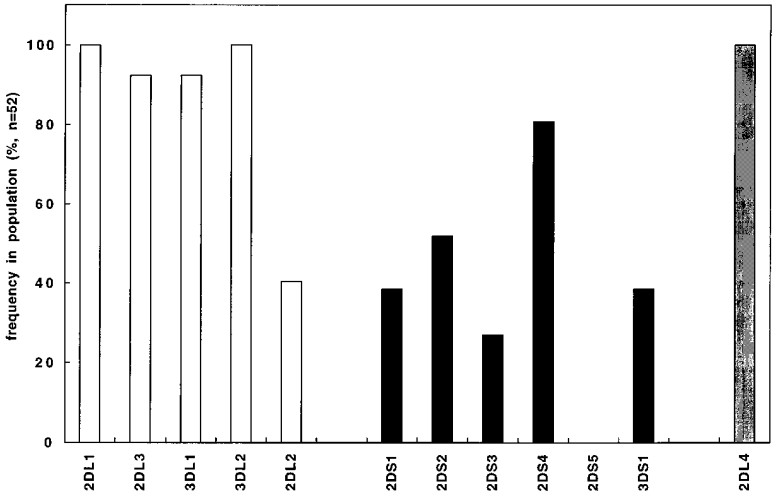

Figure 3. The Frequencies with Which Individual KIR Groups Are Expressed

Shown are the percentages of individuals within the panel (n = 52) who express each inhibitory KIR group (open bars), noninhibitory KIR group (solid bars), and the divergent KIR2DL4 group (hatched bars).

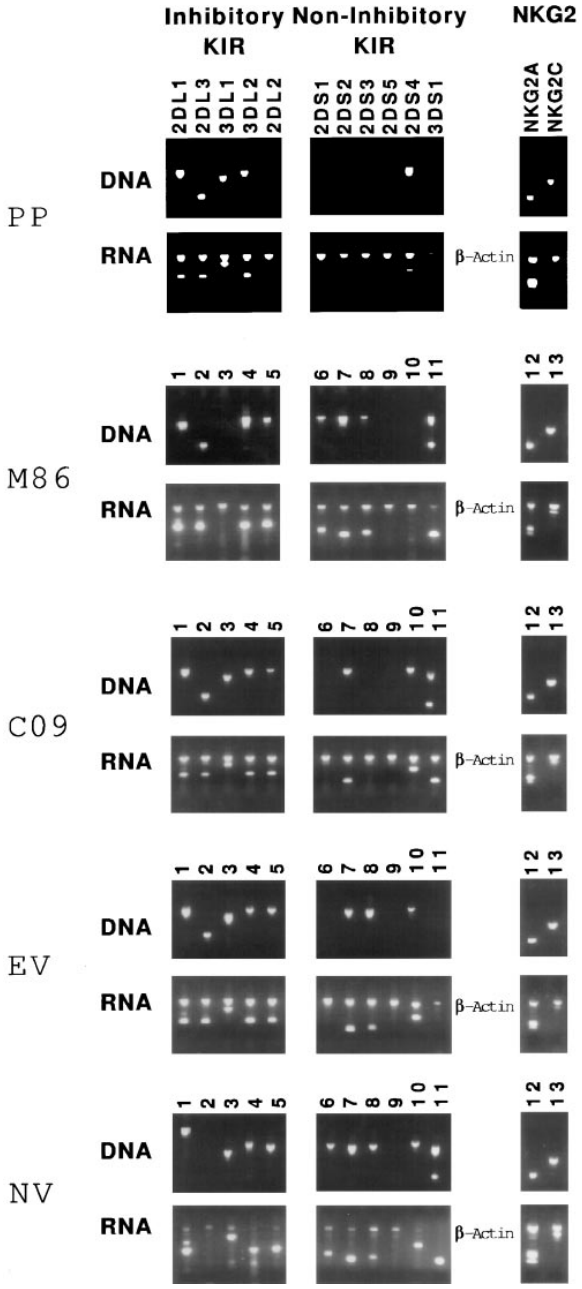

Figure 4. Variability of KIR Usage Is Determined at the Genomic Level

The presence of transcripts and genes corresponding to the indicated NK receptor groups was determined by RT-PCR and genomic PCR, respectively. A β-actin–specific primer pair was included in each RT-PCR amplification as an internal positive control and its product of 734 bp indicated. The results from 5 representative donors of a total of 12 analyzed are shown.

found in 11 of the 18 panel members, including the PP control. The remaining 7 members of the panel, including the NV control, had a variety of genotypes, generally characterized by an increased number of genes for non-inhibitory receptors. Among these were two additional genotypes that had not been found in the mostly caucasoid panel of 52 donors. The KIR genes of the ethnically more diverse panel of donors were also analyzed by Southern blotting. Those individuals with the most common KIR genotype showed the same banding pattern, as presented in Figure 5 for digestion with HindIII. Similar bands were also seen for individuals having less common KIR genotypes, but they were distinguished by an additional, large and prominent HindIII fragment of about 24 kb.

In contrast to the KIR gene family, all individuals analyzed possessed genes encoding NKG2-A, NKG2-C, NKG2-D, and NKG2-E. Southern blotting analysis showed no variation in the banding patterns for the NKG2 and CD94 genes (data not shown), although for some of the donors we were unable to detect NKG2-C transcripts.

In combination, the results obtained from KIR genotyping and Southern blotting indicate that the KIR haplotypes segregating in the human population can be divided into two broad groups. We will refer to these as the group A and the group B haplotypes. Distinguishing the two groups of haplotypes is the 24 kb HindIII band seen on Southern blots, which is present in group B haplotypes and absent from group A haplotypes. Whereas the heterogeneity within the group B haplotypes is apparent from KIR genotyping, by that method of analysis the group A haplotypes appear homogeneous. Individuals who express two group A haplotypes are those having the common KIR genotype and phenotype. However, an underlying heterogeneity in the group A haplotypes is revealed by the sequence analysis of KIR from donor PP, who possesses two group A haplotypes. Determination of complete KIR sequences shows that donor PP expresses two different but closely related alleles for five of the six different types of KIR associated with the group A haplotypes.

Based on Southern blotting and KIR typing results, we can confidently characterize 27 individuals of the panel of 68 donors as homozygous for group A haplotypes and 17 individuals as homozygous for group B haplotypes. Each of the remaining 24 donors could be either heterozygous for group A and B haplotypes or homozygous for group B haplotypes. At one end of the range of possibilities, 40% of the haplotypes would be of group A and 60% of group B, while at the other end 57% of the haplotypes would be of group A and 43% of group B. Throughout this range, the frequencies of group A and B haplotypes both are between 0.4 and 0.6 and therefore of comparable magnitude.

Discussion

Human NK cells use both lectin-like and immunoglobulin-like molecules as their inhibitory receptors for HLA class I molecules (reviewed by Lanier et al., 1997; Valiante et al., 1997b). In addition to their distinctive structures, these two kinds of receptors have complementary specificities: the lectin-like receptor CD94:NKG2-A engages most HLA-A, -B, and -C allotypes, whereas the immunoglobulin-like KIR are specific for subsets of HLA-B or -C allotypes. Because their HLA class I ligands are polymorphic, we investigated whether the receptors also exhibit diversity within the human populations. From PCR-based molecular typing and Southern blotting, no evidence for variation in the number of CD94

Table 5. KIR Genotypes of Ethnically Diverse Panel

Donor	Ethnicity	Inhibitory KIR	Noninhibitory KIR	Haplotype Group[a]
C1 (PP)	Caucasoid	2DL1, 2DL3, 3DL1-2	2DS4	A
B1	Black American	2DL1, 2DL3, 3DL1-2	2DS4	A
E1	East Indian	2DL1, 2DL3, 3DL1-2	2DS4	A
F2	Filipino	2DL1, 2DL3, 3DL1-2	2DS4	A
C1	Chinese	2DL1, 2DL3, 3DL1-2	2DS4	A
K1	Korean	2DL1, 2DL3, 3DL1-2	2DS4	A
K2	Korean	2DL1, 2DL3, 3DL1-2	2DS4	A
J3	Japanese	2DL1, 2DL3, 3DL1-2	2DS4	A
J4	Japanese	2DL1, 2DL3, 3DL1-2	2DS4	A
H1	Hispanic	2DL1, 2DL3, 3DL1-2	2DS4	A
H2	Hispanic	2DL1, 2DL3, 3DL1-2	2DS4	A
C2 (NV)	Caucasoid	2DL1-2, 3DL1-2	2DS1-4, 3DS1	B
B2	Black American	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
E2	East Indian	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
F3	Filipino	2DL1-3, 3DL1-2	2DS2	B
C2	Chinese	2DL1, 2DL3, 3DL1-2	2DS1, 2DS3, 3DS1	B
A1	American Indian	2DL1, 2DL3, 3DL1-2	2DS1, 2DS4, 3DS1	B
B3	Black American	2DL1-3, 3DL1-2	2DS1-3, 3DS1	B

[a] Donors assigned haplotype group B may also have a group A haplotype.

and NKG2 genes was obtained, whereas striking differences in the KIR genes were apparent. All of the KIR genes possessed by an individual are expressed, though not necessarily on every NK cell. At the level of the NK cell population as a whole, the KIR phenotype correlated precisely with the KIR genotype.

The molecular typing we used was designed to distinguish the major kinds of KIR that have been defined by the cloning and sequencing of cDNA. Three kinds of KIR were expressed by all 68 blood donors we examined: the inhibitory KIR2DL1, which is specific for group 2 HLA-C allotypes; KIR3DL2, an inhibitory receptor of less clear specificity, which includes certain HLA-A allotypes (Döhring et al., 1996b; Pende et al., 1996); and the divergent KIR2DL4, of unknown specificity. Most donors have genes encoding inhibitory KIR specific for the three major ligands: group 1 HLA-C, group 2 HLA-C, and Bw4 HLA-B. However, a minority (~8%) of individuals lack an inhibitory KIR (KIR3DL1) specific for Bw4 HLA-B, consistent with a previous population study of this receptor that used serological methods (Gumperz et al., 1996). In contrast to the relatively conserved phenotype of the inhibitory KIR, there is substantial variation in the number and type of noninhibitory KIR.

A minimum of 14 different KIR haplotypes can account for the 20 KIR phenotypes found for the 68 individuals analyzed in this study. Given the small size of the population analyzed and its limited coverage of the world's populations, these haplotypes probably represent a minority of the total number. The KIR haplotypes divide into two groups distinguished by the absence (group A haplotypes) or presence (group B haplotypes) of a 24 kb HindIII fragment on Southern blotting. The two kinds of haplotype have relatively even frequencies, and for the panel of individuals we analyzed there appears to be an excess of homozygotes over that expected by random association. Although this feature could well be due to the artificial nature of our sample population, it raises the possibility of a role for selection.

In PCR genotyping, haplotypes of group A type identically. However, heterogeneity was revealed by nucleotide sequencing of KIR from donor PP, who types only for group A haplotypes and expresses closely related pairs of alleles for the four inhibitory KIR genes

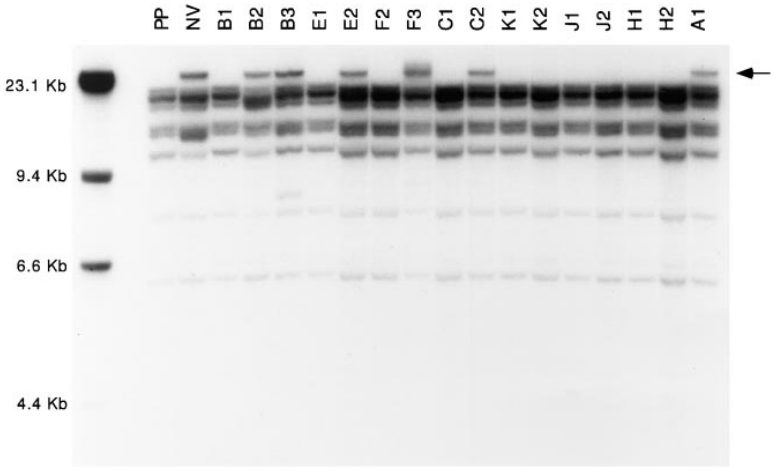

Figure 5. Genomic Polymorphism of KIR

Genomic DNA from 18 samples (see Table 5) of diverse ethnic origins were digested with HindIII and analyzed by Southern blotting using the full-length cDNA probe for KIR3DL1 (KIR-NKB1). An identical pattern was seen when a probe for KIR2DL4 (KIR-103AS), the most divergent member of the KIR gene family, was used (data not shown), indicating that all of the KIR genes hybridize with both KIR probes. The arrow indicates the 24 kb band that distinguishes the group B KIR haplotypes from the group A KIR haplotypes.

(KIR2DL1, KIR2DL3, and KIR3DL1–2). This result indicates that the four kinds of inhibitory KIR are encoded by separate genes. Of the other two kinds of KIR that characterize the group A haplotypes, two alleles for KIR2DL4 are expressed by PP and KIR2DS4 is represented by a single nucleotide sequence. Thus, these two KIR must also be encoded by separate genes. In total, the evidence supports a model in which the group A haplotypes consist of six KIR genes.

All six KIR genes that characterize group A haplotypes are also found in group B haplotypes, but individual group B haplotypes can lack certain of these genes. For example, whereas KIR2DS4 is common to all group A haplotypes, it is present on only a subset of group B haplotypes. Several lines of evidence suggest that there are additional KIR genes present on the group B haplotypes that are not present on the group A haplotypes. Indirect evidence comes from Southern blots revealing the 24 kb HindIII fragment, which is characteristic of group B haplotypes and indicative of an expansion of the KIR gene family. More direct evidence comes from consideration of the closely related KIR2DL2, KIR2DL3, and KIR2DS2, which are expressed simultaneously by certain individuals. KIR2DL3 is characteristic of group A haplotypes, whereas KIR2DL2 and KIR2DS2 are characteristic of group B haplotypes. This indicates that KIR2DL2 and KIR2DS2 derive from two different genes that are present on some group B haplotypes and probably related by gene duplication or gene deletion to the single KIR2DL3 gene on the group A haplotypes. A third piece of evidence is that the noninhibitory KIR2DS3, which is found on some group B haplotypes, is relatively divergent (Döhring et al., 1996b; Valiante et al., 1997a) and may represent a distinct locus rather than an allele of one of the other genes. KIR2DS5, which was not detected in the panel we analyzed, could be a rarer allele of the same gene as KIR2DS3.

Although genomic analyses of two KIR genes and a preliminary map of the KIR complex have been reported (Selvakumar et al., 1997; Wagtmann et al., 1997; Wilson et al., 1997), the number and organization of the KIR genes on human chromosome 19 have yet to be defined. The number of KIR genes also cannot be predicted by comparison of KIR cDNA sequences, because the cDNA sequences cannot be confidently sorted into groups corresponding to the alleles of different loci. This property of KIR sequences contrasts with those of their HLA class I ligands, for which locus assignments can readily be made on the basis of coding region sequences (Parham et al., 1995). Uncertainty in assigning KIR to loci stems in part from the apparently independent evolution of sequences encoding the extracellular and intracellular domains. Thus certain pairs of inhibitory and noninhibitory KIR are very similar in the extracellular part of the molecule but divergent in their cytoplasmic tails. KIR2DL1/KIR2DS1 and KIR3DL1/KIR3DS1 are examples of such pairs, which could represent alleles either of the same locus or of two closely related loci. For the case already discussed above, the triplet of related KIR (KIR2DL2, KIR2DL3, and KIR2DS2) that is expressed by some individuals proves that there are at least two loci involved, but for the other pairs of KIR the evidence is not so clear. Evidence in support of allelism would be the expression by certain, putatively homozygous, individuals of one member of a pair but not the other. In the panels we analyzed this prediction is met for KIR3DL1 and KIR3DS1, suggesting they could be alleles of the same locus.

For the pair KIR2DL1/KIR2DS1, the segregation appears to be different: individuals either have both of the KIR or have just KIR2DL1. No individual in the panel has KIR2DS1 without also having KIR2DL1. This pattern is consistent with encoding of KIR2DS1 and KIR2DL1 by different genes. It may also be consistent with encoding of the two KIR by alleles, providing that a role for selection is allowed. The rule governing receptor expression by NK cells is that every cell carries an inhibitory receptor that binds an autologous HLA class I allotype; in contrast, expression of noninhibitory receptors appears not to be under similar constraint (Valiante et al., 1997b). A possible effect of this requirement could be selection for individuals who have the allele encoding the inhibitory receptor KIR2DL1 and against individuals who are homozygous for the noninhibitory receptor KIR2DS1, who would then be much rarer than predicted by the square of the allele frequency. Resolution of these alternative explanations should come from pedigree studies and genomic analysis of individuals who are homozygous by consanguinity for KIR haplotypes.

Our results support a model in which the group A KIR haplotypes consist of six genes: four encoding inhibitory receptors, one encoding a noninhibitory receptor, and one encoding a divergent receptor of uncertain category. Within the population sampled, 40% of the individuals carry two group A haplotypes, showing that these six genes are sufficient to provide an adequate set of KIR for regulation of NK cells in the human immune system. The group B KIR haplotypes are more variable in their organization, containing one additional gene, and possibly more, that encode noninhibitory receptors. The functions of these additional genes have yet to be established, but the relatively even frequency of group A and B haplotypes suggests that both kinds of haplotype are actively maintained in the population.

In a previous population study we used a monoclonal antibody (MAb) to examine the cell surface expression of the HLA-Bw4–specific KIR-NKB1 (now designated KIR3DL1) by NK cells of individuals with different HLA class I types (Gumperz et al., 1996). Although variations in KIR3DL1 expression were detected, they were not correlated with expression of the Bw4 HLA-B ligand. In the present study these results are confirmed and extended to the other KIR of known HLA specificity. Expression of a KIR does not depend on expression of the HLA class I ligand, and individuals who lack expression of a particular KIR do so because they lack the gene. A considerable majority of individuals have genes for inhibitory KIR that recognize the three well-defined HLA-B and -C ligands, and they express these receptors irrespective of their HLA type. By this mechanism the KIR genes on human chromosome 19 can segregate independently from the HLA genes on chromosome 6 while still maintaining compatibility between receptors and the KIR ligands presented by a particular HLA class I type.

The number of KIR ligands an individual possesses

varies from one to three and affects the extent to which KIR are used as inhibitory receptors by their NK cells (Valiante et al., 1997b). The CD94:NKG2-A inhibitory receptor is able to compensate for such differences because the HLA class I type of most, if not all, individuals includes a ligand for CD94:NKG2-A. The broad specificity of the CD94:NKG2-A receptor for HLA-A, -B, and -C allotypes suggests that the origin of this receptor:ligand combination predates the diversification of HLA-A, -B, and -C from a common ancestral class I gene. In contrast, the specificity of KIR for subsets of HLA-B or -C allotypes suggests that these receptor:ligand combinations arose subsequent to the divergence of the HLA-B and -C genes from HLA-A (Parham, 1994; Valiante et al., 1997a). Correlating with this difference in age of the two class I receptor systems, we find that the CD94 and NKG2 genes are relatively conserved from one individual to another, whereas the KIR genes exhibit considerable polymorphism.

Our results show that humans express the products of six or more KIR genes. In addition to this diversity of KIR within an individual's immune system, there is considerable diversity in the KIR type that distinguishes individuals within the population. This polymorphism of KIR appears analogous to that seen for MHC class I and II genes but contrasts with the variability of B and T cell antigen receptors, where diversity is played out largely within the individual. At present, the functions of only a subset of the KIR are known, but if they all contribute to the regulation of the NK cell response, then the diversity of KIR and HLA class I types within the human population has the potential to modify the NK cell response in ways that are highly individualized.

Experimental Procedures

Cells

Peripheral Blood Cells

Samples were obtained from a total of 68 donors: 52 blood donors (Stanford Blood Center, Stanford, CA) and 16 unrelated donors of different ethnic origins (Cedars-Sinai Medical Center, Los Angeles, CA). PBMC were isolated from whole blood by Ficoll-Hypaque gradient separation.

NK Cells

$CD3^-CD56^+$ NK clones were generated and maintained in culture as described previously (Yssel et al., 1984; Litwin et al., 1993).

Transfected Cells

KIR-NKAT1–6 (KIR2DL1–3, KIR3DL1–2, and KIR2DS2) cDNA were transfected into the murine BaF/3 pre-B cell line (Lanier et al., 1997). The NKG2-A cDNA was transfected into the murine P815 mastocytoma (Lazetic et al., 1996).

HLA Class I Typing

Two donors, NV and PP, were HLA class I typed by cDNA cloning and sequencing as described previously (Domena et al., 1993). Donor PP typed as A*0101/0301, B*1501/0702, and Cw*0304/0702; donor NV typed as A*0201/0301, B*2702/0702, and Cw*0202/0702.

Flow Cytometry Analysis

The MAb HP-3E4 (anti-KIR2DL1) was generously provided by M. López-Botet (Melero et al., 1994). The DX27 (anti-KIR2DL2–3), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2) MAbs have been described previously (Litwin et al., 1994; Lanier et al., 1997). All anti-KIR MAbs were detected using a fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG antiserum (Caltag, Burlingame, CA). The CD94:NKG2-A–specific polyclonal rabbit antiserum was used in combination with an FITC-conjugated goat anti-rabbit IgG antiserum as the secondary reagent (Caltag, Burlingame, CA).

DNA Extraction and Southern Blot Analysis

DNA for PCR typing was prepared from 1×10^7 B lymphoblastoid cell lines (BLCL) using the QIAamp Blood Kit according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). High-molecular-weight DNA for Southern blot analysis was prepared from 3×10^8 BLCL, and 10 μg of genomic DNA was digested to completion with restriction enzymes. The resulting DNA fragments were separated by electrophoresis through 0.6% agarose gels in 0.5× TBE buffer. Hybridizations were performed as described previously (Shum et al., 1996), using full-length cDNA probes for KIR-NKB1 (KIR3DL1), KIR-103AS (KIR2DL4), CD94, and NKG2-A.

RNA Extraction and Reverse Transcription

Total cellular RNA was prepared from 1×10^6 NK cell clones or 1×10^7 PBMC using RNAzol according to the manufacturer's instructions (Tel-test, Friendswood, TX). First-strand cDNA was synthesized from NK clones and PBMC-derived RNA (0.5 μg and 5 μg RNA, respectively) by RT using oligo(dT) and Moloney murine leukemia virus reverse transcriptase (GIBCO–BRL, Gaithersburg, MD) at 42°C for 1 hr.

NK Receptor–Specific PCR Typing

PCR amplification was performed with a pair of sense and antisense primers, each possessing a 3′ residue matching a polymorphic position on a given NK receptor gene (Table 1). The primer length was adjusted to result in an annealing temperature of the primers between 56°C and 62°C. This enabled amplification of all NK receptors under the same PCR conditions. Internal control sense (5′-CGC GAG AAG ATG ACC CAG ATC -3′) and antisense (5′-TTG CTG ATC CAC ATC TGC TGG-3′) primers, specific for a 734 bp β-actin fragment, were included in each RT-PCR at a concentration of 0.1 μM for the analysis of NK clones and 0.05 μM for PBMC. Genomic PCR was performed without internal positive controls to increase amplification efficiency of the larger fragments. Each experiment included negative control reactions containing distilled water as the surrogate template. KIR typing of NK clones included as a negative control a feeder cell culture without NK clones to monitor possible amplification of residual KIR transcripts from the irradiated feeder cells.

The NK receptor–specific primers were used at a concentration of 0.5 μM and stored in ready-to-use PCR plates at −20°C. Amplification of cDNA was performed in 25 μl reactions in a model 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) using 0.625 U AmpliTaq polymerase, 2.5 μl 10× Buffer (Perkin-Elmer), and 0.2 mM dNTPs (Promega, Madison, WI) under the following conditions: initial denaturation at 95°C for 2 min; then 60 s at 62°C, 45 s at 72°C, and 60 s at 94°C for the first five cycles; and then 45 s at 60°C, 45 s at 72°C, and 30 s at 94°C for 25–30 cycles. Genomic PCR analysis of KIR and NKG2 genes was performed with 100–300 ng DNA in a model 9700 thermal cycler (Perkin-Elmer) under the following conditions: initial denaturation for 5 min at 95°C; then 20 s at 97°C, 45 s at 62°C, and 90 s at 72°C for the first five cycles; and then 20 s at 95°C, 45 s at 60°C, and 90 s at 72°C for 25 cycles. Amplification products were analyzed on ethidium bromide–prestained 1.5% (RT-PCR typing) or 0.9% (genomic typing) agarose gels.

Nucleotide Sequencing of KIR

NK receptor-specific PCR products were purified using a QIAquick PCR Purification Kit according to manufacturer's instructions (QIAGEN) and directly sequenced in both orientations using dye-labeled deoxy-terminators and a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Full-length KIR coding sequences were obtained from donor PP and NV by amplification of KIR cDNAs with primers matching sequences in the 5′ and 3′ untranslated regions (Valiante et al., 1997b). PCR products were cloned into a pBLUESCRIPT SK^+ vector and partially sequenced with a standard T7 oligonucleotide primer. Based on the partial sequences, three to four representatives of each KIR were selected and were sequenced completely on both strands to obtain a consensus sequence.

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ORIGINAL PAPER

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Definition of gene content for nine common group *B* haplotypes of the Caucasoid population: *KIR* haplotypes contain between seven and eleven *KIR* genes

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Abstract The segregation of killer cell immunoglobulin-like receptor (*KIR*) genes was determined for a panel of 21 Caucasoid families: 23 different *KIR* gene patterns were found and could be assigned to combinations of 16 different haplotypes. Four loci were held in common by all haplotypes: *KIR2DL4*, *KIR3DL2*, the putative pseudogene *KIR3DL3* and *KIR2DL2/KIR2DL3*, the latter likely being alleles of one gene. Group *A* haplotypes, which have a unique combination of seven *KIR* genes, were found at 80% frequency in the family panel, the polygenic group *B* haplotypes at 65% frequency. *KIR* gene segregation was fully determined for the nine group *B* haplotypes, which occurred at highest frequencies in both the family panel and a panel of unrelated individuals. The group *B* haplotypes carried between seven and 11 *KIR* genes and encoded inhibitory KIR for one, two, or all three major HLA class I epitopes. Analysis of human leucocyte antigen (HLA) class I genotypes revealed that most, but not all, individuals possess an inhibitory KIR for a self HLA class I epitope. The number of stimulatory *KIR* genes in group *B* haplotypes varied considerably between one and five. The data show that group *B* haplotypes possess a broad spectrum of *KIR* gene patterns, which is largely complementary to the *KIR* gene set of group *A* haplotypes. The results suggest that rapid diversification of group *B* haplotypes is the result of pathogen-mediated selection for *KIR* genotypes that have more than the set of *KIR* genes provided by the group *A* haplotype.

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Introduction

Killer cell immunoglobulin-like receptors (KIRs) (CD158) are surface receptors of the immunoglobulin superfamily with either two (KIR2D) or three (KIR3D) immunoglobulin domains that are expressed on natural killer (NK) cells and subsets of T cells (Colonna and Samaridis 1995; Phillips et al. 1995; Wagtmann et al. 1995). Currently, 14 different KIRs are known, which can be divided into inhibitory (L) and stimulatory (S) KIRs, based on functional and structural features. Seven KIRs (2DL1-2DL5, 3DL1-3DL2) have long cytoplasmic tails with immune receptor tyrosine-based inhibitory motifs (ITIMs) that are essential for the transduction of inhibitory signals (Long 1999). Several of these are specific for HLA class I products (Moretta et al. 1999). NK cells expressing KIR2DL2 and KIR2DL3 are inhibited by group 1 HLA-C allotypes (Cw1, 3, 5, and 6), whereas expression of KIR2DL1 leads to inhibition by HLA-C allotypes of group 2 (Cw2, 4, 7 and 8) (Colonna and Samaridis 1995). The third major inhibitory epitope is the HLA-B-encoded Bw4 motif, which inhibits KIR3DL1-expressing NK cells (D'Andrea et al. 1995; Gumperz et al. 1995). KIR3DL2 binds to certain HLA-A allotypes (HLA-A3, HLA-A11), but the effects on NK cell inhibition are less pronounced (Döhring et al. 1996; Valiante et al. 1997). KIR2DL4 is unusual in that it combines features of inhibitory and stimulatory KIRs (Selvakumar et al. 1996). A long cytoplasmic tail encoding one ITIM is combined with a charged amino acid residue in the transmembrane domain, a feature characteristic of stimulatory KIRs. KIR2DL4 binds to HLA-G, but the physiological relevance of this is still unclear (Cantoni et al. 1998; Rajagopalan et al. 2001). The extracellular Ig-like domains of the recently discovered KIR2DL5 are most similar to the KIR2DL4, but the ligand is currently unknown (Vilches et al. 2000c). Additionally, there are six stimulatory KIRs that possess short cytoplasmic tails, namely 2DS1-5 and 3DS1. Whereas KIR2DS1 and

Table 1 Primer sequences of generic *KIR* typing system

KIR gene	Sense primer (5′→3′)	Antisense primer (5′→3′)	Length (bp)	Reference
KIR2DL1	ACTCACTCCCCCTATCAGG	AGGGCCCAGAGGAAAGTCA	1,750	This study
KIR2DL1v	ACTCACTCCCCCTATCAGG	AGGGCCCAGAGGAAAGTT	1,750	This study
KIR2DL2	CCATGATGGGGTCTCCAAA	GCCCTGCAGAGAACCTACA	1,800	Uhrberg et al. (1997)
KIR2DL3	CCTTCATCGCTGGTGCTG	CAGGAGACAACTTTGGATCA	798	Uhrberg et al. (1997)
KIR2DL4[a]	CGGGCCCCACGGTTCGCA	AGGCAGTGGGTCACTCGC	249	This study
	GGGCCCCACGGTTCGCG	AGGCAGTGGGTCACTCGG		This study
KIR2DL5	TGCCTCGAGGAGGACAT	GGTCTGACCACTCATAGGGT	1,600	Vilches et al. (2000c)
KIR3DL1	TACAAAGAAGACAGAATCCACA	TAGGTCCCTGCAAGGGCAA	1,600	This study
	TCCCATCTTCCATGGCAGAT			This study
KIR3DL2	CGGTCCCTTGATGCCTGT	GACCACACGCAGGGCAG	1,900	Uhrberg et al. (1997)
KIR3DL3	GGACCTACAGATGTTGC	TAGTTGACCTGGGAACCCG	1,575	Rajalingam et al. (2001a)
KIR2DS1	TCTCCATCAGTCGCATGAA/G	AGGGCCCAGAGGAAAGTT	1,800	Uhrberg et al. (1997)
KIR2DS2	TGCACAGAGAGGGGAAGTA	CACGCTCTCTCCTGCCAA	1,750	Uhrberg et al. (1997)
KIR2DS3	TCACTCCCCCTATCAGTTT	GCA TCTGTAGGTTCCTCCT	1,800	Uhrberg et al. (1997)
KIR2DS4	CTGGCCCTCCCAGGTCA	GGAATGTTCCGTTGATGC	1,800	This study
KIR2DS5	AGAGAGGGGACGTTTAACC	TCCGTGGGTGGCAGGGT	1,950	This study
KIR3DS1	GGCAGAATATTCCAGGAGG	AGGGGTCCTTAGAGATCCA	1,750	This study

[a] Certain *KIR* genes were amplified using a mixture of three (*KIR3DL1*) or four (*KIR2DL4*) different primers, respectively

KIR2DS2 bind weakly to HLA-C allotypes (Biassoni et al. 1997; Vales-Gomez et al. 1998; Winter et al. 1998) the ligand specificity of other stimulatory KIRs is unknown.

In analogy to the clonotypic expression of immunoglobulins and T-cell receptors on B and T cells, respectively, NK cells express a clonally distributed repertoire of KIRs. Whereas B- and T-cell repertoires are generated through somatic recombination and subsequent expression of a single clonotypic receptor per cell, NK cell repertoires are generated through the expression of different sets of non-rearranging *KIR* genes on different NK cells (Valiante et al. 1997). The expression of *KIR* genes on a given NK cell appears to be highly stochastic: all possible combinations of two or more *KIR* genes are expressed on different NK cells. In general, all *KIR* genes possessed by an individual are expressed on overlapping subsets of NK cells. Even *KIR* genes for which a given individual provides no HLA class I ligand are expressed at substantial frequencies (Gumperz et al. 1996). Only two constraints seem to limit the combinatorial diversity: firstly, *KIR2DL4* is expressed on all NK cells in most, if not all, individuals (Rajagopalan et al. 2001; Valiante et al. 1997); secondly, each NK cell expresses at least one inhibitory NK cell receptor specific for a self HLA class I allotype (Valiante et al. 1997). Of note, proper NK cell inhibition by self HLA class I ligands can be mediated not only by inhibitory KIRs, but also by the lectin-like inhibitory receptor CD94:NKG2A, which can fully compensate for the lack of an appropriate inhibitory KIR. KIRs are not confined to NK cells, but are also expressed on subsets of T cells. The KIR repertoire on cytotoxic T cells closely resembles the repertoire found on NK cells, although the KIR T cells are apparently not selected to express a tolerogenic inhibitory receptor (Uhrberg et al. 2001; Young and Uhrberg 2002).

The *KIR* gene family is densely clustered on human Chromosome 19q13.4 and, together with the *ILT/LIR* genes, constitutes the leucocyte receptor complex (LRC) (Trowsdale et al. 2001; Wende et al. 1999). In a previous study, several different *KIR* genotypes were defined with varying numbers of inhibitory and stimulatory *KIR* genes (Uhrberg et al. 1997). The most frequent genotype was present in one third of the population and the underlying haplotypes were termed group *A* haplotypes. Group *A* haplotypes consist of six expressed *KIR* genes, which show considerable allelic variation (Trowsdale et al. 2001; Valiante et al. 1997). In contrast, a second group of *KIR* genotypes exhibit substantial variability in *KIR* gene number, possessing up to 12 different *KIR* genes. The haplotypes underlying these more complicated *KIR* haplotypes were termed group *B* haplotypes. Both groups of haplotypes have been found in all populations analyzed so far, but their ratio varies considerably between ethnic groups (Crum et al. 2000; Norman et al. 2001; Toneva et al. 2001; Witt et al. 1999). Combining the results of genomic sequencing of the *KIR* gene locus with genotyping of populations suggests that three *KIR* genes are common to group *A* and *B* haplotypes: the *KIR3DL3* pseudogene on the centromeric border, the *KIR3DL2* gene at the telomeric end and the *KIR2DL4* gene in the middle of the *KIR* gene cluster (Wilson et al. 2000). However, this conclusion remains tentative because the genomic organization of most group *B* haplotypes remains unknown. To address this question, the haplotypic variation of the *KIR* genes in Caucasoid families was studied here with a PCR-sequence-specific (SSP) typing system that permits detection of the currently known *KIR* genes and variants.

Materials and methods

Samples

Genomic DNA was obtained from families registered at the Düsseldorf Bone Marrow Donor Registry in the course of related bone marrow transplant donor searches. A group of 120 unrelated individuals was chosen by randomly selecting recruits to the Düsseldorf Bone Marrow Donor Registry.

KIR-specific PCR-SSP typing

PCR amplification of *KIR* genes was performed basically as described previously (Uhrberg et al. 1997) using a modified panel of *KIR*-gene-specific primers (Table 1). The *KIR2DL2v* variant, not included in the generic typing system, was analyzed separately using the following primers: 2DL2v1sense: 5′-AGGGGGAGGCCCATGAAT-3′ and 2DL2v1antisense: 5′-TCCGTGGGTGGCAGGGT-3′. Specificities of all new or modified *KIR*-typing reactions were verified by application of the typing system to a panel of five control samples (PP, NV, WC, RR and MU), which were previously *KIR*-typed by PCR-SSP and cDNA cloning and sequencing (Gardiner et al. 2001; Rajalingam et al. 2001a; Valiante et al. 1997; Vilches et al. 2000a). The PCR-SSP patterns were in complete agreement with the published data (data not shown).

KIR-specific PCR-SSP typing was performed by "hot start PCR" using an antibody-blocked *Taq* DNA polymerase. In several cases this prevented the occurrence of additional non-specific bands, which are caused by false primer annealing during the first PCR cycle. Aliquots of 25 µl were set up using 0.625 U HotStar TAQ (Qiagen, Hilden, Germany), 2.5 µl 10×buffer (Qiagen), 0.2 mM dNTPs (Promega, Madison, Wis.), and 50 ng DNA. Primers were used at a concentration of 0.5 µM and stored at –20 °C in ready-to-use 96-well reaction plates. PCR conditions were identical for all typing reactions: initial denaturation of the *Taq*-specific antibody at 95 °C for 15 min, then 20 s at 95 °C, 45 s at 62 °C, and 90 s at 72 °C for the first five cycles; then 30 cycles of 20 s at 95 °C, 45 s at 60 °C, and 90 s at 72 °C in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). Amplification products were analyzed on ethidium-bromide-prestained 1% agarose gels, except *KIR2DL4* and the *KIR2DL2v1* variant products, which were run on 2% gels.

HLA typing

HLA class I polymorphisms of samples included in the family study were analyzed by serological and molecular typing. HLA-A, B, and C allotypes of all samples were checked by serology. *HLA-C* polymorphisms were typed separately using a low resolution PCR-SSP kit (Dynal, AllSet SSP) discriminating Cw1-18. In samples exhibiting unusual banding patterns, exons 2 and 3 of *HLA-C* were sequenced as described (van der Vlies et al. 1998).

Results

A modified DNA-based *KIR* typing system

The PCR-SSP typing system originally developed by Uhrberg et al. (1997) was modified to accommodate the recent discovery of additional *KIR* genes, as well as the description of numerous novel alleles not included previously (Gardiner et al. 2001; Rajalingam et al. 2001a). The nomenclature used throughout the manuscript adheres to the HUGO Gene Nomenclature Committee (HGNC; http://www.gene.ucl.ac.uk/nomenclature/genefamily/kir.html). Three *KIR* genes were newly introduced: *KIR2DL4*, which had so far only been analyzed by RNA-based typing, due to its expression on all NK cells; *KIR2DL5*, which is a recently discovered member of the inhibitory *KIR* gene family (Vilches et al. 2000c); and *KIR3DL3*, which has an intact open reading frame, but is not expressed in any sample analyzed so far (Torkar et al. 1998). An additional antisense primer was added to the *KIR2DL1* specific amplification mix to enable amplification of the recently described *KIR2DL1v* allele (Shilling et al. 1998). In the case of *KIR2DS5*, the antisense primer was changed to include several previously unknown alleles (Vilches et al. 2000b). Furthermore, new primer pairs with improved amplification efficiency were introduced for the genes *KIR3DL1*, *KIR2DS4*, and *KIR3DS1*. Altogether, 14 different *KIR* groups are distinguished by the PCR-SSP typing system, which cover the currently known set of *KIR* cDNAs (Table 1). *KIR2DL2v1* was not detected in the family panel, but was detected in the African-American individual from whom this allele was originally cloned (data not shown) (Gardiner et al. 2001). Thus, *KIR2DL2v1* is rare or absent from the Caucasoid population. Additionally, one primer pair was included for the specific amplification of *KIR3DL3*, which apparently represents a pseudogene with an untranscribed *KIR*-like open reading frame. Two transcriptionally inactive gene fragments, designated *KIR2DP1* and *KIR3DP1*, were not analyzed here.

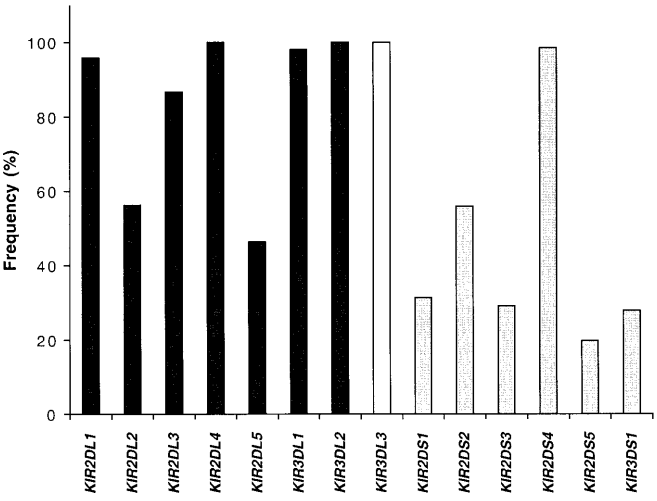

Fig. 1 Frequency of individual *KIR* genes. The frequency of 14 *KIR* genes was determined by PCR-SSP typing of 268 individuals (compiled from a family panel of 148 individuals and a panel of 120 unrelated individuals). Shown are the percentages of inhibitory *KIR* genes (*dark grey bars*), the pseudogene *KIR3DL3* (*white bar*) and stimulatory *KIR* genes (*light grey bars*)

KIR gene frequencies and genotypes

A panel of 268 samples, consisting of 21 families with 148 members, and a group of 120 unrelated individuals of mostly Caucasoid origin were subjected to *KIR*-gene typing by the modified PCR-SSP typing system. The *KIR2DL4* and *KIR3DL2* genes and the putative pseudogene *KIR3DL3* were found in all individuals, whereas all other *KIR* genes were present in subpopulations of individuals (Fig. 1). Overall, the gene frequencies of most *KIRs* in this study were similar to the frequencies found by previous analysis of *KIR* gene expression in 52 Caucasoid individuals (Uhrberg et al. 2001). Differences were found for the *KIR2DL2* and the *KIR2DS5* genes, both of which were found more frequently in the present

Table 2 *KIR* genotypes

Geno-type	*2DL1*	*2DL2*	*2DL3*	*2DL4*	*2DL5*	*3DL1*	*3DL2*	*3DL3*	*2DS1*	*2DS2*	*2DS3*	*2DS4*	*2DS5*	*3DS1*	All samples (*n*=268)[a]	Family samples (*n*=148)[a]	Unrelated samples (*n*=120)[a]
1	+		+	+		+	+	+				+			31.7	35.8	26.6
2	+	+	+	+		+	+	+		+		+			16.9	15.5	16.7
3	+	+	+	+	+	+	+	+		+	+	+			7.5	6.8	8.3
4	+		+	+	+	+	+	+	+			+	+	+	6.3	5.4	7.5
5	+	+		+	+	+	+	+		+	+	+			4.9	6.8	2.5
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	4.5	4.1	5
7	+	+	+	+	+	+	+	+	+	+	+	+		+	3	2.7	3.3
8	+	+	+	+	+	+	+	+		+		+			2.2	1.4	3.3
9	+	+		+	+	+	+	+	+	+	+	+	+	+	2.2	3.4	0.8
10		+		+		+	+	+		+		+			1.9	3.4	–
11	+		+	+	+	+	+	+	+		+	+		+	1.9	2.0	1.7
12	+	+	+	+	+	+	+	+	+	+		+	+	+	2.2	1.4	3.3
13	+	+	+	+	+	+	+	+	+	+		+		+	1.5	–	3.3
14	+		+	+	+	+	+	+	+			+		+	1.5	–	3.3
15	+	+	+	+		+	+	+				+			1.1	2.0	–
16		+	+	+		+	+	+		+		+			1.1	–	2.5
17	+	+		+	+	+	+	+	+	+		+	+	+	0.75	1.4	–
18	+	+		+	+	+	+	+	+	+	+	+		+	0.75	1.4	–
19	+	+	+	+	+	+	+	+	+	+	+	+			0.75	1.4	–
20	+		+	+		+	+	+	+			+			0.75	1.4	–
21	+	+	+	+	+	+	+	+	+	+			+	+	0.37	–	0.8
22	+	+	+	+	+	+	+	+	+	+		+			0.75	–	1.7
23		+		+	+	+	+	+	+	+		+	+	+	0.37	0.7	–
24	+	+		+	+	+	+	+		+	+	+	+	+	0.37	0.7	–
25	+	+		+	+		+	+	+	+	+		+	+	0.37	0.7	–
26	+		+	+	+	+	+	+	+			+	+		0.37	0.7	–
27	+	+	+	+	+	+	+	+		+	+	+	+		0.37	0.7	–
28	+	+		+	+	+	+	+		+		+			0.37	0.7	–
29	+		+	+	+	+	+	+	+	+		+		+	0.37	–	0.83
30	+	+	+	+	+		+	+	+	+	+		+	+	0.37	–	0.83
31	+		+	+	+	+	+	+		+	+	+			0.37	–	0.83
32	+	+		+	+	+	+	+		+	+	+	+		0.37	–	0.83
33		+	+	+	+	+	+	+	+	+	+	+			0.37	–	0.83
34	+	+	+	+	+	+	+	+	+	+		+	+		0.37	–	0.83
35	+	+	+	+	+		+	+	+	+	+			+	0.37	–	0.83
36	+		+	+	+	+	+	+				+			0.37	–	0.83
37	+		+	+	+	+	+	+			+	+			0.37	–	0.83
38	+	+	+	+	+		+	+	+	+	+	+	+	+	0.37	–	0.83
39	+	+	+	+		+	+	+	+	+	+		+		0.37	–	0.83
40		+	+	+		+	+	+	+	+		+			0.37	–	0.83

[a] Frequency of genotype (%)

study. However, this was not surprising because several recently described alleles of both genes were detected using the present DNA typing system, but not in the previous RNA-based analysis. The newly included *KIR2DL5* gene was found in close to 50% of samples.

In the family panel, 23 different *KIR* genotypes were found, based on presence or absence of *KIR* genes (Table 2). The unrelated group exhibited 16 genotypes already included in the family panel and 17 additional genotypes. As described previously, the most abundant genotype in both panels consists of six functional *KIR* genes (five inhibitory and one stimulatory gene) plus *KIR3DL3*, and corresponds to the group *A* haplotype in a homozygous state (Uhrberg et al. 1997). The second most frequent genotype had two additional genes, *KIR2DL2* and *KIR2DS2*, and again was found in comparable frequencies in the family and the unrelated panel. Together, genotypes 1 and 2 made up one half of the samples in the family panel and a little less in the unrelated group. The remaining 39 different genotypes were found at considerably lower frequencies. Genotypes contained between seven and 14 (Fig. 2a) genes, with a mean of 11. When genotype frequency is taken into account, the picture changes, with the most frequently found gene content being seven (Fig. 2b). This is almost entirely due to the high frequency of group *A* haplotype homozygotes. Most other genotypes, which are either heterozygous combinations of group *A* and *B* haplotypes or homozygous combinations of group *B* haplotypes, consist of more than seven *KIR* genes.

Most, but not all, individuals possess inhibitory *KIR* genes for a self class I ligand

KIRs and their HLA class I ligands are highly polymorphic and their genes segregate on different chromo-

Table 3 *KIR* haplotypes

Haplotype	*2DL1*	*2DL2*	*2DL3*	*2DL4*	*2DL5*	*3DL1*	*3DL2*	*3DL3*	*2DS1*	*2DS2*	*2DS3*	*2DS4*	*2DS5*	*3DS1*	Number of families (*n*=21)	Number of haplotypes (*n*=296)
A	+		+	+		+	+	+				+			21	172
B1		+		+		+	+	+		+		+			8	35
B2	+	+		+	+	+	+	+		+	+	+			6	24
B3	+	+		+	+		+	+	+	+	+		+	+	3	10
B4	+	+		+	+		+	+	+	+	+			+	3	7
B5		+		+	+		+	+	+	+			+	+	3	6
B6	+		+	+	+		+	+	+			?	+	+	2	9
B7	+	+		+		+	+	+		+		+			1	12
B8	+	+		+	+	+	+	+		+		+			1	7
B9	+	+		+	+	+	+	+	+	+	+	+			1	3
B10	?	+		+		?	+	+				?			1	3
B11	+		+	+	+		+	+	+		+	?		+	1	3
B12	+		+	+		+	+	+	+			?			1	2
B13	+		+	+	+	?	+	+	+			?	+		1	1
B14	+	+		+	+	?	+	+		+	+	?	+		1	1
B15	?	+		+	?	?	+	+		?	?	?	+	+	1	1

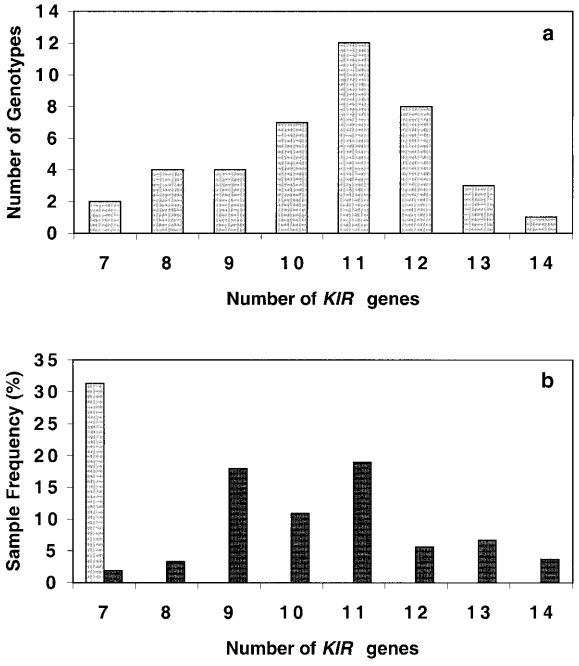

Fig. 2a, b Number of *KIR* genes found in different genotypes. **a** The number of *KIR* genes is plotted against the number of different genotypes found in the *KIR*-typing panel (*n*=268). **b** The number of *KIR* genes in samples of group *A* haplotypes (*light grey bar*) is compared to the number of *KIR* genes on samples of group *B* haplotypes (*dark grey bars*)

somes. On the KIR side, most individuals possess inhibitory *KIR* genes, encoding receptors for all three major HLA class I epitopes. KIRs for group 1 HLA-C allotypes (*KIR2DL2* and/or *KIR2DL3*) are found in all individuals; KIRs for group 2 HLA-C and the Bw4 epitope (*KIR2DL1* and *KIR3DL1*, respectively) were present in 95% of individuals. Only 5% of individuals lack either the *KIR2DL1* or *KIR3DL1* gene and individuals with only one KIR for a major HLA class I epitope were not found. In contrast, on the HLA class I side, subtyping of 14 of the 21 families with 101 members revealed that only 40% possessed all three inhibitory epitopes, 38% had two epitopes and 23% had only one epitope – in most cases a group 1 HLA-C allotype (data not shown). Of the 23 individuals that had only one HLA class-I-encoded epitope, 22 had the respective inhibitory KIR ligand. However, one individual had a group C2 epitope (A2-B18-Cw5, A3-B35-Cw4) only, but no *KIR* genes encoding the corresponding C2-specific ligand KIR2DL1 (*KIR* genotype 15). Thus, this individual appears not to possess any of the three major inhibitory KIR/HLA class I ligand combinations.

Analysis of group *B KIR* haplotypes

Analysis of *KIR* genotypes in the family panel reveals independent segregation of at least 16 different haplotypes (Table 3). As expected, group *A* haplotypes are by far the most frequent ones and were found in all 21 families. The remaining 15 different group *B* haplotypes were designated *B1–B15* according to their frequency in the family panel. Segregation of six frequently occurring group *B* haplotypes is shown in Fig. 3. Heterozygous combination of a group *A* and *B* haplotype always led to a higher number of stimulatory *KIR* genes and in most cases also of inhibitory *KIR* genes. Segregation of a group *A* and a *B3* haplotype in family 4 illustrates how two complementary *KIR* haplotypes are combined in donor 4.7 to give a complete set of *KIR* genes (Fig. 3a, b). This pair of haplotypes, which segregated in three different families, was the only combination observed to give all *KIR* genes. The haplotype composition of the three most frequently occurring genotypes is shown in family 6 (Fig. 3d). The combination of group *A* and *B1* haplotypes was the most frequent heterozygote configuration found in the family panel and was represented by genotype 2 in the unrelated panel in 16% of donors. The com-

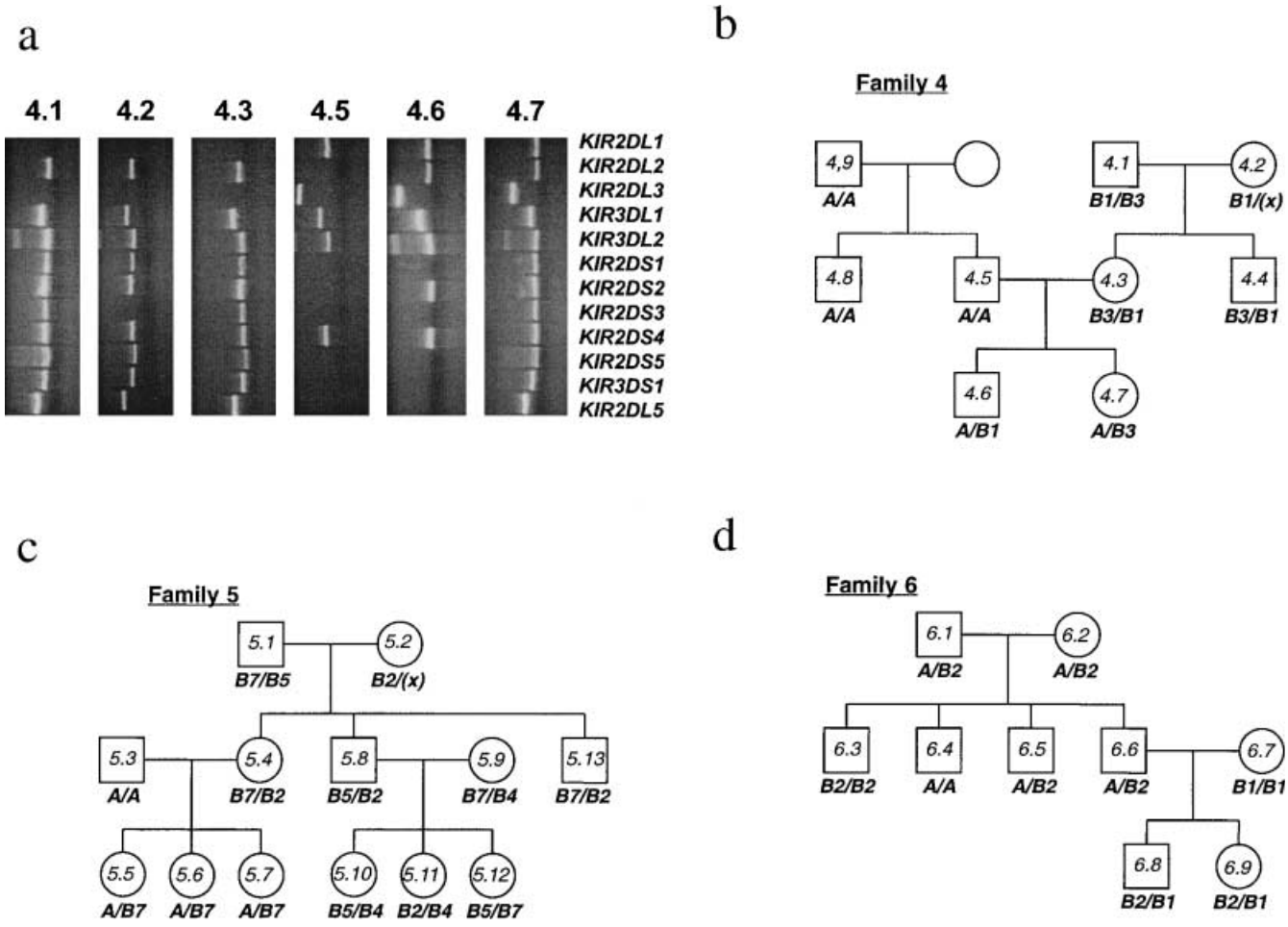

Fig. 3a–d Characterisation of different group *B KIR* haplotypes by family segregation analysis. **a** PCR-SSP-typing results of 12 *KIR* genes are shown for selected members of family 4. Samples 4.1, 4.2, and 4.3 were negative for *KIR2DL1* by generic typing (as shown here), but typed positive for the variant *KIR2DL1v* using allele-specific primers (not shown here). *KIR2DL4* and *KIR3DL3* genes were present but are not shown here. **b–d** Pedigrees for three different families, 4–6, are shown. Designations of family members were 4.1–4.9, 5.1–5.13, and 6.1–6.9 and are given within the *box/circle*. The observed haplotypes are given under each *box/circle*. Haplotypes designated *x* could not be determined

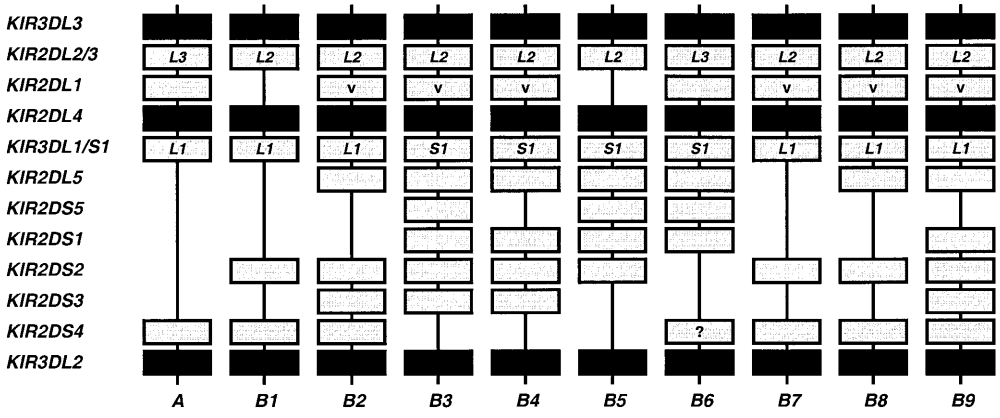

Fig. 4 Hypothetical organisation of *KIR* haplotypes. The hypothetical organisation of the group *A* (designated *A*) and nine group *B* haplotypes (designated *B1–B9*) is shown. The general structure of the *KIR* cluster was adopted from the previously determined organization of a group *A* and a group *B* haplotype (Martin et al. 2000; Wilson et al. 2000). Presence of a *KIR* gene on a given haplotype is indicated by a *rectangle*. Genes present on all haplotypes were *shaded black*; variable *KIR* genes were *shaded grey*. The presence of pairs of *KIR* genes that share the same gene locus (*KIR2D2L* and *KIR2DL3*, as well as *KIR3DL1* and *KIR3DS1*) is indicated *within rectangles*, as is the presence of the *KIR2DL1v* variant. Possession of the *KIR2DS4* gene in haplotype *B6* is hypothetical

bination of group *A* and the *B2* haplotype was the second most frequent genotype in both panels. Family 5 enabled segregation analysis of the group *B* haplotypes *B4*, *B5*, and *B7* (Fig. 3c). The hypothetical gene organization of the ten most frequent haplotypes is shown in Fig. 4. The gene content varies from seven for group *A* haplotypes to 11 in the case of the *B3* haplotype. Three *KIR* genes are shared by all haplotypes: *KIR3DL3* and *KIR3DL2* at both ends of the *KIR* cluster and in between the *KIR2DL4* gene, the so-called framework genes (Wilson et al. 2000). Moreover, all haplotypes encode an inhibitory KIR for the group 1 HLA-C epitope, either *KIR2DL3*, present on group *A* and a few group *B* haplotypes, or *KIR2DL2*, present on most group *B* haplotypes. The remaining nine different *KIR* genes are absent in some haplotypes and thus are referred to as variable *KIR* genes.

Discussion

The KIR repertoire is substantially influenced by the polymorphic and polygenic nature of the *KIR* gene family (Vilches and Parham 2002). Here, 16 *KIR* haplotypes with varying numbers and kinds of *KIR* genes were determined through segregation analyses in a panel of 21 families. Though highly divergent, all *KIR* haplotypes found in this study have several features in common that might help to define essential components of the *KIR* locus. Firstly, three *KIR* genes are held in common by all haplotypes: *KIR2DL4*, *KIR3DL2*, and the putative pseudogene *KIR3DL3*. This set of *KIR* genes was previously defined by Wilson and co-workers (2000) as framework genes, based on genomic sequencing of two *KIR* haplotypes. Secondly, all haplotypes possess a gene encoding a group 1 HLA-C-specific inhibitory KIR, either *KIR2DL2* or *KIR2DL3*. The results shown here are compatible with an allelic relationship for *KIR2DL2* or *KIR2DL3*, again consistent with the data from genomic sequencing of the *KIR* locus. Thirdly, all haplotypes contain at least one stimulatory *KIR* gene. Among the stimulatory *KIR* genes, *KIR2DS4* stands out because it is much more frequently found in the Caucasoid population than any other stimulatory *KIR*. In the three extended haplotypes lacking *KIR2DS4*, between four (haplotypes *B4* and *B5*) and five (haplotype *B3*) other stimulatory *KIR* genes were found. Apparently, *KIR2DS4* fulfils a specific function, which is not easily compensated by replacement with one of the other stimulatory *KIR* genes. Interestingly, *KIR2DS4* is the only stimulatory *KIR*, which has an ortholog in the common chimpanzee (Khakoo et al. 2000).

Two groups of *KIR* haplotypes were distinguished previously, based on the absence (group *A*) or presence (group *B*) of a 24-kb fragment in Southern blot analyses (Uhrberg et al. 1997). Here, group *A* haplotypes, which have a well-defined set of seven *KIR* genes, were found in all 21 families analyzed. This observation is consistent with several other studies showing a prevalence of group *A* haplotypes in the Caucasoid population (Crum et al. 2000; Norman et al. 2001; Toneva et al. 2001; Witt et al. 1999). Whereas diversity of group *A* haplotypes is mainly due to allelic polymorphism (Martin et al. 2000; Valiante et al. 1997; Wilson et al. 2000), group *B* haplotypes are both polymorphic and polygenic. The present study shows that group *B* haplotypes can possess between seven and 11 different *KIR* genes. Although there are no features shared exclusively by all group *B* haplotypes, several features are shared by the majority of them. Firstly, they have more *KIR* genes than group *A* haplotypes. From the nine group *B* haplotypes for which *KIR* gene content could be determined, eight had between one and four additional *KIR* genes. The frequently found haplotype *B1* is somewhat atypical, having a content of seven *KIR* genes, similar to the group *A* haplotypes. Secondly, *KIR2DL2* is usually found in place of *KIR2DL3*. In most cases, *KIR2DL2* is linked to a specific variant of the *KIR2DL1* gene, called *KIR2DL1v* (Shilling et al. 1998). Seven haplotypes containing *KIR2DL1v* could be defined and all of them are coupled with *KIR2DL2* and not *KIR2DL3*. Thirdly, the *KIR2DL5* gene is frequently present. Of note, it was recently shown that two paralogous *KIR2DL5* loci are present in certain *KIR* haplotypes and that not all *KIR2DL5* variants are transcribed (Vilches et al. 2000a, 2000c). Since the typing system used here does not discriminate between *KIR2DL5* variants, the exact number and type of *KIR2DL5* genes in the Caucasoid population remains to be determined. Finally, most group *B* haplotypes possess several stimulatory *KIR* genes (between one and five). With the exception of haplotype *B10* (and perhaps *B12*), all group *B* haplotypes have more than one and up to five different stimulatory *KIR* genes.

The segregation of *KIR* genes in the present study is compatible with the existence of 12 *KIR* loci (not considering the gene fragments *KIR2DP1* and *KIR3DP1*). Two pairs of *KIR* genes, *KIR2DL2/KIR2DL3* and *KIR3DL1/KIR3DS1* segregate as alleles, an observation which correlates with genomic sequencing results showing similar positions of both pairs of *KIR* on a physical map of the *KIR* locus (Wilson et al. 2000). For all other pairs of *KIR* genes, allelic relationships are excluded due to co-segregation on one or more of the *KIR* haplotypes defined here, except for *KIR2DS4* and *KIR2DS5*. Additional segregation analysis will be necessary to determine whether these genes never co-segregate or whether this is merely a sampling artifact of this study.

The *KIR* gene frequencies observed in this study are similar to those seen in other surveys of the Caucasoid population (Crum et al. 2000; Norman et al. 2001; Toneva et al. 2001; Uhrberg et al. 1997; Witt et al. 1999). Most of the common *KIR* gene patterns found in those studies are possibly heterozygous combinations of frequent *KIR* haplotypes determined here. However, it is likely that the 16 different haplotypes defined here represent only a small sample of all haplotypes segregating in the Caucasoid population. This is illustrated by the fact that only four out of eight unique phenotypes seen in

our previous small survey of 52 Caucasoid individuals (Uhrberg et al. 1997) are possibly due to combinations of haplotypes found in the current study of 268 individuals. Moreover, in two other studies, several partial haplotypes were defined that possess *KIR* combinations that do not appear in the current study (Crum et al. 2000; Witt et al. 1999). Nonetheless, a relatively small number of haplotypes make up the majority of genotypes found in the Caucasoid population. The ten most frequent haplotypes defined here can account for about 85% of the genotypes seen in the control group of 120 individuals. The picture changes when different ethnic populations are compared. As described by Toneva and coworkers (2001), Australian Aborigines exhibit a highly divergent pattern of *KIR* genotypes, in which *A* haplotypes are rare and the majority of individuals possess a group *B* haplotype not seen here. Similarly, an African-American family had two divergent *B* haplotypes not found in the Caucasoid population (Gardiner et al. 2001).

From phylogenetic comparison of *KIR* genes in humans and chimpanzees it is apparent that the *KIR* locus changed dramatically in a time frame of a few million years (Khakoo et al. 2000; Rajalingam et al. 2001b). No genotypes are shared by the two species and only three orthologous *KIR* genes can be identified: *KIR2DL4*, *KIR2DL5* and *KIR2DS4*. It is therefore likely that most of the haplotypic diversity described here developed after divergence of human and chimpanzee ancestors. The driving force for the generation and fixation of *KIR* haplotype diversity in humans might be simply the maintenance of interaction with their rapidly evolving HLA class I ligands. However, HLA class I polymorphism is concentrated at positions involved in peptide presentation and T-cell receptor interaction (Parham et al. 1995), whereas the amino acid motifs KIR recognize are rather conserved between humans and chimpanzees (Adams and Parham 2001). Moreover, the present study shows that haplotypic diversity of *KIR* is not primarily focused on genes encoding HLA class I-recognizing receptors. Ninety-five percent of the population possesses inhibitory *KIR* genes for the three major inhibitory HLA class I epitopes, the stimulatory *KIR* genes being the greater focus for polygenic variation. The ligands of most stimulatory KIR remain to be determined. Although KIR2DS1 and KIR2DS2 exhibit weak affinity for HLA-C allotypes, HLA class I might not be the primary ligand for most of the other stimulatory KIRs (Winter et al. 1998). One alternative possibility would be that stimulatory KIRs are involved in the recognition of pathogen structures, in a way similar to the pattern recognition receptors of the Toll receptor family (Aderem and Ulevitch 2000). Along those lines, it was recently suggested that stimulatory KIRs are involved in MHC-independent recognition of herpes simplex virus-infected cells (Pietra et al. 2000). Of relevance to this model are observations showing that an analogous stimulatory receptor in the mouse, Ly49h, confers resistance to cytomegalovirus infection (Brown et al. 2001; Lee et al. 2001). The major force driving the extensive diversification of human *KIR* haplotypes might thus have been the selection for new stimulatory receptors that recognise different pathogen structures rather than HLA class I molecules.

In summary, this study has defined several extended group *B KIR* haplotypes. These haplotypes reveal remarkable variability within the human population in terms of *KIR* gene number, especially of the stimulatory *KIR* genes, which could provide the basis for targeted and diversified NK cell responses to infection.

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Functionally and Structurally Distinct NK Cell Receptor Repertoires in the Peripheral Blood of Two Human Donors

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Summary

The expression of KIR and CD94:NKG2 receptors was determined for more than 100 natural killer (NK) cell clones obtained from two blood donors who differ in their HLA class I and KIR genes. More than 98% of the clones were inhibited by individual autologous class I allotypes, and every clone was inhibited by the combination of autologous allotypes. The patterns of inhibition correlate with expression of inhibitory receptors of defined specificity. One donor possesses three class I ligands for KIR, and a majority of NK cells use KIR as their inhibitory receptor; the second donor possesses only a single ligand for KIR, and a majority of NK cells use the more broadly reactive CD94:NKG2a as their inhibitory receptor. Because of these differences, the first donor has subpopulations of NK cells that kill cells of the second donor, whereas the NK cells of the second donor are universally tolerant of cells from the first donor.

Introduction

Kärre and coworkers proposed the missing-self model as a physiological context for their observation that cellular expression of major histocompatibility complex (MHC) class I molecules conferred resistance to lysis by natural killer (NK) cells (Kärre et al., 1986; Ljunggren and Kärre, 1990). The model predicts that circulating NK cells survey the body for MHC class I expression and kill cells in which it is abnormally down-regulated. Consistent with the missing-self model has been the discovery of receptors on rodent and human NK cells that bind to polymorphic determinants of MHC class I molecules and signal inhibition of cytolysis (Karlhofer et al., 1992; Moretta et al., 1993; Litwin et al., 1994; Moretta et al., 1994). Two kinds of cell-surface glycoprotein have these functions: one resembling C-type lectins and the other comprising immunoglobulin (Ig) domains (reviewed by Valiante et al., 1997). The NK cell class I inhibitory receptors of rodents are lectin-like, with the mouse Ly49 family of molecules being the best characterized (Yokoyama and Seaman, 1993; Dissen et al., 1996). By contrast, the class I inhibitory receptors of human NK cells include one that is lectin-like, a heterodimer of the CD94 and NKG2a polypeptides, and a family of Ig-like receptors called killer cell inhibitory receptors (KIR) (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Lazetic et al., 1996; Brooks et al., 1997; Carretero et al., 1997).

The two kinds of human receptors have distinctive specificities for human leukocyte antigen (HLA) class I molecules. More than 50 KIR family members have been identified and four inhibitory specificities determined. Two subfamilies of KIR are specific for determinants of HLA-C produced by alternative sequence motifs at positions 77 and 80 of the HLA-C heavy chain (Colonna et al., 1993a, 1993b; Moretta et al., 1993). A third subfamily of KIR is specific for the Bw4 sequence motif found at residues 77–83 on one third of HLA-B heavy chain allotypes (Litwin et al., 1994; Gumperz et al., 1995). The inhibitory specificity of the fourth subfamily of KIR has been reported to include certain HLA-A allotypes (Döhring et al., 1996; Pende et al., 1996). Whereas the individual KIR specificities are confined to the allotypes of a single HLA class I locus, the lectin-like CD94:NKG2a receptor has a broad specificity that includes a majority of HLA-A, -B, and -C allotypes and the nonclassic HLA-G molecule (Phillips et al., 1996; Pérez-Villar et al., 1997; Söderström et al., 1997). Both the KIR family and the NKG2 family contain members for which HLA class I specificities have not been defined. About half of the family members lack immune receptor tyrosine-based inhibitory motifs (ITIM) and cannot deliver inhibitory signals (Moretta et al., 1995; Biassoni et al., 1996; Houchins et al., 1997; Olcese et al., 1997). The function of these "noninhibitory" receptors remains uncertain.

NK cells can be divided into subpopulations based on the class I receptors they express (Moretta et al., 1990; Ciccone et al., 1992; Karlhofer et al., 1992; Brennan et al., 1994, 1996; Mason et al., 1995). Therefore, like T cells, there is the potential for an NK cell repertoire within an individual's immune system. Unlike T cells, individual NK cells can express more than one receptor, and expression of a receptor by an NK cell does not depend upon expression of a corresponding MHC class I ligand (Karlhofer et al., 1992; Lanier et al., 1995; Gumperz et al., 1996; Raulet et al., 1997). A corollary of the missing-self model is that NK cells do not kill autologous cells expressing the normal set of MHC class I molecules. This predicts that every circulating NK cell in an individual should express at least one receptor that has specificity for a self HLA class I ligand. We tested this prediction by determining the NK cell receptor repertoires of two healthy individuals.

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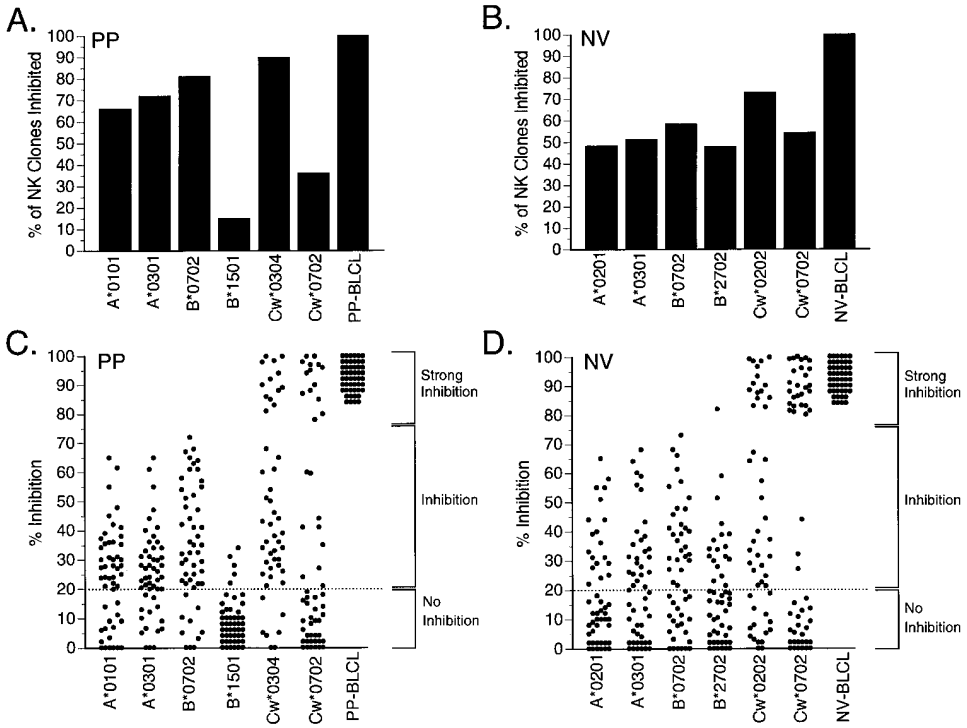

Figure 1. Inhibition of NK Cell Clones from Donors PP and NV by Self Class I Allotypes

NK cell clones from PP (A and C) and NV (B and D) were used as effectors in cell-killing assays against the autologous B cell lines and the 221 transfectants bearing autologous class I allotypes.

(A and B) The percentage of NK clones inhibited (defined as >20% inhibition compared to lysis of untransfected 221 cells) was calculated for each transfectant, and more than 100 clones (n = 108 for PP and n = 111 for NV) were analyzed for each donor.

(C and D) Fifty clones randomly selected from the clones generated in a single cloning experiment were analyzed for the extent of inhibition mediated by the autologous HLA class I allotypes. The results for each NK cell clone/target cell pair are plotted as percentage inhibition compared to lysis of the untransfected 221 cells.

Results

Human NK Cells All Can Be Inhibited by One or More Autologous HLA-A, -B, or -C Allotypes

Both of the donors studied are heterozygous for the HLA-A, -B, and -C loci. Although one HLA class I haplotype (A*0301, B*0702, and Cw*0702) is shared, the differences at the other haplotype are such that donor PP possesses only one of the well-defined ligands for KIR, the group 1 HLA-C ligand, whereas donor NV possesses all three of them, the group 1 and 2 HLA-C ligands and the Bw4 HLA-B ligand (Table 1). Transformed B cell lines were established from each donor, and from these, cDNA corresponding to the six HLA class I alleles were cloned, sequenced, and transfected into the class I–deficient B cell line 721.221 (221). More than 100 NK cell clones were established from each donor and tested for their capacity to lyse the autologous B cell line, the 221 cell line, and the panel of 221 transfectants expressing individual autologous HLA class I alleles (Figure 1).

Whereas all NK cell clones from both donors lysed the class I–deficient 221 cell line, none lysed the autologous B cell line. However, in the presence of a monomorphic anti-HLA class I monoclonal antibody (MAb), the B cell lines were lysed by NK cell clones, demonstrating the class I dependence of the inhibition (data not shown). The transfectants expressing single HLA class I allotypes were lysed to varying degrees by the different NK cell clones, but in a reproducible pattern. Almost all (>98%) of the NK cell clones were inhibited by one or more of the self HLA class I allotypes. The NK cell clones from PP were largely of two kinds: the majority of clones were inhibited by the A*0101, A*0301, B*0702, and Cw*0304 allotypes, and the minority of clones were inhibited by Cw*0304 and Cw*0702 (Figure 1A). The level of class I–mediated inhibition for NK cell clones in the latter group was significantly stronger than for those in the former group (Figure 1C). The B*1501 allotype was striking for its lack of inhibitory capacity.

By contrast to the differential inhibition seen for the HLA class I allotypes of donor PP (Figure 1A), all six of NV's allotypes inhibited a substantial number of the autologous NK cell clones, with the Cw*0202 allotype inhibiting more than 70% of the clones (Figure 1B). For donor NV, most of the NK cell clones divide into three groups: those inhibited by A*0201, A*0301, B*0702, B*2702, and Cw*0202; those inhibited strongly by Cw*0702; and those inhibited strongly by Cw*0202 (Fig-

ure 1D). These results demonstrate how every mature human NK cell can be inhibited by one or more autologous HLA class I allotypes.

Individual Humans Express Different Subsets of KIR Family Members

The functional analysis (Figure 1) shows that individual NK cells from donors PP and NV are differentially inhibited by self HLA class I allotypes. We therefore correlated these differences with the expression of NK cell receptors for HLA class I. Both the Ig-like KIR and the lectin-like CD94:NKG2 receptors were analyzed. From the published sequences for CD94 and NKG2 family members, oligonucleotide primers were designed for the amplification of cDNA, and these products were then sequenced (Houchins et al., 1991; Chang et al., 1995). Both donors were shown to express the CD94, NKG2a, and NKG2c genes (Table 1).

Using oligonucleotide primers designed from the published KIR sequences, we cloned and sequenced KIR cDNA from resting peripheral blood lymphocytes; activated, polyclonal NK cells; and selected NK cell clones from the two donors. Both individuals express 11 different KIR: 3 are held in common and 8 are different (Table 1). The inhibitory KIR can be divided into four groups (KIR2DL1, KIR2DL2/3, KIR3DL1, and KIR3DL2) associated with different HLA class I specificities, and both donors express representatives of all four groups, with PP expressing two representatives of each group and NV only one. By contrast, NV expresses five of the noninhibitory KIR, whereas PP expresses just one. In addition, both individuals express two versions of the structurally divergent KIR family member, KIR2DL4 (103AS), (Selvakumar et al., 1996).

To characterize the class I receptors of the NK cell clones used in the functional analysis (Figure 1), we typed for KIR, CD94, and NKG2 cDNA using a method of PCR-SSP (polymerase chain reaction–sequence specific priming) typing developed by Uhrberg et al. (1997 [this issue of *Immunity*]). Systematic analysis of the NV and PP panels of NK cell clones confirmed that their class I receptors expressed are included in those obtained from the sequence analysis. Moreover, except for NKG2c, which was not detected in PP's NK clones, all of the receptors for which sequences had been obtained were represented in the panel of NK cell clones. Antibody binding and flow cytometric analysis of NK cell clones from both donors revealed that whenever a transcript was detected it gave rise to receptor expression at the cell surface (data not shown and Uhrberg et al., 1997). The differential patterns of KIR gene expression in the peripheral blood NK cells from NV and PP correlated precisely with differences observed at the genomic level (Figure 2).

Human NK Cells Express 2–9 KIR and CD94:NKG2 Receptors per Cell

The results from PCR-SSP typing of the panels of NK cell clones were compiled to assess the relative number of NK cells expressing each receptor (Figures 3A and 3C). The values obtained are similar to those determined

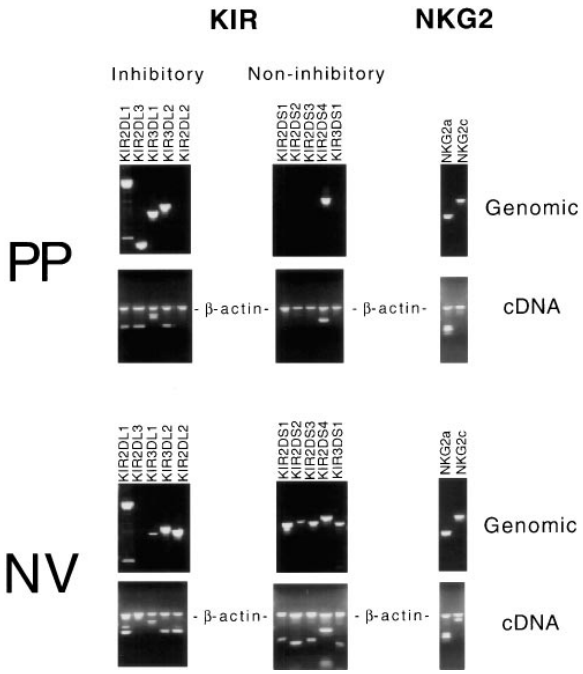

Figure 2. Donors PP and NV Possess Distinct Sets of KIR Genes

PCR-SSP typing for the NK cell receptor subfamilies indicated was performed on cDNA and genomic DNA samples isolated from PBMC or EBV-transformed BLCLs, respectively. Different pairs of oligonucleotide primers were used for the amplification of class I receptor sequences from the two DNA sources. The PCR products were resolved by agarose gel electrophoresis and analyzed under ultraviolet light for ethidium bromide staining. For amplifications with cDNA, primers specific for β-actin were included as a positive control, and the position of this product is indicated.

independently by antibody binding and flow cytometry on polyclonal, peripheral blood NK cells (Figures 3B and 3D). This concordance indicates that our method of NK cell cloning introduces little bias toward particular subpopulations of NK cells, giving confidence that assessment of the patterns of class I receptor expression obtained from NK cell clones reflects those of the NK cell populations in vivo. The exception to this generalization was that the NK cell clones obtained from NV were enriched for expression of CD94:NKG2a when compared to his peripheral blood NK cells (Figure 3D).

The two donors differ not only in their complement of KIR genes, but also in the extent to which they use them (Figure 3). The only receptor expressed by every NK cell clone is KIR2DL4 (103AS), a structurally divergent family member of unknown function. The majority of donor PP's NK cells express the inhibitory receptors KIR3DL2 or CD94:NKG2a or the noninhibitory receptor KIR2DS4, whereas no single inhibitory or noninhibitory receptor is expressed by more than half of the NK cells from NV (Figures 3A and 3C). Similar numbers of NK cells from both donors express inhibitory receptors from the KIR2DL1, KIR2DL2/3, and KIR3DL2 subfamilies even though donor PP has no class I ligand (group 2 HLA-C) for KIR2DL1. For KIR3DL1 expression, the two donors differ substantially: in PP approximately 50% and in NV

Table 1. HLA Class I Ligands and Receptors of Donors PP and NV

HLA Class I Ligands				
PP		NV		
Allotypes[a]	KIR Epitopes[b]	Allotypes[a]	KIR Epitopes[b]	
A*0101	Unknown	A*0201	Unknown	
A*0301	Unknown	A*0301	Unknown	
B*0702	Unknown	B*0702	Unknown	
B*1501	Unknown	B*2702	Bw4 HLA-B	
Cw*0304	Group 1 HLA-C	Cw*0202	Group 2 HLA-C	
Cw*0702	Group 1 HLA-C	Cw*0702	Group 1 HLA-C	
HLA Class I Receptors				
PP		NV		
Receptor Subfamily[c]	Receptors[d]	Receptor Subfamily[c]	Receptors[d]	Specificity
Inhibitory KIR		Inhibitory KIR		
KIR2DL1	NKAT1 p58cl42	KIR2DL1	KIR2DL1v	Group 2 HLA-C
KIR2DL3	NKAT2A KIR2DL3v	KIR2DL2	p58cl43	Group 1 HLA-C
KIR3DL1	NKAT3 NKB1	KIR3DL1	KIR3DL1v	Bw4 HLA-B
KIR3DL2	NKAT4 AMC5/cl5	KIR3DL2	AMC5/cl5	HLA-A?
KIR2DL4	KIR2DL4v1 KIR2DL4v2	KIR2DL4	KIR2DL4v1 KIR2DL4v3	Unknown
Noninhibitory KIR		Non-inhibitory KIR		
		KIR2DS1	KIR2DS1v	Group 2 HLA-C
		KIR2DS2	NKAT5	Group 1 HLA-C
		KIR2DS3	KIR2DS3v	Unknown
KIR2DS4	p58cl39	KIR2DS4	p58cl39	Unknown
		KIR3DS1	KIR3DS1v	Unknown
Inhibitory Lectins		Inhibitory Lectins		
CD94:NKG2a	CD94:NKG2a	CD94:NKG2a	CD94:NKG2a	HLA-A, -B, and -C
Noninhibitory Lectins		Noninhibitory Lectins		
CD94:NKG2c	CD94:NKG2C	CD94:NKG2c	CD94:NKG2C	Unknown

[a] The HLA class I alleles were identified by nucleotide sequencing.

[b] The predicted KIR epitopes for each class I allele were determined from the deduced amino acid sequences of the HLA class I heavy chain allotypes. The group 1 and 2 HLA-C epitopes are distinguished by the diallelic positions Ser 77, Asn 80 (group 1) and Asn 77, Lys 80 (group 2) (Colonna et al., 1993b).

[c] The different class I receptors (KIR and CD94/NKG2) were grouped into subfamilies as described (Uhrberg et al., 1997). The system of nomenclature used for KIR subfamilies was suggested by E. Long (National Institutes of Health, Bethesda, MD), L. Lanier (DNAX Institute, Palo Alto, CA), and M. Colonna (Basel Institute, Basel, Switzerland). This system takes into account the different number of extracellular immunoglobulin domains, the lengths of the cytoplasmic tails, and the nucleotide sequence relationships of the different KIR family members. It also correlates with the class I specificity and antibody reactivity of the different receptors. All known KIR can be divided into two groups based on the presence of two or three extracellular immunoglobulin domains; these are designated KIR2D and KIR3D, respectively. The receptors can also be divided based on the length of their cytoplasmic tails. Receptors having long tails are designated L, and those having short tails are designated S. Inhibitory receptors have long cytoplasmic tails possessing two ITIM motifs, whereas noninhibitory receptors have short tails and lack ITIMs. Similarly, the heterodimeric CD94:NKG2 receptors are divided based on the presence (inhibitory lectins) or absence (noninhibitory lectins) of ITIMs in the cytoplasmic tails of their NKG2 chains (Houchins et al., 1997).

[d] The KIR sequences characterized for each donor are listed. Variant sequences not identified previously are designated with a v. Eight variants differ from known KIR subfamily members by one or two nucleotide substitutions. The KIR2DL1 variant from donor NV differed from other KIR2DL1 subfamily members by nine or ten nucleotide changes, seven of which are shared by KIR2DL1v and the KIR2DS1 sequences: EB6Act1 and EB6Act2 (Biassoni et al., 1996). The KIR2DS1v, KIR2DS3v, KIR3DS1v, and KIR2DL4v sequences are related to EB6Act1, NKAT7, NKAT10, and 103AS sequences, respectively.

approximately 5% of NK cells express this KIR subfamily, even though NV possesses a class I ligand ($Bw4^+$ HLA-B) for this receptor but PP does not.

Individual NK cell clones from both donors were found to express between 2 and 9 different molecules of the KIR and CD94:NKG2 families combined. The frequencies of cells expressing different numbers of receptors resembles a normal distribution (Figure 4). NK cells from PP tend to express more receptors than those from NV, as seen from the modal number of receptors expressed per cell, 5 for PP and 4 for NV, as well as the mean numbers of receptors expressed per cell, 4.5 for PP and 4.2 for NV. Limiting the analysis to receptors that have inhibitory (ITIM) motifs in their cytoplasmic tails or recep-

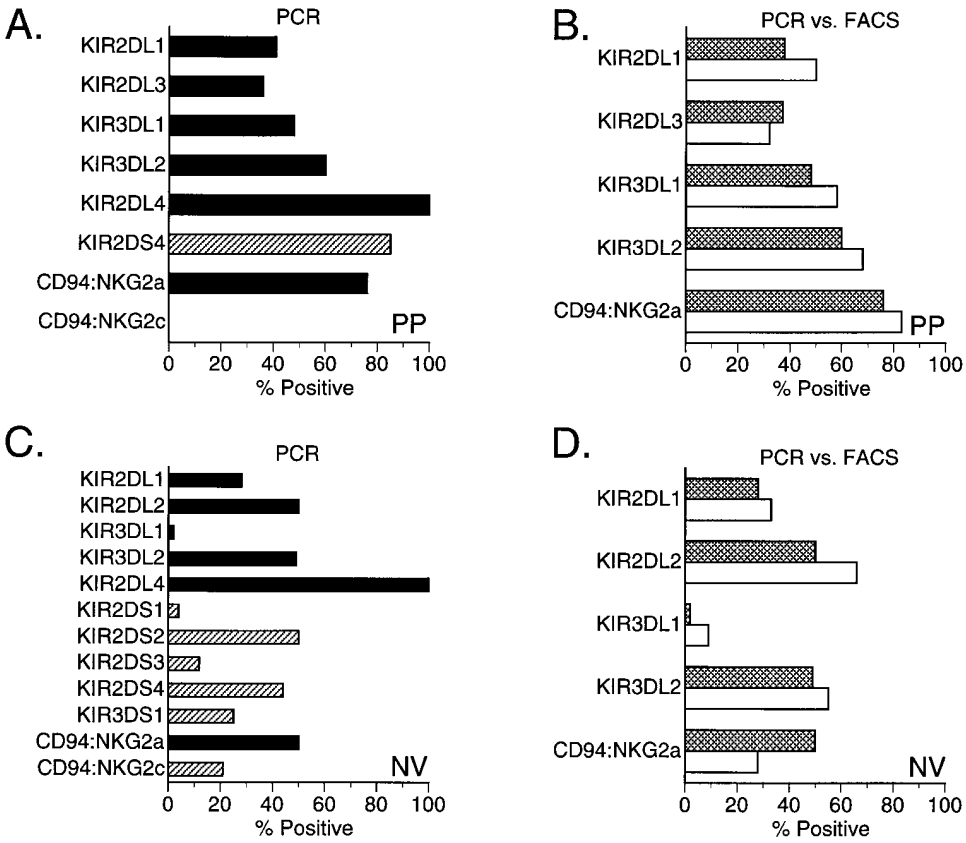

Figure 3. Frequencies of Expression of the Different Class I Receptor Subfamilies

The numbers of NK cells from donor PP (A and B) and donor NV (C and D) expressing class I receptors were determined by two methods. (A and C) PCR-SSP typing of NK cell clones (n = 108 for PP and n = 111 for NV) was used to determine the clones expressing transcripts for the indicated inhibitory (solid bars) and noninhibitory (striped bars) receptors.

(B and D) The percentages determined by PCR typing (hatched bars) are compared to the results from three-color flow cytometric analyses (open bars) obtained by staining donor-derived PBMC with antibodies specific for different class I receptor subfamilies.

For flow cytometric analyses the percentage of cells expressing each receptor was calculated on the $CD3^{-}CD56^{+}$ NK cell subset. Antibodies used were Leu4 (anti-CD3), Leu19 (anti-CD56), EB6 (anti-KIR2DL1 and anti-KIR2DS1), DX27 (anti-KIR2DL2/3 and anti-KIR2DS2), DX9 (anti-KIR3DL1), DX31 (anti-KIR3DL2), and a rabbit polyclonal antiserum specific for CD94:NKG2a. FACS, fluorescence-activated cell sorting.

tors that do not reveals a greater difference between the two donors. NK cells from PP have a range of 2–6 inhibitory receptors per cell and a mode of 4, whereas those from NV have a range of 2–5 and a mode of 3. By contrast, NV's NK cells express more noninhibitory receptors (0–5 per cell) than do those from PP (0–1 per cell).

All of PP's and NV's NK cell clones express more than one class I receptor. We calculated the observed frequencies for combinations of receptors and compared them to those expected from the products of their individual frequencies. In almost all cases the observed and expected frequencies were similar (Table 2). The significant exception was that the frequencies of NK cells expressing CD94:NKG2a in association with any inhibitory KIR were lower than expected from random association. This trend, observed for both donors, was most striking for NK cell clones bearing CD94:NKG2a and no inhibitory KIR (excluding KIR2DL4/103AS), whose observed frequency in both donors was >3 times higher than would have been predicted from random association (data not shown). Although the expression of individual KIR family members is largely stochastic, there is some limitation on the simultaneous expression of the inhibitory KIR and CD94:NKG2a receptors.

The Inhibitory Receptor Repertoire of Donor PP Is Dominated by One Lectin-like Receptor and One Ig-like Receptor

For each NK cell clone the patterns of receptor expression determined by PCR-SSP and self HLA class I–mediated inhibition of cytolysis were compared. Expression of receptors of known HLA class I specificity can account for the functional inhibition of more than 95% of the PP NK cell clones (Figure 1). That the assigned receptors were responsible for the inhibition was confirmed by showing that receptor-specific antibodies interfered with inhibition (data not shown). For donor PP, the HLA class I–mediated inhibition of NK cell clones appears mainly to be accounted for by two receptors: KIR2DL3 and CD94:NKG2a. KIR2DL3 is expressed by 36% of PP's NK cells, which constitute the subset strongly inhibited by the autologous HLA-C allotypes (Cw*0304 and Cw*0702). CD94:NKG2a is expressed by 72% of PP's NK cells, which constitute the subset inhibited by A*0101, A*0301, B*0702, and Cw*0304 (Figure

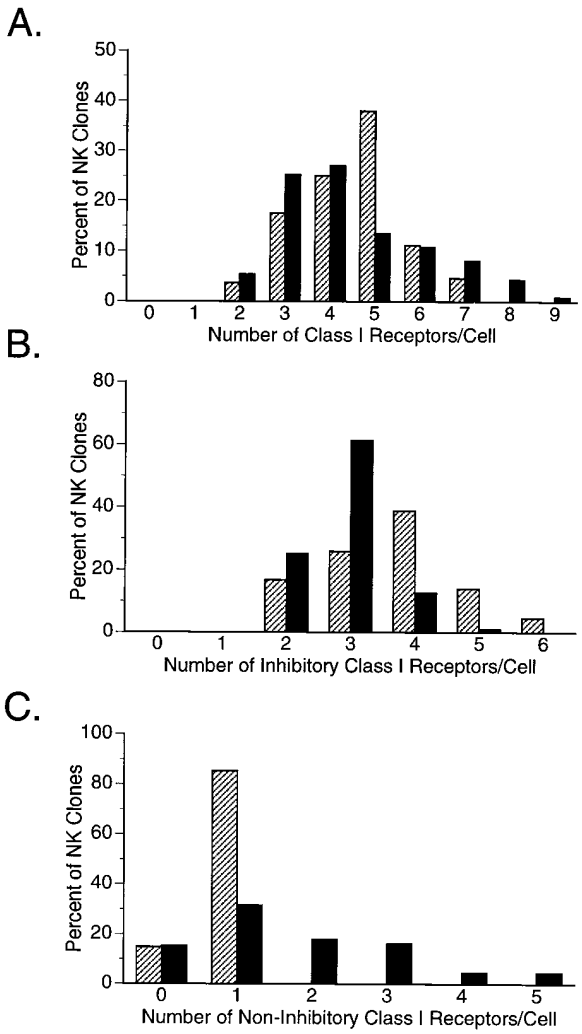

Figure 4. NK Cell Clones from PP and NV Express 2–9 KIR and CD94:NKG2 Family Members

The results from the PCR typing assays were compiled and used to determine the percentage of NK cell clones of donors PP (striped bars) and NV (solid bars) expressing different numbers of class I receptors. The receptors listed in Table 1 are included in the analysis and are divided into three groups: (A) all receptors; (B) inhibitory receptors; and (C) noninhibitory receptors. All of the NK cell clones from both donors expressed the inhibitory receptor KIR2DL4 (103AS).

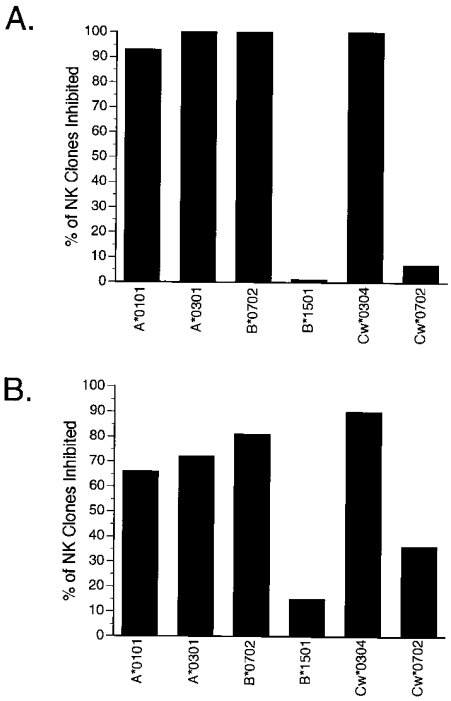

Figure 5. Specificity of the CD94:NKG2a Receptor for Four of the HLA Class I Allotypes of Donor PP

(A) Fifteen NK cell clones selected for expression of CD94:NKG2a and lack of expression of inhibitory KIR (KIR2DL1, KIR2DL3, KIR3DL1, and KIR3DL2) were tested for their inhibition by autologous HLA class I allotypes. The inhibitory pattern of these CD94:NKG2a-dependent, KIR-independent clones is compared with that of all (n = 108) NK cell clones obtained from donor PP (B).

1). The inability of B*1501 to inhibit efficiently any NK cell clone suggests that it is not a ligand for the inhibitory receptors (KIR or CD94:NKG2a) found in this individual's repertoire.

To test the hypothesis that CD94:NKG2a is responsible for the pattern of inhibition produced by A*0101, A*0301, B*0702, and Cw*0304 (Figure 1), we analyzed the inhibition by self HLA class I allotypes of NK cell clones from PP that are devoid of inhibitory KIR (excluding KIR2DL4). All these clones have CD94:NKG2a as their only inhibitory receptor, and almost all were inhibited by the self allotypes A*0101, A*0301, B*0702, and Cw*0304, but not by B*1501 and Cw*0702 (Figure 5). Thus, the CD94:NKG2a receptor accounts for all of the inhibition produced by A*0101, A*0301, and B*0702 and for the inhibition by Cw*0304 not mediated by KIR2DL3. Other investigators have proposed that KIR3DL2 (p140; NKAT4) has inhibitory specificity for A*0301 (Döhring et al., 1996; Pende et al., 1996). Although this receptor is expressed by 66% of PP's NK cells, we found no correlation between expression of KIR3DL2 and the susceptibility of an NK cell to inhibition by the self allotype A*0301. The same result was obtained from analysis of the NK cells from NV, who expresses HLA-A*0301 and has KIR3DL2 on 50% of his NK cells. Furthermore, inhibition of NK cell cytotoxicity by the A*0301-expressing transfectant was not perturbed by the addition of KIR3DL2-specific antibody to cell-killing assays (data not shown). For these two donors, inhibition by A*0301 appears to be mediated solely through the lectin-like receptor, CD94:NKG2a.

Further demonstration that inhibition of PP's NK cells by self HLA class I is mediated largely through engagement of the KIR2DL3 and CD94:NKG2a receptors came from examining the effects of different anti-receptor antibodies on the inhibition (Figure 6). The only antibodies that enabled clones of PP's NK cells to lyse the autologous B cell line were ones with specificity for KIR2DL3 or CD94:NKG2a (Figures 6A–6D). NK cell clones expressing KIR2DL3 in the absence of CD94:NKG2a lysed autologous B cells in the presence of anti-KIR2DL3, while, conversely, NK cell clones expressing CD94:NKG2a but not KIR2DL3 lysed autologous B cells in the presence of anti-CD94:NKG2a. Clones expressing both

Table 2. Comparison of Observed and Expected Frequencies of NK Cell Receptor Pairs

PP

Receptors		KIR2DL1 (0.38)	KIR2DL3 (0.36)	KIR3DL1 (0.48)	KIR3DL2 (0.66)	KIR2DS4 (0.85)
KIR2DL1	Obs.	—				
(0.38)	Exp.	—				
KIR2DL3	Obs.	0.17	—			
(0.36)	Exp.	0.14	—			
KIR3DL1	Obs.	0.20	0.19	—		
(0.48)	Exp.	0.18	0.17	—		
KIR3DL2	Obs.	0.26	0.25	0.28	—	
(0.66)	Exp.	0.25	0.24	0.32	—	
KIR2DS4	Obs.	0.32	0.31	0.43	0.55	—
(0.85)	Exp.	0.32	0.31	0.41	0.56	—
CD94:NKG2a	Obs.	0.21	0.19	0.28	0.42	0.64
(0.72)	Exp.	0.27	0.26	0.35	0.48	0.61

NV

Receptors		KIR2DL1 (0.28)	KIR2DL2 (0.51)	KIR3DL2 (0.50)	KIR2DS2 (0.50)	KIR2DS3 (0.12)	KIR2DS4 (0.44)	KIR3DS1 (0.25)	CD94:NKG2a (0.50)
KIR2DL1	Obs.	—							
(0.28)	Exp.	—							
KIR2DL2	Obs	0.10	—						
(0.51)	Exp.	0.14	—						
KIR3DL2	Obs.	0.12	0.29	—					
(0.50)	Exp.	0.14	0.25	—					
KIR2DS2	Obs.	0.20	0.25	0.29	—				
(0.50)	Exp.	0.14	0.25	0.25	—				
KIR2DS3	Obs.	0.05	0.07	0.07	0.08	—			
(0.12)	Exp.	0.03	0.06	0.06	0.06	—			
KIR2DS4	Obs	0.16	0.25	0.27	0.29	0.07	—		
(0.44)	Exp.	0.12	0.22	0.22	0.22	0.05	—		
KIR3DS1	Obs	0.11	0.16	0.14	0.16	0.06	0.14	—	
(0.25)	Exp.	0.07	0.13	0.13	0.13	0.03	0.11	—	
CD94:NKG2a	Obs	0.07	0.16	0.19	0.20	0.05	0.23	0.13	—
(0.50)	Exp.	0.14	0.25	0.25	0.25	0.06	0.22	0.13	—
CD94:NKG2c	Obs.	0.05	0.14	0.14	0.13	0.05	0.12	0.05	0.11
(0.21)	Exp.	0.06	0.11	0.10	0.10	0.03	0.09	0.05	0.10

The NK cell receptors are listed with their individual frequencies in parentheses. Analysis was not performed on CD94:NKG2c for PP or on KIR3DL1 or KIR2DS1 for NV because these receptors are expressed at too low a frequency. The observed (obs.) frequencies for each receptor pair were calculated from PCR typing of more than 100 NK clones from each donor ($n = 108$ for PP; $n = 111$ for NV). The expected (exp.) frequencies for random expression of receptor pairs are the products of each receptor's individual frequency. The underlined values differ significantly ($P < 0.01$) from that expected by random association. Statistical significance was determined using a 2×2 analysis in combination with a G test of independence.

KIR2DL3 and CD94:NKG2a lysed autologous B cells in the presence of the combination of anti-KIR2DL3 and anti-CD94:NKG2a but not in the presence of either antibody alone (Figure 6D). Antibodies specific for other inhibitory receptors had no effect on the resistance to lysis of the autologous B cell targets, even though many of the NK cell clones expressed the receptors.

Similar effects were seen in cytolytic assays using polyclonal NK cells from PP as the effector cells and the autologous B cell line as the target, a situation in which all of the inhibitory interactions between the donor's NK cell receptors and class I ligands are assessed simultaneously. Addition of antibodies specific for either KIR2DL3 or CD94:NKG2a produced substantial lysis, and addition of the two antibodies together gave an effect equivalent to that obtained with a monomorphic anti-HLA class I antibody (Figure 6E). The presence of other antibodies specific for KIR2DL1, KIR3DL1, and KIR3DL2 did not change appreciably the sensitivity of the autologous B cell targets to lysis by donor PP's NK cells.

Some 5% of the NK cell clones obtained from PP express neither KIR2DL3 nor CD94:NKG2a. For these clones, the inhibition by transfectants expressing individual self HLA class I alleles was difficult to discern, since the effects were weak and variable. However, 221 cells were consistently killed by these cells, whereas the autologous B cell line was resistant to their lysis, in a manner that could be overcome by the inclusion of anti-HLA class I MAb (data not shown). Inhibition of clones such as these may require the combined effects of two or more self HLA class I allotypes.

The Inhibitory Receptor Repertoire of Donor NV Is Dominated by One Lectin-like Receptor and Two Ig-like Receptors

The patterns of HLA class I–mediated inhibition of NV's NK cell clones (Figure 1) correlated precisely with the expression of specific receptors, and their involvement in the inhibition was demonstrated directly in antibody-blocking experiments. All three well-established KIR ligands are represented in the self HLA class I allotypes

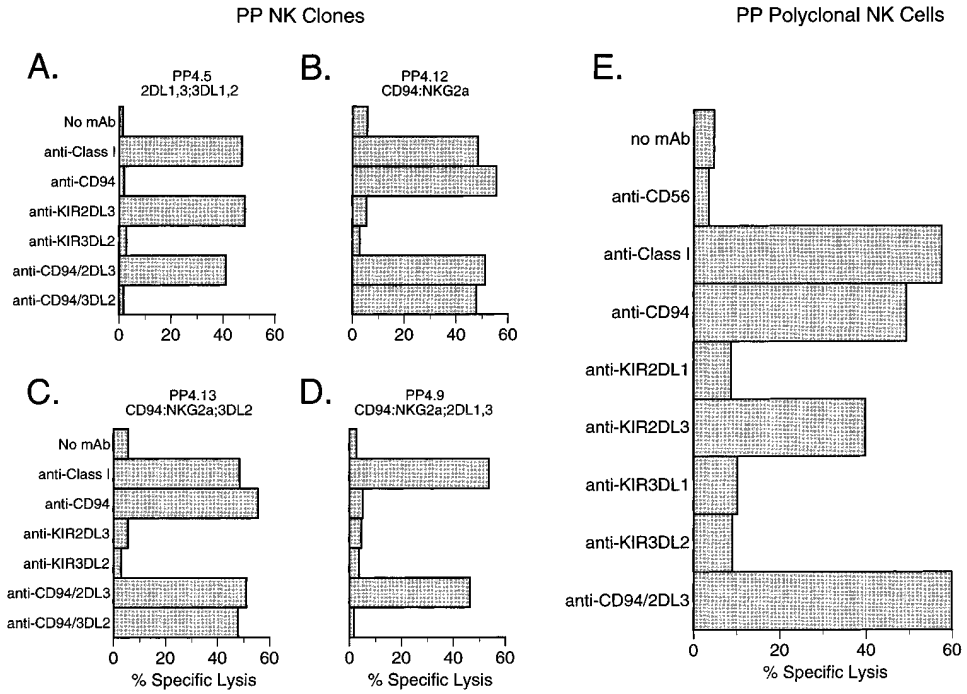

Figure 6. Antibodies Specific for CD94, KIR2DL3, and HLA Class I Permit Lysis of Autologous Cells by Donor PP–Derived NK Cells

NK clones (A–D) or a polyclonal NK cell population (E) from donor PP were used as effectors in cell-killing assays against autologous BLCL targets. Assays were performed in the presence (25 μg/ml) or absence of the indicated antibodies and the percentage specific lysis calculated. Antibodies used were Leu19 (anti-CD56), DX17 (anti-class I), DX22 (anti-CD94), EB6 (anti-KIR2DL1 and anti-KIR2DS1), DX27 (anti-KIR2DL2/3 and anti-KIR2DS2), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2).

(A–D) For the experiments with clones, addition of MAbs Leu19, EB6, and DX9 did not affect NK cell cytolysis of autologous cells (data not shown). The different combinations of inhibitory receptors expressed by the NK cell clones are listed below each clone designation.

(E) The polyclonal NK cells exhibited the same pattern of receptor expression seen for peripheral blood NK cells (data not shown).

of donor NV, who also expresses inhibitory KIR specific for each of the ligands. Of these, the KIR3DL1 receptor, which recognizes the autologous B*2702 allotype, is expressed on very few of NV's NK cells (Figure 3). Each of the receptors for the two different inhibitory epitopes of HLA-C is expressed on 30%–50% of the NK cells and is responsible for the strong inhibition by HLA-C allotypes observed in Figure 1: KIR2DL1 has specificity for Cw*0202 and KIR2DL2 has specificity for Cw*0702. By contrast, the inhibition by Cw*0202 not mediated by KIR2DL1, the inhibition by B*2702 not mediated by KIR3DL1, and all inhibition by A*0201, A*0301, and B*0702 result from interaction with the lectin-like receptor CD94:NKG2a. Only one NK cell clone obtained from NV does not express a receptor of well-established HLA class I specificity. Although expressing KIR3DL2, this clone was inhibited poorly by all of the self HLA class I allotypes when tested individually but was inhibited by their combination as expressed on the autologous B cell line (data not shown).

Antibodies specific for the different receptors were tested for their capacity to disrupt the inhibitory interactions between NV's NK cells and the autologous B cell line. Cell-killing assays with NV's NK cell clones demonstrated that antibody blocking of the predicted inhibitory receptor–class I ligand interactions resulted in lysis of the autologous targets (Figures 7A–7D). The contributions of the different inhibitory receptors to NV's NK cell repertoire is reflected in the relative effects that antibodies specific for the different receptors have on the lysis of the NV B cell line by a polyclonal population of NK cells. Whereas there is no lysis in the absence of antibody, the addition of antibodies specific for KIR2DL1, KIR2DL2, and CD94:NKG2a all resulted in substantial cytolysis (Figure 7E).

Although NV's NK cells express more noninhibitory KIR and NKG2 family members than PP's NK cells, the presence of these receptors did not affect the functions of the inhibitory receptors when presented with a compatible HLA class I allotype. Even when a single inhibitory receptor was expressed in combination with five noninhibitory receptors, as occurred for two of NV's NK cell clones, inhibition predominated. NV expresses inhibitory and noninhibitory receptors, both of which can interact with the same HLA class I allotypes. For example, KIR2DS2 (noninhibitory) and KIR2DL2 (inhibitory) are predicted to engage the self allotype Cw*0702 (Table 1). Of the more than 100 NK cell clones studied, 38 expressed such combinations of receptors, and in every instance target-cell expression of their shared class I ligand by a 221 transfectant led to inhibition (data not shown). These observations suggest that any signal

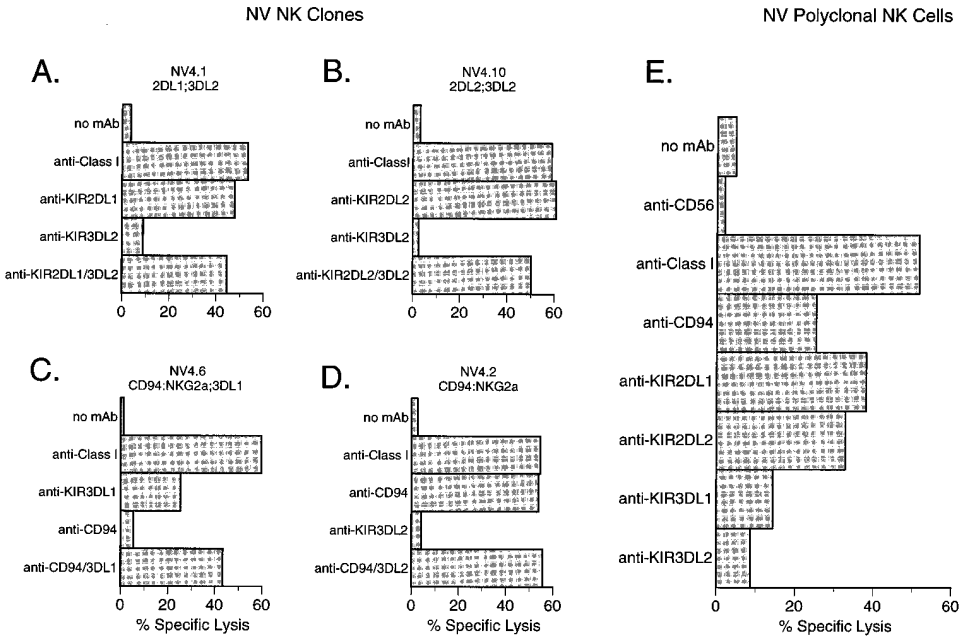

Figure 7. Antibodies Specific for CD94, KIR2DL1, KIR2DL2, KIR3DL1, and HLA Class I Permit Lysis of Autologous Targets by NK Cells from Donor NV

Donor NV–derived NK clones (A–D) or a polyclonal NK cell population (E) were used as effectors in cell-killing assays against autologous BLCL targets. Assays were performed in the presence (25 μg/ml) or absence of the antibodies indicated and the percentage specific lysis determined. Antibodies used were the same as those in Figure 6. The different combinations of inhibitory receptors expressed by the NK cell clones (A–D) are listed below each clone designation.

(A–D) For the experiments with clones, the antibodies not shown had no effect.

(E) The polyclonal NK cells exhibited the same pattern of receptor expression seen for peripheral blood NK cells (data not shown).

generated through engagement of the noninhibitory receptors is subordinate to those transduced by the inhibitory receptors, a thesis compatible with our failure to find any NK cell clone that lysed the autologous B cell line.

Human NK Cell Alloreactivity Is Determined by the Combination of HLA Class I Receptors and Ligands

Since no NK cell from either donor lyses the autologous B cell line and every NK cell is inhibitable by autologous HLA class I, it seems likely that self HLA class I imposes some selection on the receptors expressed by NK cells. At a minimum, the requirement would be that every NK cell expresses one inhibitory receptor that can be engaged by self class I. In that case NK cells from one person need not be inhibitable by the HLA class I allotypes of a second person (Moretta et al. 1990). To assess this proposition for donors PP and NV, we examined the extent to which NK cell clones from one donor could be inhibited by the HLA class I allotypes of the other donor (Figure 8). Some 15% of the NK cell clones from NV lysed the B cell line from PP, whereas none of the NK cell clones from donor PP lysed the B cell line from donor NV.

The nature of this one-way alloreaction can be readily interpreted in terms of the repertoire of class I receptors expressed by the two donors. The CD94:NKG2a and KIR2DL3 receptors are the dominant inhibitory receptors in the repertoire of PP, and since the NV B cell line has ligands for both of these receptors there is no NK cell alloreactivity of PP for NV. By contrast, NV has a subset of NK cells that use the KIR2DL1 receptor as their only inhibitory receptor for HLA class I, and among the HLA class I allotypes of PP there is no ligand for this receptor. All of the NK cell clones from NV that kill the PP B cell line express KIR2DL1 and do not express CD94:NKG2a or KIR2DL2, receptors that are inhibitable by the HLA class I allotypes of PP. These observations show that histocompatibility for NK cell alloreactions in transplantation need not be dependent on HLA identity.

Discussion

We can now describe in some detail the NK cell receptor repertoire of two healthy persons who differ both in their HLA class I type and in their KIR type. For both donors the expectations of the missing-self model were met: first, every NK cell was inhibited by the combination of self HLA class I allotypes and almost all were inhibited by individual allotypes; second, the patterns of HLA class I inhibition were highly correlated with the expression of functionally well-characterized receptors of particular HLA class I specificity.

In purely molecular terms, the human NK cell receptor repertoire is highly diverse. For example, from more than 100 NK cell clones established from each donor we identified 33 distinct receptor (KIR and CD94:NKG2) phenotypes for PP and 64 for NV. This diversity can be attributed largely to the KIR family of class I receptors

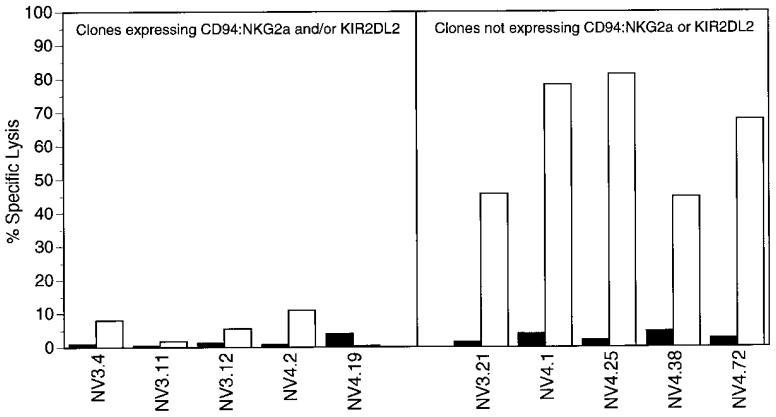

Figure 8. A Subset of NK Cell Clones from Donor NV Lyse PP BLCL

NK cell clones were tested for cytolysis of autologous (NV, solid bars) or allogeneic (PP, open bars) BLCL. Results are presented as percentage specific lysis. Clones that failed to lyse the PP BLCL express CD94:NKG2a and/or KIR2DL2, whereas clones that lyse the allogeneic target cells express neither receptor. Data from ten representative NV clones are shown. None of PP's NK cell clones lyses either NV or PP BLCL (data not shown).

and involves contributions from the following effects: (1) individuals express the products of six or more different KIR genes; (2) the KIR genes are polymorphic; (3) NK cells can express 2–9 different receptors in different combinations; and (4) persons express different combinations of HLA class I ligands, which during development select compatible NK cells from the primary repertoire and ensure that NK cells without a receptor for self HLA class I do not appear in the peripheral circulation (Uhrberg et al., 1997, and the present study).

In contrast to the complexity observed at the molecular level, the "functional" repertoire of NK cell receptors appears simple when considered in the context of inhibition by self HLA class I molecules. In PP's repertoire, 52.6% of the cells are regulated by CD94:NKG2a, 16.7% are regulated by KIR2DL3, and 19.4% are regulated by the combination of CD94:NKG2a and KIR2DL3. In NV's repertoire, 27.6% of the NK cells are regulated by CD94:NKG2a, 14.2% by KIR2DL1, 24.2% by KIR2DL2, 32.8% by any combination of two receptors, and 0.9% by all three.

Within the two repertoires we studied, inhibition of every NK cell appears to be signaled either through a lectin-like receptor or through a KIR, and from the perspective of the individual NK cell the two structurally distinct types of receptors are seen to have equivalent and independent functions. In both repertoires the lectin-like and the Ig-like receptors divide the responsibility for regulating NK cells, and in the context of an NK cell population the two types of receptor are seen to be complementary. Illustration of this complementarity is that NK cell clones ($\sim$20% in each donor) that lack all four of the major inhibitory KIR (excluding KIR2DL4/103AS) always express the CD94:NKG2a receptor. The greater use of KIR by NV correlates with NV's possessing three different ligands for KIR and PP's having only one. That a larger proportion of PP's NK cells express CD94:NKG2a, for which almost all HLA-A, -B, and -C allotypes are ligands, correlates with PP's having two fewer ligands for KIR than NV. This difference suggests that the extent to which CD94:NKG2a is used in an individual's NK cell repertoire may be regulated by the number of KIR that can be used as inhibitory receptors for the set of self HLA class I allotypes.

The expression of the CD94:NKG2 and KIR families of molecules on human NK cells does not seem to be highly constrained, beyond the requirement for each cell to express a receptor having specificity for a self HLA class I allotype. All of the genes possessed by an individual are expressed by some NK cells, and there is no apparent limitation on the expression of several receptors by a single cell or the expression of receptors for which the self HLA class I type does not provide a ligand. The frequency with which cells express a combination of KIR is the product of the individual frequencies, as has been observed similarly for the Ly49 class I receptors of mouse NK cells, leading to proposal of the "product rule" (Raulet et al., 1997). By contrast, the frequencies with which CD94:NKG2a is coexpressed with inhibitory KIR on an NK cell's surface are lower than expected from the product rule. These observations provide evidence for some coordination in expression of the two types of receptor. A hierarchy of receptor expression might also exist between the KIR specific for HLA-B and HLA-C, which would explain the observation that very few of NV's NK cells use the KIR3DL1 receptor, although the self HLA-B*2702 allotype is a good ligand for this receptor.

This investigation focused on defining the repertoire of inhibitory receptors for self class I within individual humans. The results show that it can be defined largely within the context of the receptors having well-defined HLA class I specificity. Within the KIR family are additional molecules of unknown HLA class I specificity, including ones that are noninhibitory receptors with and without known class I ligands. In this analysis any functional role of the noninhibitory receptors in binding self HLA class I was found to be subservient to that of the inhibitory receptors. However, the noninhibitory receptors are expressed to a degree comparable to that of the inhibitory receptors, with more than 80% of the NK cells in both donors expressing one or more noninhibitory receptors.

Mice seem exclusively to use lectin-like molecules as the H-2 class I inhibitory receptors of their NK cells, contrasting with the use of Ig-like and lectin-like receptors by humans (Gumperz and Parham, 1995; Valiante et al., 1997). Despite this structural dichotomy, humans and mice do show similarities. In PP and NV, the relative use of the lectin-like and the Ig-like receptors varies, and so the difference between humans and mice might better be thought of as a difference of quantity rather

than of quality. Furthermore, expression of the murine Ly49 and human KIR family members appears to contain a strong stochastic element, and the product rule governs the expression of most receptor combinations (Raulet et al. 1997, and the present study). Recently, Held and Raulet (1997) reported that the frequency of NK cells expressing an endogenous Ly49 receptor was reduced in mice transgenic for a different Ly49 receptor having overlapping H-2 specificity. This finding suggests that selection by self class I can limit the number of inhibitory receptors per NK cell, and it is consistent with our results showing that the NK cells from NV (three KIR ligands) express fewer inhibitory receptors per cell than those of PP (one KIR ligand). These parallels suggest that common principles govern the development of murine and human NK cell repertoires, despite the use of different receptors by the two species.

The one-way pattern of NK cell alloreactivity between donors PP and NV can be explained readily by differences in NK cell receptor repertoire and HLA class I type. Because NV and PP are heterozygous and homozygous, respectively, for KIR ligands, the NK cells of NV include some that cannot be inhibited by PP's HLA class I type, whereas all of PP's NK cells are inhibited by NV's HLA class I type. Such alloreactivity of a heterozygote toward a homozygote is directly analogous to the NK cell–mediated rejection of parental bone marrow transplants by F1 hybrid mice (Öhlen et al., 1989; and reviewed by Yokoyama, 1995; George et al., 1997). Although the alloreactivity of human NK cells is well known (Ciccone et al. 1988, 1990a, 1990b, 1992; Colonna et al., 1992; Bellone et al. 1993), its role in clinical bone marrow transplantation has not been worked out. The comparison of PP and NV suggests that alloreactive NK cells will, in almost all donor-recipient combinations, rely on KIR for their inhibitory receptors. Furthermore, the number of inhibitory KIR ligands provided by a person's HLA phenotype should correlate with the alloreactive potential of their NK cells: in general, a person's NK cells will be alloreactive toward cells from individuals lacking the KIR ligands that he or she possesses and, conversely, will be tolerant of cells from individuals who have the same or additional KIR ligands. For inhibitory KIR, the complexity of HLA class I polymorphism appears reduced to three ligands, which combine to produce six different KIR–ligand phenotypes. In bone marrow transplantation these six phenotypes can be combined in 72 different ways. It is therefore feasible to determine whether the principles governing the alloreactions between PP and NV are generally applicable to the population at large.

Experimental Procedures

Cloning and Sequencing of HLA Class I and NK Cell Receptor Transcripts

HLA class I cDNA clones were isolated from mRNA and sequenced as described (Ennis et al., 1990; Domena et al., 1993). cDNA clones for each allele were subcloned into the PBJ1neo expression vector (Lin et al., 1990) and transfected into 721.221 cells (a class I–deficient B lymphoblastoid cell line [BLCL]). Transfections and the selection, maintenance, and characterization of the resulting transfectants were performed as described (Shimizu and DeMars, 1989; Litwin et al., 1993).

NK cell receptor cDNAs were characterized by a similar strategy. Oligonucleotide primers based on conserved sequences of the KIR family members were used to amplify by PCR cDNA from peripheral blood mononuclear cells (PBMC), polyclonal cultured NK cells, and NK cell clones. Four different 5′ primers (2IGF: 5′-GGGCGTCGACCCATGGCGTGTGTTGGGTTCT−3′; 3IG5′: 5′-GGGCGTCGACCACTCATGGGTGGTCAGGAC−3′; NKAT1–5′UT: 5′-GCGCGTCGACTGCCTGTCTGCTCC−3′; and cl11–5′UT: 5′- GGGCGTCGACGGCGCAGCCGCCTGTCG−3′) were paired with one 3′ primer (NKR: 5′-CGCCAAGCTTGTTTGAGACAGGGCTGTTG−3′). KIR2DL4 (103AS) sequences were amplified with an additional primer set (103AS5′: 5′-GCGCGTCGACTGCACCATGTCCATGTCA-3′; and 2DL43′UT-1: 5′- CGCCGAATTCGTGAGGAAGAGTGATGCT-3′). Underlined sequences represent SalI, HindIII, and EcoRI restriction sites introduced into the primers to allow cloning of the amplification products. 5′ primers match 5′ untranslated (UT) or signal peptide sequences of KIR, and the 3′ primers match conserved sequences in the KIR 3′UT. The resulting products were subcloned into pBLUESCRIPT SK$^+$ and partially sequenced using a standard T7 or T3 primer and dye terminator automated sequencing (Applied Biosystems, Foster City, CA). Three or four clones for each KIR family member identified were fully sequenced on both strands and a consensus sequence was determined. The GenBank accession numbers for the KIR variants first identified here are KIR2DL1v (AF022045), KIR2DL3v (AF022048), KIR3DL1v (AF022049), KIR2DS1v (AF022046), KIR2DS3v (AF022047), KIR3DS1v (AF022044), KIR2DL4v1 (AF034771), KIR2DL4v2 (AF034772), and KIR2DL4v3 (AF034773).

PCR-SSP Typing for NK Cell Receptors

The RT-PCR–based typing assay for KIR and CD94:NKG2 subfamilies is described in detail by Uhrberg et al. (1997).

Antibodies

MAbs and polyclonal antibodies specific for HLA class I or NK cell receptors were used for cell sorting, for flow cytometric analyses, and as blocking reagents in cell-killing assays. The MAbs used were MAb DX17 (anti-HLA class I), MAb EB6 (anti-KIR2DL1 and anti-KIR2DS1; Coulter, Hyalea, FL), MAb DX27 (anti-KIR2DL2/3 and anti-KIR2DS2), MAb DX9 (anti-KIR3DL1), MAb DX31 (anti-KIR3DL2), MAb DX22 (anti-CD94), MAb Leu4 (anti-CD3; Becton Dickinson, Mountain View, CA), and MAb Leu19 (anti-CD56; Becton Dickinson). The specificities of the DX9, DX17, DX22, DX27, and DX31 antibodies have been described previously (Söderström et al., 1997). In flow cytometry experiments we also used a polyclonal rabbit antiserum specific for the CD94:NKG2a heterodimer (Lazetic et al., 1996).

Flow Cytometry

Three-color flow cytometric analysis was performed on CD3$^-$CD56$^+$ PBMC and polyclonal cultured NK cells using the anti-receptor reagents MAb EB6, MAb DX27, MAb DX9, MAb DX31, and an anti-NKG2a rabbit polyclonal antiserum. Samples were analyzed using a FACScan flow cytometer (Becton Dickinson). The anti-CD3 and anti-CD56 reagents were conjugated with cychrome and fluorescein isothiocyanate respectively, and the anti-KIR reagents were labeled directly with phycoerythrin. The CD94:NKG2a–specific polyclonal rabbit antiserum was detected using a phycoerythrin-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA). The percentage of NK cells expressing each class I receptor was calculated after gating on the CD3$^-$CD56$^+$ lymphocyte subset.

Generation of NK Cell Clones and Polyclonal NK Cell Cultures

NK cell clones were generated and maintained as described (Yssel et al., 1984; Litwin et al., 1993). In brief, PBMC were stained with antibodies for CD3 and CD56, and CD3$^-$CD56$^+$ NK cells were single-cell sorted using a FACStar cell sorter and cyt-clone software and hardware (Becton Dickinson). The NK cell clones were cultured in Iscove's modified Dulbecco's medium (Gibco–BRL, Gaithersburg, MD) containing 200 U/ml recombinant interleukin-2 (kindly provided by C. Reynolds, National Cancer Institute/Biological Response Modifiers Program, Frederick, MD). At the start of culture and weekly thereafter the clones were cocultured with 1×10^6/ml irradiated PBMC (allogeneic) and 1×10^5/ml Epstein-Barr virus (EBV)–

transformed BLCL (JY). Cell-killing and PCR-typing assays were performed 5–7 days after the addition of feeder cells. The PCR-typing assays always contained cells from a "feeders only" culture, without NK cell clones, to control for false-positive signals that may result from amplification of residual KIR transcripts from the irradiated feeder cells. Polyclonal NK cells were cultured using donor-derived PBMC and irradiated EBV-transformed stimulator cells (RPMI-8866) as previously described (Perussia et al., 1987), without modification.

Cell-Killing Assays

Cell-killing assays were performed using NK cell clones or polyclonal populations of NK cells as effectors and ^{51}Cr-labeled target cells: PP- and NV-derived BLCL; 721.221 (untransfected); and 221-A*0101, -A*0201, -A*0301, -B*0702, -B*1501, -B*2702, -Cw*0202, -Cw*0304, and -Cw*0702 class I transfectants. Assays were done two or three times and in duplicate and were performed for 4 hr at an effector:target ratio (E:T) of 6:1. In some assays anti-class I or anti-NK cell receptor antibodies were added (25 μg/ml), and these assays were performed for 4 hr at an E:T of 10:1. The percentage specific lysis was calculated with the following formula: (experimental release [cpm] − spontaneous release [cpm]) / (maximum release [cpm] − spontaneous release [cpm]) × 100 = percentage specific lysis. Some results are presented as percentage inhibition compared to lysis of untransfected 221 target cells, calculated using the following formula: 1 − (% specific lysis of class I–deficient 221 targets / % specific lysis of class I$^+$ targets) × 100 = percentage inhibition. All clones lysed the untransfected 221 target cells at levels exceeding 50%, whereas lysis of the autologous B cell lines never exceeded 10%.

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The Repertoire of Killer Cell Ig-Like Receptor and CD94:NKG2A Receptors in T Cells: Clones Sharing Identical $\alpha\beta$ TCR Rearrangement Express Highly Diverse Killer Cell Ig-Like Receptor Patterns[1]

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Killer cell Ig-like receptor (KIR) and CD94:NKG2A molecules were first defined as human NK cell receptors (NKR), but now are known to be expressed and to function on subpopulations of T cells. Here the repertoires of KIR and CD94:NKG2A expression by T cells from two donors were examined and compared with their previously defined NK cell repertoires. T cell clones generated from peripheral blood of both donors expressed multiple NKR in different combinations and used the range of receptors expressed by NK cells. In both donors $\alpha\beta$ T cells less frequently expressed the inhibitory receptors CD94:NKG2A and KIR2DL1 than either $\gamma\delta$ T cells or NK cells. In contrast to NK cells, not all NKR$^+$ T cells expressed an inhibitory receptor for autologous HLA class I. This lack of specific inhibitory NKR was especially apparent on $\alpha\beta$ T cells of one donor. Overall, $\alpha\beta$ T cells exhibited a distinct pattern of NKR expression different from that of $\gamma\delta$ T and NK cells, which expressed highly similar NKR repertoires. In one donor, analysis of TCR rearrangement revealed a dominant subset of NKR$^+$ T cells sharing identical TCR α- and β-chains. Remarkably, among 55 T cell clones sharing the same TCR$\alpha\beta$ rearrangement 18 different KIR phenotypes were seen, suggesting that KIR expression was initiated subsequently to TCR rearrangement. ***The Journal of Immunology,*** **2001, 166: 3923–3932.**

The physiological functions of NK cells appear to be regulated by arrays of activating and inhibitory receptors on the NK cell surface, some of which interact with oligomorphic determinants of autologous MHC class I molecules (1–3). In humans, NK cell receptors (NKR)[7] consist of two broad classes of membrane glycoprotein: the lectin-like receptors encoded by genes in the NK cell complex on chromosome 12 (4–6), and Ig-like receptors encoded by genes in the leukocyte receptor complex on chromosome 19 (7–9). The predominant HLA class I specificity of the NK cell complex-encoded receptors is determined by CD94:NKG2A (inhibitory) and CD94:NKG2C (activating) receptors that recognize composite ligands consisting of a peptide derived from the leader sequence of an HLA-A, -B, -C, or -G heavy chain bound to HLA-E (10–12). Among the leukocyte receptor complex-encoded receptors, certain members of the killer cell Ig-like receptor (KIR) family have specificity for HLA-A (13, 14), -B (15, 16), -C (17), or -G (18) molecules and are of either the inhibitory or the activating type. In addition, the ILT2 molecule (19), also called LIR 1 (20), has specificity for HLA class I.

Although first characterized on NK cells, the KIR and CD94:NKG2 families are also found on subpopulations of peripheral blood $\alpha\beta$ T cells (21–23), $\gamma\delta$ T cells (24, 25), and T cells in the liver (26). Both CD4 and CD8 $\alpha\beta$ T cells can express NKR, although the latter are much more common (27). The NKR expressed by T cells have been shown to be functional; stimulatory signals coming from the TCR can be overridden by inhibitory signals generated through a KIR or CD94:NKG2A receptor, thereby inhibiting target cell cytolysis and cytokine release (22–24, 27–33). A common feature of NKR$^+$ T cells is a cell surface phenotype that is characteristic of memory T cells; they lack CD28 and CD45RA, mostly express CD45RO, and have high levels of CD18, CD44, CD29, and CD57 (28, 34). This has suggested that induction of NKR expression on T cells occurs only after Ag stimulation.

The overall question to be addressed here is how the repertoire of expression of the different NKR in T cells compares with that which has been described for NK cells. Previous investigations have shown that individual NK cells express a variable number of different NKR (35–37), which can be as many as nine (38). Through expression of different combinations of NKR, a substantial diversity of NKR phenotype is present within an individual's NK cell population. This diversity may help NK cell populations respond to cells infected by diverse viruses and other pathogens. A

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[7] Abbreviations used in this paper: NKR, NK cell receptors; KIR, killer-cell Ig-like receptor; CDR, complementarity-determining region.

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critical factor determining an individual's repertoire of NKR expression is the cohort of NKR genes they inherit. Whereas the CD94 and NKG2 genes appear conserved within human populations, that is not the situation for the KIR gene family. KIR haplotypes differ in the number of genes they contain, with most variation being in the number (between 1 and 5) of activating receptors (9, 39, 40). Adding to the variation is polymorphism at some of the KIR genes (41). The consequence of such genetic differences is that the repertoire of NKR expression by NK cells varies between individual donors. A second factor that influences NKR expression by NK cells is the apparent requirement for each NK cell to be inhibitable by healthy cells expressing the autologous HLA class I type. This requirement for self tolerance is met by each NK cell expressing one or more inhibitory NKR with specificity for one or more autologous HLA class I allotypes (38).

Analysis of the cell surface phenotype using Abs specific for different types of NKR has shown that T cells, like NK cells, can express more than one type of NKR (21, 23). Another form of analysis has been to examine the heterogeneity of $\alpha\beta$ TCRs within cultured lines of T cells that were selected for expression of a particular KIR. Mingari et al. (34) found such lines to be restricted to particular Vβ families. A similar restriction was seen in the NKR$^+$ T cell populations in the peripheral blood. However, different Vβ gene families dominated the T cells that were derived from different donors but selected for expression of the same KIR. In contrast to the results of Mingari et al., Andrea and Lanier (27) did not find as consistent a picture when they compared Vβ usage in peripheral blood T cells that did or did not express KIR3DL1. Although in some donors they found a dominance of certain Vβ families in KIR3DL1$^+$ T cells, in others the differences were more subtle, and in no donor was there evidence for monoclonality or oligoclonality of the KIR3DL1-expressing $\alpha\beta$ T cells.

A factor limiting previous studies to assess NKR expression by T cells was the reliance on Abs for detection and discrimination of NKR. The available Abs detect only a subset of KIR; some are cross-reactive with different types of KIR, and others have polymorphic specificity, so that only the allotypes expressed by certain donors are detected. A second limiting factor was incomplete knowledge of the KIR genotypes of the donors studied. This hinders assessment of whether the observed differences between donors were a consequence of KIR genetics or of functional interactions, either with the products of other host genes or with environmental factors such as pathogens. To address these issues we have taken the following two approaches: first, to focus analysis on T cells from blood donors who have been well characterized for KIR type, HLA type, and NK cell repertoire (38); and second, to supplement Ab-based assessment of NKR expression by T cells with RT-PCR typing, which can be both more specific and more comprehensive (39). Using this overall strategy we have studied the repertoire of NKR expression by T cell clones and peripheral blood T cells from two donors with distinctive KIR genotypes.

Materials and Methods

Flow cytometry

Three-color flow cytometry was performed on PBMC, stained with FITC-coupled anti-CD16 mAb, Cy-Chrome-labeled anti-CD3 mAb (both from Becton Dickinson, Mountain View, CA), and a mixture of PE-coupled NKR-specific mAbs consisting of the anti-KIR reagents EB6 (anti-KIR2DL1, anti-KIR2DS1; Coulter, Hialeah, FL), DX27 (anti-KIR2DL2/3, anti-KIR2DS2), DX9 (anti-KIR3DL1), DX31 (anti-KIR3DL2), and the CD94-specific mAb DX22. No specific Abs were available for KIR2DL4, KIR2DS3–5, and KIR3DS1. Expression of the CD94:NKG2A heterodimer was determined by calculating the percentage of cells that stained brightly with the DX22 Ab. T cell clones and PBMC were analyzed by flow cytometry using FITC-coupled mAbs specific for the TCRVβ families 3, 5, 6, 8, 11, 12, 13, 14, 16, 17, 20, 21, and 22 (Coulter). mAbs specific for the T cell markers CD3, CD4, CD8, $\delta\gamma$ TCR, and $\alpha\beta$ TCR; the NK cell markers CD16 and CD56; the T cell memory markers CD45RO and CD57; as well as CD45RA Ag were used in different fluorochrome combinations (Becton Dickinson). Cells (2×10^5) of each T cell clone and PBMC (1×10^6) were incubated for 45 min with the appropriate Abs, washed, and analyzed with a FACScan flow cytometer using CellQuest software (Becton Dickinson).

T cell cloning

PBMC were isolated from whole blood by Ficoll-Hypaque gradient separation. CD3$^+$CD16$^-$NKR$^+$ T cells as well as CD3$^+$CD16$^-$NKR$^-$ control T cells were single-cell sorted using a FACStar cell sorter and cyt-clone software and hardware (Becton Dickinson). Sorted T cells were established in culture and maintained as previously described (42). Briefly, T cell clones were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 200 U/ml rIL-2 (provided by C. Reynolds, National Cancer Institute/Biological Response Modifier Program, Frederick, MD) and 0.1 μg/ml of PHA. Mixed allogeneic PBMC (1×10^6/ml; three donors) and cells of the JY B cell line (1×10^5/ml) were irradiated and used as feeder cells at the start of the culture and subsequently at weekly intervals. Irradiated feeder cells without T cell clones (feeders-only culture) were cultured in parallel to provide a control for background proliferation.

RNA preparation and RT

Total cellular RNA was extracted from 1×10^6 cultured T cells with feeders or with feeder cells alone using RNAzol according to manufacturer's instructions (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized from 1 μg of RNA by RT using oligo(dT) (Perkin-Elmer, Norwalk, CT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a volume of 25 μl at 42°C for 1 h.

NKR typing

PCR analysis of KIR and NKG2A expression was performed as described previously (39). In brief, a panel of PCR primer pairs was used to perform specific amplification of six groups of inhibitory KIR (KIR2DL1–4 and KIR3DL1–2) and five groups of noninhibitory KIR (KIR2DS1–4 and KIR3DS1) as well as the NKG2A gene. Amplifications were performed with NKR-specific primers at a concentration of 0.5 μM in 25-μl reactions for 30 cycles using 1 μl of cDNA. Internal control primers specific for β-actin were included in each PCR at a concentration of 0.05 μM. Feeders-only cultures were used as controls for RT-PCR that monitored the presence of residual NKR expression from irradiated feeder cells.

Typing for TCR Vα and Vβ families

TCRVβ family-specific PCR was performed as described previously (43). For each of 24 Vβ families a specific sense-primer and a common antisense primer matching the TCRβ constant region were used. Similarly, Vα family-specific PCR was performed as previously described (44) using sense primers for the specific amplification of 27 Vα families in combination with a common Cα-specific antisense primer. T cell clones were amplified for 30 cycles, and PBMC were amplified for 35 cycles using 1 μl of cDNA/25 μl reaction.

Nucleotide sequencing of TCR$\alpha\beta$ rearrangements

TCRα and TCRβ rearrangements in T cell clones were characterized by direct sequencing of template obtained by TCR family-specific PCR. Due to the monoclonality of the established T cell clones, no subcloning step was necessary to obtain unambiguous complementarity-determining region 3 (CDR3) sequences. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Subsequently, products were cycle-sequenced employing dye-labeled deoxynucleotide terminators and a 373A automated sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences of TCR α- and β-chain rearrangements were determined in both directions using as sequencing primers the family-specific and constant region-specific primers used for amplification.

Results

NKR$^+$ subsets of $\alpha\beta$ and $\gamma\delta$T cells express a diverse repertoire of NKR combinations

T cell clones from two healthy donors were cultured from PBMC that had been isolated by single-cell sorting using CD3-specific mAbs in combination with a cocktail of KIR-specific and CD94:

NKG2A-specific mAbs. Eighty-two NKR^+ T cell clones from donor NV and 71 clones from donor PP were obtained. As controls, 20 T cell clones expressing no NKR were also established from NKR^- T cells of each donor using the same protocol. Flow cytometric analysis showed that 68 (83%) of the NKR^+ T cell clones from donor NV expressed $\alpha\beta$ T cell receptors, and 14 (17%) expressed $\gamma\delta$ TCRs. In contrast, 30 (42%) of the NKR^+ T cell clones from donor PP expressed $\alpha\beta$ TCRs, and 41 (58%) expressed $\gamma\delta$ TCRs. The numbers of $\alpha\beta$ vs $\gamma\delta$ T cell clones obtained from each donor reflected the relative abundance of $\alpha\beta$ and $\gamma\delta$ cells in the NKR^+ T cell population in their peripheral blood as determined by flow cytometry. For donor NV the NKR^+ population of peripheral blood T cells comprised 76% $\alpha\beta$ T cells and 24% $\gamma\delta$ T cells; for donor PP it was 45% $\alpha\beta$ cells and 55% $\gamma\delta$ T cells. For both donors, the $\alpha\beta$ T cell clones obtained were all $CD4^-CD8^+$, while the $\gamma\delta$ T cell clones were mostly $CD4^-CD8^-$, with a few being $CD4^-CD8^+$. All NKR^+ T cell clones from both donors had the T cell memory phenotype, $CD28^-CD45RA^-CD45RO^+$.

RNA was isolated from each T cell clone and typed for KIR and NKG2A by RT-PCR-based methods we described previously (39). Clones derived from NKR^+ T cells expressed NKR, whereas those derived from NKR^- T cells did not, indicating that the culture conditions we used did not induce NKR expression in T cells. The expression of NKR by T cells (Fig. 1, *A* and *B*) was compared with that previously determined for NK cell clones obtained from the same two donors (38). For each donor all of his KIR genes are used by T cells as well as NK cells. None of the KIR was restricted to one or the other cell type. As for NK cells, the differential expression of KIR and CD94:NKG2A on T cells leads to a diverse repertoire of 31 and 24 different phenotypes in the 82 and 71 clones studied from donors NV and PP, respectively. The frequency of expression for most KIR genes was similar on NKR^+ T cells and NK cells. The KIR2DL4 gene was expressed on every NKR^+ T cell and NK cell. The HLA-Bw4-specific KIR3DL1 was infrequently expressed on the NK and NKR^+ T cells of donor NV, but was abundant on both kinds of lymphocytes from PP. The five noninhibitory KIR of donor NV (missing tyrosine-based inhibitory motifs in the cytoplasmic chain) were expressed at comparable frequencies on NK cells and NKR^+ T cells, as was the only noninhibitory KIR of donor PP, KIR2DS4.

KIR2DL1 was the only KIR expressed differentially by NKR^+ T cells and NK cells of both donors. Although NV expresses the HLA-C ligand for KIR2DL1, while PP does not, this receptor was expressed much more frequently on NK cells than on NKR^+ T cells of both donors. Differences were also seen for KIR2DL2 and KIR3DL1, which were found more frequently on NKR^+ T cells of NV and PP, respectively. Whereas the KIR repertoire of NKR^+ T and NK cells was mostly similar, CD94:NKG2A was expressed less frequently on NKR^+ T cells than on NK cells in both donors.

The expression of NKR by the $\alpha\beta$ and $\gamma\delta$ subsets of NKR^+ T cell clones obtained from the same individual showed specific differences (Fig. 1, *C* and *D*). Particularly impressive was the high frequency of expression of NKG2A by $\gamma\delta$ T cell clones compared with $\alpha\beta$ T cell clones; this difference was seen for both donors, but was more pronounced in NV. In expression of CD94:NKG2A, the $\gamma\delta$ T cells resemble NK cells, and it is the $\alpha\beta$ T cells that are different. Similarly, the higher frequency of KIR2DL2 expression by NV's T cell clones and that of KIR3DL1 by PP's T cell clones are due to $\alpha\beta$ T cells, while the expression of these receptors by $\gamma\delta^+$ cells resembles that of each individual's NK cell clones. By contrast, the low frequency of KIR2DL1 expression by T cell clones from both donors is a feature that distinguishes both $\alpha\beta$ and $\gamma\delta$ T cell clones from NK cell clones.

NKR^+ T cell clones from NV and PP expressed multiple NKR (two to eight receptors per cell for NV and two to seven receptors per cell for PP). For both donors a majority of the T cell clones expressed either three or four NKR (Fig. 2, *A* and *B*). In comparison with the autologous NK cell clones, the T cell clones expressed slightly fewer NKR, on the average. This can be seen in the reduction by one of the range in receptor number and in the reduction of the mean number of receptors expressed per cell (3.9 for NV's T cell clones compared with 4.2 for his NK cell clones, 3.7 for PP's T cell clones compared with 4.5 for his NK cell clones). These differences between NKR^+ T cells and NK cells are principally due to differences in the expression of inhibitory NKR (Fig. 2, *C* and *D*) rather than noninhibitory NKR (Fig. 2, *E* and *F*).

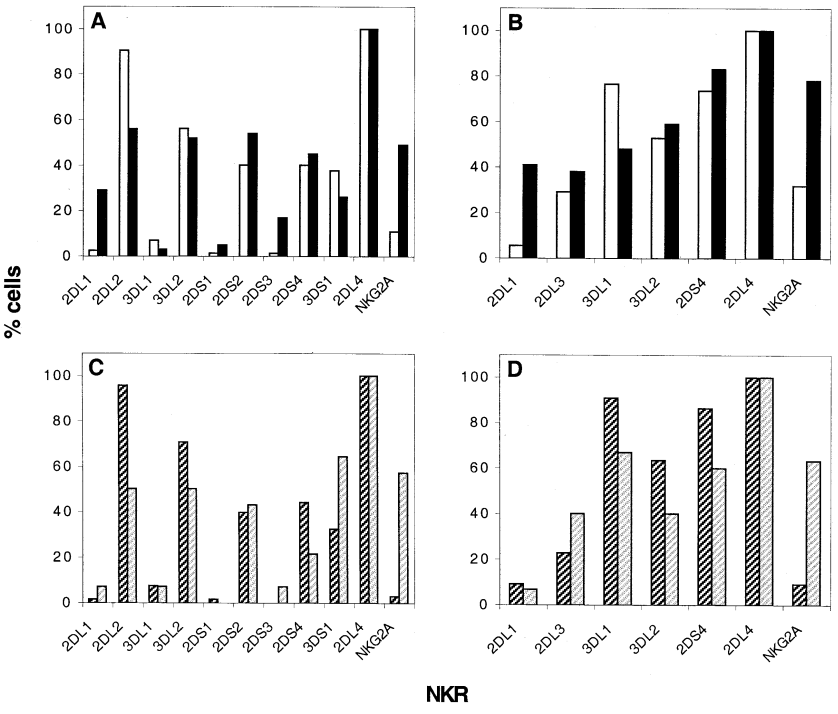

FIGURE 1. Comparisons of the frequencies with which individual KIR and NKG2A are expressed in the panels of NKR^+ T cell clones and NK cell clones from two healthy donors. In *A* (donor NV) and *B* (donor PP) the percentage of NKR^+ T cell clones (□) and NK cell clones (■) expressing each receptor are given. In *C* (donor NV) and *D* (donor PP) the percentage of NKR^+ $\alpha\beta$ T cells expressing each receptor (▨), and the percentage of NKR^+ $\gamma\delta$ T cells (▨) are shown. For donor NV, the data are from 82 T cell clones (68 $\alpha\beta$ and 14 $\gamma\delta$) and 111 NK cell clones; for donor PP, the data are from 71 T cell clones (30 $\alpha\beta$ clones and 41 $\gamma\delta$ clones) and 108 NK cell clones. Receptor expression was assessed by RT-PCR. The two donors have distinct sets of KIR genes, and the NK cell data are from the report by Valiante et al. (38).

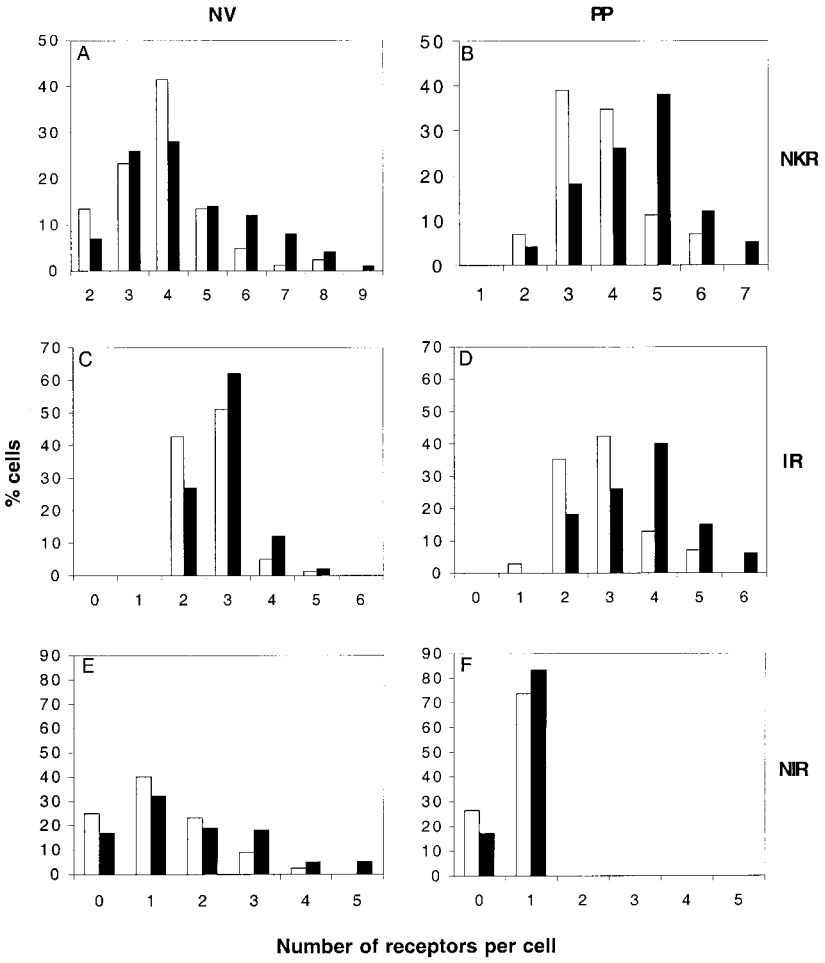

FIGURE 2. Clones of NKR$^+$ T cells express multiple NKR in different combinations. The percentage of NKR$^+$ T cell clones expressing the given numbers of receptors (□) are compared with the percentage of NK cell clones expressing those same numbers of receptors (■). In *A* (donor NV) and *B* (donor PP) all of the receptors analyzed are considered together. In *C* (donor NV) and *D* (donor PP) only inhibitory receptors (IR) are considered, while in *E* (donor NV) and *F* (donor PP) only the noninhibitory (NIR) or activating receptors are considered. A description of the clones analyzed is given in Fig. 1.

The two donors differ in their KIR types, with NV having genes for several noninhibitory KIR that are not possessed by PP. This disparity results in NV's NKR$^+$ T cells expressing up to five noninhibitory receptors, whereas PP's cells express only one (Fig. 2, *E* and *F*).

From knowledge of the NKR expressed by the NKR$^+$ T cell clones and of the HLA class I allotypes expressed by donors PP and NV, we assessed the extent to which each NKR$^+$ T cell clone expressed an inhibitory receptor that reacted with an autologous (self) HLA class I ligand (Fig. 3). For donor NV, every NKR$^+$ T cell clone expressed an inhibitory receptor specific for an autologous HLA class I allotype. By contrast, this criterion was met by only 41 of the 71 NKR$^+$ T cell clones from donor PP. Of the 30 clones that did not express a receptor for autologous HLA class I, 24 were $\alpha\beta$ T cells, and six were $\gamma\delta$ cells. Whereas 80% of PP's $\gamma\delta$ T cell clones had an inhibitory receptor, this was only true for 27% of the $\alpha\beta$ T cell clones. This difference between the two types of T cell clone can largely be attributed to the more frequent expression of CD94:NKG2A (Fig. 1, *C* and *D*). Similar differences in CD94:NKG2A expression between peripheral blood $\gamma\delta$ and $\alpha\beta$ cells were seen using flow cytometry; CD94:NKG2A was expressed by 25% (NV) and 17% (PP) of NKR$^+\alpha\beta$ T cells, and by 58% (NV) and 62% (PP) of NKR$^+\gamma\delta$ T cells (data not shown).

Cloned NKR$^+\alpha\beta$ T cells with identical $\alpha\beta$ TCR can be highly diversified for KIR expression

To examine the clonal diversity of NKR$^+$ T cells, their expression of TCR Vβ segments was analyzed by family-specific PCR. Each of the NKR$^+\alpha\beta^+$ T cell clones from donor NV expressed the Vβ16 gene segment. None of the control NKR$^-$ T cells expressed Vβ16; instead, they used various other Vβ segments (data not shown). NKR$^+\alpha\beta$ T cell clones from donor PP expressed one of three different Vβ families: Vβ11 (eight clones), Vβ14 (eight clones), and Vβ21 (six clones). Cell surface expression of the Vβ-chains assigned by PCR typing was confirmed by flow cytometric analysis of the T cell clones using Vβ family-specific mAbs (data not shown).

To determine the extent to which the restricted use of Vβ segments found in the NKR$^+$ $\alpha\beta$ T cell clones reflects the in vivo situation, PBMC from the two donors were analyzed by flow cytometry (Fig. 4*A*). For donor NV, 35% of NKR$^+$ T cells expressed Vβ16 (Fig. 4*D*), and 85% of the peripheral T cells expressing Vβ16 were NKR$^+$. Vβ16$^+$ T cells were also predominantly of CD8$^+$ type (Fig. 4*C*). The subset of peripheral T cells expressing both Vβ16 and NKR (Fig. 4*D*) was estimated to comprise approximately 4% of NVs PBMC. No other Vβ family was found on peripheral blood NKR$^+$ T cells of donor NV at such high frequency, although other Vβ families were clearly represented. Flow cytometry was also used to assess the expression of different KIR within the subset of peripheral blood NKR$^+$Vβ16$^+$ T cells, and the results were similar to those obtained by KIR typing of the cultured Vβ16$^+$ T cell clones by RT-PCR typing (Fig. 5). Thus, in pattern of NKR and TCR expression, the panel of NKR$^+$ T cell clones from donor NV represents the dominant Vβ family expressed by NKR$^+$ T cells in the peripheral blood.

For donor PP the results were less clear cut. Of the three Vβ families expressed by the NKR$^+$ T cell clones, only Vβ21 was expressed by substantial numbers (15%) of NKR$^+$ T cells in the

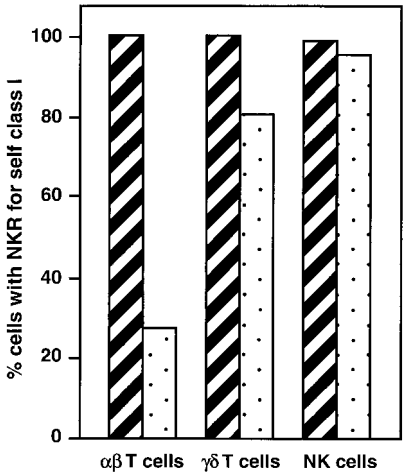

FIGURE 3. NKR$^+$ T cell clones need not express an inhibitory NKR with specificity for autologous class I. The percentages of NKR$^+$ $\alpha\beta$ T cell clones, NKR$^+$ $\gamma\delta$ T cell clones, and NK cell clones with one or more inhibitory NKR for autologous HLA class I are shown for donors NV (▨) and PP (⊡).

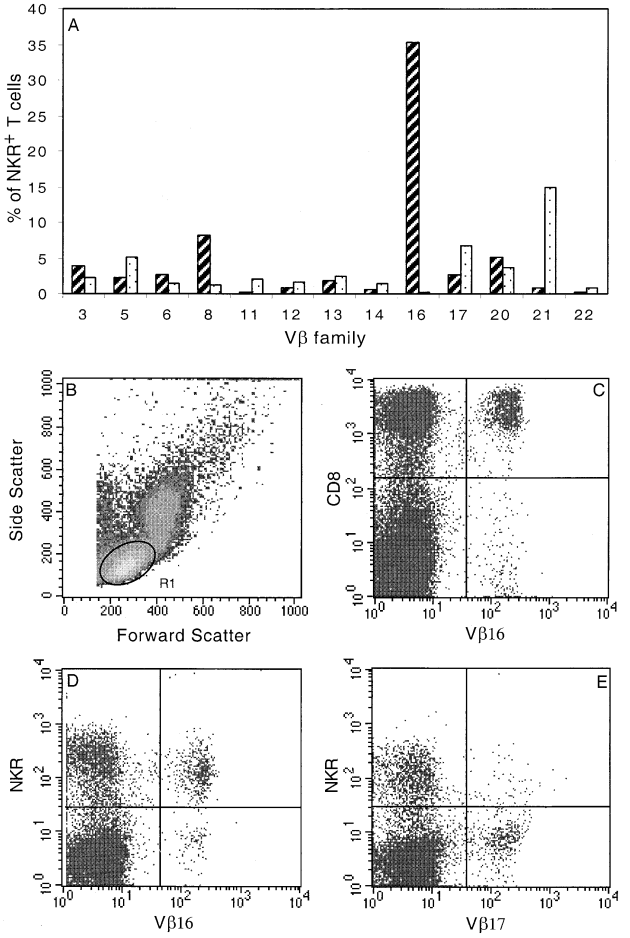

FIGURE 4. The Vβ16 family is highly expressed in donor NV's peripheral blood NKR$^+$ T cells. *A*, The relative expression of different Vβ families within peripheral blood NKR$^+$ T cells of donors NV (▨) and PP (⊡). *B–E*, Flow cytometric data from analysis of PBMC from donor NV. *B*, The population of lymphocytes selected for analysis of cell surface phenotypes. *C*, Staining with anti-Vβ16 and anti-CD8. *D*, Staining with anti-Vβ16 and anti-NKR. *E*, Staining with anti-Vβ17 and anti-NKR. Anti-NKR was a mixture of five Abs specific for different KIR or CD94:NKG2A.

peripheral blood. By contrast, the Vβ11 and Vβ14 families, which were the two Vβ families most commonly expressed by PP's NKR$^+$ T cell clones, were expressed by relatively few NKR$^+$ T cells in the peripheral blood (Fig. 4*A*).

Since NKR$^+$$\alpha\beta^+$ T cell clones from donor NV were strongly biased toward usage of the Vβ16 gene segment, we were interested to determine the heterogeneity of their TCR β-chain gene rearrangements. Five different Vβ rearrangements were represented in the panel of 68 $\alpha\beta$ T cell clones (Fig. 6*A*). A predominant rearrangement (II) was common to 55 clones, while four less frequent rearrangements (IV, V, I, and III) were represented by one, two, three, and seven clones, respectively. All five gene rearrangements led to shared characteristic features in the CDR3 region of the TCR Vβ-chain. Aspartic acid followed by arginine was found in all five rearrangements at positions 97–98 encoded by the D gene-segment and its flanking N nucleotides. This motif is determined either by the D1 germline segment (probably rearrangements II, III, and IV) or by N nucleotide addition (rearrangements I and V). Moreover, three of the five rearrangements share the same J element, and they differ from each other by no more than two amino acids in the CDR3 region. In the clones expressing rearrangement II, the Vβ19 gene segment was also rearranged and expressed at the mRNA level (data not shown). However, its nucleotide sequence showed this to be an unproductive rearrangement, resulting in a stop codon in the CDR3 region, consistent with the assignment of Vβ19 as a pseudogene (45). Thus, in clones with rearrangement II, Vβ16 is the only functionally rearranged TCR-Vβ gene segment.

To define the complete TCR $\alpha\beta$ clonotype for each NKR$^+$ T cell clone in the NV panel, their Vα gene rearrangements were determined. Among the 66 NKR$^+$ T cell clones having Vβ rearrangement I, II, III, or IV, different Vα gene rearrangements were found in combination with each Vβ rearrangement, and all clones with identical Vβ rearrangements had identical Vα rearrangements. Clones with the type I, II, or III Vβ16 rearrangement expressed two different α-chains. Thus, a total of seven different α rearrangements were expressed by the 66 T cell clones, and these involved five different Vα segments and five different Jα segments (Fig. 6*B*). The seven Vα rearrangements had no shared characteristics, contrasting with their Vβ counterparts. Even in cases where one Vα segment was held in common among cells having the same Vβ rearrangement, they differed substantially in their Vα CDR3 region and were also joined to different Jα segments. All seven rearrangements are in-frame and could give rise to full-length α-chains. Not known is whether any of the T cell clones of clonotype I, II, or III expresses both the α-chains at their cell surfaces, since their simultaneous cell surface expression could not be analyzed with the Abs available.

Finding that only four different $\alpha\beta$ TCR clonotypes were represented in 66 NKR$^+$ $\alpha\beta$ T cell clones provided an unprecedented opportunity to assess the extent to which NKR expression distinguished clones of identical clonotype. Typing was performed by both RT-PCR and flow cytometry, and both techniques gave concordant results (Fig. 7). Whereas only the single $\alpha\beta$ T cell of clonotype IV was distinguished by expression of NKG2A:CD94, there were many differences in the expression of KIR (Fig. 8). Most informative was the analysis of clonotype II that accounted for 55 NKR$^+$$\alpha\beta$ T cell clones from donor NV. Among these cells with identical TCR were 18 different KIR phenotypes that varied in frequency and number of KIR expressed. All the clones expressed KIR2DL2 and KIR2DL4, and diversity was mediated by the differential expression and combination of seven other KIR. The frequency of expression of these KIR was in the order

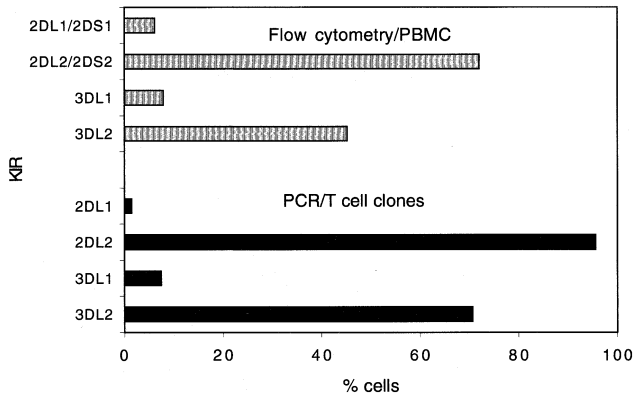

FIGURE 5. Peripheral blood Vβ16$^+$ T cells and cloned NKR$^+$ T cells from donor NV have similar patterns of KIR expression. The frequencies of KIR expression determined by three-color flow cytometry of PBMC are compared with the results obtained from RT-PCR typing of the panel of NKR$^+$ T cell clones. For flow cytometric analyses the percentages of cells expressing KIR were calculated relative to the total Vβ16$^+$ T cell subpopulation. The KIR-specific mAbs used were EB6 (anti-KIR2DL1), DX27 (anti-KIR2DL2/3 and anti-KIR2DS2), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2).

KIR3DL2>2DS4>2DS2>3DS1>3DL1>2DL1~2DS1. KIR-mediated diversification of T cell clones with clonotypes I, II, and V was also seen (Fig. 8), although to a lesser extent because of the fewer numbers of clones analyzed.

Discussion

Previous investigation has shown that CD94:NKG2A and KIR receptors (NKR) are expressed on subpopulations of human peripheral blood T cells with cell surface phenotypes that are characteristic of memory T cells. Signals generated through HLA class I ligands binding to NKR can influence the effects of Ag engagement through the TCR, a modulatory activity that has been likened to T cell costimulation. The results described here demonstrate that T cells can express diverse combinations of NKR involving as many as eight different receptors. More specifically, we have characterized T cell clones from one donor that have identical an $\alpha\beta$ TCR clonotype, but different KIR phenotypes. Particularly striking are the 55 clones of clonotype II that divide into 18 distinct groups on the basis of differential expression of five KIR genes. These data reveal the considerable potential for NKR expression to modulate the functional activities of T cells.

We previously showed that mature NK cells express at least one inhibitory KIR or CD94:NKG2A receptor with specificity for self class I (38). This restriction in the NKR repertoire is in accord with the missing self model and ensures self tolerance of the peripheral NK cell compartment (46). A similar restriction was not apparent in our panel of NKR$^+$ T cell clones. Whereas NV's T cell clones all express an inhibitory receptor for autologous HLA class I determinants, this is only true for some 60% of PP's clones. This suggests either that there is no requirement for T cells to express an inhibitory NKR for autologous class I or that additional receptors that we have not considered here are fulfilling that role.

ILT2, also called LIR1, is another type of inhibitory receptor with Ig-like domains that is distinct from KIR (19, 20). ILT2/LIR1 is specific for the viral homologue UL18, but exhibits also weak binding to relatively nonpolymorphic sites on HLA class I molecules (47). In peripheral blood of both donors NV and PP, expression of ILT2/LIR1 and KIR was found on distinct but overlapping T cell subsets. Similarly, ILT2/LIR1 expression was found on a substantial fraction of NKR$^+$ T cell clones by flow cytometry. However, in studies with a soluble LIR1 fusion protein, LIR1 was found to bind to one of NV's HLA-B allotypes, but none of PP's class I allotypes (data not shown). It therefore seems unlikely that those NKR$^+$ T cells in PP that lack KIR and CD94:NKG2A receptors for autologous class I allotypes are functionally inhibited by ILT2/LIR1. On the other hand, the existence of mature NKR$^+$ T cells without self-specific inhibitory NKR should not pose a principal problem to the host. In contrast to NK cells that rely on engagement of their inhibitory receptors by self class I epitopes to be self tolerant, T cells are already selected for self tolerance by TCR-driven processes in the thymus.

Although the differences in NKR expression by the two donors are many, within each individual the expression of NKR by NK cell clones and that by T cell clones from each individual are largely similar, but with a few potentially important differences. The biggest difference is the reduced frequency with which NKR$^+$ T cells express CD94:NKG2A compared with NK cells, which approaches zero for NKR$^+$ $\alpha\beta$ T cells. It is this difference that largely accounts for the absence of an inhibitory receptor for autologous HLA class I on 40% of PP's NKR$^+$ T cells. Also common to both donors was a reduced frequency of KIR2DL1, the inhibitory receptor for HLA-C allotypes with the N77, K80 amino acid sequence motif. Although for NV the frequency of KIR2DL2 expression by $\alpha\beta$ T cells was higher than that for either NK cells or $\gamma\delta$ T cells, this may not be a general property because of the limited number of T cell clonotypes represented in the clones and their ubiquitous expression of KIR2DL2. Overall, the similarities in frequency with which KIR are expressed by NK cells and NKR$^+$ T cells indicate that the mechanisms that diversify the expression of KIR in populations of these cells are very similar.

Much of the analysis we report was made upon NKR$^+$ T cell clones that were stimulated and cultured in vitro, and in this regard T cells expressing NKRs are more difficult to isolate than NK cells because of their lack of a generic marker. The extent to which the clones reflect the in vivo populations is therefore of importance; to address this question we have, wherever possible, compared the clones with the NKR$^+$ T cells in peripheral blood. Such comparison shows that clones we have isolated and analyzed do not provide complete representation of the peripheral blood NKR$^+$ T cells of either donor. One possible source of bias is that cells expressing certain combinations of NKR were not captured by the Ab cocktail used in isolating NKR$^+$ T cells. This cocktail did not include Abs that bind to either KIR2DL4 or the recently described KIR2DL5 (9, 48), and the weak affinity of the CD94-specific Ab for the CD94:NKG2C heterodimer may have meant that this Ab was inefficient in capturing cells via interaction with CD94:NKG2C. A second potential source of bias is that the in vitro cloning procedure did not equally promote the growth of all cells isolated with the Ab cocktail.

For donor NV, one-third of the peripheral blood NKR$^+$ T cells and all 68 of the $\alpha\beta$ T cell clones express Vβ16. Clearly, the in vitro culture was strongly biased toward furthering the growth and survival of Vβ16-expressing cells; however, this selection involved the dominant Vβ family of the peripheral blood NKR$^+$ T cells, and the data collected from these cells are therefore likely to have physiological relevance to this particular donor's history of immune response. The dominance of Vβ16 NKR$^+$ T cells of the type seen in donor NV is a phenomenon that may not have generality, being seen in just a fraction of the human population, or may even be unique to this donor. Indeed, the high diversity and polymorphism of KIR genotype and the low frequency of unrelated individuals with identical KIR genes provide a genetic context in which individuality in the patterns of NKR expression by human T cells could actually be the common theme (39, 40).

A

No	Rearrangement	n	BV16	N1-DB-N2	BJ	BC
			A S S H	D R L	E A F F G Q G T R L T V V	E D L N
I	BV16S1J1S1BC1	3	GCC AGC AGC CA	C GAC AGA CTA	GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA G	AG GAC CTG AAC
			A S S Q	D R F	Y E Q Y F G P G T R L T V T	E D L K
II	BV16S1J2S7BC2	52	GCC AGC AGC CAA	GAC AGG TT	C TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	AG GAC CTG AAA
			A S S	P D R	T Y E Q Y F G P G T R L T V T	E D L K
III	BV16S1J2S7BC2	7	GCC AGC AGC C	CG GAC AGG A	CC TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	AG GAC CTG AAA
			A S S Q	D R A	Y E Q Y F G P G T R L T V T	E D L K
IV	BV16S1J2S7BC2	1	GCC AGC AGC CAA	GAC AGG GCG	TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	AG GAC CTG AAA
			A S S Q	D R V L	Q F F G P G T R L T V L	E D L K
V	BV16S1J2S1BC2	2	GCC AGC AGC CAA	GAT CGG GTA CT	G CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA G	AG GAC CTG AAA

B

No	Rearrangement	AV	N	AJ	AC
		C A V S	V S T	N T G K L I F G Q G T T L Q V K P	D I Q N
I	AV8S6J37AC1	TGT GCT GTG AGT G	TG TCT AC	C AAC ACA GGC AAA CTA ATC TTT GGG CAA GGG ACA ACT TTA CAA GTA AAA CCA G	AT ATC CAG AAC
		C A M	K	D S G Y A L N F G K G T S L L V T P	H I Q N
	ADV14S1J41AC1	TGT GCA ATG A	AA G	AT TCC GGG TAT GCA CTC AAC TTC GGC AAA GGC ACC TCG CTG TTG GTC ACA CCC C	AT ATC CAG AAC
		A L	I Y N Q G G K L I F G Q G T E L S V K P	N I Q N
II	AV16S1J23AC1	GCT CT	T ATC AT	G ATT TAT AAC CAG GGA GGA AAG CTT ATC TTC GGA CAG GGA ACG GAG TTA TCT GTG AAA CCC A	AT ATC CAG AAC
		C A A	T R L R	D N T D K L I F G T G T R L Q V F P	N I Q N
	ADV23S1J34AC1	TGT GCA GCA A	CC CGT CTA AGG G	AT AAC ACC GAC AAG CTC ATC TTT GGG ACT GGG ACC AGA TTA CAA GTC TTT CCA A	AT ATC CAG AAC
		C A M R E G	S	S N T G K L I F G Q G T T L Q V K P	D I Q N
III	ADV14S1J37AC1	TGT GCA ATG AGA GAG GG	C TCT A	GC AAC ACA GGC AAA CTA ATC TTT GGG CAA GGG ACA ACT TTA CAA GTA AAA CCA G	AT ATC CAG AAC
		C A	Q A T R	L S F G A G T T V T V R A	N I Q N
	AV19S1J20AC1	TGT GC	C CAG GCC ACA CG	G CTC AGC TTT GGA GCC GGA ACC ACA GTA ACT GTA AGA GCA A	AT ATC CAG AAC
		C A L S E A	G G	D N T D K L I F G T G T R L Q V F P	N I Q N
IV	AV19S1J34AC1	TGT GCT CTG AGT GAG GC		AT AAC ACC GAC AAG CTC ATC TTT GGG ACT GGG ACC AGA TTA CAA GTC TTT CCA A	AT ATC CAG AAC
V	n.d.				

FIGURE 6. Five combinations of Vβ and Vα rearrangements (clonotypes) account for the TCRs expressed by the 68 NKR$^+$ $\alpha\beta$ T cell clones from donor NV. Nucleotide and amino acid sequences encompassing the recombinatorial regions of the TCR Vβ-chain (*A*) and Vα-chains (*B*) of NKR$^+$ $\alpha\beta$ T cell clones from donor NV are shown. The five clonotypes are designated I, II, III, IV, and V. The α- and β-chain rearrangements given the same Roman numeral are expressed together. Conserved nucleotides at positions 97–98 of the CDR3 region of TCRVβ are indicated in bold. The borders of the V and J segments were based on published germline sequences (56, 57). TCR rearrangements were named using World Health Organization-International Union of Immunological Societies nomenclature (58). The Vα rearrangement of clonotype V could not be determined unequivocally because of background amplification of Vα rearrangements from feeder cells.

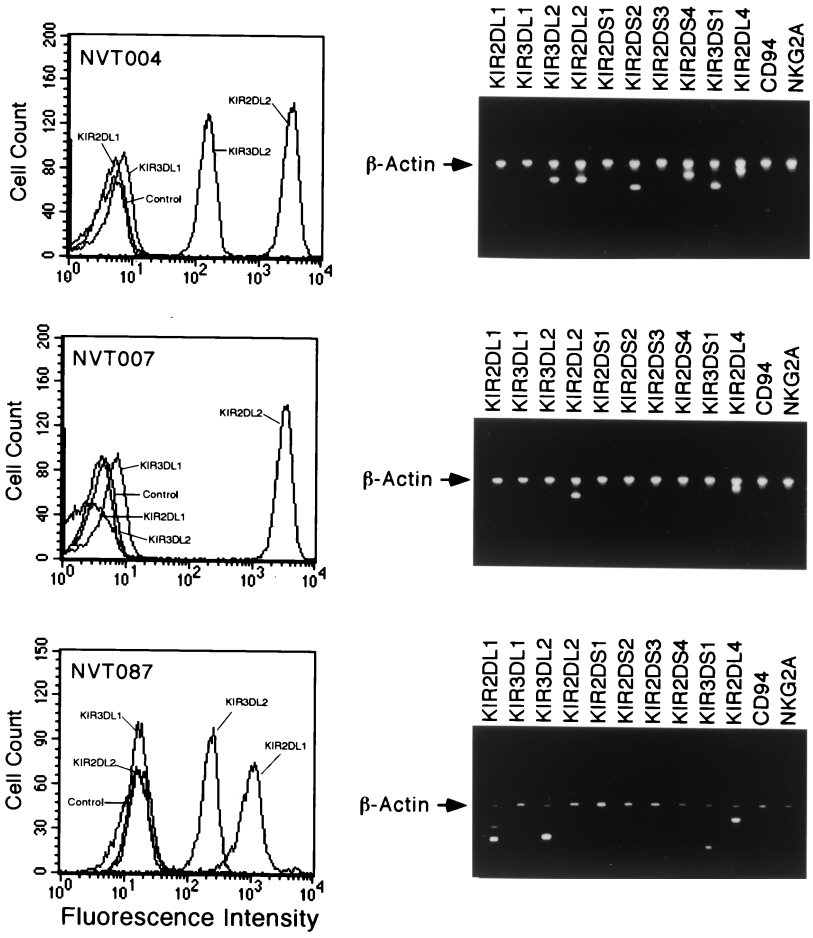

FIGURE 7. NKR gene expression in NKR$^+$ T cell clones with identical $\alpha\beta$ TCR correlates with NKR cell surface phenotype. Three representative NKR$^+$ T cell clones (NVT004, NVT007, and NVT087) with $\alpha\beta$ TCR of clonotype II were typed for expression of NKR by RT-PCR (*right panels*) and for cell surface expression of NKR as determined by flow cytometric analysis (*left panels*) using mAbs specific for KIR2DL1 and KIR2DS1 (EB6), KIR2DL2/3 and KIR2DS2 (DX27), KIR3DL1 (DX9), and KIR3DL2 (DX31).

In donor NV, five $\alpha\beta$ TCR clonotypes account for the 68 clones, and for all four clonotypes that were represented by more than one clone, there is diversification due to differential KIR expression. That different combinations of KIR are expressed by T cells with the same TCR strongly indicates that KIR expression was turned on in mature T cells that had already undergone TCR gene rearrangement and thymic selection. This model is consistent with the memory phenotype of Vβ16$^+$NKR$^+$ T cells in peripheral blood. The alternative model, that KIR expression preceded TCR gene rearrangement, is not consistent with the memory phenotype and is inherently unlikely because it requires the occurrence of 18 independent, but identical, sets of TCR α and β gene rearrangements in immature T cells with different KIR phenotypes.

It is possible that KIR-mediated diversification of T cells with identical clonotype occurred during in vitro culture. It was recently reported that murine CD8 T cells acquire Ly-49 receptors upon in vitro culture with IL-2, IL-4, or IL-15 (49). For several reasons we consider an analogous induction of KIR genes during the cloning procedure unlikely. First none of the control clones cultured from NKR$^-$ T cells expressed KIR at any stage during culture. Second stimulation of CMV-specific NKR$^-$ T cells with stimulatory cytokines and cognate peptide did not result in acquisition of KIR. Many other deliberate attempts to induce KIR expression in T cells in vitro have failed, although Mingari et al. have reported the induction of CD94:NKG2A (50). Recently, down-regulation of KIR was reported on NKR$^+$ T cell clones that were deprived of specific Ag (51). Since we do not know the Ag specificity of our NKR$^+$ T cell clones, we were unable to perform a similar experiment with our T cell clones. However, analysis of KIR expression levels over time did not reveal any signs of KIR gene down-regulation during culture. Therefore, it is likely that the diverse KIR phenotypes seen on single-cell sorted T cells with identical TCR are not a consequence of in vitro changes, but represent changes in the NKR expression status that occurred in vivo.

The memory phenotype of all NKR$^+$ T cells in peripheral blood implies that induction of NKR expression occurs only after T cells have been stimulated by Ag, a model supported by the absence of KIR on T cells in fetal thymus and cord blood (27). That all $\alpha\beta$ T cell clones from donor NV comprise five clonotypes using Vβ16 chains with common residues in their CDR3 loops is also evidence for clonal expansion driven by some form of Ag. Vβ16 was shown to dominate the clonal expansions of CD8$^+$CD57$^+$ T cells that occurred in four patients following transplantation of allogeneic bone marrow from an HLA-identical sibling (52). Steinle et al. showed that an HLA-B35-specific alloreactive T cell expressed a similar type of Vβ16 chain (53). Such associations raise the possibility that T cells bearing such Vβ16-containing receptors could have specificity for alloantigens. In healthy donors expansions of such cells might arise as a result of blood transfusion or pregnancy. Alternatively, some types of superantigen or specific Ag could be responsible for the expansion of Vβ16-expressing cells, and these might be associated with infections that either occur or are reactivated following transplantation. In this context it is important to note that the culture system we used to generate T cell clones could have favored the growth of NKR$^+$ T cells with allospecificity, because the feeder cells were pooled PBMC from allogeneic donors. Selection by alloantigens could also explain why a majority of the $\alpha\beta$ T cell clones from donor PP expressed Vβ families that were represented at relatively low frequency in the NKR$^+$T cell population of the peripheral blood.

T cell clonotype II

KIR Type	2DL1	2DL2	3DL1	3DL2	2DS1	2DS2	2DS3	2DS4	3DS1	2DL4	NKG2A	No
1		■		■						■		9
2		■								■		7
3		■						■		■		5
4		■		■				■		■		5
5		■		■		■				■		5
6		■						■	■	■		4
7		■		■		■		■	■	■		3
8		■				■		■		■		3
11		■				■			■	■		3
9		■		■		■		■		■		2
10		■				■		■	■	■		2
12		■		■				■	■	■		1
13		■	■	■						■		1
14		■	■	■				■	■	■		1
15		■		■	■	■		■		■		1
16		■							■	■		1
17		■				■				■		1
18	■	■		■					■	■		1
frequency	1.8	100.0	3.6	52.7	1.8	36.4	0.0	49.1	30.9	100.0	0.0	

T-cell clonotype I

1		■								■		2
2		■						■		■		1

T-cell clonotype III

1		■		■		■				■		3
2		■		■						■		1
3		■		■				■		■		1
4		■		■					■	■		1
5		■		■		■			■	■		1

T-cell clonotype IV

1		■	■	■		■		■	■	■	■	1

T-cell clonotype V

1		■	■	■					■	■		1
2		■		■		■			■	■		1

FIGURE 8. NKR$^+$ T cell clones with an identical TCR $\alpha\beta$ clonotype can be highly diversified for KIR expression. The results of typing the 68 NKR$^+$ $\alpha\beta$ T cell clones from donor NV for TCR α- and β-chains and for NKR expression are summarized. Expression of a particular NKR is indicated by stippled shading. The number of T cell clones sharing a particular NKR expression pattern is indicated in the *right column*.

The results of this study demonstrate that individual NKR$^+$ $\alpha\beta$ and $\gamma\delta$ T cells express variable combinations of receptors that collectively have a complexity approaching that of NK cells. This complexity can also introduce considerable heterogeneity within the populations of memory-type T cells that constitute a clone as defined by the TCR. The functional consequences of differential KIR expression on clones of NKR$^+$ T cells in the course of an immune response are largely unknown. Redirected lysis experiments show that both types of inhibitory NKR, KIR and CD94: NKG2A, are functional in the analyzed T cell clones (data not shown). Other studies have shown that inhibitory signaling through NKR can affect TCR-mediated functions, and in the case of a melanoma-specific response this property has the deleterious effect of preventing T cell-mediated killing of tumor cells (31). Very little is known about the role of noninhibitory NKR on T cells. It was recently shown that the adapter molecule DAP12, also known as KARAP, is involved in the activation of NK cells through noninhibitory KIR (54, 55). RT-PCR analyses show that a fraction of approximately 20% of donor NVs NKR$^+$ T cells expresses DAP12. Differential expression of DAP12 was also seen within T cells with identical KIR phenotype and TCR (data not shown). If DAP12 is essential for signal transduction through noninhibitory KIR, its differential expression would introduce another level of heterogeneity into clones of NKR$^+$ T cells. In summary, the present study shows that expression of inhibitory and noninhibitory KIR in a clone of T cells produces 18 different KIR phenotypes, each of which involves two to seven different receptors that individually or in combination have the potential to modify T cell responses to Ag.

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Differential Expression of Leukocyte Receptor Complex-Encoded Ig-Like Receptors Correlates with the Transition from Effector to Memory CTL[1]

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The human leukocyte receptor complex (LRC) on chromosome 19q13.4 encodes Ig superfamily receptors expressed on hemopoietic cells. Killer Ig-like receptors (KIR) are expressed in cytotoxic lymphocytes but other LRC molecules (Ig-like transcript(ILT)/leukocyte Ig-like receptor (LIR)) are more ubiquitous. We investigated expression of the ILT2/LIR1 inhibitory receptor compared with the related KIR. Both ILT2/LIR1 and KIR were expressed by peripheral $CD8^+$ T cells with a memory/effector phenotype. ILT2/$LIR1^+$ T cells demonstrated diverse TCRBV repertoires in contrast to KIR^+ T cells, while numbers of peripheral ILT2/$LIR1^+$ T cells were greater than KIR^+ T cells and the majority of ILT2/$LIR1^+$ T cells did not coexpress KIR. Analysis of $CD8^+$ T cells with specific HLA class I tetramers confirmed this pattern of expression, indicating differential regulation of LRC gene expression in T lymphocytes. Only a minor proportion of ILT2/$LIR1^+$ KIR^- clones survived in vitro cloning, were more susceptible to anti-CD3 or cognate peptide induced cell death than KIR^+ T cells and exhibited lower levels of the Bcl-2 survival molecule. Our results indicate a sequential program of LRC-encoded receptor expression with initial ILT2/LIR1 expression in effector T cells and KIR gene transcription in the minor proportion of expanded clones which survives activation-induced cell death to become long term memory T cells. *The Journal of Immunology,* **2001, 166: 3933–3941.**

Killer cell Ig-like receptor (KIR)[5] molecules are members of the Ig superfamily, containing either two (KIR2D) or three (KIR3D) extracellular Ig domains (1). The genes for these receptors are located in a polygenic cluster on chromosome 19q13.4 (2). Further molecules with homology to KIR have been described and shown to be encoded by genes located in the same genomic region, now designated the leukocyte receptor complex (LRC) (3). This additional family of receptors, known either as Ig-like transcripts (ILT) (4), leukocyte Ig-like receptors (LIR) (5), or monocyte inhibitory receptors (MIR) (6, 7) have structural and functional similarities to KIR including multiple isoforms. ILT/LIR possess either four or two extracellular Ig-like domains and can be classified as inhibitory forms with long cytoplasmic tails containing immunoreceptor tyrosine inhibitory motifs (ITIMs) which recruit SHP-1 phosphatase (8) or short-tailed, activating forms which associate with the FcRIγ signaling subunit (9). ILT/LIR proteins differ from KIR by being principally expressed on cells with a phagocytic and Ag-presenting function such as monocytes, macrophages, dendritic cells, and B lymphocytes, although a subset of these inhibitory receptors are expressed on peripheral NK and T lymphocytes (4, 5).

The functional activity of human NK cells and a subset of CTL is influenced by the interaction of KIR with epitopes of HLA class I molecules. Upon binding their specific ligands, KIR recruit SHP-1 phosphatase via ITIMs located in their cytoplasmic tail, inhibiting the signaling processes associated with cellular activation (1). In NK cells, KIR genotype and expression repertoire determine self tolerance (10) and allow these cells to detect "missing self," i.e., the loss of cell surface HLA class I expression resulting from infection with certain viruses or malignant transformation. Isoforms of the KIR molecules that have short cytoplasmic tails lacking ITIM motifs may trigger effector cell activation through association with the adaptor molecule DAP12 (11). The precise role of KIR expression in CTL is unclear, although these receptors can modulate signals transmitted through the TCR/CD3 complex (12–14). KIR expression in T lymphocytes appears to be restricted to cells with a differentiated "memory" phenotype (15).

As the KIR and ILT/LIR receptors are encoded within the same genomic cluster, it is relevant to assess the features of ILT/LIR expression in peripheral lymphocytes and the extent of any coincidence with KIR expression, particularly as inhibitory receptors such as ILT2/LIR1 appear to be expressed on both NK and T lymphocytes and recognize HLA class I ligands (16). Investigation of these features will provide information on cell-type specific expression of genes within the LRC and may have important implications for the functional repertoires of cells with cytotoxic capacity. We have thus analyzed characteristics of the ILT2/LIR1 inhibitory receptor on T lymphocytes in comparison with expression of the related KIR. Previous studies have demonstrated the

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[6] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; ILT, Ig-like transcript(s); LRC, leukocyte receptor complex; LIR, leukocyte Ig-like receptor(s); CDR3, complementarity-determining region 3.

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expression of ILT2/LIR1 by a proportion of T cells but have not addressed the coordination of ILT2/LIR1 and KIR expression or defined the specific types of T cells that express ILT2/LIR1. Our results indicate a certain overlap in expression of the two types of receptors but also demonstrate a differential regulation of Ig-like receptor acquisition which correlates with the development of resistance to activation-induced cell death in memory CTL.

Materials and Methods

Normal donor leukocytes

Buffy coats obtained by leukapheresis of normal blood donors were purchased from Stanford Medical Center Blood Bank. Cord blood samples were obtained from normal term deliveries at the Maternity Unit, Packard Children's Hospital, Stanford. Mononuclear cells were obtained from these products by Ficoll-Hypaque gradient centrifugation. HLA class I typing was performed on genomic DNA derived from PBMC using PCR followed by direct sequencing and sequence-specific primer amplification.

Flow cytometry

Three-color flow cytometry was performed on mononuclear cells stained in a two-stage procedure with the ILT2/LIR1-specific mAb HPF1 (16) followed by FITC- or PE-labeled goat anti-mouse reagent. After blocking with normal mouse serum (Sigma, St. Louis, MO), cells were counterstained with directly conjugated (FITC or CyChrome) Abs specific for the T cell markers CD3, CD4, CD8, TCR$\gamma\delta$, TCR$\alpha\beta$, CD45RO, CD45RA, CD57, CD28, CD27, CD38, and the NK cell markers CD16 and CD56. A mixture of PE-coupled KIR-specific mAbs consisting of the anti-KIR reagents EB6 (anti-KIR2DL1, anti-KIR2DS1; Coulter, Hialeah, FL), DX27 (anti-KIR2DL2/L3, anti-KIR2DS2), DX9 (anti-KIR3DL1), and DX31 (anti-KIR3DL2) was also used. T cell clones as well as peripheral mononuclear cells were analyzed by flow cytometry using FITC-coupled mAbs specific for the TCRV region families AV2, AV12.1, BV3.1, BV5a, BV5b, BV5c, BV6.7, BV8a, BV12, BV13, BV14, BV16, BV21, BV22, and BV23 (Coulter and Serotec, Raleigh, NC). For analysis of intracellular Bcl-2 levels by four color flow cytometry, PBMC were first stained with appropriate PE, Cychrome or APC-labeled Abs specific for cell surface markers, permeabilized, and stained with FITC-labeled anti-Bcl-2 or IgG1 isotype control (Becton Dickinson, San Jose CA).

Ag-specific T lymphocytes were identified using synthetic HLA-A*0201 tetrameric complexes labeled with PE fluorochrome. HLA-A2 tetramers were refolded with HCMV pp65 peptide NLVPMVATV or human EBV BZLF1 peptide GLCTLVAML. Tetramer staining was performed at room temperature to minimize low affinity binding.

Cells (2×10^5) of each T cell clone and 1×10^6 PBMC were incubated at 4°C for 45 min with the appropriate Abs, washed, and analyzed on a FACScan or FACSort flow cytometer using CellQuest (Becton Dickinson) and FlowJo (TreeStar, San Carlos, CA) analysis software.

T cell cloning

Single $CD3^+$ $HPF1^+$ T cells were sorted using a FACStar or FACSVantage cell sorter and Cyt-clone software (Becton Dickinson). Single-cell sorted T cell clones were established and maintained as described (17). Briefly, T cell clones were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 200 U/ml recombinant IL-2 (provided by C. Reynolds, National Cancer Institute/Biological Response Modifier Program, Frederick, MD) and 5% human T-STIM (Becton Dickinson). Mixed, allogeneic PBMC (1×10^6/ml; three donors) and 1×10^5/ml JY cells (EBV-transformed B lymphoblastoid cell line) were irradiated and used as feeder cells at the start of the culture and subsequently at weekly intervals. Irradiated feeder cells without T cell clones ("feeders-only" culture) were cultured in parallel to control for background proliferation.

RNA preparation and reverse transcription

Total cellular RNA was extracted from 3–5 $\times 10^6$ T cell clones and the "feeders-only" culture using RNAzol according to the manufacturer's instructions (Tel-Test, Friendswood, TX).

First strand cDNA was synthesized from 1 μg RNA by reverse transcription using oligo(dT) (Perkin-Elmer, Norwalk, CT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) in a volume of 50 μl at 42°C for 1.5 h.

PCR amplification

RT-PCR analysis of KIR expression on T cell clones was performed as described (18) using a panel of PCR primer pairs to enable the specific amplification of six groups of inhibitory KIR (KIR2DL1–4 and KIR3DL1–2) and six groups of noninhibitory KIR (KIR2DS1–5 and KIR3DS1). Amplifications were performed with specific primers at a concentration of 0.5 μM in 25-μl reactions for 30 cycles using 2 μl of cDNA per reaction. Internal control primers specific for β-actin were included in each PCR at a concentration of 0.05 μM. Feeders-only cultures were used as RT-PCR controls to monitor the presence of residual transcripts from irradiated feeder cells. The use of this RT-PCR analysis allowed us to detect expression of all KIR genes, including those receptors that cannot be detected by specific Abs.

TCRBV family-specific PCR was performed as previously described (19). For each of 25 TCRBV families a specific sense primer and a common antisense primer matching the TCRB constant region were used. T cell clones were amplified for 25 cycles, PBMC for 35 cycles using 1 μl of cDNA per 25-μl reaction.

TCRB gene rearrangements of T cell clones were determined by direct sequencing of products obtained by TCR family-specific PCR. Due to the monoclonality of the established T cell clones, no subcloning step was necessary to obtain unambiguous complementarity-determining region 3 (CDR3) sequences. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA) and cycle-sequenced using dye-labeled deoxynucleotide terminators and a 373A automated sequencer (Applied Biosystems, Foster City, CA). TCRB rearrangements were determined in both directions using the family-specific and constant region-specific primers used for PCR amplification.

Functional analysis

T cell clones (2×10^4) reactive with tetrameric HLA complexes were incubated at 37°C in 96-well plates with irradiated peptide-pulsed stimulator cells at a ratio of 5:1 for the indicated time periods in IL-2 medium. Stimulator cells comprised the 721.221 HLA class I-negative B lymphoblastoid cell line transfected with cDNA for HLA-A*0201 and incubated in the presence of relevant nonamer peptides at 37°C overnight before washing and irradiation. Cultures for each time point were established in duplicate. Peptides were synthesized by F-moc chemistry and purified to greater than 90% purity by reverse phase HPLC (Sigma Genosys, Austin TX). Cells were analyzed by flow cytometry with fluorochrome-labeled anti-CD8 and anti-CD3 Abs. Cell viability was assessed by forward and side scatter characteristics and staining with FITC-labeled annexin V (Becton Dickinson). For anti-CD3 stimulation, T cell clones (2×10^5) were incubated in the presence of 1 μg/ml anti-CD3 mAb (clone UCHT1; Coulter) and 2 μg/ml recombinant protein G (Sigma) in IL-2 medium at 37°C for 6 h, washed, and assayed for Annexin V binding and propidium iodide exclusion by flow cytometry. Control incubations consisted of T cell clones incubated in the presence of protein G or medium alone. For analysis of PBLs, $CD3^+$ $CD8^+$ T cells were sorted into $ILT2/LIR1^+$ KIR^- and $ILT2/LIR1^+$ KIR^+ populations by flow cytometry before anti-CD3 stimulation and flow cytometric analysis as described above.

Statistical analysis

Analysis of differences in Ig-like receptor expression between flow cytometry-defined subsets was accomplished using a two-tailed Student *t* test and ANOVA.

Results

Variation in ILT2/LIR1-inhibitory receptor expression in PBLs

Flow cytometric analysis of ILT2/LIR1 expression in PBMC of 20 normal donors using the specific mAb HPF1 demonstrated that the principal cell types expressing this inhibitory receptor were monocytes and B lymphocytes (Fig. 1*A*), as previously reported (16). In all individuals analyzed, ILT2/LIR1 was expressed on close to 100% of $CD3^-$ $CD19^+$ B lymphocytes and $CD14^+$ $CD16^+$ or $CD13^+$ $CD16^+$ monocytes. In contrast to the ubiquitous expression by B lymphocytes and monocytes, ILT2/LIR1 was expressed on a subset of peripheral adult $CD3^+$ T lymphocytes and $CD3^-$ $CD56^+$ NK lymphocytes (Fig. 1*A*) and a very small proportion of cord blood T lymphocytes (mean = 1.7% SD = 1.2, $n = 5$). ILT2/LIR1 was expressed on a mean of 48% (SD = 21.4) of $\gamma\delta$ T lymphocytes and a mean of 23.5% (SD = 14.9) of $\alpha\beta$ T-lymphocytes in the 20 normal adults. Expression of both ILT2/LIR1 and KIR was restricted to $CD8^+$ T lymphocytes, with $<1\%$ of $CD4^+$ T lymphocytes expressing Ig superfamily inhibitory receptors (Fig. 1*B*). Although the values for $CD3^+$ cells appear quantitatively

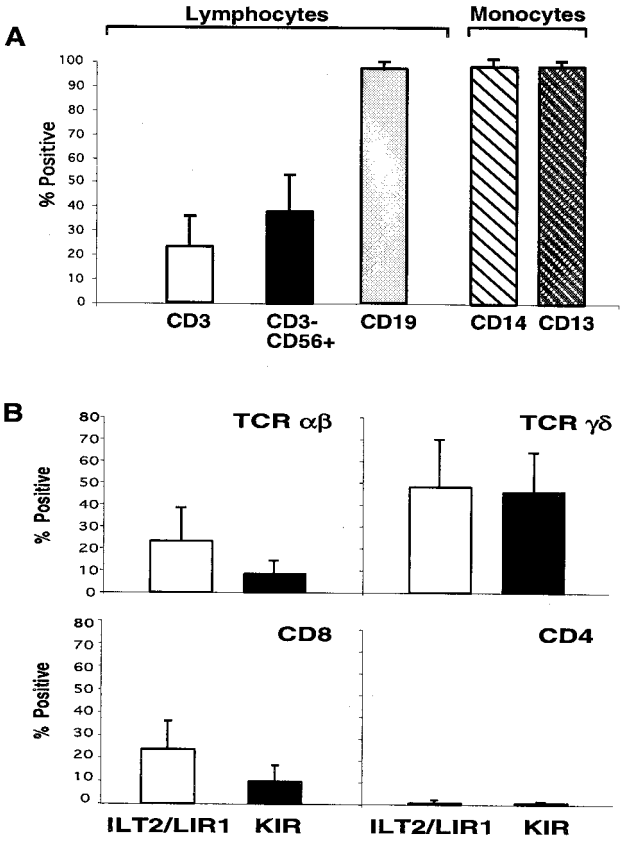

FIGURE 1. ILT2/LIR1 receptor is expressed on a subset of peripheral blood T and NK lymphocytes. *A*, Mean percentage of ILT2/LIR1$^+$ cells within each of the major mononuclear cell populations as determined by flow cytometric analysis of 20 normal adult individuals. Errors bars depict SD of the mean. Open columns, CD3$^+$ T lymphocytes; solid black columns, CD3$^-$ CD56$^+$ NK lymphocytes; solid gray columns, CD19$^+$ B lymphocytes; hatched white columns, CD14$^+$ monocytes; hatched black columns, CD13$^+$ monocytes. *B*, Mean percentage of ILT2/LIR1$^+$ cells within each of the major T cell subsets as determined by flow cytometric analysis of 20 normal adult individuals. Errors bars depict SD of the mean. Within the peripheral lymphocyte population, ILT2/LIR1 is principally expressed on cytotoxic cells. Open columns, ILT2/LIR1 (HPF1 Ab); filled columns, KIR (a mixture of EB6, DX27, DX9, and DX31 Abs).

similar to that for CD8$^+$ cells, this is an artifact of presenting the results as mean values. Analysis of individual donors demonstrates that these values are not correlated and are apparently influenced by variations in numbers of CD8$^+$ T cells between donors (data not shown).

ILT2/LIR1-inhibitory receptor is principally expressed by memory CTLs

In view of the low numbers of ILT2/LIR1$^+$ T lymphocytes observed in cord blood samples and the previously reported restriction of KIR expression to memory phenotype adult T lymphocytes, we performed a subset analysis of T cells in the panel of 20 normal individuals using the HPF1 Ab and a pool of Abs recognizing KIR2D and KIR3D molecules. This analysis demonstrated that both ILT2/LIR1$^+$ and KIR$^+$ T cells are predominantly contained within the CTL memory/effector population i.e., CD8$^+$ CD56$^+$ CD57$^+$ CD27$^-$ CD28$^-$ T cells (Fig. 2*A-D*).

However, analysis of subsets defined by markers previously recognized as distinguishing Ag-naive and Ag-experienced T cells showed no significant differences in ILT2/LIR1 or KIR expression between either CD45RA/RO or CD38$^+$/CD38$^-$ T cells (Fig. 2, *E* and *F*). ILT2/LIR1$^+$ T cells were of a higher proportion in the CD45RA$^+$ population, which may correlate with the reported effector function of this subset of cells (20). By comparison with KIR expression in the populations defined by CD8, CD56, CD57, CD27, or CD28, ILT2/LIR1 was expressed by a significantly higher number of T cells within each subset ($p < 0.0005$).

Coexpression of ILT2/LIR1 and KIR on CTLs

Our analysis of inhibitory receptor expression in T lymphocyte subsets revealed a consistently greater number of cells positive with the HPF1 Ab than the mixture of KIR-specific Abs within all subsets tested. This raised the possibility that ILT2/LIR1 and KIR expression may not be concomitant in these cells. To analyze this further, we tested coexpression of ILT2/LIR1 and KIR in the peripheral lymphocytes of twenty individuals. Although a consistent proportion of KIR$^+$ NK (gated on CD56$^+$ or CD16$^+$ lymphocytes) or T lymphocytes (gated on CD3$^+$ lymphocytes) coexpressed ILT2/LIR1 (Fig. 3*A*), this feature was not apparent when ILT2/LIR1$^+$ CD3$^+$ T-lymphocytes were analyzed for coexpression of KIR (Fig. 3*B*). Although equivalent levels of KIR expression were observed on ILT2/LIR1$^+$ NK lymphocytes, the majority of ILT2/LIR1$^+$ T cells expressed only this receptor and the minority coexpressed KIR. A comparison of the relative proportions of ILT2/LIR1 and KIR coexpression in the CD3$^+$ population is shown in Fig. 3, *C* and *D*.

This difference between ILT2/LIR1 and KIR expression in T lymphocytes and NK cells was strikingly apparent when the twenty individuals were analyzed individually (Fig. 4). In both CD3$^+$ CD8$^+$ (Fig. 4*A*) and TCR$\alpha\beta^+$ (Fig. 4*B*) populations, 19 of 20 individuals expressed ILT2/LIR1 on a higher percentage of T cells than KIR molecules. However, the majority of these individuals expressed ILT2/LIR1 on a lower percentage of their NK (CD3$^-$ CD56$^+$) cells (Fig. 4*C*) than KIR molecules with $\gamma\delta$ T cells demonstrating an intermediate phenotype (Fig. 4*D*).

Clonal analysis of ILT2/LIR1-positive T lymphocytes

To precisely investigate the characteristics of ILT2/LIR1 expression in T lymphocytes, we derived T cell clones by single cell sorting of CD3$^+$ ILT2/LIR1$^+$ lymphocytes from the peripheral blood of two donors who were previously analyzed in detail for the features of KIR expression in both NK (10) and T lymphocytes (21). The phenotype and TCR clonotype of the clones were analyzed in detail using flow cytometric and molecular techniques. Because the pool of KIR-specific Abs we used in the flow cytometric analysis detect all but four of the 12 known KIR molecules, a precise molecular analysis of T cell clones was required to unambiguously determine the full extent of overlap in Ig-like receptor expression.

ILT2/LIR1$^+$ T cell clones are representative of peripheral blood T lymphocytes

The panels of ILT2/LIR1$^+$ T cell clones derived from donors NV and PP have similarities to the populations of KIR$^+$ T cell clones we have previously analyzed (21) but also have significant differences (Table I). In short, although these cells typically displayed the phenotypes of activated memory cells, the panels comprised not only CD8$^+$ T cells but also included CD4$^-$ CD8$^-$ TCR$\alpha\beta$ cells and rare CD4$^+$ cells. Similar proportions of TCR$\alpha\beta$ and TCR$\gamma\delta$ cells were derived from cloning ILT2/LIR1$^+$ T cells as were found in KIR$^+$ cloning.

ILT2/LIR1$^+$ T cell clones exhibit a diverse TCR repertoire

The most striking finding of our previous study of KIR$^+$ T cell clones was the almost complete restriction of TCRBV region gene usage in TCR$\alpha\beta$ clones from donor NV. All such clones were

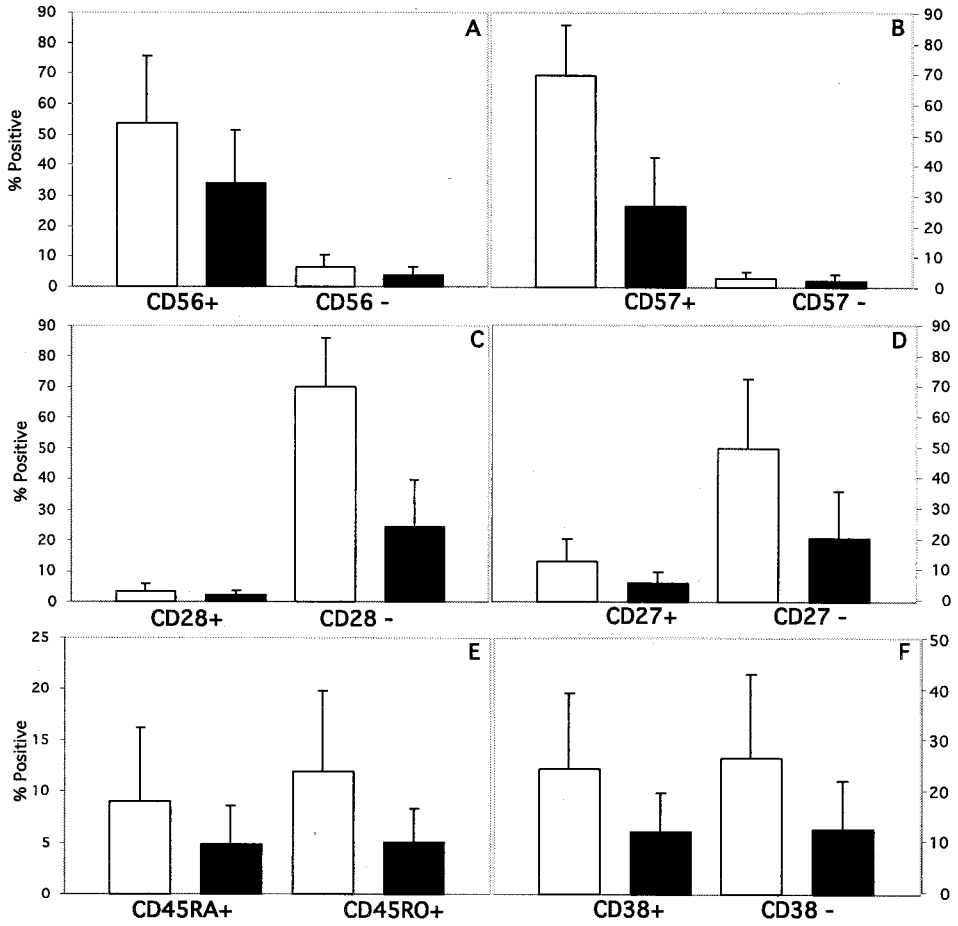

FIGURE 2. ILT2/LIR1 receptor expression is principally restricted to memory/effector T lymphocytes. ILT2/LIR1 and KIR expression was determined by flow cytometry in $CD3^+$ T lymphocytes positive or negative for the following markers of T cell memory and activation: *A*, CD56; *B*, CD57; *C*, CD28; *D*, CD27; *E*, CD45; *F*, CD38. Mean percentage of positive cells within each subset is shown ($n = 20$). Error bars depict SD of the mean. Open columns, ILT2/LIR1 (HPF1 Ab); filled columns, KIR (a mixture of EB6, DX27, DX9, and DX31 Abs).

TCRBV16 and expressed a highly restricted CDR3 sequence (21). We used PCR amplification and nucleotide sequencing of TCRBV gene transcripts from ILT2/$LIR1^+$ T cell clones from donors NV and PP to assess the extent of TCR diversity in these cells. A proportion of the ILT2/$LIR1^+$ TCR$\alpha\beta$ clones from donor NV represented the population cloned previously as they expressed the same TCRBV16 clonotype as the KIR^+ T cell clones. However, a further population of ILT2/$LIR1^+$ T cell clones expressed TCRB chain genes of diverse families and clonotypes including TCRBV2, BV6, BV17, BV21, and BV23 (Table II). No motifs common to all represented TCRBV families were found when the CDR3-encoding regions of these transcripts were sequenced, suggesting that these clones originally proliferated in response to unique peptide Ags. Similar results were found in clones derived from donor PP, where clonal TCRBV transcripts included TCRBV1, BV2, BV3, BV6, BV10, BV13, BV14, BV16, BV17, and BV21 (Table II).

In addition, flow cytometric analysis of peripheral blood $CD8^+$ lymphocytes with TCRBV-specific mAbs in six normal donors indicated that ILT2/LIR1 was expressed on a proportion of all families tested (mean = 25.4% 1SD = 10.3) whereas the mixture of KIR-specific Abs only detected low percentages in each family (mean = 6.1% 1SD = 4.1) or expansions within a small number of families.

Clonal analysis of ILT2/LIR1 and KIR coexpression

Typing of KIR gene expression in each of the T cell clones by RT-PCR and flow cytometry revealed a surprising finding. Although all clones were ILT2/$LIR1^+$, only a very small number displayed the Ig-like receptor phenotype of the cells that predominated in peripheral blood, i.e., ILT2/$LIR1^+$ KIR^-. From a total of 104 clones (including 20 from a third donor), only 19 did not react with KIR-specific mAbs and the majority of these expressed KIR2DL4, KIR2DS4, or KIR3DS1, which are not detectable by currently available Abs but can be detected at the cDNA level (Fig. 5). Thus, only 3 of the 104 clones displayed a truly KIR^- phenotype. The remaining 101 clones displayed KIR phenotypes, as determined by RT-PCR typing, similar to those described in our previous study (21). These results indicate that our use of the pooled KIR-specific Abs in the analysis of PBLs detected the majority (~80%) of the KIR-expressing T cells.

To examine this apparent differential expression of LRC-encoded receptors more precisely, we analyzed Ag-specific peripheral blood $CD8^+$ cells of normal donors with tetrameric HLA class I molecules (Fig. 6). Using an HLA-A*0201-HCMVpp65 tetramer, it is evident that the majority of these peptide-specific $CD8^+$ T cells express ILT2/LIR1 (Fig. 6*B*) but only a very small proportion express KIR or CD94 receptors (Fig. 6*C*). Similar results were detected in another five donors

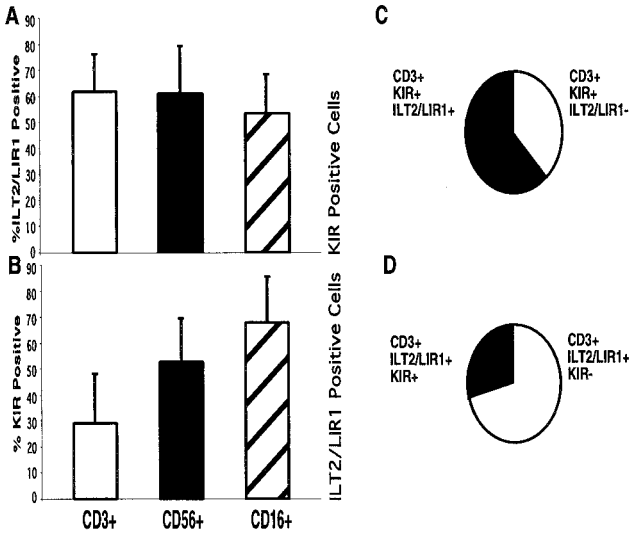

FIGURE 3. The majority of $CD3^+$ ILT2/LIR1-expressing T lymphocytes do not coexpress KIR. Flow cytometric analysis of receptor coexpression in peripheral lymphocyte subsets reveals differences in ILT2/LIR1 and KIR coexpression in the $CD3^+$ T cell subset. Mean percentage of positive cells within each subset is shown ($n = 20$). Error bars depict SD. Open columns, $CD3^+$ cells; filled columns, $CD56^+$ cells; hatched columns, $CD16^+$ cells. *A*, Percentage of $ILT2/LIR1^+$ cells of each subset within the KIR^+ population. *B*, Percentage of KIR^+ cells of each subset within the $ILT2/LIR1^+$ population. *C* and *D*, Proportions of single and double positive $CD3^+$ cells within either KIR^+ or $ILT2/LIR1^+$ populations, respectively.

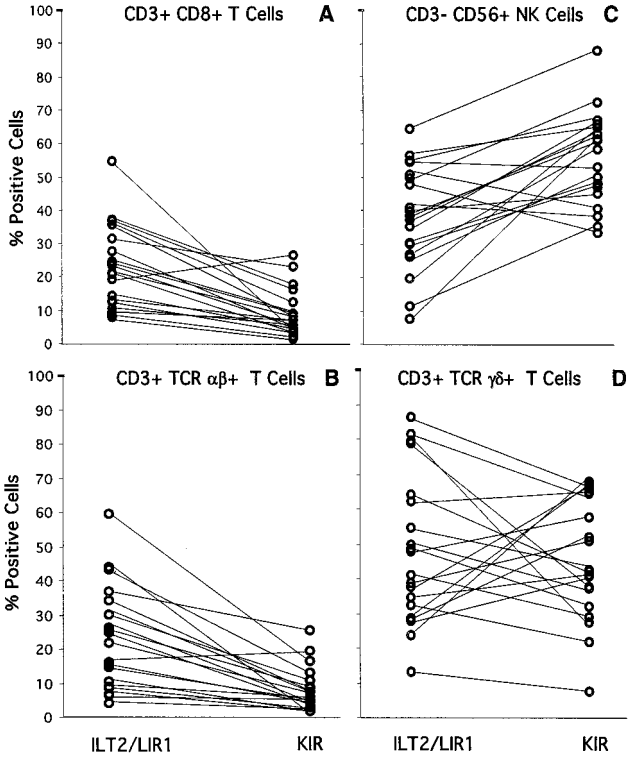

FIGURE 4. Discordant expression of ILT2/LIR1 and KIR in peripheral blood cytotoxic lymphocytes. Each panel depicts the percentage of PBL subsets (*A*, $CD8^+$ T cells; *B*, TCR$\alpha\beta$ T cells; *C*, $CD3^-$ $CD56^+$ NK cells; *D*, TCR$\gamma\delta$ T cells) expressing either ILT2/LIR1 (*left*, HPF1 Ab) or KIR (*right*, a mixture of EB6, DX27, DX9, and DX31 Abs) in 20 normal individuals. Values for each receptor are linked for individual donors and demonstrate a consistently higher proportion of ILT2/LIR1 expression in $CD8^+$ TCR$\alpha\beta$ T cells that is not observed in NK or TCR$\gamma\delta$ cells.

using tetramers containing either HCMV- or EBV-specific peptides (data not shown) confirming the validity of the LRC receptor phenotype found in our analysis of peripheral blood $CD8^+$ lymphocytes (Fig. 4).

$ILT2/LIR1^+$ KIR^- clones are prone to activation-induced cell death

In view of the discrepancy observed between the phenotype of peripheral blood $ILT2/LIR1^+$ T cells and that of the in vitro expanded panel of T cell clones, we reasoned that the majority of $ILT2/LIR1^+$ T cells did not survive the cloning procedure and only those clones, which were also KIR^+, exhibited long-term in vitro growth characteristics. To investigate whether possible differences in the survival ability of T cell clones correlated with their expression of particular Ig-like receptors we performed another two experiments. After isolation and limited expansion of HCMVpp65 peptide-specific T cell clones using tetramers and single cell sorting, we tested the response of these $ILT2/LIR1^+$ KIR^- clones to in vitro stimulation with their cognate peptide/HLA class I restriction element in the presence of IL-2.

As shown in Fig. 7*A*, incubation of T cell clones with their cognate peptide resulted in the death of ~80–90% of these cells by 48 h of culture, with a slightly more rapid rate of cell death induced by stimulator cells pulsed with higher peptide concentrations. In contrast, incubation with stimulators pulsed with an equivalent concentration of irrelevant peptide resulted in a decrease of viable $CD8^+$ cells of only 5–10%. To compensate for any potential differences in the affinity of clonally distributed TCRs for peptide-HLA complexes, we also stimulated $ILT2/LIR1^+$ KIR^- and $ILT2/LIR1^+$ KIR^+ T cell clones with soluble anti-CD3 Ab in the presence of protein G and IL-2. After 6 h of incubation under these conditions, ~50% of cells of the KIR^- clone bound annexin V, indicating the translocation of plasma membrane phospholipids characteristic of the early stages of apoptosis (22). In contrast, $KIR2DL4^+$ T cell clones displayed only a background level of such annexin V binding (Fig. 7*B*).

Flow cytometric sorting of peripheral $CD8^+$ T cells expressing ILT2/LIR1 alone or in combination with KIR demonstrated a similar finding, with the KIR^- cells being more susceptible to anti-CD3-induced cell death (Fig. 8*A*). This population also contained significantly ($p < 0.005$) lower intracellular levels of the survival molecule Bcl-2 (Fig. 8*B*). The results of these two experiments

Table I. *Phenotypic characteristics of $ILT2/LIR1^+$ T cell clones derived from two normal donors*[a]

Phenotype	NV Clones ($n = 45$)	PP Clones ($n = 39$)
TCR$\alpha\beta$	80[b]	59[b]
TCR$\gamma\delta$	20	41
$CD8^+$	67	33
$CD4^+$	0	8
$CD4^-8^-$	13[c]	21[c]
$CD56^+$	71	28
$CD57^+$	67	77
$CD28^+$	0	5
$CD16^+$	20	5
$CD45RO^+$	100	100

[a] Clones were derived by single cell sorting of $CD3^+$ $ILT2/LIR1^+$ peripheral blood lymphocytes and expanded in vitro. Clones were phenotyped by flow cytometry and T cell receptor type was confirmed by RT-PCR.
[b] Percentage of total clones expressing the indicated cell surface marker.
[c] Percentage calculated from the number of TCR$\alpha\beta$ $CD4^-8^-$ clones.

Table II. *Comparison of the range of TCRBV gene usage in T cell clones selected on the basis of KIR expression or ILT2/LIR1 expression*[a]

Donor NV		Donor PP	
KIR[b]	ILT2/LIR1[c]	KIR[b]	ILT2/LIR1[c]
BV16	BV16	BV11	
	BV2	BV14	BV14
	BV6	BV21	BV21
	BV12		BV1
	BV17		BV2
	BV21		BV3
	BV23		BV6
			BV10
			BV13
			BV16
			BV17

[a] TCRBV gene usage was determined by RT-PCR and nucleotide sequencing. The overlap between the two populations is apparent but ILT2/LIR1$^+$ clones display a greater diversity of TCR usage.
[b] KIR$^+$ T cell clones. Data from Ref. 21.
[c] ILT2/LIR1$^+$ T cell clones derived in the present study.

indicate that expression of KIR by T cells correlates with the acquisition of resistance to activation-induced cell death by apoptosis.

Discussion

The LRC contains a large number of genes encoding Ig-superfamily proteins that have both structural and functional homologies.

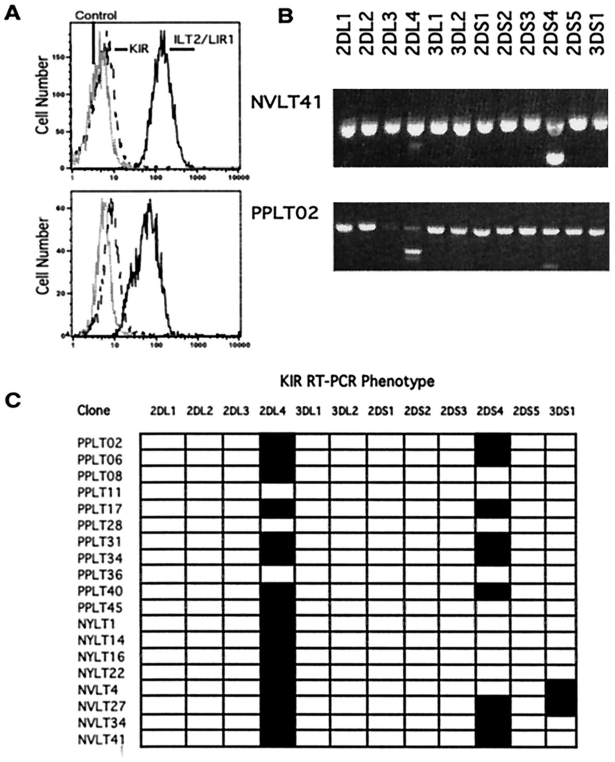

FIGURE 5. Phenotypic analysis of CD3$^+$ ILT2/LIR1$^+$ T cell clones detects KIR2DL4 expression. Flow cytometric analysis of a panel of T cell clones demonstrates only a minor proportion display the commonest phenotype observed in peripheral blood. *A* and *B*, Flow cytometric and RT-PCR analysis of KIR expression in two representative clones. *C*, KIR phenotype defined by RT-PCR in 19 clones (from a total of 104) that did not react with KIR-specific Abs, demonstrating that the majority of these cells express KIR2DL4. Open boxes indicate a negative typing result, and filled boxes represent a positive result.

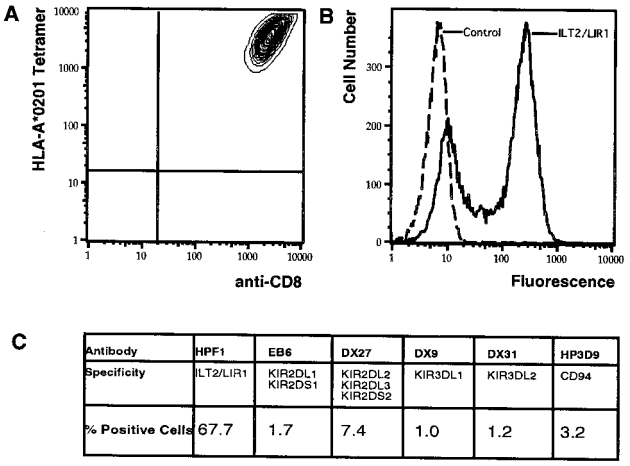

FIGURE 6. Analysis of peripheral Ag-specific CD8$^+$ T cells confirms the ILT2/LIR1$^+$ KIR$^-$ phenotype. Ex vivo analysis of bulk-sorted CD8$^+$ cells reactive with an HLA-A*0201-HCMVpp65 tetramer (*A*) demonstrates a phenotype consistent with the investigation of peripheral blood T cells. These cells display a proportion of ILT2/LIR1 expression (*B*) at least 10-fold greater than KIR expression (*C*).

Although certain of these receptors (e.g., ILT2/LIR1) are expressed on several hemopoietic cell types, particular genes such as KIR are only expressed in cells with a specific function. The mechanisms that control this cell type-specific gene expression are currently unknown. Our initial results demonstrated the expression of the ILT2/LIR1 inhibitory receptor on virtually all B lymphocytes and monocytes but emphasized the differences between individuals in terms of T lymphocyte expression. This feature suggested clonal variation in ILT2/LIR1 expression and prompted us to investigate the detailed characteristics of ILT2/LIR1 expression in T lymphocytes in regard to that of the related KIR genes. Unlike certain KIR genes (18), haplotypic diversity in the presence of the ILT2/LIR1 gene did not contribute to the apparent clonal variation because all donors typed positively for ILT2/LIR1 at the level of genomic DNA (data not shown).

The phenotypic assessment of peripheral blood T cell subsets definitively shows that ILT2/LIR1 and KIR genes are expressed in the same types of T cells, i.e., differentiated, Ag-experienced cytotoxic cells which may have lost the requirement for costimulation. However, this overlap in expression of the related genes is not necessarily coordinate as the proportions of ILT2/LIR1$^+$ cells always exceeded that of KIR$^+$ cells within each subset. The more widespread expression of ILT2/LIR1 is also supported by phenotypic analysis of T cell clones and Ag-specific T cells. ILT2/LIR1$^+$ clones and peripheral T cells displayed a diverse assortment of TCRV region gene usage in contrast to KIR$^+$ cells. Also, ILT2/LIR1$^+$ clones included CD4$^-$CD8$^-$ and rare CD4$^+$ T cells in addition to the CD8$^+$ cells characteristically expressing KIR.

Analysis of coexpression of the two receptor types demonstrated the predominance of ILT2/LIR1$^+$ KIR$^-$ T cells in peripheral blood and the use of tetrameric HLA class I constructs confirmed this pattern of receptor expression within a population of CD8$^+$ T cells specific for distinct viral peptides.

The derivation of ILT2/LIR1$^+$ T cell clones allowed us to define a major functional difference between cells expressing ILT2/LIR1 alone or in concert with KIR. The use of established in vitro cloning procedures failed to produce clones representative of the major phenotype observed ex vivo, as the only cells that displayed significant in vitro proliferation were KIR$^+$ and not ILT2/LIR1$^+$ KIR$^-$. Further functional analysis of these clones demonstrated

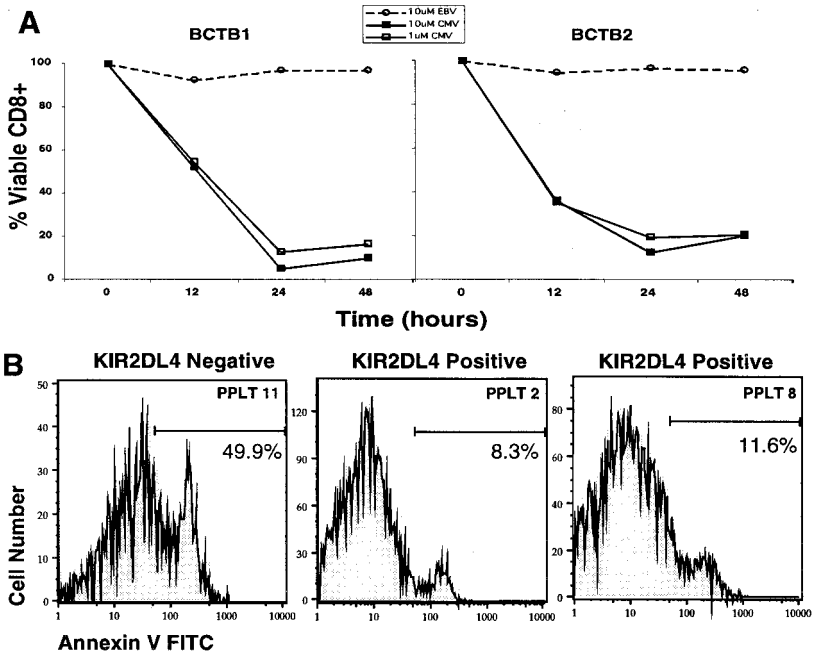

FIGURE 7. Stimulation of TCR of T cell clones defines resistance to activation-induced cell death associated with expression of KIR. *A*, HLA-A*0201-HCMVpp65 tetramer-reactive ILT2/LIR1$^+$ KIR$^-$ T cell clones (BCTB1 and BCTB2) were incubated with 721.221-HLA-A*0201 cells pulsed with 10 μM HCMVpp65 peptide (■), 1 μM HCMVpp65 peptide (□), or 10 μM EBV BZLF1 peptide (○), and the viability of CD8$^+$ cells was determined by flow cytometry at indicated time points. *B*, ILT2/LIR1$^+$ KIR2DL4$^-$ and ILT2/LIR1$^+$ KIR2DL4$^+$ T cell clones were incubated with 1 μg/ml soluble anti-CD3 in the presence of 2 μg/ml protein G for 6 h and analyzed for binding of annexin V by flow cytometry.

that ILT2/LIR1$^+$ KIR$^-$ clones were susceptible to apoptosis when activated through the TCR/CD3 complex, whereas KIR$^+$ clones were not. This result was confirmed by analysis of these T cell populations derived from peripheral blood, which additionally demonstrated a lower level of the cell survival molecule Bcl-2 in ILT2/LIR1$^+$ KIR$^-$ CD8$^+$ T cells.

It could be argued that without an Ab to specifically identify KIR2DL4, we do not currently know the true extent of KIR2DL4 expression in the serologically KIR$^-$ population. However, if all of the ILT2/LIR1$^+$ KIR$^-$ cells expressed KIR2DL4 we would expect a far higher proportion of the in vitro expanded clones to display this phenotype than was actually detected (~18% of ILT2/LIR1$^+$ clones compared with ~60% of peripheral blood ILT2/LIR1$^+$ T cells). This suggests that the data on KIR expression we obtained by flow cytometry are not a gross underestimate of peripheral KIR$^+$ T cells.

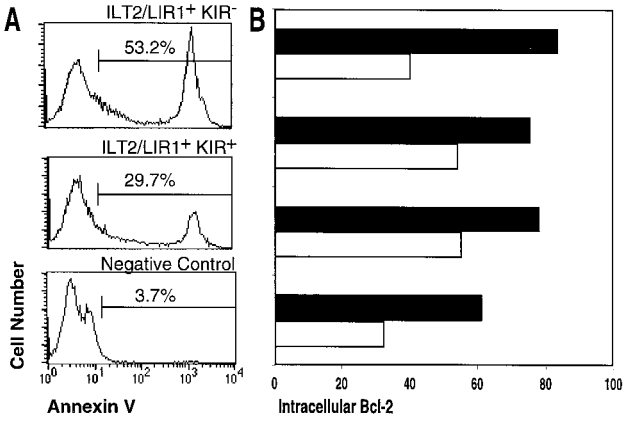

FIGURE 8. Peripheral CD8$^+$ lymphocytes expressing ILT2/LIR1 are more susceptible to activation-induced cell death and have lower intracellular levels of Bcl-2. *A*, Bulk-sorted ILT2/LIR1$^+$ KIR$^-$ (*top*) and ILT2/LIR1$^+$ KIR$^+$ (*middle*) peripheral CD3$^+$ CD8$^+$ lymphocytes were incubated with 1 μg/ml soluble anti-CD3 in the presence of 2 μg/ml protein G for 6 h and analyzed for binding of annexin V by flow cytometry. *Bottom*, Profile of cells incubated with protein G alone. Results shown are representative of four donors. *B*, PBLs were stained to identify CD3$^+$ CD8$^+$ ILT2/LIR1$^+$ (open columns) and CD3$^+$ CD8$^+$ KIR$^+$ (filled columns) T cells, permeabilized, and stained with a labeled anti-Bcl-2 Ab. The graph shows intracellular Bcl-2 levels (measured in arbitrary fluorescence units) in each subset of CD8$^+$ T cells of four donors. Significantly ($p < 0.005$) lower levels of Bcl-2 are found in the ILT2/LIR1$^+$ population.

These phenotypic and functional results allow us to propose a scheme for the sequential acquisition of LRC-encoded receptors by activated CTLs. In our previous study of KIR$^+$ T cell clones (21), we suggested that the diverse KIR phenotypes displayed by a dramatically expanded clone of T lymphocytes represented the end result of a program of KIR gene activation which began with the expression of KIR2DL4, a receptor which has been demonstrated to be expressed in all KIR$^+$ NK or T cells (23).

Our present study supports such a serial mechanism of LRC receptor expression and allows this to be extended to a stage before the expression of KIR2DL4. The dramatically increased proportion of activated T cells that are ILT2/LIR1$^+$ KIR$^-$, as exemplified by the tetramer binding population, can be regarded as effector CTL because they comprise the greater number of Ag-specific cells, are CD56$^+$ (24) and are prone to apoptosis when stimulated (25). This association of ILT2/LIR1 gene expression with T cell activation may be related to our finding of an NCAM (CD56)-associated transcription element in the 5′-untranslated region of ILT2/LIR1 cDNA clones (N.Y., unpublished data). However, the precise signals determining Ig-like receptor gene expression are currently unknown. Although certain cytokines, such as IL-15 (26), TGF-β (27), and IL-10 (28), have been reported to induce or modulate expression of the lectin-like receptors CD94 and NKG2A on T cells, no effects on LRC-encoded Ig-like receptor expression have been observed. The relevance of KIR2DL4 expression at the initiation of KIR gene expression in T cells is unclear. Although this receptor has been detected at the mRNA level in all KIR$^+$ cells, its expression on the cell surface is currently controversial. One study reports KIR2DL4 expression on all peripheral NK cells (23), whereas another suggests that KIR2DL4 is only expressed on the surface of decidual NK cells in pregnancy (29). The significance of the apparent specificity of KIR2DL4 for the nonclassical HLA-G class I molecule (23, 29) is also unclear because HLA-G expression is highly tissue restricted (30). Specific

reagents to reliably detect KIR2DL4 are required before these matters can be fully resolved.

The relatively small population of KIR^+ cells represent clones that have made the transition to a state of long-lived memory T cells in a scheme that is consistent with the recently demonstrated linear differentiation of specific memory CTL in transgenic mice (31). The proportions of KIR^+ and KIR^- cells within the ILT2/$LIR1^+$ T cell population are consistent with the numbers of effector and memory cells reported in the anti-HY response of these mice. This situation is likely to be observed in chronic viral infections because the pool of responsive peptide-specific T cells will continually be exposed to Ag (32), becoming activated and expanding in number before maintaining dynamic homeostasis through apoptosis, with a small percentage of cells remaining as KIR^+ memory cells. Such a predominance of effector cells may not be observed where viral infections are cleared and only the relatively scarce Ag-specific memory cells will be detectable (33).

Recently, it has been suggested that memory T cells can be distinguished into two types (effector and central) based on the expression of CCR7 chemokine receptors, correlating with their in vivo trafficking (34). Due to the unavailability of the Abs defining the CCR7 chemokine receptor we have been unable to directly assess how this correlates with the expression of specific Ig-like receptors. We would suggest that the major population of ILT2/$LIR1^+$ KIR^- cells represent the effector cells characteristic of an initial immune expansion, while future studies should allow us to define whether the ILT2/$LIR1^+$ KIR^+ cells we have identified are comparable with the effector memory subset defined by Sallusto et al. (34).

Our results also indicate a possible role for KIR in the maintenance of memory T cell survival and resistance to activation-induced cell death. Effector $CD8^+$ cells have lower levels of the cell protective molecule Bcl-2 (35), which results in sensitivity to apoptosis induced by fratricide (36) or TNF-α (37). Our findings support these reports and suggest that KIR expression in T cells is associated with resistance to activation-induced cell death mediated through stimulation of the TCR. At present, we cannot assign this anti-apoptotic role directly, as it is possible that KIR expression in memory cells is simply coordinate with that of another molecule(s) that confers this survival feature (38). However, KIR have been suggested to bind the p85α subunit of PI3-kinase leading to activation of the anti-apoptotic AKT kinase (39), and the functionally equivalent Ly49 receptors in mice may be involved in the selection and survival of individual NK clones (40).

The differential expression of ILT2/LIR1 and KIR in activated T lymphocytes is consistent with differences in the structure and ligand binding specificities of these molecules. Whereas KIR bind defined epitopes in the α1 domain of HLA-C or HLA-B molecules, the larger size of the four Ig-domain ILT2/LIR1 molecule is compatible with its binding to the common α3 domain of class I in an extended conformation, similar to that of CD8 (41). If such a model is correct, the molecular dimensions of ILT2/LIR1, in addition to the nature of its ligand binding site, would allow concomitant binding of both TCR and ILT2/LIR1 to the same HLA class I ligand molecule. This would enable the recruitment of phosphatases directly to the site of TCR signaling and the subsequent diminution of cellular activation.

In conclusion, our study indicates a program of sequential expression of Ig-superfamily receptors encoded within the LRC, with ILT2/LIR1 expression as an initial event in early stage, activated cytotoxic effector T cells followed by the acquisition of KIR at the stage of transition to differentiated memory cells. The expression of KIR appears to correlate with a resistance to activation-induced cell death in these cell populations, although the requirement for KIR interaction with self-HLA class I in this process is currently unknown.

In view of the close developmental and functional relationships between NK and $CD8^+$ T lymphocytes (42), further investigation of the events occurring in activated $CD8^+$ T cells should enable the use of such cells as an appropriate working model for analysis of LRC gene transcription and expression. Future studies will allow us to determine the potential role of these Ig-like receptors in the long-term survival of memory T cells.

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KIR expression shapes cytotoxic repertoires: a developmental program of survival

Neil T. Young and Markus Uhrberg

We hypothesize a sequential program of expression of the leukocyte-receptor complex (LRC) in CD8$^+$ T cells, associated with cellular activation and the subsequent establishment of immune homeostasis through resistance to apoptosis. This program, which is consistent with the linear development of memory CD8$^+$ T cells, represents an ordered expression of genes during differentiation, analogous to expression of the homeobox- or globin-gene clusters. Our model not only has implications for the development and maintenance of T-cell memory but also, relates to the formation of LRC repertoires in other cell types, particularly, the development of killer-cell Ig-like receptor (KIR) repertoires in natural-killer-cell precursors.

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The leukocyte-receptor complex (LRC) is a polymorphic cluster of related genes located on human chromosome 19q13.4. The genes within the LRC encode Ig-superfamily proteins that are expressed in hematopoietic-cell lineages. The majority of these receptors contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail, which recruit phosphatase enzymes and inhibit intracellular activatory signaling pathways. By contrast, isoforms of certain receptors have short cytoplasmic tails with no intrinsic signaling capacity; however, they associate with an immunoreceptor tyrosine-based activation motif (ITAM)-containing adapter molecule and, consequently, might activate cellular processes. Thus, LRC receptors are able to exert fine control of cellular effector functions in the context of expression of their relevant ligands. In mice, the LRC is present in a minimal form, lacking the genes encoding killer-cell Ig-like receptors (KIRs); this region has undergone extensive diversification during primate evolution, suggesting that it plays an important role in immunological surveillance (reviewed in Ref. [1]).

LRC receptors

The first genes to be characterized within the LRC were those encoding the KIRs, expressed on natural killer (NK) cells and CD8$^+$ T cells [2,3]. Specific epitopes of HLA class I molecules are the ligands for these receptors and receptor–ligand interactions transmit inhibitory signals, resulting in NK-cell self-tolerance. The cytotoxic function of NK cells is inhibited by KIR ligation, enabling the recognition of virally infected or malignant cells that down-regulate their expression of HLA class I antigens. Thus, NK cells recognize 'missing' self rather than 'altered' self. A further set of genes, encoding molecules known as Ig-like transcripts (ILTs) or leukocyte Ig-like receptors (LIRs), is situated also in the LRC. These receptors have structural and functional homology with KIRs but are expressed in NK cells, T and B cells, and myelomonocytic cells. Two of these receptors (ILT2/LIR1 and ILT4/LIR2) are known to recognize a wide range of HLA class I molecules, but the ligands for the majority of ILT/LIR proteins are unknown at present.

Currently, the molecular and cellular factors controlling the expression of LRC receptors are unclear. At a basic level, expressed KIR repertoires are determined by the encoding genes within the LRC [4], which show genetic variability between individuals [5]. However, other factors are implicated because donors with identical KIR genotypes show variation in the proportions of peripheral NK cells expressing particular KIRs. The majority of individuals possesses a full set of the ILT/LIR loci [6] and although ILT/LIR proteins are expressed on the majority of peripheral B cells and monocytes, clonal variation is observed for ILT/LIR expression on NK cells and T cells [7].

'…differential expression of LRC receptors by human CD8$^+$ T cells correlates with the linear development of memory T cells…'

Unlike the loci encoding T-cell and B-cell receptors, LRC genes are nonrearranging and it is unclear whether educational or selective processes are involved in the formation of LRC-receptor repertoires, or whether the maturation of NK cells, in particular, simply follows an instructional, 'hardwired' genetic program. This latter concept is compatible with the known independence of KIR expression from HLA type, because many individuals express KIRs for which they do not express the relevant MHC ligand. Although NK cells can express several KIRs, the only requirement for self-tolerance is that individual clones must express at least one functional receptor recognizing self-HLA. Thus, selection of immature NK-cell precursors must operate, if only at the level of establishing self-tolerance. However, attempts to study LRC-gene expression during the development of NK-cell precursors have not proven successful. Cytokine-mediated, *in vitro* maturation of NK cells from hematopoietic stem cells or immature, thymus-derived NK cells results in the expression of the lectin-like CD94 receptor, but not KIRs [8,9].

LRC-receptor expression in CD8$^+$ T cells

KIR expression in CD8$^+$ T cells occurs after the rearrangement of the T-cell receptor (TCR) and

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thymic education [10,11], suggesting that the development of LRC-receptor repertoires in this cell type occurs in the periphery. T cells expressing KIRs have been reported to be mainly $CD8^+$ T cells with an activated, memory phenotype [12]. Recently, we have studied the characteristics of LRC-receptor expression by $CD8^+$ T cells in comparison with that of peripheral NK cells [7,11]. By analyzing both peripheral lymphocytes and T-cell clones in individuals with well-defined HLA and LRC genotypes and repertoires, we demonstrated that LRC-encoded receptors (ILTs/LIRs and KIRs) are the major family of inhibitory receptors expressed in TCR$\alpha\beta$ $CD8^+$ T cells. The CD94/NKG2 lectin-like receptors are expressed preferentially in TCR$\gamma\delta$ T cells and NK cells.

Effector and memory $CD8^+$ T cells

Once activated in the periphery, by recognition of an HLA-class-I–peptide ligand of sufficient affinity, naive $CD8^+$ T cells begin a differentiation process [13], proliferate in number and execute their effector functions of cellular cytotoxicity and cytokine production. To ensure that the immune system does not retain a large number of redundant cells, the vast majority of these effector $CD8^+$ T cells is deleted by activation-induced cell death (AICD), with a small proportion remaining as memory cells [14]. Many studies have attempted to address the differences between the effector and memory phases of $CD8^+$ T-cell differentiation, and the signals involved in the maintenance of T-cell homeostasis. Although some progress has been made in the definition of cytokine- or costimulation-mediated survival signals, many proposed schemes remain controversial, as does the significance of numerous cell-surface markers reported to be associated with the acquisition of effector or memory status [15,16]. Indeed, it is not clear that there exists only one definitive pathway of memory T-cell development [17].

Our proposed mechanism of sequential LRC-receptor expression in activated $CD8^+$ T cells is based on both phenotypical and functional data. Both ILT2/LIR1 and KIRs are expressed on $CD8^+$ T cells with a characteristic effector- or memory-cell phenotype ($CD56^+CD57^+CD28^-CD27^-$) [7], which indicates that these differentiated cells are activated and might have lost the requirement for costimulation. Within a population of viral-peptide-specific $CD8^+$ T cells, the proportion of cells expressing ILT2/LIR1 alone is approximately tenfold greater than the proportion expressing KIRs [7]. These numbers are highly consistent with the proportions of effector and memory $CD8^+$ T cells, respectively, in the anti-peptide response of TCR-transgenic mice [18], suggesting that the differential expression of LRC receptors by human $CD8^+$ T cells correlates with the linear development of memory T cells from the activated effector subset. Clonal analysis of human T cells indicates that the KIR^- population (effector subset) is susceptible to AICD, whereas the KIR^+ population (memory subset) is resistant to AICD and has higher levels of expression of the Bcl-2 survival molecule [7]. In accordance with this finding, we have identified a CD56-associated transcription element in the 5′-untranslated region of ILT2/LIR1 cDNA clones [6], suggesting that the expression of the genes encoding CD56 and ILT2/LIR1 is a co-ordinated event in activated $CD8^+$ T cells, correlating with their effector status [19]. The association between KIR expression, interaction with HLA ligand and survival of a subset of memory $CD8^+$ T cells has been confirmed recently in KIR-transgenic mice *in vivo* [20].

Sequential expression of LRC receptors in $CD8^+$ T cells

We propose that LRC-gene expression begins with the ILT2/LIR1 receptor during the expansion phase of effector $CD8^+$ T-cell proliferation and is associated with the expression of CD56 and cellular activation (Fig. 1). Subsequently, the majority of this population is removed by AICD, and the minor proportion of clones destined to become the memory population initiates expression of genes encoding KIRs and acquires resistance to apoptosis through increased levels of expression of Bcl-2 [21]. This KIR-encoding gene-transcription program, which might be modulated by post-transcriptional regulation, begins with the gene encoding KIR2DL4. This receptor has been detected in all KIR^+ cells examined [22] and is present on a population of $ILT2/LIR1^+$ T-cell clones that express no other KIRs [7]. Although LRC haplotypes vary in their exact complement of KIR-encoding genes, all haplotypes possess genes encoding both KIR2DL4 and ILT2/LIR1 [5,6], allowing a common program of initial receptor expression. Successive expression of individual KIR-encoding genes from polymorphic haplotypes, possibly associated with further stages of cellular differentiation, results in a varied, genetically determined repertoire of KIR phenotypes within a clonal population of $CD8^+$ T cells [11].

A role for KIRs in T-cell survival

KIRs are best known for their inhibitory function in NK cells. Ligation of a KIR results in Lck-mediated phosphorylation of the tyrosine residues within the ITIM motif of the KIR cytoplasmic tail, which allows the recruitment of Src-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP-1) to the phosphorylated ITIM [23]. In addition to reducing cellular activation, this ITIM phosphorylation might also link to alternative signaling pathways, particularly the pathway connecting phosphatidylinositol 3-kinase (PI 3-K) to cellular proliferation [24]. Such KIR-mediated activation of PI 3-K results in phosphorylation and activation of the anti-apoptotic protein kinase B/Akt [24], a survival factor involved in maintaining cellular resistance to apoptosis through the regulation of expression of Bcl-family members [25] and caspase inhibitors [26]. Studies of an activated NK-cell line [24] and

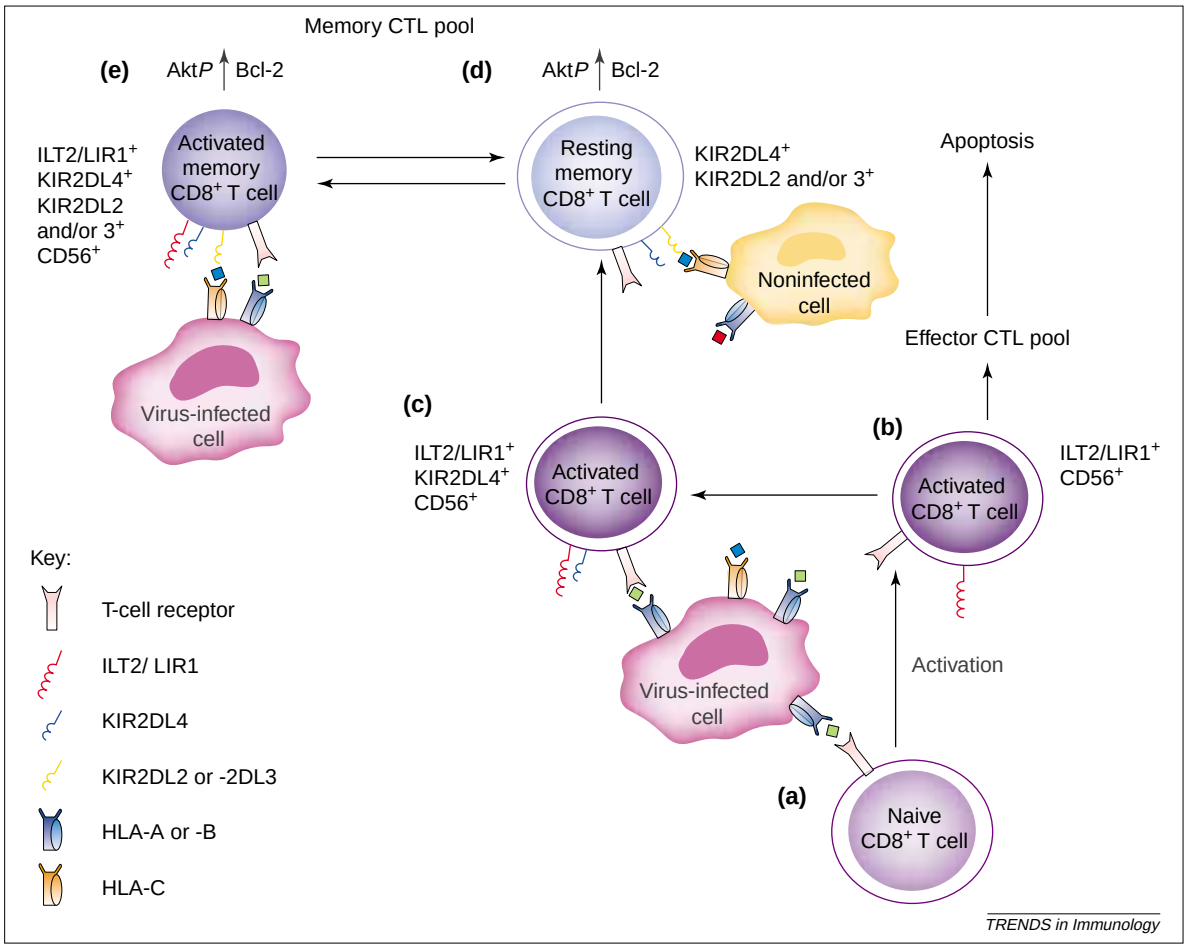

Fig. 1. A program of leukocyte-receptor complex (LRC) gene expression during the differentiation of CD8+ T cells to a memory state. (a) Naive peripheral CD8+ T cells are triggered to enter a program of differentiation when their clonotypic T-cell receptors bind with sufficient avidity to self-HLA class I molecules presenting antigenic peptides on a virus-infected cell. (b) Activation of the CD8+ T cell leads to expression of both CD56 and the LRC-encoded inhibitory receptor Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1), coupled with the acquisition of effector function. The majority of these proliferating cells is destined to die through activation-induced cell death (AICD), but a proportion switches on the expression of genes encoding killer-cell Ig-like receptors (KIRs), beginning with (c) KIR2DL4 and, subsequently, KIR2DL2 and/or KIR2DL3. (d) These latter KIRs will engage their self-HLA-C ligands expressed on noninfected cells, resulting in the activation of Akt by phosphorylation (Akt*P*), up-regulation of expression of Bcl-2 and resistance to AICD at the memory-cell stage of differentiation. (e) Subsequent exposure to the original stimulating antigen is associated with re-expression of CD56 and ILT2/LIR1, but in contrast to (b), KIRs provide a survival signal, allowing the activated memory cell to return to a resting memory state (d). Expression of additional KIRs, such as the HLA-B-specific KIR3DL1, is related possibly to further stages of cellular differentiation, allowing diversification of the clonal response.

transfected B cells [27] have shown that the cytoplasmic tail of KIRs can transmit a positive signal upon receptor crosslinking, which might confer resistance to cell death. Evidence that SHP-1 might be involved in T-cell homeostasis comes from a study in *motheaten* mice. These animals lack SHP-1 and exhibit a T-cell proliferative disease characterized by a lack of AICD in the expanded T-cell population [28]. This suggests that the balance between SHP-1- and PI-3-K-mediated signaling pathways, resulting from KIR engagement of an HLA class I ligand, might determine or maintain cell survival in memory T cells. The differences in CD8+ T-cell resistance to AICD that we observed [7] imply a distinct variance between the ILT2/LIR1 receptor and KIRs in their ability to signal through possible cell-survival pathways. This difference might be due to the variation in protein sequence and number of ITIM motifs located in their cytoplasmic tails [29], influencing the ability of individual receptors to activate PI 3-K [24].

Implications of LRC-receptor expression in memory CD8+ T cells

Several studies of both CD4+ and CD8+ T cells have shown that LRC-receptor expression can influence cellular functions resulting from activation of the TCR, including cytoskeletal reorganization [30], cytokine release [31] and cytotoxicity [32]. The functional effects of these receptors might be clear but the purpose of their expression in peripheral T cells is counterintuitive. Why should peripheral CD8+ T cells express receptors that, principally, inhibit their reactivity?

In this context, it is relevant to consider LRC-receptor expression as an extension of the processes occurring during the selection and education of immature thymocytes. Thymic emigrants are the result of an extensive process that selects T cells for their reactivity to the peptide ligands bound in the

groove of self-HLA molecules. This procedure sets the baseline levels defining nonself in T cells that emigrate to the periphery, where they are destined to differentiate further based on the affinity of their TCR for nonself peptides presented by self-HLA. Thus, these selection events can be thought of as a continual process, with the extent of proliferation of naive peripheral $CD8^+$ T cells in response to nonself peptide ligands influencing the proportion of individual TCRs that constitute the peripheral repertoire [33]. Responses of $CD8^+$ T cells to certain viral infections are characterized by dramatic expansions of one or a few specific clones [34,35], a feature which is characteristic also of KIR^+ T-cell populations [11,12]. This suggests that these mono- or oligoclonal KIR^+ $CD8^+$ T cells are the residual memory-cell population derived from the grossly expanded clones that responded in the initial infection. Thus, a KIR-mediated survival mechanism might influence the extent to which particular TCR sequences dominate the $CD8^+$ peripheral T-cell pool.

Once activated, memory $CD8^+$ T cells retain their TCR signaling complexes in a pre-assembled state in membrane microdomains, resulting in an increased avidity for antigen in the periphery [36]. Because the TCR binds with varying degrees of avidity to peptides that are structurally similar to its cognate ligand, this pre-activated state might present difficulties in terms of peripheral self-tolerance [37]. Expression of LRC receptors raises the threshold for TCR stimulation by self-HLA-class-I, constituting a mechanism for both the maintenance of peripheral tolerance and the transmission of survival signals to memory cells. The diversity of KIR expression in clonal T cells [11], potentially allows a range of responses, through the capacity for fine-tuning of cellular activation thresholds, in what would otherwise constitute oligo- or monoclonal T-cell responses. Thus, the diversity of KIRs expressed in an expanded clonal population might represent a mechanism to introduce a degree of affinity maturation in peripheral $CD8^+$ T cells.

Implications of a sequential LRC-receptor expression program in NK cells

Our proposed mechanism of a sequential program of LRC-receptor expression associated with cellular differentiation and survival has implications for the selection of NK-cell repertoires. It is probable that NK-cell precursors maturing in the bone marrow are initially self-tolerant, owing to their expression of the broadly self-specific ILT2/LIR1 or CD94/NKG2A receptors. Then, this generic receptor expression would be followed by the expression of individual KIRs [38], which, when crosslinked by ligation with the relevant self-class-I molecule, induce the proliferation and survival of individual clones to form the repertoire of mature peripheral NK cells. The further expression of inhibitory or activatory KIR isoforms in individual clones allows a diversification of signaling thresholds in these cells, resulting in a polymorphic receptor repertoire in peripheral NK cells.

Conclusions

Understanding the full implications of this novel function of KIR molecules will require technical advances in the *in vitro* culture of immature NK-cell precursors. The definition of ligands for those LRC receptors that do not bind to HLA class I [39] and the molecular events controlling the expression of individual KIR-encoding genes is also needed. In particular, the role of the ubiquitously expressed KIR2DL4 receptor requires clarification [40]. The apparent specificity of KIR2DL4 for HLA-G (a class I molecule with a highly restricted tissue distribution); the potential role of KIR2DL4 as either an inhibitory or activatory receptor; and the possible existence of KIR2DL4-associated co-receptors or adapter proteins are all points that need to be addressed. However, the use of activated $CD8^+$ T cells as a model system for unraveling the molecular and cellular processes involved in a program of LRC-gene expression should allow the subsequent definition of the development of the LRC repertoires of NK cells and memory $CD8^+$ T cells.

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The origin of *CD95*-gene mutations in B-cell lymphoma

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CD95 (Apo-1/Fas) is crucial for the negative selection of B cells within the germinal center (GC). Impairment of CD95-mediated apoptosis results in defective affinity maturation and the persistence of autoreactive B-cell clones. *CD95* was defined recently as a tumor-suppressor gene and is silenced in many tumor entities. In contrast to other malignancies, in GC-derived B-cell lymphomas, inactivation of the *CD95* gene is often a result of deleterious mutations. Such mutations occur also at a low frequency in normal GC, but not naive, B cells. We propose that *CD95* mutations in B-cell lymphomas originate from the GC reaction and are introduced most probably as targeting errors of the somatic hypermutation machinery, which bears – besides its physiological role – an inherent risk of malignant transformation and the persistence of autoreactive B-cell specificities.

The CD95 (Apo-1/Fas) molecule belongs to the tumor necrosis factor receptor (TNFR) family [1] and is an almost ubiquitously expressed transmembrane death receptor [2]. Usually, the induction of apoptosis by CD95 requires crosslinking of CD95 by CD95 ligand (CD95L), which is expressed only in a few anatomically well-defined structures, including germinal centers (GCs) [3]. Crosslinking of CD95 by CD95L leads to the assembly of a death-inducing signaling complex (DISC), which includes trimerized CD95, CD95/Fas-associated death-domain-containing protein (FADD) and procaspase-8 (Fig. 1). The DISC is assembled around the cytoplasmic death domain (DD) of CD95, which thus, plays a pivotal role in the transduction of the death signal [2].

'…the somatic-hypermutation machinery can act occasionally outside of the Ig loci.'

CD95 mediates negative selection of B cells within the germinal center

In the B-cell lineage, expression levels of CD95 peak at the GC stage of differentiation [4], which contributes to the susceptibility of GC B cells to apoptosis [3,4] (Fig. 1). Indeed, human GC B cells carry a preformed DISC that is maintained in an inactive configuration by FADD-like interleukin-1β-converting-enzyme-inhibitory protein (c-FLIP) (Fig. 1) [5]. CD40 stimulation and Ig crosslinking – mimicking the T-cell–B-cell interaction – prevent the degradation of c-FLIP, suggesting that inhibition of the CD95 pathway is involved in positive selection and affinity maturation within the GC [3,5]. This idea is supported by the analysis of

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