UNIVERSITAT POMPEU FABRA
FACULTAT DE BIOLOGIA
DEPARTAMENT DE CIENCIES EXPERIMENTALS I DE LA SALUT

# ANÁLISIS DEL REPERTORIO DE RECEPTORES DE CÉLULAS NK EN LA INFECCIÓN POR CITOMEGALOVIRUS HUMANO

**TESIS DOCTORAL**

**Mònica Gumà Uriel**

**Barcelona, diciembre 2005**

# ANÁLISIS DEL REPERTORIO DE RECEPTORES DE CÉLULAS NK EN LA INFECCIÓN POR CITOMEGALOVIRUS HUMANO

Mònica Gumà Uriel

Memoria presentada para optar al título de Doctora por la Universitat Pompeu Fabra. Este trabajo se ha realizado bajo la supervisión del Dr Miguel López-Botet Arbona y la Dra Ana Angulo Aguado en la Unidad de Inmunopatología Molecular, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra.

Miguel López-Botet Arbona,
Director

Ana Angulo Aguado
Director

Mònica Gumà Uriel

Barcelona, diciembre 2005

# ÍNDICE

# CAPÍTULO 1

**Receptores específicos para moléculas MHC de clase I en la infección por citomegalovirus**

# 1. RECEPTORES ESPECÍFICOS PARA MOLÉCULAS MHC DE CLASE I

Las funciones de los leucocitos están reguladas por el equilibrio establecido entre señales inhibidoras y activadoras, que contribuyen a mantener la homeostasis del sistema inmunitario[1-4]. Los receptores y co-receptores estimuladores determinan la respuesta frente a antígenos extraños o patógenos mientras que los receptores inhibidores bloquean la cascada de señalización iniciada por los activadores, contribuyendo a limitarla y previniendo una respuesta contra antígenos propios Varios grupos de receptores están implicados en el control de los diferentes tipos celulares del sistema inmunitario. En los últimos años se han ido identificando varias familias de receptores de membrana, de las superfamilias de las inmunoglobulinas (Ig) o de las lectinas de tipo C que se resumen en la tabla 1 [5-9].

La mayoría de estas familias de receptores incluyen tanto miembros con función activadora como inhibidora y con excepciones se expresan exclusivamente en células de origen hematopoyético. Algunos receptores como ILT/LIR (*Ig-like transcripts o leucocyte Ig-like receptors*), PIR-B (*Paired Ig-like receptors-B*), CD66a y LAIR ( *inhibitory leukocyte-associated Ig-like receptor-1*) se encuentran ampliamente distribuidos en diferentes linajes de leucocitos, mientras que otros como KIR (*killer Ig-like receptors*), CD94/NKG2, FcγRIIB, CD22, CD72 y KLRG1 (*killer cell lectin-like receptor G1*) tienen una distribución más restringida. Gran parte de los genes del grupo de receptores tipo Ig se localizan en el cromosoma 19 humano (19q13.1-19q13.4), mientras que sus ortólogos se encuentran en la región sinténica del cromosoma 7 del ratón. A su vez, los genes que codifican para los receptores de tipo lectina se localizan en el brazo corto del cromosoma 12 humano, y en la región sinténica del cromosoma 6 de ratón.

Tabla 1. Receptores activadores e inhibidores en leucocitos humanos

| Receptor | Expresión | Ligando | Familia |
|---|---|---|---|
| **Receptores inhibidores y activadores** | | | |
| FcγR | Basófilos, mastocitos, monocitos, macrófagos, células B y NK | IgG | Ig-SF |
| KIR | Células NK y subpoblaciones de linfocitos T | MHC de clase I | Ig-SF |
| ILT/LIR | Linfocitos y células linfoides | MHC de clase I, UL18 del citomegalovirus humano (HCMV) y ¿? | Ig-SF |
| CMRF35 | Células NK, B, mieloides y subpoblaciones de linfocitos T | ¿? | Ig-SF |
| CD94/NKG2 | Células NK y subpoblaciones de linfocitos T | HLA-E | Lectina |
| NKR-P1 | Células NK y subpoblaciones de linfocitos T | ¿? | Lectina |
| SIRP | Células hematopoyéticas y no hematopoyéticas | CD47 | Ig-SF |
| **Receptores inhibidores** | | | |
| LAIR-1 | Linfocitos y células mieloides | ¿? | Ig-SF |
| CD22 | Células B | a. siálico | Ig-SF |
| SIGLECs | Células mieloides, linfocitos T, B, NK | a. siálico | Ig-SF |
| CD66a | Neutrófilos, células T y NK activadas, células epiteliales y endoteliales | CD66, CD62E | Ig-SF |
| KLRG1 | Mastocitos, basófilos, células T y NK | ¿? | Lectina |
| CD72 | Células B | ¿? | Lectina |
| **Receptores activadores** | | | |
| NKG2D | Células NK, mieloides y subpoblaciones de linfocitos T | MICA/B, ULBP1/2/3/4 | Lectina |
| NKP46/44/30 | Células NK | Hemaglutinina víricas, pp65 (HCMV) y ¿? | Ig-SF |

Un mismo tipo de leucocito puede expresar simultáneamente varios de estos receptores activadores y/o inhibidores, confiriendo a la célula mecanismos alternativos/complementarios para controlar el proceso de activación en respuesta a diferentes ligandos. Así, las células NK presentan

KIR, ILT2 y/o CD94/NKG2, mientras que las células B emplean FcγRIIB, CD72, PIR-B, ILT2 y CD22.

Los receptores activadores, especialmente el receptor de células T (TCR) y el receptor de célula B (BCR), son esenciales para el reconocimiento de antígenos extraños o patógenos por el sistema inmunitario. Estos receptores se componen de la unidad de unión al ligando y de proteínas trasmembrana asociadas tales como CD3ξ, cadena γ del FcεRI o DAP12 (*DNAX activating protein of 12 kDa*), que inician la cascada de señalización. Los dominios citoplasmáticos de estas proteínas adaptadoras contienen dominios ITAM (*immunoreceptor tyrosine-based activation motif*) cuya secuencia consenso es Yx(2)Lx(6-8)Yx(2)L (donde x representa cualquier aminoácido, y los otros residuos están representados por la letra código correspondiente). Estas proteínas adaptadoras son fosforiladas en sus ITAMs, probablemente por una quinasa de la familia src, reclutando las tirosina quinasas ZAP70 y/o Syk que transducen señales activadoras a través de la fosforilación de proteínas tales como fosfolipasa C-g (PLC-g), c-Cbl, p85 PI3K (*phosphatidylinositol 3-kinase*), Grb2, LAT (*linker of activation of T cells*), Vav-1 y Vav-2, Rho, p38 MAPK (*mitogen activated protein kinase*) y ERK (*extracellular signal-regulated kinase*), que activan las funciones efectoras de las células. Algunos receptores activadores también se pueden asociar a la molécula adaptadora DAP10 (*DNAX activating protein of 10 kDa*) que contiene el motivo YxxM, que al fosforilarse recluta la subunidad p85 de la PI3K, implicando también a las proteínas Vav1, Rho, GTPasas y PLC (Figura 1) [10, 11].
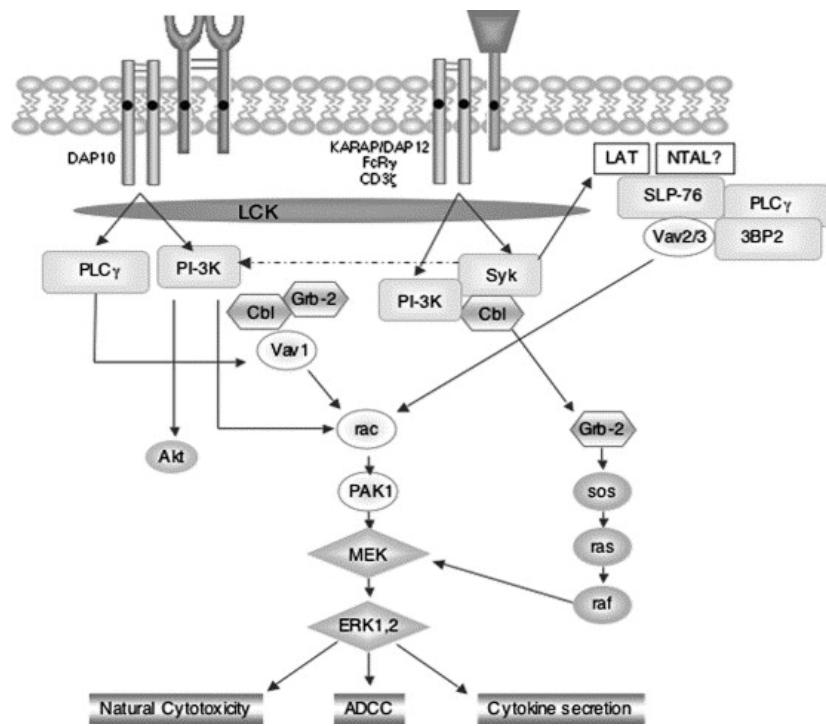
Figura 1. Señalización en las células NK. Representación esquemática de las principales vías de transducción de señales tras la estimulación de los receptores activadores acoplados a DAP10 o a adaptadores con dominios ITAM [11]

La característica común de los receptores inhibidores es su capacidad para atenuar las señales iniciadas por los receptores activadores [4, 12]. El mecanismo de transducción de señales inhibidoras es análogo para todos estos receptores, que contienen en su tallo citoplásmico un numero variable de secuencias denominadas ITIM (*immunoreceptor tyrosine-based inhibitory motif*) cuya secuencia consenso es V/IxYxxL/V. Al ser fosforilados por una tirosina quinasa de la familia src, los ITIM ensamblan fosfatasas con dominios de homología a src tipo II (SH2) que intervienen en la función represora. De ellas, SHP-1 y SHP-2 tienen como substratos otras proteínas fosforiladas en tirosina, mientras que SHIP actúa sobre fosfatos de inositol [10,11,13] (Figura 2).
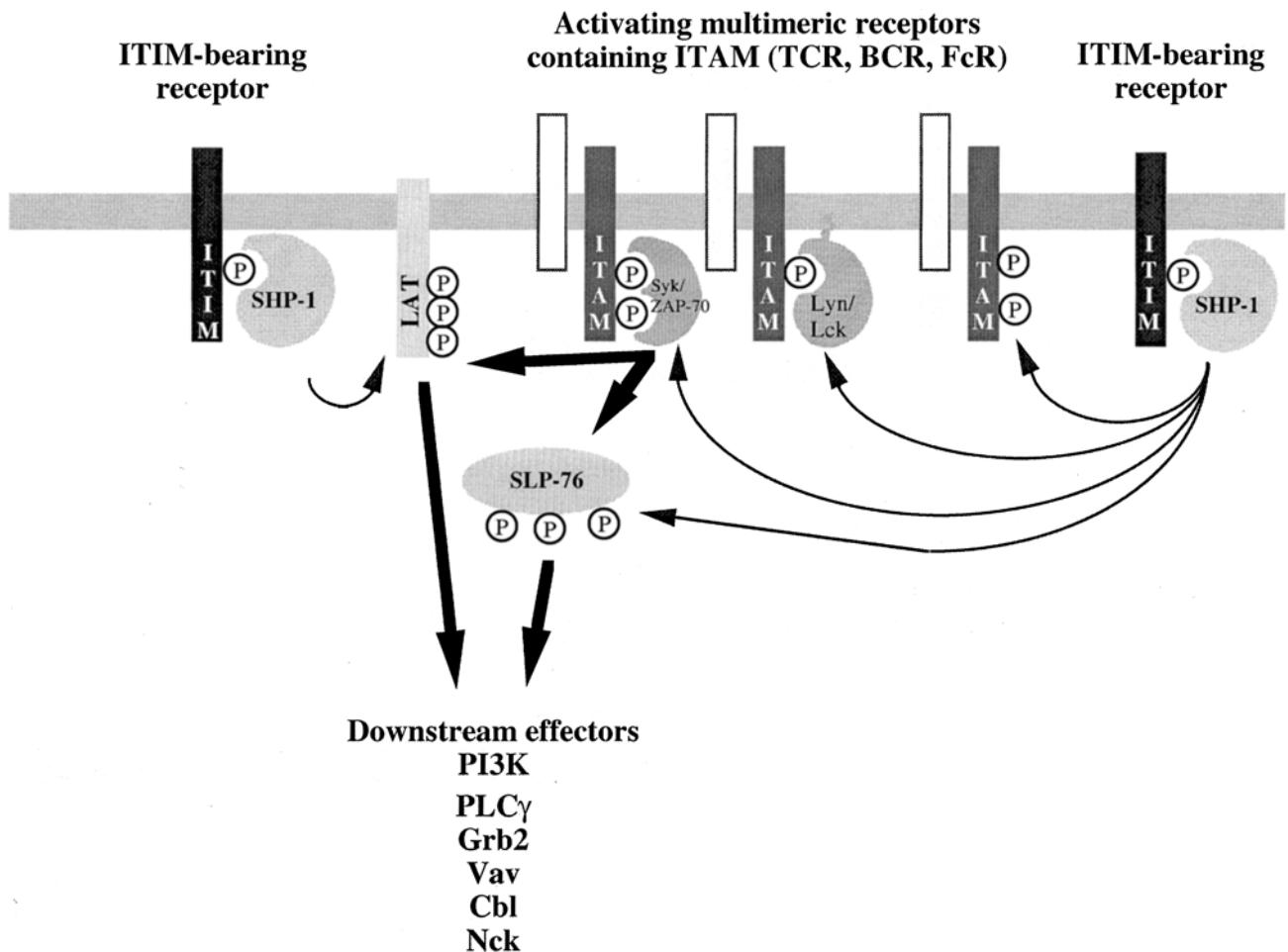
Figura 2. Esquema del funcionamiento de receptores que contienen dominios ITIM. Después de la activación de estos receptores, los dominios ITIM se fosforilan y reclutan fosfatasas como SHP-1. El sustrato de la SHP-1 incluye varias proteínas fosforiladas en las tirosinas, tales como receptores que contienen dominios ITAM, quinasas de la familia src o syk, o proteínas adaptadoras. La desfosforilación de estas proteínas conlleva a la extinción de las vías de señalización activadoras [13].

Aunque los receptores activadores e inhibidores reconocen ligandos diferentes en la superficie celular, en ocasiones se encuentran pares de receptores activadores e inhibidores con dominios extracelulares tan relacionados que unen ligandos similares aunque con distinta afinidad.

Este es el caso de algunos receptores específicos para moléculas del complejo principal de histocompatibilidad (MHC) de clase I que se describen a continuación [14] [15,] [16](figura 3A y 3B). La función de estos receptores fue estudiada en primer lugar en las células natural killer, aunque también controlan la respuesta adaptativa mediada por otros linfocitos y regulan parte del equilibrio entre las señales inhibidoras y activadoras de diferentes tipos celulares tales como macrófagos, monocitos y células dendríticas que también expresan estos receptores [4, 17].
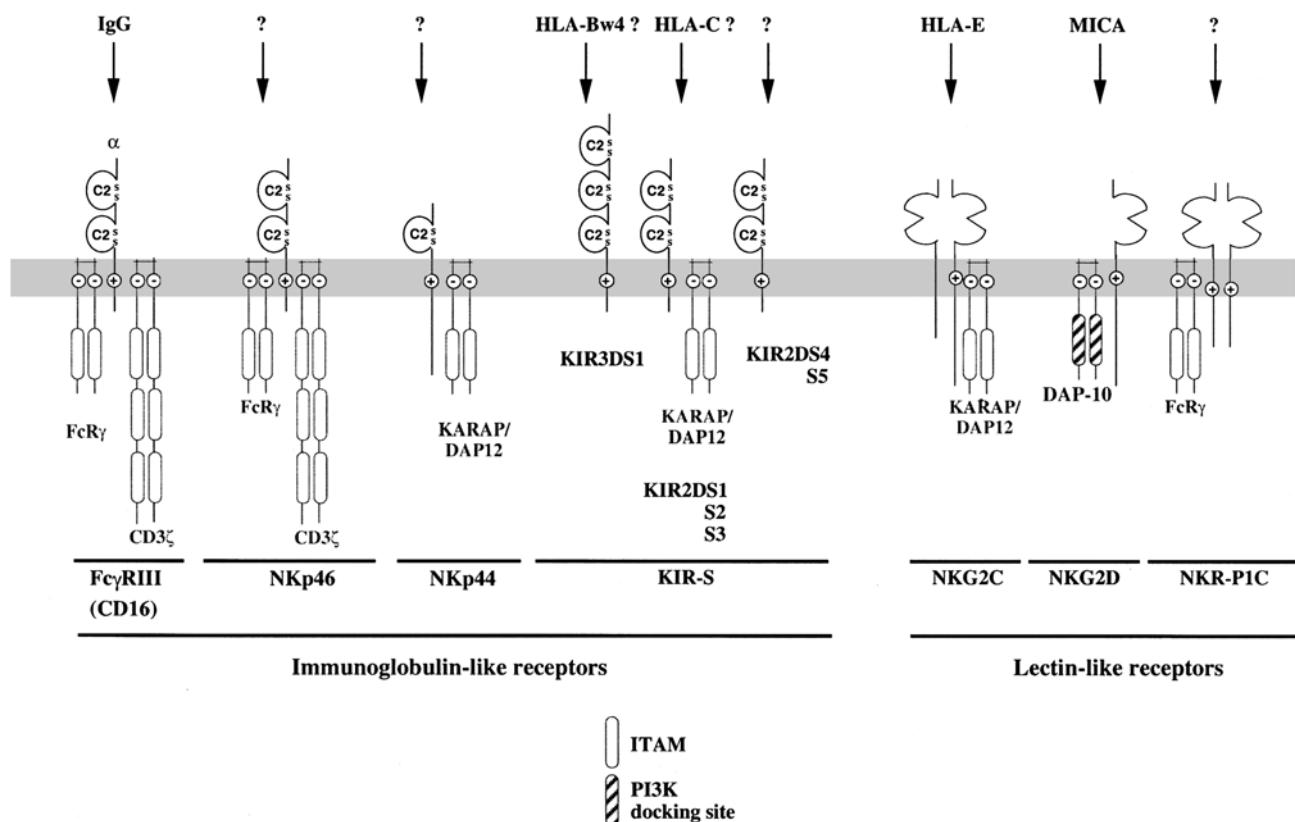


Figura 3A. Receptores activadores expresados en células NK humanas [13].
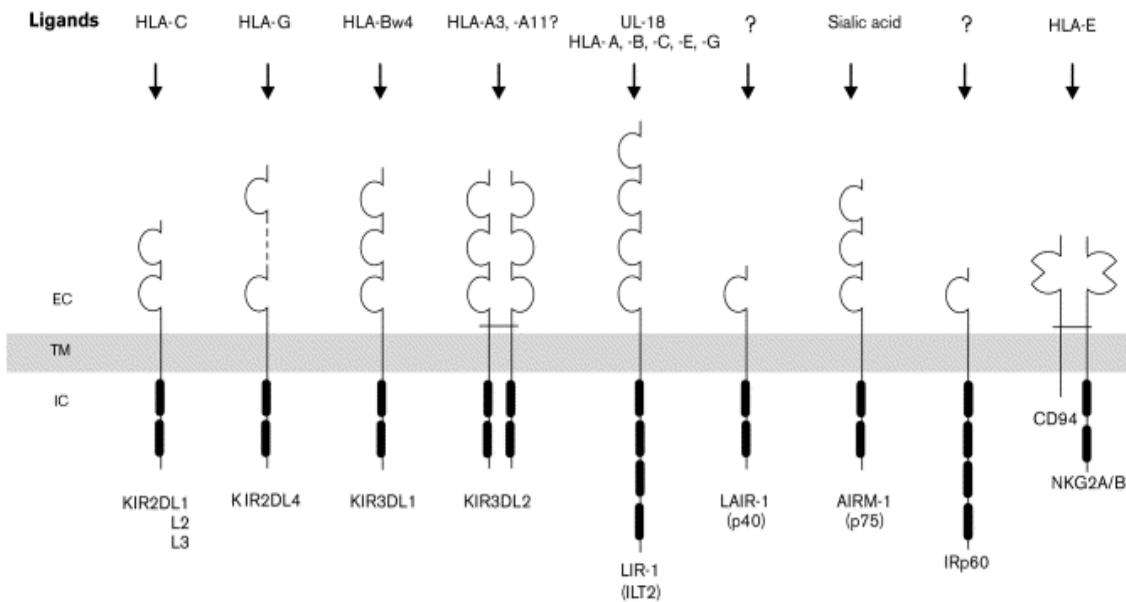
Figura 3B. Receptores inhibidores específicos para moléculas del complejo de histocompatibilidad de clase I [18].

## 1.1 RECEPTORES DE LA SUPERFAMILIA DE LAS INMUNOGLOBULINAS

### 1.1.1 KIR

Los receptores de la familia KIR son proteínas tipo Ig cuyos genes se agrupan en el cromosoma 19q13.4, y que se expresan en células NK así como en una subpoblación de linfocitos T [19-21]. Se han identificado 15 genes y dos pseudogenes pertenecientes a esta familia, algunos de los cuales presentan polimorfismo alélico y diferentes isoformas generadas por procesamiento alternativo. Se ha propuesto una clasificación basada en sus características estructurales (Tabla 2). Según el número

de dominios de tipo Ig en la región extracelular se distinguen dos grupos de KIR, denominados KIR2D y KIR3D; mientras que según la longitud citoplasmática, estos receptores se subclasifican en S (corta) o L (larga). Generalmente, los KIR con regiones cortas son receptores activadores, mientras que aquellos con tallos citoplasmáticos largos son inhibidores. Los receptores con una región citoplasmática larga donde se localizan dos motivos ITIM responsables de su función inhibidora se llaman KIR2DL y KIR3DL. Los receptores de tallo citoplasmático corto se denominan KIR2DS y KIR3DS, carecen de motivos ITIM y tienen un aminoácido cargado (Lys o Arg) en su región transmembrana a través del cual se asocian electrostáticamente a la molécula adaptadora DAP12, que contiene en su estructura ITAMs.

Se ha descrito que los clones NK pueden expresar varias combinaciones de diferentes receptores KIR, definiéndose así varias subpoblaciones de células NK cuyas proporciones varían en diferentes individuos. Además el número de genes KIR en el genoma de cada individuo varía en la población. Se han definido dos amplios grupos de haplotipos KIR. El haplotipo A contiene pocos KIR y la mayoría incluyen KIR2DS4, y posiblemente KIR2DL4, como únicos genes de receptores activadores. El haplotipo B contiene diversas combinaciones de KIR activadores y muchos se caracterizan por la presencia de KIR2DL5. En poblaciones caucasianas, los dos haplotipos se hallan en frecuencias comparables [19].

Algunos KIR reconocen moléculas MHC de clase I que comparten determinados rasgos estructurales en el domino α1; en general, los KIR2D reconocen moléculas del locus C y los KIR3D de los loci B y A; mientras que en otros casos, especialmente los activadores, no se ha identificado el ligando. Los KIR activadores que interaccionan con moléculas MHC de clase I lo hacen con una afinidad menor que sus homólogos inhibidores [19]. Existen indicios de que el reconocimiento por los KIR podría estar condicionado por la naturaleza de los péptidos unidos al HLA de clase I, aunque no está clara la relevancia biológica de esta observación [22].

Tabla 2. Principales características de los receptores KIR

| KIR | Nombre alternativo | Función | Ligando |
|---|---|---|---|
| 2DL1 | CD158a-forma larga, p58.1 | Inhibidora | HLA-C$^{Lys80}$ |
| 2DL2 | CD158b-forma larga, p58.2 | Inhibidora | HLA-C$^{Asn80}$ |
| 2DL3 | CD158b, p58.2 | Inhibidora | HLA-C$^{Asn80}$ |
| 2DL4 | P49 | Inhibidora (¿?) | HLA-G1 |
| 2DL5 | Ninguno | Inhibidora | ¿? |
| 2DS1 | CD158a-forma corta, p50.1 | Activadora | HLA-C$^{Lys80}$ |
| 2DS2 | CD158b-forma corta, p50.2 | Activadora | HLA-C$^{Asn80}$ |
| 2DS3 | Ninguno | Desconocida | ¿? |
| 2DS4 | p50.3 | Activadora | ¿? |
| 2DS5 | Ninguno | Desconocida | ¿? |
| 3DL1 | p70, NKB1 | Inhibidora | HLA-B$^{Bw4}$ |
| 3DL2 | p140 | Inhibidora | HLA-A3, A11 |
| 3DS1 | Ninguno | Desconocida | ¿? |

Durante los últimos años se ha observado que existen células T, tanto CD8+TCRαβ+ como TCRδγ+, que pueden expresar KIR [23, 24]. Las células T KIR+CD8+ se pueden aislar en el bazo, amígdalas, ganglios linfáticos y sangre periférica donde en un individuo adulto sano representan hasta un 5% de las células T. Este porcentaje aumenta con la edad alcanzando hasta el 30% en la población anciana. La mayoría de las células KIR+CD8+TCRαβ+ tienen un fenotipo de memoria (CCR7- CD28- y CD27-). En donantes sanos las células T KIR+ representan expansiones mono u oligoclonales, atendiendo al reordenamiento de la cadena β del TCR, variable en diferentes individuos, lo que probablemente refleja un proceso de estimulación crónica.

En roedores, que carecen de KIR, se ha encontrado una familia de receptores de la superfamilia de las lectinas tipo C, denominada Ly49, homóloga funcionalmente de los KIR[25, 26]. Esta familia incluye tanto receptores activadores como inhibidores. Algunos tienen especificidad

para moléculas MHC de clase I  (H-2), y otros (Ly49H, Ly49I) reconocen la proteína m157 del citomegalovirus murino (MCMV) [27].

## 1.1.2 ILT/LIR/LILR

Se ha identificado otro grupo de receptores de la superfamilia de las Ig denominados ILT o LIR que se caracterizan por tener cuatro dominios extracelulares de tipo Ig, excepto ILT3 que tiene dos, y cuyos 13 genes se localizan adyacentes a los KIR en el cromosoma 19q13.4 (Tabla 3) [28, 29]. Al igual que en la familia KIR, se encuentran moléculas ILT inhibidoras (con 2 o 4 ITIMs citoplásmicos), junto a otras con función activadora que se asocian a dímeros de la cadena γ del  FcεRI, que contiene ITAM [30]; ILT6 corresponde a una molécula soluble [31]. A diferencia de los KIR, los receptores ILT/LIR no son específicos de células NK y linfocitos T, sino que se expresan en otros linajes hematopoyéticos, predominantemente en células del sistema mononuclear fagocítico como monocitos, macrófagos y células dendríticas; algunos de estos receptores se encuentran también en linfocitos B y granulocitos [32].

Se ha determinado que algunos ILT muestran una importante variabilidad alélica; por ejemplo, se han descrito hasta 15 alelos diferentes de ILT5 [28]. En ratones se han encontrado dos grupos de receptores homólogos a los ILT denominados PIR [33] y gp49 [34] que también incluyen receptores inhibidores y activadores.

Entre los ILT mejor caracterizados se encuentran los receptores inhibidores ILT2 e ILT4. Mientras que ILT2 se expresa en todas las células B y monocitos/macrófagos, y en una subpoblación de linfocitos T y células NK [32], ILT4 se expresa selectivamente en monocitos/macrófagos [35]. Ambos receptores reconocen un amplio espectro de moléculas MHC de clase I  y, además, ILT2 también interacciona con la proteína del citomegalovirus humano (HCMV) UL18, homóloga a MHC de clase I con una alta afinidad  hasta 1000 veces superior que la de los ligandos MHC de clase I [36, 37]. Sin embargo, se desconoce todavía la naturaleza de los ligandos para la mayoría de los LIR. La

distribución celular de ILT-2 e ILT-4 indica que las moléculas de HLA de clase I no sólo modulan la respuesta inmunitaria de las células NK y T [38].

Tabla 3. Principales características de algunos de los receptores LIR

| LIR/ILT | Nombre alternativo | Señalización | Ligando |
|---------|-------------------|--------------|---------|
| ILT2 | LIR1, MIR1, CD85 | 4 ITIMs | HLA-A, B y G1, UL18(HCMV) |
| ILT4 | LIR2, MIR10 | 3 ITIMs | HLA-A, B y G1 |
| ILT5 | LIR3, HL9 | 4 ITIMs | ¿? |
| LIR8 | | 2 ITIMs | ¿? |
| ILT3 | LIR5, HM18 | 3 ITIMs | ¿? |
| ILT1 | LIR7 | FcRγ | ¿? |
| ILT7 | | FcRγ | ¿? |
| LIR6a | | FcRγ | ¿? |
| ILT8 | | FcRγ | ¿? |
| ILT6 | LIR4, HM31, HM43 | | ¿? |

Las células T también pueden expresar el receptor LIR1/ILT2. Como en las células T KIR+, LIR1 se detecta en células de memoria y por consiguiente se expresa tras estimulación por el antígeno [24]. El patrón de reordenamiento del TCR de las células T LIR1+ es más diverso que el de las células T KIR+ sugiriendo que la expresión de LIR1 precede a la expresión de los KIR inhibidores en las células T.

### 1.1.3  Receptores y co-receptores activadores no específicos para moléculas del MHC

Se han descrito 3 receptores activadores no específicos para moléculas MHC de clase I denominados NKp46, NKp44 y NKp30, que pertenecen a la Ig-SF [39, 40]. Estos "receptores de citotoxicidad natural" (NCR) se expresan en células NK en reposo y activadas, excepto NKp44 que se expresa sólo en las últimas, y son los principales responsables de la lisis de células tumorales mediada por

células NK. Cada NCR tiene un residuo cargado en el dominio transmembrana con capacidad para unirse a diversas moléculas adaptadoras para iniciar la señalización. También se han descrito otras moléculas de superficie tales como 2B4, NTBA, NKp80, DNAM-1 (*DNAX accessory molecule-1*) y CD59 cuya función activadora depende de la estimulación simultánea de otro receptor activador [41] (tabla 4).

Existe una relación directa entre la expresión en superficie de los NCR y la capacidad de las células NK para lisar a células tumorales que no expresan moléculas MHC de clase I [39]. Los ligandos celulares todavía se desconocen pero se han implicado en el reconocimiento de células infectadas por virus. Se ha publicado que NKp46 y NKp44 reconocen la hemaglutinina del virus de la influenza y la hemaglutinina-neuraminidasa del virus de la parainfluenza [42, 43]. Recientemente se ha descrito que el receptor NKp30 reconoce la proteína pp65 del HCMV [44]. Se requieren mas estudios que confirmen estos ligandos víricos.

Tabla 4. Principales características de los receptores/coreceptores activadores no específicos para moléculas MHC

| Receptor | Dominio extracelular | Proteína adaptadora | Ligando |
|----------|---------------------|---------------------|---------|
| NKp46 | 2IgC | CD3ζ/FcεRIγ | Hemaglutinina vírica y ¿? |
| NKp30 | IgV | CD3ζ/FcεRIγ | pp65 (HCMV) y ¿? |
| NKp44 | IgV | DAP12 | Hemaglutinina vírica y ¿? |
| 2B4 | IgC+IgV | LAT | CD48 |
| NTBA | IgC+IgV | | NTBA |
| DNAM-1 | IgC+IgV | | CD155; Nectina-2 |
| NKp80 | Lectina | | ¿? |
| CD59 | | | ¿? |

## 1.2 RECEPTORES DE LA SUPERFAMILIA DE LAS LECTINAS DE TIPO C

1.2.1 <u>CD94/NKG2</u>

CD94 y NKG2 son glicoproteínas tipo II con un dominio extracelular de reconocimiento de carbohidratos cuyos genes se agrupan en el c.12p12-p13 y que se expresan en células NK y en algunas subpoblaciones de linfocitos T [16, 45].

La subunidad común CD94 está codificada por un gen único no polimórfico y se une covalentemente por enlaces disulfuro a diferentes glicoproteínas de la familia NKG2 [46, 47]. Su tallo citoplasmático carece de dominios funcionales conocidos por lo que se ha sugerido que actúa como una chaperona, permitiendo el transporte y ensamblaje de las moléculas NKG2 a la superficie celular[46].

La familia NKG2 consta de cuatro genes NKG2A, C, E y F. El procesamiento alternativo de los RNA mensajeros A y E da lugar a las proteínas NKG2A/B y NKG2E/H respectivamente[48, 49]. La proteína NKG2F se expresa en el compartimento intracelular y no se asocia a CD94[50].

Algunos receptores CD94/NKG2 tienen una función antagónica. Así, aunque NKG2A y NKG2C comparten un 94% de homología en su región extracelular, al dimerizar con CD94 forman respectivamente un receptor inhibidor y otro activador. La función inhibidora del receptor CD94/NKG2A está determinada por secuencias ITIM presentes en la región citoplasmática del NKG2A [51, 52] (figura 4). Al contrario, NKG2C carece de ITIM y tiene en su región transmembrana un aminoácido cargado (Lys) que le permite asociarse con el adaptador DAP12 [53, 54] (figura 4). El entrecruzamiento del receptor CD94/NKG2C con AcM en clones NK activa tirosina quinasas como p56lck y ZAP-70, así como la PI3K y la PLC-$\gamma$1 que promueve la generación de inositol fosfato y diacílglicerol[55, 56]. Los heterodímeros CD94/NKG2E y CD94/NKG2H también forman receptores activadores (figura 4). Estos al igual que NKG2C, carecen de secuencias ITIM y se pueden asociar a

moléculas señalizadoras a través de un residuo cargado presente en sus regiones transmembrana. En este sentido se ha descrito la asociación de CD94/NKG2H a DAP12 [57]. Hasta el momento, no se han descrito AcM específicos contra estos receptores que permitan estudiar su expresión y función.

Aunque en general, las células expresan sólo las formas activadoras o inhibidoras del heterodímero CD94/NKG2, en algunos clones se ha comprobado por RT-PCR la co-expresión de ambos tipos de receptores.
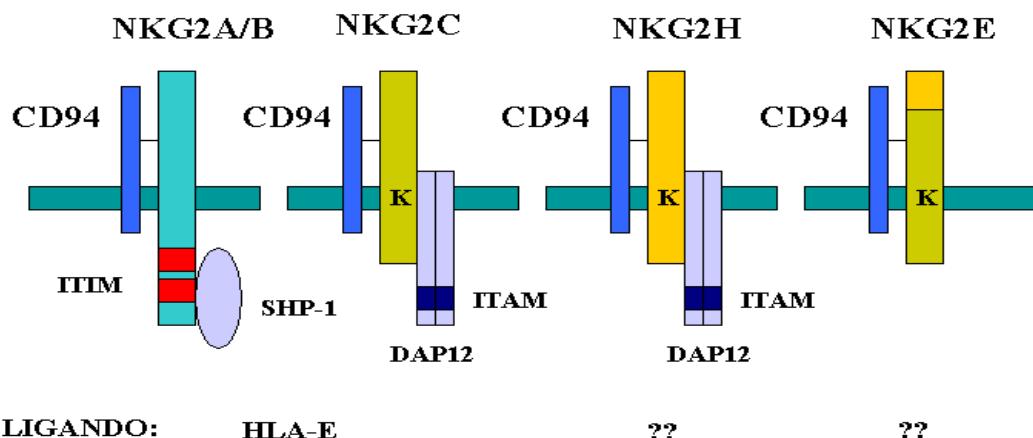


Figura 4. Principales características de los receptores CD94/NKG2

1.2.1.1 CD94/NKG2 y HLAE

Se ha demostrado que los receptores CD94/NKG2A y CD94/NKG2C reconocen la molécula del MHC de clase Ib HLA-E [58-60]. A diferencia de los KIR, este sistema de reconocimiento está conservado en primates y roedores. Se han aislado los ortólogos de CD94/ NKG2 de ratón y rata; en ratón su ligando es la molécula Qa-1b, funcionalmente homóloga a HLA-E [61]. Aunque no se conoce el ligando de los receptores CD94/NKG2E/H, se ha sugerido que podría ser diferente al de CD94/NKG2C, ya que ambas moléculas difieren de su extremo carboxilo-terminal[62].

HLA-E es un gen de clase Ib del MHC que se transcribe en todos los tejidos y líneas celulares humanas analizadas [63] aunque con un nivel de expresión bajo. Es muy poco polimórfico y

se caracteriza por presentar un dimorfismo alélico ya que las variantes identificadas se diferencian por tener una arginina HLA-E$^R$ (E*0103) (-E$^R$) o una glicina HLA-E$^G$ (E*0101) (E$^G$) en la posición 107 [64].

HLA-E se estabiliza en la superficie tras asociarse en el retículo endoplásmico, por un mecanismo dependiente de transportadores en la presentación de antígenos (TAP), a nonámeros hidrofóbicos derivados de la secuencia señal de otras moléculas HLA de clase I[65, 66]; la presencia de una metionina en posición 2 del péptido parece crítica para la asociación y, de hecho, los alotipos HLA que contienen treonina no estabilizan adecuadamente HLA-E. Los péptidos que estabilizan a HLA-E se encuentran en la secuencia señal de la mayoría de alotipos de los loci HLA-A y -C así como en un tercio de los HLA–B y HLA-G. Sin embargo, están ausentes en HLA-F, HLA-E y en la mayoría de los alotipos del locus HLA-B.

Los ensayos de citotoxicidad con transfectantes de HLA de clase I indican que HLA-E es reconocido por el receptor CD94/NKG2A y que la estructura del péptido asociado a HLA-E puede condicionar la eficiencia del reconocimiento, afectando la afinidad de la interacción con los receptores [59, 60].

Los heterodímeros CD94/NKG2C también reconocen HLA-E. Sin embargo el aumento de la actividad citotóxica de clones CD94/NKG2C+ es muy modesto comparado con el efecto inhibidor de clones CD94/NKG2A+ frente a los mismos péptidos estabilizadores de HLA-E, a excepción del efecto activador observado con el péptido derivado de la secuencia señal de HLA-G[67]. Estos datos indicativos de que el KLR (receptor de células citotóxicas tipo lectina) activador CD94/NKG2C presenta menor afinidad que su homólogo CD94/NKG2A por los mismos ligandos fueron verificados estudiando la cinética de interacción de HLA-E con CD94/NKG2A y CD94/NKG2C [68].

Los alelos de HLA-E se expresan de forma diferente en la superficie celular. En la mayoría de los casos, e independientemente del péptido, el alelo HLA-E$^G$ se expresa más en la superficie celular que el alelo HLA-E$^R$ en células humanas transfectadas [69, 70]. Sin embargo no se observan

peculiaridades en la estructura de ambos alelos que sugieran una interacción diferente con los heterodímeros CD94/NKG2 [70].

Se han descrito otros péptidos que estabilizan HLA-E y que no derivan de las secuencias señal de moléculas HLA de clase I. Estos péptidos provienen de proteínas víricas o de otras proteínas endógenas, y su capacidad de interaccionar con el receptor CD94/NKG2A e inhibir a la célula es variable. Uno de estos péptidos, derivado de la HSP-60 (*heat shock protein*-60), compite con los nonámeros endógenos derivados de moléculas MHC de clase I para unirse y estabilizar HLA-E. Este complejo no es reconocido por el receptor CD94/NKG2A dejando a las células sometidas a estrés, que expresan HSP-60, vulnerables al ataque por las células NK [71]. De la misma manera un péptido derivado de la proteína BZLF1 del virus Epstein Barr (EBV) que interacciona con HLA-E no inhibe a la célula NK [72]. Recientemente se ha descrito que el péptido aa35-44 de la proteína core del virus de la hepatitis C (HCV) estabiliza HLA-E y es reconocido por el receptor CD94/NKG2A inhibiendo a la célula NK [73].

1.2.2 <u>NKG2D</u>

NKG2D es otra molécula tipo lectina, conservada en humanos y ratones, cuyo gen se localiza junto a la familia NKG2 en el cromosoma 12. Sin embargo la homología entre este receptor y el resto de moléculas NKG2 es muy limitada, y no se asocia con la molécula CD94 sino que se expresa como homodímeros en células NK, células NKT (*natural killer T*), T γδ, T CD8+ y macrófagos [74].

Recientemente se ha demostrado en células NK de ratón la existencia de isoformas de NKG2D que se pueden asociar a DAP12 o a DAP10, que señaliza por la PI3K. Así pues, el mismo receptor puede ejercer diferentes funciones (activación primaria o coestimulación) [75, 76]. En las células NK y T humanas el receptor NKG2D se asocia únicamente a DAP10.

El receptor NKG2D interacciona con varias moléculas relacionadas con el MHC de clase I inducidas por estrés [77]. Al receptor humano se le conocen seis ligandos: MICA, MICB, ULBP1,

ULBP2, ULBP3, ULBP4. MICA y MICB están codificadas por genes en la región de HLA de clase I próximos al locus HLA-B. Se describieron en el epitelio intestinal, pero su expresión puede ser inducida en otros tipos celulares en situación de estrés, así como por infecciones víricas o transformación tumoral. La familia de proteínas llamadas "UL-16 binding protein/ retinoic acid early induced transcript-1" (ULBP/RAET1) comprende 10 genes relacionados, seis de los cuales codifican glicoproteínas potencialmente funcionales y cuatro son pseudogenes.

### 1.2.3 Otros receptores de la superfamilia de las lectinas de tipo C

KLRG1 es un receptor inhibidor que contiene dominios ITIM en la región citoplasmática, que se expresa en células T CD4+ y CD8+, así como en las células NK, y cuyo ligando se desconoce. Se ha descrito que la mayoría de células T CD8+ específicas para infecciones víricas crónicas (EBV, HCMV) expresan este receptor[78].

## 2. REGULACIÓN DE LA RESPUESTA INMUNITARIA EN CÉLULAS NK POR RECEPTORES ESPECÍFICOS DE MHC DE CLASE I

Las células NK se identificaron originalmente por su capacidad de lisar algunos tumores sin necesidad de una inmunización previa. Se definen como linfocitos citotóxicos que no expresan en su superficie receptores para antígeno (TCR o BCR), y pueden diferenciarse a partir de un tipo de progenitor compartido con el linaje linfoide [79]. A diferencia de las células T, su desarrollo es extratímico y no se afecta en ratones SCID (*Severe Combined Immunodeficiency Disease*) ni en ratones deficientes en RAG (*Recombinase Activator Gene*), lo que indica que durante su diferenciación los procesos de reordenamiento génico no son esenciales[62]. De las múltiples citocinas que están implicadas en el desarrollo de las células NK, la interleucina 15 (IL-15) juega un papel

crucial. Esta citocina también está implicada en la homeostasis de las células NK maduras, siendo necesaria para su proliferación y supervivencia[80-82].

Las células NK se hallan sobre todo en la sangre periférica, bazo y médula ósea, pero pueden migrar hacia los tejidos inflamados, participando en la primera línea defensiva frente a infecciones por algunos virus, parásitos y bacterias [83, 84]. Además participan en el control del crecimiento tumoral y previenen la diseminación de tumores metastáticos en modelos murinos[85, 86].

Una vez activadas, las células NK desarrollan funciones efectoras e inmunoreguladoras. Su actividad citotóxica depende principalmente de la secreción de proteínas como la perforina y las granzimas (serina esterasas) así como de Fas-L. Sus funciones inmunomoduladoras se basan en la secreción de citocinas (IFN-γ (interferón-γ), TNF-α (*tumor necrosis factor-α)* y GM-CSF (*granulocyte/macrophage colony stimulation factor)*) y quimiocinas (MIB-1β (*beta chemokines macrophage inflammatory protein 1β)*, MIP-1α (*macrophage inflammatory protein 1α)* y RANTES (*factor regulated on activation normal T cell expressed and secreted)*) que promueven una respuesta inflamatoria, modulan la hematopoyesis, controlan la función de monocitos y granulocitos, y favorecen el desarrollo de una respuesta Th1[82, 87-89]. Recientemente se ha demostrado que las células NK interaccionan con las células dendríticas (DC) influyendo tanto la respuesta inmunitaria innata en los tejidos periféricos inflamados, como en la respuesta inmunitaria adaptativa en los órganos linfoides secundarios [90-92]. Las DC activadas por los patógenos contribuyen a la activación de las células NK mediante citocinas. Se ha propuesto que la interacción de las células NK con las células dendríticas inmaduras (iDC) puede promover efectos contrapuestos. Por una parte secretan citocinas que favorecen la maduración de las DC, aumentando la expresión de HLA-E, que protege a las DC de las lisis por parte de las células NK CD94-NKG2A+ y, por otra, pueden lisar a las iDC que expresan bajos niveles de HLA-E, a través del receptor NKP30, interfiriendo indirectamente con la respuesta de las células T.

Lanier et al han descrito que la expresión de ligandos coestimuladores OX40L en las células NK activadas tras el entrecruzamiento con los receptores CD16, KIR2DS2 o NKG2D pueden ayudar a la proliferación y producción de citocinas inducida por el TCR en clones T CD4+ autólogos [93]. La posibilidad de que las células NK puedan interaccionar directamente con las células T CD4+ se sugirió al observar que las células NK activadas expresan MHC de clase II y que clones NK humanos son capaces de internalizar un antígeno y presentarlo vía MHC clase II a las células T CD4+ [94, 95].

Además se ha descrito que algunas células NK humanas están presentes en los órganos linfoides secundarios. Se han localizado en áreas parafoliculares de ganglios linfáticos humanos donde sería posible su interacción con las células T. Se ha sugerido que el IFN-γ secretado por las células NK induce la secreción de IL-12 por las células dendríticas maduras (mDC) en los órganos linfoides secundarios lo que favorecería el desarrollo de una respuesta Th1[92]. Recientemente se ha descrito un subgrupo especializado de células NK que pueden ir al ganglio linfático y participar directamente en el proceso de estimulación antigénica de las células T mediante la secreción de IFN-γ [96].

La activación de las células NK se produce por tres mecanismos principales. La citotoxicidad celular dependiente de anticuerpo (ADCC) se desencadena mediante el entrecruzamiento del receptor FcγR-III (CD16) por anticuerpos unidos a células infectadas, tumorales o alogénicas [97]. Por otra parte las citocinas secretadas por macrófagos o células dendríticas tales como IFNα/β, IL-12, IL-15, IL-18 o IL-21 inducen la proliferación, citotoxicidad y secreción de citocinas[88]. Sin embargo, la activación de las células NK se produce sobre todo por contacto directo con la célula diana mediante receptores que desencadenan la citotoxicidad natural [7, 22, 40, 98]. A diferencia de las células T y B pero de manera similar a otras células del sistema inmunitario innato, las células NK pueden ser activadas a través de diferentes receptores que pueden actuar individualmente o en combinación, dependiendo de los ligandos presentes en la célula diana. No

existe un único receptor activador dominante en las células NK, sino que participan múltiples receptores y co-receptores (ej. NKp30, NKG2D, NKG2C) que reconocen diferentes ligandos: algunos ligandos se expresan basalmente en células normales, como las moléculas MHC de clase I; otros se inducen en células sometidas a estrés; y otros son todavía desconocidos[98]. En la activación también participan moléculas de adhesión tales como la integrina LFA-1 y CD2 [99].

Los receptores específicos para MHC de clase I se definieron en primer lugar en las células NK [100]. La función de las células NK está controlada por un delicado equilibrio entre señales activadoras e inhibidoras. Los receptores activadores estimulan la citotoxicidad natural y secreción de citocinas, mientras que los receptores inhibidores reconocen principalmente moléculas MHC de clase I [22, 101]. Así, los niveles normales de moléculas MHC de clase I protegen frente al ataque por las células NK que son controladas por los receptores inhibidores. El reconocimiento de moléculas MHC no opera a través de un único receptor universal que detecta todas las moléculas de MHC sino que las células NK tienen en su superficie múltiples receptores de distintas familias descritas que reconocen diferentes grupos de moléculas de HLA de clase I tanto clásicas (HLA-A, B, C) como no clásicas (HLA-E, G...). Se admite que para prevenir la autorreactividad contra células normales cada célula NK madura debe adquirir al menos un receptor inhibidor (KIR o CD94/NKG2A) específico para una molécula MHC de clase I del propio individuo [102].

Sin embargo, las células NK no lisan eritrocitos, que carecen de moléculas MHC de clase I, ni otros tipos celulares con niveles bajos de expresión de las mismas (ej. hepatocitos, fibroblastos). Se interpreta que la célula NK puede ser inhibida por otros receptores cuyos ligandos no son moléculas MHC (ej. CEACAM) y/o no se activa porque la célula diana no presenta ligandos para receptores activadores no específicos para HLA [22, 103]. Así pues, la célula NK puede activarse (figura 5): a) cuando en las células tumorales o infectadas se incrementa la expresión o la afinidad de ligandos para receptores activadores, b) cuando intervienen receptores activadores para ligandos que se expresan constitutivamente en las células y éstas pierden/reducen la expresión de moléculas MHC

de clase I en condiciones patológicas, o carecen de moléculas HLA reconocibles por los receptores inhibidores que expresa la célula NK (células alogénicas o xenogénicas).
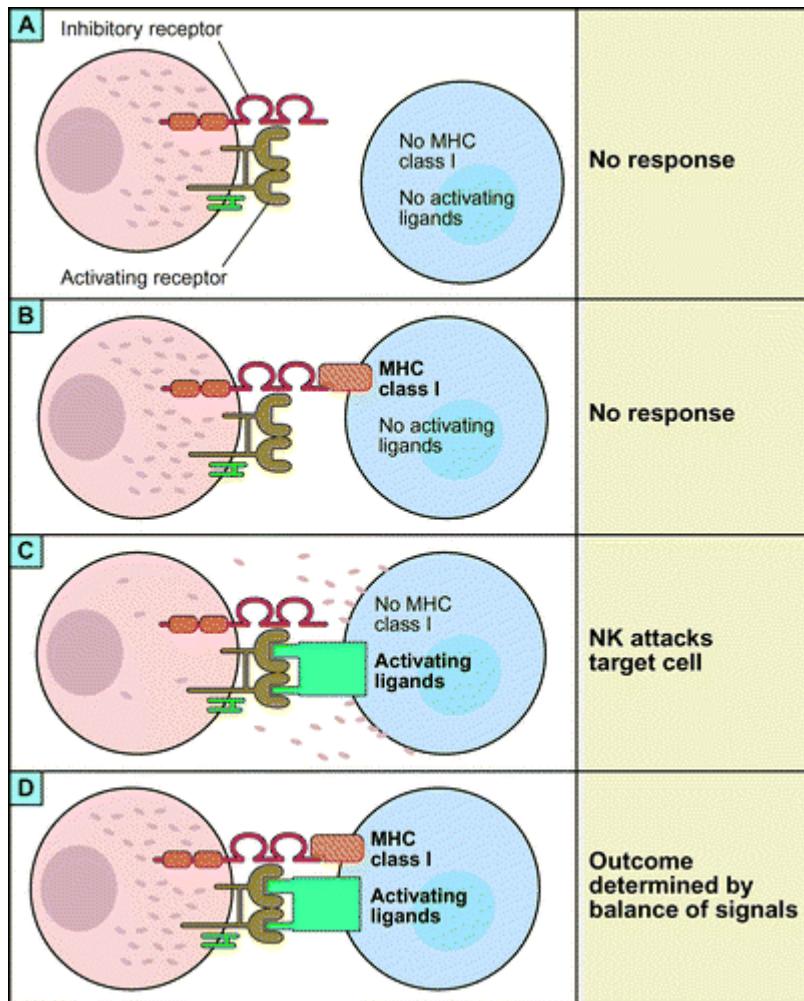


Figura 5. Descripción de las posibles respuestas tras el encuentro de la célula NK y la célula diana. La cantidad de receptores activadores e inhibidores en las células NK y la cantidad de ligandos en la célula diana así como las diferencias cualitativas en las señales transducidas, determinan la respuesta de la célula NK[22].

El papel de los receptores activadores que reconocen las moléculas de HLA es incierto. Dado que la afinidad de los receptores activadores por las moléculas MHC es baja, se supone que sólo pueden operar en situaciones en las que el control por los receptores inhibidores disminuya bajo un

umbral crítico, o en el caso de que se produzca un incremento de la avidez del receptor activador por su ligando. La primera situación puede ocurrir en células infectadas por virus o células tumorales, por lo que estos receptores activadores contribuirían a eliminar estas células patológicas; no hay datos que respalden la segunda posibilidad.

# 3. REGULACIÓN DE LA RESPUESTA INMUNITARIA EN LINFOCITOS T POR RECEPTORES ESPECÍFICOS DE MHC DE CLASE I

3.1 <u>Regulación de la respuesta inmunitaria por receptores inhibidores específicos de MHC de clase I</u>

Se han propuesto varias teorías no excluyentes para interpretar la función de los receptores inhibidores específicos de HLA en las células T[18, 23, 24]. Por una parte estos receptores servirían para modular la respuesta de las células T, aumentando o disminuyendo la respuesta a través del TCR. Muchos estudios han demostrado que la función efectora –citotóxica o secretora de citocinas- o la reorganización del citoesqueleto inducida por el TCR puede ser regulada por los receptores KIR, ILT2 o CD94/NKG2A.

Otras teorías sugieren que los receptores inhibidores se inducen en los linfocitos T citotóxicos (CTL) como un mecanismo de tolerancia periférica; también se ha propuesto que se expresan en células T como consecuencia de una estimulación prolongada como es el caso de células T específicas de virus tras una infección crónica, y que representaría un mecanismo para evitar la sobreestimulación de esas células. Por otra parte se ha descrito la posibilidad que algunas células T CD8+ puedan estimularse a través de receptores activadores diferentes al TCR (ej. KIR, CD94/NKG2C). En este caso la expresión paralela de receptores inhibidores tendría la misma función que en las células NK previniendo la agresión contra células sanas. Finalmente, se ha sugerido que la expresión de estos receptores podría contribuir a prevenir la apoptosis, propiciando el mantenimiento de las CTL de memoria.

Se sabe poco de los mecanismos que controlan la expresión de los NKR (*natural killer receptors*) inhibidores en las células T. La de los KIR en células NK maduras parece constitutiva y estable, ya que el fenotipo no se modifica como consecuencia de la activación y proliferación in vitro. Se desconocen los mecanismos que regulan la expresión de los receptores tipo Ig (KIR, ILT2) en células T. Por el contrario, la del complejo CD94/NKG2A se induce en células T humanas después de su activación mediante el TCR y estimulación por citocinas tales como TGFβ, IL15, IL-2, IL-12 o IL-10 [104, 105], y hay indicios de que puede también inducirse en células NK [106].

### 3.2 Regulación de la respuesta inmunitaria por receptores activadores específicos de MHC de clase I

En general se acepta la idea de que la función de estos receptores es diferente en las células T y NK. En las células NK cada NKR activador es una unidad independiente que puede activar directamente la citotoxicidad y la producción de citocinas. Sin embargo, en las células T los NKR actúan como moléculas coestimuladoras potenciando la respuesta a una estimulación subóptima por el TCR [107-109].

Las subpoblaciones de linfocitos T CD8+ que expresan NKR son efectoras y de memoria, y se caracterizan por ser CD28-, tener un repertorio del TCR restringido y telómeros cortos lo que implica una extensa expansión clonal [24]. Estos linfocitos NKR+ pueden recibir señales coestimuladoras de receptores activadores específicos de moléculas de clase I tales como NKG2D, CD94/NKG2C o receptores de la familia KIR [110]. Sin embargo en algunas circunstancias se ha observado que los NKR funcionan en células T como unidades independientes al TCR [111, 112].

El mecanismo por el que los KIR actúan como estimuladores o coestimuladores parece relacionado con el adaptador al que se asocian [110]. Las células NK expresan tanto DAP10 como DAP12, mientras que las células T en general sólo expresan DAP10, por lo que se ha sugerido que DAP12 media la función activadora en las células NK, mientras que DAP10 es responsable de la actividad coestimuladora en las células T. En las células NK los KIR activadores se asocian a

DAP12 y, mediante la participación de tirosina quinasas, estimulan la citotoxicidad y secreción de citocinas implicando la activación de la PLC-g y ERK. Por el contrario, la estimulación de los KIR en la mayoría de las células T que no expresan DAP12, no induce fosforilación de ERK pero activa JNK (*cJun N-terminal kinase)*, que constituye una vía de señalización asociada a otras moléculas coestimuladoras; por el momento se desconoce el mecanismo de acoplamiento entre KIR y la ruta de JNK. Aunque la mayoría de células T no expresan DAP12, existen ciertas subpoblaciones en donde se ha descrito su expresión por RT-PCR[113] o FACS[112]. Se ha sugerido que la expresión de DAP12 en esas células T proporciona a los NKR activadores el potencial para funcionar como receptores autónomos [110].

## 4. RECEPTORES ESPECIFICOS PARA HLA EN LA RESPUESTA INMUNITARIA A CITOMEGALOVIRUS.

### 4.1 La infección por HCMV

El citomegalovirus humano forma parte de la familia herpesvirus y posee un genoma de DNA de doble cadena lineal de 230 kpb, que presenta aproximadamente 200 fases de lectura abiertas (ORF) [114]. Las ORF se enumeran secuencialmente y se denominan por su localización en las regiones únicas (UL, US) o repetidas (TRL/IRL, TRS/IRS) del genoma viral (figura 6).

TRL                                           IRL/IRS          TRS

Región UL                  ◄ Región US ►

Figura 6. Estructura del genoma del HCMV. Las secuencias únicas larga (UL) y corta (US) están flanqueadas por las secuencias repetidas terminales (TR) e internas (IR).

El virión que contiene 30 a 40 proteínas consiste en una cápside icosahédrica (7 proteínas), tegumento (unas 25 proteínas entre las cuales está la UL83 (pp65)) y una envuelta lípidica (que contiene una serie de glicoproteínas) (figura 7).
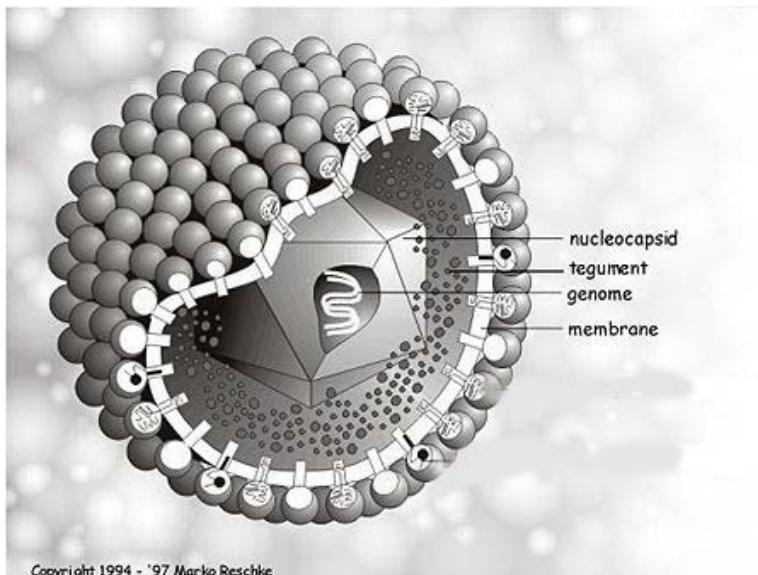


Figura 7. Estructura del virión del HCMV.

El HCMV infecta diferentes tipos celulares tales como fibroblastos, células endoteliales, epiteliales, del músculo liso y células hematopoyéticas. La expresión de los genes en las células permisivas es secuencial, en tres fases denominadas inmediata-temprana, temprana y tardía, causando al final un efecto citopático. Las células de origen mielomonocítico y algunas células endoteliales son consideradas como el principal reservorio del HCMV.

La prevalencia de la infección por HCMV en la población adulta es elevada (50-100%) y a menudo se adquiere en la infancia transmitida por secreciones [115]. La sintomatología clínica es en general leve aunque puede causar una embriopatía grave durante el embarazo. La característica principal de la respuesta inmunitaria frente a este virus, al igual que frente a otros herpesvirus, es que es capaz de controlar al patógeno pero no de erradicarlo, manteniéndose latente en los individuos inmunocompetentes que se convierten en portadores. En sujetos sanos se pueden observar reactivaciones ocasionales del HCMV con incrementos de DNA vírico y linfocitos T específicos en la sangre. Cabe señalar que algunas citocinas proinflamatorias (ej. TNF-$\alpha$ e IFN-$\gamma$) pueden favorecer el proceso de reactivación en macrófagos. La persistencia de células T específicas después de la primoinfección posiblemente refleja el impacto de este proceso, aunque las reactivaciones pasen clínicamente desapercibidas. Esta situación de control se altera en el caso de inmunodepresión, donde la reactivación puede causar patología grave como retinitis en enfermos con SIDA o neumonitis intersticial en receptores de transplantes de progenitores hematopoyéticos alogénicos. El HCMV también puede participar en el rechazo crónico de transplantes de órganos sólidos, y contribuir como cofactor en el desarrollo de la aterosclerosis y de la reestenosis coronaria tras una angioplastia.

El modelo murino se ha usado extensamente para estudiar la biología y patogénesis del HCMV. A pesar de su clara divergencia, el MCMV comparte muchas características con el HCMV como la replicación en la infección aguda, su tropismo tisular y el establecimiento de latencia y reactivación [116]. Además el HCMV y MCMV comparten una organización similar del genoma y codifica productos génicos homólogos [117, 118].

Los estudios realizados en modelos murinos han mostrado que el control de la infección por CMV requiere la participación de la respuesta inmunitaria tanto innata como adaptativa, implicando respectivamente a células NK y linfocitos T citotóxicos específicos [89, 119, 120]. Por su parte, el virus ha adoptado diferentes estrategias para evadir la respuesta inmunitaria del huésped [121-124] (tabla 5).

Entre las complejas interacciones que se establecen entre el sistema inmunitario y las células infectadas por CMV el reconocimiento específico de MHC de clase I y de moléculas relacionadas, derivadas del huésped o del propio virus, parecen particularmente relevantes en la interfase de estos dos sistemas[125].

Tabla 5. Genes del HCMV y MCMV que participan en la evasión de la respuesta inmunitaria

| FUNCIÓN | Gen HCMV | Gen MCMV |
|---|---|---|
| Disminución de MHC clase I | US2,US3,US6, US11 | m04, m06, m152 |
| Disminución de MHC clase II | US2 | - |
| Inhibir la presentación de IE1 a células T | UL83 | - |
| Homóloga MHC clase I | UL18 | m144 |
| Evasión células NK | UL40, UL16, UL141 | m155, m145, m152, m157 |
| Receptor de quimiocina | US28 | M33 |
| Receptores 7TM adicionales | UL33, UL78, US27 | M78 |
| Homólogo receptor TNF | UL144 | - |
| Homólogo IL-10 | UL111a | - |
| Quimiocina | UL146, UL147 | m131 |
| Interferencia vía del IFNI, II | - | M27 |
| Inhibidor de apoptosis | UL36, UL37 | M36, m41 |

4.2 Control de las infecciones víricas por la respuesta inmunitaria innata y adaptativa

La relación entre el virus y el huésped es un proceso dinámico en el cual el virus intenta minimizar su visibilidad mientras que el huésped intenta prevenir y erradicar la infección con un daño colateral mínimo. Al principio el virus debe reconocer, unirse y entrar en la célula diana, migrando al compartimiento celular apropiado. Aquí el genoma se transcribe, se traduce y se replica, permitiendo el ensamblaje y exportación de nuevos viriones necesarios para que la infección se pueda extender a otras células y diseminarse a otros huéspedes. La mayoría de tipos celulares son capaces de detectar moléculas de doble cadena de RNA (dsRNA) que se producen durante la replicación de muchos virus DNA o RNA. La detección de dsRNA implica la inducción de citocinas antivíricas tales como los IFN tipo I (IFN-$\alpha/\beta$) que inhiben uno o más pasos del ciclo vírico y

contribuyen a activar la respuesta inmunitaria. La inducción de IFN-$\alpha$/$\beta$ ocurre a las pocas horas después de la infección vírica. Algunos virus activan rápidamente los macrófagos y la secreción de otras citocinas tales como quimiocinas y TNF-$\alpha$. Las citocinas reclutan leucocitos en el tejido infectado, activan sus funciones efectoras, incrementan la expresión de moléculas MHC, así como el procesamiento y transporte de péptidos víricos para que puedan ser presentados eficientemente por las moléculas MHC en la superficie de las células infectadas. Las células que son atraídas por las citocinas y que constituyen la primera línea de defensa son los granulocitos, células NK y probablemente las células NKT [126].

Los macrófagos y las DC del huésped también juegan un papel en el control temprano de la infección secretando citocinas, tales como la IL-12 y IL-18, que pueden inducir IFN-$\gamma$, y otras (IL-1, IL-6). Todos estos procesos contribuyen al desarrollo de la respuesta inmunitaria adaptativa al virus que tiene lugar en los ganglios linfáticos regionales donde los antígenos han sido transportados por las células dendríticas. La población T específica puede migrar otra vez al tejido infectado y realizar sus funciones efectoras, incluyendo la producción de citocinas tales como IFN-$\gamma$ y TNF-$\alpha$ que juegan un papel importante en el control de la infección. Finalmente, los anticuerpos específicos para antígenos víricos se detectan mas tardíamente en la mayoría de infecciones y se cree que son responsables de prevenir la diseminación y de la resistencia a las reinfecciones[126].

En ocasiones el huésped no es capaz de eliminar completamente al patógeno que puede desarrollar una infección crónica o quedar latente, como es el caso del citomegalovirus.

## 4.3 Respuesta frente al HCMV mediada por linfocitos T. Mecanismos de evasión

En la primoinfección por HCMV las células CD4+ específicas para CMV se hacen patentes en sangre periférica a los diez días de la detección de DNA vírico, la cual es posible a la semana de la infección. Posteriormente, se detectan anticuerpos específicos y finalmente células T CD8+ específicas [127].

Tanto los anticuerpos neutralizantes como los CTL son importantes en el control de la infección por citomegalovirus. Ambas respuestas son dependientes de las células T CD4+ cooperadoras y, en modelos murinos, se ha demostrado que un déficit selectivo de éstas determina la persistencia de la replicación vírica. La participación de los linfocitos T CD4+ durante la respuesta primaria asegura el establecimiento de la población de memoria [128]. Durante la infección por CMV se produce una respuesta sostenida que conlleva la presencia en sangre de células T CD4+ y CD8+ específicas. Estimulando PBL con antígenos de HCMV y detectando la producción de IFN-γ intracelular se ha estimado que un 1-4% de los linfocitos T, CD4+ y CD8+, son específicos para CMV[129], aumentando el porcentaje con la edad hasta un 10% en individuos mayores de 60 años [130].

Estos linfocitos presentan un fenotipo CD45RO+, CCR7-, CD62L-, perforina+, CD28-, CD27- correspondiente a células efectoras/memoria [128, 131]. Se ha descrito que las células T CD8+ específicas de CMV expresan en su superficie ILT2 y NKG2D, pero carecen de KIR y CD94[132].[133]

Los linfocitos T específicos reconocen epítopos derivados de diferentes antígenos del HCMV tales como las proteínas estructurales UL83 (pp65) y UL32 (pp150), así como el transactivador inmediatamente-temprano UL123 (IE-1; pp72)[114]. Recientemente se ha descrito en algunos donantes un subgrupo de células T NKR+CD8+TCRαβ+, denominado NK-CTL, que reconocen a través del TCR la molécula HLA-E asociada con péptidos endógenos[134]. La secuencia de uno de estos péptidos coincide con un péptido de la secuencia señal de la proteína UL40 del HCMV[135]. Estas células T presentan un patrón de reordenamiento del TCR oligoclonal, se caracterizan por tener un fenotipo de memoria (CD45R0+, CD28-), expresan en superficie NKR inhibidores (CD94/NKG2A, ILT2, KIR2DL1, KIR2DL2/3, KIR3DL2, KIR3DL1) y ejercen una actividad tipo NK[136].

Para interferir con la presentación antigénica a las células T el citomegalovirus dispone de varios mecanismos. En primer lugar la expresión de la proteína pp65 en la célula infectada por HCMV inhibe la generación de células T específicas para epítopos derivados de la proteína IE1 [137]. Por otra parte el HCMV reduce la expresión de moléculas de HLA. Varias proteínas están

implicadas en el proceso actuando a través de distintos mecanismos en diferentes estadios del ciclo replicativo [138]. US6, que se expresa tardíamente, se une a TAP impidiendo el transporte de los péptidos al retículo endoplásmico (RE) y su ensamblaje con moléculas de HLA de clase I [139]. US3, es una proteína inmediatamente temprana que retiene las moléculas de HLA de clase I en el RE [140]. Finalmente US2 y US11, que se expresan en las fases temprana y tardía del ciclo también se unen a las cadenas pesadas de las moléculas de clase I en el RE, translocándolas al citosol donde son degradadas por el proteosoma [141]. En un sistema en el que se comparó el efecto de US2, US6 y US11 en la expresión de HLA de clase Ia y HLA-E en una línea celular B (RPMI 8866), se observó que no todos los genes US afectan de manera comparable la expresión de las moléculas de HLA de clase I[142]. En este sistema, US6 disminuyó la expresión de todas las moléculas de HLA, mientras que US11 preservó selectivamente la expresión de HLA-E, y US2 no afectó HLA-B ni HLA-E [142]; otros estudios en sistemas diferentes obtienen resultados distintos [143, 144]. Otros dos genes adicionales adyacentes, US8 y US10, codifican glicoproteínas que unen cadenas pesadas de MHC de clase I, aunque no parecen alterar su expresión en superficie [145]. US2 y US3 también pueden interferir con la presentación antigénica por MHC de clase II [146].

La redundancia de los mecanismos que tienen como objetivo disminuir la expresión de MHC de clase I para interferir con la presentación antigénica a linfocitos T probablemente refleja la importancia de esta estrategia de evasión de la respuesta inmunitaria.

## 4.4 Respuesta frente al HCMV mediada por células NK. Mecanismos de evasión

Las células NK parecen jugar un papel importante en el control de algunas infecciones víricas en particular por herpesvirus[147]. Se ha descrito que los raros casos de deficiencias de células NK sufren infecciones graves recurrentes por este grupo de virus [148]. Igualmente, los ratones deficientes en células NK tiene mayor susceptibilidad a las infecciones por citomegalovirus [149]. Las células NK utilizan al menos dos funciones efectoras que pueden contribuir al control de la infección: pueden

matar a las células infectadas, usando perforina/granzima y/o FAS-L, y secretan citocinas inflamatorias con actividad antivírica, especialmente IFN-γ [150]. En ratones se ha observado como se inducen rápidamente la proliferación, secreción de IFN-γ, y citotoxicidad de las células NK después de la infección por MCMV. Biron et al describieron que la activación de las células NK, valorada por el incremento del tamaño celular ("blastogenesis"), durante la infección por el citomegalovirus murino se correlacionaba con el incremento del número de células NK en el bazo y el hígado. Estudios más recientes usando el método de marcaje con BrdU han demostrado una proliferación general de células NK al inicio de la infección por MCMV atribuible a la acción de la IL-15, seguida de una expansión preferente de la población Ly49H+, sugiriendo que en esa fase las células NK que expresan el receptor activador reconocen y responden frente a las células infectadas, desarrollando una expansión clonal análoga a que experimentan los linfocitos T [150].

Aunque se ha demostrado que el IFN α/β incrementa la capacidad citotóxica de las células NK, no está clara la importancia de la citotoxicidad en la respuesta frente al CMV. Por el contrario, la producción de IFN-γ juega un papel importante. Ciertas funciones del IFN-γ tales como incrementar el procesamiento y transporte antigénico, y la expresión del MHC se superponen a las del IFN-α/β. Otras funciones con acción antivírica directa son específicas del IFN-γ, como la inducción del enzima NOS (*nitric oxide synthase)* que promueve la producción de NO el cual puede modificar moléculas necesarias para la replicación vírica; o la inducción de MIP-1α que induce la secreción de la quimiocina CX3CL1 muy efectica en el reclutamiento de linfocitos activados[147, 151].

Tanto la proliferación, como la citotoxicidad y la secreción de IFN-γ están reguladas por otras citocinas tales como IFN-α/β, IL-12, IL-15 y IL-18 que son secretadas por células infectadas, o por DC activadas y macrófagos, y que activan las respuestas de las células NK[147, 152]. En ratones se ha visto que la interacción coordinada entre diferentes citocinas que incrementan sus niveles durante las infecciones víricas activa la respuesta mediada por las células NK. Así, la IL-12 es crítica para la expresión de IFN-γ, mientras que IFN-α/β se requiere para estimular la citotoxicidad. La

acumulación/supervivencia de las células NK que proliferan es independiente de la IL-12, pero requiere la inducción de IL-15 por IFN-$\alpha/\beta$ [153].

Como se ha expuesto anteriormente, las funciones de las células NK están también sometidas al control de receptores específicos expresados en la superficie de las células NK que, tras la interacción con sus ligandos, regulan su capacidad citotóxica, proliferación y/o producción de IFN-$\gamma$. Éstos incluyen receptores activadores que reconocen una gran variedad de ligandos, muchos de ellos desconocidos y receptores inhibidores que reconocen moléculas de clase I del MHC. Las células NK pueden reconocer a los patógenos intracelulares mediante uno o más de los siguientes mecanismos: a) reconocimiento directo de una proteína vírica; b) disminución de ligandos para receptores inhibidores de las células NK; c) inducción o incremento de la expresión de ligandos propios para receptores activadores de la célula NK. Sin embargo, la respuesta de las células NK durante la infección por CMV puede ser contrarrestada por mecanismos de evasión específicos (tabla 6 y Figura 8) [121-124, 154].

Tabla 6. Genes del HCMV y MCMV que participan en la evasión de la respuesta de las células NK

| Virus | Gen vírico | Ligando del receptor en NK | Efecto en la célula diana | Consecuencia en las células NK |
|-------|-----------|----------------------------|---------------------------|-------------------------------|
| HCMV | UL40 | | HLA-E↑ | |
| | UL16 | | MIC-B↓; ULBP-1,2↓ | ↓ activación por NKG2D |
| | UL18 | ILT2 | | Inhibición por ILT2 |
| | UL141 | | CD155↓ | ↓ activación por DNAM-1 |
| | | | | ↓ adhesión por CD96 |
| MCMV | m157 | | | Inhibición por Ly49I |
| | m152 | | Rae1↓; H60↓ | ↓ activación por NKG2D |
| | m155 | | H60↓ | ↓ activación por NKG2D |
| | m145 | | MULT-1↓ | ↓activación por NKG2D |
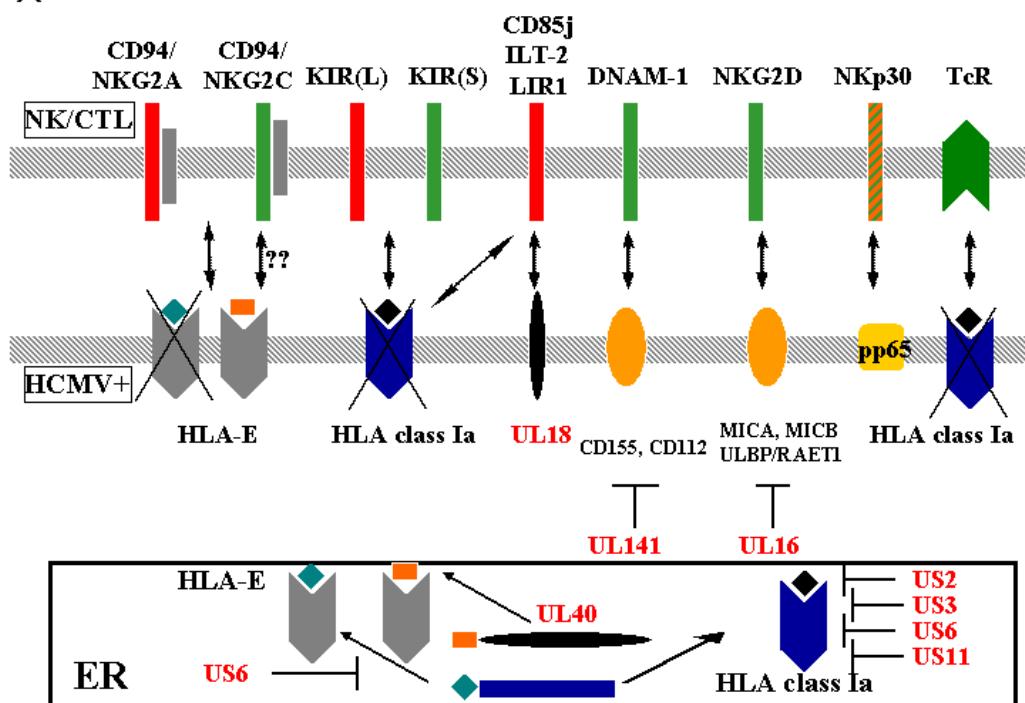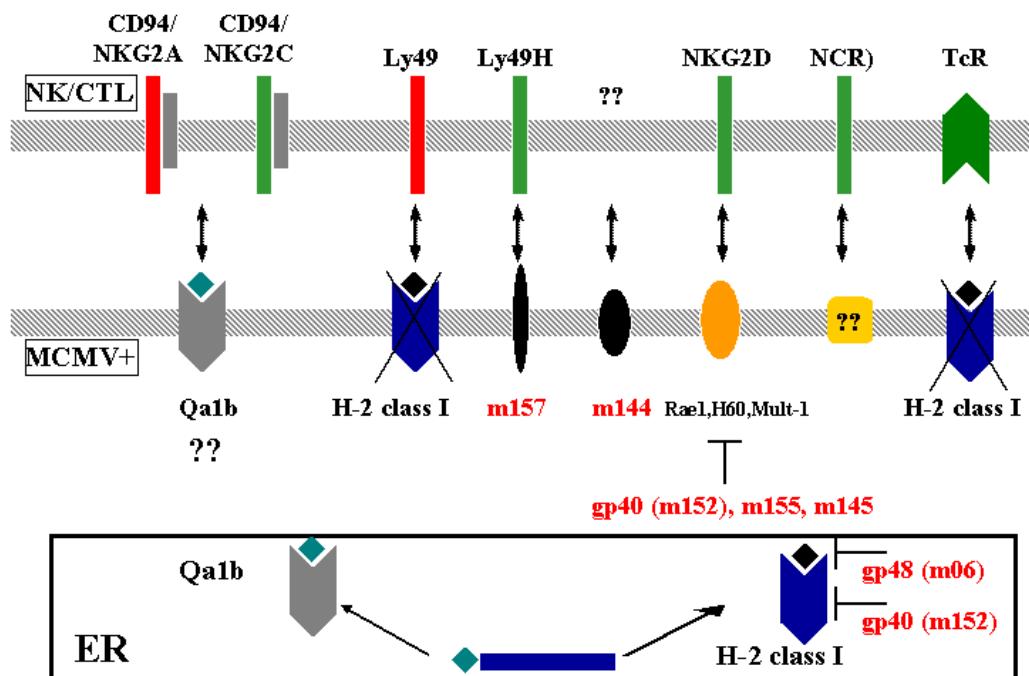
Figura 8. Posibles interacciones entre los diferentes receptores inhibidores (rojos) y activadores (verdes) expresados en células NK y CTL, con sus ligandos, en células infectadas por HCMV (A) o MCMV (B)

4.4.1 <u>Reconocimiento directo de una proteína vírica</u>

El sistema inmunitario innato está dotado de diferentes tipos de receptores que le permiten responder frente a patrones moleculares asociados a patógenos (PAMP), tales como los TLR (*Toll-like receptores)*[155]. Se ha descrito que las células NK, independientemente de su estado de activación, expresan TLR3 y 9 que reconocen respectivamente RNA de doble hebra (dsRNA) y secuencias ricas en CpG desmetiladas, abundantes en el DNA bacteriano y vírico [156], por lo que las células NK pueden responder a esas moléculas.

En células NK de ratón se describió un receptor activador, denominado Ly49H, que reconoce directamente una proteína vírica (m157) que se expresa durante la infección del MCMV [157, 158]. El carácter hereditario denominado Cmv-1[159], que determinaba la susceptibilidad a la infección por MCMV en algunas cepas de ratón, se identificó con el gen Ly49H[160]. Este miembro de la familia Ly49 se asocia a DAP12 y juega un papel importante en la defensa contra células infectadas por MCMV[161], estimulando las funciones NK al interaccionar con la proteína vírica m157. Se ha sugerido que este receptor Ly49H activador representaría una adaptación evolutiva del huésped para contrarrestar el efecto de m157 como mecanismo de evasión, ya que se ha constatado que también se une al receptor inhibidor Ly49I[162]. Por otra parte, hay evidencia de que bajo la presión de las células NK Ly49H+ se seleccionan variantes del virus con mutaciones en la proteína m157, que impiden el reconocimiento por Ly49H [163]. La combinación de Ly49P con H-2D$^k$ también confiere resistencia la infección por MCMV aunque se desconoce por el momento el posible ligando de Ly49P [164].

Recientemente se ha descrito el reconocimiento de la proteína pp65 del HCMV por el receptor NKp30. Pese a tratarse de un receptor activador, la interacción con el ligando pp65 inhibe a la célula promoviendo, aparentemente, la disociación del receptor y su adaptador [44].

4.4.2 Disminución de ligandos para receptores inhibidores específicos para HLA

El CMV disminuye la expresión de moléculas MHC para evadirse de la respuesta mediada por células T. Como consecuencia las células NK quedan liberadas del control que ejercen los receptores inhibidores específicos para moléculas de clase I y pueden mediar citotoxicidad y secreción de citocinas contra las células infectadas. Sin embargo, en estudios in vitro los fibroblastos infectados con CMV mantienen cierta resistencia a la lisis por células NK lo que sugiere que existen mecanismos de evasión [165].

Para escapar del control por las células NK, el virus puede mantener los receptores inhibidores activos, interfiriendo de manera selectiva con la expresión de las moléculas MHC de clase I o presentando moléculas homólogas a éstas en las células infectadas [124].

El CMV podría escapar tanto de las células NK y T preservando la expresión de moléculas con un papel limitado en la presentación antigénica pero que constituyen ligandos importantes para receptores inhibidores (HLA-E y HLA-C). Al respecto, se ha demostrado que las proteínas US2 o US11 interfieren selectivamente con la expresión de moléculas MHC de clase I, preservando HLA-E [142]. La posibilidad de que HCMV pueda preservar HLA-C para escapar del control de KIR, como se propuso originalmente para la proteína nef del virus de la inmunodeficiencia humana (VIH) [166], no se ha demostrado.

Respecto al segundo mecanismo de evasión mencionado se han descrito dos proteínas del HCMV que pueden contribuir a evadir la respuesta mediada por las células NK:

a) UL-40 y CD94/NKG2A

Dos laboratorios describieron que un nonámero derivado de la secuencia señal de la proteína del HCMV UL40 es idéntico al proveniente de la molécula del HLA-Cw03 que se une a HLA-E, por lo que puede estabilizar su expresión [167, 168]. Se ha postulado que la síntesis de UL40 durante la

infección vírica puede preservar la expresión de HLA-E, confiriendo protección contra las células NK que expresan el receptor inhibidor CD94/NKG2A. Esta hipótesis se vio respaldada al observar que fibroblastos infectados con un mutante de la cepa AD169 deficiente en la proteína UL40 eran más susceptibles a la lisis por líneas primarias y células NK CD94/NKG2A+ [169]. Sin embargo, otro grupo ha cuestionado que la molécula HLA-E se mantenga a lo largo de la infección por HCMV y que efectivamente permita evadir la respuesta de las células NK [170].

Para que las células infectadas por HCMV preserven HLA-E, ésta debería ser refractaria a la acción de otras proteínas víricas que inhiben la expresión de moléculas de clase I (ej. US2, US3, US6 y US11). La presentación del nonámero derivado de UL40 por HLA-E es independiente de TAP y resistente a US6 [167]. Además tanto US2 como US11 preservan los niveles de superficie de HLA-E[142] por lo que, junto a la presentación del péptido derivado de UL40, pueden contribuir a mantener la expresión de HLA-E en la superficie durante la infección y proteger a los fibroblastos infectados de la lisis mediada por células NK CD94/NKG2A+.

Se ha observado que un péptido derivado de la proteína HSP-60, que se expresa en células sometidas a estrés, puede estabilizar HLA-E pero impide su unión a CD94/NKG2A por lo que la célula es susceptible a la lisis [71]. La HSP-60 al competir con los péptidos endógenos propiciaría la lisis de células en situaciones patológicas.

b) UL18-ILT2

La proteína del HCMV UL18 es homóloga a HLA de clase I, se une a b2-microglobulina, puede unir péptidos y se expresa en la superficie de las células [171]. La glicoproteína UL18 se une al receptor inhibidor ILT2 con una afinidad superior que las moléculas MHC de clase I [36, 37]. Aunque resulta razonable, la hipótesis de que UL18 pueda interferir con la actividad de las células NK no se ha demostrado. Algunos trabajos han propuesto que la reducida susceptibilidad a la lisis mediada por células NK de las células infectadas por HCMV es independiente de la expresión de UL18 [172] y que,

paradójicamente, UL18 podría incrementar la susceptibilidad a la actividad NK [173]. Recientemente, Ciccone et al, han publicado que los linfocitos T CD8+ pueden lisar de manera independiente del TCR células UL18+, interpretando que la interacción UL18-ILT2 podría estimular las funciones efectoras en células T [174]. Se requieren más datos experimentales para evaluar la hipótesis.

El hecho de que no todas las variantes polimórficas de UL18 expresadas en diferentes aislados víricos se unan con la misma afinidad a ILT2 [175], la posibilidad que UL18 pueda ser ligando de otro receptor activador o que opere sobre otros tipos celulares ILT2+, como monocitos/macrófagos, deben tenerse en cuenta para resolver las incógnitas sobre su papel en la infección.

### 4.4.3 Inducción o incremento de la expresión de ligandos propios para receptores activadores de células NK

Hay datos a favor de que NKG2D está implicado en la respuesta al HCMV. Por una parte, la infección por HCMV aumenta la expresión en superficie de MICA/B y ULBP, ligandos del receptor NKG2D [77] y por otra el receptor, que se expresa en la mayoría de células T CD8+ específicas para HCMV, funciona como una molécula coestimuladora aumentando la citotoxicidad y secreción de citocinas mediada por el TCR [176]. Además, la existencia de mecanismos tanto en el HCMV como en MCMV para disminuir el efecto del receptor NKG2D sugiere que este receptor tiene un papel importante en la respuesta al virus. La glicoproteína del HCMV UL16, que se expresa temprano durante la infección, retiene a nivel intracelular MICB, ULBP1 y ULBP2, inhibiendo su expresión en superficie e interfiriendo con la respuesta mediada por NKG2D[177-179]; por el contrario MICA, ULBP3 y ULBP4 parecen resistentes a UL16. Los fibroblastos infectados con un mutante de la cepa AD169 que no expresa la proteína UL16 son más susceptibles a la lisis por células NK [180]. En el MCMV las glicoproteínas m152, m155 y m145 disminuyen los ligandos del receptor NKG2D murino [181-184].

Se ha descrito recientemente que la proteína UL141 del HCMV disminuye la expresión de los ligandos CD112 y CD155 del coreceptor activador DNAM1 [185], lo que sugiere que este receptor también está implicado en la respuesta frente al HCMV.

# CAPÍTULO 2

## NK Cell Receptors Involved in the Response to Human Cytomegalovirus Infection

Gumà M, Angulo A, López-Botet M

**Summary**

Human cytomegalovirus (HCMV) infection is a paradigm of the complexity reached by host-pathogen interactions. To avoid recognition by cytotoxic T lymphocytes (CTL) HCMV inhibits the expression of HLA class I molecules. As a consequence, engagement of inhibitory KIR, CD94/NKG2A and CD85j (ILT2 or LIR-1) natural killer receptors (NKR) specific for HLA class I molecules is impaired, and infected cells become vulnerable to an NK cell response driven by activating receptors. In addition to the well-defined role of the NKG2D lectin-like molecule, the involvement of other triggering receptors (i.e. activating KIR, CD94/NKG2C, NKp46, NKp44 and NKp30) in the response to HCMV is being explored. To escape from NK cell-mediated surveillance, HCMV interferes with the expression of NKG2D ligands in infected cells. In addition, the virus may keep NK inhibitory receptors engaged preserving HLA class I molecules with a limited role in antigen presentation (i.e. HLA-E) or, alternatively, displaying class I surrogates. Despite a considerable progress in the field, a number of issues regarding the involvement of NKR in the innate immune response to HCMV remain uncertain.

Human cytomegalovirus (HCMV) is a prototypic betaherpesvirus that infects with a high prevalence all human populations (PASS 2001). Primary infection in healthy immunocompetent individuals is usually mild or asymptomatic. Replicating HCMV is eventually cleared by the host immune response, but the virus remains in a lifelong latent state. Reactivation of HCMV, associated with unapparent shedding, can sporadically occur in seropositive carriers facilitating the spread of the virus to additional hosts. The long-term persistence after primary infection of circulating T cells specific for viral antigens, presumably reflects the impact of this recurrent process on the immune system. By contrast, primary infection, reinfection or reactivation of HCMV may cause a significant morbidity in individuals with immature or compromised immune systems. In transplant recipients, HCMV infection can lead to severe complications such as pneumonia, hepatitis or graft failure. Retinitis is a common HCMV-induced pathology in human immunodeficiency virus (HIV)-infected patients. In addition, HCMV is the leading viral cause of congenital disorders such as hearing loss, chorioretinitis or mental retardation. Several studies have also implicated HCMV infection as a co-factor contributing to atherosclerosis and coronary restenosis following angioplasty.

HCMV is a large enveloped double-stranded DNA virus; its 230-kb genome encodes for around 200 open reading frames, a vast number of which have not yet been related to any specific functional role during infection (MOCARSKI and COURCELLE 2001). HCMV exhibits strict species specificity, a relatively slow replication cycle, and a narrow cell tropism in tissue culture. Despite that HCMV infects different cell types in the host (i.e. fibroblasts, hepatocytes, epithelial, endothelial, smooth muscle, stromal, neuronal and hematopoietic cells), complete productive infection in vitro is mainly sustained in fibroblasts and, less efficiently, in endothelial and differentiated myelomonocytic cells. Expression of HCMV genes in fully permissive cells follows a temporally ordered cascade in which three phases, designated as immediate-early, early and late, can be distinguished. Viral gene expression is limited in some cell types, where only a restricted/abortive HCMV infection takes place, thus representing potential sites of viral persistence in vivo. In

particular, myelomonocytic cells harboring HCMV genomes are thought to serve as reservoirs of latent virus. Under specific stimuli, such as proinflammatory cytokines, monocytes may differentiate into mature macrophages allowing productive HCMV replication and dissemination.

The majority of in vitro studies on HCMV have been performed with laboratory strains (i.e. AD169, Towne) which have been subjected to extensive passages on human fibroblast cell lines. This manipulation results in genetic deletions and, in fact, at least an extra 15 kb region containing more than 19 ORFs is only present in freshly isolated clinical strains of HCMV (CHA et al. 1996). Genetic polymorphisms have also been reported in HCMV clinical isolates (PIGNATELLI et al. 2004); yet, the relevance of HCMV genetic variability in the context of viral immunopathogenesis and disease outcome is still poorly understood (CERBONI et al. 2000). A powerful approach to study the function of individual CMV genes is the generation and analysis of viral mutants carrying specific genome deletions. The introduction of full-length CMV genomes into E. coli as an artificial chromosome (BAC) clone (ADLER et al. 2003) has facilitated efficient and reliable targeted mutagenesis.

Experimental animal models, mainly using murine CMV (MCMV), have been extensively employed to provide insights into HCMV biology and pathogenesis. Despite a significant divergence, MCMV shares many features with its human counterpart in terms of replication during acute infection, tissue tropism, establishment of latency and reactivation (HO 1991). In addition, human and murine viruses exhibit a similar genetic organization and encode homologous gene products (CHEE et al. 1990; RAWLINSON et al. 1996).

Early studies in animal models revealed that an effective defence against CMV requires the coordinated participation of the innate and adaptive immune responses, mainly involving NK cells and specific CTL (BIRON and BROSSAY 2001; FRENCH and YOKOYAMA 2003; SCALZO 2002); reciprocally, CMV have adopted a variety of immune evasion strategies. Human CTL recognize peptide epitopes derived from different HCMV antigens such as the UL83 (pp65) and

UL32 (pp150) structural proteins, as well as the immediate-early transactivator UL123 (IE-1; pp72) (MOCARSKI and COURCELLE 2001). In order to interfere with antigen presentation, HCMV impairs the expression of HLA class I molecules, employing several proteins encoded by a gene cluster located in the unique short (US) region of the HCMV genome (HENGEL et al. 1999; TORTORELLA et al. 2000). Among them, US2 and US11 are expressed at early-late stages of the viral replication cycle and translocate class I heavy chains from the ER to the cytosol where they are degraded. US3 is an immediate-early protein that retains class I molecules at the endoplasmic reticulum (ER), whereas the late US6 protein impairs TAP-mediated peptide transport. Two additional genes in the US region, US8 and US10, encode glycoproteins that bind MHC class I heavy chains, although they do not appear to drastically alter processing and cell surface expression of the MHC class I molecules (FURMAN et al. 2002; TIRABASSI and PLOEGH 2002). Remarkably, US2 and US3 may also interfere with MHC class II antigen presentation (HEGDE et al. 2003).

The redundant mechanisms aimed to inhibit HLA class I expression likely reflect the importance of this immune evasion strategy. As a consequence, NK cells are released from the control exerted by inhibitory receptors specific for class I molecules, and can mediate cytotoxicity and cytokine production against infected cells. To escape from NK cell-mediated surveillance, HCMV impairs the expression of ligands for activating receptors. Alternatively, the virus may keep NK inhibitory receptors engaged, either preserving ligands with a limited role in antigen presentation (i.e. HLA-E and HLA-C) or displaying class I surrogates in infected cells (LOPEZ-BOTET et al. 2004).


**Involvement of inhibitory NKR in the response to HCMV**

NK cells express several inhibitory receptors such as killer immunoglobulin-like receptors (KIR), the CD94/NKG2A killer lectin-like receptor (KLR) and CD85j (ILT2 or LIR-1) (COLONNA et al.

1999; LOPEZ-BOTET and BELLON 1999; MORETTA and MORETTA 2004), that are also expressed by some T lymphocytes (VIVIER and ANFOSSI 2004). The spectra of class I HLA molecules covered by inhibitory KIR and, indirectly, by CD94/NKG2A are partially overlapping. Both receptor systems complement each other to monitor the surface expression of most class I molecules, which are also broadly recognized by CD85j. The heterogeneous distribution of NK cell receptors (NKR) in distinct NK cell subsets enables the system to react against variable alterations of HLA class I expression, provided that activating signals do overcome the inhibitory threshold.


**CD94/NKG2A**

CD94 and NKG2 are lectin-like membrane glycoproteins encoded at the NK gene complex (NKC) in human chromosome 12 (CHANG et al. 1995; HOUCHINS et al. 1991). The CD94/NKG2A heterodimer constitutes an inhibitory receptor that recruits the SHP-1 tyrosine phosphatase through the Immunoreceptor Tyrosine-based inhibition motif (ITIM)-bearing NKG2A subunit. By contrast, CD94/NKG2C forms a triggering receptor linked to KARAP/DAP12, an Immunoreceptor Tyrosine-based Activation Motif (ITAM)-bearing adapter molecule that connects these receptors to a protein tyrosine kinase (PTK) activation pathway (LANIER 2003; LOPEZ-BOTET and BELLON 1999). The function of other putative activating molecules encoded by the NKG2E gene (YABE et al. 1993) remains unknown. HLA-E was shown to be a specific ligand for both CD94/NKG2A and CD94/NKG2C receptors, presenting peptides derived from the signal sequences of other HLA class I molecules (BORREGO et al. 1998; BRAUD et al. 1998; LEE et al. 1998). HLA-E is dimorphic at position 107, where the few allotypes identified display either an Arg (HLA-E$^R$) or a Gly (HLA-E$^G$) (STRONG et al. 2003). Resolution of the crystal structure of HLA-E revealed the basis of its affinity for hydrophobic leader sequence-derived peptides (O'CALLAGHAN et al. 1998).

Detection of HLA-E by CD94/NKG2A is currently viewed as a sensor mechanism that probes the status of HLA class I biosynthesis. Yet, there is evidence supporting that HLA-E may bind

to hydrophobic peptides from other proteins; some of these sequences are very similar to those derived from HLA class I molecules whereas others appear completely unrelated. Among the first group, a peptide from the HSP60 was shown to potentially compete with endogenous class I-derived nonamers for binding to HLA-E (MICHAELSSON et al. 2002); the complex was not recognized by CD94/NKG2A, rendering stressed cells vulnerable to an NK-mediated attack. The biological relevance of this observation in the context of the immune response against virus-infected targets is still uncertain.

On the other hand, a nonamer derived from the leader sequence of the UL40 HCMV protein was also reported to interact with HLA-E (TOMASEC et al. 2000; ULBRECHT et al. 2000). Expression of the class Ib molecule was preserved in infected cells, conferring protection against a CD94/NKG2A+ NK cell line. Moreover, fibroblasts infected by a UL40-deletion mutant of the AD169 HCMV strain were killed by CD94/NKG2A+ primary NK cell lines more efficiently than cells infected by the wild-type virus (WANG et al. 2002). The possibility that HLA-E may be maintained along HCMV infection to effectively evade the NK response has been questioned by another study (FALK et al. 2002); some differences between both experimental approaches may explain the discrepancy.

In order to be preserved in HCMV-infected cells, HLA-E should be refractory to the action of viral proteins that target class I molecules (i.e. US2, US3, US6 and US11). Indeed, HLA-E presentation of the UL40-derived nonamer was confirmed to be TAP-independent and resistant to US6 (TOMASEC et al. 2000). We compared the effect of US2, US6 or US11 on the endogenous HLA-E and HLA class Ia expression in a human B cell lymphoma line, assessing in parallel their influence on target susceptibility to NK cell clones (LLANO et al. 2003). In this system, US6 downregulated all class I molecules, whereas US11 selectively preserved HLA-E. This rendered US11+ cells sensitive to NK clones under the control of KIR2DL2 and/or CD85j receptors, maintaining resistance to CD94/NKG2A+ KIR2DL2- cells. US2 also spared HLA-E though it selectively targeted class Ia molecules, inhibiting HLA-A and HLA-C but not HLA-B expression. Altogether these observations support the hypothesis that US6-resistant presentation of the UL40-

derived peptide together with the restricted action of US2 and US11 may contribute to maintain HLA-E expression during the infection. In that way, the virus should be able to interfere with HLA class Ia antigen presentation to CD8+ lymphocytes, concomitantly protecting infected cells against CD94/NKG2A+ effectors.

Recent observations suggest that the immune system may counteract this evasion strategy. In this regard, it has been shown that some CTL may specifically recognize HLA-E (GARCIA et al. 2002; MORETTA et al. 2003; PIETRA et al. 2001). We originally demonstrated binding of HLA-E tetramers to the TCR of a T cell clone, which specifically killed cells expressing the class Ib molecule associated to some HLA class I and virus-derived peptides (i.e. BZLF1 from EBV) (GARCIA et al. 2002). Mingari et al. have extended these studies, proposing that CTL recognizing HLA-E bound to the UL40 nonamer might have a relevant role in the response against HCMV-infected cells (PIETRA et al. 2003).

## KIR

The killer immunoglobulin-like receptor (KIR) gene family is located in human chromosome 19q13.4; different KIR haplotypes that include partially overlapping sets of genes have been identified (MORETTA and MORETTA 2004; VILCHES and PARHAM 2002). A group of KIRs (i.e. KIR2DL and KIR3DL) display cytoplasmic ITIM which once phosphorylated become docking sites for the SHP-1 protein tyrosine phosphatase involved in inhibitory signalling. Other KIR bear shorter intracytoplasmic domains lacking ITIMs (i.e. KIR2DS/3DS) and, similarly to NKG2C, contain a charged transmembrane residue (Lys) interacting with KARAP/DAP12. Some KIR specifically interact with sets of HLA class Ia allotypes that share structural features at the α1 domain, whereas the ligands for other KIR still remain unknown.

The possibility that HCMV infected cells might preserve HLA-C to escape from KIR-mediated surveillance, as originally proposed for HIV (COHEN et al. 1999), remains unclear. On

one hand, US2 was shown to bind HLA-A but not HLA-E, -B7, -B27 nor HLA-Cw4 molecules (GEWURZ et al. 2001); moreover, HLA-C appeared resistant to US2 and US11 when expressed in a trophoblast cell line (SCHUST et al. 1998). By contrast, endogenous HLA-Cw7 was downregulated in US2+ and US11+ transfected cells (LLANO et al. 2003), and Huard et al described that US11+ targets were sensitive to KIR2DL+ NK cells, indirectly supporting that HLA-C expression was inhibited (HUARD and FRUH 2000). In the same line, Falk et al. reported that downregulation of HLA class I molecules, including HLA-C, did not occur in fibroblasts infected with a deletion mutant lacking the US2-US11 region (FALK et al. 2002).

**CD85j (ILT2, LIR-1)**

The Ig-like transcript (ILT) or leukocyte Ig-like receptor (LIR) gene family flanks the KIR locus at chromosome 19p13.4, encoding for molecules preferentially expressed by the myeloid lineage (COLONNA et al. 1999). Some ILT (LIR, CD85) molecules contain cytoplasmic ITIM that recruit SHP phosphatases, whereas others display a charged transmembrane residue (Arg) and associate to the FcεRγchain. Among the first group, ILT2 (LIR-1, CD85j) and ILT4 (LIR-2) broadly interact with HLA class I molecules (COLONNA et al. 1997; COSMAN et al. 1997). ILT2 is detected on NK and T cell subsets, as well as on B cells and monocytes/macrophages, whereas ILT4 expression is restricted to the latter.

Engagement of inhibitory receptors by HLA class I surrogates expressed in CMV-infected cells constitutes a potential way to subvert the NK cell response. In this regard, the ILT2 receptor was reported to bind the UL18 HCMV HLA class I-like molecule with an affinity higher than that for class I molecules (CHAPMAN et al. 2000; COSMAN et al. 1997). Yet, the hypothesis that UL18 may interfere with NK cell activity during HCMV infection has not received consistent experimental support. Moreover, a reduced susceptibility to NK cell-mediated lysis of HCMV-infected cells was

shown to be independent of UL18 expression (ODEBERG et al. 2002). The possibility that UL18 may act on other CD85j+ cell types (i.e. monocytes/macrophages) should be envisaged.

Remarkably, Leong et al. observed that, rather than conferring protection, UL18 increased susceptibility to NK-mediated lysis; yet, ILT2 was not analysed in that study (LEONG et al. 1998). More recently, Ciccone et al. (SAVERINO et al. 2004) reported that CD8+ T lymphocytes killed in an MHC-unrestricted and TCR-independent manner UL18+ cells; moreover, fibroblasts infected by an HCMV deletion mutant lacking UL18 were resistant to lysis. Strikingly, T cell-mediated cytotoxicity of HCMV+ fibroblasts was inhibited by UL18 and CD85j-specific mAbs though, though only at very late stages of infection (i.e. 6 days). These functional data were interpreted as an indirect indication that a cognate UL18-CD85j interaction might trigger T cell effector functions. This hypothesis requires further experimental support to confirm the ability of CD85j to activate T lymphocytes and, eventually, to define the signalling pathway(s) involved. The identification of other triggering receptor(s) specific for UL18 might also contribute to explain the observations. It is of note that CD85j expression was shown to be increased in PBL from patients undergoing HCMV infection after lung transplantation (BERG et al. 2003); in the same line, we observed that the proportions of CD85j+ T lymphocytes were raised in HCMV+ individuals (GUMA et al. 2004). The mechanisms underlying the impact of HCMV infection on CD85j expression should be explored. UL18 polymorphisms have been reported to influence its interaction with ILT2 (VALES-GOMEZ et al. 2005)

Preliminary reports point out that HCMV may synthesize additional class I-like molecules to interfere with the NK-mediated response (Sissons et al. and Wang et al. reported at the 29th Annual International Herpesvirus Workshop, Reno, Nevada. July 2004); whether these proteins engage inhibitory NKR is as yet unknown. It is of note that the corresponding genes (i.e. UL141 and UL142) can be found in HCMV clinical isolates but are deleted in commonly used laboratory strains

(i.e. AD169 and Towne). Recently, UL141 has been described to exert a blocking effect on the expression of CD155, a ligand for the DNAM-1 activating receptor (TOMASEC et al. 2005)

## Activating NK cell receptors in the response to HCMV

The nature of the cellular ligands for triggering human NK cell receptors has been only partially unraveled. Some of them appear to be constitutively expressed by target cells (i.e. HLA class I molecules), others are inducible under stress conditions and can be detected in virus-infected and tumour cells (i.e. MICA/B), whereas a third category remains unknown. Although the possibility that MHC class I molecules bound to foreign peptides may be efficiently recognized by triggering NKR remains theoretical, studies in mice point out that some NK activating receptors may recognize pathogen-derived molecules.

## NKG2D

Human NKG2D (hNKG2D) is a lectin-like molecule expressed by NK and T cells, that is coupled to a PI3K signalling pathway through the DAP10 adapter (VIVIER et al. 2002; WU et al. 1999). NKG2D has been reported to function either as a triggering receptor (BILLADEAU et al. 2003), or a costimulatory molecule in conjunction with other PTK-linked receptors (GROH et al. 2001; WU et al. 2002). Like its murine homologue, hNKG2D interacts with stress-inducible molecules, which are also detected in some transformed and virus-infected cells. Several class I-related ligands have been defined for human NKG2D, including the polymorphic MICA/B molecules and a family of proteins termed "UL16-binding proteins" (ULBP) or retinoic acid early inducible-1 (RAE-1)-like (BACON et al. 2004; BAUER et al. 1999; CHALUPNY et al. 2003; COSMAN et al. 2001; RAULET 2003).

NKG2D ligands are expressed in CMV-infected cells and co-stimulate virus-specific CTL (GROH et al. 2001; RAULET 2003). The existence of viral escape mechanisms that target NKG2D function indirectly illustrates the importance of this mechanism of response to HCMV. In this

regard, the UL16 glycoprotein inhibits surface expression of MICB, ULBP1, ULBP2 (VALES-GOMEZ et al. 2003; WELTE et al. 2003) and also interacts with RAET1G (BACON et al. 2004), thus potentially interfering with the NKG2D-mediated response. Upon infection with a UL16 deletion mutant, all ULBP molecules were expressed at the cell surface leading to an increase in NKG2D-mediated lysis (ROLLE et al. 2003). On the other hand, Oderberg et al. have proposed that UL16 may also exert a direct protective effect against cytolytic mediators released by NK cells (ODEBERG et al. 2003).

**Natural cytotoxicity receptors (NCR)**

Several Ig-like natural cytotoxicity receptors (NCR) connected to PTK signaling pathways have been shown to trigger NK cell functions (MORETTA et al. 2001). NKp46 is coupled to the $\zeta$ or $\gamma$ adapters, activating cytotoxicity and cytokine production upon recognition of still undefined cellular ligand(s). The nature of the molecules recognized by the DAP12-associated NKp44 and the $\zeta$-linked NKp30 NCR also remain unknown. Although the role of NCR in the defense against HCMV remains uncertain, the putative expression of NCR ligands by different cell types suggests that these receptors might contribute to the response against virus-infected cells that have downregulated HLA class I molecules. On the other hand, the possibility that quantitative/qualitative changes in expression of NCR ligands may take place during HCMV infection cannot be excluded. The involvement of additional activating receptors (MORETTA et al. 2004) in the response to HCMV should be also explored. Recently, pp65 has been shown to interact with NKp30, inhibiting NK cell function (ARNON et al. 2005).

**CD94/NKG2C**

Most inhibitory receptor families include activating molecules whose physiological role remains unclear. It has been hypothesized that KIR2DS/3DS and CD94/NKG2C receptors may contribute to

trigger cytotoxicity and cytokine production when the dominant control by inhibitory receptors falls beneath a critical threshold (LOPEZ-BOTET et al. 2000). As the affinity of stimulatory NKR for class I molecules appears lower than that of the inhibitory counterparts, either a selective down-modulation of the inhibitory ligand and/or an increase of the activating NKR avidity for their ligands would be required. The first situation may take place in HCMV-infected cells, where HLA-E molecules appear to be selectively spared from the action of US proteins. With regard to the second possibility, there is no evidence for the existence of class I-peptide complexes or other ligands recognized with high affinity by the activating NKR. Nevertheless, the hypothesis that some activating NKR may directly interact with microbial products has gained experimental support. Ly49H associates to DAP12 and plays a pivotal role in the defence against MCMV infected cells, triggering NK cell functions upon its interaction with the m157 viral protein (ARASE et al. 2002; SMITH et al. 2002).

As observed for Ly49H expression during MCMV infection (DOKUN et al. 2001), it is conceivable that HCMV might shape the NKR repertoire and the distribution of NK cell subsets. In this regard, increased proportions of CD94/NKG2C+ NK and T cells were detected in HCMV+ individuals (GUMA et al. 2004), presumably reflecting the challenge exerted by the virus on the innate immune system, and suggesting that they might participate in the response to the pathogen. In contrast to the CD94/NKG2A+ subset, most CD94/NKG2C+ cells co-expressed KIR and CD85j, displaying lower levels of NCR. Detection of CD85j+ CD94/NKG2C- cells suggests that both phenotypic features are independently associated to HCMV infection.

CD94/NKG2C+ T lymphocytes populations generally displayed a TCRαβ+ CD8+ CD56+ CD28- phenotype and appear to be oligoclonal; yet, NKG2C+ TCRαβ and rare NKG2C+ CD4+ cells were also detectable in some donors (GUMA et al. 2005). The antigen specificity of NKG2C+ cells is uncertain. It is of note that most HCMV-specific CTL identified with HLA-A*0201/pp65 tetramers did not express NKG2C (GUMA et al. 2004). Moreover, despite that some CD94/NKG2C+ cells may

correspond to HLA-E-specific CTL (GARCIA et al. 2002), this association is not a general finding as NKG2C was reported to be undetectable in HLA-E-specific CTL (PIETRA et al. 2003).

Comparably to the response induced in NK cells, specific engagement of the CD94/NKG2C lectin-like receptor was observed to trigger the proliferation and effector functions of a subset of CD94/NKG2C+ CD8+ T cells; moreover, the KARAP/DAP12 adapter protein was detected in CD94/NKG2C+ T cell clones (GUMA et al. 2005). Altogether these results supported that the KLR may potentially constitute an autonomous activation pathway alternative to the TCR, stimulating the response of NKG2C+ T lymphocytes against HCMV infected cells.

Several mechanisms may account for the variable increase of CD94/NKG2C+ cells in HCMV+ individuals. First, changes in the NKR repertoire might result from alterations in the cytokine network secondary to the viral infection. IL-21 has been shown to promote the expression of NCR and NKR during the NK cell differentiation from CD34+ precursors (SIVORI et al. 2003); moreover, TGF ₋ and IL-15 induce CD94/NKG2A expression in T cells (MINGARI et al. 1998). On the other hand, as observed for Ly49H+ cells, CD94/NKG2C-mediated recognition of HCMV-infected cells could promote the expansion/survival of the corresponding NK and T cell subsets. As stressed above, this would require an increased avidity of the KLR-ligand interaction and/or a selective loss of ligands for inhibitory receptors co-expressed by CD94/NKG2C+ cells (i.e. KIR, ILT2). The preservation of HLA-E bound to the UL40-derived peptide in HCMV-infected cells could favour a response of CD94/NKG2C+ lymphocytes; studies are in progress to address these key questions.

Though HCMV and MCMV are quite disparate, CD94/NKG2 receptors are conserved in mice and specifically recognize Qa1[b], a functional homologue of HLA-E (VANCE et al. 1999). Studies are required to evaluate whether MCMV infection may target the expression of Qa1 or have any impact on the expression of NKG2C.

**Concluding remarks**

A number of questions regarding the involvement of NKR in the innate immune response to HCMV remain open. Among them, the existence of human triggering receptors capable of driving the NK cell response to HCMV upon recognition of virus-encoded proteins or peptides is uncertain. The identification of NCR ligands becomes essential to define their putative participation in the response against HCMV. The analysis of CD94/NKG2C expression may become an additional useful parameter to explore the host-pathogen relationship, and studies of the NKR repertoire in clinical settings involving HCMV are warranted. On the other hand, further studies are required to understand the implications of the UL18-CD85j interaction in the response to HCMV, and the role in immune evasion of the other class I-like genes deserves attention.

**REFERENCES**

Adler H, Messerle M, Koszinowski UH (2003) Cloning of herpesviral genomes as bacterial artificial chromosomes. Rev Med Virol 13:111-121

Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL (2002) Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 296:1323-1326

Arnon TI, Achdout H, Levi O, Markel G, Saleh N, Katz G, Gazit R, Gonen-Gross T, Hanna J, Nahari E, Porgador A, Honigman A, Plachter B, Mevorach D, Wolf DG, Mandelboim O (2005) Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol 6:515-523

Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J (2004) Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. J Immunol 173:1078-1084

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress- inducible MICA. Science 285:727-729

Berg L, Riise GC, Cosman D, Bergstrom T, Olofsson S, Karre K, Carbone E (2003) LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients. Lancet 361:1099-1101

Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ (2003) NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat Immunol 4:557-564

Biron CA, Brossay L (2001) NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 13:458-464

Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med 187:813-818

Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391:795-799

Cerboni C, Mousavi-Jazi M, Linde A, Soderstrom K, Brytting M, Wahren B, Karre K, Carbone E (2000) Human cytomegalovirus strain-dependent changes in NK cell recognition of infected fibroblasts. J Immunol 164:4775-4782

Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR (1996) Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol 70:78-83

Chalupny NJ, Sutherland CL, Lawrence WA, Rein-Weston A, Cosman D (2003) ULBP4 is a novel ligand for human NKG2D. Biochem Biophys Res Commun 305:129-135

Chang C, Rodríguez A, Carretero M, López-Botet M, Phillips JH, Lanier LL (1995) Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. Eur J Immunol 25:2433-2437

Chapman TL, Heikema AP, West AP, Bjorkman PJ (2000) Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). Immunity 13:727-736

Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchison CA, III, Kouzarides T, Martignetti JA, . (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol 154:125-69.:125-169

Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, Baltimore D (1999) The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity 10:661-671

Colonna M, Nakajima H, Navarro F, Lopez-Botet M (1999) A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. J Leukoc Biol 66:375-381

Colonna M, Navarro F, Bellón T, Llano M, García P, Samaridis J, Angman L, Cella M, López-Botet M (1997) A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med 186:1809-1818

Cosman D, Fanger N, Borges L, Kubin M, Chin W, Peterson L, Hsu ML (1997) A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity 7:273-282

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14:123-133

Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM (2001) Specific and nonspecific NK cell activation during virus infection. Nat Immunol 2:951-956

Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, Hahn G (2002) NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. J Immunol 169:3257-3266

French AR, Yokoyama WM (2003) Natural killer cells and viral infections. Curr Opin Immunol 15:45-51

Furman MH, Dey N, Tortorella D, Ploegh HL (2002) The human cytomegalovirus US10 gene product delays trafficking of major histocompatibility complex class I molecules. J Virol 76:11753-11756

Garcia P, Llano M, de Heredia AB, Willberg CB, Caparros E, Aparicio P, Braud VM, Lopez-Botet M (2002) Human T cell receptor-mediated recognition of HLA-E. Eur J Immunol 32:936-944

Gewurz BE, Wang EW, Tortorella D, Schust DJ, Ploegh HL (2001) Human cytomegalovirus US2 endoplasmic reticulum-lumenal domain dictates association with major histocompatibility complex class I in a locus- specific manner. J Virol 75:5197-5204

Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T (2001) Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol 2:255-260

Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M (2004) Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood 104:3664-3671

Guma M, Busch LK, Salazar-Fontana LI, Bellosillo B, Morte C, Garcia P, Lopez-Botet M (2005) The CD94/NKG2C killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8(+) T cells. Eur J Immunol 35:2071-2080

Hegde NR, Chevalier MS, Johnson DC (2003) Viral inhibition of MHC class II antigen presentation. Trends Immunol 24:278-285

Hengel H, Reusch U, Gutermann A, Ziegler H, Jonjic S, Lucin P, Koszinowski UH (1999) Cytomegaloviral control of MHC class I function in the mouse. Immunol Rev 168:167-176

Ho M (1991) Cytomegaloviruses: biology and infection. Plenum Publishing Corp., New York

Houchins JP, Yabe T, McSherry C, Bach FH (1991) DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. J Exp Med 173:1017-1020

Huard B, Fruh K (2000) A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells. Eur J Immunol 30:509-515

Lanier LL (2003) Natural killer cell receptor signaling. Curr Opin Immunol 15:308-314

Lee N, Llano M, Carretero M, Ishitani A, Navarro F, López-Botet M, Geraghty D (1998) HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci U S A 95:5199-5204

Leong CC, Chapman TL, Bjorkman PJ, Formankova D, Mocarski ES, Phillips JH, Lanier LL (1998) Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog [published erratum appears in J Exp Med 1998 Aug 3;188(3):following 614]. J Exp Med 187:1681-1687

Llano M, Guma M, Ortega M, Angulo A, Lopez-Botet M (2003) Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. Eur J Immunol 33:2744-2754

Lopez-Botet M, Angulo A, Guma M (2004) Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens 63:195-203

Lopez-Botet M, Bellon T (1999) Natural killer cell activation and inhibition by receptors for MHC class I. Curr Opin Immunol 11:301-307

Lopez-Botet M, Bellon T, Llano M, Navarro F, Garcia P, de Miguel M (2000) Paired inhibitory and triggering NK cell receptors for HLA class I molecules. Hum Immunol 61:7-17

Michaelsson J, Teixeira dM, Achour A, Lanier LL, Karre K, Soderstrom K (2002) A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. J Exp Med 196:1403-1414

Mingari MC, Moretta A, Moretta L (1998) Regulation of KIR expression in human T cells: a safety mechanism that may impair protective T-cell responses. Immunol Today 19:153-157

Mocarski ES, Courcelle CT (2001) Cytomegaloviruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA (eds) Lippincott Williams & Wilkins, Philadelphia, pp 2629-73

Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19:197-223.:197-223

Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A (2004) Different checkpoints in human NK-cell activation. Trends Immunol 25:670-676

Moretta L, Moretta A (2004) Killer immunoglobulin-like receptors. Curr Opin Immunol 16:626-633

Moretta L, Romagnani C, Pietra G, Moretta A, Mingari MC (2003) NK-CTLs, a novel HLA-E-restricted T-cell subset. Trends Immunol 24:136-143

O'Callaghan CA, Tormo J, Willcox BE, Braud V, Jakobsen BK, Stuart DI, McMichael A, Green DR (1998) Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E. Mol Cell 1:531-541

Odeberg J, Browne H, Metkar S, Froelich CJ, Branden L, Cosman D, Soderberg-Naucler C (2003) The human cytomegalovirus protein UL16 mediates increased resistance to natural killer cell cytotoxicity through resistance to cytolytic proteins. J Virol 77:4539-4545

Odeberg J, Cerboni C, Browne H, Karre K, Moller E, Carbone E, Soderberg-Naucler C (2002) Human cytomegalovirus (HCMV)-infected endothelial cells and macrophages are less susceptible to natural killer lysis independent of the downregulation of classical HLA class I molecules or expression of the HCMV class I homologue, UL18. Scand J Immunol 55:149-161

Pass RF (2001) Cytomegalovirus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA (eds) Lippincott, Williams & Wilkins, Philadelphia, pp 2675-705

Pietra G, Romagnani C, Falco M, Vitale M, Castriconi R, Pende D, Millo E, Anfossi S, Biassoni R, Moretta L, Mingari MC (2001) The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR alpha/beta-mediated recognition. Eur J Immunol 31:3687-3693

Pietra G, Romagnani C, Mazzarino P, Falco M, Millo E, Moretta A, Moretta L, Mingari MC (2003) HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. Proc Natl Acad Sci U S A 100:10896-10901

Pignatelli S, Dal Monte P, Rossini G, Landini MP (2004) Genetic polymorphisms among human cytomegalovirus (HCMV) wild-type strains. Rev Med Virol 14:383-410

Raulet DH (2003) Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 3:781-790

Rawlinson WD, Farrell HE, Barrell BG (1996) Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol 70:8833-8849

Rolle A, Mousavi-Jazi M, Eriksson M, Odeberg J, Soderberg-Naucler C, Cosman D, Karre K, Cerboni C (2003) Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol 171:902-908

Saverino D, Ghiotto F, Merlo A, Bruno S, Battini L, Occhino M, Maffei M, Tenca C, Pileri S, Baldi L, Fabbi M, Bachi A, De Santanna A, Grossi CE, Ciccone E (2004) Specific recognition of the viral protein UL18 by CD85j/LIR-1/ILT2 on CD8+ T cells mediates the non-MHC-restricted lysis of human cytomegalovirus-infected cells. J Immunol 172:5629-5637

Scalzo AA (2002) Successful control of viruses by NK cells--a balance of opposing forces? Trends Microbiol 10:470-474

Schust DJ, Tortorella D, Seebach J, Phan C, Ploegh HL (1998) Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. J Exp Med 188:497-503

Sivori S, Cantoni C, Parolini S, Marcenaro E, Conte R, Moretta L, Moretta A (2003) IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur J Immunol 33:3439-3447

Smith HR, Heusel JW, Mehta IK, Kim S, Dorner BG, Naidenko OV, Iizuka K, Furukawa H, Beckman DL, Pingel JT, Scalzo AA, Fremont DH, Yokoyama WM (2002) Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A 99:8826-8831

Strong RK, Holmes MA, Li P, Braun L, Lee N, Geraghty DE (2003) HLA-E Allelic Variants.Correlating differential expression, peptide affinities, crystal structures and termal stabilities. J Biol Chem 278:5082-5090

Tirabassi RS, Ploegh HL (2002) The human cytomegalovirus US8 glycoprotein binds to major histocompatibility complex class I products. J Virol 76:6832-6835

Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, Gadola S, Cerundolo V, Borysiewicz LK, McMichael AJ, Wilkinson GW (2000) Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science 287:1031

Tomasec P, Wang EC, Davison AJ, Vojtesek B, Armstrong M, Griffin C, McSharry BP, Morris RJ, Llewellyn-Lacey S, Rickards C, Nomoto A, Sinzger C, Wilkinson GW (2005) Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol 6:181-188

Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL (2000) Viral subversion of the immune system. Annu Rev Immunol 18:861-926

Ulbrecht M, Martinozzi S, Grzeschik M, Hengel H, Ellwart JW, Pla M, Weiss EH (2000) Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. J Immunol 164:5019-5022

Vales-Gomez M, Browne H, Reyburn HT (2003) Expression of the UL16 glycoprotein of Human Cytomegalovirus protects the virus-infected cell from attack by natural killer cells. BMC Immunol 4:4

Vales-Gomez M, Shiroishi M, Maenaka K, Reyburn HT (2005) Genetic variability of the major histocompatibility complex class I homologue encoded by human cytomegalovirus leads to differential binding to the inhibitory receptor ILT2. J Virol 79:2251-2260

Vance RE, Jamieson AM, Raulet DH (1999) Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. J Exp Med 190:1801-1812

Vilches C, Parham P (2002) KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol 20:217-251

Vivier E, Anfossi N (2004) Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. Nat Rev Immunol 4:190-198

Vivier E, Tomasello E, Paul P (2002) Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? Curr Opin Immunol 14:306-311

Wang EC, McSharry B, Retiere C, Tomasec P, Williams S, Borysiewicz LK, Braud VM, Wilkinson GW (2002) UL40-mediated NK evasion during productive infection with human cytomegalovirus. Proc Natl Acad Sci U S A 99:7570-7575

Welte SA, Sinzger C, Lutz SZ, Singh-Jasuja H, Sampaio KL, Eknigk U, Rammensee HG, Steinle A (2003) Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol 33:194-203

Wu J, Groh V, Spies T (2002) T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. J Immunol 169:1236-1240

Wu J, Song Y, Bakker AB, Bauer S, Spies T, Lanier LL, Phillips JH (1999) An activating immunoreceptor complex formed by NKG2D and DAP10. Science 285:730-732

Yabe T, McSherry C, Bach FH, Fisch P, Schall RP, Sondel PM, Houchins JP (1993) A multigene family on human chromosome 12 encodes natural killer- cell lectins. Immunogenetics 37:455-460

# CAPÍTULO 3

## Objetivos

1.- Analizar la expresión de receptores de células NK en relación con la infección por citomegalovirus

2.- Estudiar el papel del receptor CD94/NKG2C en la infección por citomegalovirus.

3.- Estudiar la expresión y función del receptor CD94/NKG2C en células Tαβ+CD8+.

# CAPÍTULO 4

## Imprint of human cytomegalovirus infection on the NK cell receptor repertoire

Gumà M, Angulo A, Gómez-Lozano N, Malats N, Vilches C, López-Botet M

**ABSTRACT**

Expression of the activating CD94/NKG2C killer lectin-like receptor (KLR) specific for HLA-E was analysed in peripheral blood lymphocytes (PBL) from healthy adult blood donors; the expression of other NK cell receptors (i.e. CD94/NKG2A, KIR, CD85j, CD161, NKp44, NKp46, NKp30 and NKG2D) was also studied. Human cytomegalovirus (HCMV) infection, as well as the HLA-E and KIR genotypes were considered as potentially relevant variables associated with CD94/NKG2C expression. The proportion of NKG2C+ lymphocytes varied within a wide range ($\leq$0.1-22.1%), and a significant correlation (r=0.83; p<0.001) between NKG2C+ NK and T cells was noticed. The HLA-E genotype and the number of activating KIR genes of the donors were not significantly related to the % of NKG2C+ lymphocytes. By contrast, a positive serology for HCMV, but not for other herpesviruses (i.e. Epstein-Barr and Herpes Simplex), turned out to be strongly associated (p<0.001) with increased proportions of NKG2C+ NK and T cells. Remarkably, the CD94/NKG2C+ population expressed lower levels of NCR (i.e. NKp30, NKp46) and included higher proportions of KIR+ and CD85j+ cells than CD94/NKG2A+ cells. Altogether, these data support that HCMV infection selectively shapes the NKR repertoire of NK and T cells from healthy carrier individuals.

**INTRODUCTION**

Killer immunoglobulin-like receptors (KIR), CD94/NKG2 killer lectin-like receptors (KLR) and CD85j (ILT2, LIR1, LILRB1) specifically recognize HLA class I molecules and are expressed by Natural Killer and T cell subsets [1-4]. Single cells bear variable combinations of these NK cell receptors (NKR), presumably resulting from stochastic gene activation/silencing events that take place during their maturation [5]. The diversity of NKR observed in different individuals is in part genetically determined, as distinct KIR haplotypes include variable sets of genes [1]. On the other hand, there is evidence that microbial infections may also influence the NKR repertoire. In this regard, murine cytomegalovirus (MCMV) promotes an expansion of NK cells bearing the Ly49H receptor specific for the m157 viral glycoprotein [6], that play a crucial role in the immune response to infection [7-9]. Moreover, increased proportions of CD8+ T cells with an effector/memory phenotype bearing inhibitory NKR (i.e. CD94/NKG2A) have been observed in mice infected by different viruses [10-12] as well as in human immunodeficiency virus (HIV)-infected patients [13].

CD94, NKG2A and NKG2C are C-type lectins encoded at the NK gene complex (NKC) in human chromosome 12 [14]; surface expression of NKG2A/C molecules requires their covalent assembly with CD94 [4, 15, 16]. The CD94/NKG2A heterodimer constitutes an inhibitory receptor that recruits the SHP-1 tyrosine phosphatase through the ITIM-bearing NKG2A subunit, whereas CD94/NKG2C is coupled to a tyrosine kinase activation pathway through the DAP12 adapter [17, 18]. In humans, both NKR specifically recognize HLA-E, that presents peptides derived from the signal sequences of other HLA class I molecules [19-22]; HLA-E allotypes contain either an Arg (HLA-E$^{R107}$) or a Gly (HLA-E$^{G107}$) at position 107 [23]. The biological relevance of such structural dimorphism remains unclear but it may affect surface expression levels of the class Ib molecule [24, 25] and its interaction with CD94/NKG2 receptors [26].

A number of studies have addressed the characterization of CD94/NKG2A in NK and T cells, whereas little is known about the biological role of the CD94/NKG2C dimer. According to the

current hypotheses proposed to interpret the function of activating NKR, either a selective down-modulation of the ligands for inhibitory receptors expressed by CD94/NKG2C+ cells or/and an increased avidity of CD94/NKG2C interaction with infected cells might favour NK cell activation through that pathway [4]. In support of the first possibility, HLA-E appears selectively preserved from the action of some human cytomegalovirus (HCMV) proteins that interfere with HLA class Ia expression [27, 28]. Moreover, the class Ib molecule can be stabilized by a peptide derived from the UL40 HCMV protein [29, 30], potentially allowing the pathogen to evade the response mediated by CD94/NKG2A+ cells.

In the present study we analysed CD94/NKG2C expression in peripheral blood mononuclear cells (PBL) from healthy adult blood donors; moreover, other NKR (i.e. CD94/NKG2A, KIR, CD85j, CD161, NKG2D) and triggering NKp46 and NKp30 Natural Cytotoxicity Receptors (NCR) [31] were also studied. HCMV infection as well as the HLA-E and KIR genotypes were assessed as potentially relevant variables related to CD94/NKG2C expression. Our observations support that HCMV, but not other herpesviruses (Epstein-Barr, EBV; and Herpes Simplex, HSV), shapes the NKR repertoire of healthy carrier individuals promoting an expansion of CD94/NKG2C+ NK and T cells.

## MATERIALS AND METHODS

### Subjects.

Blood samples derived from a cohort of 70 healthy adult individuals, including 51 males and 19 females (age:20-56 years, median=27) were analysed. Written informed consent was obtained from every donor, and the study protocol was fully approved by the Ethics Committee. Standard clinical diagnostic tests were used to analyse serum samples from blood donors for circulating IgG antibodies against CMV (Abbot Laboratories, Abbot Park, IL), HSV and EBV (Trinity Biotech Plc, Jamestown, NY).


### Antibodies and reagents.

HP-3B1 anti-CD94, HP-1F7 anti-HLA-class I, HP-MA3 anti-KIR2DL1/S1, HP-3G10 anti CD161 and HP-F1 anti-CD85j mAbs were generated in our laboratory and have been previously reported [32,33]. Z199 anti-CD94/NKG2A [15], p25 anti-CD94/NKG2A/NKG2C [34], C218 anti-CD56, KD1 anti-CD16, AZ20 anti-NKp30, Bab281 anti-NKp46, and OM72 anti-NKG2D mAbs were generously provided by Dr. A. Moretta (University of Genova, Italy) [31]. Dx9 anti-KIR3DL1 mAb was provided by Dr. L. Lanier (UCSF, San Francisco, CA, USA). CH-L anti-KIR2DL2/S2/L3 was provided by Dr. S. Ferrini (University of Genova, Italy). Anti NKG2C mAb (MAB1381) was from R&D systems Inc (Minneapolis, MN); biotin-labeling of MAB1381 was carried out using EZ-Link Sulfo-NHS-Biotin (Pierce. Rockford, IL) according to the manufacturer instructions. Z199 (anti-NKG2A) mAb was conjugated to Fluorescein isothiocyanate (FITC) (Sigma, St Louis, MO). Indirect immunofluorescence analysis was carried out with Phycoerythrin (PE) or Fluorescein isothiocyanate (FITC)-tagged F(ab')2 rabbit anti-mouse Ig antibodies (Dakopatts, Glostrup, Denmark). Anti CD3-Pcep, CD56-PE and streptavidin-FITC were from BD Biosciences Pharmingen (San Jose, CA).

CMV-specific T cells were detected with an R-phycoerythrin-labelled HLA-A*0201 tetramer bound to the HLA A*0201-restricted NLVPMVATV peptide (amino acids 495-503 of the lower matrix protein, pp65) (Proimmune, Oxford, UK).

**Immunofluorescence and flow cytometry analysis.**

Heparinized peripheral blood was obtained by venous puncture. Peripheral blood mononuclear cell (PBMC) were isolated using a Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation. For indirect immunofluorescence staining, cells were pretreated with saturating concentrations of human aggregated Ig to block FcR, and subsequently labeled with saturating concentrations of the different mAb according to the protocols detailed below. Samples were subsequently analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). For multicolor staining the following procedures were used: Protocol 1- Cells were incubated with anti NKG2A/C (p25) mAb followed by washing and labelling with PE-tagged F(ab')2 rabbit anti-mouse Ig antibody (Dako); subsequently, samples were incubated with anti the Z199 anti NKG2A-FITC and anti CD3Pcep (BD Biosciences Pharmingen). Protocol 2- Cells were incubated with individual anti NKR or NCR MAbs followed by washing and labelling with FITC-tagged F(ab')2 rabbit anti-mouse Ig antibody (Dako); subsequently samples were incubated with anti CD56-PE and anti CD3Pcep (BD Biosciences Pharmingen). Protocol 3- Cells were incubated with either anti NKp46, NKp30, CD85j, NKG2D or a mixture of anti KIR (2DL1/S1, 2DL2/S2/L3 and 3DL1) mAbs, followed by washing and labelling with PE-tagged F(ab')2 rabbit anti-mouse Ig antibody (Dako); subsequently samples cells were incubated with either anti-NKG2A-FITC or anti-NKG2C-Biotin+streptavidin-FITC (BD Biosciences Pharmingen) and with anti CD3-Pcep (BD Biosciences Pharmingen).

**Extraction, amplification of genomic DNA and DNA Sequencing**

DNA was isolated from total blood using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, Minnesota). An HLA-E gene sequence comprised between exons 2 and 3 was amplified by the polymerase chain reaction (PCR) using the following primers: 2FHLAE 5' CGC ACA GAT TTT CCG AGT GAA 3' and 382-ALL-AS 5' CCG CCT CAG AGG CAT CAT TTG 3' [23]. PCR reactions were run at 94ºC for 1 min, at 64º for 30 seconds, and 72º C for 30 seconds for 35 cycles, with a final 10-min extension at 72ºC. DNA sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and a 3100 ABI automatic sequencer.

The NKG2C gene sequence comprised between intron1 and exon 3 was amplified using the following primers: FNKG2C 5' GGC ATT GTT CAA CTG TAA TCT GCG 3' and RNKG2C 5' ACC TTT CTG CGT TCT TGT ATT CGG 3'. PCR amplifications were run at 94ºC for 1 min, at 61º for 30 seconds, and 72º C for 1 min 30 for 35 cycles, with a final 10-min extension at 72ºC.

**KIR and HLA genotyping**

KIR gene typing was performed using the PCR-SSP method (PCR with sequence-specific primers) as previously described [35]. Mutant KIR2DS4 alleles bearing a 22-bp deletion in exon 5 were distinguished from normal ones by PCR, using primers that produce amplicons of different lengths for each of these allotypes (Carlos Vilches, unpublished). The mutant KIR2DS4 alleles were not taken into account for estimating the number of activating KIR of each donor. The HLA-A alleles were analysed using the PCR-SSO method (Dynal Biotech Ltd., Wirral, UK).

**Statistical analysis**

Kolmogorov-Smirnov non-parametric test was applied to check the normal distribution of the continuous variables, and either Pearson or Spearman correlation coefficients were computed to

compare levels of continuous variables. To assess the relationship between a categorical variable with two levels and normally or non-normally distributed quantitative variables, Student's and Mann-Whitney U tests were applied, respectively. To determine the independence of HLA-E and CD94/NKG2C expression and the serological status for HCMV, the Kruskall-Wallis and chi-square tests were applied, respectively. Multivariate lineal regression was chosen to assess the relationship between CD94/NKG2C expression and HCMV status considering age as potential confounding variable. The model was then stratified by HLA-E genotypes. Analyses were performed with SPSS 9.0 (SPSS Inc., Chicago, IL, 1999) statistical package. Results were considered significant at the two-sided p of 0.05 level.

**RESULTS**

CD94/NKG2C expression was studied in PBL from a cohort of 70 healthy adult blood donors (51 males and 19 females; age: 20-56, median: 27). To circumvent the unavailability of NKG2C specific reagents, we initially used a combination of anti NKG2A mAb (Z199) and NKG2A/C (p25) mAbs; in that way the relative expression of the inhibitory and triggering CD94/NKG2 receptors was simultaneously assessed. As the NKG2C gene is deleted in some individuals [36], genomic analysis was required to interpret the phenotypic data; NKG2C appeared undetectable by PCR in 2 out of 70 donors that were thus separately considered.

NKG2C+ cells, indirectly defined as p25+ Z199-, were observed to vary within a wide range (≤0.1-22%; mean±SD=1.9±3.5%), being virtually undetectable in approximately 40% of the donors. Three-color immunofluorescence analysis confirmed that the lectin-like dimer was expressed by both CD3+ (≤0.1-5%; 0.6±0.8%) and CD3- (≤0.1-45%; 4.5±8.2%) lymphocyte subsets, the latter including NK cells. Figure 1A shows the distribution of p25+Z199+ and p25+Z199- lymphocytes in two representative donors, illustrating the marked variability of CD94/NKG2C expression.

A commercial NKG2C-specific mAb (MAB1381) became recently available, allowing to compare its staining pattern with that obtained with the Z199/p25 mAb pair in samples from 21 individuals. MAB1381 brightly stained a cell population that did not co-express NKG2A (Fig 1B), and no significant differences were substantiated between the proportions of NKG2C+ cells, defined either as p25+ Z199- or MAB1381$^{bright}$. NKG2C+ NK cells, identified as CD3-CD56+ MAB1381$^{bright}$, represented 25.3±25.1% of total NK cells (range: 2.5-80). This approach revealed that most NKG2A+ PBL do not detectably co-express the NKG2C protein, an important question that could not be solved using the p25/Z199 mAb combination. A minor fraction of dull-stained MAB1381+ cells was also observed even in samples in which p25+Z199- lymphocytes were undetectable (Fig 1B); yet, the significance of this observation is unclear and requires further characterization of the commercial mAb. Interestingly, a significant correlation (r=0.83, p<0.001)

between the proportions of NKG2C+CD3+ and either NKG2C+CD3- or NKG2C+CD3-CD56+ cells was observed, suggesting that the regulatory event(s) controlling the expression of the activating KLR act concomitantly on both NK and T cell lineages; conversely, such a correlation was not substantiated for NKG2A+ cells.
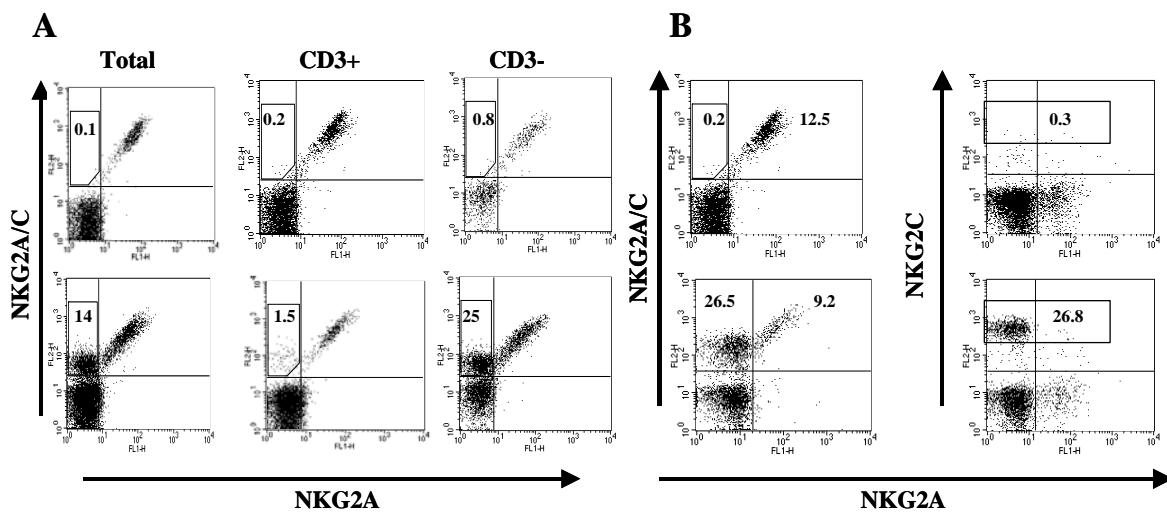


**Figure 1. Comparative analysis of CD94/NKG2C and CD94/NKG2A expression in PBL from normal blood donors**. (A) PBL were stained with anti NKG2A/C mAb (p25), NKG2A (Z199) and CD3 mAbs (see protocol 1). Samples were analyzed by flow cytometry and the proportions of NKG2A+ and NKG2C+ cells (p25+ Z199-) were calculated in total PBL, as well as in gated CD3+ and CD3- populations. The staining patterns in samples from two different individuals (upper and lower histograms), representative of the variability in NKG2C expression are displayed. (B) PBL stained with a combination of anti NKG2A (Z199) and either NKG2A/C (p25) or NKG2C-specific (MAB1381) mAbs (see protocols 1 and 2) were comparatively analyzed by flow cytometry. The staining patterns observed in two different individuals (upper and lower panels) are displayed.

Blood donors were classified according to the detection of HCMV-specific circulating IgG into two groups: HCMV+ (n=34) and HCMV- (n=34); it is of note that according to previous studies some carriers may appear seronegative [37, 38]. A striking association between the detection of NKG2C+ cells and HCMV-specific antibodies was noticed (p<0.001). As shown in Table I and Figure 2, the proportions of NKG2C+ cells, including CD3- and CD3+ subsets, were significantly increased in HCMV+ donors; by contrast, no relationship was observed between the serological status for HCMV and the percentage of NKG2A+ or CD94+ cells (data not shown). Consequently, the NKG2A/NKG2C ratio also varied within a broad range and appeared significantly reduced among HCMV+ individuals (Table I). Despite the strong association between the serological status for HCMV and expression of the activating KLR, NKG2C+ cells were <0.5% in 9 HCMV+ and ≥0.5% in 5 HCMV- donors, the latter eventually corresponding to seronegative carriers. Thus, a clear-cut threshold in the proportions of NKG2C+ cells discriminating HCMV+ and HCMV- subjects could not be precisely established. A significant correlation was observed between donor age and the percentage of NKG2C+ cells (p=0.026), although it disappeared when the HCMV variable was jointly considered in the model (p=0.232).

The clear-cut relationship between HCMV serology and increased proportions of circulating NKG2C+ NK and T cells indirectly supported that the viral infection may shape the NKR repertoire in healthy carriers. Remarkably, analyses performed in PBL from 31 individuals, including HCMV+ (n=17) and HCMV- (n=14), indicated that the numbers of circulating NKG2C+ cells were unrelated to the serological status for EBV or HSV (Table II) and, in fact, all HCMV- cases were either EBV+ and/or HSV+. These results point out that the driving force(s) that underlie the expansion of NKG2C+ cells do not operate as a general response to all latent herpesvirus infections.

**A**



**C**

**B**
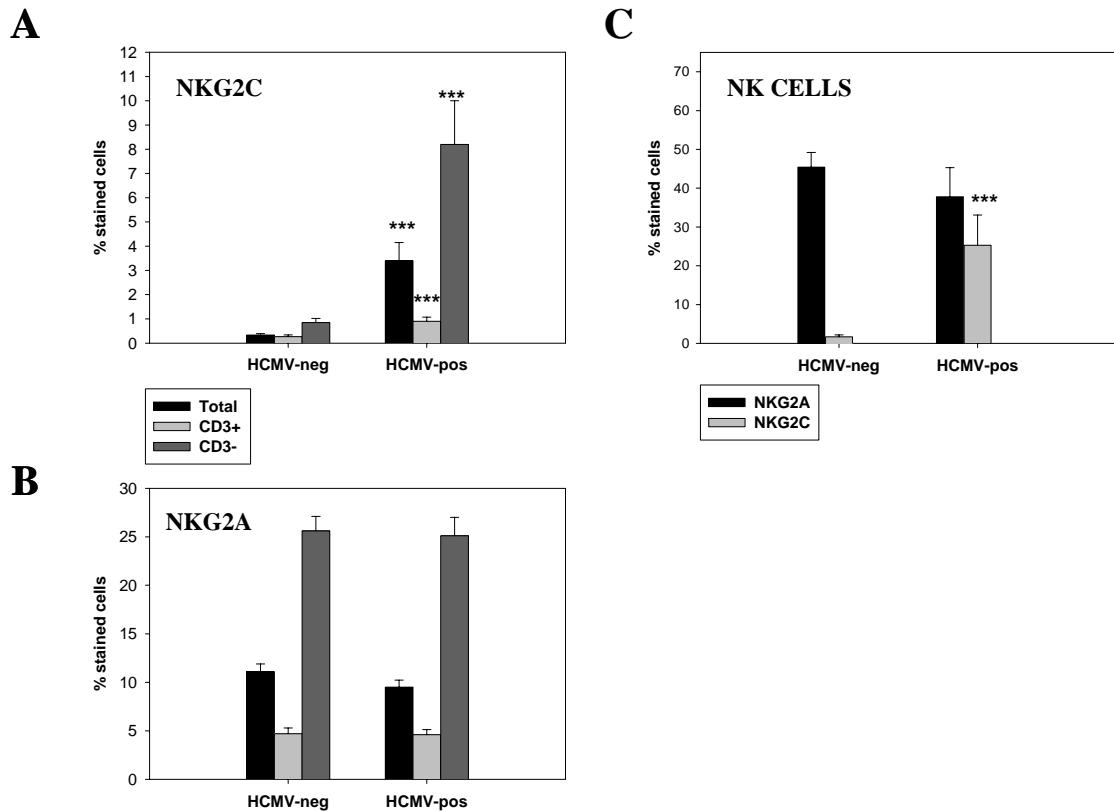


**Figure 2. Expansion of CD94/NKG2C+ cells in PBL from HCMV+ donors.** Blood donors were classified in two groups (HCMV+ and HCMV-) according to the detection of circulating HCMV-specific IgG; PBL samples were analyzed as described (see Figure 1). (A-B) The proportions of NKG2A+ (Z199+) and NKG2C+ (p25+Z199-) cells detected in total PBL, as well as in gated CD3+ and CD3- subsets, from 68 different individuals are shown (mean ± SEM). (C) The % of NK cells (CD3- CD56+) expressing NKG2A or NKG2C in PBL from 21 HCMV+ and HCMV- donors are displayed (mean ± SEM). Statistical analysis was carried out as described in Material and Methods; (*) $p<0.05$, (**) $p<0.01$, (***) $p <0.001$ .

**Table I. Relation of CD94/NKG2C expression and HCMV serology.**

| Receptor[1] | Subset[1] | HCMV status[2] | n | %stained cells[3] | p[4] |
|---|---|---|---|---|---|
| **NKG2A** | Total | Negative | 34 | 11.1±4.5 (4.3-23) | |
| | | Positive | 34 | 9.5±4.2 (1.3-18) | 0.2 |
| | CD3+ | Negative | 34 | 4.7±3.4 (1.2-18) | |
| | | Positive | 34 | 4.6±3 (1-14) | 0.8 |
| | CD3- | Negative | 34 | 25.6±8.7 (10-44) | |
| | | Positive | 34 | 25.1±11.3 (1.6-56) | 0.8 |
| | CD3-CD56+ | Negative | 11 | 45.4±12.5 (23.5-62.7) | |
| | | Positive | 8 | 37.8±21 (12.3-75) | 0.4 |
| **NKG2C** | Total | Negative | 34 | 0.33±0.4 (0.1-1.7) | |
| | | Positive | 34 | 3.5±4.4 (0.1-22.1) | <0.001 |
| | CD3+ | Negative | 34 | 0.3±0.5 (0.1-1.9) | |
| | | Positive | 34 | 0.9±1(0.1-5) | <0.001 |
| | CD3- | Negative | 34 | 0.9±1 (0.1-5.9) | |
| | | Positive | 34 | 8.2±10.4 (0.1-45) | <0.001 |
| | CD3-CD56+ | Negative | 11 | 1.7±1.6 (0.1-6) | |
| | | Positive | 10 | 25.3±25.1 (2.5-80) | <0.001 |
| **NKG2A/NKG2C** | Total | Negative | 34 | 60.7±44.5 (6.4-163) | |
| **ratio** | | Positive | 34 | 16.1±29.4 (0.29-161) | <0.001 |

(1) NKG2A+ (Z199+) and NKG2C+ (p25+ Z199- or MAB1381+) cell subsets were defined by flow cytometry.
(2) Donors were classified in two groups according to the detection of serum HCMV-specific IgG
(3) Mean ± sd (range)
(4) Statistical analysis according to the Mann Whitney U test

**Table II. Expression of CD94/NKG2C is unrelated to HSV or EBV serology**

| Serological status[1] | N | % CD94/NKG2C+[2] | p[3] |
|---|---|---|---|
| HCMV | | | |
| Negative | 34 | 0.3±0.4 0.2 [0.1 – 1.7] | <0.001 |
| Positive | 34 | 3.5±4.4 2.2 [0.1 – 22.3] | |
| HSV | | | |
| Negative | 8 | 3.0±4.8 0.6 [0.1 – 11] | 0.39 |
| Positive | 23 | 1.5±2.1 0.3 [0.1 – 6.5] | |
| EBV | | | |
| Negative | 6 | 3.4±4.1 1.9 [0.1 – 10.4] | 0.21 |
| Positive | 25 | 1.5±2.6 0.2 [0.1 – 11] | |

(1) Detection of serum IgG specific for HCMV, HSV and EBV
(2) NKG2C+ PBL were defined by flow cytometry. Mean ± sd / median [range]
(3) Analysis according to the Mann Whitney U test.

HLA-E allotypes contain either arginine (HLA-E$^{R107}$) or glycine (HLA-E$^{G107}$) at position 107. It has been reported that these allotypes differ in their expression levels, their sensitivity to the action of the US6 HCMV protein, as well as in their ability to interact with CD94/NKG2 receptors [24-26]. Thus, the possibility that the HLA-E genotype might influence the expression of CD94/NKG2C was also addressed. The HLA-E allotypes were defined by sequencing the specific PCR products amplified from genomic DNA. While HLA-E genotypes were not associated either with the percentage of NKG2C+ cells nor with HCMV serology (Table III), the correlation between NKG2C expression and HCMV serology among HLA-E$^{G107}$ donors was not as strong as among the other HLA-E genotypes: β=1.3 (p=0.097) versus β=3.1 (p=0.010) for HLA-E$^{R107}$, and β=4.4 (p=0.020)

for HLA-E$^{G107}$ HLA-E$^{R107}$. Yet, the small sample size of the HLA-E$^{G107}$ group impairs the interpretation of this result, further studies in a larger population being required to precisely evaluate this effect.

**Table III. CD94/NKG2C expression and HCMV serology according to the HLA-E genotypes**

**HLA-E genotypes[1]**

|  | HLA-E$^R$ (n=25) | HLA-E$^R$ HLA-E$^G$ (n=29) | HLA-E$^G$ (n=10) | p[4] |
|---|---|---|---|---|
| %NKG2C[2] Mean (sd) | 1.97 (2.88) | 2.54 (4.77) | 0.60 (1.10) | 0.15 |
| HCMV[3] | | | | |
| Pos. | 11 | 16 | 6 | 0.60 |
| Neg. | 14 | 13 | 4 | |

(1) HLA-E typing was carried out as described in Methods
(2) % NKG2C+ cells defined by flow cytometry.
(3) Donors were classified in two groups according to the detection of serum HCMV-specific IgG
(4) Analysis according to the to the Kruskal-Wallis test.

The number and identity of KIR encoded in the human genome varies greatly in different individuals, the variation being greatest for activating KIR [1]. Whilst the combination of KIR expressed by NK cell clones appears to be completely stochastic, a bias towards increased expression of CD94/NKG2A has been reported in individuals having lower numbers of functional inhibitory KIR (i.e., lower numbers of self-HLA ligands for the inhibitory KIR encoded in their genomes). To test the possibility of a reciprocal increase of NKG2C expression in individuals having lower numbers of activating KIR, we determined the KIR genotypes of 31 individuals (16 HCMV- and 15 HCMV+) having diverse proportions of NKG2C+ cells (range: ≤0.1–22.3%). Neither a correlation between the number of activating KIR genes of each individual and the proportions of NKG2C+ cells, nor

appreciable deviations between the KIR-gene frequencies of HCMV+ and HCMV- subjects were observed (data not shown). KIR2DS3 was underrepresented among donors carrying <0.5% NKG2C+ cells in comparison with those having higher numbers of these cells (3/29 vs. 11/25), but this deviation was not statistically significant.
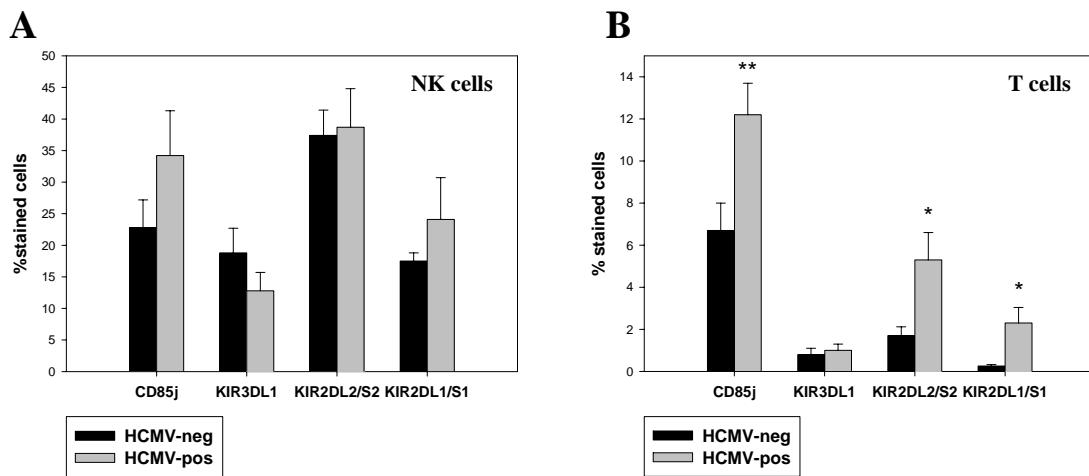


**Figure 3. CD85j and KIR expression in lymphocytes from HCMV+ individuals.** PBL from HCMV+ (n=11) and HCMV- donors (n=13) were stained with CD3 and CD56- specific mAbs in combination with either anti CD85j, KIR3DL1, KIR2DL1/S1 or KIR2DL2/S2/L3 mAbs (see protocol 2). Samples were analyzed by flow cytometry, and the proportions of T (CD3+) and NK (CD3- CD56+) cells expressing the different NKR were calculated (mean ± SEM). According to their KIR genotype, donors lacking KIR2DL1/S1 or KIR2DL2/S2/L3 genes were excluded. Statistical analysis was carried out as described in Material and Methods; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

To explore whether HCMV infection was associated with additional features of the NKR expression pattern, three-colour FACS analysis was performed employing anti CD3 and CD56 mAbs in combination with a panel of reagents specific for NKR (i.e. CD85j, KIR2DL1/S1, KIR2DL2/S2/L3, KIR3DL1, NKG2D, CD161) and NCR (i.e. NKp46, NKp30). The impossibility to

discriminate by flow cytometry between homologous KIR2DL and KIR2DS molecules, due to the cross-reactivity of available mAbs, somehow limits the interpretation of the phenotypic data. The proportions of NK and T cells bearing KIR3DL1 (Figure 3), NKG2D (Figure 4) and CD161 (data not shown) were comparable in HCMV- and HCMV+ subjects. By contrast, a significant increase in the minor fraction of T cells expressing CD85j or KIR2D was observed in samples from HCMV+ individuals (Figure 3B), that tended to display as well higher proportions of CD85j+ and KIR2D+ NK cells (Figure 3A).
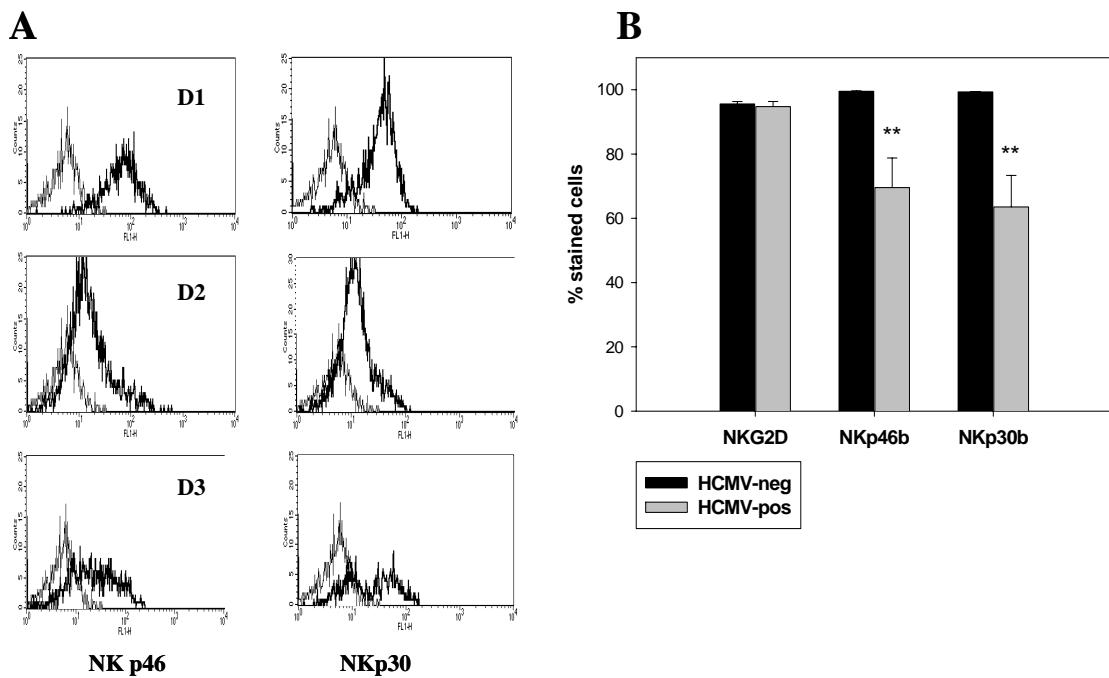


**Figure 4. Expression of NKG2D and NCR in NK cells from HCMV+ individuals.** PBL from HCMV+ (n=11) and HCMV- donors (n= 13) were stained with anti CD3 and CD56- specific mAbs in combination with either NKG2D, NKp46 or NKp30-specific mAbs and subsequently analyzed by flow cytometry (see protocol 2). (A) The different staining patterns of CD3- CD56+ (NK) cells observed with anti NKp46 and NKp30 mAbs are shown, corresponding to samples from three representative donors (D1-D3). (B) The proportions (mean ± SEM) of NKG2D+, NKp46$^{bright}$+ and NKp30$^{bright+}$ NK cells are displayed for each group. The % of NKG2D+ T cells were comparable in HCMV+ and HCMV- donors (data not shown). Statistical analysis was carried out as described in Material and Methods; (*) p<0.05, (**) p<0.01, (***) p <0.001.

NKp46 and NKp30 have been reported to be expressed on virtually all NK cells, but some NK cell subsets have been shown to bear low levels of these NCR and to mediate inefficient cytotoxicity against tumor cells [39]. Thus, the staining intensity with NCR-specific mAbs was considered; Figure 4A shows the different staining patterns observed with NKp46 and NKp30 mAbs. It is of note that NK cells from all HCMV- donors expressed pattern D1, whereas samples from HCMV+ individuals displayed either patterns D1, D2 or D3. Altogether, HCMV+ donors tended to display significantly lower proportions of NKp46 $^{bright}$ and NKp30 $^{bright}$ NK cells (Figure 4B).
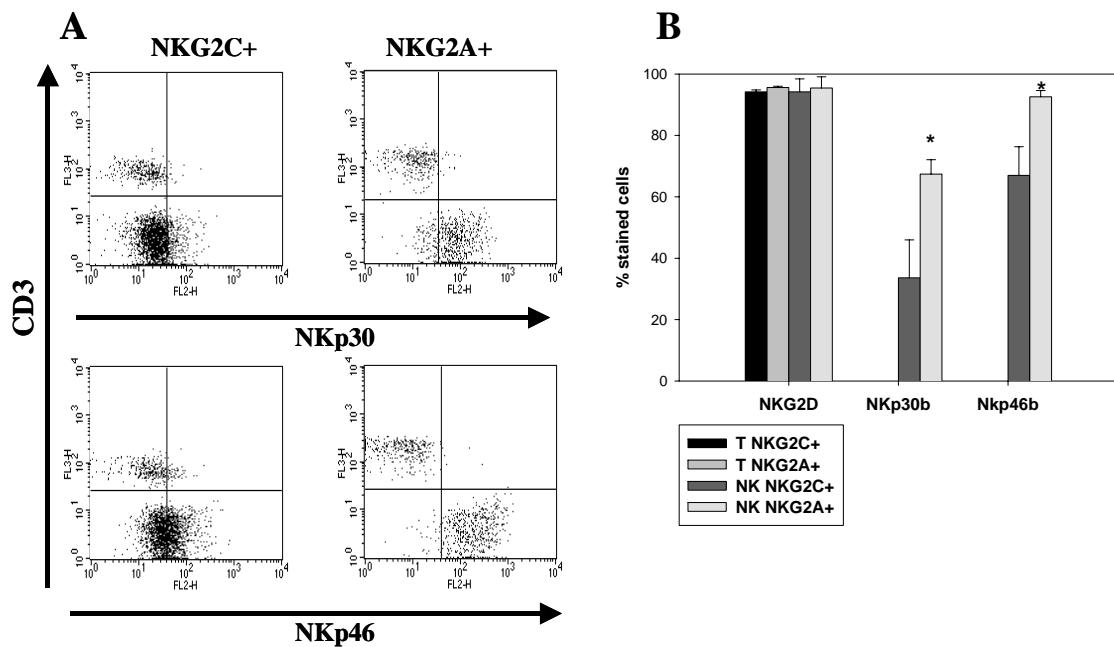


**Figure 5. Comparative analysis of NCR expression in NKG2C+ and NKG2A+ NK cells.** PBL were stained with anti CD3 and either anti NKG2C or NKG2A mAbs, in combination with anti NKp46 or NKp44-specific mAbs. NCR expression was analysed gating on CD3-NKG2C+ and CD3-NKG2A+ cells (see protocol 3). (A) The staining pattern observed in representative case is displayed. (B) The proportions (mean ± SEM) of NKp46$^{bright}$ and NKp30$^{bright+}$ NK cells in PBL samples from 5 different donors are shown. Statistical analysis was carried out as described in Material and Methods; (*) p<0.05, (**) p<0.01, (***) p <0.001.

These data raised the question as to whether the features observed in the NKR repertoire from HCMV+ donors might constitute independent events or rather reflect a coordinated distribution of the other receptors with NKG2C. To address this issue, three-colour flow cytometry analysis was carried out in PBL from 5 HCMV+ donors using the biotin-labeled anti NKG2C mAb in combination with anti CD3 and either NCR, CD85j or KIR-specific mAbs. Remarkably, as compared to NKG2A+ lymphocytes from the same individuals, NKG2C+ cells displayed lower levels of NKp30 and NKp46 NCR (Figure 5) and included higher proportions of KIR+ and CD85j+ lymphocytes (Figure 6), thus supporting the second possibility.
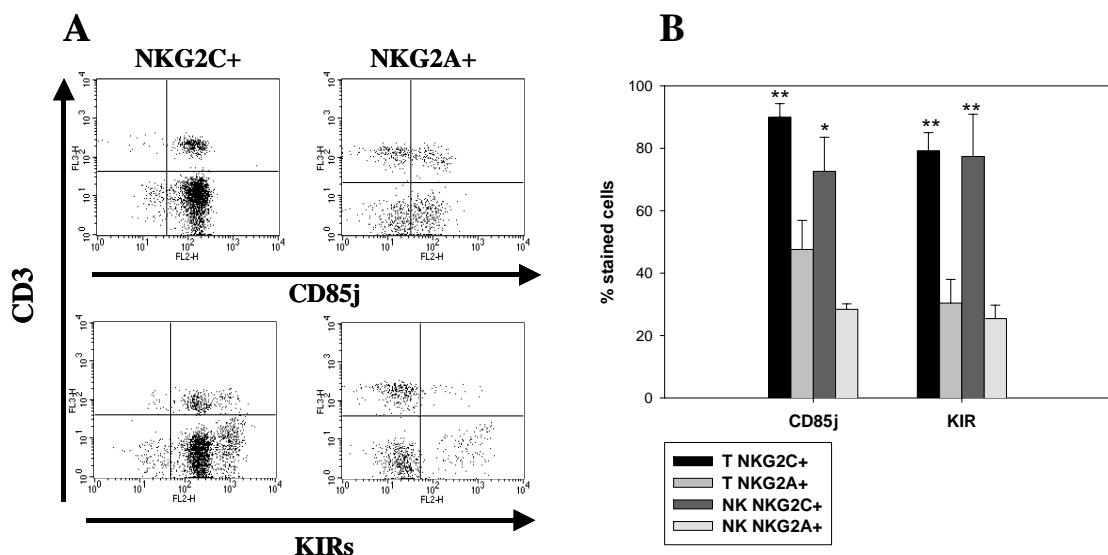


**Figure 6. Comparative analysis of CD85j and KIR expression in NKG2C+ and NKG2A+ NK cells.** PBL were stained with anti CD3 and either anti NKG2C or NKG2A mAbs, in combination with anti CD85j or a mixture of KIR-specific mAbs (KIR3DL1, 2DL1/S1, and 2DL2/S2/L3). NKR expression was selectively analysed in NKG2C+ and NKG2A+ cells, gating on CD3+ and CD3- cells (see protocol 3). (A) The staining pattern observed in a representative case is displayed. (B) The proportions (mean ± SEM) of CD85j+ and KIR+ cells detected in PBL samples from 5 different donors are shown. Statistical analysis was carried out as described in Material and Methods; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

The detection of CD94/NKG2C in T lymphocytes from HCMV+ donors raised the question as to whether such cells might correspond to an expansion of CTL specific for viral antigens. To address this issue, we selected 10 HLA-A*0201 HCMV+ donors and analysed by flow cytometry the expression of NKG2C in T cells stained by HLA-A*0201 tetramers refolded with an immunodominant peptide epitope from the pp65 HCMV lower matrix protein. In every case, most T lymphocytes binding the tetramer did not co-express NKG2C (data not shown). These data do not entirely exclude that the CD94/NKG2C might be preferentially co-expressed by CTL subsets specific for other viral antigens, but strongly suggest that the NKG2C+ cell expansion may occur independently of the TcR specificity.

**DISCUSSION**

HCMV infection is quite prevalent (50-100%) in most populations and it generally follows an indolent course. Yet, HCMV may cause a severe congenital disease and important disorders in immunocompromised individuals; moreover, the virus has been proposed to constitute a co-factor in the development of artheriosclerosis [38]. HCMV infects different cell types and tends to remain latent in immunocompetent hosts, where occasional reactivation allows its dissemination. An effective defence against CMV requires the participation of both NK cells and T cells [40, 41]. HCMV-specific antibodies and circulating CTL specific for viral peptides reflect the adaptive immune response to the virus, thus constituting conventional parameters to assess exposure and response to the pathogen [38, 42, 43]. Our data provide a first evidence indicating that HCMV infection may selectively shape the NKR repertoire of healthy individuals and, moreover, that the driving force(s) leading to the expansion of NKG2C+ cells may act co-ordinately on NK and T cell lineages. These observations suggest that both lymphocyte subsets could participate in the response to HCMV, possibly involving the KLR itself.

The increased proportions of NKG2C+ cells likely reflect the challenge exerted by HCMV on the innate immune system, and thus may become another useful parameter to explore the complex host-pathogen relation during the course of infection and latency. The selective imprint of HCMV on the NKR repertoire is reminiscent of the expansion of CTL specific for viral antigens during the adaptive immune response. Preliminary longitudinal analyses carried out in some donors pointed out that the distribution pattern of NKG2C+ and NKG2A+ subsets tended to remain rather stable along time (data not shown). Yet, an increase of the KLR expression should predictably follow primary infection, and oscillations in the proportions of circulating NKG2C+ cells may occur in HCMV+ donors. The basis for the wide variability in the numbers of NKG2C+ cells observed among HCMV+ individuals needs to be addressed. Furthermore, a prospective follow-up of the NKR repertoire should be carried out in the context of different clinical settings in which HCMV is

involved. Little is known about the expression and function of the murine CD94/NKG2C receptor specific for Qa1$^b$ [44], the functional homologue of HLA-E; moreover, the influence of MCMV infection on Qa1$^b$ and CD94/NKG2C expression is also uncertain.

There is limited information on the function played by activating NKR (i.e. CD94/NKG2C and KIR) in CTL, and it has been proposed that they might play a co-stimulatory role, rather than directly triggering T cell effector functions [45]. The expansion of NKG2C+ T cells in HCMV+ donors raised the question as to whether they might be CTL specific for viral antigens. Arguing against that possibility, we observed in HCMV+ HLA-A*0201 donors that most T cells specifically stained by HLA-A*0201 tetramers bound to an immunodominant pp65-derived epitope peptide did not co-express CD94/NKG2C; consistent with this observation others have reported that HCMV-specific CTL were CD94-negative [42]. As an alternative, we also considered the possibility that CD94/NKG2C+ cells might correspond to HLA-E-specific T cells. We previously described a CD94/NKG2C+ T cell clone that recognized HLA-E via the TcR [46]. Moreover, Mingari and colleagues also identified HLA-E-specific T cells, bearing the CD94/NKG2A inhibitory receptor, and provided evidence supporting that they recognize peptides derived from the UL40 HCMV protein [47, 48]. CD94/NKG2C+ T cell clones were tested for their ability to kill the HLA-E-transfected 721.221 cells (.221-AEH) [22]. In every case, the enhanced lysis against .221-AEH cells was completely prevented by an anti CD94 mAb, consistent with an involvement of the activating KLR rather than of the TcR (M. Gumá and M. López-Botet, unpublished results); yet, further studies are required to precisely assess the frequency of CD94/NKG2C+ T cells bearing an HLA-E-specific TcR.

Several mechanisms may account for the variable increase of CD94/NKG2C+ cells in HCMV+ individuals. First, changes in the NKR distribution might result from alterations in the cytokine network secondary to the viral infection. In this regard, CD94/NKG2A has been reported to be inducible in T cells by TGFβ and IL-15 [49]. Recently, IL-21 was shown to promote the

differentiation of CD34+ precursors to NK cells and their sequential acquisition of NCR and NKR [50]; yet, there is no evidence that cytokines may regulate differential NKR expression during maturation. Alternatively, signalling by NKR may control not only lymphocyte effector functions but also the proliferation and/or survival of NK cells subset(s) that participate in ligand recognition, as shown for the expansion of Ly49H+ cells in MCMV-infected mice [6]. Thus, NKG2C-mediated recognition of HCMV-induced alterations in infected cells could lead to the expansion of the corresponding NK and T cell subsets. Based on the current view of NKR biology, this might happen either upon an increased avidity of the KLR-ligand interaction, or/and secondarily to a selective loss of the ligands for inhibitory receptors (i.e. KIR, ILT2) expressed by CD94/NKG2C+ cells. Thus far, there is no evidence supporting that CD94/NKG2C may recognize HLA-E-bound microbial peptides nor viral molecules, as shown for Ly49H [7, 8]. Though HLA-E bound to an HSP60-derived peptide interacts with CD94/NKG2A [51], there is no either information as to whether this complex may efficiently engage CD94/NKG2C. Regarding the second possibility, a nonamer derived from the UL40 HCMV protein binds to HLA-E and favors its TAP-independent and US6-resistant expression [29, 30]. Moreover, the US11 and US2 HCMV proteins reduced the surface levels of class Ia molecules but did not affect HLA-E [28]. Thus, preservation of the class Ib molecule to maintain HCMV-infected cells resistant against CD94/NKG2A+ subsets might promote their recognition by NKG2C+ cells.

Differences between the surface levels of HLA-E allotypes and their interaction with CD94/NKG2 receptors have been reported. Moreover, the HLA-E$^{G107}$ transfected in the HLA-negative K562 cell line was shown to be insensitive to the US6 HCMV protein [24-26]. When individually analysed, the HLA-E allotypes/genotypes were not significantly associated either with the proportions of circulating NKG2C+ cells nor to the serological status for HCMV. Yet, the possibility that the HLA-E genotype may influence the impact of HCMV infection on the proportions of NKG2C+ cells cannot be entirely excluded, requiring further studies in a larger population.

The effect of HCMV infection on the expression of other NKR and NCR was also addressed. Human NKG2D is coupled to PI3-K signalling pathways through the DAP10 adapter and interacts with stress-inducible class I-related molecules, including MICA/B and "UL16 binding proteins" (ULBP) or RAET1 (Rae-1 like transcripts) [52, 53]. Expression of NKG2D ligands in CMV infected cells has been shown to co-stimulate virus-specific CTL [54]. Moreover, the HCMV UL16 glycoprotein impairs the expression of MICB, ULBP1 and ULBP2, presumably constituting a mechanism to escape from the NKG2D-mediated response [55, 56, 57]. According to our data, the NKG2D expression was comparable in HCMV+ and HCMV- donors, as well as among the NKG2C+ and NKG2A+ cell subsets.

A putative involvement of natural cytotoxicity receptors (NCR) of the IgSF (i.e. NKp46, NKp44 and NKp30) [31] in the response to HCMV is uncertain. Though their cellular ligands have not yet been defined, there is indirect evidence that they are widely distributed in different tissues and, thus, NCR might contribute to the NK cell response against some virus-infected cells in which the expression of HLA class I molecules has been down-regulated. Paradoxically, lower proportions of NKp30+ and NKp46+ "bright" NK cells were detected in HCMV+ individuals; consistent with this observation, NKG2C+ NK cells displayed significantly lower levels of these NCR than NKG2A+ cells from the same donors. This is in line with a previous study showing that the NKG2A+ cells were confined to the NKp46$^{bright}$ subset [39]. A low expression of NCR has been recently reported in NK cells from viremic HIV+ patients, and the putative influence of HCMV infection on the NKR repertoire of HIV+ individuals should be addressed [58].

CD85j (ILT2/LIR-1) is an inhibitory receptor of the Ig-SF expressed by different leukocyte lineages that interacts with a broad spectrum of HLA class I molecules and binds with high affinity to the class I-like UL18 HCMV glycoprotein [59-61]. The hypothesis that UL18 may interfere with NK cell activity during HCMV infection has not yet received a convincing experimental support [27]. However, CD85j expression was reported to increase in lymphocytes from patients undergoing HCMV

infection after lung transplantation [62]. In line with this finding, we observed that the proportions of CD85j+ and KIR2D+ cells tended to be raised in HCMV+ individuals. This association reflected the higher co-expression of CD85j and KIR in NKG2C+ cells, as compared to the NKG2A+ subset. Such coordinated distribution of NKR suggests that CD94/NKG2C+ lymphocytes displaying low levels of NCR that co-express inhibitory NKR (i.e. CD85j and KIR) may be preferentially selected during maturation, preventing their potential autoreactivity against normal HLA-E+ cells. Further studies are required to assess whether indeed NKG2C preferentially associates to inhibitory KIR. No relation could be established between the KIR genotype and either HCMV infection or CD94/NKG2C expression, ruling out that the activating KLR may be preferentially associated to haplotypes containing fewer activating KIR genes.

Altogether, our results support that HCMV shapes the NKR repertoire expanding NKG2C+ NK and T cell subsets that co-express CD85j and KIR and display low levels of NCR. The driving force(s) that determine the HCMV imprint on the NKR repertoire are being currently explored at the cellular and molecular levels, with a special attention to the role played by CD94/NKG2C itself.

# REFERENCES

1. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol. 2002;20:217-251.

2. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A. Human NK-cell receptors. Immunol Today. 2000;21:420-422.

3. Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003;15:308-314.

4. López-Botet M, Bellón T, Llano M, Navarro F, García P, de Miguel M. Paired inhibitory and triggering NK cell receptors for HLA class I molecules. Hum Immunol. 2000;61:7-17.

5. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. Annu Rev Immunol. 2001;19:291-330.:291-330.

6. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. Nat Immunol. 2001;2:951-956.

7. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002;296:1323-1326.

8. Smith HR, Heusel JW, Mehta IK, et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A. 2002;99:8826-8831.

9. Daniels KA, Devora G, Lai WC, O'Donnell CL, Bennett M, Welsh RM. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. J Exp Med. 2001;194:29-44.

10. Kambayashi T, Assarsson E, Michaelsson J, et al. Emergence of CD8+ T cells expressing NK cell receptors in influenza A virus-infected mice. J Immunol. 2000;165:4964-4969.

11. Moser JM, Gibbs J, Jensen PE, Lukacher AE. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. Nat Immunol. 2002;3:189-195.

12. McMahon CW, Zajac AJ, Jamieson AM, et al. Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells. J Immunol. 2002;169:1444-1452.

13. De Maria A, Moretta L. HLA-class I-specific inhibitory receptors in HIV-1 infection. Hum Immunol. 2000;61:74-81.

14. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. J Exp Med. 1991;173:1017-1020.

15. Carretero M, Cantoni C, Bellón T, et al. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. Eur J Immunol. 1997;27:563-567.

16. Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol. 1996;157:4741-4745.

17. Carretero M, Palmieri G, Llano M, et al. Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. Eur J Immunol. 1998;28:1280-1291.

18. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity. 1998;8:693-701.

19. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci U S A. 1998;95:5199-5204.

20. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391:795-799.

21. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med. 1998;187:813-818.

22. Llano M, Lee N, Navarro F, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. Eur J Immunol. 1998;28:2854-2863.

23. Grimsley C, Kawasaki A, Gassner C, et al. Definitive high resolution typing of HLA-E allelic polymorphisms: Identifying potential errors in existing allele data. Tissue Antigens. 2002;60:206-212.

24. Strong RK, Holmes MA, Li P, Braun L, Lee N, Geraghty DE. HLA-E Allelic Variants.Correlating differential expression, peptide affinities, crystal structures and termal stabilities. J Biol Chem. 2003;278:5082-5090.

25. Ulbrecht M, Couturier A, Martinozzi S, et al. Cell surface expression of HLA-E: interaction with human beta2- microglobulin and allelic differences. Eur J Immunol. 1999;29:537-547.

26. Maier S, Grzeschik M, Weiss EH, Ulbrecht M. Implications of HLA-E allele expression and different HLA-E ligand diversity for the regulation of NK cells. Hum Immunol. 2000;61:1059-1065.

27. López-Botet M, Angulo A, Gumá M. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens. 2004;63:195-203.

28. Llano M, Gumá M, Ortega M, Angulo A, López-Botet M. Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. Eur J Immunol. 2003;33:2744-2754.

29. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science. 2000;287:1031.

30. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. J Immunol. 2000;164:5019-5022.

31. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.:197-223.

32. Navarro F, Llano M, Bellón T, Colonna M, Geraghty DE, López-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. Eur J Immunol. 1999;29:277-283.

33. de Miguel M, López-Botet M. Characterization of monoclonal antibodies specific for receptors of the KIR family. Inmunología. 2002;21:187-193.

34. Cantoni C, Biassoni R, Pende D, et al. The activating form of CD94 receptor complex: CD94 covalently associates with the Kp39 protein that represents the product of the NKG2-C gene. Eur J Immunol. 1998;28:327-338.

35. Gómez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: An update. Tissue Antigens. 2002;59:184-193.

36. Hikami K, Tsuchiya N, Yabe T, Tokunaga K. Variations of human killer cell lectin-like receptors: common occurrence of NKG2-C deletion in the general population. Genes Immun. 2003;4:160-167.

37. Larsson S, Soderberg-Naucler C, Wang FZ, Moller E. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. Transfusion. 1998;38:271-278.

38. Pass RF. Cytomegalovirus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, eds. Fields Virology. Philadelphia: Lippincott, Williams & Wilkins; 2001:2675-2705.

39. Sivori S, Pende D, Bottino C, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol. 1999;29:1656-1666.

40. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol. 2001;13:458-464.

41. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin Immunol. 2003;15:45-51.

42. Gamadia LE, Rentenaar RJ, Baars PA, et al. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. Blood. 2001;98:754-761.

43. Aubert G, Hassan-Walker AF, Madrigal JA, et al. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. J Infect Dis. 2001;184:955-963.

44. Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. J Exp Med. 1999;190:1801-1812.

45. Snyder MR, Weyand CM, Goronzy JJ. The double life of NK receptors: stimulation or co-stimulation? Trends Immunol. 2004;25:25-32.

46. García P, Llano M, de Heredia AB, et al. Human T cell receptor-mediated recognition of HLA-E. Eur J Immunol. 2002;32:936-944.

47. Moretta L, Romagnani C, Pietra G, Moretta A, Mingari MC. NK-CTLs, a novel HLA-E-restricted T-cell subset. Trends Immunol. 2003;24:136-143.

48. Pietra G, Romagnani C, Mazzarino P, et al. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. Proc Natl Acad Sci U S A. 2003;100:10896-10901.

49. Mingari MC, Moretta A, Moretta L. Regulation of KIR expression in human T cells: a safety mechanism that may impair protective T-cell responses. Immunol Today. 1998;19:153-157.

50. Sivori S, Cantoni C, Parolini S, et al. IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur J Immunol. 2003;33:3439-3447.

51. Michaelsson J, Teixeira dM, Achour A, Lanier LL, Kärre K, Soderström K. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. J Exp Med. 2002;196:1403-1414.

52. Cerwenka A, Lanier LL. NKG2D ligands: unconventional MHC class I-like molecules exploited by viruses and cancer. Tissue Antigens. 2003;61:335-343.

53. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3:781-790.

54. Gröh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol. 2001;2:255-260.

55. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol. 2003;33:194-203.

56. Dunn C, Chalupny NJ, Sutherland CL, et al. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med. 2003;197:1427-1439.

57. Rolle A, Mousavi-Jazi M, Eriksson M, et al. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol. 2003;171:902-908.

58. De Maria A, Fogli M, Costa P, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). Eur J Immunol. 2003;33:2410-2418.

59. Colonna M, Navarro F, Bellón T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186:1809-1818.

60. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity. 1997;7:273-282.

61. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. Immunity. 1999;11:603-613.

62. Berg L, Riise GC, Cosman D, et al. LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients. Lancet. 2003;361:1099-1101.

# CAPÍTULO 5

## The CD94/NKG2C Killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8+ T cells

Gumà M, Busch LK, Salazar-Fontana LI, Bellosillo B, Morte C, García P, López-Botet M

# CAPÍTULO 6

# Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts.

Gumà M, Budt M, Sáez A, Brckalo T, Hengel H, Angulo A, López-Botet M

**ABSTRACT**

CD94/NKG2C+ NK cells are increased in healthy individuals infected with human cytomegalovirus (HCMV), suggesting that HCMV infection may shape the NK cell receptor repertoire. To address this question, we analysed the distribution of NK cell subsets in PBL co-cultured with HCMV-infected fibroblasts. A substantial increase of NK cells was detected by day 10 in samples from a group of HCMV+ donors, and CD94/NKG2C+ cells outnumbered the CD94/NKG2A+ subset. Fibroblast infection was required to induce the preferential expansion of CD94/NKG2C+ NK cells, that was comparable with allogeneic or autologous fibroblasts, and different virus strains. A CD94-specific mAb abrogated the effect, supporting an involvement of the lectin-like receptor. Purified CD56+ populations stimulated with HCMV-infected cells did not proliferate, but the expansion of the CD94/NKG2C+ subset was detected in the presence of IL-15. Experiments with HCMV deletion mutants indicated that the response of CD94/NKG2C+ NK cells was independent of the UL16, UL18 and UL40 HCMV genes, but was impaired when cells were infected with a mutant lacking the US2-11 gene region. Taken together the data support that the interaction of CD94/NKG2C with HCMV-infected fibroblasts, concomitant to the inhibition of HLA class I expression, promotes an outgrowth of CD94/NKG2C+ NK cells.

# INTRODUCTION

Human cytomegalovirus (HCMV) infection generally follows a subclinical course  but may lead to severe disorders in immunocompromised individuals and is a main cause of infectious congenital diseases. HCMV remains latent in immunocompetent hosts, undergoing occasional reactivation[1]. Studies in murine models revealed that an effective defence against CMV requires the participation of NK and T cells[2; 3]. Detection of antibodies and CD8+ T lymphocytes specific for HCMV antigens allow to assess the adaptive immune response to the pathogen[4; 5]. To escape from CD8+ T cells, HCMV inhibits the expression of HLA class I molecules and interferes with antigen presentation using a set of glycoproteins (US2, US3, US6, US10 and US11) whose genes are clustered within the unique short (US) region of the virus genome[6-8]. The loss of HLA class I molecules in HCMV-infected cells impairs the engagement of inhibitory receptors and prompts the activation of NK cell effector functions; reciprocally the virus has developed several strategies to evade NK-mediated recognition[9].

The nature of receptor-ligand interactions involved in the NK cell response to CMV-infected cells is incompletely understood. In strains of mice expressing the Ly49H receptor, NK cell functions are triggered upon recognition of the m157 mouse CMV (MCMV) glycoprotein, becoming essential to control replication[10; 11]; by contrast, human activating NK cell receptors (NKR) specific for HCMV molecules have not been identified. The involvement of activating killer Ig-like receptors (KIR) and natural cytotoxicity receptors (NCR) (i.e. NKp46, NKp30 and NKp44) in the response to HCMV is uncertain. The interaction of the pp65 HCMV tegument protein with NKp30 has been reported to inhibit rather than to activate NK cell functions[12]. The ability of the UL16 HCMV molecule to interfere with the surface expression of NKG2D ligands[13-15], and the evidence that similar evasion mechanisms operate in MCMV infection[16; 17], support an important role for this killer lectin-like receptor (KLR) in the anti-viral defence[18]. Recently, the UL141 HCMV molecule has been shown to inhibit the expression in infected cells of CD155, a ligand for the DNAM-1 stimulating receptor[19].

HCMV may also escape NK-mediated surveillance keeping inhibitory receptors for HLA class I molecules engaged. The viral UL18 molecule binds with high affinity to the ILT2 (CD85j) inhibitory receptor[20; 21], though its role in immune evasion has not been precisely elucidated[9]. On the other hand, HLA-E appears constitutively resistant to the action of US2 and US11[22], and it becomes as well refractory to the action of US6 when bound to a peptide from the leader sequence of the HCMV UL40 protein[23; 24]. Thus, the HLA class Ib molecule may be preserved in infected cells and interfere with the NK cell response by engaging the inhibitory CD94/NKG2A KLR[25].

We recently reported[26] that healthy HCMV-seropositive individuals displayed increased proportions of NK and T cells that expressed the triggering CD94/NKG2C KLR, which binds HLA-E with a lower affinity than CD94/NKG2A[27; 28]. Increased numbers of circulating CD85j+ T lymphocytes were also significantly associated to HCMV infection. Taken together, the data supported that the challenge exerted by HCMV on the innate immune system might shape the NKR repertoire. Signalling by NCR and KIR may control the proliferation and/or survival of NK cells[29; 30]; we recently reported that CD94/NKG2C+ NK and T cell subsets divided in response to stimulation with an HLA class I-deficient tumour cell line transfected with HLA-E[31]. On that basis, we hypothesized that CD94/NKG2C-mediated recognition of virus-infected cells might promote the expansion of NKG2C+ cell subsets, as described for Ly49H+ cells in MCMV-infected mice[32]. To address this issue we analysed the NKR repertoire in PBL co-cultured with HCMV-infected fibroblasts; little information is available on the NK cell response in this system, widely used to study CTL specific for viral antigens. Our results indicate that stimulation of PBL from HCMV+ donors with virus-infected fibroblasts elicited a preferential expansion of CD94/NKG2C+ NK cells, and that a cognate interaction of the activating KLR with HCMV-infected cells may contribute to drive the proliferation of this lymphocyte subset.

## MATERIALS AND METHODS

### Subjects.

Heparinized blood samples were obtained from 15 healthy adult individuals (age: 23-51 years); written informed consent was obtained, and the study protocol was approved by the institutional Ethics Committee (CEIC-IMAS). Standard clinical diagnostic tests were used to analyse serum samples for circulating IgG antibodies against CMV (Abbot Laboratories, Abbot Park, IL); four donors were seronegative (HCMV-) and 11 seropositive (HCMV+).

### Cell lines

MRC-5 fetal human lung fibroblasts and human foreskin fibroblasts (HFF) cell lines were obtained from the American Type Culture Collection (Manassa, VA) and grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin (referred to as complete medium). Primary fibroblast lines were established from skin biopsies obtained from two subjects following standard procedures; briefly, tissue samples were minced, treated with 0.2% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany), washed and grown in complete medium.

Cell lines were screened and shown to be negative for *Mycoplasma* contamination by PCR using primers as described[33] . Fibroblast lines used were between passages 8 and 15 (HFF), 20 and 30 (MRC-5 cells) and 4 and 6 (primary fibroblast lines).

### HCMV preparations and infection of fibroblasts

Stocks of HCMV strains AD169, Towne, and Toledo, and HB5-derived mutants were prepared, and titers of infectious virus determined by standard procedures on HFF or MRC-5 cells. Viral preparations were screened for Mycoplasma as indicated above. The Towne strain of HCMV was purified as described[34] with slight modifications. UV-inactivation of virus was performed using a 30

W Phillips lamp (model G13) and confirmed by monitoring expression of the *IE1* protein on MRC-5 cells by indirect immunofluorescence.

Recombinant HCMV genomes were generated in E. coli. The AD169-derived HCMV bacterial artificial chromosome plasmid (BACmid) HB5[35], which lacks the US2-6 gene region was used to generate all HCMV mutants described in the study. The HB5-ΔUS2-11 mutant was constructed by homologous recombination, as described[36], with some modifications. Briefly, 5': TCACACATACCTTTGTGCATACGGTTTATATATGA CCATCCACGCTTATAACGAACCTACGATTTATTCAACAAAGCCACG and 3': TGCTATAAGACAGCCTTACAGCTTTTGAGTCTAGACAGGGTAACAGCCTTCCCTTGTAA GGCCAGTGTTACAACCAATTAACC, PCR primers were used to amplify the *kanamycin* gene, adding sequences homologous to regions upstream of *US7* and downstream of *US11*, respectively. The PCR product was transformed into *E. coli* carrying the HB5 BACmid and expressing the Red recombinase system from the plasmid pkD46[37]. Recombinant clones were selected at 43°C under chloramphenicol and kanamycin selection, and correct mutagenesis was confirmed by restriction digest analysis, PCR and southern blot analysis of isolated BACmid DNA. Reconstitution of the virus mutant was carried out by transfecting 5 μg of BACmid into MRC-5 cells using superfect (Quiagen, Hilden, Germany). Human CMV recombinants HB5-ΔUL14-20 and HB5-ΔUL40-42 have been previously described [36].

Human fibroblasts in 24 or 96-well plates at 70% confluence were mock- or HCMV-infected (moi=1). After a 2-h adsorption period, the inoculum was removed, cells were washed with PBS and incubated in complete medium; in some experiments infection was carried out at a different moi (0.01-5).

**Lymphocyte cultures**

PBL were obtained by centrifugation of heparinized blood on Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). PBL were incubated in 24-well ($2x10^6$/well) or 96-well ($2x10^5$/well)

plates in complete medium in the presence of mock- or HCMV-infected fibroblasts; in some experiments PBL were stimulated with free HCMV (0.05 infectious particles/cell). PBL were added to infected fibroblasts immediately after the 2-h adsorption period; in time-course experiments co-culture was delayed 24-72 hours post-infection. All cultures were supplemented with IL-2 (10 U/ml) (Eurocetus) at day 3, and in some experiments with IL-15 (10 U/ml) (Peprotech Ec, London, UK). Cells were incubated for 10-12 days, fed every 3 days and eventually split. Some experiments were set up in a Transwell permeable support (0.4 μ) (Corning Inc, NY); infected fibroblasts were cultured with and without PBL, and the phenotype of PBL incubated separately in the upper chamber was analysed. CD94/NKG2C+ NK cell clones were derived as previously described [31] .

**Antibodies. Immunofluorescence and flow cytometry analysis.**

Monoclonal antibodies (mAbs) specific for CD56, CD94, NKG2A, KIR, ILT2, NCR and NKG2D have been previously detailed[26]. HP-1F7 anti-HLA-class I was generated in our laboratory[38]; 3D12 anti-HLA-E was provided by Dr. D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle,WA); anti-NKG2C (MAB1381) and anti-NKG2C-PE was from R&D Systems Inc (Minneapolis, MN); CD25-PE was from Immunotools (Friesoythe, Germany) NKG2C-PE (R&Dsystems Inc),

For immunofluorescence staining, cells were pretreated with human aggregated Ig (10 μg/ml) to block FcR, and subsequently labeled with the different mAb and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA), as described[26]. In experiments where blocking mAbs (CD94 or CD56) were used, harvested cells were stained with anti CD3-PerCP, anti NKG2A-FITC, or anti NKG2C-PE. In some experiments, cells were sorted (FACSvantage, Becton Dickinson, Mountain View, CA) after labeling with CD56-PE and NKG2C-PE.

Detection of HCMV IE1 was performed with MAB 8130 from Chemicon (Temecula, CA) and a secondary antibody, FITC conjugate anti-mouse IgG (Sigma, St. Louis, MO).

**Proliferation and cytotoxicity assays**

As described[31], PBL were resuspended in RPMI-1640 ($10^7$/ml) and incubated for 10 min at 37°C with the intracellular fluorescent dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) (2 μM). After two washes, CFSE-labeled PBL were cultured on mock- and HCMV- infected MRC-5. FACS analysis was performed at 7 days, after staining cells with anti NKG2C or anti NKG2A mAbs by indirect immunofluorescence with PE-tagged F(ab')2 rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). The proportions of dividing cells within each subset were calculated as described[39]. In some experiments CD25 expression was assessed after 72 h incubation with mock and HCMV-infected MRC-5.

CD94/NKG2C+ NK cell clones were tested in a 4h $^{51}$Cr-release assay against mock- and HCMV (Towne)-infected (24 or 72 h) autologous or HFF fibroblasts[40], at different effector/target ratios. Cells were treated with Trypsin-EDTA (Invitrogen Gibco, Grand Island, NY) labeled with $^{51}$Cr and used in standard cytotoxicity assays[41]. In parallel samples, effector cells were pre-incubated with CD94 or CD56- specific mAbs for 30 min before adding targets. All assays were set up in triplicate and specific lysis was calculated as described[41].

**Statistical analysis**

Statistical analysis was performed by the Mann-Whitney *U* test, using the SPSS 9.0 software (SPSS, Chicago, IL). Results were considered significant at the 2-sided *P* level of 0.05.

# RESULTS

## Preferential expansion of NKG2C+ NK cells in PBL stimulated with HCMV-infected fibroblasts.

We previously reported that increased proportions of CD94/NKG2C+ NK cells and ILT2+ (CD85j+) T lymphocytes were detectable in PBL from HCMV seropositive (HCMV+) donors, indicating that the viral infection might shape the NK cell receptor repertoire[26]. To address whether HCMV could promote the expansion of CD94/NKG2C+ lymphocytes, PBL from HCMV+ donors were co-cultured either with mock- or HCMV (Towne)-infected MRC-5 fibroblasts, in the presence of an exogenous supply of IL-2 (10 U/ml); at different time-points the proportions and phenotype of NK cells were comparatively assessed. In vitro stimulation of PBL with HCMV-infected fibroblasts has been extensively used to analyse CTL specific for viral antigens, but NK cells have not been systematically studied in this system.
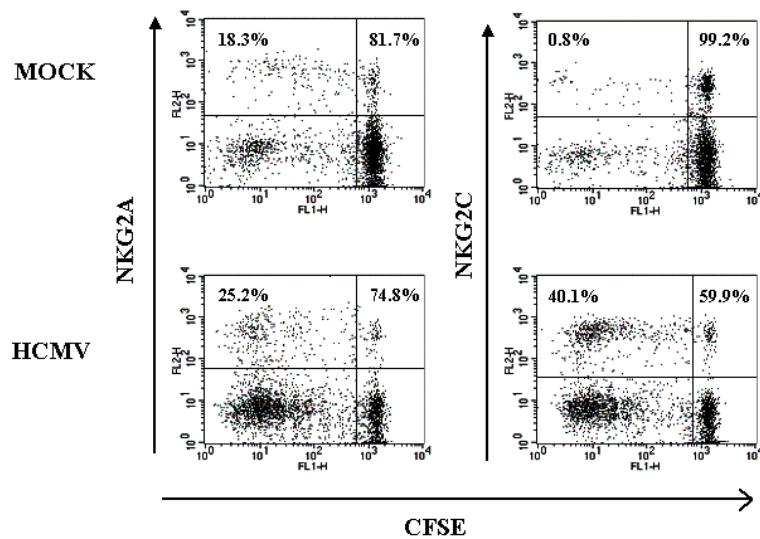


**Figure 1A.** NK cell proliferation in response to HCMV-infected fibroblasts. CFSE-labeled PBL from an HCMV+ donor were co-cultured with mock- and Towne-infected (moi=1) MRC-5 cells as described in Methods. Flow cytometry analysis was carried out at day 7, after staining cells with anti NKG2C or NKG2A mAbs; numbers correspond to the proportions of dividing and non-dividing cells in NKG2C+ and NKG2A+ populations, calculated as described in Methods. The experiment is representative of 6 performed.
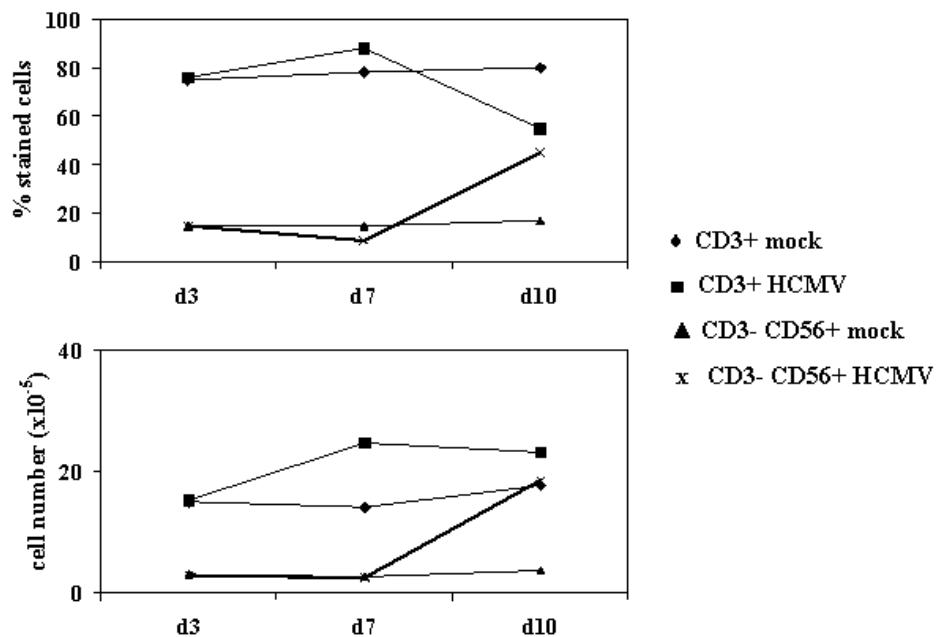
**Figure 1B.** NK cell proliferation in response to HCMV-infected fibroblasts. PBL were stimulated with Towne-infected fibroblasts as described above, and flow cytometry analysis was carried out at different time points with anti CD3-PerCP and anti CD56-PE, counting the numbers of recovered cells. The % and the calculated numbers of NK and T cell populations are represented.

A proliferative response of PBL to HCMV-infected cells, was observed by phase-contrast microscopy. Cell recovery after 10-12 days, referred to the input, was 1.5-5 fold in cultures with HCMV-infected fibroblasts and 0.8-1.5 fold with mock-infected cells in different experiments (n=25). Studies carried out with CFSE-labelled PBL also showed that cell proliferation stimulated by HCMV-infected MRC-5 cells exceeded that induced by mock-infected cultures, allowing a phenotypic analysis of the dividing cells (Figure 1A). As compared to NKG2A+ lymphocytes, NKG2C+ cells mainly divided upon stimulation by HCMV-infected MRC-5 cells; yet, only a fraction proliferated.

Analysis at different time-points of PBL co-cultured with HCMV-infected fibroblasts indicated that T cells were predominant during the first week (Figure 1B), but a shift in the distribution of lymphocyte populations was observed by day 10-12. Remarkably, NK cells

substantially increased (Figure 1B), becoming up to 35-80 % of the population in different experiments (n=25), and NKG2C+ lymphocytes outnumbered the NKG2A+ subset (Figure 2A); the proportions of cells co-expressing both KLR were negligible (data not shown). The NKG2C+ cell recovery in cultures with HCMV-infected cells was 5-25 fold that obtained with mock-infected fibroblasts; the NKG2C+/NKG2A+ ratios were 0.3-1.3 and 2.2-6.7 in mock- and HCMV-infected cultures, respectively. Together with a majority of NKG2C+ NK cells, small proportions of CD3+ NKG2C+ and NKG2A+ T lymphocytes were also identified (figure 2A). By contrast, the phenotype of PBL from HCMV-seronegative donors (n=4) was comparable upon incubation with virus- or mock- infected MRC-5 cells (Figure 2A). It is of note that the late outgrowth of NKG2C+ cells was not perceived in samples from a group of HCMV+ donors (5 out of 11), in which T cells remained the predominant population all over the culture. As compared to responders (R), fresh PBL from the non-responder (NR) group displayed significantly lower proportions of NKG2C+ cells (NR=2.2±1.5 vs R=10.1±7.3; p=0.03) and reduced NKG2C/NKG2A ratios (NR=0.3±0.3 vs R=1.7±1.1; p=0.02).

The expansion of NKG2C+ cells was detectable when PBL were co-cultured with HCMV-infected HFF (data not shown) or autologous skin fibroblasts (Figure 2B). Together with the limited NK cell proliferation in response to mock-infected MRC-5 cells, these results ruled out alloreactivity as the main stimulus responsible for driving the proliferation of NKG2C+ NK cells, and strongly supported a central role for the virus. The effect was comparable upon infection with different HCMV strains (i.e. AD169 and Toledo), as well as with a purified Towne preparation, thus excluding a role for cellular products present in crude virus stocks (not shown); subsequent experiments were performed using either Towne or AD169, as specified in every case.
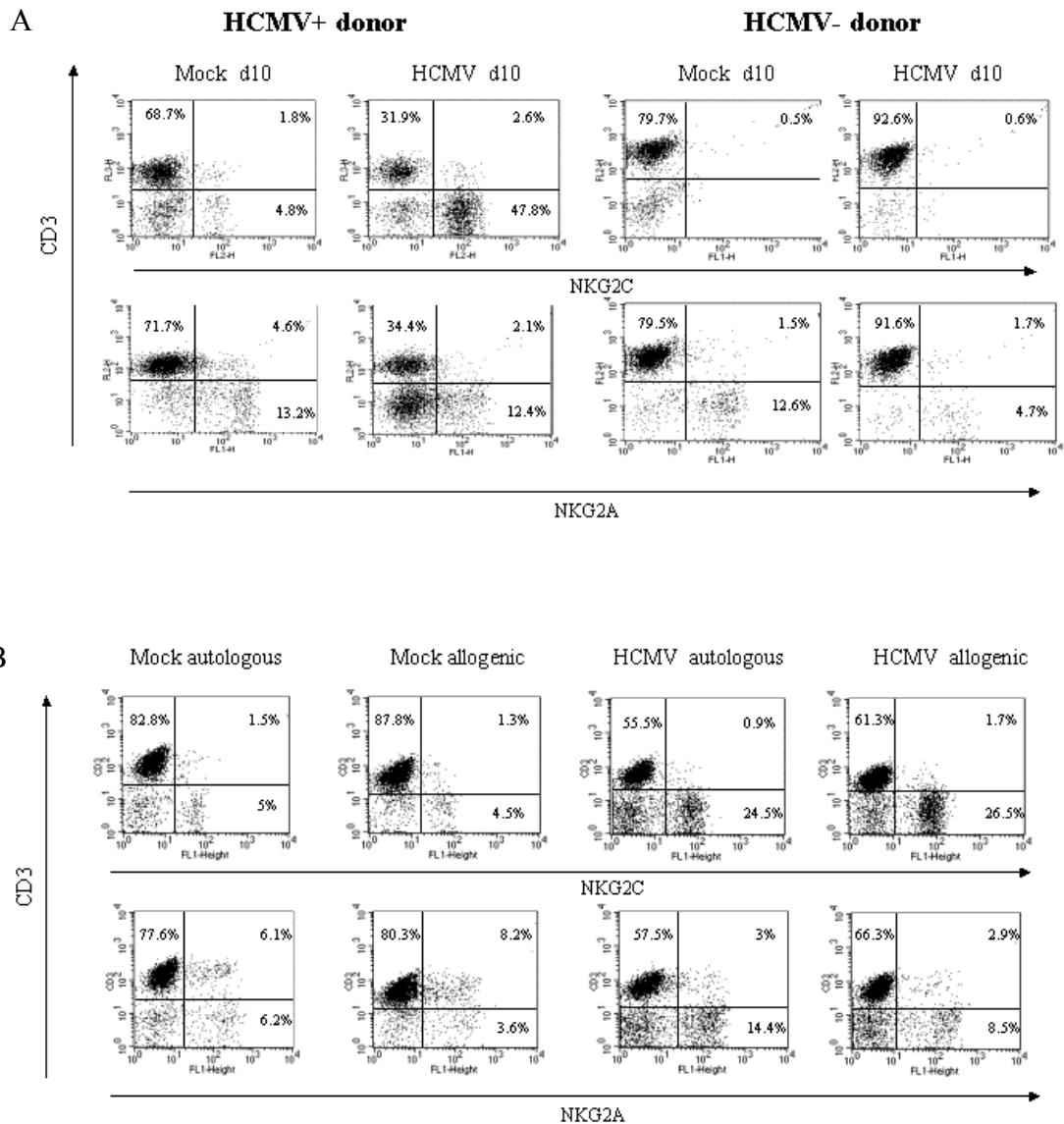
**Figure 2.** Preferential expansion of CD94/NKG2C+ NK cells upon stimulation with HCMV-infected autologous or allogeneic fibroblasts. (A) PBL from HCMV+ and HCMV- donors were co-cultured with mock- and AD169-infected (moi=1) MRC-5 cells. Two-colour analysis was carried out at day 10 with an anti CD3 mAb combined to anti NKG2C or NKG2A mAbs. The results are representative of the patterns of response observed in 6 out of 11 HCMV+ individuals, and in 4 HCMV- donors. (B) PBL from an HCMV+ donor were co-cultured with mock- and AD169-infected allogeneic (MRC-5) or autologous fibroblasts, and samples were analysed at day 10 as described in (A). Similar results were obtained in 6 different experiments with PBL from two different individuals.

**Distribution of NKR and NCR in PBL stimulated with HCMV-infected fibroblasts**.

The expression of other NKR (i.e. KIR2D, KIR3D, NKG2D, ILT2) and NCR (i.e. NKp46 and NKp30) was studied in NK and T cells stimulated with HCMV-infected fibroblasts, as previously done in PBL from HCMV+ donors. Expression of NKG2D, NKp46 and NKp30 was similar in mock- and HCMV-infected cultures (Figure 3), except for a slight reduction of NKp46 and NKp30 in the latter; it is of note that NKG2C+ NK cells were reported to express lower levels of these receptors than the NKG2A+ subset[26]. Some differences in the distribution of KIR were also noticed, but did not follow a reproducible pattern in samples from different donors. By contrast, the proportions of ILT2+ T lymphocytes were found to be systematically increased upon stimulation with HCMV-infected fibroblasts.
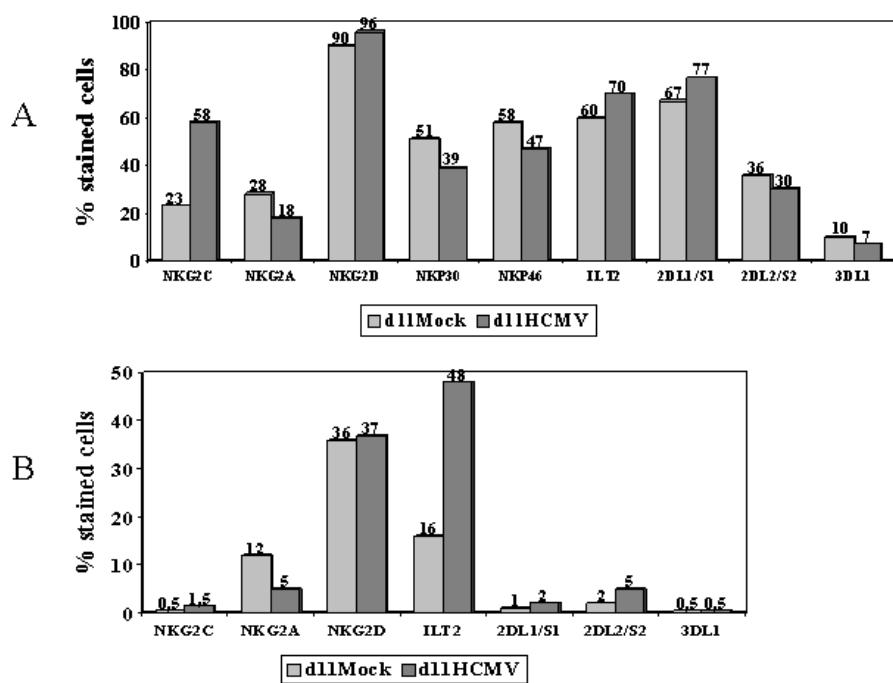


**Figure 3.** NKR and NCR distribution in PBL stimulated with HCMV-infected fibroblasts. PBL from an HCMV+ donor were co-cultured with mock- and Towne-infected (moi=1) MRC-5 cells. Cells harvested at day 11 were stained by indirect immunofluorescence with mAbs specific for different NKR or NCR, followed by labelling with anti CD56-PE and CD3-PerCP mAbs. Three-colour flow cytometry analysis was carried out gating on CD3- CD56+ (A) and CD3+ (B) populations. The data are representative of four different experiments.

**Fibroblast infection is essential to promote the expansion of NKG2C+ cells.**

An additional set of experiments was carried out to define the conditions required for HCMV to promote the expansion of NKG2C+ cells, that was dependent on the infectious dose used (Figure 4A). Treatment of MRC-5 with UV-inactivated HCMV (Figure 4B) abolished the effect. On the other hand, incubation of PBL with the virus in the absence of fibroblasts induced a proliferative response of T lymphocytes, including the ILT2+ subset, but did not expand NK cells (figure 4B). Infected fibroblasts, either alone or with PBL, did not stimulate NK cell proliferation in PBL samples cultured separately in a transwell system (not shown). Taken together, these findings indicate that a direct interaction between PBL and HCMV-infected fibroblasts is required to induce the proliferation of NKG2C+ cells.
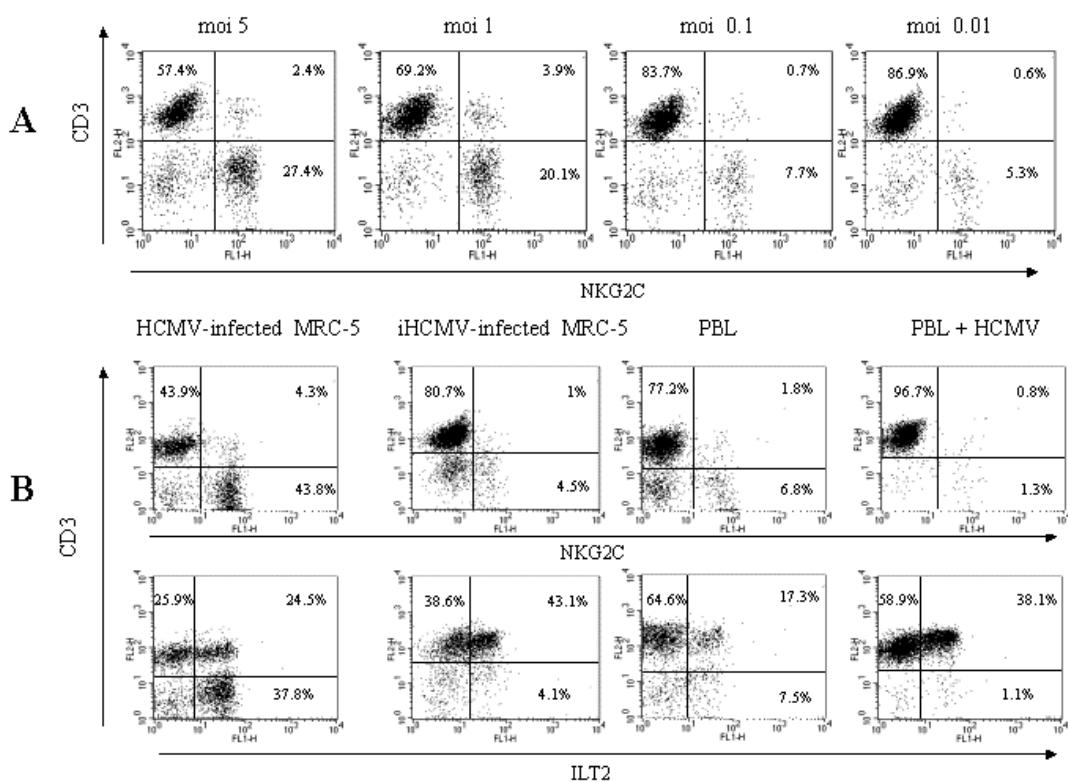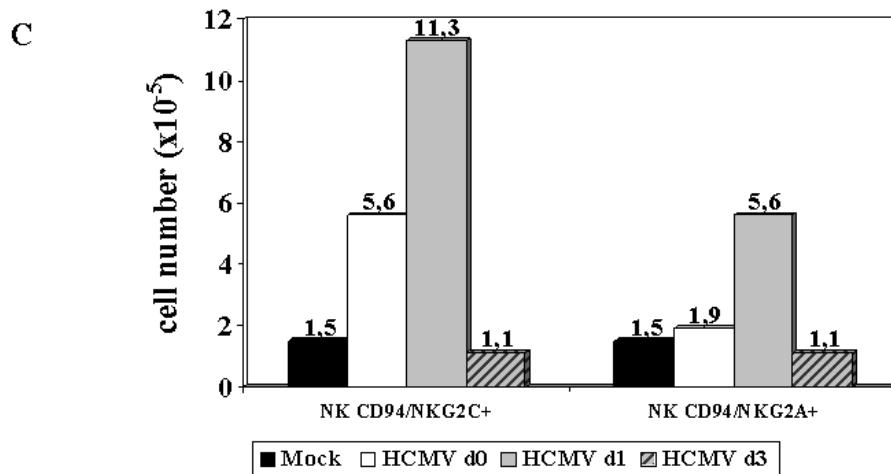
**Figure 4.** Expansion of NKG2C+ NK cells is dependent on the HCMV-infection of fibroblasts and on the time of their interaction. (A) The distribution of NKG2C+ cells in response to HCMV-infected MRC-5 cells at different moi was analysed. The % of NKG2C at moi 0.01 was comparable to mock-infected samples (not shown). (B) PBL from HCMV+ donors were co-cultured with mock- or Towne-infected MRC-5 fibroblasts; in parallel, PBL were incubated with the virus alone (HCMV) or fibroblasts infected with UV-inactivated Towne (iHCMV). Two-colour flow cytometry analysis was performed at day 10 with anti CD3, NKG2C and ILT2 mAbs. Data are representative of 5 different experiments. (C) PBL were incubated with Towne-infected MRC-5 cells at different time points post-infection (days 0, 1 and 3). At day 10, cells were harvested, counted and analysed by flow cytometry; the numbers of NKG2C+ and NKG2A+ cells recovered are shown. A similar pattern of response was observed in 3 different experiments.

Time-course experiments were carried out culturing PBL with MRC-5 cells at different stages after HCMV-infection. As shown in figure 4C, the expansion of NKG2C+ cells was optimal upon stimulation of PBL at day 1, but was reduced when the co-culture was delayed to day 3 post-infection, revealing the existence of an optimal temporal window for the cellular interactions required to stimulate the NKG2C+ subset.

**The expansion of NKG2C+ NK cells involves the CD94/NKG2C receptor and is enhanced by IL-15.**

Depletion of NKG2C+ lymphocytes by cell sorting abolished the effect, pointing out that the responding cells constitutively express the activating KLR (not shown); positive selection of NKG2C+ cells was not considered reliable due to the potential agonistic/antagonistic effects mediated by the receptor-specific mAb. Thus, to further dissect the process, CD56+ and CD56- populations were sorted and incubated with HCMV-infected fibroblasts under the same conditions used for PBL; NKG2C+ CD56- cells were undetectable. Remarkably, the CD56+ population, that included both NKG2C+ and NKG2A+ subsets, did not divide despite the presence of exogenous IL-2 (10 U/ml); in fact, IL-2Rα (CD25) expression remained negative after 48-72 h (not shown). By contrast, cell proliferation was observed among the CD56- population, and the proportions of CD56+ NKG2C+ cells increased, becoming detectable (not shown). These findings indicated that additional signals, missing in the CD56+ population, were required to stimulate the proliferation of NK cells in response to HCMV-infected fibroblasts. Such complementary stimuli, provided by cells contained within the CD56- fraction, likely accounted for the expansion of residual NKG2C+ CD56+ cells (<1-2 %) after sorting.

IL-15 plays a primordial role in regulating NK cell proliferation and differentiation, contributing to the accumulation of NK cells in CMV-infected mice[42]. Additional experiments were conducted to analyse the influence of this cytokine in the response of NKG2C+ cells. Purified CD56+ populations were cultured either with HCMV- or mock-infected MRC-5 cells, and replicate samples were supplemented with IL-15 at different time points. Experiments with CFSE-labelled cells indicated that, in both cases, NK cells divided by day 8 in response to IL-15 (Figure 5A). Yet, the cytokine promoted a preferential expansion of the NKG2C+ subset only in response to HCMV-infected MRC-5 when cultures were supplemented by day 4 (Figure 5B) but not earlier (day 0). Altogether, these observations supported that the interaction of NKG2C+ cells with HCMV-infected

fibroblasts is insufficient to induce IL-2Rαexpression and proliferation in response to IL-2, but enhances their responsiveness to IL-15. Further studies are required to dissect the influence of the cytokine network in the response of NKG2C+ cells to HCMV.
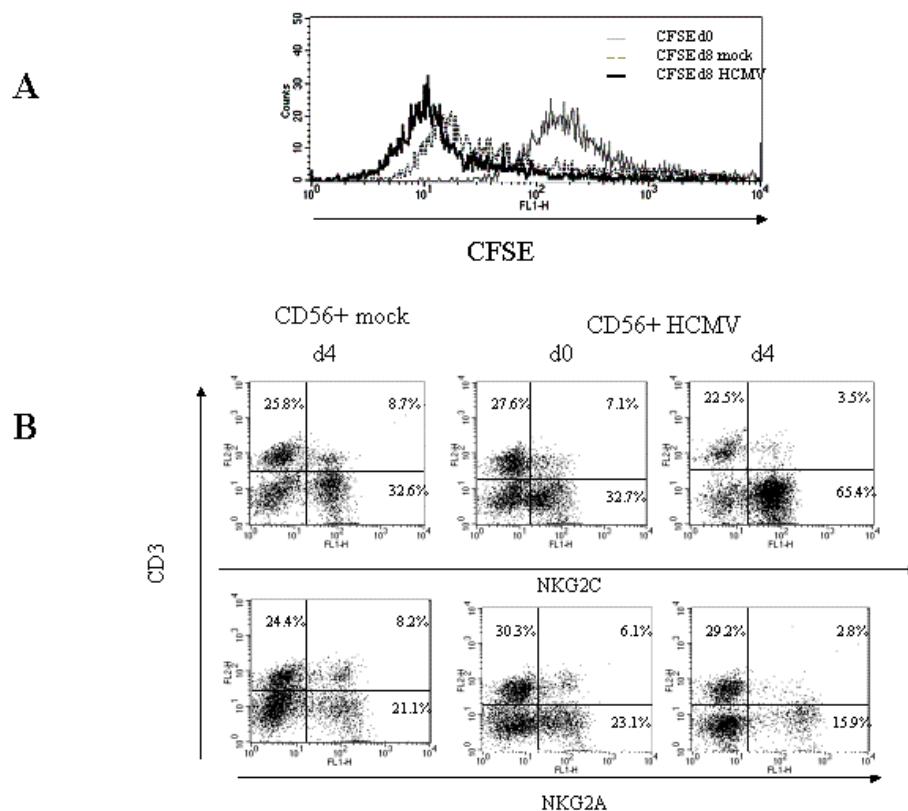


Figure 5. IL-15 promotes the preferential expansion of NKG2C+ cells in CD56+ populations stimulated with HCMV-infected cells. CD56+ populations sorted from PBL of HCMV+ donors were cultured with mock- or HCMV (AD169)-infected MRC-5 cells, infected 24 h before, in the presence of IL-2 (10 U/ml). (A) Proliferation of CFSE-labelled CD56+ cells cultured for 8 days with mock- or HCMV-infected cells supplemented with IL-15 at day 4. (B) Distribution of NKG2A+ and NKG2C+ subsets in CD56+ lymphocytes co-cultured for 8 days with mock- or HCMV-infected cells in the presence of IL-15, added at days 1 or 4. The data are representative of 6 experiments performed.

To approach whether the CD94/NKG2C receptor participated in the response to HCMV-infected cells, experiments were carried out in the presence of an F(ab')2 anti CD94 mAb (HP-3B1). As compared to the control (anti-CD56), the HP-3B1 mAb hampered the expansion of NKG2C+ cells when added early during co-culture of PBL with HCMV-infected fibroblasts (Figure 6A).

Interestingly, the inhibitory effect vanished if the mAb supply was delayed to day 4 (Figure 6B), when infected fibroblasts co-cultured with PBL were no longer detectable (not shown), further arguing against a non-specific effect of the reagent. The blocking effect of anti CD94 mAb supported that an interaction of the CD94/NKG2C receptor with HCMV-infected fibroblasts was required, though insufficient, for driving the expansion of NKG2C+ cells. By contrast, the cytolytic activity of NKG2C+ NK clones in response to HCMV-infected MRC-5 or autologous fibroblasts was not consistently inhibited by the anti-CD94 mAb, thus indicating that the KLR did not play a dominant role in triggering NK effector functions against virus-infected fibroblasts (data not shown).
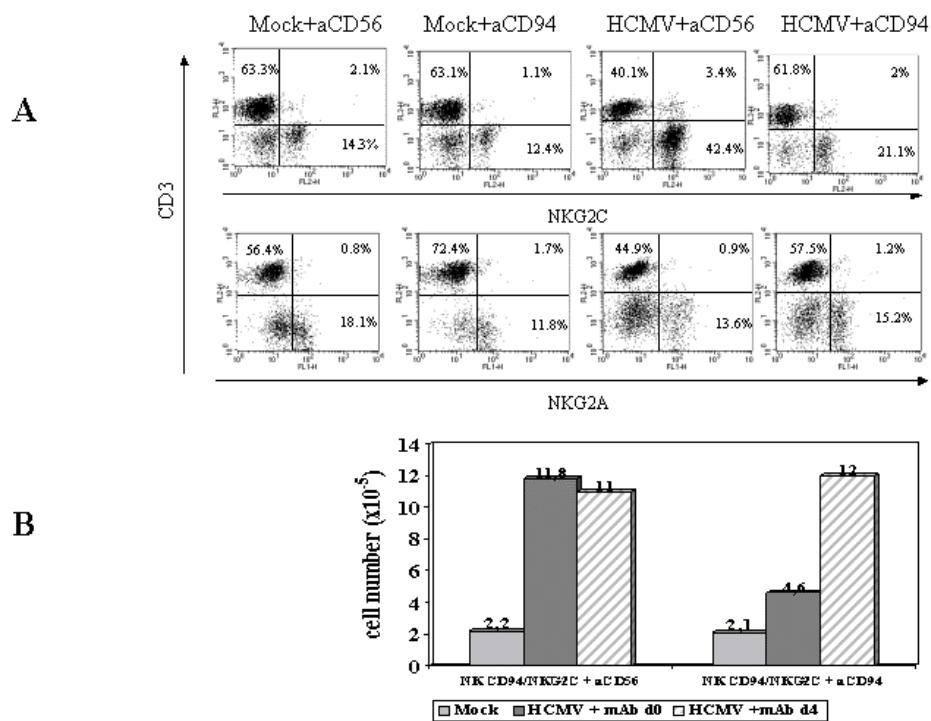


**Figure 6.** Anti CD94 mAb blocks the expansion of NKG2C+ NK cells stimulated by HCMV-infected fibroblasts. (A) PBL from an HCMV+ donor were cultured with mock- and Towne-infected (moi=1) MRC-5 cells. Either F(ab')2 anti CD94 or CD56 mAbs (10 μg/ml) were added at day 0. Flow cytometry analysis with anti CD3, NKG2C and NKG2A mAbs was carried out at day 10. (B) The differential effects in the numbers of recovered cells in the presence of the anti CD94 mAb added at day 0 or day 4 are compared. The data are representative of 5 experiments performed.

**Response of CD94/NKG2C+ cells to fibroblasts infected with HCMV deletion mutants.**

The CD94/NKG2C receptor specifically interacts with HLA-E bound to peptides derived from the leader sequences of other class I molecules, though with a lower affinity than NKG2A[27; 28]. It has been proposed that HLA-E may be selectively preserved during HCMV infection, impairing the response of CD94/NKG2A+ NK cells[23; 24]. Thus, the HLA class Ib molecule was considered a first candidate ligand for CD94/NKG2C in HCMV-infected cells. To address whether UL40 was required for the expansion of CD94/NKG2C+ cells, a targeted deletion mutant of HCMV missing the UL40-42 genes[36] was studied. The HB5ΔUL40-42 mutant was constructed from a HCMV BACmid pHB5, that lacks the US2-US6 gene region. Thus, HB5 and HB5ΔUL40-42 viruses retain only US11 to inhibit HLA class I expression. In these experiments, PBL were stimulated with MRC-5 cells infected with either AD169, HB5, HB5ΔUL40-42, or another HB5-derived targeted deletion mutant (HB5ΔUL14-20) missing the UL16 and UL18 genes[36]. In line with a previous report[23], surface HLA-E was undetectable by flow cytometry in both mock- and HCMV-infected fibroblasts (Figure 7A). Infection of MRC-5 cells with either HB5, HB5ΔUL40-42 or HB5ΔUL14-20 resulted in significantly higher levels of HLA class I molecules (Figure 7A, panel c), as compared to the inhibition exerted by AD169. Albeit reaching a slightly lower yield compared with AD169, the late outgrowth of NKG2C+ NK cells was observed in every case, indicating that it was independent of UL40, and excluding as well a critical involvement of UL18 or UL16 (Figure 7B). By contrast, the expansion of NKG2C+ cells was impaired in response to another HCMV mutant lacking all MHC I downregulating genes, i.e. US2 through US11 (HB5ΔUS2-11) (Figure 7B) which, as expected, did not alter the expression of HLA class I in infected fibroblasts (Figure 7A). These results suggested that normal levels of HLA class I may dampen the stimulation of CD94/NKG2C+ cells, presumably engaging the inhibitory receptors (i.e. KIR and/or ILT2) expressed by this cell subset. Further studies are required to determine whether the low levels of surface HLA-E in HCMV-infected fibroblasts participate in engaging CD94/NKG2C and inducing the response.
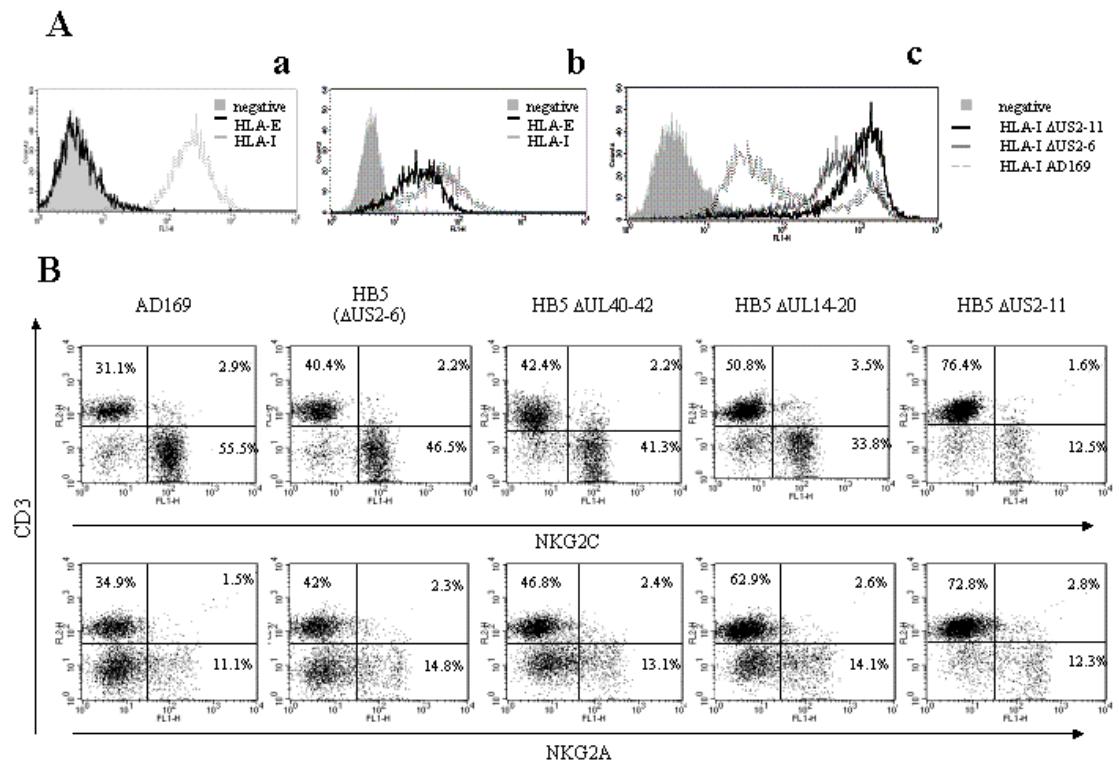
**Figure 7.** Expansion of NKG2C+ NK cells in response to fibroblasts infected with HCMV deletion mutants. (A) (a) The expression of total class I molecules (HLA-I) and HLA-E was analysed by flow cytometry in MRC-5 fibroblasts. The expression of HLA-E was also undetectable in AD169-infected MRC-5 cells (not shown). (b) Staining of an HLA-E+ cell line (.221-AEH) is shown for comparison. (c) Expression of HLA-I in MRC-5 cells infected for 72 with either AD169, HB5 (ΔUS2-6) or HB5-ΔUS2-11 mutants; HLA expression in cells infected with HB5-ΔUS40-42 or HB5-ΔUS14-20 was comparable to HB5 (not shown). It is of note that the histogram of AD169-infected cells includes a fraction of uninfected cells. (B) PBL from an HCMV+ donor were cultured in parallel with MRC-5 cells infected (moi=1) with the wild type AD169 HCMV strain or different deletion mutants generated from the HB5 BACmid clone (ΔUS2-6). Two-colour flow cytometry analysis was performed by day 10. Data are representative of 6 different experiments. The proportion of NKG2C+ cells in mock-infected cultures (not shown) was 10.5%

## DISCUSSION

The detection of increased proportions of CD94/NKG2C+ cells in PBL from healthy HCMV+ donors suggested that the viral infection might shape the NKR repertoire[26]. In the present report we provide direct in vitro evidence supporting that the CD94/NKG2C+ NK cell subset preferentially expands upon interaction with HCMV-infected fibroblasts, and that the KLR itself may participate in driving the proliferation. The requirements for HCMV-infection to promote this effect have been defined, and its complexity partially dissected.

To assess the influence of HCMV on the NKR repertoire, PBL were stimulated with infected fibroblasts, an experimental system conventionally employed to study virus-specific CTL[43]. T lymphocytes were predominant during the first week, but a substantial increase of NK cells was detected by day 10-12. NKG2C+ cells systematically outnumbered the NKG2A+ subset and a minor proportion of NKG2C+ T lymphocytes was also identified. The proliferation of NKG2C+ cells was confirmed by two-colour analysis of CFSE-labelled samples. This pattern of response observed in PBL samples from a group of HCMV+ donors, but not in HCMV- individuals, was similar regardless of the origin of fibroblasts (allogeneic vs autologous) and the virus strains tested (i.e. Toledo, Towne and AD169). Fibroblast infection was essential for the expansion of NKG2C+ NK cells, as it was dependent on the infectious dose and was not substantiated with UV-inactivated HCMV, nor when PBL were incubated with the virus alone.

Anti CD94 mAb F(ab')2 markedly inhibited the response of NKG2C+ cells, supporting that signalling by the KLR was required. Nevertheless, purified CD56+ populations, containing NKG2C+ and NKG2A+ cells, did not proliferate in response to HCMV-infected fibroblasts despite the presence of exogenous IL-2, pointing out the requirement of additional signals. The outgrowth of NKG2C+ cells became evident when the CD56+ subset was stimulated with HCMV-infected cells in the presence of exogenous IL-15, added at day 4 after the culture onset. IL-15 has been reported to be required for the accumulation of NK cells in MCMV infection[42; 44]. Altogether the observations are consistent with the

view that signalling by the CD94/NKG2C receptor, upon interaction with infected fibroblasts, enhances the responsiveness to IL-15 and promotes the proliferation of the corresponding subset. The molecular basis for this effect deserves further attention, and the contribution of other signals in the expansion of NKG2C+cells is not excluded.

The nature of the putative ligand(s) expressed by HCMV-infected fibroblasts responsible for engaging CD94/NKG2C is a key open question. The observed phenomenon was reminiscent of the activation of NKG2C+ NK and T cells in response to a transfectant of the 721.221 HLA class I-deficient cell line overexpressing HLA-E+ (.221-AEH)[31]. This class Ib molecule was reported to be refractory to the action of US6 when bound to a peptide from the leader sequence of the HCMV UL40 protein, and appeared constitutively resistant to the action of US2 and US11[22-24]. On that basis it was proposed that surface HLA-E expression may be preserved in infected cells protecting them against CD94/NKG2A+ NK cells; a CD8+ T cell subset that specifically recognizes HLA-E via the TcR might counteract this evasion mechanism[45; 46]. In another report [47], downregulation of MHC class I by the HCMV US2-11 gene region was reported to dominate UL40 mediated effects in infected fibroblasts attacked by NK cells.

The analysis of HCMV deletion mutants provided a valuable information. First, the response to HB5ΔUL40-42 unequivocally demonstrated that UL40 was not required to promote the expansion of NKG2C+ cells. Moreover, cells infected with the HB5ΔUL14-20 elicited a response of NKG2C+ cells comparable to that observed with wild-type HCMV, also excluding a critical role of UL16 and UL18. This is relevant considering that most NKG2C+ cells express the ILT2 (CD85j) receptor which binds with high affinity to UL18. Moreover, 19 viral genes, present in the UL/*b'* region of HCMV strain Toledo but missing in AD169 and Towne[48], are obviously dispensable for the expansion of CD94/NKG2C cells.

HCMV mutants derived from AD169(HB5) lack the US2-US6 genes, and inhibit HLA class Ia expression using only US11, which was reported to preserve the constitutive expression of HLA-E. The

interaction of CD94/NKG2C with HLA-E, concomitant to the inhibition of HLA class Ia expression, might contribute to drive the proliferation of NKG2C+ cells. In our hands, surface expression of HLA-E was virtually undetectable by flow cytometry in mock- and HCMV-infected fibroblasts using a *bona fide* specific mAb (3D12). Tomasec et al.[23] reported similar difficulties to detect the class Ib molecule in fibroblasts employing a mAb cross-reactive with HLA-E and HLA-C. Thus, if HLA-E bound to class I-derived nonamers is indeed the ligand responsible for engaging CD94/NKG2C in infected fibroblasts, it should be effective despite its limiting expression levels and low affinity for the activating KLR. This is conceivable if signalling by inhibitory receptors is prevented by a reduction of HLA class Ia expression, even minimal as in cells infected with the HB5-derived mutants. The inability to expand NKG2C+ cells upon stimulation with cells infected with HB5ΔUS2-11, which fully preserved HLA class I molecules, supported this view; yet, a direct role for other gene(s) in the US7-11 region is not excluded. Alternatively, it cannot be ruled out that infected fibroblasts may display HLA-E/peptide complexes with high affinity for CD94/NKG2C or a viral ligand capable of directly engaging the receptor.

CD94 mAb did not consistently block cytotoxicity of NKG2C+ NK clones against HCMV-infected fibroblasts, questioning a dominant role of the KLR in triggering this NK effector function. Nevertheless, these observations do not rule out a relevant participation of CD94/NKG2C+ cells in the response to HCMV-infected cells. First, this NK subset is not susceptible to HLA-E-mediated inhibition. Moreover, the KLR may act in concert with other NKR (i.e. NKG2D, DNAM-1) and/or NCR; in this regard, it has been reported that the NK response to some tumor cells could not be inhibited by blocking individual receptor-ligand pairs [49]. On the other hand, NKG2C may be relevant in the response against other cell types susceptible to HCMV infection in vivo, not represented by fibroblasts.

Experiments with CFSE-labeled PBL indicated that only a fraction of NKG2C+ cells proliferated. Moreover, the expansion was not perceived in PBL from a group of HCMV+ donors that displayed lower proportions of NKG2C+ cells. Thus, it is conceivable that this NK cell subset

may be heterogeneous in terms of its replication capacity, and that the proliferating "progenitor" pool responsible for the expansion may be variably represented in different individuals. Lower proportions of this subset, together with the intrinsic complexity of the co-culture system where T lymphocytes respond in parallel to HCMV-infected cells, may render undetectable the proliferation of NKG2C+ cells accounting for the "non-responder" phenotype. The possibility that other signals contributing to the expansion of NKG2C+ cells may be defective in these samples, and/or that regulatory mechanisms interfere with their response, are not ruled out. In this regard, preliminary experiments indicate that the expansion of NKG2C+ cells may be perceived stimulating CD56+ populations from "non-responders" with HCMV-infected cells in the presence of IL-15.

Together with the skewed distribution of NKG2C+ cells, a marked expansion of ILT2+ T lymphocytes was systematically reproduced in PBL from HCMV+ donors stimulated with infected fibroblasts. It is noteworthy that ILT2+ T cells were also significantly increased in fresh PBL from HCMV+ individuals[26], and in transplant patients undergoing HCMV infections[50]. Studies are in progress to characterize the relationship between ILT2+ T cells and HCMV.

The impact of HCMV infection on the CD94/NKG2C+ subset is reminiscent of the oligoclonal expansion of CTL specific for viral antigens, that display an effector/memory phenotype and tend to substantially increase in elderly individuals[5; 51]; the relationship between both events deserves attention. The basis for the variability in the numbers of NKG2C+ cells observed in HCMV+ donors is uncertain, but likely reflects the challenge exerted by the virus on the innate immune system. The frequency of reactivation episodes, the extension of latent infection, and/or genetic differences in HCMV clinical isolates may be relevant in this context. The analysis of NKG2C+ cells becomes a potentially useful novel parameter to monitor the host-pathogen relationship.

**ACKNOWLEDGEMENTS:**

## REFERENCES

1.  Pass RF. Cytomegalovirus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, eds. Fields Virology. Philadelphia: Lippincott, Williams & Wilkins; 2001:2675-2705.

2.  Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol. 1999;17:189-220.

3.  Yokoyama WM, Scalzo AA. Natural killer cell activation receptors in innate immunity to infection. Microbes Infect. 2002;4:1513-1521.

4.  Gamadia LE, Rentenaar RJ, Baars PA, et al. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. Blood. 2001;98:754-761.

5.  Moss P, Khan N. CD8(+) T-cell immunity to cytomegalovirus. Hum Immunol. 2004;65:456-464.

6.  Hengel H, Brune W, Koszinowski UH. Immune evasion by cytomegalovirus--survival strategies of a highly adapted opportunist. Trends Microbiol. 1998;6:190-197.

7.  Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. Annu Rev Immunol. 2000;18:861-926.

8.  Furman MH, Dey N, Tortorella D, Ploegh HL. The human cytomegalovirus US10 gene product delays trafficking of major histocompatibility complex class I molecules. J Virol. 2002;76:11753-11756.

9.  López-Botet M, Angulo A, Gumá M. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens. 2004;63:195-203.

10. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002;296:1323-1326.

11. Smith HR, Heusel JW, Mehta IK, et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A. 2002;99:8826-8831.

12. Arnon TI, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol. 2005;6:515-523.

13. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 2001;14:123-133.

14. Valés-Gómez M, Browne H, Reyburn HT. Expression of the UL16 glycoprotein of Human Cytomegalovirus protects the virus-infected cell from attack by natural killer cells. BMC Immunol. 2003;4:4.

15. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol. 2003;33:194-203.

16. Krmpotic A, Hasan M, Loewendorf A, et al. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J Exp Med. 2005;201:211-220.

17. Lodoen MB, Abenes G, Umamoto S, Houchins JP, Liu F, Lanier LL. The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J Exp Med. 2004;200:1075-1081.

18. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3:781-790.

19. Tomasec P, Wang EC, Davison AJ, et al. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol. 2005;6:181-188.

20. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity. 1997;7:273-282.

21. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. Immunity. 1999;11:603-613.

22. Llano M, Gumá M, Ortega M, Angulo A, López-Botet M. Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. Eur J Immunol. 2003;33:2744-2754.

23. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science. 2000;287:1031.

24. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. J Immunol. 2000;164:5019-5022.

25. Wang EC, McSharry B, Retiere C, et al. UL40-mediated NK evasion during productive infection with human cytomegalovirus. Proc Natl Acad Sci U S A. 2002;99:7570-7575.

26. Gumá M, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood. 2004;104:3664-3671.

27. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391:795-799.

28. Valés-Gómez M, Reyburn HT, Erskine RA, López-Botet M, Strominger JL. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. EMBO J. 1999;18:4250-4260.

29. André P, Castriconi R, Espeli M, et al. Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. Eur J Immunol. 2004;34:961-971.

30. Ugolini S, Arpin C, Anfossi N, et al. Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. Nat Immunol. 2001;2:430-435.

31. Gumá M, Busch LK, Salazar-Fontana LI, et al. The CD94/NKG2C killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8(+) T cells. Eur J Immunol. 2005;35:2071-2080.

32. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. Nat Immunol. 2001;2:951-956.

33. Wong-Lee JG, Lovett M. Rapid and sensitive PCR method for identification of mycoplasma species in tissue culture. In: Persing D, ed. Diagnostic Molecular and Microbiology Principles and Applications. Washington DC: American Society for Microbiology; 1993:257-260.

34. Theiler RN, Compton T. Characterization of the signal peptide processing and membrane association of human cytomegalovirus glycoprotein O. J Biol Chem. 2001;276:39226-39231.

35. Borst EM, Hahn G, Koszinowski UH, Messerle M. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in Escherichia coli: a new approach for construction of HCMV mutants. J Virol. 1999;73:8320-8329.

36. Atalay R, Zimmermann A, Wagner M, et al. Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor homologs. J Virol. 2002;76:8596-8608.

37. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A. 2000;97:6640-6645.

38. Colonna M, Navarro F, Bellón T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186:1809-1818.

39. Warren HS. Using carboxyfluorescein diacetate succinimidyl ester to monitor human NK cell division: analysis of the effect of activating and inhibitory class I MHC receptors. Immunol Cell Biol. 1999;77:544-551.

40. Carr WH, Little AM, Mocarski E, Parham P. NK cell-mediated lysis of autologous HCMV-infected skin fibroblasts is highly variable among NK cell clones and polyclonal NK cell lines. Clin Immunol. 2002;105:126-140.

41. Pérez-Villar JJ, Melero I, Rodríguez A, et al. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. J Immunol. 1995;154:5779-5788.

42. Nguyen KB, Salazar-Mather TP, Dalod MY, et al. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J Immunol. 2002;169:4279-4287.

43. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.

44. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001;97:14-32.

45. García P, Llano M, de Heredia AB, et al. Human T cell receptor-mediated recognition of HLA-E. Eur J Immunol. 2002;32:936-944.

46. Pietra G, Romagnani C, Mazzarino P, et al. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. Proc Natl Acad Sci U S A. 2003;100:10896-10901.

47. Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, Hahn G. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. J Immunol. 2002;169:3257-3266.

48. Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol. 1996;70:78-83.

49. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190:1505-1516.

50. Berg L, Riise GC, Cosman D, et al. LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients. Lancet. 2003;361:1099-1101.

51. Almanzar G, Schwaiger S, Jenewein B, et al. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. J Virol. 2005;79:3675-3683.

# CAPÍTULO 7

## Expansión de células CD4+ ILT2+ en PBL estimulados con citomegalovirus

## MATERIAL Y MÉTODOS

**Donantes**

Se obtuvieron muestras de sangre heparinizada de 8 donantes sanos. Se detectaron los anticuerpos circulantes IgG anti HCMV como hemos descrito previamente[186]. Dos donantes eran seronegativos (HCMV-) y 6 seropositivos (HCMV+).

**Fibroblastos**

La línea celular MRC-5 (fibroblastos humanos fetales) se obtuvo de la American Type Culture Collection (Manassa, VA). Se establecieron las líneas de fibroblastos primarios a partir de biopsias de piel: brevemente, las muestras de tejido se disgregaron y trataron durante 1 hora a 37ºC con colagenasa A (Roche Diagnostics GmbH, Mannheim, Germany) al 0.2% en PBS que contenía penicilina 100U/ml, estreptomicina 100μg/ml y anfotericina B 2,5μg/ml. Los fibroblastos se cultivaron en Dulbecco's modified essential medium (DMEM) suplementado con suero fetal bovino (10%), L-glutamina 2 mM, penicilina 100U/ml y estreptomicina 100μg/ml (medio completo). Las líneas se usaron entre el pase 20 y 30 (células MRC-5) y entre el pase 4 y 6 (fibroblastos primarios).

**Preparación del HCMV e infección de los fibroblastos**

El HCMV (cepa Towne) se preparó infectando las células MRC-5 a baja multiplicidad. Se recogió el sobrenadante cuando se observó un efecto citopático en el 100% de las células, y se centrifugó a 1750 x g durante 10 minutos recuperando el sobrenadante. El título viral se determinó siguiendo ensayos convencionales de formación de placa de lisis[187]. La inactivación del virus mediante luz ultravioleta se realizó usando una lámpara Phillips de 30W (modelo G13) y se confirmó controlando la expresión de la proteína IE1 en células MRC-5 por inmunofluorescencia indirecta.

Los fibroblastos humanos sembrados en placas de 24 o 96 pocillos con una confluencia del 70%, se infectaron con HCMV (moi=1). Tras dos horas de adsorción en un volumen reducido de medio, se

retiró el inóculo, las células se lavaron con PBS y se cultivaron en medio completo. En paralelo y como control, fibroblastos humanos fueron tratados del mismo modo pero en ausencia de virus.

## Cultivos de linfocitos de sangre periférica

Los PBL se obtuvieron centrifugando sangre heparinizada en Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) y se incubaron en placas de 24 ($2x10^6$/2ml) o 96 pocillos ($2x10^5$/200$\mu$l) en medio completo en presencia de fibroblastos sin infectar o infectados por HCMV, inmediatamente después del periodo de adsorción. Los PBL también se estimularon con el HCMV (0.05 unidades formadoras de placa/célula) o con la cantidad equivalente de virus inactivado. Todos los cultivos se suplementaron con IL-2 (10 U/ml) (Eurocetus) desde el día 1 y se incubaron durante 10-12 días.

## Anticuerpos. Inmunofluorescencia y análisis por citometría de flujo.

El anticuerpo HP-F1 específico para ILT2 se ha descrito previamente [188]. Para el análisis por inmunofluorescencia las células se preincubaron con Ig humana agregada (10 $\mu$g/ml) para bloquear los receptores Fc, y seguidamente se marcaron con diferentes AcM y analizaron por citometría de flujo (FACScan, Becton Dickinson, Mountain View, CA) tal como hemos descrito previamente [186]. En algunos experimentos las células se separaron tras el marcaje por citometría de flujo preparativa (FACSvantage, Becton Dickinson, Mountain View, CA).

## Extracción del DNA y estudio clonal

El DNA se extrajo mediante el QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) siguiendo las instrucciones del fabricante. La clonalidad de la población T se evaluó mediante amplificación por PCR con oligonucleótidos específicos para la cadena $\gamma$ del TCR [189]. En algunas

muestras se aplicó el protocolo consensuado para el estudio de la cadena β del TCR [190]. El análisis se realizó en un secuenciador automatizado de DNA (ABIPrism 3100, Applied Biosystems, Foster City, CA).

**Expansión preferente de células T ILT2+ en PBL estimulados con fibroblastos infectados por HCMV**

Como se describe en el artículo anterior (Guma y cols, enviado a publicación), en los cultivos de PBL con fibroblastos MRC-5 infectados con HCMV en presencia de IL-2 exógena (10U/ml), observamos que la proporción de linfocitos T ILT2+ se incrementó respecto al control (fibroblastos no infectados) en las muestras de los donantes HCMV+ (figura 1), mientras que no se alteró en las de los individuos HCMV- (datos no mostrados).
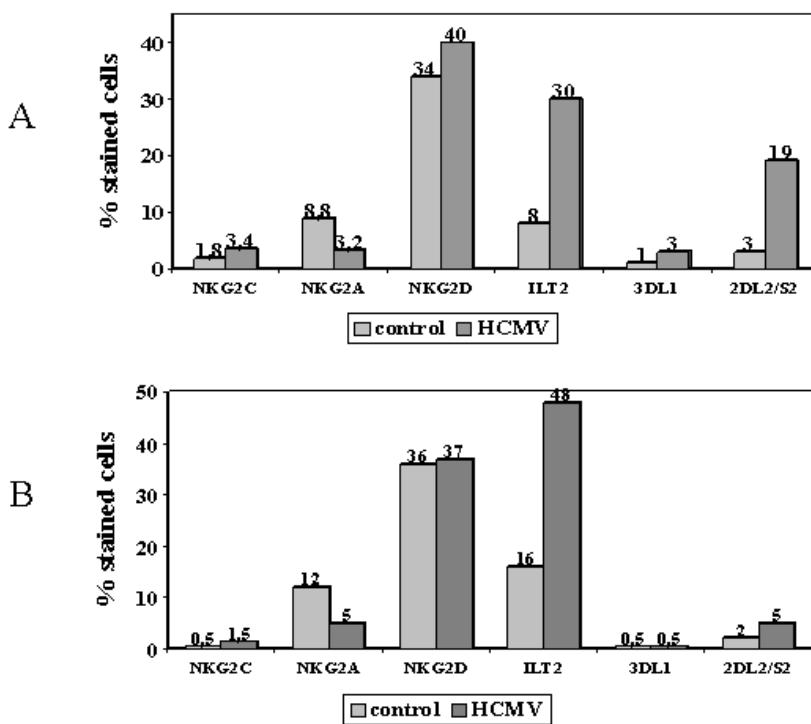


Figura 1. Los PBL de dos donantes HCMV+ (A y B) se cultivaron con fibroblastos MRC-5 sin infectar o infectados con HCMV. Las células se marcaron el día 11 con AcM específicos para diferentes NKR o NCR por inmunofluorescencia indirecta y con el AcM anti CD3-PE, analizando por FACS la población CD3+. Los datos son representativos de cuatro donantes diferentes.

**El HCMV estimula la expansión de las células T ILT2+ independientemente de la infección de los fibroblastos.**

La expansión de las células T ILT2+ se detectó en presencia de fibroblastos autólogos infectados (figura 2A) o de fibroblastos tratados con HCMV inactivado, así como incubando los PBL sólo con el virus (figura 2B). Los datos indican que las partículas de HCMV son suficiente para estimular la respuesta proliferativa de las células T que incluyen la población ILT2+.
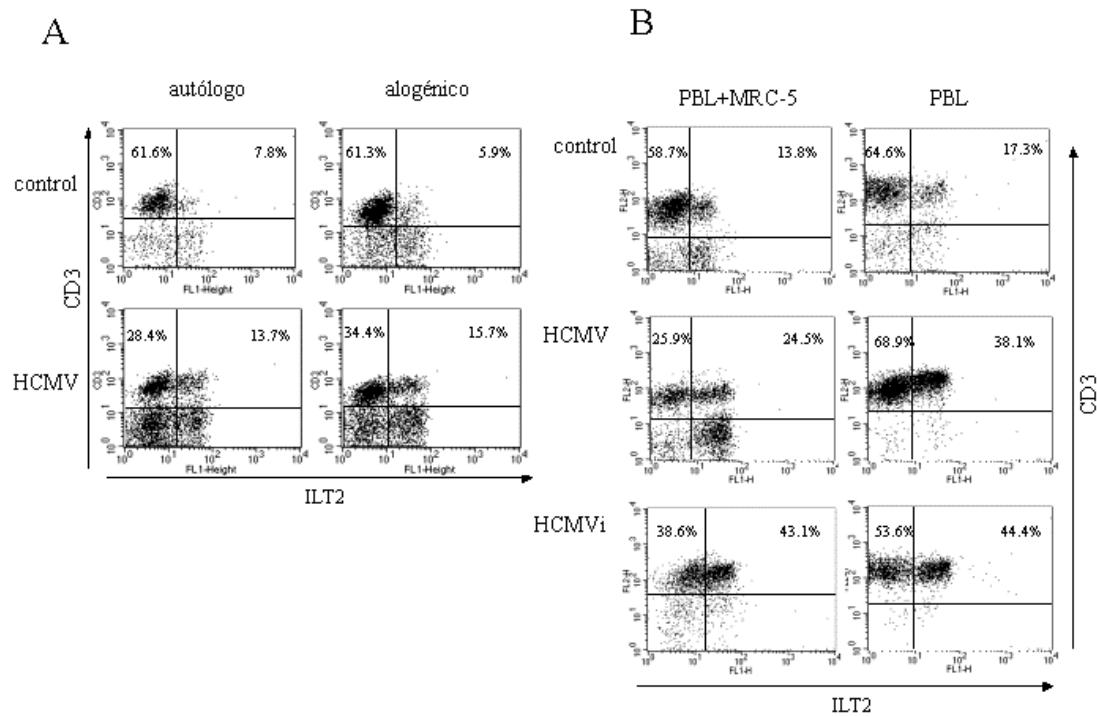


Figura 2. A) Los PBL de donantes HCMV+ se cultivaron con fibroblastos autólogos o alogénicos sin infectar (control) o  infectados con el HCMV. Las células se marcaron el día 10 con el AcM específico para ILT2 por inmunofluorescencia indirecta y con el AcM anti CD3-PE. B) Los PBL de donantes HCMV+ se incubaron en presencia de fibroblastos MRC-5 sin infectar (control), tratados con el HCMV (HCMV) o con el HCMV inactivado (iHCMV); en paralelo los PBL se incubaron solos con cada una de las preparaciones del HCMV indicadas. Las muestras se analizaron como se describe en la figura A. Los datos son representativos de 5 experimentos diferentes.

**La población T ILT2+ expandida tras la estimulación de los PBL con el HCMV es mayoritariamente CD4+.**

Por inmunofluorescencia y citometría de flujo empleando AcM anti CD4 y CD8 se constató que el fenotipo de las células T al final del cultivo era predominantemente CD4+, tanto si estimulamos los PBL con los fibroblastos infectados como sólo con el HCMV. En algunos donantes detectamos al final del cultivo una población CD4+ que coexpresaba débilmente CD8 (CD8$^{débil}$).
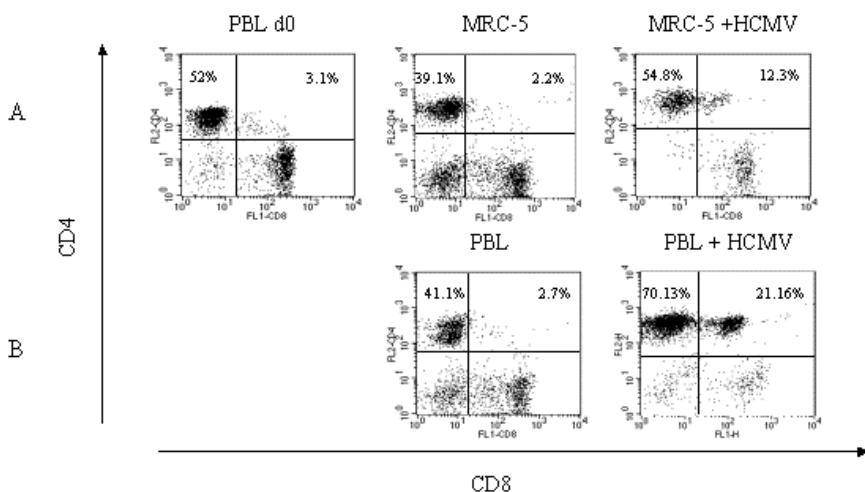


Figura 3.  A) Se cultivaron los PBL de donantes HCMV+ con fibroblastos MRC-5 sin infectar o infectados con HCMV. Las células se marcaron el día 10 con un AcM específico para CD8 y un AcM anti CD4-PE. La proporción de células CD4+ aumentó hasta un 67.1% (55-70%), siendo del 41.3% (40-50%) en el control. B) Los PBL de donantes HCMV+ se incubaron solo con el virus y se analizaron como en (A). La proporción de células CD4+ aumentó hasta un 91.29% (75-95%) en presencia del virus respecto al 43.8% (40-50%) del control. Los datos son representativos de 6 experimentos diferentes.

El incremento de células T ILT2+ se detectó tanto en la población CD4+ como CD8+. Las células CD8+ recién separadas o estimuladas con fibroblastos sin infectar expresaban ILT2 hasta en un 35%, y la proporción de CD8+ILT2+ aumentó hasta un 50% tras la incubación con el HCMV. En la población CD4+ el incremento fue mayor, ya que sólo 4-10% de las células CD4+ expresaban

ILT2 basalmente, mientras que tras la estimulación con el virus la proporción de células CD4+ILT2+ se elevó hasta un 40% (figura 4).
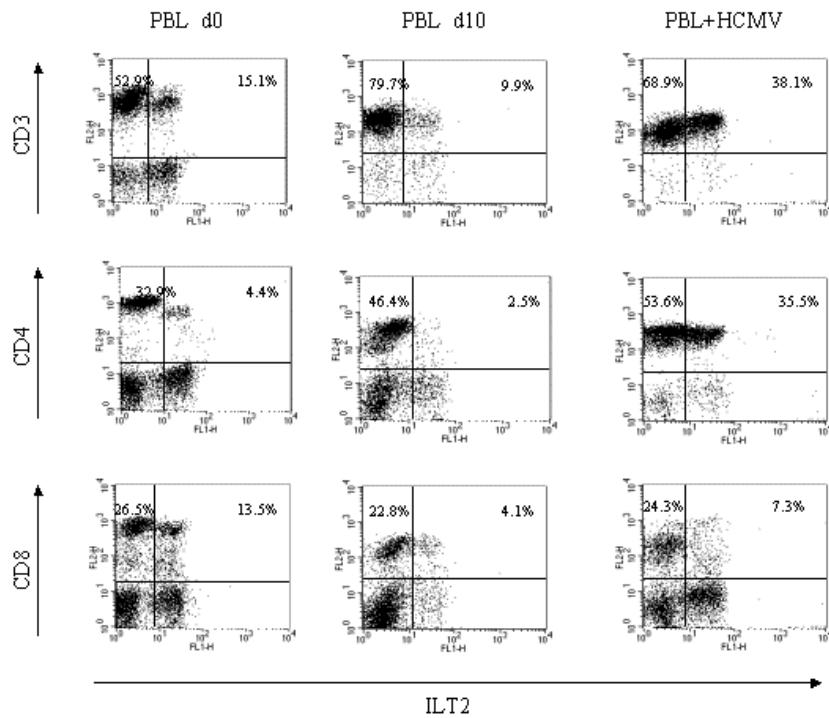


Figura 4. Se estimularon los PBL de donantes HCMV+ con el HCMV. Las células se marcaron el día 10 con el AcM específico para ILT2 y con los AcM anti CD3-PE, CD4-PE, CD8-PE. Los datos son representativos de 5 experimentos diferentes.

**La expansión de la población T en respuesta al HCMV es oligoclonal**

Estudiamos el patrón de reordenamiento del TCR $\gamma$ en la población T obtenida tras la incubación de los PBL con el HCMV. En los donantes HCMV- las células presentaron un patrón policlonal, mientras que en los donantes HCMV+ la población T al final del cultivo fue oligoclonal reflejando presumiblemente la respuesta específica al HCMV (figura 5). El pico dominante fue muy reproducible en diferentes experimentos y distinto en cada donante. Además, en tres donantes se estudió el reordenamiento V$\beta$ en la población obtenida tras estimular los PBL con HCMV,

observando un Vβ dominante diferente en cada caso (Vβ12a, 13 y 6a). El mismo pico se observó incubando los PBL con el virus inactivado o con fibroblastos autólogos/alogénicos infectados con HCMV, así como en las poblaciónes CD4+ e ILT2+ purificadas al final del cultivo (Figura 6).
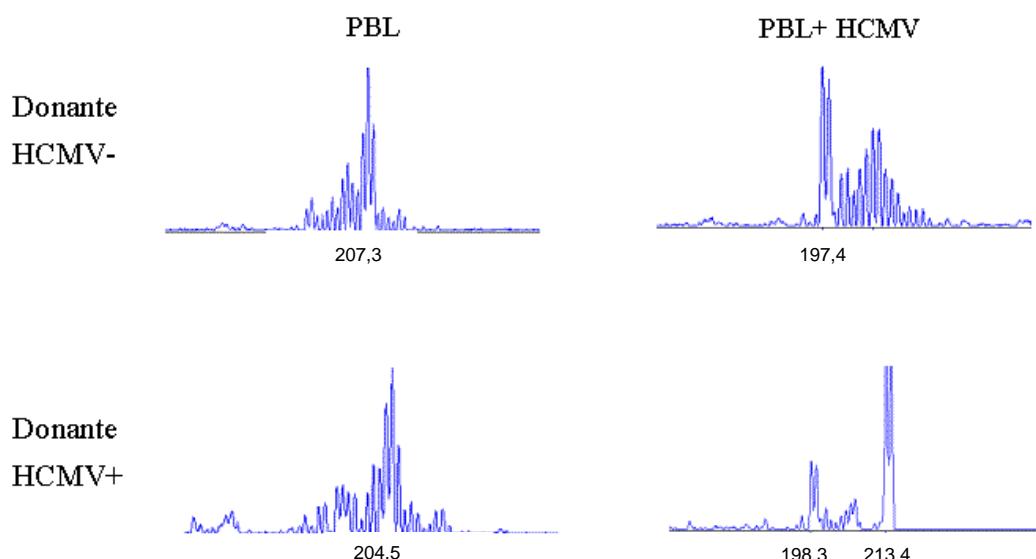


Figura 5. Se incubaron los PBL de donantes HCMV- y HCMV+ solos o en presencia del HCMV. A los 10 días se recogieron las células, y se analizó el reordenamiento del TCRγ. En el donante HCMV- se observó un patrón policlonal en ambas condiciones, a diferencia del individuo HCMV+ en el que se apreció un patrón oligoclonal tras la estimulación con el virus. Los datos son representativos de las observaciones en 2 donantes HCMV- y 6 HCMV+
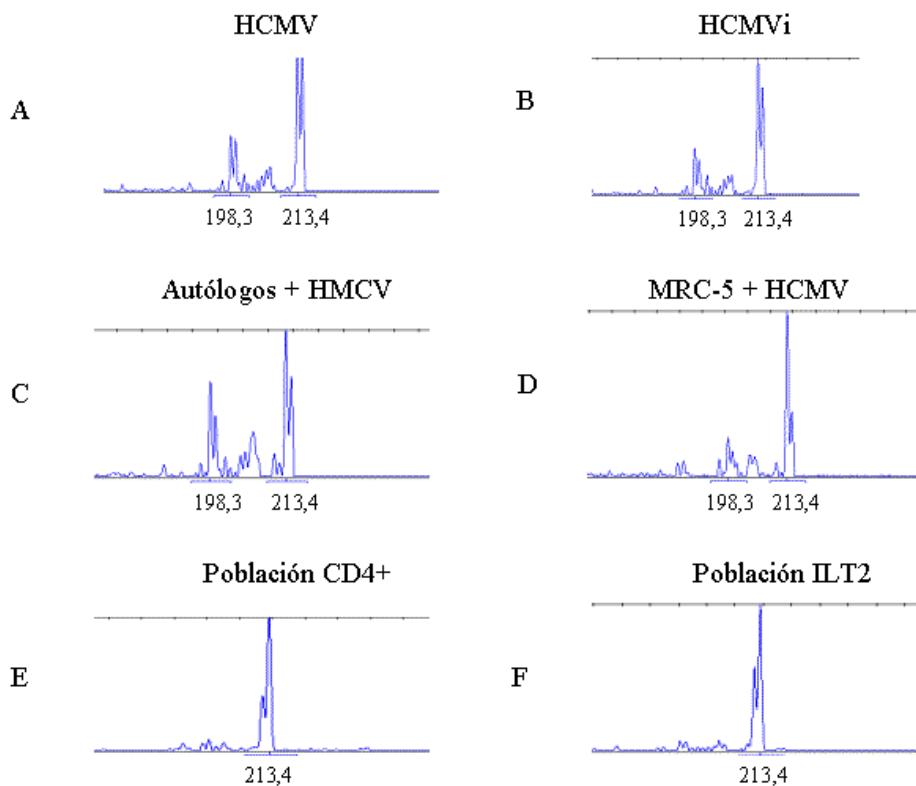
Figura 6. Los PBL de donantes HCMV+ se estimularon: con el virus (HCMV) (A), el virus inactivado (iHCMV) (B), así como con fibroblastos autólogos (C) o alogénicos (D) infectados por HCMV. A los 10 días se recogieron las células, y se analizó el reordenamiento del TCRγ en la población total (A-D) o en las subpoblaciones CD4+ (E) e ILT2+ (F) purificadas (>99%) por FACS.

**Conclusión**

Hemos observado que la población de células T ILT2+ que se expande tras la estimulación de PBL de donantes HCMV+ con fibroblastos infectados por HCMV corresponde mayoritariamente a células T CD4+. La expansión fue independiente de la infección de los fibroblastos, y se observó estimulando los PBL directamente con el HCMV o el virus inactivado. El patrón oligoclonal del

reordenamiento del TCR $\gamma$ en la población recuperada sugiere que corresponde a linfocitos T CD4+ específicas para el antígeno del CMV. Se prosigue el estudio para conocer si el receptor ILT2 expresado en estas células CD4+ es funcional.

# CAPÍTULO 8

## Discusión

Los resultados descritos constituyen la primera evidencia de que la infección por HCMV puede alterar el repertorio de NKR. El incremento en la proporción de células NK y T CD94/NKG2C+ en donantes HCMV positivos (HCMV+) sugiere que ambos subgrupos de linfocitos pueden participar en la respuesta al patógeno. El receptor no sólo estimula las funciones efectoras y la proliferación de las células NK, sino que también activa a una subpoblación minoritaria de células T CD8+. El estudio de la expansión preferente de la población NK CD94/NKG2C+ tras la interacción in vitro con fibroblastos infectados con HCMV, sugiere que el propio KLR está implicado en el proceso.

Las células NK contribuyen junto a los linfocitos T al control de la infección por HCMV [88]. Pese a los estudios realizados persisten múltiples incógnitas acerca de la naturaleza de los receptores/ligandos implicados en la respuesta innata frente al virus. Recientemente, se ha demostrado que un NKR activador reconoce directamente una proteína de MCMV [157, 158]. El cmv-1 se definió como un carácter hereditario determinante en la susceptibilidad a la infección por MCMV[159, 160], y fue identificado como el producto del gen Ly49H . Este miembro de la familia Ly49 se asocia a DAP12 y desempeña un papel crucial en la defensa frente a la infección por MCMV, activando la función de las células NK al reconocer la proteína vírica m157. Este receptor no controla únicamente las funciones efectoras (citotoxicidad y secreción de citocinas) sino que puede promover una expansión selectiva de la población Ly49H+, con posibles implicaciones en el control de la reactivación del virus[161].

Es concebible que algún receptor activador humano pueda jugar un papel comparable al de Ly49H en ratón, desencadenando la respuesta a HCMV al reconocer una proteína vírica o péptidos presentados por moléculas del MHC. La mayoría de las familias de receptores inhibidores incluyen miembros con función activadora (ej. KIR2DS/3DS, CD94/NKG2C), cuyo papel fisiológico permanece incierto; en algunos casos reconocen los mismos ligandos que los receptores inhibidores

aunque con menor afinidad. Aunque se desconocen los ligandos celulares de los NCR (NKp46, NKp30, NKp44), hay evidencia indirecta de que están distribuidos ampliamente en diferentes tejidos[40] y algún NCR podría propiciar la respuesta contra las células infectadas por HCMV, al disminuir la expresión de las moléculas MHC de clase I. Por otra parte, los ligandos del receptor NKG2D se detectan en células infectadas por CMV y el receptor coestimula la actividad de los linfocitos T citotóxicos específicos para HCMV [176].

Al analizar el repertorio de NKR en individuos HCMV+ y HCMV-, no observamos diferencias en la expresión del receptor NKG2D y detectamos una disminución de las células NKp30+ y NKp46+ 'bright' en los individuos HCMV+. Por el contrario, se observó un incremento de células CD94/NKG2C+ en los donantes HCMV+[186]. El incremento de células NK y T CD94/NKG2C+ en los donantes HCMV+, sugiere que los mismos procesos relacionados probablemente con la infección influyen en la expresión del KLR en ambas poblaciones.

Para que la interacción del receptor CD94/NKG2C con su ligando pudiera influir en el repertorio de NKR y en la distribución de las subpoblaciones de células NK, el receptor debería controlar la proliferación y/o la supervivencia celular, tal como se ha descrito en el caso de Ly49H[161] y de otros NKR [191, 192]. Aunque los conceptos de expansión clonal y memoria están reservados a las células T y B, el aumento de células NK Ly49H+ en la infección por MCMV, que persiste durante días y que contribuye a eliminar el virus, recuerda a los procesos de activación y expansión de las células T, si bien no parece conferir 'memoria' a la respuesta de las células NK [193]. Según nuestros resultados, el receptor CD94/NKG2C puede activar a las células NK así como a la subpoblación minoritaria de linfocitos T αβ+CD8+ que probablemente hayan adquirido DAP12 durante su diferenciación, controlando su proliferación y funciones efectoras [194].

Los datos indican que este receptor funciona en células T CD94/NKG2C+ DAP12+ de manera autónoma, como lo hace en las células NK, y que podría constituir una vía de activación alternativa al TCR, además de potenciar la respuesta específica de los linfocitos T. Sin embargo, es

improbable que pueda desencadenar el desarrollo de autoinmunidad, considerando los niveles relativamente bajos de HLA-E en la superficie de tejidos normales y su baja afinidad por CD94/NKG2C. Además, las células NK CD94/NKG2C+ presentan bajos niveles de NCR y, al igual que las células T NKG2C+, expresan NKR inhibidores específicos para moléculas HLA de clase I. Por otra parte, hemos observado que la modulación del TCR inducida por el AcM anti-CD3 se asocia a una inhibición de la respuesta a la estimulación tanto a través del TCR como del receptor CD94/NKG2C, indicando que puede existir una regulación cruzada entre ambas vías que impediría la activación de células T anérgicas a través del KLR (datos no publicados).

Los datos obtenidos indican que la infección por HCMV promueve la expansión de las células NKG2C+. El efecto del HCMV sobre el repertorio de NKR recuerda la generación de CTL específicos frente a antígenos víricos durante la respuesta adaptativa. Es verosímil que tras la infección primaria se incremente el número de células que expresan el KLR y que las proporciones de células NKG2C varíen como consecuencia de los episodios de reactivación. El aumento de las células NKG2C+ y de linfocitos T específicos para HCMV durante la latencia probablemente reflejan la sobrecarga a la que se ve sometido el sistema inmunitario para controlar la infección a lo largo de la vida del individuo.

Aunque el aumento de linfocitos CD94/NKG2C+ en individuos HCMV+ puede ser la consecuencia de las alteraciones en la secreción de citocinas en el curso de la infección vírica [105], la interpretación más atractiva es que la interacción del KLR con un ligando expresado en las células infectadas por HCMV controle la proliferación y/o supervivencia de los linfocitos NKG2C+ y, por consiguiente que el KLR juegue un papel activo en la defensa contra el patógeno.

El receptor CD94/NKG2C podría funcionar como coestimulador en linfocitos T específicos para CMV tal como se ha demostrado en el caso de NKG2D [176]. La coestimulación mediada por los NKR en linfocitos específicos compensaría la disminución de la expresión de las moléculas de MHC

de clase I en las células infectadas. Sin embargo, hemos observado en individuos HCMV+ HLA -A*0201 que la mayoría de las células T marcadas con tetrámeros HLA-A*0201 unidos a un epítopo derivado de la proteína inmunodominante pp65, no co-expresa CD94/NKG2C. Estos resultados coinciden con estudios que muestran que los CTL específicos para HCMV son CD94- [125, 126]. Sin embargo, no se puede descartar que las células T NKG2C+ puedan ser específicas para otros antígenos de HCMV.

La caracterización de un clon T que reconocía HLA-E a través del TCR y que expresaba niveles bajos del receptor CD94/NKG2C [134] apuntó la posibilidad de que las células T CD94/NKG2C+ pudieran corresponder a CTL específicos para HLA-E. Pietra et al, han identificado linfocitos T específicos para HLA-E, que reconocen péptidos derivados de la proteína UL40 del HCMV[135]. Sin embargo, la asociación con el receptor CD94/NKG2C no parece ser una característica general de estas células ya que se ha descrito que expresan bajos niveles de CD94/NKG2A, siendo NKG2C indetectable [136, 195]. En nuestra experiencia, la lisis de las células 221.AEH por los clones T CD94/NKG2C+ se inhibió por el AcM anti- CD94, indicando que la activación se produce a través del KLR y no vía el TCR.

Alternativamente, los linfocitos T NKG2C+ pueden representar poblaciones de CTL oligoclonales específicos para antígenos no relacionados con el CMV que han adquirido a lo largo de la diferenciación la expresión del receptor CD94/NKG2C, y que podrían ser activadas a través del mismo en el contexto de la infección por HCMV.

Para valorar la posibilidad que la interacción del KLR con un ligando expresado en las células infectadas por HCMV controle la proliferación y/o supervivencia de las células NKG2C+, analizamos el repertorio de NKR en PBL cultivados con fibroblastos infectados por HCMV. En este sistema, que se ha usado para estudiar CTL específicos de antígenos víricos [196], se detectó un marcado incremento de las células NK NKG2C+ en fases tardías. Analizando muestras marcadas

con CFSE, se confirmó que una fracción de las células NKG2C+ proliferaba. El AcM F(ab')2 anti-CD94 inhibió la respuesta de las células NKG2C+, sugiriendo que se requiere la señalización a través del KLR. Sin embargo, el AcM anti-CD94 no bloqueó la citotoxicidad de los clones NK NKG2C+ contra fibroblastos infectados por HCMV, cuestionando un papel dominante del receptor en la regulación de las funciones efectoras. A este respecto, conviene señalar que el mecanismo principal por el que la célula NK lisa al fibroblasto infectado no está bien definido y se han publicado resultados aparentemente contradictorios. Algunos trabajos proponen que el fibroblasto infectado por CMV es más susceptible al ataque por parte de las células NK[170], mientras que otros concluyen que es más resistente [165]. Por otra parte, se ha relacionado el aumento de la susceptibilidad de la células infectada con el incremento de la expresión de LFA-3, un ligando de CD2 [197].

Por consiguiente no puede descartarse que las células CD94/NKG2C+ tengan una participación relevante en la defensa frente a la infección por HCMV. En primer lugar, esta subpoblación de células NK no resulta susceptible a la inhibición mediada por la interacción de HLA-E con CD94/NKG2A. Además, el KLR podría actuar en conjunción con otros NKR (ej. NKG2D, DNAM-1), NCR y/o TLR; al respecto, se ha descrito que para inhibir la respuesta NK a algunos tumores se debe bloquear simultáneamente varios pares receptor-ligando [198]. Finalmente, el receptor CD94/NKG2C podría intervenir en la respuesta contra otros tipos celulares susceptibles a la infección in vivo (ej. células dendríticas o macrófagos).

La población CD56+ purificada no proliferó en respuesta a los fibroblastos infectados por HCMV, pese a la presencia de IL-2 exógena, sugiriendo que se requieren señales adicionales. Por el contrario, la expansión preferente de la población NKG2C+ se observó cuando las células CD56+ se estimularon por fibroblastos infectados con HCMV en presencia de IL-15 exógena. Se ha descrito que la IL-15 es necesaria para la acumulación de células NK en la infección por MCMV [80, 150, 153]. Estas observaciones sugieren que tras la interacción del receptor con los fibroblastos infectados y en

presencia de IL-15, se promueve la proliferación de la subpoblación correspondiente. No se puede descartar la posibilidad que otras señales/citocinas puedan participar en la expansión de las células NKG2C+ durante la respuesta a la infección vírica.

El aumento de las células NKG2C+ no se percibió en los PBL de un grupo de individuos HCMV+ que expresaban bajas proporciones de células NKG2C+. Es concebible que la subpoblación de células NK NKG2C+ sea heterogénea en términos de su capacidad de replicación. La variabilidad en la proporción de células responsable de la expansión, y la complejidad intrínseca del propio sistema experimental dificultarían detectar la expansión de las NK NKG2C+ en algunos individuos.

Para estudiar el papel de las células NKG2C+, realizamos algunos estudios preliminares durante la infección por MCMV. Se sabe poco acerca de la expresión y función del receptor CD94/NKG2C murino que es específico para Qa1[b], homólogo funcional de HLA-E[61]; tampoco se conoce la influencia de la infección por MCMV en la expresión de Qa1[b] ni del receptor CD94/NKG2C. En estos experimentos se infectaron ratones C57BL/6 con la cepa Smith del MCMV y se analizó la expresión del receptor NKG2C, que basalmente no se detecta, en los linfocitos del bazo y a diferentes tiempos tras la infección que se comprobó analizando la presencia del virus en las glándulas salivares a los 12 días. Aunque se observó un incremento de células NK NKG2A+, no se percibió en un ningún momento de la infección variación alguna en la proporción de linfocitos NKG2C+. Este resultado negativo no descarta que el receptor pueda estar implicado en la respuesta al CMV en otras cepas de ratones o que su expresión se incremente como resultado de los repetidos procesos de reactivación.

Se requiere, por lo tanto, determinar si realmente existe un ligando expresado en los fibroblastos infectados por HCMV, responsable de la activación vía el receptor CD94/NKG2C. La expansión de la población NKG2C+ podría acontecer si hubiera un aumento de la avidez en la

interacción KLR-ligando, y/o secundariamente a una pérdida selectiva de los ligandos para los receptores inhibidores (ej. KIR, ILT2) expresados en las células CD94/NKG2C+.

No hay datos indicativos de que el receptor CD94/NKG2C pueda reconocer alguna molécula del HCMV como en el caso de Ly49H [157, 158] ni un péptido de origen vírico asociado a HLA-E. HLA-E unida a un péptido derivado de la HSP-60 interacciona con el receptor CD94/NKG2A [71], pero no se ha demostrado que este complejo pueda activar vía CD94/NKG2C. No se puede descartar que péptidos provenientes de patógenos puedan estabilizar HLA-E y activar preferentemente vía el receptor CD94/NKG2C como se sugiere en el caso de KIR activadores [39, 199, 15].

Otra posibilidad es que una pérdida selectiva de los ligandos para los receptores inhibidores (ej. KIR, ILT2) expresados en las células CD94/NKG2C+ permita la señalización a través del receptor tras la interacción con su ligando natural. Al respecto cabe señalar que las proteínas del HCMV US2 y US11 reducen los niveles en superficie de las moléculas HLA de clase I sin afectar a HLA-E[142]; además, el nonámero derivado de la proteína UL40 del HCMV estabiliza HLA-E, favoreciendo su expresión de manera independiente a TAP y resistente a US6[167, 168]. Al preservar la molécula de clase Ib se confiere a las células infectadas por HCMV resistencia a la lisis mediada por la población CD94/NKG2A+, pero a la par podría favorecer la respuesta de las células NKG2C+. Se ha propuesto que algunos patógenos han adquirido ligandos para receptores inhibidores como mecanismo de evasión de la respuesta inmunitaria y que el huésped, como consecuencia de la presión evolutiva, puede expresar un receptor activador para ese mismo ligando[162]. Esta idea se apoya en la interacción de los receptores murinos Ly49H (activador) y Ly49I (inhibidor) con la proteína m157 del MCMV. Cabe especular que el receptor CD94/NKG2C podría haber evolucionado para contrarrestar el efecto de mecanismos de evasión que actuen a través de CD94/NKG2A.

En nuestra experiencia, la expresión en la superficie de HLA-E fue indetectable empleando un AcM específico tanto en fibroblastos sin infectar como infectados por HCMV. Tomasec et al.

describieron dificultades parecidas para detectar la molécula de clase Ib en fibroblastos usando un AcM que reconocía HLA-E y HLA-C[167]. Tampoco observamos activación en la línea RBL transfectada con el receptor CD94/NKG2C y el adaptador DAP12 cuando la cultivamos con fibroblastos infectados por HCMV (datos no publicados). Sin embargo, este resultado negativo no es valorable ya que tampoco pudimos estimularla mediante la línea 221.AEH, que sobreexpresa HLA-E en ausencia de otras moléculas MHC de clase Ia, sugiriendo que la activación fisiológica requiere la contribución de moléculas adicionales de adhesión/coestimulación. En cualquier caso, la respuesta al mutante HB5ΔUL40-42, que carece de las proteínas UL40 a UL42, demuestra inequívocamente que la proteína UL40 no es necesaria para promover la expansión de las células NKG2C+.

Los estudios con otros mutantes del HCMV proporcionaron información adicional sobre la naturaleza del hipotético ligando expresado en los fibroblastos infectados por HCMV. Las células infectadas con el mutante HB5ΔUL14-20, que carece de las proteínas UL14 a UL20, promovieron una respuesta de las células NKG2C+ comparable a la observada tras la infección con la cepa salvaje, excluyendo la influencia de UL16 y UL18. Aunque no está bien definido el papel de la interacción entre ILT2 y UL18 en la evasión de la respuesta inmunitaria, este dato es relevante considerando que la mayoría de células NKG2C+ expresan el receptor ILT2. Los resultados también descartan que UL18 actue como ligando para el receptor CD94/NKG2C, lo que se corroboró al observar que la proteína de fusión de UL18 no se unía al receptor CD94/NKG2C expresado en la línea RBL (datos no publicados).

Los mutantes de HCMV derivados de la cepa AD169 HB5, no expresan los genes US2 a US6 y disminuyen la expresión de HLA de clase Ia sólo por medio de la proteína US11, que preserva la expresión basal de HLA-E[142]. Estas observaciones indican que si la molécula HLA-E unida a nonámeros derivados de clase I fuera el ligando responsable de activar al receptor CD94/NKG2C en fibroblastos infectados, debería hacerlo a pesar de su bajo nivel de expresión (indetectable por FACS) y su reducida afinidad por el KLR. Esta situación sólo es concebible si los receptores

inhibidores no están operativos como consecuencia de la reducción de la expresión de HLA de clase Ia, incluso mínima como ocurre en las células infectadas con el mutante HB5. La incapacidad para expandir las células NKG2C+ cuando estimulamos con células infectadas con el mutante HB5ΔUS2-11, que no expresa los genes US2 a US11, apoyaría esta idea. Por otra parte, no se puede descartar que los fibroblastos infectados puedan presentar un complejo HLA-E/péptido, con mayor afinidad por el receptor CD94/NKG2C, o incluso un ligando vírico capaz de interaccionar directamente con el receptor. A este respecto debe estudiarse el posible papel de los genes de la región US7-11.

Si las células NKG2C+ juegan un papel en la defensa contra HCMV por algunos de los mecanismos mencionados, los individuos seropositivos para otros virus con mecanismos de evasión similares podrían presentar también una expansión de la población NKG2C+. En nuestra serie hemos identificado algunos casos NKG2C+ seronegativos para CMV. Aunque la explicación más probable es que sean falsos negativos de la serología[200], no se puede descartar que presenten otra infección vírica latente diferente a EBV o HSV, con las que no observamos una asociación significativa.

Se ha descrito que la proteína nef del VIH disminuye selectivamente la expresión de las moléculas HLA-A y HLA-B sin afectar a la expresión de HLA-C y HLA-E. Se ha sugerido que la expresión de éstas últimas inhibiría la función de las células NK respectivamente a través de los KIR y CD94/NKG2A[166]. Hemos iniciado en colaboración con el grupo de la Dra. Cabrera (Laboratorio de Retrovirología IrsiCaixa, Hospital Universitari Germans Trias i Pujol) un estudio para analizar la población CD94/NKG2C en pacientes VIH+, considerando la infección por CMV entre otras variables. Los datos preliminares sugieren que la infección por VIH no se relaciona directamente con la expansión de células NKG2C+, y que su incremento en estos pacientes sería también secundario a la infección por HCMV. Recientemente se ha demostrado que el péptido aa35-44 de la proteína core del virus de la hepatitis C (HCV) estabiliza HLA-E y es reconocido por el receptor CD94/NKG2A

inhibiendo a la célula NK[73]. Se requieren estudios de la población CD94/NKG2C+ en individuos HCV+.

La infección por HCMV también influye sobre el repertorio de los NKR inhibidores. Se ha descrito que la expresión de ILT2 está aumentada en pacientes con transplante de pulmón que sufren una infección por HCMV[201]. Hemos observado un incremento de linfocitos T ILT2+ tanto en PBL de donantes HCMV+[186] como tras su cultivo con fibroblastos infectados por el virus o con el virión. Un trabajo reciente [202] ha descrito que en individuos HCMV+ la expresión de ILT2 en las células T está aumentada tanto en células CD8+ (media ±SD, 35.5% ± 15.6% vs 17.2% ± 8.1%; p=0.012) como CD4+ (2.8% ± 3.1% vs 0.6% ± 1%; p=0.007). Esta asociación no se explica sólo por la co-expresión de ILT2 en las células NKG2C+ y hay que considerar la posibilidad de que ILT2 se exprese en los linfocitos T específicos para CMV. La población T CD8+ específica para CMV se ha estudiado usando tetrámeros HLA-A*0201 unidos a epítopos derivados de antígenos inmunodominantes del virus [123]. Se ha descrito que hasta un 45-55% de las células CD8+ específicas para CMV son ILT2+[202]. Este receptor, que también se expresa en linfocitos T CD8+ específicos para otros patógenos[203], e incluso en células T CD4+ específicas para M. tuberculosis[204], puede inhibir las funciones de los mismos [203, 204]. Hemos observado que la población oligoclonal T que se expande tras la estimulación de PBL de donantes HCMV+ con fibroblastos infectados por el virus o con el virus solo, corresponden a células T CD4+ que expresan ILT2+, presumiblemente linfocitos específicos para HCMV.

Se desconoce como se regula la expresión del receptor ILT2 en las células T. Se ha sugerido que la estimulación de la expresión de receptores inhibidores en células T durante las infecciones víricas, podría atenuar la respuesta inmunitaria. Sin embargo, el progresivo aumento de células T NKR+ específicas para virus que se asocia a la eliminación del patógeno, indica que los NKR no interfieren con la inmunidad antivírica [205].

En resumen, la población de células NK CD94/NKG2C+ parece implicada en el reconocimiento de células infectadas por CMV. El efecto de la infección sobre la población CD94/NKG2C+ recuerda la expansión oligoclonal que experimentan los CTL específicos para antígenos víricos, con un fenotipo efector/memoria, que tienden a aumentar en individuos de edad avanzada. La variabilidad en el número de células NKG2C+ que se observa en los individuos HCMV+ probablemente refleja el grado de presión que ejerce el virus sobre el sistema inmunitario innato. La frecuencia de los episodios de reactivación, la extensión de la infección latente, y/o diferencias genéticas en los aislados clínicos de HCMV pueden ser relevantes en este contexto. Desde un punto de vista práctico consideramos que el análisis de las células CD94/NKG2C+ puede constituir un nuevo parámetro potencialmente útil para monitorizar la relación huésped-patógeno.

# CAPÍTULO 9

## Conclusiones

1.- El receptor CD94/NKG2C se expresa en una población oligoclonal de células Tαβ+ CD8+ CD56+ CD28-, así como en células Tγδ+ y CD4+ de algunos donantes.

2.- En algunos clones Tαβ+CD8+ el receptor CD94/NKG2C se asocia a la proteína adaptadora DAP12, activando la proliferación, citotoxicidad y secreción de citocinas.

3.- En los PBL de donantes HCMV+ se detectó un incremento en las proporciones de células T y NK CD94/NKG2C+, así como de linfocitos T ILT2+, indicando que la infección por el HCMV puede alterar el repertorio de receptores NKR.

4.- La población CD94/NKG2C+ expresa más débilmente los NCR e incluye mayores proporciones de células ILT2+ y KIR+ que la población CD94/NKG2A+.

5.- No se detecta la expresión de CD94/NKG2C en los linfocitos T específicos para HCMV marcados con tetrámeros de HLA-A*0201 unidos a un péptido del antígeno pp65.

6.- Se observa una expansión preferente de la subpoblación de células NK CD94/NKG2C+ al estimular los PBL de donantes HCMV+ con fibroblastos infectados por el virus pero no con el virión. Por el contrario, la respuesta de la población de linfocitos T ILT2+ se produce en ambas condiciones.

7.- La expansión de las células CD94/NKG2C+ se bloquea con un AcM F(ab')2 anti-CD94, sugiriendo que el propio receptor puede estar implicado en el proceso.

8.- La expansión de los linfocitos CD94/NKG2C+ en respuesta a los fibroblastos infectados por HCMV es independiente de los genes UL16, UL18 y UL40. Por el contrario, no se aprecia al infectar con un mutante del HCMV que carece de la región US2-US11, donde se codifican las preoteínas que inhiben la expresión de las moléculas HLA de clase I.

9.- En conjunto, los resultados indican que las célula CD94/NKG2C pueden desempeñar un papel activo en la respuesta a las infecciones por HCMV.

# Referencias bibliográficas

1. Yokoyama WM. Natural killer cell receptors. Curr Opin Immunol. 1995;7:110-120.

2. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol. 1999;17:875-904.

3. Healy JI, Goodnow CC. Positive versus negative signaling by lymphocyte antigen receptors. Annu Rev Immunol. 1998;16:645-70.:645-670.

4. Leibson PJ. The regulation of lymphocyte activation by inhibitory receptors. Curr Opin Immunol. 2004;16:328-336.

5. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science. 2000;290:84-89.

6. Colonna M, Nakajima H, Navarro F, Lopez-Botet M. A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. J Leukoc Biol. 1999;66:375-381.

7. Diefenbach A, Raulet DH. Innate immune recognition by stimulatory immunoreceptors. Curr Opin Immunol. 2003;15:37-44.

8. Colucci F, Di Santo JP, Leibson PJ. Natural killer cell activation in mice and men: different triggers for similar weapons? Nat Immunol. 2002;3:807-813.

9. McQueen KL, Parham P. Variable receptors controlling activation and inhibition of NK cells. Curr Opin Immunol. 2002;14:615-621.

10. Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003;15:308-314.

11. Chiesa S, Tomasello E, Vivier E, Vely F. Coordination of activating and inhibitory signals in natural killer cells. Mol Immunol. 2005;42:477-484.

12. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol. 1999;17:875-904.

13. Tomasello E, Blery M, Vely E, Vivier E. Signaling pathways engaged by NK cell receptors: double concerto for activating receptors, inhibitory receptors and NK cells. Semin Immunol. 2000;12:139-147.

14. Moretta A, Moretta L. HLA class I specific inhibitory receptors. Curr Opin Immunol. 1997;9:694-701.

15. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225-74.:225-274.

16. Lopez-Botet M, Bellon T. Natural killer cell activation and inhibition by receptors for MHC class I. Curr Opin Immunol. 1999;11:301-307.

17. Colonna M, Navarro F, Lopez-Botet M. A novel family of inhibitory receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. Curr Top Microbiol Immunol. 1999;244:115-122.

18. Ugolini S, Vivier E. Regulation of T cell function by NK cell receptors for classical MHC class I molecules. Curr Opin Immunol. 2000;12:295-300.

19. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol. 2002;20:217-251.

20. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005;5:201-214.

21. Moretta L, Moretta A. Killer immunoglobulin-like receptors. Curr Opin Immunol. 2004;16:626-633.

22. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225-74.:225-274.

23. McMahon CW, Raulet DH. Expression and function of NK cell receptors in CD8+ T cells. Curr Opin Immunol. 2001;13:465-470.

24. Vivier E, Anfossi N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. Nat Rev Immunol. 2004;4:190-198.

25. Mason LH, Ortaldo JR, Young HA, Kumar V, Bennett M, Anderson SK. Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2) [see comments]. J Exp Med. 1995;182:293-303.

26. Stoneman ER, Bennett M, An J, et al. Cloning and characterization of 5E6(Ly-49C), a receptor molecule expressed on a subset of murine natural killer cells. J Exp Med. 1995;182:305-313.

27. Dimasi N, Biassoni R. Structural and functional aspects of the Ly49 natural killer cell receptors. Immunol Cell Biol. 2005;83:1-8.

28. Colonna M, Navarro F, Lopez-Botet M. A novel family of inhibitory receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. Curr Top Microbiol Immunol. 1999;244:115-122.

29. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. Tissue Antigens. 2004;64:215-225.

30. Nakajima H, Samaridis J, Angman L, Colonna M. Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor gamma-chain. J Immunol. 1999;162:5-8.

31. Torkar M, Haude A, Milne S, Beck S, Trowsdale J, Wilson MJ. Arrangement of the ILT gene cluster: a common null allele of the ILT6 gene results from a 6.7-kbp deletion. Eur J Immunol. 2000;30:3655-3662.

32. Samaridis J, Colonna M. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. Eur J Immunol. 1997;27:660-665.

33. Kubagawa H, Burrows PD, Cooper MD. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells [see comments]. Proc Natl Acad Sci U S A. 1997;94:5261-5266.

34. Wang LL, Mehta IK, LeBlanc PA, Yokoyama WM. Mouse natural killer cells express gp49B1, a structural homologue of human killer inhibitory receptors. J Immunol. 1997;158:13-17.

35. Colonna M, Samaridis J, Cella M, et al. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. J Immunol. 1998;160:3096-3100.

36. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity. 1997;7:273-282.

37. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. Immunity. 1999;11:603-613.

38. Fanger NA, Cosman D, Peterson L, Braddy SC, Maliszewski CR, Borges L. The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fc receptor- mediated signaling in monocytes [In Process Citation]. Eur J Immunol. 1998;28:3423-3434.

39. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Different checkpoints in human NK-cell activation. Trends Immunol. 2004;25:670-676.

40. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.:197-223.

41. Bottino C, Castriconi R, Moretta L, Moretta A. Cellular ligands of activating NK receptors. Trends Immunol. 2005;26:221-226.

42. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. 2001;409:1055-1060.

43. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol. 2001;31:2680-2689.

44. Arnon TI, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol. 2005;6:515-523.

45. Aramburu J, Balboa MA, Ramírez A, et al. A novel functional cell surface dimer (Kp43) expressed by natural killer cells and T cell receptor-gamma/delta+ T lymphocytes. I. Inhibition of the IL-2-dependent proliferation by anti-Kp43 monoclonal antibody. J Immunol. 1990;144:3238-3247.

46. Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol. 1996;157:4741-4745.

47. Carretero M, Cantoni C, Bellón T, et al. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. Eur J Immunol. 1997;27:563-567.

48. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. J Exp Med. 1991;173:1017-1020.

49. Yabe T, McSherry C, Bach FH, et al. A multigene family on human chromosome 12 encodes natural killer- cell lectins. Immunogenetics. 1993;37:455-460.

50. Kim DK, Kabat J, Borrego F, Sanni TB, You CH, Coligan JE. Human NKG2F is expressed and can associate with DAP12. Mol Immunol. 2004;41:53-62.

51. Carretero M, Palmieri G, Llano M, et al. Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. Eur J Immunol. 1998;28:1280-1291.

52. Le Drean E, Vely F, Olcese L, et al. Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. Eur J Immunol. 1998;28:264-276.

53. Cantoni C, Biassoni R, Pende D, et al. The activating form of CD94 receptor complex: CD94 covalently associates with the Kp39 protein that represents the product of the NKG2-C gene. Eur J Immunol. 1998;28:327-338.

54. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity. 1998;8:693-701.

55. Pérez-Villar JJ, Melero I, Rodríguez A, et al. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. J Immunol. 1995;154:5779-5788.

56. Brumbaugh KM, Pérez-Villar J, Dick CJ, Schoon RA, López-Botet M, Leibson PJ. Clonotypic differences in signaling from CD94 (kp43) on NK cells lead to divergent cellular responses. J Immunol. 1996;157:2804-2812.

57. Bellon T, Heredia AB, Llano M, et al. Triggering of effector functions on a CD8+ T cell clone upon the aggregation of an activatory CD94/kp39 heterodimer. J Immunol. 1999;162:3996-4002.

58. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature. 1998;391:795-799.

59. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med. 1998;187:813-818.

60. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci U S A. 1998;95:5199-5204.

61. Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. J Exp Med. 1999;190:1801-1812.

62. Lanier LL. NK cell receptors. Annu Rev Immunol. 1998;16:359-93:359-393.

63. Koller BH, Geraghty DE, Shimizu Y, DeMars R, Orr HT. HLA-E. A novel HLA class I gene expressed in resting T lymphocytes. J Immunol. 1988;141:897-904.

64. Grimsley C, Kawasaki A, Gassner C, et al. Definitive high resolution typing of HLA-E allelic polymorphisms: Identifying potential errors in existing allele data. Tissue Antigens. 2002;60:206-212.

65. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur J Immunol. 1997;27:1164-1169.

66. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. J Immunol. 1998;160:4951-4960.

67. Llano M, Lee N, Navarro F, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. Eur J Immunol. 1998;28:2854-2863.

68. Vales-Gomez M, Reyburn HT, Erskine RA, Lopez-Botet M, Strominger JL. Kinetics and peptide dependency of he binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. EMBO J. 1999;18:4250-4260.

69. Ulbrecht M, Couturier A, Martinozzi S, et al. Cell surface expression of HLA-E: interaction with human beta2- microglobulin and allelic differences. Eur J Immunol. 1999;29:537-547.

70. Strong RK, Holmes MA, Li P, Braun L, Lee N, Geraghty DE. HLA-E Allelic Variants.Correlating differential expression, peptide affinities, crystal structures and termal stabilities. J Biol Chem. 2003;278:5082-5090.

71. Michaelsson J, Teixeira dM, Achour A, Lanier LL, Karre K, Soderstrom K. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. J Exp Med. 2002;196:1403-1414.

72. Ulbrecht M, Modrow S, Srivastava R, Peterson PA, Weiss EH. Interaction of HLA-E with peptides and the peptide transporter in vitro: implications for its function in antigen presentation. J Immunol. 1998;160:4375-4385.

73. Nattermann J, Nischalke HD, Hofmeister V, et al. The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. Am J Pathol. 2005;166:443-453.

74. Wu J, Song Y, Bakker AB, et al. An activating immunoreceptor complex formed by NKG2D and DAP10. Science. 1999;285:730-732.

75. Zompi S, Hamerman JA, Ogasawara K, et al. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. Nat Immunol. 2003;4:565-572.

76. Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat Immunol. 2003;4:557-564.

77. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3:781-790.

78. Vivier E, Anfossi N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. Nat Rev Immunol. 2004;4:190-198.

79. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. Annu Rev Immunol. 2004;22:405-29.:405-429.

80. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001;97:14-32.

81. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity. 2001;14:105-110.

82. Colucci F, Caligiuri MA, Di Santo JP. What does it take to make a natural killer? Nat Rev Immunol. 2003;3:413-425.

83. Yokoyama WM, Scalzo AA. Natural killer cell activation receptors in innate immunity to infection. Microbes Infect. 2002;4:1513-1521.

84. Tay CH, Welsh RM. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. J Virol. 1997;71:267-275.

85. Kawamura T, Seki S, Takeda K, et al. Protective effect of NK1.1(+) T cells as well as NK cells against intraperitoneal tumors in mice. Cell Immunol. 1999;193:219-225.

86. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Human natural killer cells: Molecular mechanisms controlling NK cell activation and tumor cell lysis. Immunol Lett. 2005;100:7-13.

87. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. Proc Natl Acad Sci U S A. 2000;97:2731-2736.

88. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol. 1999;17:189-220.

89. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol. 2001;13:458-464.

90. Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol. 2002;2:957-964.

91. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol. 2005;5:112-124.

92. Moretta A. The dialogue between human natural killer cells and dendritic cells. Curr Opin Immunol. 2005;17:306-311.

93. Zingoni A, Sornasse T, Cocks BG, Tanaka Y, Santoni A, Lanier LL. Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions. J Immunol. 2004;173:3716-3724.

94. Hanna J, Gonen-Gross T, Fitchett J, et al. Novel APC-like properties of human NK cells directly regulate T cell activation. J Clin Invest. 2004;114:1612-1623.

95. Zingoni A, Sornasse T, Cocks BG, Tanaka Y, Santoni A, Lanier LL. NK cell regulation of T cell-mediated responses. Mol Immunol. 2005;42:451-454.

96. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004;5:1260-1265.

97. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.:187-376.

98. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Different checkpoints in human NK-cell activation. Trends Immunol. 2004;25:670-676.

99. Robertson MJ, Manley TJ, Donahue C, Levine H, Ritz J. Costimulatory signals are required for optimal proliferation of human natural killer cells. J Immunol. 1993;150:1705-1714.

100. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition [see comments]. Immunol Today. 1990;11:237-244.

101. Chiesa S, Tomasello E, Vivier E, Vely F. Coordination of activating and inhibitory signals in natural killer cells. Mol Immunol. 2005;42:477-484.

102. Raulet DH. Development and tolerance of natural killer cells. Curr Opin Immunol. 1999;11:129-134.

103. Kumar V, McNerney ME. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. Nat Rev Immunol. 2005;5:363-374.

104. Mingari MC, Moretta A, Moretta L. Regulation of KIR expression in human T cells: a safety mechanism that may impair protective T-cell responses. Immunol Today. 1998;19:153-157.

105. Mingari MC, Pietra G, Moretta L. Human cytolytic T lymphocytes expressing HLA class-I-specific inhibitory receptors. Curr Opin Immunol. 2005;17:312-319.

106. Draghi M, Yawata N, Gleimer M, Yawata M, Valiante NM, Parham P. Single-cell analysis of the human NK cell response to missing self and its inhibition by HLA class I. Blood. 2005;105:2028-2035.

107. Huard B, Karlsson L. A subpopulation of CD8+ T cells specific for melanocyte differentiation antigens expresses killer inhibitory receptors (KIR) in healthy donors: evidence for a role of KIR in the control of peripheral tolerance. Eur J Immunol. 2000;30:1665-1675.

108. Mandelboim O, Kent S, Davis DM, et al. Natural killer activating receptors trigger interferon gamma secretion from T cells and natural killer cells. Proc Natl Acad Sci U S A. 1998;95:3798-3803.

109. Namekawa T, Snyder MR, Yen JH, et al. Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. J Immunol. 2000;165:1138-1145.

110. Snyder MR, Weyand CM, Goronzy JJ. The double life of NK receptors: stimulation or co-stimulation? Trends Immunol. 2004;25:25-32.

111. Snyder MR, Lucas M, Vivier E, Weyand CM, Goronzy JJ. Selective Activation of the c-Jun NH(2)-terminal Protein Kinase Signaling Pathway by Stimulatory KIR in the Absence of KARAP/DAP12 in CD4(+) T Cells. J Exp Med. 2003;197:437-449.

112. Snyder MR, Nakajima T, Leibson PJ, Weyand CM, Goronzy JJ. Stimulatory killer Ig-like receptors modulate T cell activation through DAP12-dependent and DAP12-independent mechanisms. J Immunol. 2004;173:3725-3731.

113. Uhrberg M, Valiante NM, Young NT, Lanier LL, Phillips JH, Parham P. 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. J Immunol. 2001;166:3923-3932.

114. Mocarski ES, Courcelle CT. Cytomegaloviruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, eds. Fields Virology. Philadelphia: Lippincott Williams & Wilkins; 2001:2629-2673.

115. Pass RF. Cytomegalovirus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, eds. Fields Virology. Philadelphia: Lippincott, Williams & Wilkins; 2001:2675-2705.

116. Ho M. Cytomegaloviruses: biology and infection. New York: Plenum Publishing Corp.; 1991.

117. Chee MS, Bankier AT, Beck S, et al. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol. 1990;154:125-69.:125-169.

118. Rawlinson WD, Farrell HE, Barrell BG. Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol. 1996;70:8833-8849.

119. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin Immunol. 2003;15:45-51.

120. Scalzo AA. Successful control of viruses by NK cells--a balance of opposing forces? Trends Microbiol. 2002;10:470-474.

121. Farrell HE, Degli-Esposti MA, Davis-Poynter NJ. Cytomegalovirus evasion of natural killer cell responses. Immunol Rev. 1999;168:187-97.:187-197.

122. Hengel H, Brune W, Koszinowski UH. Immune evasion by cytomegalovirus--survival strategies of a highly adapted opportunist. Trends Microbiol. 1998;6:190-197.

123. Vossen MT, Westerhout EM, Soderberg-Naucler C, Wiertz EJ. Viral immune evasion: a masterpiece of evolution. Immunogenetics. 2002;54:527-542.

124. Alcami A. Viral mimicry of cytokines, chemokines and their receptors. Nat Rev Immunol. 2003;3:36-50.

125. Lopez-Botet M, Angulo A, Guma M. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens. 2004;63:195-203.

126. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol. 2001;19:65-91.:65-91.

127. Harari A, Zimmerli SC, Pantaleo G. Cytomegalovirus (CMV)-specific cellular immune responses. Hum Immunol. 2004;65:500-506.

128. Gamadia LE, Rentenaar RJ, van Lier RA, ten Berge IJ. Properties of CD4(+) T cells in human cytomegalovirus infection. Hum Immunol. 2004;65:486-492.

129. Lozza L, Lilleri D, Percivalle E, et al. Simultaneous quantification of human cytomegalovirus (HCMV)-specific CD4+ and CD8+ T cells by a novel method using monocyte-derived HCMV-infected immature dendritic cells. Eur J Immunol. 2005;35:1795-1804.

130. Komatsu H, Sierro S, Cuero V, Klenerman P. Population analysis of antiviral T cell responses using MHC class I-peptide tetramers. Clin Exp Immunol. 2003;134:9-12.

131. Moss P, Khan N. CD8(+) T-cell immunity to cytomegalovirus. Hum Immunol. 2004;65:456-464.

132. Gamadia LE, Rentenaar RJ, Baars PA, et al. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. Blood. 2001;98:754-761.

133. Young NT, Uhrberg M, Phillips JH, Lanier LL, Parham P. Differential expression of leukocyte receptor complex-encoded Ig-like receptors correlates with the transition from effector to memory CTL. J Immunol. 2001;166:3933-3941.

134. Garcia P, Llano M, de Heredia AB, et al. Human T cell receptor-mediated recognition of HLA-E. Eur J Immunol. 2002;32:936-944.

135. Pietra G, Romagnani C, Mazzarino P, et al. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. Proc Natl Acad Sci U S A. 2003;100:10896-10901.

136. Romagnani C, Pietra G, Falco M, Mazzarino P, Moretta L, Mingari MC. HLA-E-restricted recognition of human cytomegalovirus by a subset of cytolytic T lymphocytes. Hum Immunol. 2004;65:437-445.

137. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature. 1996;383:720-722.

138. Tortorella D, Gewurz B, Schust D, Furman M, Ploegh H. Down-regulation of MHC class I antigen presentation by HCMV; lessons for tumor immunology. Immunol Invest. 2000;29:97-100.

139. Hengel H, Koopmann JO, Flohr T, et al. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. Immunity. 1997;6:623-632.

140. Ahn K, Angulo A, Ghazal P, Peterson PA, Yang Y, Fruh K. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. Proc Natl Acad Sci U S A. 1996;93:10990-10995.

141. Machold RP, Wiertz EJ, Jones TR, Ploegh HL. The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complex (MHC) class I heavy chains. J Exp Med. 1997;185:363-366.

142. Llano M, Guma M, Ortega M, Angulo A, Lopez-Botet M. Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. Eur J Immunol. 2003;33:2744-2754.

143. Gewurz BE, Wang EW, Tortorella D, Schust DJ, Ploegh HL. Human cytomegalovirus US2 endoplasmic reticulum-lumenal domain dictates association with major histocompatibility complex class I in a locus- specific manner. J Virol. 2001;75:5197-5204.

144. Schust DJ, Tortorella D, Seebach J, Phan C, Ploegh HL. Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. J Exp Med. 1998;188:497-503.

145. Furman MH, Dey N, Tortorella D, Ploegh HL. The human cytomegalovirus US10 gene product delays trafficking of major histocompatibility complex class I molecules. J Virol. 2002;76:11753-11756.

146. Hegde NR, Chevalier MS, Johnson DC. Viral inhibition of MHC class II antigen presentation. Trends Immunol. 2003;24:278-285.

147. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol. 1999;17:189-220.

148. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med. 1989;320:1731-1735.

149. Bukowski JF, Woda BA, Welsh RM. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. J Virol. 1984;52:119-128.

150. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. Annu Rev Immunol. 2004;22:405-29.:405-429.

151. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol. 2001;13:458-464.

152. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin Immunol. 2003;15:45-51.

153. Nguyen KB, Salazar-Mather TP, Dalod MY, et al. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J Immunol. 2002;169:4279-4287.

154. Diefenbach A, Raulet DH. Innate immune recognition by stimulatory immunoreceptors. Curr Opin Immunol. 2003;15:37-44.

155. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. Nature. 2004;430:257-263.

156. Sivori S, Falco M, Della CM, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci U S A. 2004;101:10116-10121.

157. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002;296:1323-1326.

158. Smith HR, Heusel JW, Mehta IK, et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A. 2002;99:8826-8831.

159. Scalzo AA, Fitzgerald NA, Simmons A, La Vista AB, Shellam GR. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. J Exp Med. 1990;171:1469-1483.

160. Scalzo AA, Fitzgerald NA, Wallace CR, et al. The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. J Immunol. 1992;149:581-589.

161. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. Nat Immunol. 2001;2:951-956.

162. Arase H, Lanier LL. Specific recognition of virus-infected cells by paired NK receptors. Rev Med Virol. 2004;14:83-93.

163. Voigt V, Forbes CA, Tonkin JN, et al. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. Proc Natl Acad Sci U S A. 2003;100:13483-13488.

164. Desrosiers MP, Kielczewska A, Loredo-Osti JC, et al. Epistasis between mouse Klra and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell-mediated innate resistance to cytomegalovirus infection. Nat Genet. 2005;37:593-599.

165. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat Rev Immunol. 2001;1:41-49.

166. Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. 1999;10:661-671.

167. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science. 2000;287:1031.

168. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. J Immunol. 2000;164:5019-5022.

169. Wang EC, McSharry B, Retiere C, et al. UL40-mediated NK evasion during productive infection with human cytomegalovirus. Proc Natl Acad Sci U S A. 2002;99:7570-7575.

170. Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, Hahn G. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. J Immunol. 2002;169:3257-3266.

171. Fahnestock ML, Johnson JL, Feldman RM, Neveu JM, Lane WS, Bjorkman PJ. The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides. Immunity. 1995;3:583-590.

172. Odeberg J, Cerboni C, Browne H, et al. Human cytomegalovirus (HCMV)-infected endothelial cells and macrophages are less susceptible to natural killer lysis independent of the downregulation of classical HLA class I molecules or expression of the HCMV class I homologue, UL18. Scand J Immunol. 2002;55:149-161.

173. Leong CC, Chapman TL, Bjorkman PJ, et al. Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog [published erratum appears in J Exp Med 1998 Aug 3;188(3):following 614]. J Exp Med. 1998;187:1681-1687.

174. Saverino D, Ghiotto F, Merlo A, et al. Specific recognition of the viral protein UL18 by CD85j/LIR-1/ILT2 on CD8+ T cells mediates the non-MHC-restricted lysis of human cytomegalovirus-infected cells. J Immunol. 2004;172:5629-5637.

175. Vales-Gomez M, Shiroishi M, Maenaka K, Reyburn HT. Genetic variability of the major histocompatibility complex class I homologue encoded by human cytomegalovirus leads to differential binding to the inhibitory receptor ILT2. J Virol. 2005;79:2251-2260.

176. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol. 2001;2:255-260.

177. Vales-Gomez M, Browne H, Reyburn HT. Expression of the UL16 glycoprotein of Human Cytomegalovirus protects the virus-infected cell from attack by natural killer cells. BMC Immunol. 2003;4:4.

178. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol. 2003;33:194-203.

179. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3:781-790.

180. Rolle A, Mousavi-Jazi M, Eriksson M, et al. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol. 2003;171:902-908.

181. Lodoen MB, Abenes G, Umamoto S, Houchins JP, Liu F, Lanier LL. The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J Exp Med. 2004;200:1075-1081.

182. Hasan M, Krmpotic A, Ruzsics Z, et al. Selective down-regulation of the NKG2D ligand H60 by mouse cytomegalovirus m155 glycoprotein. J Virol. 2005;79:2920-2930.

183. Krmpotic A, Hasan M, Loewendorf A, et al. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J Exp Med. 2005;201:211-220.

184. Lodoen M, Ogasawara K, Hamerman JA, et al. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J Exp Med. 2003;197:1245-1253.

185. Tomasec P, Wang EC, Davison AJ, et al. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol. 2005;6:181-188.

186. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood. 2004;104:3664-3671.

187. Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA. Human cytomegalovirus productively infects primary differentiated macrophages. J Virol. 1991;65:6581-6588.

188. Colonna M, Navarro F, Bellón T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186:1809-1818.

189. Dippel E, Assaf C, Hummel M, et al. Clonal T-cell receptor gamma-chain gene rearrangement by PCR-based GeneScan analysis in advanced cutaneous T-cell lymphoma: a critical evaluation. J Pathol. 1999;188:146-154.

190. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257-2317.

191. Ugolini S, Arpin C, Anfossi N, et al. Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. Nat Immunol. 2001;2:430-435.

192. Andre P, Castriconi R, Espeli M, et al. Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. Eur J Immunol. 2004;34:961-971.

193. Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. Nat Immunol. 2004;5:996-1002.

194. Guma M, Busch LK, Salazar-Fontana LI, et al. The CD94/NKG2C killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8(+) T cells. Eur J Immunol. 2005;35:2071-2080.

195. Moretta L, Romagnani C, Pietra G, Moretta A, Mingari MC. NK-CTLs, a novel HLA-E-restricted T-cell subset. Trends Immunol. 2003;24:136-143.

196. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.

197. Fletcher JM, Prentice HG, Grundy JE. Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA. J Immunol. 1998;161:2365-2374.

198. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190:1505-1516.

199. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet. 2002;31:429-434.

200. Larsson S, Soderberg-Naucler C, Wang FZ, Moller E. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. Transfusion. 1998;38:271-278.

201. Berg L, Riise GC, Cosman D, et al. LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients. Lancet. 2003;361:1099-1101.

202. Antrobus RD, Khan N, Hislop AD, et al. Virus-specific cytotoxic T lymphocytes differentially express cell-surface leukocyte immunoglobulin-like receptor-1, an inhibitory receptor for class I major histocompatibility complex molecules. J Infect Dis. 2005;191:1842-1853.

203. Ince MN, Harnisch B, Xu Z, et al. Increased expression of the natural killer cell inhibitory receptor CD85j/ILT2 on antigen-specific effector CD8 T cells and its impact on CD8 T-cell function. Immunology. 2004;112:531-542.

204. Merlo A, Saverino D, Tenca C, Grossi CE, Bruno S, Ciccone E. CD85/LIR-1/ILT2 and CD152 (cytotoxic T lymphocyte antigen 4) inhibitory molecules down-regulate the cytolytic activity of human CD4+ T-cell clones specific for Mycobacterium tuberculosis. Infect Immun. 2001;69:6022-6029.

205. Moser JM, Byers AM, Lukacher AE. NK cell receptors in antiviral immunity. Curr Opin Immunol. 2002;14:509-516.

# Abreviaturas

AcM       Anticuerpo monoclonal

BCR       Receptor de células B

CTL       Linfocitos T citotóxicos

CMV       Citomegalovirus

DC       Célula dendrítica

DAP10       Proteína activadora DNAX de 10 kDa

DAP12       Proteína activadora DNAX de 12 kDa

EBV       Virus de Epstein-Barr

FcR       Receptor del fragmento cristalizable de las inmunoglobulinas

HCMV       Citomegalovirus humano

HCMV+       Donante seropositivo para HCMV

HCMV-       Donante seronegativo para HCMV

HCV       Virus de la hepatitis C

HLA       Complejo principal de histocompatibilidad humano

HSV       Virus del herpes virus

IFN       Interferón

IL       Interleucina

Ig       Inmunoglobulina

Ig-SF       Superfamilia de las inmunoglobulinas

ILT       *Ig-like transcripts*

KIR       Receptor de células citotóxicas con dominios Ig

KLR       Receptores de células citotóxicas tipo lectina

LIR       Receptores de leucocitos con dominio Ig

| | |
|---|---|
| MCMV | Citomegalovirus murino |
| MHC | Complejo principal de histocompatiblidad |
| NCR | Receptores de citotoxicidad natural |
| NK | Célula citotóxica natural |
| NKR | Receptores de las células citotóxicas naturales |
| PBL | Linfocitos de sangre periférica |
| PCR | Reacción en cadena de la polimerasa |
| rADCC | Citotoxicidad natural dependiente de anticuerpo reversa |
| SHP | Fosfatasas con dominios SH2 |
| VIH | Virus de la inmunodeficiencia humana |
| TAP | Transportadores en la presentación de antígenos |
| TCR | Receptor de células T |
| TNF | *Tumor necrosis factor* |

# Publicaciones

1 **Gumà M**, Gómez-Lozano N, Solé FX, Santoli D, Vilches C, López-Botet M. Variegated expression of functional Killer Immunoglobulin-like Receptors in a human TCRgd+ cell line. Submitted.

2 **Gumà M**, Budt M, Sáez A, Brckalo T, Hengel H, Angulo A, López-Botet M. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. Submitted.

3 **Gumà M**, Angulo A, López-Botet M. NK cell receptors involved in the response to human cytomegalovirus infection. Curr Top Microbiol Immunol 2005; VIII: 207-224↓.

4 **Gumà M**, Busch LK, Salazar-Fontana LI, Bellosillo B, Morte C, García P, López-Botet M. The CD94/NKG2C killer lectin-like receptor constitutes an alternative activation pathway for a subset of CD8+ T cells. Eur J Immunol. 2005;35:2071-80.

5 **Gumà M**, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood. 2004;104:3664-71.

6 López-Botet M, Angulo A, **Gumà M**. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens. 2004;63:195-203.

7 Llano M, **Gumà M**, Ortega M, Angulo A, López-Botet M. Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. Eur J Immunol. 2003;33:2744-54.

## Agradecimientos

Quisiera agradecer a todos los donantes de sangre, también de fibroblastos, su ayuda para realizar estos experimentos, especialmente a aquellos que han accedido de forma repetida durante estos dos últimos años. Esta tesis no se hubiera podido realizar sin su colaboración.