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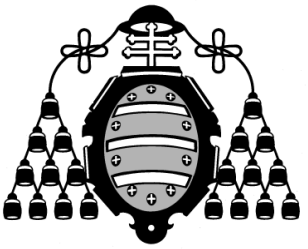

Programa de Doctorado
Biología Funcional y Molecular

CARACTERIZACIÓN DE LAS VÍAS DE SEÑALIZACIÓN ACTIVADAS POR NKG2D Y SU PAPEL EN LA REGULACIÓN DE LA MIGRACIÓN CELULAR

Tesis Doctoral
Esther Serrano Pertierra
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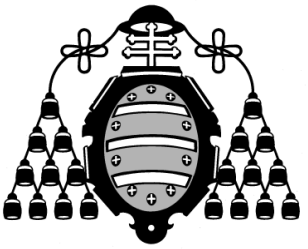

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español: Caracterización de las vías de señalización activadas por NKG2D y su papel en la regulación de la migración celular	Inglés: Characterization of NKG2D-mediated signaling pathways and its role in the regulation of cell migration

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RESUMEN (en español)

NKG2D es un receptor transmembrana expresado principalmente en células NK y T CD8+. Se asocia a la proteína DAP10 para la transducción de señales, la cual contiene un motivo de señalización YINM, similar al de la molécula CD28. Los ligandos de NKG2D en humanos son MICA, MICB y las ULBP1-5, cuya expresión está restringida en condiciones fisiológicas, y regulan los niveles en superficie de NKG2D. Las células tumorales pueden liberar los ligandos de NKG2D en su forma soluble, lo cual promueve la internalización del receptor y la disfunción en la actividad de células NK y T CD8+. Además, los ligandos de NKG2D también pueden ser secretados mediante exosomas que son capaces de modular los niveles en superficie de NKG2D y la actividad de las células efectoras. El sistema de reconocimiento de NKG2D se ha postulado como un mecanismo importante no sólo en la respuesta inmune, sino también en la vigilancia inmunológica o en la reactividad autoinmune de las células T. Sin embargo, el papel de NKG2D en la regulación de otros procesos que puedan estar implicados en estas respuestas no se ha explorado completamente.

En este proyecto se estudió el papel de NKG2D en la regulación de la migración de células NK y T CD8+, dado que la capacidad migratoria de las células del sistema inmune es esencial para llegar adecuadamente a los tejidos diana. Esta tesis demuestra que la activación de NKG2D o la señalización simultánea por CD3 y NKG2D inhiben la quimiotaxis de las células NK y las células T CD8+, respectivamente. También se investigaron las vías de señalización implicadas en este

proceso. NKG2D activa las Rho GTPasas Rac1 y Cdc42 en las células NK, mientras que la actividad de RhoA disminuye. Además, la proteína del síndrome de Wiskott-Aldrich (WASp) y N-WASp están implicadas en la regulación de la migración de células NK mediada por NKG2D. En células T CD8+, Rac1 se activa tras la estimulación de CD3 y CD28. Sin embargo, sólo la activación de CD3 y NKG2D incrementa adicionalmente la actividad de Cdc42. N-WASp es también necesaria para la migración de células T CD8+ mediada por CD3 y NKG2D. Por tanto, la coestimulación vía NKG2D induce cascadas de señalización diferentes de aquellas inducidas por la coestimulación mediada por CD28.

Este proyecto también evaluó el papel de las balsas lipídicas en la señalización mediada por NKG2D. Las balsas lipídicas son compartimentos especiales en la membrana plasmática que actúan como plataformas de señalización. NKG2D se recluta a estas balsas lipídicas, y el análisis proteómico de estas fracciones mostró que el citoesqueleto de actina está implicado en este proceso. Además la proteína L-plastina, identificada en el análisis proteómico, es esencial en el reclutamiento de NKG2D a las balsas lipídicas y es necesaria en la inhibición de la quimiotaxis de las células NK mediada por NKG2D.

También se ha estudiado la capacidad migratoria de las principales poblaciones linfocitarias (células CD4+, CD8+, NK y B) en pacientes con esclerosis múltiple, una enfermedad autoinmune. Se ha observado la expresión de MICA/B en lesiones activas de EM, sugiriendo un posible papel de NKG2D en la enfermedad. La migración de las células NK está incrementada en pacientes durante la fase de remisión o en pacientes con un primer evento desmielinizante (síndrome clínicamente aislado), pero no durante el brote. Este incremento correlaciona con un mayor porcentaje de células NK que expresan el receptor de quimiocina CXCR4. El papel de las células NK en las enfermedades autoinmunes es aún controvertido, sin embargo los resultados descritos en esta tesis sugieren que en el inicio de la enfermedad se induce una respuesta de células NK, incrementándose así su capacidad migratoria y facilitando la regulación de células autorreactivas o de la microglía activada en el sistema nervioso central.

RESUMEN (en Inglés)

NKG2D is a transmembrane receptor mainly expressed on NK cells and CD8+ T cells. It couples to the adaptor protein DAP10 for signal transduction. DAP10 contains a YINM signaling motif similar to the YMNM motif of CD28. In humans, the ligands for NKG2D are MICA, MICB, and ULBP1-5, whose expression is normally restricted under physiological conditions. NKG2D surface levels are regulated by these ligands. Tumor cells can release NKG2D ligands into their soluble form, which promotes the internalization of the receptor and the impairment of NK cell and CD8+ T cell activities. In addition, NKG2D ligands may also be secreted via exosomes that are able to modulate NKG2D surface levels and effector cell activities. Thus, the NKG2D recognition system has been postulated as an important mechanism not only in the immune response but also in immune surveillance or in autoimmune reactivity of T cells. However, the possible role of NKG2D in the regulation of other mechanisms that may be involved in these responses has not been fully explored.

In this project, the role of NKG2D in the regulation of cell migration of NK cells and CD8+ T cells was studied since the migratory ability of the cells of the immune system is essential to properly reach the target tissues. This thesis demonstrates that activation of NKG2D alone or simultaneous signaling through CD3 and NKG2D inhibit NK cell and CD8+ T cell chemotaxis, respectively. The signaling pathways that participate in this process have been also investigated. NKG2D cross-linking activated the RhoGTPases Rac1 and Cdc42 in NK cells, while the activity of RhoA decreased. Furthermore, the Wiskott-Aldrich syndrome protein (WASp) and N-WASp are involved in the regulation of NKG2D-mediated NK cell migration. In CD8+ T cells, Rac1 is activated upon CD3/CD28 stimulation. However, CD3/NKG2D activation, but not CD3/CD28, additionally activates Cdc42. Moreover, N-WASp is required for CD3/NKG2D-mediated CD8+ T cell migration. Thus, costimulation via NKG2D induces different signaling pathways from those arising from CD28-mediated costimulation.

This project also evaluated the role of membrane rafts in NKG2D-mediated signaling. Membrane rafts are special compartments within the plasma membrane that act as signaling platforms. NKG2D is recruited into membrane rafts, and the proteomic analysis of these fractions showed that the actin cytoskeleton is involved in this process. Furthermore, the actin-bundling protein L-plastin that was identified in the

proteomic analysis is essential for NKG2D recruitment into membrane rafts. L-plastin was found to be required for NKG2D-mediated inhibition of NK cell chemotaxis as well.

In addition, the migratory ability of the main lymphocyte subsets (CD4+, CD8+, NK and B cells) was studied in patients with multiple sclerosis, an autoimmune disease. The expression of MICA/B in active MS lesions has been observed, suggesting a possible role of NKG2D in the disease. NK cell migration is increased in patients in the remission phase or in patients with a first demyelinating event (called clinically isolated syndrome), but not during relapse. This increment correlated with an increased percentage of NK cells expressing the chemokine receptor CXCR4. The role of NK cells in autoimmune diseases is still controversial, however the results described in this thesis suggest that in the onset of the disease a NK cell response is induced, thus increasing the ability of NK cells to migrate and facilitating the regulation of autoreactive T cells or activated microglia in the central nervous system.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNCIONAL Y MOLECULAR

This research thesis was done under the supervision of Professor Carlos López-Larrea (HUCA; Immunology Department, Universidad de Oviedo, SPAIN) and Dr. Eva Cernuda-Morollón (HUCA; Neurology Department, Oviedo, SPAIN).

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Federico García Lorca dijo: "El teatro es poesía que se levanta del libro y se hace humana. Y al hacerse, habla y grita, llora y se desespera".

Esta tesis doctoral, al hacerse, también.

Como si el porvenir no viniera nunca, añorado Ángel González. Y sin embargo, *siempre queda, un poco más, un minuto, la lanza, un segundo, la fiebre, y otro segundo, el sueño -la lanza, la fiebre, mi dolor y la palabra, el sueño-.*

"¿Y ahora qué pasa, eh?"

Pues ahora sólo vértigo y gratitud. Muchísima gratitud…

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A la gente del laboratorio de Terapia Celular, donde nacieron mis primeros experimentos: María, Yolanda, Amaya, Silvia y Marcos.
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Y a Corto Maltés.

Por nuestros delirios en la Casa Dorada de Samarkanda que ahora habitamos.

Por nuestras carreras para coger el tren del oro.

Porque has sido testigo y víctima a la vez de esta tesis.

Y esto último no lo merecías.

Sácame de esta fábula.

„Brenn' doch, Fantasie!

Du, die so lange im Untergrund meines Gedächtnisses arbeitete."

Offener Brief (unter dem Licht der Schiffslaterne)

TABLES

FIGURES

ABBREVIATIONS (in alphabetical order)

7-AAD	7-amino-actinomycin D
Ab	antibody
Aβ	beta-amyloid peptide
ACN	acetonitrile
ANOVA	analysis of the variance
APC	antigen presenting cell / allophycocyanin
APP	amyloid precursor protein
Arp 2/3	actin-related protein 2/3
β-OG	ß-octylglucoside
BACE1	ß-site APP cleavage enzyme
BBB	blood-brain barrier
BCECF	2′-7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
BSA	bovine serum albumin
CEC	cerebral endothelial cell
CD	cluster of differentiation
CIP4	Cdc42-interacting protein 4
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DMSO	dimethyl sulphoxide
DRM	detergent resistant membranes
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EBP50	ezrin/radixin/moesin-binding phosphoprotein of 50 kDa
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
ERK	extracellular signal regulated kinase
ERM	ezrin-radixin-moesin
FBS	fetal bovine serum
FcεRI	high affinity receptor for IgE
FcRγ	Fc receptor gamma
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GDI	GDP-dissociation inhibitor

GDP	guanosine di-phosphate
GEF	guanosine nucleotide exchange factor
GLM	general linear model
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound 2
GST	glutathione-S-transferase
GTP	guanosine tri-phosphate
hCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HPLC	high-performance liquid chromatography
ICAM-1	intercellular adhesion molecule
IL	interleukin
IFN-γ	interferon-gamma
Ig	immunoglobulin
IPTG	isopropyl ß-D-1-thiogalactopyranoside
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KIR	killer immunoglobulin-like receptor
LAT	linker for activation of T cells
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LFA-1	lymphocyte function-associated antigen 1
LIMK	LIM kinase
mAb	monoclonal antibody
MAG	myelin associated glycoprotein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	MAPK/ERK kinase
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIC	MHC class I-related chain
MOG	myelin oligodendrocyte protein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MTOC	microtubule organizing center
NCR	natural cytotoxicity receptor
NK	natural killer
NKG2DL	NKG2D ligands
NKL	natural killer leukemia
NKT	natural killer T cell

NOD	non-obese diabetic
N-WASp	neural-WASp
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PAGE	polyacrylamide gel electrophoresis
PBD	p21-binding domain
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PLP	proteolipidic protein
PMSF	phenylmethanesulfonylfluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPMS	primary progressive multiple sclerosis
RBD	Ras-binding domain
Rho	Ras homology
RRMS	relapsing-remitting multiple sclerosis
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	small interfering RNA
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
SLT	secondary lymphoid tissue
SPMS	secondary progressive multiple sclerosis
TBS-T	Tris buffered saline with Tween-20
TcR	T cell receptor
TGF-β	transforming growth factor b
Th	T helper cell
TNF-α	tumor necrosis factor-alpha
Treg	regulatory T cell
TRITC	tetramethylrhodamine isothiocyanate
ULBP	UL-16 binding protein
VCAM	vascular cell adhesion molecule
VLA-4	very late antigen-4
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
ZAP70	zeta-chain-associated protein kinase 70 kDa

I. INTRODUCTION

The immune system involves a complex network of different cell types, tissues, and organs that together protect the body against external pathogens. To this end, the immune system has developed two defense strategies: the innate immunity and the adaptive immunity.

The innate responses are triggered when a pathogen is identified by pattern recognition receptors that recognize conserved molecules in microorganisms, as well as other components released by damaged or injured cells. Activation of the complement system, inflammation, and the activation of the cells of the immune system (neutrophils, basophils, eosinophils, macrophages, dendritic cells, mast cells and natural killer cells) constitute the main innate responses.

The adaptive immune system is much more specific and allows the generation of an immunological memory to remember and respond quickly each time the pathogen is identified. The cells of the adaptive immune system are the lymphocytes, mainly B cells and T cells. B cells are involved in the humoral responses, whereas T cells are involved in cell-mediated immune responses. There are two types of T lymphocytes: T helper cells, which regulate both innate and adaptive immune responses, and cytotoxic T cells, which kill infected cells or clear pathogens directly.

In immunological and pathological processes, the migratory ability of cells of the immune system is essential. Dendritic cells (DC) process potentially dangerous antigens and migrate to lymphoid organs; lymphocytes continuously enter lymphoid tissues from the blood, moving randomly and scaning the surface of DC; activated T lymphocytes migrate to the site of inflammation. Cell motility is also crucial in the dissemination of tumor cells and the invasion of normal tissues. Thus, understanding the signaling pathways that regulate cell migration is important not only in the pathogenesis of the disease, but could also provide new targets for future therapeutics.

1. NATURAL KILLER CELLS

Natural killer (NK) cells are lymphocytes of the innate immune system. Approximately 10% NK cells are found in peripheral blood and the rest in secondary lymphoid tissues (SLT) (Caligiuri, 2008). NK cells constitute one of the first host defenses against intracellular pathogens, such as parasites and viruses, and play a major role in anti-tumor responses (Cerwenka and Lanier, 2001). They respond by releasing cytotoxic granules containing perforins, which form pores in the plasma membrane of the target cells, and granzymes, which together with other molecules induce apoptosis. NK cells also secrete cytokines and chemokines that influence the immune response.

In contrast to T cells or B cells, NK cell development is still not clear. Pre-NK cells derived from hematopoietic progenitor cells (phenotypically characterized by the expression of CD34 and CD45 on the cell surface) have been identified in bone marrow and blood, and also in SLT in a greater proportion. This fact has led to consider the SLT a site for NK-cell development (Caligiuri, 2008). Although NK cells are a heterogeneous population, the conventional phenotype that defines them is the expression of the CD56 surface antigen and the absence of CD3 (CD3-CD56+) (Papamichail *et al.*, 2004). In peripheral blood, approximately 90% of circulating NK cells express CD16. Conversely, NK cells lacking CD16 (CD16-) are enriched in SLT and only 10% are present in peripheral blood (Ferlazzo *et al.*, 2004).

NK cells can discriminate between healthy and abnormal cells. When they encounter a target cell, NK cell receptors reorientate to recognize potential ligands and to form an immunological synapse (IS) (Davis, 2002). This is similar to the interaction between T cells and antigen presenting cells (APC). However, NK cells are able to respond immediately (Lanier, 2005). Mechanisms for NK cell recognition are complex and involve a wide repertory of activating and inhibitory receptors expressed on their surface. Several NK

cell receptors have been described to date. Inhibitory receptors contain a tyrosine-based inhibitory motif (ITIM), whereas activating NK cell receptors have different cytoplasmic signaling domains, tyrosine-based activation motif (ITAM) or non-ITAM (Bryceson *et al.*, 2011) (Table I.1).

ITAM motif	**Non-ITAM**
CD16	NKG2D
NKp30	2B4
NKp44	**ITIM motif**
NKp46	KIR2DL1
KIR2DS1	KIR2DL2/3
KIR2DS2	KIR3DL1
NKG2C	NKG2A
Integrins	
LFA-1 (αLβ2, CD11a/18)	
VLA-4 (α4β1, CD49d/29)	

Table I.1. NK cell receptors. Representative NK cell activating and inhibitory receptors according to the signaling motifs present in their cytoplasmic domains and adhesion receptors that contribute to NK cell activity.

The killer immunoglobulin (Ig)-like receptors (KIR) are monomeric type I glycoproteins with 2 or 3 Ig-like extracellular domains. This family includes both activating and inhibitory receptors that recognize human leukocyte antigen (HLA)-A, HLA-B and HLA-C proteins. Inhibitory KIRs contain an ITIM motif, whereas activating KIRs signal through the immunoglobulin Fc

receptor gamma (FcRɔ) or through the ITAM-bearing adaptor protein DAP12 (Thielens *et al.*, 2012).

CD94/NKG2 receptors are type II transmembrane proteins of the C-type lectin-like family. CD94 forms heterodimers with NKG2A or NKG2C (Pegram *et al.*, 2011). CD94/NKG2A serves as inhibitory receptors, whereas CD94/NKG2C function as activating receptors that associate with DAP12 for signal transduction (Lanier *et al.*, 1998).

Natural cytotoxicity receptors (NCR) are type I transmembrane activating receptors whose members are NKp30, NKp46 and NKp44. Unlike other NK cell receptors mentioned above, NCRs recognize pathogen-derived molecules and non-major histocompatibility complex (MHC) self-molecules that are expressed on stressed cells (Hudspeth *et al.*, 2013).

The NK cell receptor 2B4 belongs to the Ig-superfamily and has two Ig-like domains in the extracellular region. It apparently behaves as an activating receptor in humans (Lanier, 2005) altough it is thought that the outcome of 2B4 triggering depends on the stage of NK cell maturation.

NK cells are able to kill target cells lacking MHC molecules or cells expressing allogeneic MHC molecules, which is called the 'missing self' hypothesis. However, in the last years several studies have described further mechanisms in addition to this hypothesis. For instance, inhibitory receptors that recognize other ligands rather than class I MHC molecules (MHC-I) may suppress the NK cell response. When both activating and inhibitory signals exist, the final outcome of NK cell activity depends on the strength of the signal (Lanier, 2005) (Figure I.1).

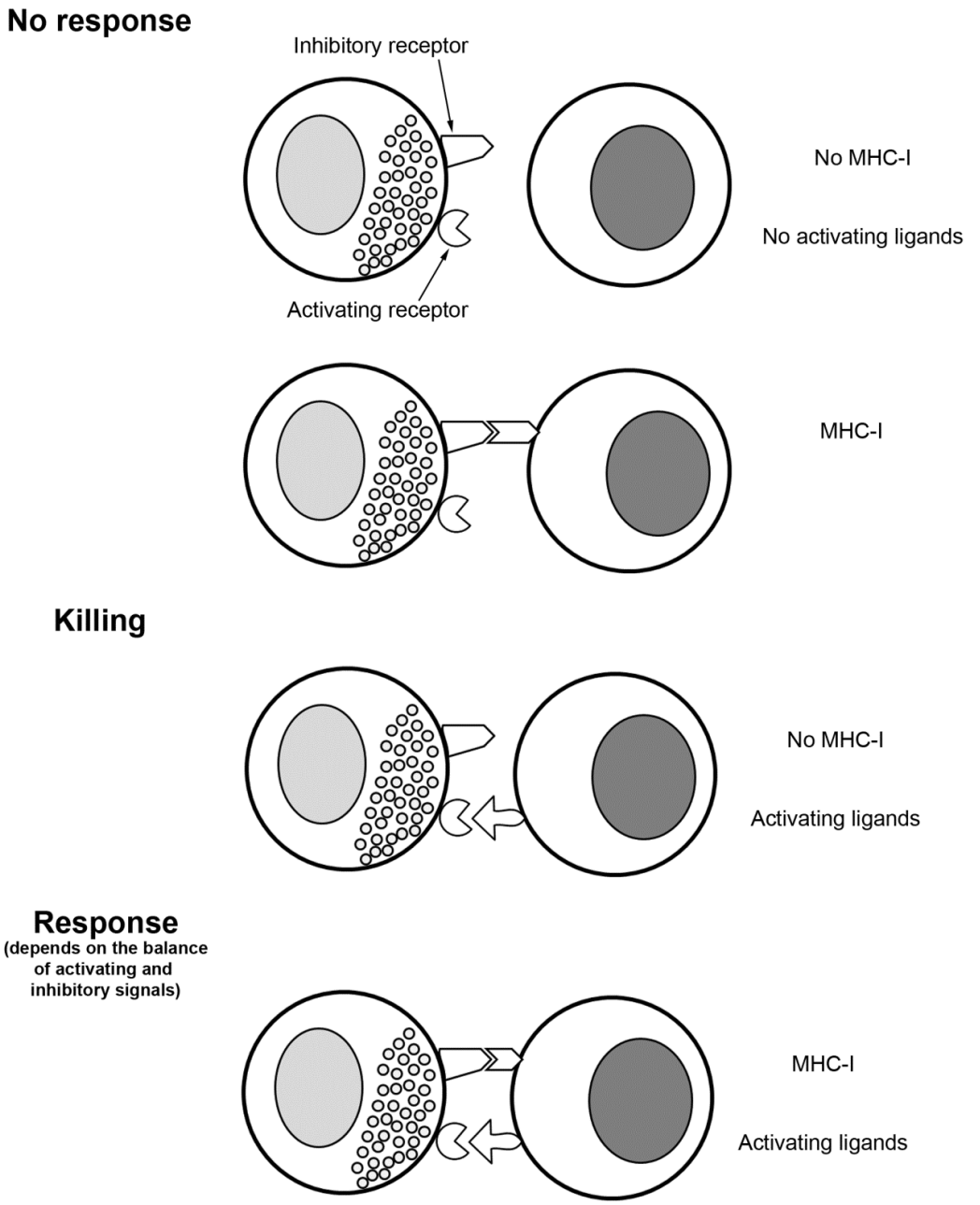


Figure I.1. Mechanisms of NK cell recognition. MHC class I inhibitory receptors suppress NK cell responses. Cells lacking MHC-I proteins and expressing ligands for activating receptors trigger NK-cell cytotoxicity and killing. When NK cells interact with target cells expressing ligands for activating and inhibitory receptors, the NK cell response is determined by the balance of both signals. Adapted from Lanier, 2005.

2. CYTOTOXIC T LYMPHOCYTES

Cytotoxic T lymphocytes (CTL), also called CD8+ T cells, are a subpopulation of Ŭƀ-T lymphocytes of the adaptive immune system. Approximately 13-32% CD8+ T cells are found in peripheral blood. Most CD8+ T cells usually express T-cell receptors (TcR) that recognize a specific 'non-self' antigen, and the CD8 co-receptor. The main functions of CD8+ T cells are killing infected and tumor cells and the secretion of interferon-gamma (IFN-γ) to activate macrophages.

After maturation in the thymus, naïve CD8+ T cells circulate throughout the bloodstream and SLT. When they recognize an antigen, CD8+ T cells undergo clonal expansion and differentiate into effector T cells and central and effector memory T cells. Memory CD8+ T cells can elicit a much more rapid response against the same antigen. On one hand, stimulation of central memory T cells leads to the differentiation of effector memory T cells and the next generation of central memory T cells. On the other hand, stimulation of effector memory T cells leads to their proliferation and the exertion of their effector functions (Kaech *et al.*, 2002). The functional attributes of these T cell subsets are associated with the expression of diverse surface receptors (Appay *et al.*, 2008). Amongst these surface markers, the most commonly used are CD45RA and CCR7 (Figure I.2).

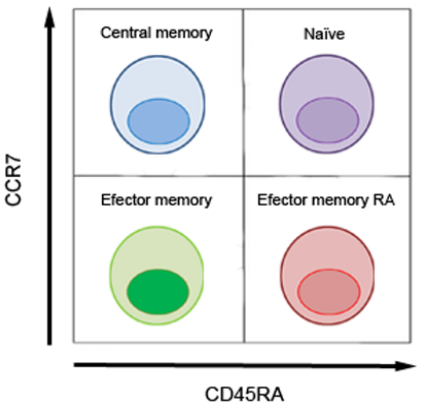


Figure I.2. CD8+ T cell subsets. Different circulating CD8+ T cell subsets according to the expression of CD45RA and CCR7.

Cytotoxic T cells recognize the target cells through the binding of the TcR with a MHC-I expressed on the target cell surface. This recognition must be accompanied by additional signals delivered by the interaction of costimulatory receptors (*e.g.* CD2 or CD28) with their ligands (CD58 or CD80/86, respectively) to get fully activated. CD8+ T cells release cytotoxic granules containing perforins and granzymes that eventually induce apoptosis (Radoja *et al.*, 2006). In the absence of a second stimulus, CD8+ T cells enter into a state of unresponsiveness or anergy, and even apoptosis may be induced (Figure I.3).

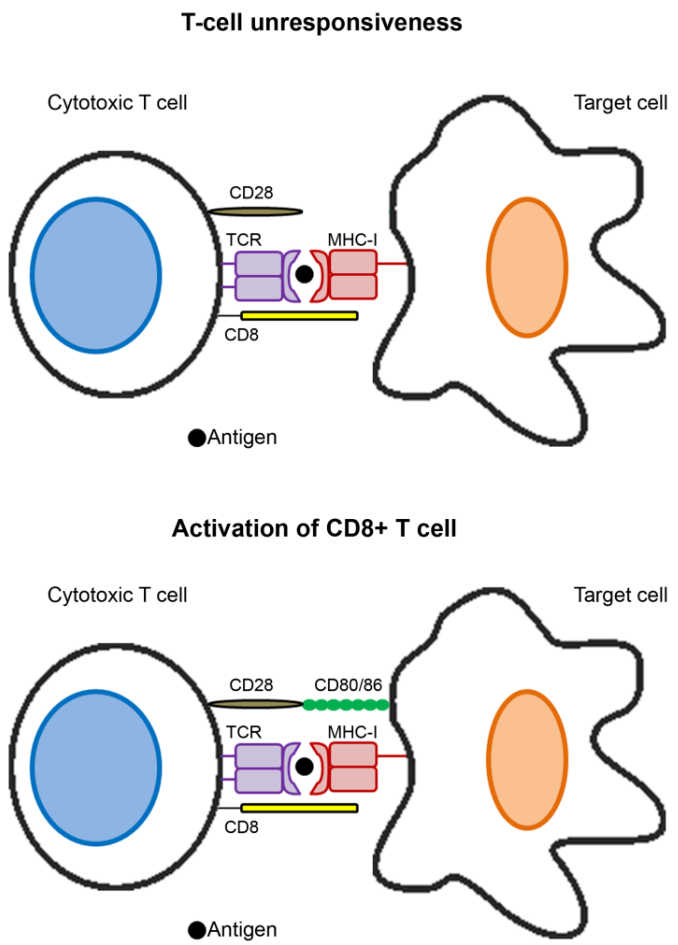


Figure I.3. Schematic model of costimulation of CD8+ T cells.

3. THE NKG2D RECEPTOR

NKG2D is a type II transmembrane protein with an extracellular C-type lectin-like domain (Li *et al.*, 2001). It is expressed not only on all NK cell subsets, but also on CD8+ T lymphocytes, ɔǔT cells, and natural killer T cells (NKT) (López-Larrea *et al.*, 2008).

The NKG2D gene is located within the 'NK complex' on human chromosome 12 and mouse chromosome 6 (Vance *et al.*, 1997; Ho *et al.*, 1998). The NKG2D receptor couples to the adaptor protein DAP10 in humans and DAP10 or DAP12 in mice for signal transduction (Rosen *et al.*, 2004; Wu *et al.*, 1999). NKG2D and DAP10 constitute a hexameric complex, containing 2 NKG2D and 4 DAP10 molecules (Garrity *et al.*, 2005). DAP10 contains a Tyr-Ile-Asn-Met (YINM) motif (Wu *et al.*, 1999). The phosphorylation of this motif by a Src family kinase leads to DAP10 binding to either the p85 regulatory subunit of the phosphoinositide 3-kinase (PI3K) or to the growth factor receptor-bound 2 (Grb2) adaptor (Chang *et al.*, 1999). These binding sites overlap, which means that one DAP10 chain can bind p85 or Grb2, but not both. Recruitment of p85 and Grb2 triggers two main signaling pathways with different downstream consequences (Upshaw *et al.*, 2006) (Figure I.4):

-DAP10-PI3K pathway: PI3K activation leads to phosphorylation of MAPK/ERK kinase (MEK) and extracellular signal regulated kinases (ERK), which are required for cytotoxicity and calcium release. PI3K activation also leads to Akt phosphorylation, activating survival pathways.

-DAP10-Grb2 pathway: Binding of DAP10 to Grb2 leads to the recruitment of Vav1, which is necessary to induce F-actin polymerization and microtubule organizing center (MTOC) polarization (Graham *et al.*, 2006).

Both signaling pathways are required for full calcium release and cytotoxicity, since the mutation of p85 or Grb2 binding sites impairs the cytolytic activity (Upshaw *et al.*, 2006).

In humans, the ligands for NKG2D (NKG2DL) are encoded in two different regions of chromosome 6 and can be subdivided into 2 groups (Burgess *et al.*, 2008) (Figure I.5):

-MHC class I-related chain (MICA and MICB): they contain Ǔ1, Ǔ2, and Ǔ3 extracellular domains similar to MHC-I molecules, but they do not associate with ß2-microglobulin or antigenic peptides.

-UL-16 binding proteins (ULBP) 1-6: they lack the Ǔ3 domain. ULBP 1-3 and ULBP-6 are bound to the cell membrane by glycosylphosphatidylinositol (GPI) anchors, whereas ULBP 4-5 are transmembrane proteins.

This diversity of NKG2DL has been investigated to find whether the different ligands equally trigger NKG2D signaling. NKG2DL differ in sequence, structure, and affinity and this could mean distinct functional implications (Mistry and O'Callaghan, 2007). However, this question remains to be elucidated.

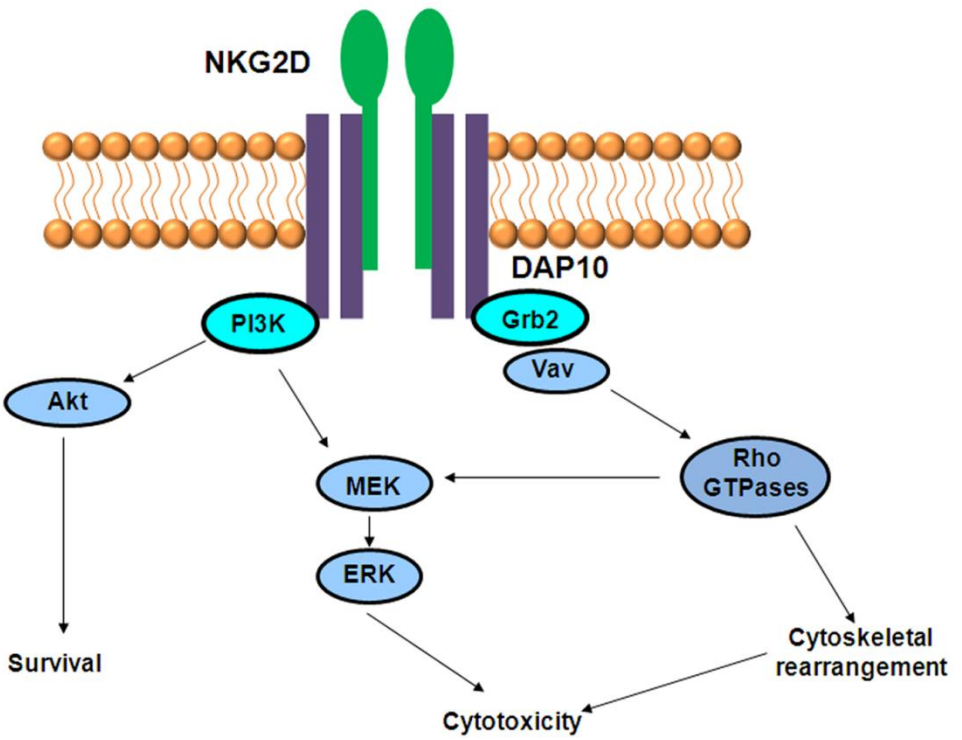


Figure I.4. NKG2D-mediated signaling pathways.

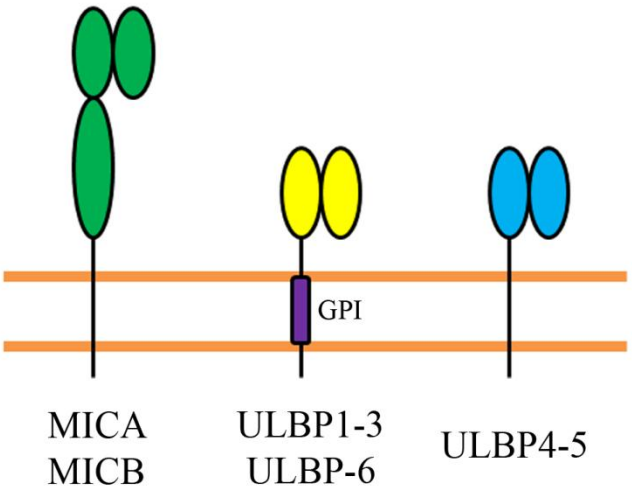


Figure I.5. Representative diagram of human NKG2D ligands. MICA and MICB have three extracellular and one cytoplasmic domains, similar to MHC-I molecules. ULBP proteins have two extracellular domains; ULBP4 and ULBP5 are transmembrane proteins, whereas ULBP1-3 and ULBP-6 are GPI-linked proteins.

3.1. The NKG2D receptor in NK cells

NKG2D is one of the best characterized receptors on NK cells. It serves as a primary activation receptor on activated NK cells. The outcome of NKG2D-mediated cell activation is regulated by the integration of activating and inhibitory signals. The state of activation of the NK cell, as well as the microenvironment surrounding the target cell, also determines the degree of NKG2D response.

Engagement of the NKG2D-DAP10 receptor complex triggers cell-mediated cytotoxicity, but the involvement in other effector functions, such as cytokine production, is less well understood. Crosslinking of NKG2D in human NK cells triggers cytotoxicity but not cytokine secretion (Billadeau *et al.*, 2003). However, stimulation of NKG2D with soluble recombinant NKG2DL or with an agonist antibody leads to the secretion of cytokines (*e.g.* IFN-ɔ or granulocyte macrophage colony-stimulating factor /GM-CSF) in human NK cells (André *et al.*, 2004). The influence of NKG2D on other NK cell functions is less well understood. It has been recently described that the balance of activating and inhibitory signals regulates NK cell contact with the target cell. Thus, coengagement of NKG2D and lymphocyte function-associated antigen 1

(LFA-1) promotes a stop signal and the formation of the IS. Conversely, when inhibitory signals dominate, the stop signal is reverted and allows NK cells to migrate (Culley *et al.*, 2009).

3.2. The NKG2D receptor in CD8+ T cells

As mentioned above, NKG2D is expressed in CD8+ T cells. Since the YINM motif of DAP10 is similar to the Tyr-Met-Asn-Met (YMNM) motif of CD28, the prototypical T cell costimulatory molecule, it has been postulated that NKG2D could serve as a costimulatory receptor (Figure I.6). However, there is still controversy about its function in CD8+ T cells.

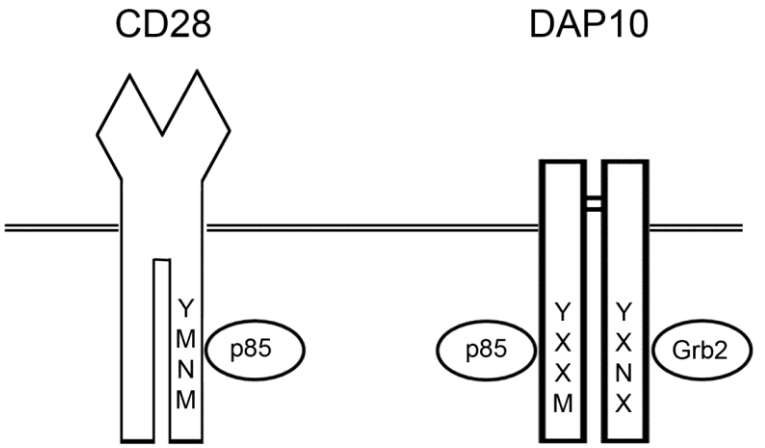


Figure I.6. Signaling motifs in CD28 and DAP10. The YMNM motif binds to the p85 subunit of PI3K. DAP10 have a YINM signaling motif and is able to recruit either p85 (YXXM) or the adaptor Grb2 (YXNX).

NKG2D may require additional cofactors to costimulate cell proliferation, cytokine production, and cytotoxicity (Ehrlich *et al.*, 2005). Verneris *et al.* (2004) showed that NKG2D may trigger cytotoxicity alone in expanded CD8+ T cells cultured in the presence of interleukin-2 (IL-2). However, other studies reported that NKG2D acts as a costimulatory molecule in human and murine CD8+ T cells (Groh *et al.*, 2001; Jamieson *et al.*, 2002; Maasho *et al.*, 2005; Markiewicz *et al.*, 2005). In human naïve CD8+ T cells,

simultaneous signaling through the TcR and NKG2D leads to cytotoxicity, cell proliferation and production of IFN-ɔ, tumor necrosis factor-alpha (TNF-Ŭ) and IL-2 (Maasho *et al.*, 2005). In effector CTL from human intestinal epithelium that do not express CD28, NKG2D acts as a potent costimulatory receptor, and its levels are upregulated by the cytokine IL-15, which is released by the inflamed intestinal epithelium (Roberts *et al.*, 2001). Markiewicz *et al.* (2005) described the role of NKG2D costimulation in the regulation of murine CTL responses. Although both costimulatory molecules triggered cytotoxic, proliferative, and cytokine responses only CD28 enhanced CTL survival. Indeed, differences between CD28 and NKG2D in naïve and effector human T cells have also been described (Rajasekaran *et al.*, 2010), finding a costimulatory function for NKG2D in effector CD8+T cells that lack CD28 on their cell surface. Moreover, costimulation via NKG2D triggers different signaling pathways from CD28, reducing the expression of anti-inflammatory cytokines (Barber and Sentman, 2011).

Together these studies indicate that the intracellular signaling pathways mediated by CD28 and NKG2D are different.

3.3. The NKG2D/NKG2DL system in health and disease

Several studies have highlighted the importance of modulating NKG2D receptor expression and signaling in both health and disease. Although NKG2D has been related to a decrease in the incidence and progression of tumors induced by carcinogenic agents (Smyth *et al.*, 2005), an inappropriate NK cell or CD8+T cell activation by NKG2D may lead to aberrant responses. Indeed, NKG2D receptor has been involved in autoimmune diseases, such as rheumatoid arthritis (Groh *et al.*, 2003), celiac disease (Hüe *et al.*, 2004), or autoimmune diabetes in non-obese diabetic (NOD) mice (Ogasawara *et al.*, 2003). Moreover, the treatment with a blocking anti-NKG2D antibody has

been shown not only to prevent autoimmune diabetes in NOD mice (Ogasawara *et al.*, 2004) but also to prevent transplant rejection (Ogasawara *et al.*, 2005), and to ameliorate disease in a murine model of rheumatoid arthritis (Andersson *et al.*, 2011).

The levels of NKG2D on the cell surface, as well as NKG2D function, are regulated by various cytokines and NKG2DL. Exposure of NK cells to transforming growth factor beta- (TGF-β) containing plasma of patients with lung or colorectal cancer induces a decrease in NKG2D expression and suppresses NK cell cytotoxicity (Lee *et al.*, 2004). Other cytokines, such as IL-2 or IL-12, enhance NKG2D-mediated tumor rejection (Smyth *et al.*, 2004). IL-21 may downmodulate NKG2D surface expression whilst it increases the levels of other NK activating receptors, including NKp30, NKp46, and 2B4 (Burgess *et al.*, 2006). IL-12 or IFN-β1 may also inhibit NKG2D expression and NK cell cytotoxicity during human cytomegalovirus (hCMV) infection (Muntasell *et al.*, 2010).

Expression of NKG2DL is normally restricted under physiological conditions. MICA is expressed constitutively at low levels in the intestinal epithelium (Groh *et al.*, 1996). MICA and ULBPs have also been found on primary bronchial epithelial cells (Kraetzel *et al.*, 2008). The upregulation of NKG2DL under stress situations like viral infections or tumors leads to a rapid immune response by NK cells and CD8+ T cells (Champsaur and Lanier, 2010). However, the chronic exposure to NKG2DL and their release from tumor cells into their soluble form (Salih *et al.*, 2002; Salih *et al.*, 2006; Waldhauer *et al.*, 2006) result in an impairment of NK and CD8+ T cell activities by inducing the internalization of the receptor (Groh *et al.*, 2002; Coudert *et al.*, 2005; Wiemann *et al.*, 2005; Salih *et al.*, 2008). In fact, several studies have demonstrated the implication of NKG2DL expression in a variety of pathologies, such as autoimmune diseases and epithelial tumors or even in transplantation. High expression of MICA and ULBP2 has been detected in

ovarian cancer tissue and related to a poor prognosis (Li *et al.*, 2009), as well as on the cell surface of leukemia cells (Salih *et al.*, 2003). The soluble form of MICA has been detected after heart transplantation and its levels inversely correlate with allograft rejection (Suárez-Álvarez *et al.*, 2006). More recently, it has been described that NKG2D ligands may also be secreted via exosomes, being able to modulate NKG2D surface levels (Hedlund *et al.*, 2009). Cancer-derived exosomes downregulate receptor expression and reduce NK and CD8+ T cells functional responses (Clayton and Tabi, 2005; Clayton *et al.* 2008; Ashiru *et al.*, 2010).

Given the importance of the NKG2D recognition system not only in the immune response but also in immune surveillance or in autoimmune reactivity of T cells (Coudert and Held, 2006; Brycesson and Ljunggren, 2008), a detailed description of NKG2D signaling pathways might have great potential in clinical applications.

4. MEMBRANE RAFTS AND SIGNAL TRANSDUCTION

4.1. Structure and function of membrane rafts

Membrane rafts (also called 'lipid rafts') are microdomains of the plasma membrane that are enriched in cholesterol, glycolipids, phospholipids and other lipids containing preferentially saturated fatty acids. This enrichment in sphingolipids and cholesterol allows raft domains to be more tightly packed than the bulk of the plasma membrane (Simons and Ikonen, 1997; Rajendran and Simons, 2005). These domains also contain lipid-modified signaling proteins, GPI-anchored proteins, phospholipid binding proteins (*e.g.* annexins), and transmembrane proteins (most of them palmitoylated) or transmembrane adaptor proteins, without extracellular domain (Lingwood *et al.*, 2009) (Figure I.7). A special subtype of lipid rafts is the caveola. Caveolae are small

invaginations of the plasma membrane. They also have a high content of cholesterol and sphingolipids, but unlike lipid rafts, they contain the cholesterol-binding protein caveolin, which is responsible for stabilizing the invaginated structure of caveolae (Parton and Simons, 2007; Lajoie and Nabi, 2010).

Due to their specific lipid composition, membrane rafts are relatively resistant to certain detergents (*e.g.* Triton X-100, some of Brij-series, octyl-glucoside or NP-40), and upon solubilization produce detergent-resistant membrane complexes (DRMs) (Brown and London, 2000) that are believed to correspond more or less exactly to the raft microdomains in intact membranes. Thus, DRMs are widely used as an approximate biochemical equivalent of membrane rafts. The size of these domains is not yet well established since they are too small and cannot be directly measured or resolved by conventional optical microscopy. It has been estimated that elemental raft size may vary from 25 nm to 50 nm (Varma and Mayor, 1998; Pralle *et al.*, 2000), although it increases by coalescence of rafts upon protein crosslinking.

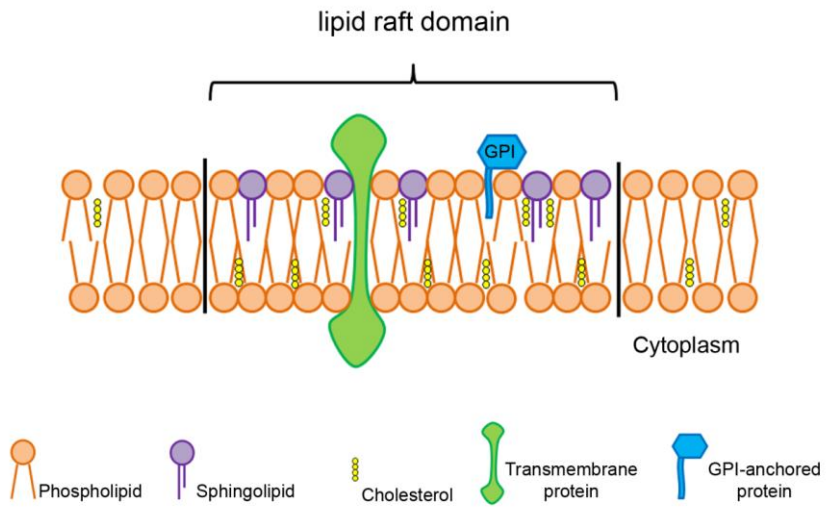


Figure I.7. Schematic model of the structure of lipid rafts. These domains are highly enriched in cholesterol and sphingolipids immersed in a phospholipid-rich environment, which together interact to form a liquid-ordered phase. Several types of proteins associate with raft domains, such as transmembrane proteins and GPI-anchored proteins.

Only a small group of proteins are localized constitutively in these microdomains, whereas other proteins are outside rafts but upon ligation are recruited to lipid rafts. For this reason, membrane rafts have been proposed as being important platforms for the enrichment of molecules involved in signal transduction and other aspects of leukocyte biology (Dykstra *et al.*, 2003). In response to cellular stimuli, lipid rafts can alter their composition, excluding or including proteins that promote the activation of different signaling pathways (Simons and Toomre, 2000). Thus, membrane rafts are involved in receptor signaling, intracellular transport and protein sorting, and the interaction of cells with pathogens. In addition, membrane rafts may be important in several pathologies, such as Alzheimer disease. Lipid rafts are involved in the generation of the ß-amyloid peptide (Aß). The amyloid precursor protein (APP) and the ß-site APP cleavage enzyme (BACE1) may associate with rafts and disruption of these domains by cholesterol depletion decreases Aß production (Ehehalt *et al.*, 2003). Moreover, due to the composition of membrane rafts with high content of cholesterol and sphingolipids it has been postulated that lipid rafts may be involved in the development of atherosclerosis (Lemaire-Ewing *et al.*, 2012).

4.2. Interactions of the actin cytoskeleton with membrane rafts

The studies that have addressed the structure of membrane rafts have demonstrated the implication of raft-associated proteins in the formation of raft domains. Interactions of these proteins with the actin cytoskeleton are able to control membrane raft dynamics, formation, and maintainance of these microdomains (Chichili and Rodgers, 2009). An example is the high affinity receptor for IgE (FcεRI), which interacts with lipid rafts in an actin-dependent manner (Holowka *et al.*, 2000; Andrews *et al.*, 2008). In RBL-2H3 mast cells,

crosslinking of FcεRI induces coalescence of membrane rafts and the redistribution of Lyn kinase to these domains together with F-actin. This association of FcεRI with Lyn is regulated by the actin cytoskeleton (Holowka *et al.*, 2000). In line with this, FcεRI recruitment to rafts is defined by actin bundles that prevent the diffusion of membrane proteins and favor the maintenance of these clusters and the activation of downstream signaling pathways (Andrews *et al.*, 2008). Moreover, lipid rafts associate with the actin cytoskeleton upon platelet activation. This interaction depends on the αIIbβ3 integrin, which upregulates levels of phosphatidylinositol 4,5-biphosphate (PIP_2) in membrane microdomains (Bodin *et al.*, 2005). PIP_2 induces raft dependent vesicle transport through the stimulation of actin polymerization in fibroblasts (Rozelle *et al.*, 2000). Chichili and Rodgers (2007) demonstrated using fluorescence resonance energy transfer (FRET) that actin cytoskeleton promotes clustering of raft-associated proteins in Jurkat T cells, which in turn regulates the signaling of these proteins.

Together, these studies demonstrate that there is a synergistic interaction between the actin cytoskeleton and membrane rafts in which actin cytoskeleton participates in regulating and activating raft associated signaling events.

4.3. Membrane rafts in immunoreceptor signaling

A functional role of membrane rafts has arisen from studies in hematopoietic cells. Indeed, the importance of these structures was demonstrated in a number of studies in T lymphocytes (Horejsi, 2005). Upon engagement of the TcR, lipid rafts aggregate and bring together signaling molecules like the Src-like tyrosine kinases Fyn and Lck. On the contrary, CD45, which is a phosphatase that inhibits Lck kinases, is excluded, thus promoting Lck activity. This activation leads to the recruitment of the zeta-

chain-associated protein kinase 70 (ZAP70) and the phosphorylation of other molecules such as linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76 kDa (SLP-76), which eventually induce the amplification of TcR signaling (Janes *et al.*, 2000) (Figure I.8).

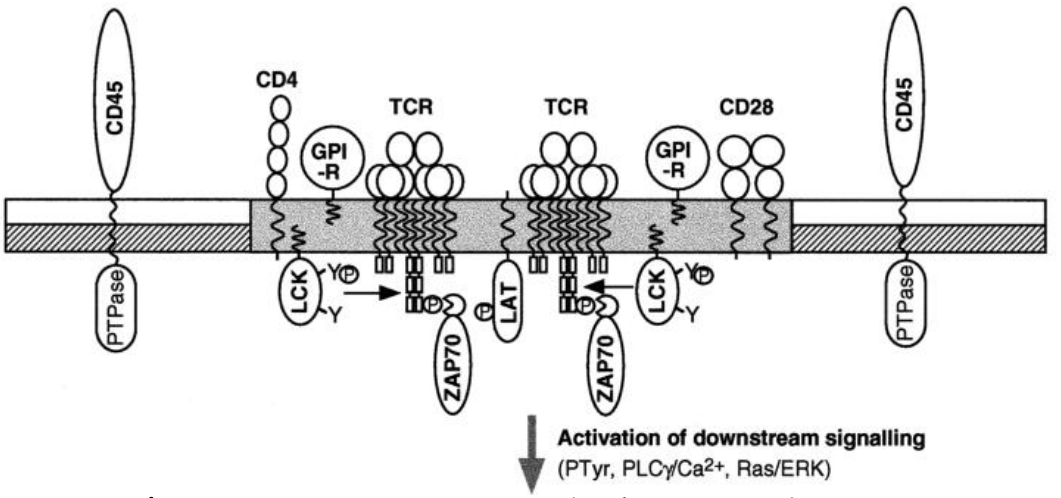


Figure I.8. Raft aggregation upon TcR activation. Taken from Janes *et al.* (2000).

The clustering of membrane rafts in TcR signaling not only allows the formation of a mature immunological synapse, but is also functionally linked to actin cytoskeleton. On one hand, regulation of actin dynamics is essential to recruit cytoskeletal associated proteins that modulate cell polarization and stabilize the IS. On the other hand, actin cytoskeleton reorganization contributes to the maintainance of membrane raft clustering for an appropriate activation of T cells (Gomez and Billadeau, 2008). CD48, a GPI-anchored protein that is present in lipid rafts (Cinek and Horejsi, 1992), enhances TcR-induced rearrangement of the cytoskeleton by associating membrane rafts to TcR clusters (Moran and Miceli, 1998). An association of the phosphoprotein associated with glycosphingolipid-enriched membrane domains (PAG) and ezrin/radixin/moesin-binding phosphoprotein of 50 kDa (EBP50) to form a complex that associates F-actin with lipid rafts has been described (Brdickova *et al.*, 2001). The Vav1/Rac pathway regulates actin reorganization upon

activation of the TcR and contributes to lipid raft clustering in T cells induced by antigen stimulation (Villalba *et al.*, 2001).

4.3.1. Membrane rafts in NK cell activation

Whilst the majority of the studies have focused on elucidating the roles of membrane rafts in T cell activation, less is known about these compartments in NK cells. NK cell cytotoxicity has been shown to require CD2AP, a protein that localizes into lipid rafts (Ma *et al.*, 2010). The phosphorylation of ERK kinase and the production of IFN-ɔ in NK cells are enhanced by the recruitment of the IL-12 receptor and the FcɔRIIIa receptor to membrane rafts (Kondadasula *et al.*, 2008). Moreover, the adhesion between the NK cell and the target cell, based on the LFA-1 – intercellular adhesion molecule 1 (ICAM-1) interaction, results in Vav phosphorylarion, inducing cytoskeletal rearrangements that lead to clustering of 2B4 in lipid rafts. Coengagement of 2B4 with LFA-1 induces activation of NK cell cytotoxicity and activation of a MHC-I inhibitory receptor abrogates 2B4 recruitment, thereby preventing NK cell activation (Riteau *et al.*, 2003). Statins are specific inhibitors of the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase that decrease cholesterol levels. Membrane rafts can be disrupted by statins due to these changes (Hillyard *et al.*, 2004) and could explain the pleiotropic effect of these molecules. Disruption of cholesterol by statins impairs NK cell cytotoxicity and this reduction is similar to that produced by cyclodextrins, which are known to deplete cholesterol and avoid raft clustering (Hillyard *et al.*, 2007). In contrast, ligation of inhibitory receptors on NK cells results in the immobilization of the receptors at the contact sites, and is accompanied by lipid rafts exclusion from these areas (Figure I.9). For instance, ligation of the CD94/NKG2A inhibitory receptor leads to polarization and stabilization of the receptor at the contact site, but it does not associate with lipid rafts, thus impeding the development of activating

signals (Sanni *et al.*, 2004). Consistent with these reports, it has been described that inhibitory signaling via NKG2A disrupts raft recruitment and actin reorganization at the IS, thus impairing NK cell activation (Masilamani *et al.*, 2006). Furthermore, the inhibitory KIR is able to block actin-dependent recruitment of the activating receptor 2B4 to lipid rafts, preventing the initiation of activating signaling cascades (Watzl and Long, 2003).

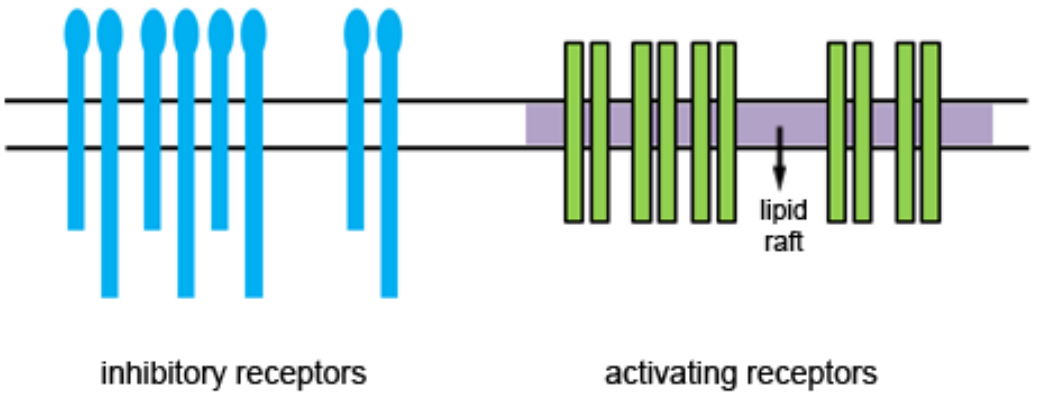


Figure I.9. Proposed distribution of activating and inhibitory receptors in NK cells. Ligation of activating receptors results in their recruitment to lipid rafts whereas ligation of inhibitory receptors results in their immobilization at the contact sites and the exclusion of lipid rafts from these areas.

However, less well known is the role of membrane rafts in NKG2D-mediated signaling. It has been demonstrated that NKG2D is recruited to DRMs upon receptor activation, and that inhibitory signals triggered by the CD94/NKG2A receptor can prevent NKG2D association with membrane rafts and the formation of the IS (Endt *et al.*, 2007). Thus, interaction of membrane rafts with cytoskeleton is important for NKG2D-mediated NK cell activation. Nevertheless, a specific approach about the role of membrane rafts in NK cell migration has not been carried out to date.

5. CELL MIGRATION AND TRAFFICKING

Cell migration is an essential process in both health and disease. It is required not only for normal development or tissue repair, but also occurs in pathologic situations like metastasis, chronic inflammatory diseases, or atherosclerosis. Cell migration is also required for a correct functioning of the immune system since recruitment of cells of the immune system from the bloodstream and the extravasation to the target tissues are the first step in the inflammatory response and against infections (Ridley, 2001).

The movement of individual cells may be classified into mesenchymal or ameboid migration. Mesenchymal migration is characteristic of fibroblast-like cells (fibroblasts, endothelial cells, smooth muscle cells, cancer cells) and is associated with the remodeling of the extracellular matrix (ECM) by proteolysis and low migration speed. Amoeboid movement refers to the primary mode of migration of rounded cells, such as hematopoietic cells (neutrophils, DCs, lymphocytes) and is characterized by the lack of mature focal adhesions and stress fibers due to the high motility of these cells (Friedl and Wolf, 2010).

Cell migration consists of the repetition of four consecutive steps: formation and extension of a lamellipodium, cell adhesion to the substrate, contraction of the cell body, and detachment of the rear end of the cells. An essential requirement for cell migration is the acquisition of a polarized morphology with a cell advancing front and a trailing edge or uropod (Figure I.10). During leukocyte migration, chemokine receptors and adhesion molecules are redistributed in these two different regions (Sánchez-Madrid and del Pozo, 1999). Cell migration is affected by a diversity of extracellular signals, which in turn originates different cellular responses that must be coordinated. For this reason, several proteins and signaling pathways are involved in cell

migration (*e.g.* calcium regulated proteins, MAPK kinases, tyrosine kinases or Ras homology/Rho GTPases).

The reorganization of the actin cytoskeleton and the microtubule dynamics in order to interact with the cell substrate and the ECM is also indispensable during cell polarization and migration. These processes strongly depend on the Rho GTPase family of proteins since they are involved in the regulation of actin polymerization and depolymerization, microtubule dynamics, and myosin activity associated with actin (Wittmann and Waterman-Storer, 2001).

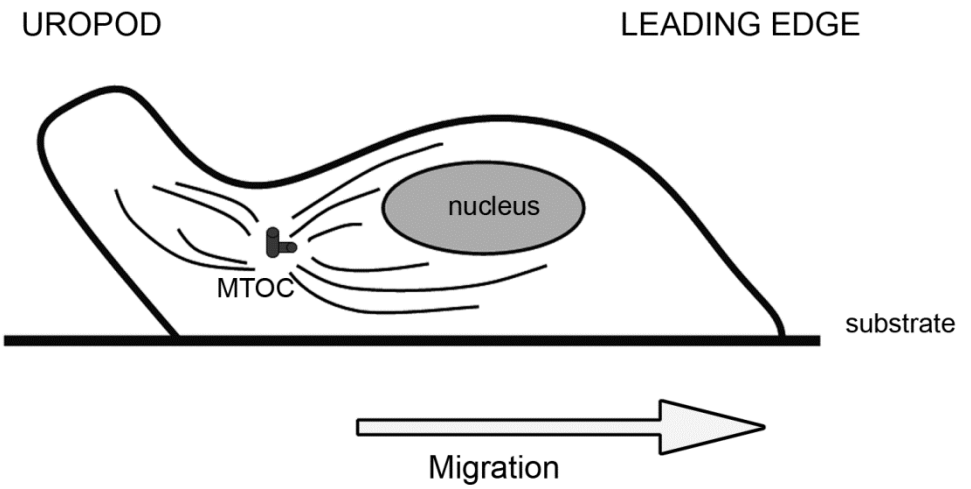


Figure I.10. Leukocyte migration. Schematic model of a migrating leukocyte with a polarized morphology that distinguishes a leading edge and the uropod.

5.1. The Rho GTPase family of proteins

Rho GTPases are monomeric proteins with molecular masses between 20-40 KDa. The majority of Rho GTPases cycle between an active form bound to guanosine tri-phosphate (GTP) and an inactive form bound to guanosine di-phosphate (GDP). Under basal conditions, they are located in the cytosol bound to GDP and form complexes with guanine nucleotide dissociation inhibitor (GDI) proteins, which prevent their interaction with the plasma membrane. In

response to extracellular stimuli, Rho GTPases and GDI proteins dissociate and their activation is catalysed by guanine nucleotide exchange factor (GEF) proteins. Active Rho GTPases bound to GTP interact with effector proteins to induce downstream responses. Finally, interaction with GTPase activating proteins (GAP) inactivates Rho GTPases by catalyzing the hydrolysis of GTP to GDP (Takai *et al.*, 2001) (Figure I.11).

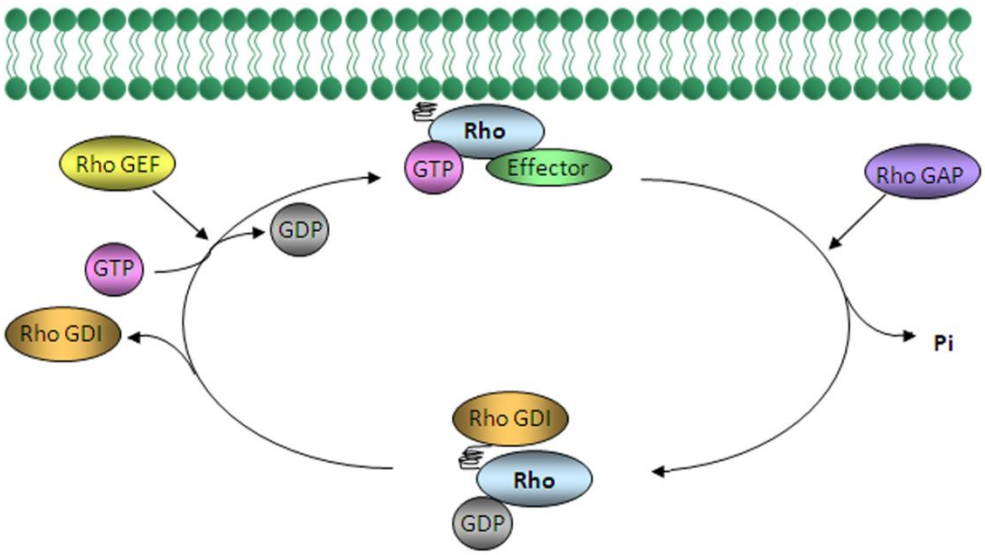


Figure I.11. Regulation of Rho GTPase activity. GEF proteins promote Rho GTPases activation by exchanging GDP for GTP. GAP proteins enhance their GTPase activity and GDI proteins inhibit the GDP/GTP exchange and prevent the binding of Rho GTPases to the plasma membrane.

At least 25 members of the Rho-family have been described. Rho GTPases regulate several processes, including cell migration, cell-cell and cell-substrate adhesion, proteins secretion, and vesicle trafficking (Ridley, 2001). Thus, Rho GTPases signaling pathways are key players in the immune system responses, regulating *e.g.* the release of cytotoxic granules, T cell activation, and the formation of the IS. Among the Rho GTPases, the majority of studies have focused on the role Rac1, RhoA and Cdc42 in T cell activation, adhesion, polarization and migration (Rougerie and Delon, 2012). Indeed, the use of

activated mutants or dominant negative forms of these Rho GTPases has allowed knowing in detail their role in the regulation of actin dynamics during cell migration (del Pozo *et al.*, 1999) (Figure I.12).

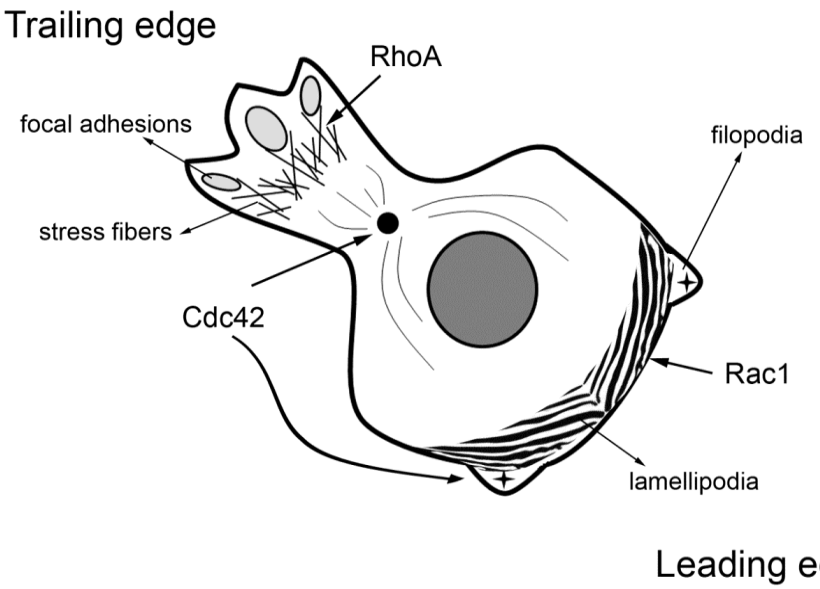


Figure I.12. Rho GTPases in cell migration. A schematic illustration indicating the main Rho-family members involved in the regulation of different assemblies of actin filaments: Rac1, lamellipodia; Cdc42, filopodia and MTOC localization; RhoA, stress fibers bundles and focal adhesions.

At the leading edge, Rac1 regulates lamellipodia and membrane-ruffle formation by modulating actin dynamics. Rac1 can signal actin assembly via WASP-family verprolin-homologous (WAVE) proteins and the actin-related protein 2/3 (Arp2/3) complex (Miki *et al.*, 1998) or it can increase the amount of actin monomers for actin polymerization by regulating cofilin (Delorme *et al.*, 2007). It has been shown that membrane ruffling and migration is impaired in dominant-negative Rac1 in several cell types (Ridley, 2001). Although the main function of Rac1 has been defined in the establishment and maintenance of the leading edge, its activity in the retracting tail is required as well during neutrophil chemotaxis (Gardiner *et al.*, 2002). In T cells, Rac1 is also necessary

in cell adhesion since dominant-negative Rac1 has been found to abolish adhesion to integrin ligands (García-Bernal *et al.*, 2005).

RhoA regulates the contraction of the cell body in the uropod, promoting the formation of stress fibers and the assembly/disassembly of the focal adhesions (Etienne-Manneville and Hall, 2002). RhoA activity is required in leukocytes for tail retraction (Alblas *et al.*, 2001). Knockdown of RhoA in T cells leads to a defective uropod formation and retraction (Haesman *et al.*, 2010). RhoA and Rac1 have antagonic activities (Rottner *et al.*, 1999; Sander *et al.*, 1999; Xu *et al.*, 2003), thus there exists a balance between them that determines the formation of the different actin-rich protrusions. In addition, the regulation of RhoA activity by Rac1 within the uropod has been described, in which Rac1-mediated activation of RhoA is essential to maintain the cell polarity during neutrophil chemotaxis (Pestonjamasp *et al.*, 2006).

Cdc42 promotes filopodia (finger-like actin protrusions) that form focal adhesions with the substrate and act as sensors of the external environment. The formation of these protrusions may be disrupted in cells expressing either constitutively active Cdc42 or dominant-negative Cdc42 (Gupton and Gertler, 2007). Cdc42 activates the Arp2/3 complex through Wiskott-Aldrich syndrome protein (WASp) and neural-WASp (N-WASp) to induce actin filaments branching and the formation of filopodia (Figure I.13). Cdc42 is also involved in the establishment of the cell polarity during migration as well as in microtubule reorganization and regulation of the MTOC localization in the immunological synapse. The MTOC relocates behind the nucleus, away from the leading edge, in fast-moving cells, such as lymphocytes (del Pozo *et al.*, 1998). On the contrary, the MTOC localizes in front of the nucleus in low-motility cells (*e.g.* macrophages or fibroblasts) (Gomes and Gundersen, 2006).

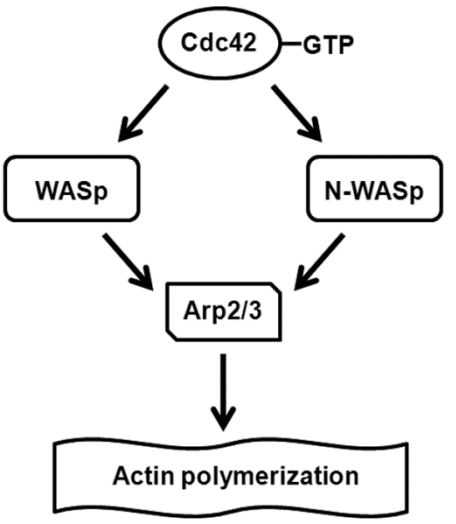


Figure I.13. Actin assembly regulation by Cdc42/WASp – N-WASp. Binding of GTP-bound Cdc42 to either WASp or to N-WASp results in the activation of the Arp2/3 complex, which in turn promotes actin polymerization.

5.2. Regulation of cell trafficking: Integrins and chemokines

Integrins constitute a family of heterodimeric transmembrane receptors which consist of an Ŭ and a ɓ subunit. Each subunit contains a large extracellular domain that binds ECM and a short cytoplasmic tail that engages the actin cytoskeleton (Hynes, 2002). Thus, integrins mediate the interactions over the plasma membrane in both directions (*i.e.* outside-in and inside-out signaling) during cell adhesion and motility. Activation of integrins by ligand binding promotes their clustering and the formation of multiprotein complexes containing intracellular signaling proteins that link to the actin cytoskeleton (Huttenlocher and Horwitz, 2011). This organization of the signaling networks makes integrins important in regulating other processes, such as cell proliferation and cell survival. Indeed, integrins have been associated with several diseases like cancer and immunodeficiency disorders, thus becoming potential therapeutic targets.

Chemokines are a large family of small proteins which regulate leukocyte trafficking through interactions with G-protein coupled receptors.

They are classified into four families based on their structural characteristics: CXC, which have a single amino-acid residue (X) between the first two cysteines; CC-chemokines, with the first two of the four cysteine residues adjacent; CX3C, with three amino-acids between the cysteines; C-chemokines, which have a single cysteine in the N-terminus. The nomenclature of the chemokines is based on these families followed by 'R' for receptors and 'L' for ligands and a number that indicates the position of the gene encoding each chemokine (Zlotnik and Yoshie, 2012).

Although a binding redundancy between many ligands and receptors exists, some chemokines can also show high tissue and receptor specificity. Other chemokines are able to regulate both homeostasis and inflammatory responses. Taken together, chemokines orchestrate different processes pivotal for the immune system, including DC and lymphocyte trafficking and recirculation throughout the SLT, or cell recruitment to target tissues during immune responses. Moreover, they are involved in the development of the central nervous system (CNS) (Jin *et al.*, 2008), and can increase the binding activity of integrin adhesion receptors (Dustin *et al.*, 2004; Laudanna *et al.*, 2002).

Given the importance of chemokines in these physiological processes, a deregulation on cell chemotaxis may cause several pathologies, such as cancer metastasis, inflammatory diseases, and neurological diseases (Jin *et al.*, 2008). Indeed, the importance of these molecules in inflammatory responses and homeostasis makes them targets for clinical application. An antagonist of the CXCR4 receptor has been recently approved for stem-cell mobilization and collection to peripheral blood for subsequent autologous transplantation in non-Hodgkin's lymphoma and multiple myeloma (Keating, 2011). Pharmaceutical agents against CCR5 have been developed as well for inhibition of entry of human immunodeficiency virus (HIV) into the host cell (Gilliam *et al.*, 2011).

6. MULTIPLE SCLEROSIS AND THE IMMUNE SYSTEM

The immune system protects the body against external pathogens. To this end, it must not only recognize this foreign agents (antigens), but also has to distinguish these antigens from the own components of the organism (self antigens). The failure in this recognition is called autoimmunity, and an aberrant immune response against a self antigen is termed an autoimmune disease.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS that is believed to be an autoimmune disorder. MS most commonly affects young adults. The usual age at onset is between 15 and 50 years and it affects females twice as often as males. MS has an incidence of 7 per 100,000 every year and a prevalence of around 120 per 100,000 (Compston and Coles, 2008). The reactivity of immune cells against myelin components (myelin associated glycoprotein, MAG; myelin oligodendrocyte glycoprotein, MOG; myelin basic protein, MBP; proteolipidic protein, PLP) is considered the main cause of the development and persistence of the disease. However, the etiology of the disease remains to date unknown and is considered as a complex interplay between environmental and genetic factors.

6.1. Clinical courses

The revised McDonald criteria are currently used for the diagnosis of MS (McDonald *et al.*, 2001; Polman *et al.*, 2011), which are based on the detection of lesions by magnetic resonance imaging (MRI) and the analysis of cerebrospinal fluid (CSF) in search of oligoclonal IgG bands. Clinically, MS is classified into relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) subtypes. The RRMS subtype is characterized by relapses followed by variable periods of remission. SPMS is an initial RRMS, but then the progression of disability begins without periods of remission.

PPMS is the most aggressive subtype, as it describes a steady neurologic decline from the onset without remissions. The RRMS subtype usually begins with a clinically isolated syndrome (CIS), a term which describes a first neurological episode suggestive of demyelination that is compatible with MS (Miller *et al.*, 2005).

6.2. Role of the immune cells in multiple sclerosis

It has been considered that autoreactive CD4+ T cells play the main role in the pathogenesis of multiple sclerosis. In MS patients, these cells differentiate to a T helper cell type 1 (Th1) phenotype, which maintains a sustained immune response against antigens of the nervous system (Minagar *et al.*, 2007). However, administration of an antibody against this subpopulation (anti-CD4, cM-T412) has been found to be ineffective in MS patients (van Oosten *et al.*, 1997). IL-17-producing Th cells (Th17) are a novel CD4+ T effector subset that has been proposed to be implicated in MS (Bettelli *et al.*, 2008). High levels of the IL-17 receptor have been detected in the surface of cerebral endothelial cells (CEC) (Kebir *et al.*, 2007), and RRMS patients during relapse showed increased frequency of Th17 cells, suggesting a pathogenic role in the disease (Brucklacher-Waldert *et al.*, 2009). The role of CD8+ T cells remains to be elucidated as they can mediate suppression on CD4+ T cells but also induce injury through the recognition of MHC-I molecules on the surface of axons (Kasper and Shoemaker, 2010). Several pieces of evidence support a pivotal role of B cells on MS pathogenesis (Franciotta *et al.*, 2008). B lymphocytes are considered of high interest in the course of MS as well since an increased IgG synthesis detected in CSF is currently a clinical feature. Moreover, synthesis of oligoclonal IgM against myelin lipids in CSF has been found to correlate with a worse progression of the disease (Villar *et al.*, 2005), and the presence of these lipid-specific IgM oligoclonal bands is related to an

increase in lesion load and brain atrophy in patients with CIS (Magraner *et al.*, 2012).

The innate immune system has recently emerged as an important factor in the pathogenesis and progression of MS due to its role in regulating the effector functions of T and B cells (Gandhi *et al.*, 2010). However, further investigations are required to better define the role of the different cells of the innate immunity in the disease.

6.2.1. NK cells in multiple sclerosis

Up to date, it is still not fully elucidated whether NK cells contribute to the progression or to the remission of the disease (Lünemann and Münz, 2008) (Figure I.14). However, the most recent data point to a beneficial effect in humans. Circulating NK cells have shown a decreased functional activity in MS patients during relapse (Kastrukoff *et al.*, 2003). The frequency of NK cells from peripheral blood expressing CD95 (Fas receptor) is increased in patients with RRMS in the remission phase, whereas this subpopulation is absent in healthy individuals and RRMS patients during relapses. Moreover, depletion of this population led to an increased secretion of the proinflammatory molecule IFN-ɔ by autoreactive T cells. These data suggest that this population is able to prevent relapses (Takahashi *et al.*, 2004). The frequency of NK cells was also found to be increased in patients that showed a good response to the administration of daclizumab, a humanized monoclonal antibody against the IL-2RŬ chain (Bielekova *et al.*, 2006). In addition to these studies, it might be possible that the location of NK cells and the regulation of NK cell activity by other subpopulations (*e.g.* NKT cells and regulatory T cells/T regs) could determine the effect on the disease (Morandi *et al.*, 2008). Thus, the neuroprotective role of NK cells in MS requires more detailed studies.

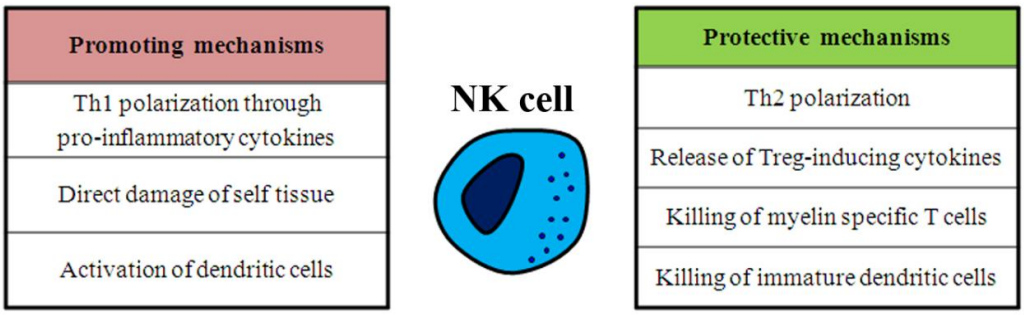


Figure I.14. Roles of NK cells in multiple sclerosis. It has been suggested that NK cells may promote or prevent the progression of MS. NK cells could promote the disease by inducing Th1 phenotype through the release of pro-inflammatory cytokines (IFN-ɔ), direct damage to the tissue or activating DCs. NK cells may play a protective role by inducing Th2 or Treg phonotype trough the release of anti-inflammatory cytokines (IL-5, IL-13, IL-10) or the killing of myelin specific T cells or immature dendritic cells.

6.3. Cellular trafficking in the central nervous system

The leukocyte trafficking across the blood brain barrier (BBB) is strictly regulated in the CNS. The cerebral endothelial cells of the BBB have unique features that prevent the migration of leukocytes by creating a network of tight junctions that blocks cell and molecule movement into the CNS (Minagar and Alexander, 2003; Minagar *et al.*, 2007). This control may be disrupted during neuroinflammatory diseases. In the case of MS, the expression of adhesion molecules and chemokines in the surface of CEC is upregulated, which allows T and B lymphocytes, as well as macrophages and NK cells, to enter the CNS (Engelhardt, 2006).

Chemokines and chemokine receptor expression patterns have been widely studied in MS (Sørensen *et al.*, 1999; Misu *et al.*, 2001; Uzawa *et al.*, 2010). It is worth mentioning the chemokine CXCL12 and its receptor CXCR4 that are constitutively expressed in the CNS and regulate several processes, including neurotransmission, neurotoxicity, migration, proliferation, and survival (Li and Ransohoff, 2008). The chemokine CXCL12 has been found to play an important role in the pathogenesis of MS since its levels are increased

in astrocytes and blood vessels (Ambrosini *et al.*, 2005; Krumbholz *et al.*, 2006). CXCL12 may also favor an appropriate microenvironment for B-cell differentiation and survival in the CNS of MS patients (Corcione *et al.*, 2004). In active lesions high levels are found in macrophages in perivascular infiltrates, whereas they are detected in CECs and astrocytes in non-active lesions (Calderón *et al.*, 2006). CXCL12 is located in the basolateral surface of CECs, but a redistribution of the protein to the apical side in active lesions and in the progressive forms of MS has been observed. This promotes the activation of CXCR4 of the mononuclear cells and therefore an inappropriate trafficking of cells of the immune system into the CNS (McCandless *et al.*, 2008).

II. OBJECTIVES

OBJECTIVES

The activating receptor NKG2D is required for optimal responses against viral infections and tumors. However, an inappropriate NK cell or CD8+ T cell activation by NKG2D may induce aberrant responses. Thus, NKG2D has been related to several autoimmune diseases, and tumor cells releasing NKG2D ligands into their soluble form or via microvesicles induce receptor internalization, which in turn impairs cell effector functions. Nevertheless, alternative mechanisms that might contribute to the immune response or the immune evasion, as well as the signaling pathways involved, have not been fully explored. Cell migration is particularly important in leukocyte function and immune response. However, the role of NKG2D in this process has rarely been investigated. Therefore, the present project addresses the following specific objectives:

–To analyze the role of the NKG2D receptor in the regulation of NK cell and CD8+ T cell migration.

–To explore the possible role of membrane rafts in NKG2D-mediated signaling. This research is part of collaboration with Dr. Václav Horejsi in the Laboratory of Molecular Immunology (Institute of Molecular Genetics, Academy of Sciences of Czech Republic, Prague).

–To study the migratory ability of different cell subsets (CD4+ T cells, CD8+ T cells, NK cells and B cells) in patients with multiple sclerosis.

OBJETIVOS

El receptor activador NKG2D es necesario para una respuesta óptima frente a infecciones víricas y tumores. Sin embargo, una activación inapropiada de células NK y células T CD8+ por NKG2D puede inducir respuestas anómalas. De esta manera se ha relacionado a NKG2D con varias enfermedades autoinmunes, y las células tumorales que liberan ligandos de NKG2D en su forma soluble o mediante microvesículas inducen la internalización del receptor, que a su vez afecta a las funciones de las células efectoras. No obstante, mecanismos alternativos que puedan contribuir a la respuesta inmunológica o a la evasión del sistema inmune, así como las vías de señalización implicadas, no han sido completamente estudiados. La migración celular es particularmente importante en la función leucocitaria y la respuesta inmune. Sin embargo, el papel de NKG2D en este proceso apenas se ha estudiado. Por tanto, el presente proyecto aborda los siguientes objetivos específicos:

–Analizar el papel del receptor NKG2D en la regulación de la migración de células NK y células T CD8+.

–Explorar el posible papel de las balsas lipídicas en la señalización mediada por NKG2D. Este estudio es parte de una colaboración con el Dr. Václav Horejsi en el Laboratorio de Inmunología Molecular (Instituto de Genética Molecular, Academia de las Ciencias de República Checa, Praga).

–Estudiar la capacidad migratoria de diferentes poblaciones celulares (células T CD4+, células T CD8+, células NK y células B) en pacientes con esclerosis múltiple.

III. MATERIAL AND METHODS

1. CELL LINES AND PRIMARY CELLS

The human NK leukemia cell line (NKL) was kindly donated by Dr. Jerome Ritz (Dana-Farber Cancer Institute, Boston, Massachusetts) and cultured as described (Robertson *et al.*, 1996). Briefly, NKL cells were maintained in RPMI medium (PAA; Pasching, Austria) supplemented with 15% fetal bovine serum (FBS; PAA), 50 U/mL IL-2 (Roche; Mannheim, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin and 25 ng/mL amphoterycin (PAA). Cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2. The medium was replaced every 48 h.

For isolation of human NK cells or CD8+ T cells, peripheral blood mononuclear cells (PBMC) were obtained from buffy coats by centrifugation on a Lymphoprep gradient. NK cells were isolated by negative selection using Dynabeads NK cell kit (Invitrogen, California) according to the manufacturer's instructions. Isolation of CD3+CD8+ T cells was done by magnetic bead sorting with directly conjugated CD8 microbeads, according to the manufacturer´s instructions (MACS, Miltenyi Biotec). The purity of isolated NK cells and CD8+ T cells was tested by flow cytometry (Figure III.1). Cells were used when purity was > 90%. Human NK cells and CD8+ T cells were cultured for 48 h in the absence (resting) or presence of IL-2 (50 U/mL).

2. RECEPTOR ACTIVATION

NKL or NK cells were stimulated with 10 µg/mL of plate-bound monoclonal antibodies (mAb) against NKG2D (R&D Systems; Minneapolis, USA), anti-NKG2A (R&D), IgG (Jackson ImmunoResearch; Suffolk, UK) as control or 5 µg/mL recombinant human ULBP-2/Fc chimera (R&D). CD8+ T cells were stimulated with plate-bound antibodies to CD3 (OKT-3; 1 µg/mL;

eBioscience; San Diego), CD28 (CD28.2; 5 µg/mL; eBioscience) and NKG2D alone or in combinations; IgG was used as antibody control.

For detergent extraction methods, NKL cells were stimulated with 10 µg/mL anti-NKG2D Ab in medium for 30 min on ice, then 2 µg/mL goat anti-mouse Ab was added and cells were transferred to 37ºC for 5 min.

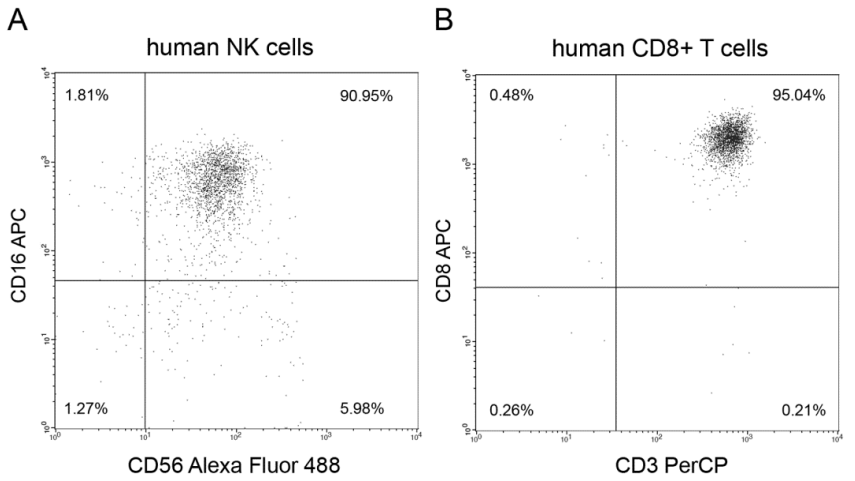


Figure III.1. Purity of isolated cells. Representative dot plots of the purity of **(A)** human NK cells (gated on CD3-) and **(B)** CD8+ T cells.

3. MIGRATION ASSAYS

All assays were performed in Transwell chambers (6.5-mm diameter, 3.0-µm or 5-µm pore size; Costar Corporation, New York). Experiments were performed as duplicates.

3.1. NKL cells

NKL cells ($3x10^5$- $5x10^5$) were incubated with plate-bound antibodies, then removed from the plates and allowed to migrate through transwell membranes coated with ICAM-1/Fc chimera (5 µg/mL; R&D Systems) or fibronectin (5 µg/mL; Sigma-Aldrich; St Louis, MO, USA) in starving medium

(random migration) or medium containing 50 ng/mL CXCL12 or 1 nM CX3CL1/fractalkine (Peprotech; London, UK). Migrated cells were counted after 3.5 h in a Casy Counter (Roche Innovatis AG; Reutlingen, Germany). Where indicated, cells were pretreated for 30 minutes with chemical inhibitors of Rac1 (NSC23766, 0.1 µM in H_2O; Calbiochem EMD Chemicals, San Diego, CA, USA) and N-WASp/WASp (wiskostatin, 1 µM in DMSO; Enzo Life Sciences; Lausen, Switzerland).

3.2. Human NK cells

NK cells ($3x10^5$– $5x10^5$) were incubated with plate-bound antibodies, then removed from the plates and allowed to migrate through transwell membranes coated with fibronectin (5 µg/mL) in medium containing 80 ng/mL CXCL12. Migrated NK cells were counted after 16 h in a Casy Counter.

3.3. Human CD8+ T cells

For chemotaxis assays, cells (10^6/100 µL) were added to transwell membranes coated with human fibronectin (10 µg/mL). CXCL12 (50 ng/mL) was added to the lower chamber. Thereafter, CD8+ T cells were allowed to migrate for 2.5 h at 37ºC in 5% CO_2 atmosphere. Then, the migrated cells were recovered from the lower part of the chemotaxis chamber and counted by flow cytometry or in a Casy Counter. Where indicated, the absolute number of cells pre- and postmigration was estimated using TruCount tubes (BD Biosciences; Erembodegem, Belgium), through the formula: [cell] = (nº events counted per test * nº beads per test) / (nº total of beads * test volume). 5000 beads were routinely collected to analyze the populations.

4. ADHESION ASSAYS

NKL, NK cells and CD8+ T cells were washed and adjusted to 10^6 cells/mL. Then BCECF (Invitrogen-Molecular Probes, Oregon, USA) was added to the cell suspension and the mixture was incubated for 20 min. Labeled cells were washed twice with medium containing 1% BSA and activated with plate-bound antibodies. Then cells were added to ICAM-1- or fibronectin-coated wells (5 μg/mL) at approximately 10^5 cells/well and incubated for 1.5 h at 37°C with 5% CO_2. Non-adherent cells were removed by washing twice with PBS. Fluorescence was quantified using a LS 55 Luminescence Spectrometer (PerkinElmer). Experiments were performed as triplicates.

5. DETERMINATION OF F-ACTIN CONTENT

F-actin levels were determined by flow cytometry using phalloidin conjugated with tetramethylrhodamine isothiocyanate (TRITC; Sigma) as previously described (Burns *et al.*, 2010). Briefly, after activation cells were fixed with IC Fixation buffer (eBioscience) for 20 min at room temperature. After being washed with PBS, cells were permeabilized with IC Permeabilization buffer (eBioscience) and thereafter incubated with phalloidin-TRICT (1:1000) in the dark for 30 min at 4°C. Cells were washed and analyzed by flow cytometry on a FACS Calibur (Becton Dickinson) using CellQuest Pro analysis software.

6. IMMUNOFLUORESCENCE STAINING

10^5 – $1.5x10^5$ cells treated as indicated were added to slides coated with Poly-L-lysine (Sigma). Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 5 min at 4°C. Cells were blocked in 1% BSA in PBS for 20 min at room

temperature, and then stained with the indicated primary antibodies for 1 h at room temperature, followed by three washes in PBS. Cells were then incubated with the appropriate secondary antibodies also containing phalloidin-TRITC to label F-actin for 1 h at room temperature. After three washes in PBS, the slides were mounted and imaged using a Olympus BX41 Fluorescence Microscope. The following antibodies were used: rabbit anti-ICAM-3 (Abcam; Cambridge, UK) and fluorophore-conjugated secondary antibodies were from Invitrogen.

7. FLOW CYTOMETRY

For surface staining, mAbs used are listed in Table III.1. NKL cells were incubated with the indicated mAbs or their respective isotype controls according to the manufacturer's instructions and analyzed on a FACSCalibur (Beckton Dickinson). Viability of the cells after activation with plate-bound antibodies was determined using 7-amino-actinomycin D (7-AAD; Immunostep) and was > 95%.

Marker	Clone	Conjugate	Manufacturer
CD45	D3/9	FITC	Immunostep
CD3	33-2A3	PerCP	Immunostep
CD8	143-44	APC	Immunostep
CD28	CD28.2	FITC	eBioscience
CD16	3G8	APC	Immunostep
CD56	HCD56	Alexa Fluor 488	BioLegend
NKG2D (CD314)	1D11	PE	BioLegend
NKG2D (CD314)	1D11	APC	BioLegend
CXCR4 (CD184)	12G5	APC	eBioscience
CD11a	TP1/40	FITC	Immunostep
CD49d	ALC1/1	FITC	Immunostep
CD49d	9F10	PerCP-Cy5.5	BioLegend

Table III.1. List of monoclonal antibodies used for flow cytometry analysis. Location of the manufacturers: Immunostep (Salamanca, Spain); eBioscience (San Diego, USA); BioLegend (San Diego, USA).

8. EXPRESSION AND PURIFICATION OF GST-FUSION PROTEINS

Bacterial strain BL21 transformed with the plasmids GST-PAK-PBD, GST-WASP-PBD and GST-Rtk-RBD was kindly donated by Dr. Anne Ridley (King's College London, UK). BL21 cultures were grown overnight in LB-medium containing 100 µg/mL ampicillin. The protein expression was induced at an OD_{600} of 0.7 with 0.4 mM IPTG. Subsequently, bacteria were harvested by centrifugation and the pellet was resuspended in STE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) and lysed for 15 min at 4ºC with 100 µg/mL lysozime and addition of 5 mM DTT, 1% Tween-20 and 0.03% SDS. After centrifugation of the lysate, GST-fused proteins were purified by affinity with Glutation Sepharose 4B and stored at 4ºC in STE buffer for pull down purposes. Protein concentration was estimated by SDS-PAGE and Coomassie staining.

9. WESTERN BLOTTING

Cell lysates were separated in SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk in TBS-T for 1 h at room temperature, and then incubated with the indicated primary antibodies (Table III.2) overnight at 4ºC with gently rocking. Membranes were washed three times with TBS-T and horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako; Glostrup, Denmark) were added for 1 h at room temperature. Membranes were washed again with TBS-T and blots were developed with the enhanced chemiluminescence (ECL) detection system (Amersham; GE Healthcare, Munich, Germany).

Primary antibodies			
Antibody	**Source**	**Dilution**	**Manufacturer**
anti-NKG2D	mouse	1:500	Upstate
4G10	mouse	1:1000	Millipore
anti-Rac1	mouse	1:1000	Upstate
anti-RhoA	mouse	1:250	Santa Cruz
anti-β-actin	mouse	1.2000	Sigma
anti-Annexin 2	mouse	1:2000	BD Bioscience
anti-WASp	mouse	1:500	Santa Cruz
anti-Cdc42	rabbit	1:1000	Cell signaling
anti-Vav	rabbit	1:500	Santa Cruz
anti-phosphoVav	rabbit	1:500	Santa Cruz
anti-ERM	rabbit	1:1000	Cell signaling
anti-phosphoERM	rabbit	1:1000	Cell signaling
anti-Cofilin	rabbit	1:5000	Abcam
anti-phosphoCofilin	rabbit	1:2000	Abcam
anti-N-WASp	rabbit	1:250	Santa Cruz
anti-ERK1/2	rabbit	1:500	Santa Cruz
anti-phosphoERK1/2	rabbit	1:250	Santa Cruz
anti-Lyn	rabbit	1:500	Santa Cruz
anti-GAPDH	rabbit	1:5000	Santa Cruz
anti-DAP10	goat	1:250	Santa Cruz
anti-L-Plastin	goat	1:500	Santa Cruz

Table III.2. Antibodies used for western blotting. Location of the manufacturers: Upstate (NY, USA), Millipore (Billerica, MA, USA), SantaCruz Biotech (Santa Cruz, CA, USA), BD Biosciences (Erembodegem, Belgium), Cell Signaling Technology (Danvers, MA, USA), Abcam (Cambrigde, UK).

10. RHO GTPASE ACTIVITY ASSAYS

10.1. NKL cells

Activation of members of the Rho GTPase family of proteins Rac1, RhoA and Cdc42 in NKL cells was analyzed in pull down assays as previously described (Cernuda-Morollón *et al.*; 2010). Briefly, NKL were activated and lysed in 50 mM Tris pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 0.5% ß-mercaptoethanol, 500 mM NaCl and 2 mM DTT containing phosphatases and proteases inhibitors (0.4 mM Na_3VO_4, 2 mM PMSF, 2 mM NaF and 2 εg/mL aprotinin). Cell lysates were incubated with GST-fusion proteins on glutathione-sepharose beads. Bound proteins were solubilized in Laemmli

buffer (10% SDS, 50% glycerol, 300 mM Tris pH 6.8, 25% ß-mercaptoethanol and 0.04% bromophenol blue) and Rho GTPases were detected by western blot. The relative levels of total and active Rho GTPases were quantified by densitometric analysis.

10.2. Human CD8+ T cells

For CD8+ T cells, the activation of Rac1 and Cdc42 was determined using the G-LISA Cdc42/Rac1 Activation Assay kit (colorimetric format) from Cytoskeleton (Denver, CO, USA), according to the manufacturer's instructions. The signal was quantified by using a microplate reader (BioRad; Hercules, CA, USA).

11. TRANSIENT GENE KNOCKDOWN

11.1. NKL cells

NKL were transfected using specific ON-TARGETplus SMARTpool small interfering RNA (siRNA) for N-WASp (L-006444-00-0005), for WASp (L-028294-00-0005), for L-plastin (L-011716-00-0005) or ON-TARGETplus non-targeting pool (Dharmacon; Thermo Fisher Scientific., Lafayette, CO.) according to the manufacturer´s instructions. Twenty-four hours after transfection, cells transfected with N-WASp or WASp siRNA were used for transwell migration assays and for determination of F-actin content. Cells transfected with L-plastin siRNA were used for raft fractionation by ß-OG. Viability of the cells was determined by flow cytometry using 7-AAD and dead cells were removed by centrifugation before each experiment. The effect of siRNA on levels of N-WASp, WASp and L-plastin was analyzed by western blotting. Anti-ß-actin or anti-GAPDH blots were included as a protein loading control.

11.2. Human NK cells

Human NK cells were transfected using ON-TARGETplus SMARTpool siRNA for L-plastin (L-011716-00-0005) or ON-TARGETplus non-targeting pool. Twenty-four hours after transfection, cells were used for transwell migration assays and for measuring F-actin levels. The effect of siRNA on levels of L-plastin was analyzed by western blotting. Anti-GAPDH blots were included as a protein loading control.

11.3. Human CD8+ T cells

CD8+ T cells were transfected using ON-TARGETplus SMARTpool siRNA for N-WASp (L-006444-00-0005). Twenty-four hours after transfection, CD8+ T cells were used for chemotaxis assays and for determination of the F-actin content. Viability of the cells was determined by flow cytometry using 7-AAD and dead cells were removed by centrifugation before each experiment. The effect of siRNA on levels of WASp was analyzed by western blotting and anti-β-actin blots were included as a protein loading control.

12. DETERGENT EXTRACTION PROCEDURES

12.1. Density gradient ultracentrifugation

NKL cells ($5x10^7$) were solubilized in 50 mM Tris pH 7.5, 150 mM NaCl and 1% Brij98 containing phosphatases and proteases inhibitors (10 mM NaF, 2 mM EDTA, 1 mM Pefabloc, 1 mM Na_3VO_4 and 5 mM iodoacetamide) for 30 min on ice. Lysates were mixed 1:1 with 80% sucrose and placed at the bottom of a pollyallomer centrifuge tube (Beckman, Palo Alto, CA), then carefully overlaid with 35% sucrose and lysis buffer without sucrose (Figure III.2).

Samples were subjected to ultracentrifugation (50,000 xg/18 h/2ºC). Eight fractions (0.5 mL each) were collected from the top of the gradient and analyzed by western blot or subjected to proteomic analysis.

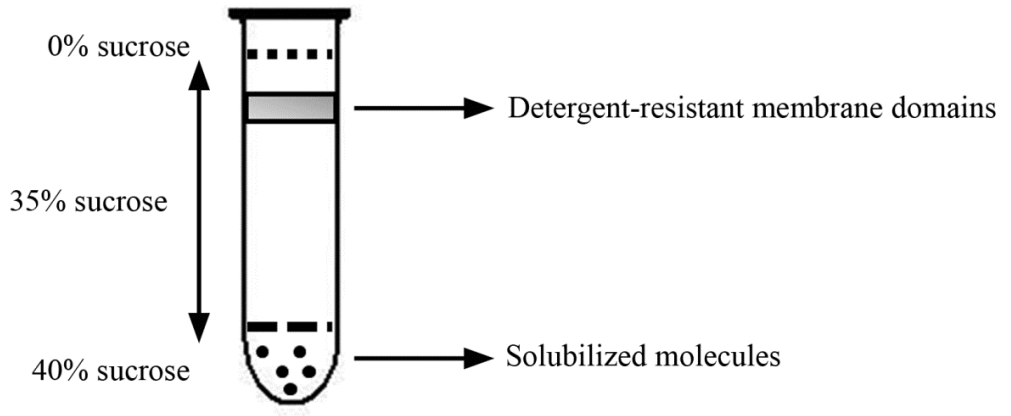


Figure III.2. Density gradient ultracentrifugation.

12.2. Gel filtration chromatography

Gel filtration chromatography experiments were performed using Sepharose 4B (Sigma), a gel prepared from agarose with beads from 45-165 µm diameters, as previously described (Draber *et al.*, 2011). After activation, cells were lysed as described in 12.1. Then, 0.1 mL of cell lysates were separated on a 1 mL Sepharose 4B column and washed with lysis buffer. Twelve fractions (0.1 mL each) were collected and analyzed by western blotting.

12.3. Raft fractionation by β-octylglucoside selective solubility

After stimulation, NKL cells were lysed in 25 mM Tris pH 7.4, 150 mM NaCl and 0.2% Triton X-100 containing protease and phosphatase inhibitors (1 mM DTT, 0.2 mM Na_3VO_4, 1 mM NaF, 1 mM Pefabloc, 1 mM PMSF and 1 µg/mL aprotinin) and then the pellet fraction was solubilized with 0.2% Triton

X-100 and 60 mM ß-octylglucoside (ß-OG) at 37ºC to analyze surface rafts associated with the cytoskeleton. Solubilized raft components were separated from nuclei and protein cytoskeletal network by centrifugation. The raft fractions were then subjected to western blot or proteomic analysis.

13. PROTEIN DIGESTION AND MASS SPECTROMETRY

Membrane raft fractions from sucrose density gradient ultracentrifugation experiments were precipitated in methanol-chloroform (Wessel and Flügge, 1984), subjected to SDS-PAGE utilizing a 12.5% gel, and stained with Coomassie Blue (Bio-Rad). Protein bands were excised in 10 pieces of the gel (Figure III.3), which were washed for 5 min in 50% acetonitrile (ACN) for three times and with 100% ACN once. Gel pieces were then dried in a speed-centrifuge under vacuum. Proteins were reduced with 10 mM DTT for 1 h at 50ºC and alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. Washing and dehydration steps were repeated and then proteins digested with 15 ng/µl of trypsin. The peptides were analyzed by LC-MS/MS in a Q-Trap Mass Spectrometer (Applied Biosystems, Foster City, CA, USA) coupled to a nano-HPLC (NanoLC Ultimate, LC Packings, Dionex, Sunnyvale, CA, USA). The same procedure was followed with the raft fractions obtained by solubilization in ß-OG.

13.1. Data analysis

All MS data sets were searched using the MASCOT search engine (Matrix Science Ltd. London, v2.4.01) under the *Homo sapiens* taxonomy. Trypsin was selected as the enzyme (one missed cleavage allowed). The peptide mass tolerance was set at ± 0.5 atomic mass units (amu), and the fragment mass tolerance was set at ± 0.3 amu. Mascot score obtained for each protein was greater than 32, where score >32 indicates identity or extensive homology at a

significance level ($p<0.05$). Protein functions and characteristics were obtained from UniProt Knowledgebase (www.expasy.org).

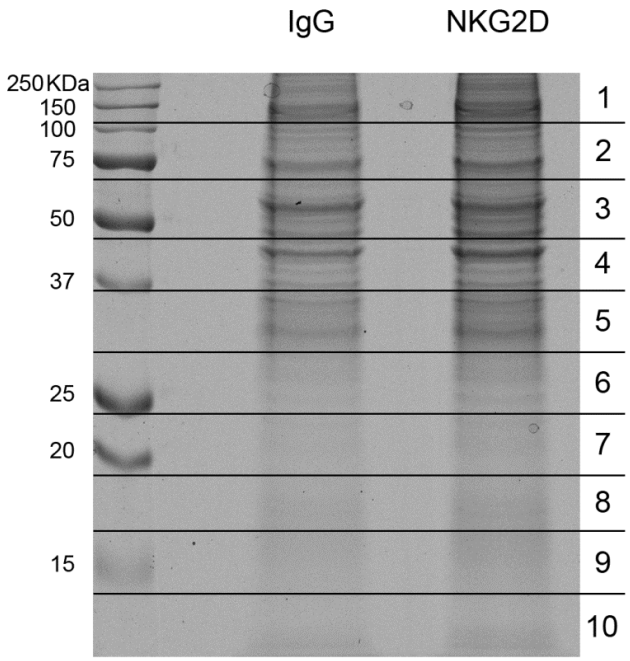


Figure III.3. Representative Coomassie Blue-stained gel for protein digestion, with sizes of the molecular marker in KDa on the left and numbers of the excided bands on the right.

14. STATISTICAL ANALYSIS

Differences in the mean observations were analyzed with Student´s t test. Where indicated, comparisons between more than two groups were analyzed by using a general linear model (GLM), with treatments (IgG, mAbs against CD3, CD28 and NKG2D alone or in combination) and/or pretreatments as fixed factors and experiments as a random factor. Interactions between the factors were considered. When significant differences were found, comparison between treatments was done using the post hoc Student-Newman-Keuls test. Statistical significance was set at $p < 0.05$. Values are expressed as mean + SEM. Data were analyzed using the free statistical software R version 2.13 (www.r-project.org).

15. MULTIPLE SCLEROSIS METHODS

15.1. Study population

This project was approved by the Ethics Committee of Hospital Central de Asturias (HUCA). Blood samples were obtained with written informed consent. For migration assays, peripheral blood lymphocytes (PBL) from untreated RRMS patients, 11 in the remitting phase (7 females, 4 males, aged 27-54, mean 37.73) and 7 during relapses (4 females, 3 males, aged 28-56, mean 41), and 7 untreated CIS patients (6 females, 1 male, aged 21-49, mean 36.43) were used. Twenty-five age- and sex-matched healthy donors (17 females, 8 males, aged 22-58, mean 38.65) were recruited as controls. For the analysis of CXCR4 and CD49d expression, 19 healthy volunteers (11 females, 8 males, aged 20-56, mean 36.47), 19 RRMS in the remitting phase (12 females, 7 males, aged 27-52, mean 40.47) and 9 patients with CIS (3 females, 6 males, aged 20-49, mean 33.11) were analyzed. MS was clinically defined using the revised McDonald criteria (McDonald *et al.*, 2001; Polman *et al.*, 2011). None of the patients had received any treatment or taken any corticosteroids for at least three months prior to study entry.

15.2. Cell isolation

Peripheral venous blood was collected in Vacutainer (Beckton Dickinson) tubes containing EDTA as an anticoagulant and processed within 30 min of collection. PBMC were isolated from blood samples by density gradient centrifugation over Lymphoprep and cryopreserved until use in liquid nitrogen in FBS containing 10% DMSO. For chemotaxis assays cells were recovered on RPMI medium containing 10% heat inactivated FBS and depleted of monocytes by adhesion to plastic for 2 hours.

15.3. Chemotaxis assays

All assays were performed in a Costar transwell system with a pore size of 5.0 µm coated with 20 µg/mL human fibronectin (Sigma). PBMC were thawed, counted with a Casy Counter and the viability measured using 7-AAD (> 90%). Cells were added (10^6 cells in 0.1 mL/well) in the upper chamber; the lower chambers were filled up with 0.6 mL RPMI containing CXCL12 (80 ng/mL; Peprotech). Two replicates were made for each condition. The same number of cells was plated on wells without the insert, in order to determine the initial composition of the subpopulations (premigration). After 16 h of incubation at 37ºC 5% CO_2, the content of the bottom chamber was collected to perform the flow cytometry analysis and determine the number of migrated cells (postmigration).

15.4. Flow cytometry

Peripheral blood lymphocytes from patients and healthy donors used in transwell assays were stained to perform a 6-color assay with a panel of fluorochrome-conjugated mAbs as indicated (Table III.3). Whole-blood samples from patients and healthy controls were collected and stained for 4-color cytometry with the fluorescently conjugated monoclonal antibodies listed in Table III.3. Samples from patients and healthy donors were incubated with the fluorochrome-conjugated mAbs according to the manufacturer's instructions for 30 min at 4ºC in the dark.

15.4.1. Flow cytometry analysis

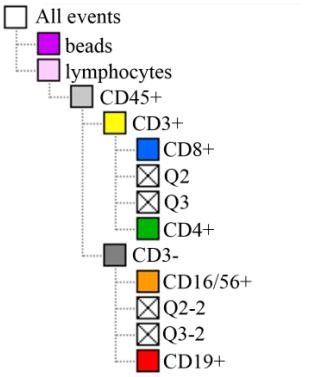
For migration assays, data acquisition and analysis were performed on a FACSAria I flow cytometer (Beckton Dickinson), equipped with two lasers: 488-nm (blue) laser and the 633-nm (red) laser.

6-color assay				
Description	**Marker**	**Clone**	**Conjugate**	**Manufacturer**
Leukocytes	CD45	D3/9	FITC	Immunostep
T cells	CD3	SK7	PerCP-Cy5.5	BD Biosciences
Helper T cells	CD4	RPA-T4	APC	BD Pharmingen
Cytotoxic T cells	CD8	SK1	APC-H7	BD Pharmingen
NK cells	CD16	3G8	PE	BD Pharmingen
NK cells	CD56	B159	PE	BD Pharmingen
B cells	CD19	HIB19	PE-Cy7	eBioscience
4-color assay				
Leukocytes	CD45	D3/9	FITC	Immunostep
T cells	CD3	33-2A3	PerCP	Immunostep
NK cells	CD16	3G8	PE	BD Pharmingen
NK cells	CD56	B159	PE	BD Pharmingen
Helper T cells	CD4	HP2/6	PE	Immunostep
Helper T cells	CD4	HP2/6	FITC	Immunostep
Cytotoxic T cells	CD8	HIT8a	PE	BioLegend
Cytotoxic T cells	CD8	143-44	APC	Immunostep
CXCR4	CD184	12G5	APC	eBioscience
α4 integrin	CD49d	ALC1/1	FITC	Immunostep

Table III.3. List of monoclonal antibodies used for 4-color or 6-color flow cytometry analysis.

Individual compensation settings were performed with single-stained controls using the BD FACSDiVa™ software (BD Biosciences). Further compensation adjustments were made based on fluorescence minus one (FMO) controls, which consist of all the reagents but the one of interest (Baumgarth and Roederer, 2002). To determine the fluorescence background, conjugated isotypic mAbs for each fluorochrome that were not reactive to human cells were used. For pre- and post-migration analysis, a systematic gating strategy consisting of a first gate on the lymphocyte cell population (CD45+) was used. Then PBL were identified based on positive or negative staining for CD3. Gated on CD3+, CD4+ and CD8+ cells were identified, and CD16/56+ and CD19+ populations were defined on gate CD3- (Figure III.4). The absolute number of cells pre- and post-migration was estimated using TruCount tubes, through the formula: [cell] = (nº events counted per test * nº beads per test) / (nº total of beads * test volume). 10000 beads were routinely collected to analyze the populations.

Flow cytometry to measure CXCR4 and CD49d surface levels from patients and healthy subjects in CD4+, CD8+ and CD16/56+ cells was performed on a FACSCalibur flow cytometer. PBMC were first gated based on forward and side light scatter properties. CD4+ T cells were gated as CD3+CD4+CD8- PBMC; CD8+ T cells as CD3+CD8+CD4-, and NK cells as CD45+CD3-CD16+CD56+. Isotype and FMO controls were employed to define positive and negative cell populations for CXCR4 and CD49d in each lymphocyte subset. A total of 10000 events in the PBMC gate were acquired to analyze the populations.



Markers	Description
CD45+, CD3+, CD8-, CD4+	Helper T cells
CD45+, CD3+, CD4-, CD8+	Cytotoxic T cells
CD45+, CD3-, CD16/56+	Natural killer cells
CD45+, CD3-, CD19+	B cells

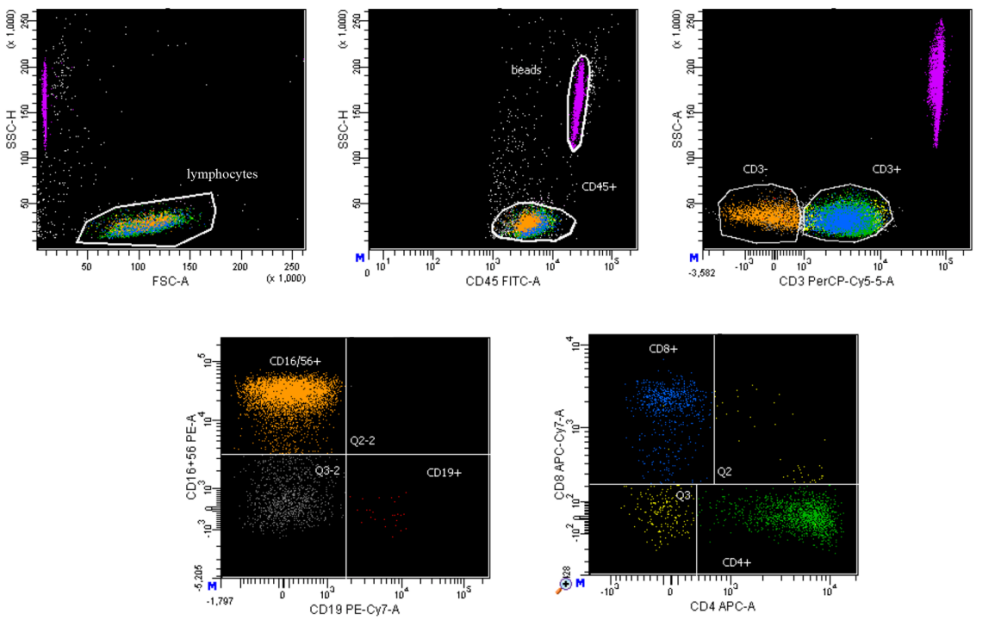


Figure III.4. Gating strategy employed to identify the different subpopulations.

15.5. Statistical analysis

In chemotaxis assays, comparisons of migration rates between groups were analyzed by GLM, with type of subjects (healthy subjects, MS and CIS patients) as fixed factors and experiments as random factor. Interactions between the factors were considered. To analyze differences in the frequencies of lymphocyte subsets prior to chemotaxis assays, analysis of the variance (ANOVA) was used. To determine the differences in either the percentage of integrin- or chemokine receptor-expressing cells or their median fluorescence intensity (MFI), the Welch test of equality of means was used. When the P-value was <0.05, Duncan's post-hoc test was done. Data were analyzed using the free statistical software R version 2.13 (www.r-project.org).

IV. RESULTS

1. ROLE OF NKG2D IN NK-CELL MIGRATION

1.1. NKG2D inhibits cell migration of human NK cells and NKL cells

The NKG2D/NKG2DL recognition system has been postulated as a relevant mechanism in the immune response to cancer or in the tumor evasion from immunosurveillance. However, the possible role of NKG2D in other processes that may be involved in these responses is not fully explored. NK cell recruitment during an immune response through chemotactic cues is essential to properly reach the target tissues. Therefore, the involvement of NKG2D receptor in the migratory ability of NK cells was examined.

To this end, human NK cells were isolated from peripheral blood and were cultured with complete medium (resting) or activated with 50 U/mL IL-2 for 48 h. Cells were stimulated with immobilized anti-NKG2D or IgG as a control for 20 min and then were subsequently removed from the plates and added to transwell filters. Cells were allowed to migrate through fibronectin, a protein of the ECM involved in cell adhesion and chemotaxis, and towards the chemokine CXCL12, whose receptor CXCR4 is expressed in NK cells (Inngjerdingen et al., 2001). Number of migrating cells was initially monitored every 2 h for up to 16 h and then counted with a Casy Counter.

NKG2D activation inhibited cell migration of IL-2-activated NK cells compared with the control condition (Figure IV.1A; $^{**}p<0.005$). In contrast, migration rates of non-stimulated (resting) NK cells showed no differences upon receptor activation in comparison with IgG-treated cells. Similar results were observed when the IL-2-dependent NKL cell line was employed. In this case, migration assays were performed through fibronectin in the presence or absence (randomized) of a CXCL12 gradient. Additionally, chemotaxis assays

were performed employing the chemokine CX3CL1/Fraktalkine, which is known to act as chemoattractant for NK cells as well (Inngjerdingen et al., 2001). As shown in Figure IV.1B, NKG2D activation significantly decreased cell motility in random, CXCL12- or CX3CL1-induced migration (*p<0.05). Thus, these results indicate that the effect of receptor activation on cell migration is independent of the chemoattractant employed. In addition, this decrease in chemotactic and random migration of NKL cells is not exclusive of the fibronectin/alpha4 integrin axis. When a different adhesion molecule was employed as substrate, such as ICAM-1, NKG2D stimulation also inhibited random migration and chemotaxis (**p<0.005; *p<0.05; Figure IV.1C).

To gain insight into the mechanism involved in the inhibition of NK cell migration upon NKG2D stimulation, it was next explored whether this effect was due to significant changes in the surface levels of the proteins implicated in cell adhesion or in the chemotactic response. The levels of these molecules were determined by flow cytometry in NKL cells upon NKG2D activation (Figure IV.2). Fibronectin and ICAM-1 bind to their counter receptors Ŭ4ƀ1 (CD49d/29) and ŬLƀ2 (CD11a/CD18), respectively. Therefore, the surface expression of the integrins CD49d and CD11a were measured. The percentage of NKL cells expressing these molecules showed no significant changes after 20 min of NKG2D cross-linking. Their surface levels, indicated by the MFI, were not altered compared with the control condition. Similar results were found when analyzing the percentage of cells expressing the chemokine receptor CXCR4 and its levels on the cell surface. Moreover, expression of NKG2D also remained unchanged upon receptor activation in comparison with IgG-treated cells.

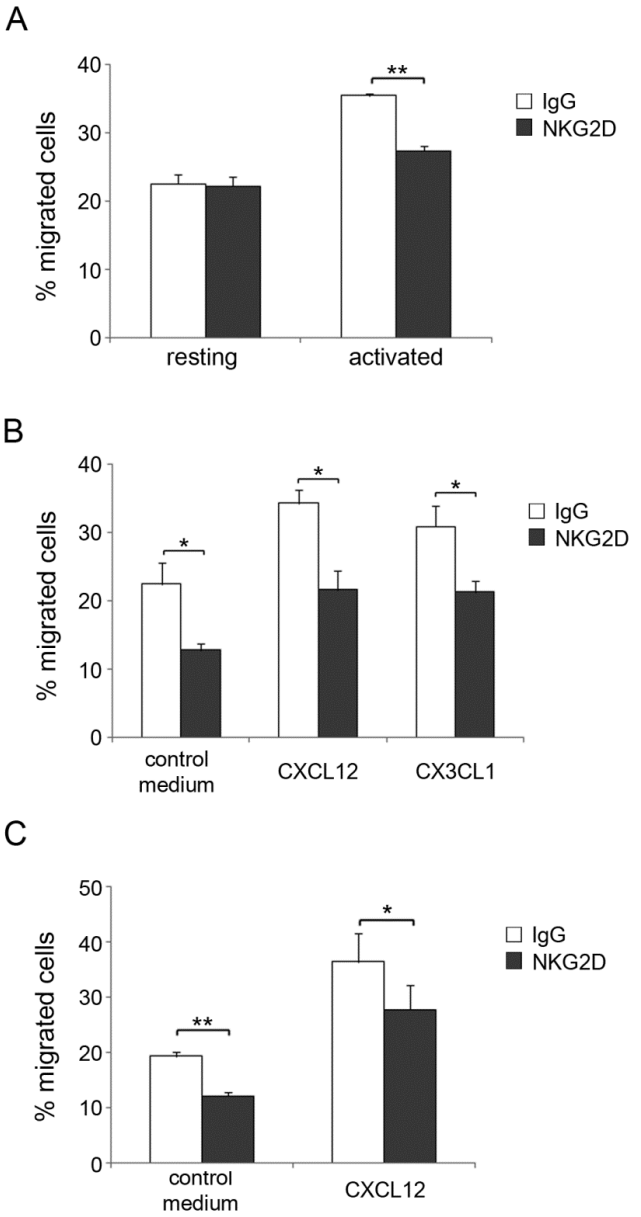


Figure IV.1. NKG2D activation in NK cell migration. **(A)** Isolated human resting or IL-2-activated NK cells were assayed for their ability to migrate through fibronectin-coated inserts (5 µg/mL), using CXCL12 as chemoattractant. After incubation with plate-bound antibodies against NKG2D or IgG as a control, cells were allowed to migrate for 16 h. Results shown are the mean + SEM of two replicates pooled from four independent experiments (**p<0.005). **(B)** NKL cells were incubated with plate-bound antibodies, added to fibronectin-coated inserts, and assessed in random, CXCL12- and CX3CL1-directed migration assays. Mean + SEM of two replicates pooled from three independent experiments is shown (*p<0.05). **(C)** Random and CXCL12-mediated migration assays in NKLs trough ICAM-1. Graphs show the mean + SEM of two replicates pooled from three independent experiments (*p<0.05, **p<0.005).

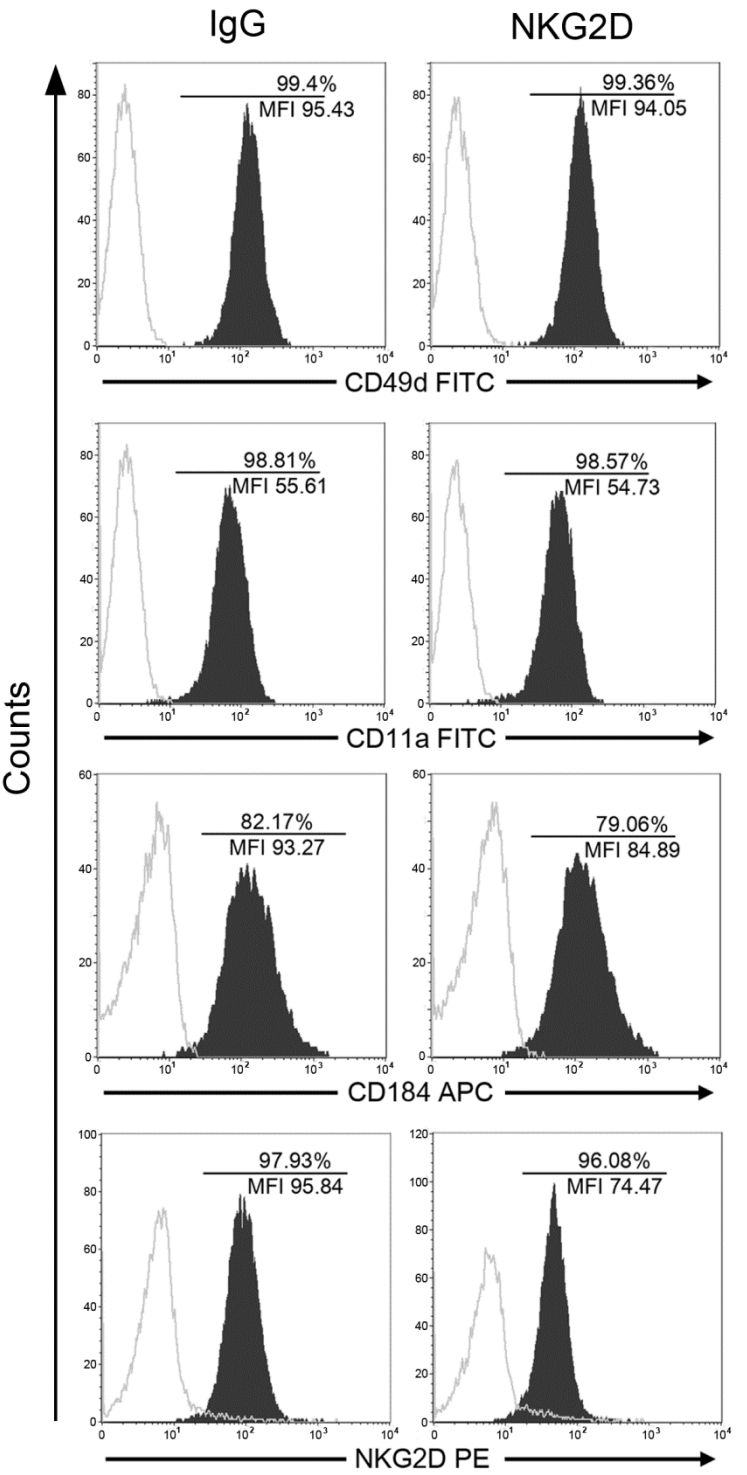


Figure IV.2. CD49d, CD11a, CXCR4 and NKG2D expression upon receptor activation. NKL cells were incubated with plate-bound anti-NKG2D or IgG as a control, stained with the indicated mAbs (filled histograms) or conjugated isotypic mAbs for each fluorochrome (unfilled histograms) and analyzed by flow cytometry. Histograms shown are representative of four independent experiments, with the percentage of cells expressing the indicated molecules and the MFI.

The adhesion of the cells to their substrate plays a key role in migrating cells and involves the clustering of integrins and the reorganization of the actin cytoskeleton. Therefore, adhesion of primary NK cells or NKL cells to fibronectin and changes in the actin dynamics upon receptor activation were analyzed. The adhesion ability of NK cells and NKL cells to the substrate was not altered after NKG2D activation in comparison with the control condition (Figure IV.3A). Thus, differences on cell migration are not attributable to an increase in cell adhesion to the substrate. However, when changes in F-actin content were measured by flow cytometry, a significant increase was found upon NKG2D engagement (*$p<0.05$; Figure IV.3B).

Cell motility involves a highly dynamic actin polymerization and depolymerization and important changes in the cell shape. Polarization of the cell is indispensable for a proper migration of leukocytes, and this process implicates an asymmetrical cluster of proteins on the plasma membrane. In T cells chemokine receptors and integrins redistribute to the leading edge, whereas adhesion molecules such as ICAM-1, ICAM-3, CD43 and CD44 are recruited to the uropod (Krummel and Macara, 2006). Cell polarity upon NKG2D activation was analyzed by studying the localization of ICAM-3 and the distribution of F-actin (Figure IV.4A). NKG2D-activated cells showed more rounded morphologies than control-treated cells, without a well defined leading edge or uropod (Figure IV.4B).

Since NKG2D activation altered neither CXCR4 nor integrin surface levels, and cells adhere normally to fibronectin, the impairment observed in cell motility is not due to a defect on chemokine engagement or an increase in cell adhesion. However, the fact that NKG2D cross-linking correlated with an increase in F-actin content suggests a role for the Rho GTPase family of proteins.

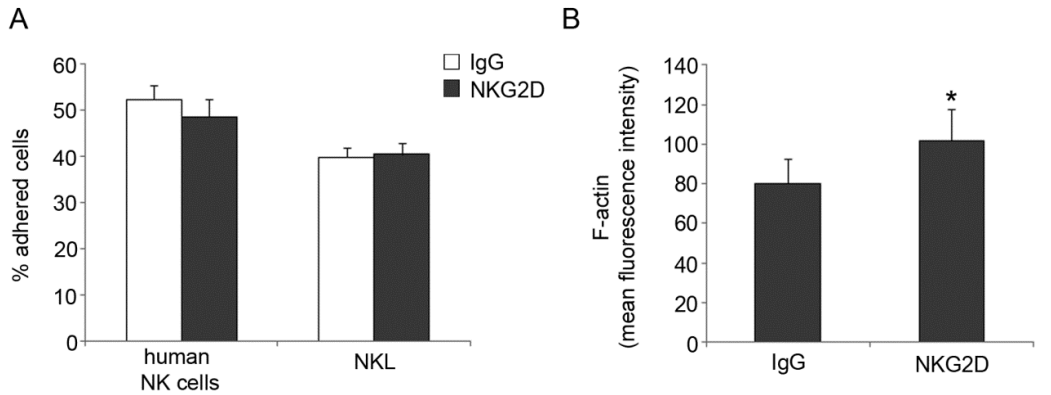
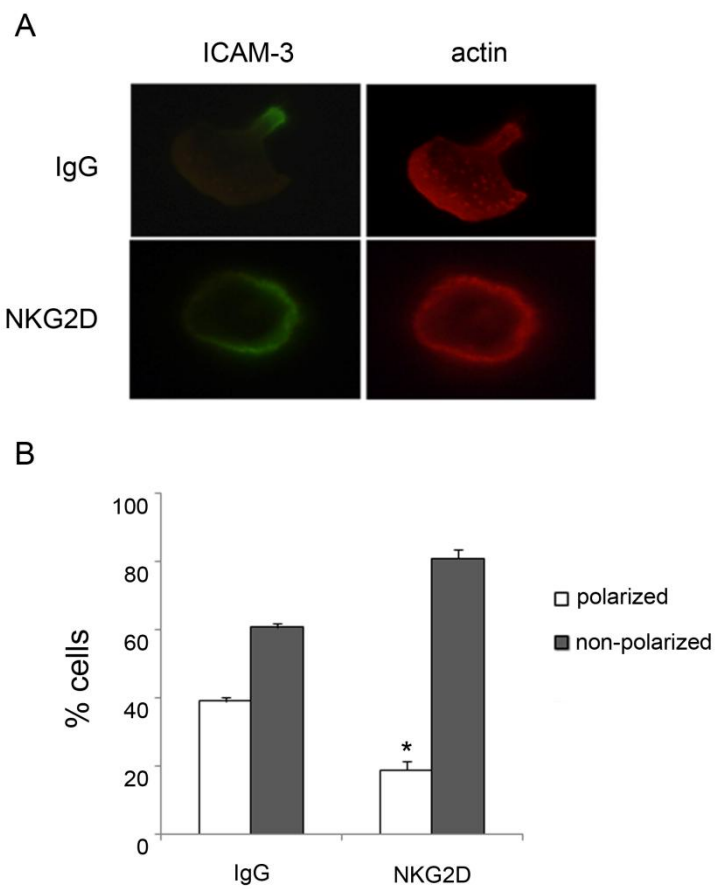


Figure IV.3. Cell adhesion and F-actin dynamics after NKG2D activation. (A) BCECF-labeled human NK cells or NKL cells were plated on fibronectin-coated wells and adhesion was determined after 1.5 h. Percentage of adhesion was estimated relative to the total number of plated cells. Results represent the mean + SEM of three replicates pooled from four independent experiments. **(B)** NKL cells were incubated with the indicated antibodies, fixed, permeabilized, and stained with phalloidin-TRITC. The mean fluorescence intensity was determined. The graph shows the mean of four independent experiments + SEM (*p<0.05).

Figure IV.4. Cell polarity upon NKG2D activation (A) NKL cells were stimulated with the indicated plate-bound antibodies and plated on fibronectin, then fixed, permeabilized and stained with ICAM-3-FITC and with phalloidin-TRITC to localize F-actin. **(B)** Cells displaying a polarized or non-polarized morphology after activation were counted. Three fields per experiment were analyzed with 50 cells/field. The graph shows the mean + SEM of three independent experiments (*p<0.05).



1.2. Integration of activating and inhibitory signals

Since NK cell function is regulated by a balance of activating and inhibitory signals, it was investigated whether an inhibitory receptor could rescue the cell motility. To address this issue, NKL cells were incubated with anti-NKG2D, anti-NKG2A, or both together and then were assayed for their ability to migrate. NKG2D activation leads to a decrease in the migration rate (*$p<0.05$; Figure IV.5A). This effect was abrogated by co-stimulation of the NKG2A receptor, while the activation of this receptor alone did not affect cell migration. These results are consistent with the study of Culley *et al.* (2009), which describes that activating signals in NK cells, such as interaction of NKG2D with MICA promotes a stop signal to form the IS, while inhibitory signals may reverse this effect.

Recent works have shown the remarkable role of ULBP2 as a marker for disease progression (Salih *et al.* 2003; Paschen *et al.* 2009). In addition, it has been reported that proteasome inhibitor treatment leads to a higher sensitivity to NK cell mediated killing by upregulation of ULBP2 expression (Valés-Gómez *et al.* 2008), supporting its potential relevance in the development of anticancer therapy. It was next evaluated whether NKG2D/ULBP-2 interaction exerted similar effects than NKG2D cross-linking. NKL cells were activated with plate-bound anti-NKG2D, human recombinant ULBP2-Fc chimera or control IgG, then removed from the plates and chemotaxis assays were performed. Activation of NKG2D receptor with ULBP-2 efficiently inhibited NK cell migration (*$p<0.05$; Figure IV.5B), and both plate-bound anti-NKG2D and ULBP-2 equally impaired cell migration, since no significant differences between their migration rates were found.

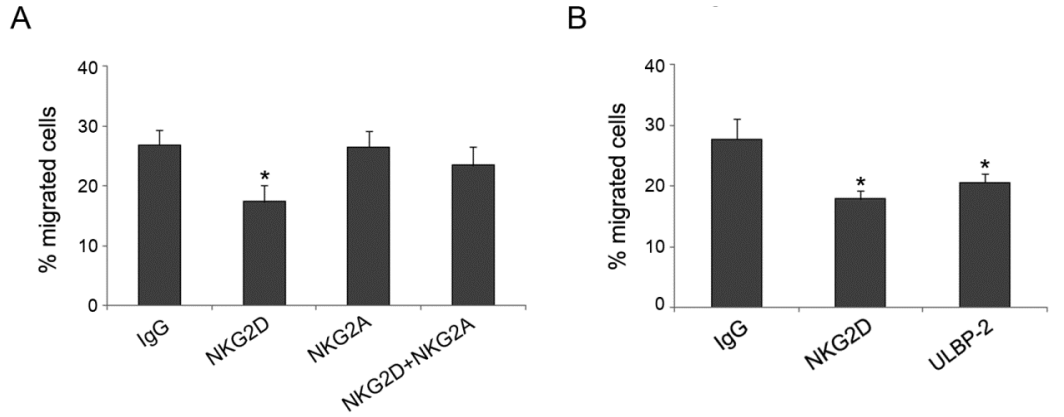


Figure IV.5. Activating and inhibitory signals in NK cell chemotaxis. (A) NKL cells were activated with anti-NKG2D, anti-NKG2A or both mAb and assessed for their migratory ability on fibronectin coated inserts. The graph shows the mean rates + SEM of two replicates pooled from three independent experiments (*p<0.05). **(B)** Migration rates of pretreated NKL cells with plate-bound IgG, anti-NKG2D or recombinant ULBP-2/Fc chimera. Results are the mean + SEM of two replicates pooled from four independent experiments (*p<0.05).

1.3. Involvement of Rho GTPases in NKG2D signaling

Rho GTPases are key molecular switches that modulate the required changes in the cytoskeleton and microtubule architectures during cell polarization and migration. In NK cells, Rho GTPases play an important role in their activity, as they regulate cell polarization, adhesion and vesicle trafficking of the cytotoxic granules towards the target cell (Wittman and Waterman-Storer, 2001; Ridley, 2001). The regulation of the activity of the best characterized members of Rho GTPases, Rac1, RhoA and Cdc42 upon NKG2D stimulation was studied by pull down assays. NKL cells were activated via plate-bound antibodies to NKG2D and IgG as a control. Then cells were lysed and incubated with purified GST-PAK-PBD, GST-WASp-PBD and GST-Rtk-RBD, which bind to active Rac1, Cdc42 and RhoA, respectively. GTP-bound proteins and total levels of Rho GTPases were detected by western blot. As shown in Figure IV.6, the activity of Rac1 was increased (1.75-fold over control; *p<0.05), as well as the activity of Cdc42 (2.25-fold over control; *p<0.05), whereas a decrease in the activity of RhoA was observed (1.97-fold decrease;

*p<0.05). Rac1 and Cdc42 control cytoskeletal reorganization at the leading edge of the migrating cell and are essential for the establishment of cell polarity (Etienne-Manneville and Hall, 2002). Since NKG2D activation regulates the activity of these Rho GTPases and induces an increase in F-actin levels, the involvement of Rac1 and Cdc42 in NKG2D-mediated migration and actin dynamics was further studied.

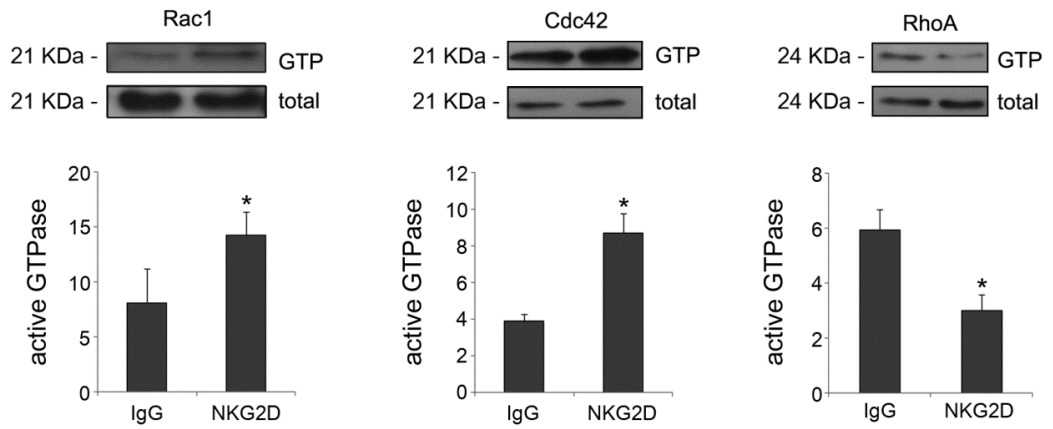


Figure IV.6. Regulation of Rho GTPases activity by NKG2D activation. NKL cells were incubated with plate-bound anti-NKG2D or control IgG. After activation cells were lysed and pull down assays were performed to analyze Rac1, Cdc42 and RhoA activity. A representative experiment for each Rho GTPase is shown. Results indicate the mean active GTPase/total GTPase ratio of five independent experiments + SEM (*p<0.05).

To address this issue, the Rac inhibitor NSC23766 or wiskostatin, a selective inhibitor of N-WASp (Peterson *et al.*, 2004), the effector of Cdc42, were employed. Human NK cells cultured with IL-2 for 48 h were pretreated for 30 min with NSC23766 (0.1 µM) or wiskostatin (1 µM). Then cells were incubated with plate-bound anti-NKG2D antibody or control IgG and tested for their chemotactic response to CXCL12. Activation of the receptor inhibited cell migration in control- and NSC23766-treated cells (*p<0.05; Figure IV.7A). Thus, inhibition of Rac1 was not sufficient to rescue NK cell migration. Pretreatment of NK cells with wiskostatin impaired NK cell motility under control conditions, while anti-NKG2D-treated cells showed a partial rescue of

the migratory ability (*p<0.05). Similar results were found in the NKL cell line. NSC23766 pretreatment increased NKL migration in control conditions, suggesting that a basal over-activation of Rac1 may be impairing cell motility, but the Rac1 inhibitor did not rescue the NKG2D-induced inhibition of NKL cell migration (*p<0.05; Figure IV.7B). Regarding wiskostatin, cells pretreated with this agent exhibited decreased chemotaxis in control conditions (*p<0.05; Figure IV.7C), whereas triggering of the NKG2D receptor with wiskostatin pretreatment showed a recovery of cell migration (*p<0.05). These results suggest that the Cdc42/N-WASp axis is involved in the regulation of NK cell migration upon NKG2D activation.

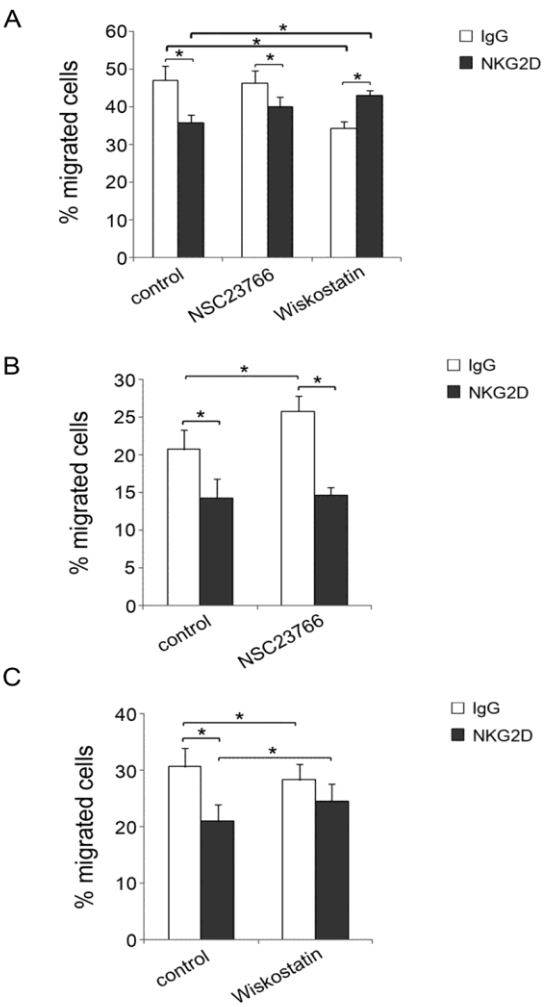


Figure IV.7. Involvement of Rac1 and Cdc42 in NKG2-mediated cell migration.(A) Human NK cells were pretreated with the Rac1 inhibitor NSC23766 (0.1 µM) and the inhibitor of N-WASp activity, wiskostatin (1 µM) or vehicle. Cells were incubated with IgG or anit-NKG2D and their ability to migrate was assayed. Results shown are the mean + SEM of two replicates pooled from three independent experiments (*p<0.05). A similar procedure was followed with NKL cells treated with **(B)** NSC23766 and **(C)** wiskostatin. Data shown are the mean + SEM of two replicates pooled from four independent experiments (*p<0.05).

1.4. WASp and N-WASp are involved in NKG2D-mediated NK cell migration

WASp-family proteins activate the Arp2/3 complex, leading to the formation of F-actin structures that are necessary in several cellular processes, including cell migration. Because wiskostatin selectively inhibits N-WASp, it was therefore investigated whether this protein was involved in the inhibition of NKG2D-mediated migration. N-WASp was knocked down using siRNA. The expression of the homolog WASp was additionally checked to rule out that silencing N-WASp was affecting its levels (Figure IV.8A). CXCL12-induced chemotaxis assays were then performed after activation. Depletion of N-WASp in NKL cells partially rescued CXCL12-induced migration, as the percentage of inhibition decreased from 26.99% in cells transfected with control siRNA to 11.29% in cells transfected with N-WASp siRNA (*$p<0.05$; Figure IV.8B).

As mentioned above, the chemical inhibitor wiskostatin is selective for N-WASp, but could also interfere in WASp activity. In addition, in human NK cells WASp is required for cytotoxic activity and the formation of the IS (Orange *et al.* 2002). For this reason, a similar approach was performed silencing WASp with a WASp siRNA and confirming that depletion of the protein was not affecting N-WASp (Figure IV.9A). Under control conditions, cells transfected with siRNA for WASp showed a dramatic reduction in CXCL12-induced chemotaxis (*$p<0.05$; Figure IV.9B), in agreement with Stabile *et al.* (2010). However, activation of NKG2D in transfected cells did not impair cell migration compared with cells transfected with scrambled siRNA (2% of inhibition), indicating that both N-WASp and WASp proteins are involved in the regulation of NK cell migration upon NKG2D stimulation.

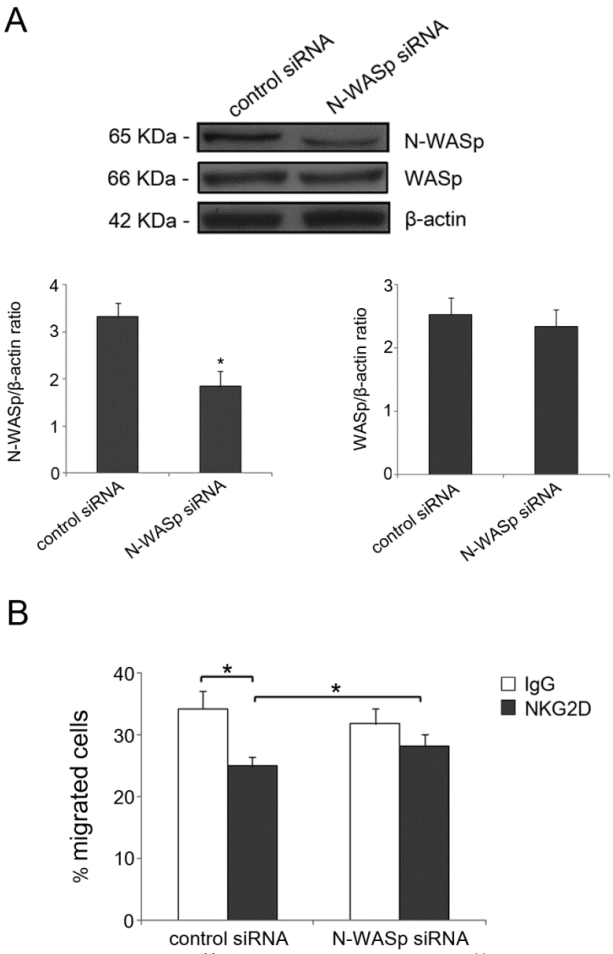


Figure IV.8. Role of N-WASp in NK cell migration. (A) NKL cells were transfected with siRNA targeting N-WASp or a siRNA control. Cells were lysed and N-WASp and WASp expression levels were analyzed by western blotting. Anti-ß-actin blots were included as protein loading controls. **(B)** Twenty four hours after transfection, cells were incubated with anti-NKG2D or IgG and their ability to migrate was assayed. Data shown are the mean + SEM of two replicates pooled from three independent experiments (*$p<0.05$).

Given the essential role of N-WASp and WASp in actin cytoskeleton reorganization, changes in F-actin content upon NKG2D cross-linking in the presence of the inhibitors and in N-WASp- and WASp-deficient cells were explored. Human NK cells were pretreated with NSC23766 or wiskostatin prior to receptor activation. Inhibition of Rac showed no significant changes in the F-actin content compared with untreated cells (Figure IV.10A). In fact, NKG2D

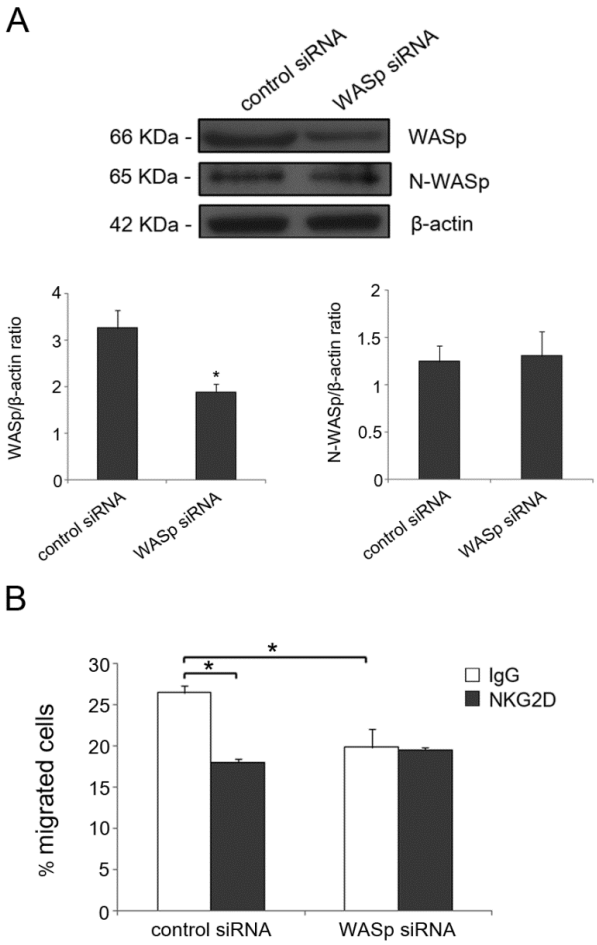


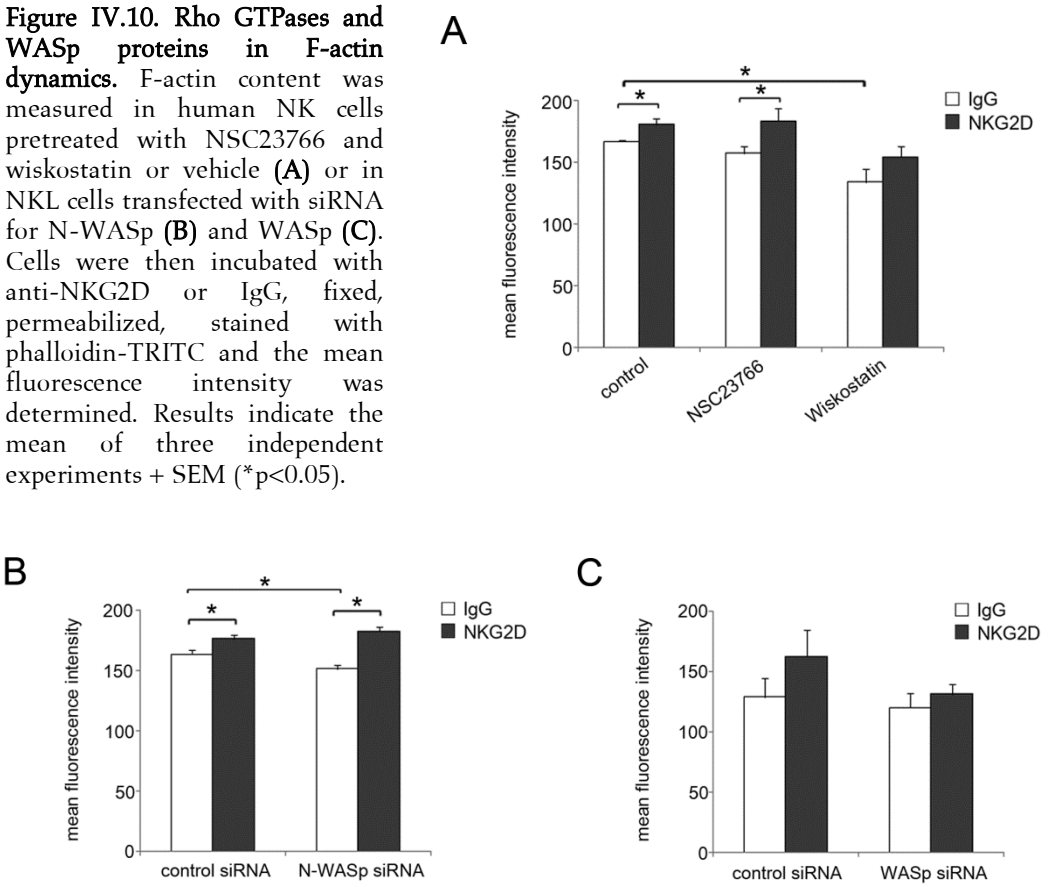
Figure IV.9. Role of WASp in NK cell migration. (A) NKL cells were transfected with siRNA targeting WASp or a siRNA control. Cells were lysed and WASp and N-WASp expression levels were analyzed by western blotting. Anti-ß-actin blots were included as protein loading controls. **(B)** Twenty four hours after transfection, cells were incubated with anti-NKG2D or IgG and their ability to migrate was assayed. Data shown are the mean + SEM of two replicates pooled from three independent experiments (*p<0.05).

crosslinking increased the amount of F-actin (*p<0.05). In contrast, wiskostatin pretreatment reduced F-actin levels under control conditions (IgG) in comparison with untreated cells (*p<0.05), and a decrease in NKG2D-treated cells was observed although not statistically significant. F-actin levels also declined in NKL cells transfected with siRNA for N-WASp under control conditions, while NKG2D activation still increased the amount of F-actin (*p<0.05; Figure IV.10B). Knockdown of WASp showed a decrease in F-actin

levels in both IgG- and NKG2D-treated cells, although it did not reach statistical significance (Figure IV.10C). Therefore, a lack of correlation between the effect of NKG2D activation on F-actin content and on cell chemotaxis was observed in knock-down experiments.

Altogether, these data indicated that WASp and N-WASp are involved in the inhibition of NK cell migration upon NKG2D activation, but these effects are not dependent on the regulation of actin dynamics by the Cdc42/WASp/Arp2/3 pathway.

Figure IV.10. Rho GTPases and WASp proteins in F-actin dynamics. F-actin content was measured in human NK cells pretreated with NSC23766 and wiskostatin or vehicle **(A)** or in NKL cells transfected with siRNA for N-WASp **(B)** and WASp **(C)**. Cells were then incubated with anti-NKG2D or IgG, fixed, permeabilized, stained with phalloidin-TRITC and the mean fluorescence intensity was determined. Results indicate the mean of three independent experiments + SEM (*p<0.05).



2. ROLE OF NKG2D AS A COSTIMULATORY MOLECULE IN CD8+ T CELL MIGRATION

2.1. Costimulation of CD8+ T cells via NKG2D inhibits cell migration

The activation state or the microenvironment determines NKG2D-mediated responses in effector cells. Responses are enhanced in the presence of IL-12, IL-18 or IL-2 in NK cells and CD8+ T cells (Verneris *et al.*, 2004; Markiewicz *et al.*, 2005; Ortaldo *et al.*, 2006). Analogous to human NK cells, human CD8+ T cells were stimulated after isolation from peripheral blood with IL-2 for 48 h. Expression patterns of CD28, NKG2D, the chemokine receptor, CXCR4, and the integrin CD49d were analyzed by flow cytometry after isolation (t = 0 h) and before chemotaxis assays (t = 48 h), and are shown in Table IV.1. A representative histogram for each protein is shown at t = 0 and at t = 48 h, indicating the MFI (Figure IV.11). The percentage of cells expressing CD28 and NKG2D was similar, and showed no changes after 48 h. However, stimulation with IL-2 for 48 h upregulated the surface expression of the two receptors, as indicated by the higher MFI of the molecules after 48 h. Similar results were found when analyzing the percentage of cells expressing CXCR4 and CD49d and their respective levels on the cell surface.

After the activation of CD8+ T cells with IL-2, chemotaxis assays towards CXCL12 were performed. Cells were stimulated with anti-CD3-, anti-CD28-, and anti-NKG2D-coated surfaces, alone or in combination for 30 min. Cells were then removed from the plates and added to fibronectin-coated or uncoated transwell filters. The same number of cells was added to wells without insert for the analysis of initial cells. They were then analyzed by flow cytometry to estimate the number of initial and migrated cells. Dead cells were

	t=0h	t=48h	p-value
%CD28+	79.81 ± 3.16	78.29 ± 3.30	0.110
%NKG2D	85.80 ± 1.67	90.72 ± 1.43	0.073
%CXCR4	77.92 ± 3.25	85.73 ± 2.87	0.072
%CD49d	95.74 ± 1.08	97.05 ± 0.89	0.074
MFI CD28	128.74 ± 20.16	177.80 ± 21.59	0.023
MFI NKG2D	93.34 ± 6.54	162.96 ± 6.40	0.0007
MFI CXCR4	98.85 ± 11.86	398.26 ± 28.22	0.0002
MFI CD49d	250.74 ± 19.45	438.09 ± 16.24	0.0003

Table IV.1. CD28, NKG2D, CXCR4 and CD49d in CD8+ T cells. Percentages and MFI for each molecule after isolation of CD8+ T cells (t = 0 h) and after 48 h of stimulation with IL-2 (t = 48 h). Data shown are the mean ± SEM of four independent experiments.

excluded from the analysis using 7-AAD to identify the viable cells. Migration rates were calculated relative to total number of CD8+ T cells. The analysis of the variance showed that CD8+ T cell chemotaxis through fibronectin was differentially affected depending on the treatment (Figure IV.12A; $p<0.001$). Post hoc comparisons using the Student-Newman-Keuls test revealed that CD3-stimulated cells showed a decreased chemotactic response. Chemotaxis of CD28-activated cells was no different from that under control conditions (IgG), whereas CD28-costimulation further inhibited cell migration compared with IgG- and CD3-activated cells. Similarly, engagement of NKG2D alone had no effect on migration rates, whilst CD3/NKG2D-activation impaired cell motility relative to control-treated cells and CD3-treated cells. Both costimulatory molecules equally impaired cell migration, as no significant differences between their migration rates were found. Similar results were found when chemotaxis assays in the absence of fibronectin were performed (Figure IV.12B). It is worth noting that neither NKG2D or CD28 crosslinking alone nor costimulation via NKG2D or via CD28 was sufficient to inhibit CD8+ T cell CXCL12-induced chemotaxis when cells were cultured in the absence of IL-2, as migration rates were similar to that under control conditions (Figure IV.13A-B).

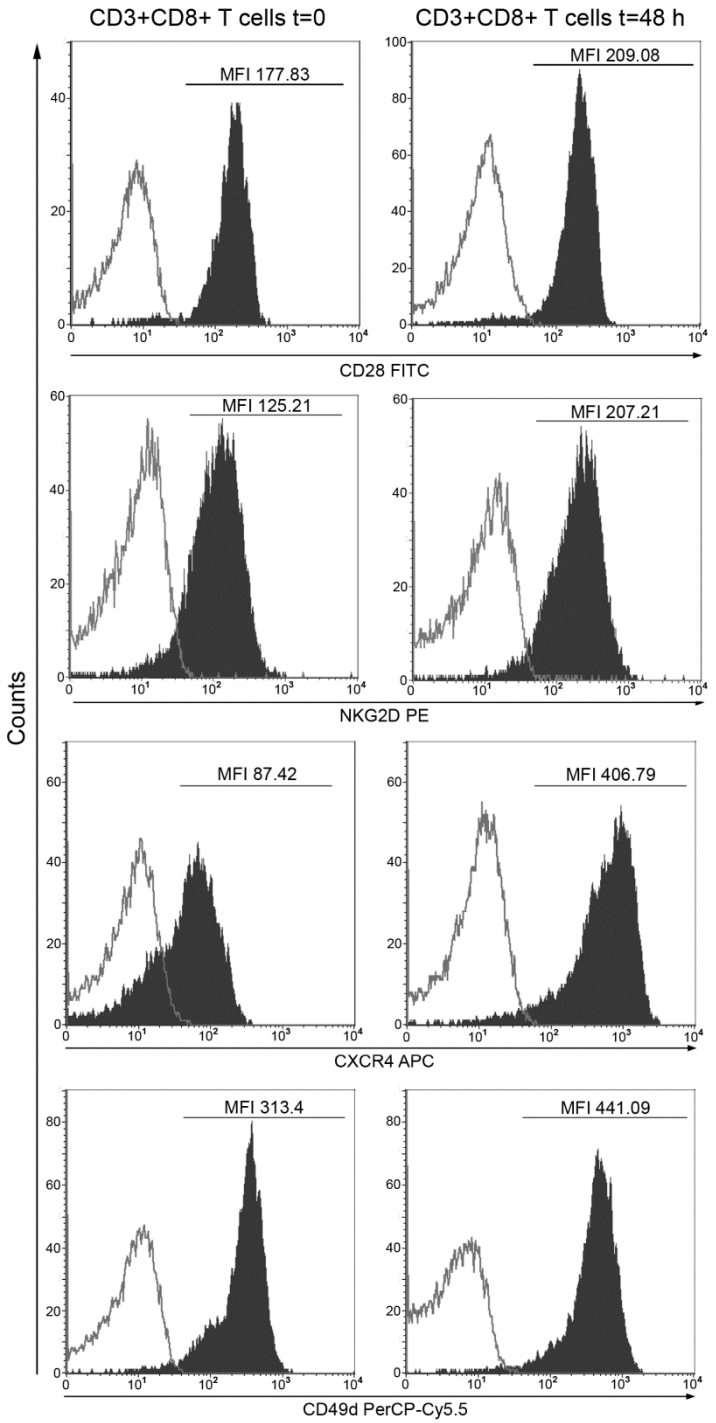


Figure IV.11. Flow cytometry analysis of CD28, NKG2D, CXCR4 and CD49d expression. CD8+ T cells were incubated with the indicated mAbs (filled histograms) or isotypic mAbs (unfilled histograms) for each fluorochrome. A representative histogram for each protein is shown at t = 0 h and t = 48 h, indicating the MFI.

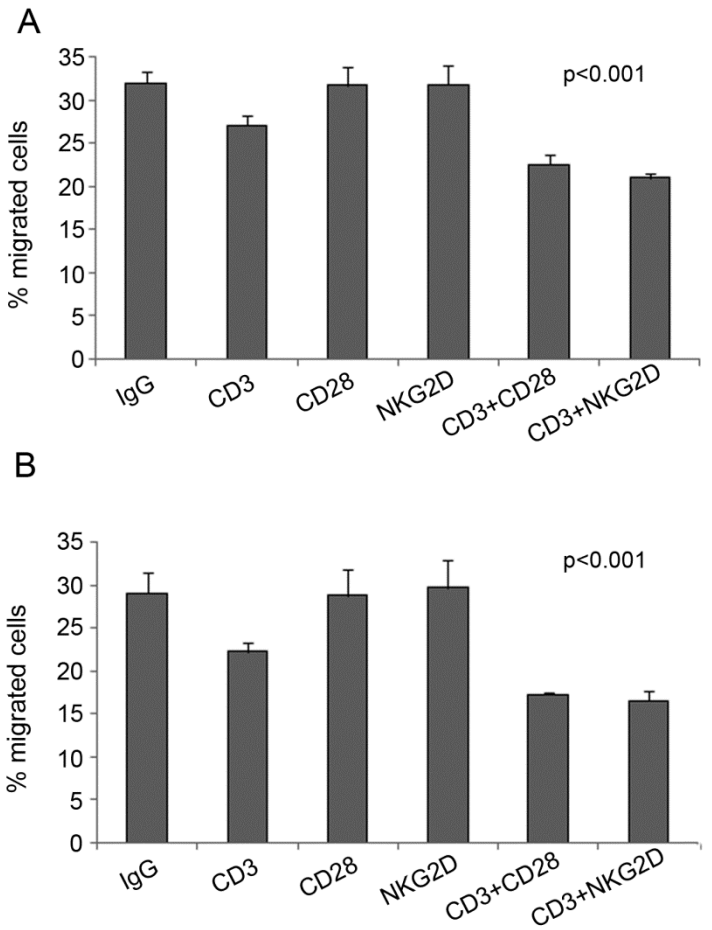


Figure IV.12. Effect of CD28- and NKG2D-mediated costimulation on CD8+ T cell migration. CD8+ T cells were activated with the indicated plate-bound antibodies for 30 min. The migratory ability of the cells toward a CXCL12 gradient was then assayed in **(A)** fibronectin-coated or uncoated **(B)** transwell inserts. Migrated cells were collected after 2 h, counted and analyzed by flow cytometry. Results shown are the mean + SEM of two replicates pooled from four independent experiments. Statistical analysis was performed using a GLM and Student-Newman-Keuls post hoc test. Statistical significance for the effect of receptor activation on cell chemotaxis: $p < 0.001$.

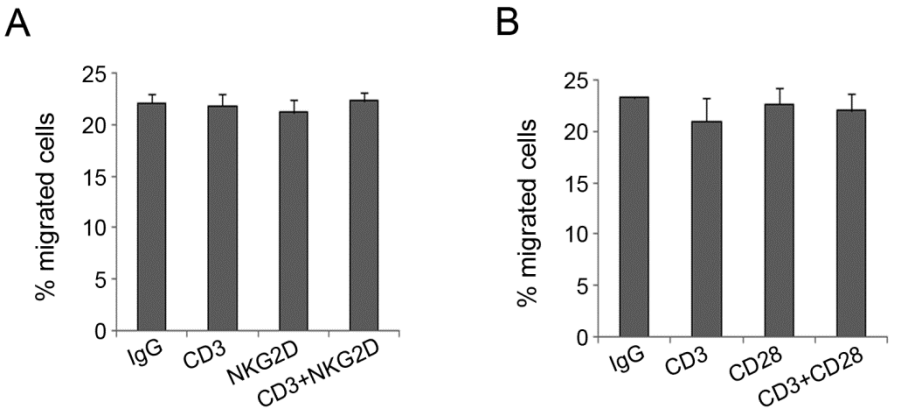


Figure IV.13. NKG2D- and CD28-mediated costimulation in chemotaxis of unstimulated CD8+ T cells. The effect of NKG2D-mediated costimulation **(A)** or CD28-mediated costimulation **(B)** on cell migration was studied in human CD8+ T cells cultured for 48 h under basal conditions (*i.e.,* without IL-2). Results shown are the mean + SEM of two replicates pooled from four **(A)** and three **(B)** independent experiments.

Analogous to NK cells, adhesion of CD8+ T cells to fibronectin was examined. The ability of the cells to adhere to the substrate was not affected by the treatments, except in cells activated with CD3/NKG2D, in which adhesion of CD8+ T cells decreased (p=0.002; Figure IV.14).

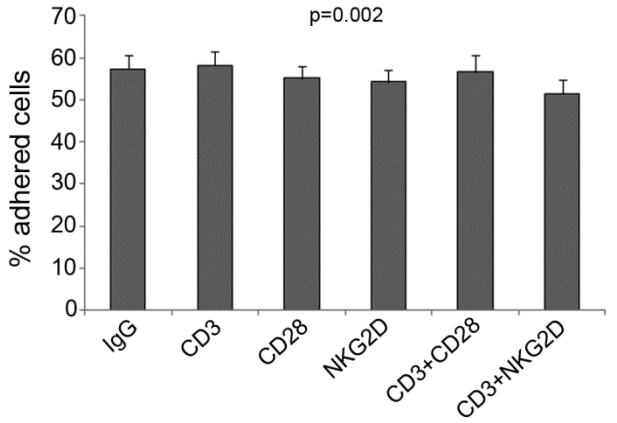


Figure IV.14. CD8+ T cell adhesion to fibronectin. BCECF-labeled CD8+ T cells were plated on fibronectin-coated wells after receptor activation and adhesion was determined after 1.5 h. Percentage of adhesion was estimated relative to the total number of plated cells. Results shown represent the mean + SEM of three replicates pooled from four independent experiments Statistical analysis was performed using a GLM and Student-Newman-Keuls post hoc test. Statistical significance for the effect of receptor activation on cell adhesion: p = 0.002.

2.2. Costimulation via NKG2D, but not via CD28, activates Cdc42

Activation of T cells, cell motility towards chemotactic cues and immunological synapse formation comprise a complex remodeling of the actin cytoskeleton (Gómez and Billadeau, 2008). Rho GTPases play a key role in the regulation of these processes. As outlined above, Rac1, RhoA and Cdc42 are the best characterized members in T cells. However, a comparative study between CD28 and NKG2D in the activity of Rho GTPases in cell migration has not been fully addressed. Since the activities of Rac1 and Cdc42 augmented upon NKG2D stimulation in NK cells and Cdc42 was found to be essential in

NKG2D-mediated cell migration, it was therefore examined whether CD3/CD28 and CD3/NKG2D-mediated signaling could differentially modulate the activity of these Rho GTPases by analyzing the effects of CD28 and NKG2D costimulation on Rac1 and Cdc42 activities. Active Rac1 and Cdc42 concentrations were determined by G-LISA following stimulation of CD8+ T cells (Figure IV.15).

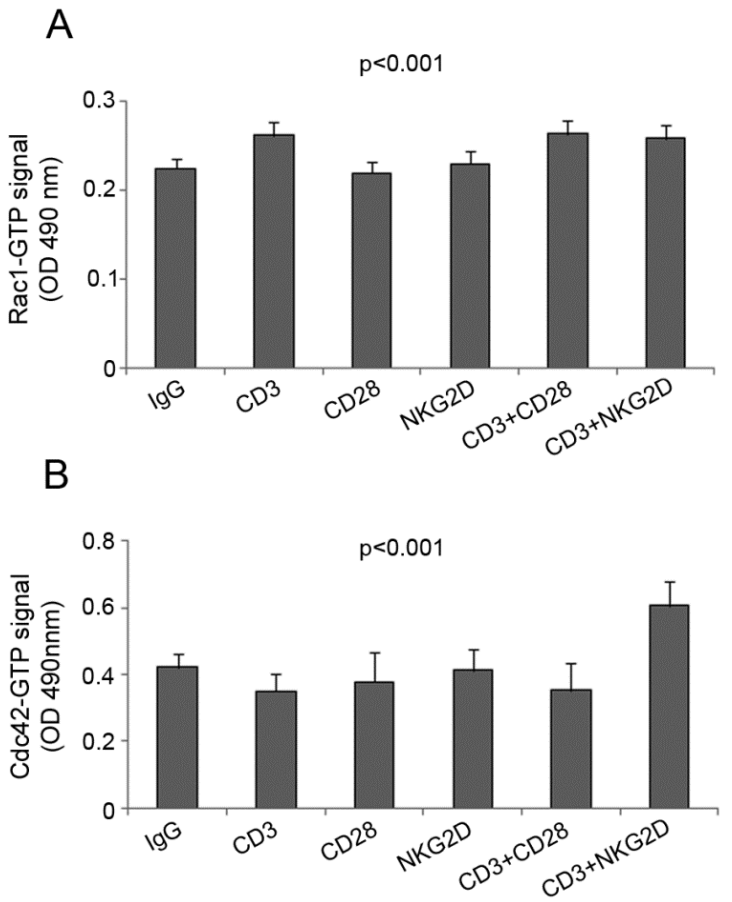


Figure IV.15. Rac1 and Cdc42 activities in CD8+ T cells. Activation of the Rho GTPases Rac1 **(A)** and Cdc42 **(B)** upon stimulation of CD8+ T cells was analyzed by G-LISA assays. Cells were activated with the indicated antibodies and lysed. Cell lysates were then subjected to the G-LISA assays according to the manufacturer's instructions and the GTP-bound Rac1 and Cdc42 was quantified. Data given are the mean + SEM (Rac1: n=4; Cdc42: n=3). Statistical analyses were performed using a GLM and Student-Newman-Keuls post hoc test (effect of the treatment on Rac1 and Cdc42 activities: $p<0.001$)

The activity of both Rho GTPases changed in response to some of the treatments ($p<0.001$). A post hoc Student-Newman-Keuls test showed that stimulation of the cells through CD3 induced Rac1 activation (Figure IV.15A), consistent with previous reports (Cernuda-Morollón *et al.*, 2010). Activation of CD8+ T cells through CD28 or NKG2D receptors along with the TcR equally induced Rac1 activation, as same levels of GTP-bound active form were found. In contrast, Cdc42 was activated strictly upon stimulation through CD3 plus NKG2D ($p<0.001$), as no change in Cdc42 activity was observed under other experimental conditions (Figure IV.15B).

Although both costimulatory molecules impair CD8+ T cell chemotaxis, these data suggest that they induce distinct signaling pathways through the activation of different Rho GTPases.

2.3. Knockdown of N-WASp expression rescues CD8+ T cell migration upon NKG2D costimulation

As mentioned above, the WASp family of proteins, which includes WASp, N-WASp and WAVE/Scar1, 2 and 3, is a key regulator of cytoskeleton dynamics and cell migration (Takenawa and Miki, 2001). WASp is expressed exclusively in leukocytes, while N-WASp is ubiquitously expressed. In T cells, the role of WASp has been thoroughly described in F-actin reorganization following TcR ligation (Thrasher, 2002), and its deficiency is known to impair leukocyte migration (Snapper *et al.*, 2005). However, the possibility that N-WASp may have a redundant or a complementary role in T cell migration, or that it might be involved in the CD3/NKG2D-mediated migration of CD8+ T cells has not been fully explored. Therefore, it was investigated whether the disruption of Cdc42/N-WASp interaction affected CD3/NKG2D-induced chemotaxis. To this end, expression of N-WASp was silenced by an N-WASp

siRNA in CD8+ T cells, then checked that WASp expression was not affected (Figure IV.16A). CXCL12-induced chemotaxis assays were then performed after receptor activation. Activation of CD28 or NKG2D alone were not performed in these experiments, since these conditions showed no significant effects neither in migration and adhesion assays nor in Rho GTPase activity assays. In agreement with the results found in section 2.1., CD3 activation impaired cell migration, whereas CD28- and NKG2D-mediated costimulation further decreased migration rates compared with the control condition (IgG) (Figure IV.16B). The inhibitory effect of costimulation via CD28 and via NKG2D compared with CD3 activation alone was also significant.

Reduction of N-WASp levels affected CD8+ T cell chemotaxis in a different manner depending on the experimental condition ($p<0.001$). The post hoc analysis revealed that knockdown of N-WASp dramatically impaired CD8+ T cell chemotaxis of control-treated cells, whereas it did not alter CD3-mediated migration, which was similar to that found in cells transfected with control siRNA. N-WASp depletion did not affect CD8+ T cell chemotaxis upon CD3/CD28 activation either. In contrast, CD3/NKG2D-mediated CD8+ T cell chemotaxis was significantly higher in comparison with the control condition and with costimulation via CD28. Therefore, the partial depletion of N-WASp differentially affects CD3/CD28- and CD3/NKG2D-mediated chemotaxis.

N-WASp is an activator of the Arp2/3 complex, which mediates actin polymerization (Weaver *et al.*, 2003). Therefore, the effect of knocking down N-WASp on the cytoskeleton dynamics by measuring the F-actin content in activated CD8+ T cells was examined by flow cytometry (Figure IV.16C). Differences in the F-actin content were observed in control siRNA transfected cells ($p=0.002$). The post hoc test showed that CD3 stimulation resulted in an increase in F-actin levels, as described in previous studies (Parsey and Lewis,

1993; Bunnell *et al.*, 2001). Costimulation of CD8+ T cells through CD28 or NKG2D led to a decrease in the F-actin content. In contrast, partial depletion of N-WASp was not sufficient to significantly affect F-actin content, although there was a tendency of F-actin levels to decrease in all the conditions compared with cells transfected with control siRNA.

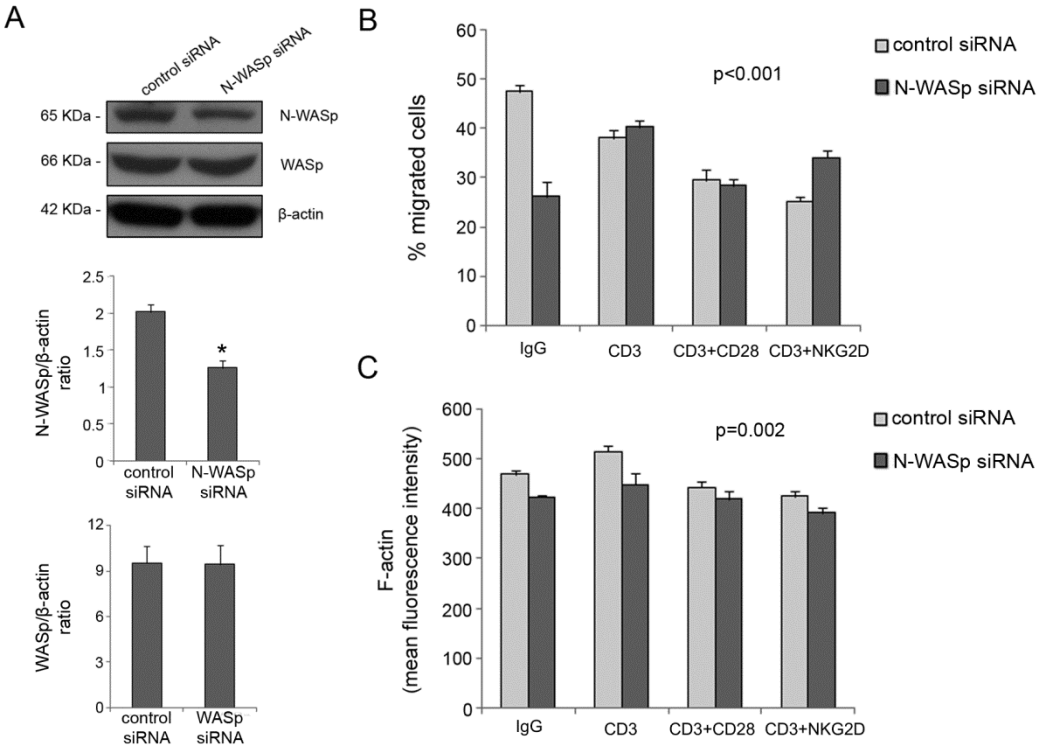


Figure IV.16. Knockdown of N-WASp in CD8+ T cell migration. (A) Cell lysates from CD8+ T cells transfected with control siRNA or N-WASp siRNA were prepared and immunoblotted for N-WASp, WASp and ß-actin as loading control. Representative blots of three independent experiments are shown. Graphs indicate the N-WASp/β-actin or WASp/β-actin ratio (*p<0.05). **(B)** Transfected cells were activated with the indicated antibodies and their ability to migrate was assayed. Data are given as mean + SEM of two replicates pooled from three independent experiments. The effect of N-WASp depletion was analyzed using a GLM and Student-Newman-Keuls post hoc test ($p < 0.001$).**(C)** Levels of F-actin content in transfected CD8+ T cells were measured by flow cytometry after activation. Cells were fixed, permeabilized, and stained with phalloidin-TRITC. The mean fluorescence intensity was determined. Results indicate the mean of three independent experiments + SEM. Data were analyzed using a GLM and Student-Newman-Keuls post hoc test (treatment effect for control siRNA, $p = 0.002$).

2.4. Differential cofilin phosphorylation in CD3/CD28 *vs.* CD3/NKG2D signaling

Despite the extensive knowledge of the signaling pathways triggered by TcR stimulation, differences between CD3/CD28 and CD3/NKG2D signaling are not well understood. Given that Cdc42 is specifically activated upon NKG2D-mediated costimulation and depletion of N-WASp abolishes CD3/NKG2D-mediated inhibition of cell migration, other downstream molecules that could give rise to other significant differences between both costimulatory receptors were considered. To this end, the N-WASp inhibitor wiskostatin was employed. CD8+ T cells were pretreated with 1 μM wiskostatin or vehicle prior to stimulation. Cells were lysed and proteins involved in TcR signaling and actin cytoskeleton dynamics were analyzed by western blot (Figure IV.17).

Vav is a GEF protein that activates several biochemical pathways downstream the TcR and cytoskeleton-dependent processes (Tybulewicz, 2005). Vav is known to activate both Rac1 and Cdc42. CD3 stimulation and CD3 costimulation through CD28 and NKG2D phosphorylated Vav relative to control conditions (Figure IV.18A). However, phosphorylation levels remained unchanged after pretreatment with wiskostatin.

ERK pathway is one of the most important mitogen-activated protein kinase (MAPK) pathways in the regulation of T-cell activation. It is activated by Ras GTPase and Raf upon TcR stimulation. Under our experimental conditions, an increase in the phosphorylation of the MAPK ERK1/2 following CD3 activation was observed (Figure IV.18B). CD3/CD28 and CD3/NKG2D-mediated costimulation equally promoted ERK1/2 phosphorylation. Administration of wiskostatin and subsequent T cell stimulation or

costimulation did not affect phosphorylation levels of ERK1/2, as no significant differences were detected.

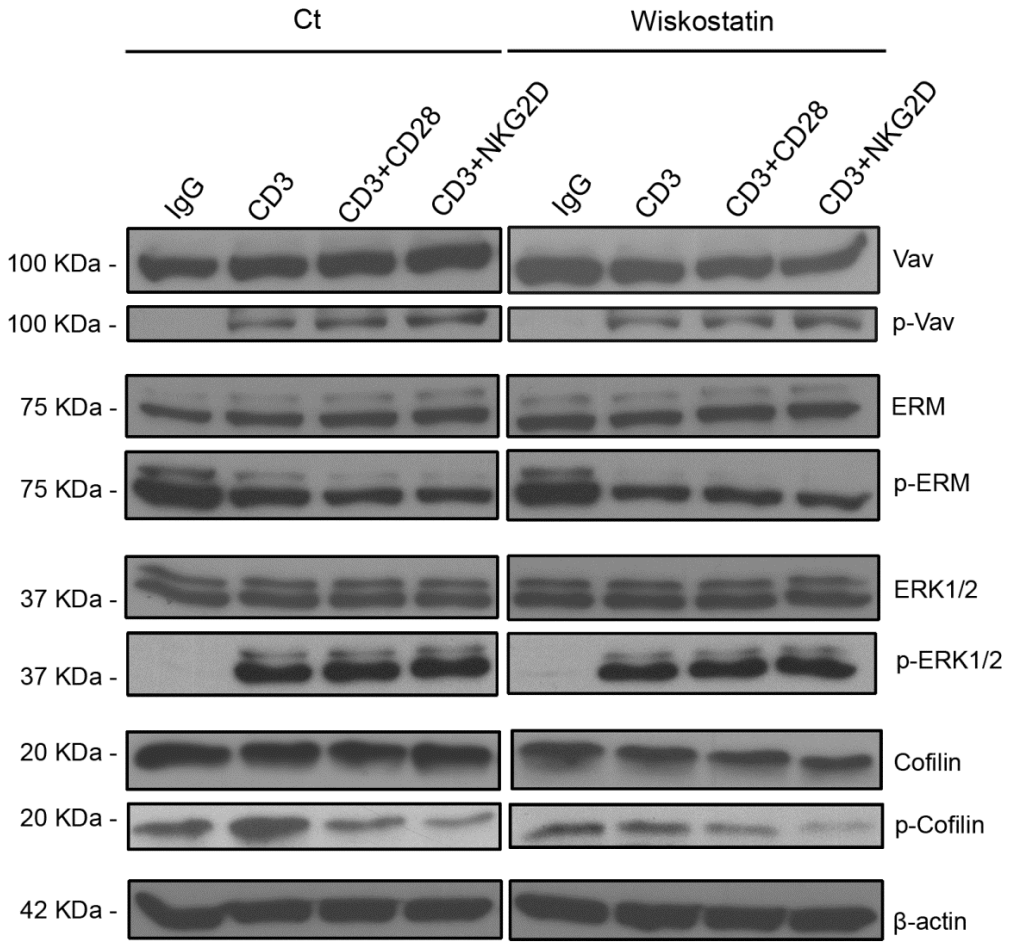


Figure IV.17. Determination of phosphorylation levels of Vav, ERM, ERK and cofilin by western blot. CD8+ T cells cultured for 48 h in the presence of IL-2 were preincubated with the N-WASp inhibitor wiskostatin (1μM) or vehicle. Subsequently, cells were stimulated with plate-bound antibodies against CD3, CD28 or NKG2D alone, or in combination and with an isotype control (IgG). Then cells were lysed and the phosphorylation states of Vav, ERK1/2, ERM and cofilin were analyzed by western blot. Immunoblot with actin was included as a loading control.

Ezrin-radixin-moesin (ERM) proteins act as linkers between TcR signaling and actin cytoskeleton. Rho GTPases are known to regulate ERM activity in the establishment of cell polarity in migrating lymphocytes or

during cell-cell contact (Ivetic and Ridley, 2004). TcR engagement results in a reduction of ERM proteins phosphorylation through Rac (Faure *et al.*, 2004; Cernuda-Morollón *et al.* 2010). Consistent with these studies, phosphorylation levels of ERM proteins dramatically decreased in response to CD3 stimulation and CD28 or NKG2D crosslinking (Figure IV.18C), whilst N-WASp inhibition by wiskostatin did not alter the phosphorylation status of ERM proteins.

Although Vav, ERK1/2 and ERM proteins are regulated by CD3-mediated signaling, costimulation via CD28 or NKG2D did not modify phosphorylation levels of these proteins. Regulation of their activity is not mediated by Cdc42 either, as wiskostatin pretreatment did not affect phosphorylation levels.

Cofilin is an actin-binding protein that controls both F-actin depolymerization and formation of new actin filaments. Costimulation of the TcR together with CD28, but not TcR triggering alone, induces dephosphorylation of cofilin, resulting in its activation (Lee *et al.*, 2000). We found that cofilin undergoes dephosphorylation not only after costimulation via CD28 but also via NKG2D (Figure IV.18D). Administration of wiskostatin affected the phosphorylation status of cofilin upon CD3/CD28 and CD3/NKG2D activation ($p<0.001$). Moreover, post hoc comparisons revealed that the decrease was even more severe after CD3/NKG2D engagement than after CD3/CD28 activation.

In summary, these results suggest that both costimulatory molecules regulate CD8+ T cell chemotaxis but through different signaling pathways, since costimulation via NKG2D preferentially activated Cdc42/N-WASp axis. Moreover, costimulation mediated via NKG2D differentially regulated actin cytoskeleton dynamics through N-WASp and cofilin.

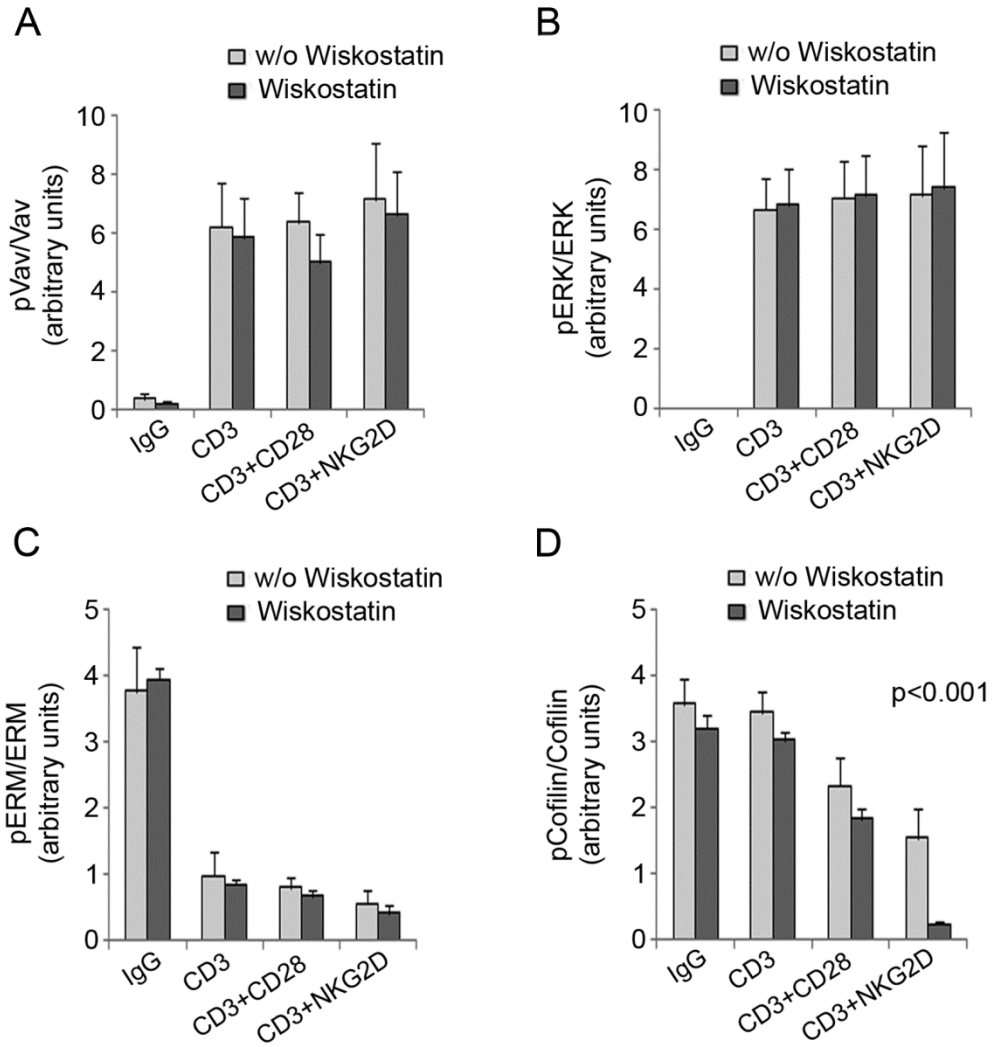


Figure IV.18. Phosphorylation levels of Vav, ERK1/2, ERM and cofilin. Densitometry was used to determine levels of pVav **(A)**, pERK1/2 **(B)**, pERM **(C)** and pCofilin **(D)**. Data are expressed as phosphorylated protein:total protein ratio. Graphs show the mean + SEM of five independent experiments. The effect of wiskostatin upon receptor activation was analyzed using a GLM and Student-Newman-Keuls post hoc test ($p < 0.001$).

3. ROLE OF LIPID RAFTS IN NKG2D-MEDIATED SIGNALING

3.1. NKG2D-DAP10 complex is recruited to DRMs upon ligation

Signaling downstream the NKG2D receptor involves the phosphorylation of the YINM motif of the adaptor protein DAP10. This process is analogous to phosphorylation of the ITAM-containing receptors by Src kinases present in membrane rafts. Thus, it was considered of interest to gain insight into the function of these structures in NKG2D-mediated signaling in NK cells.

One of the characteristic features of membrane rafts is their insolubility in non-ionic detergents. It was necessary first to verify that NKG2D is localized in lipid rafts. To this end, NKL cells were stimulated with 10 µg/mL anti-NKG2D Ab in medium for 30 min on ice, then 2 µg/mL goat anti-mouse Ab was added and cells were transferred to 37ºC for 5 min. Cells were then solubilized in lysis buffer containing 1% Brij-98 and fractions were separated in a sucrose density gradient (Figure IV.19). Ab-mediated crosslinking induced the recruitment of the NKG2D receptor into rafts, as it is recovered in the low-density DRM fractions, whereas under control conditions the majority NKG2D is located in the lowest fractions of the gradient. The adaptor protein DAP10 also associated with DRMs after NKG2D activation. Moreover, the phosphorylated form of Vav (pVav), which regulates actin polymerization and clustering of NKG2D (Endt *et al.*, 2007), was found in the low density fractions upon NKG2D activation. In contrast, only a small fraction of pVav was detected under control conditions.

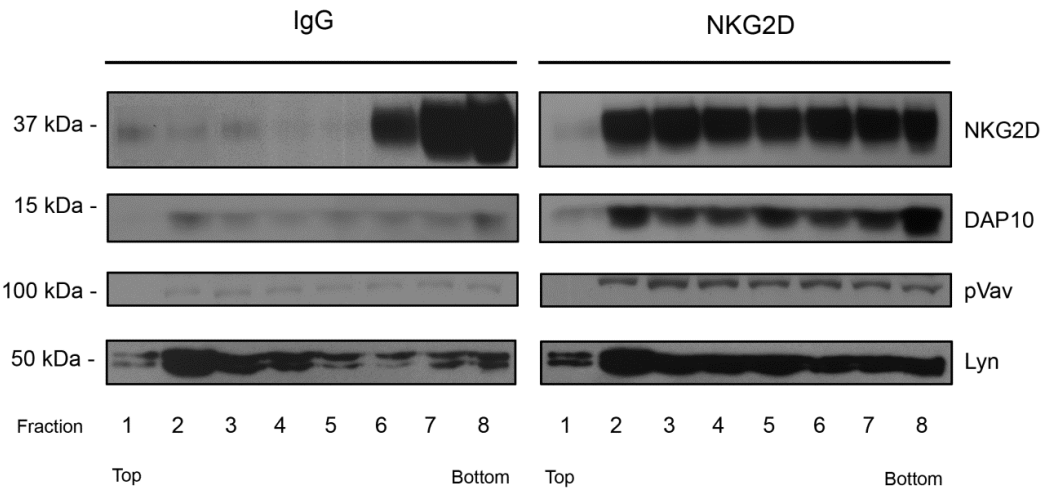


Figure IV.19. Activation-induced recruitment of the NKG2D-DAP10 complex and pVav to DRMs. NKL cells were stimulated by anti-NKG2D crosslinking or an isotype control (IgG) for 5 min at 37ºC. Cells were lysed and DRM fractions were isolated by sucrose gradient ultracentrifugation. Fractions were analyzed by western blotting using the indicated antibodies, and Lyn served as control for membrane fraction.

Furthermore, similar results were obtained in gel filtration chromatography experiments. This technique allows the separation of molecules or complexes in solution according to differences in their size as they pass through a column containing a gel matrix. Therefore, the largest protein complexes move down the column first, followed by the smaller ones. NKL cells were stimulated and lysed as indicated above. The lysates were separated on a Sepharose 4B column. Collected fractions were then analyzed by western blot. Both NKG2D-DAP10 complex and pVav were recovered in large detergent-resistant complexes upon lysis in Brij98 (Figure IV.20).

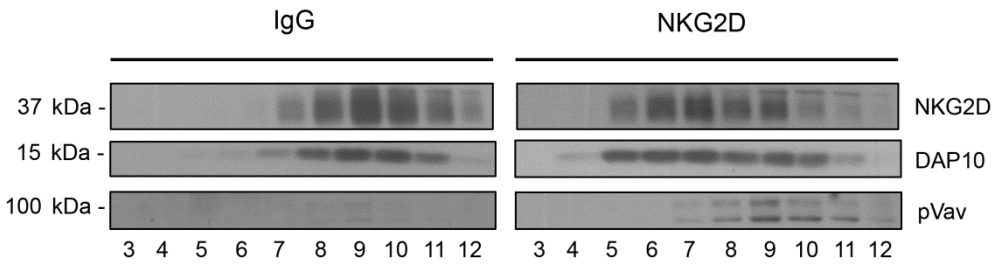


Figure IV.20. Gel filtration experiments upon NKG2D cross-linking. NKL cell lysates after NKG2D activation were fractionated by gel filtration chromatography on Sepharose 4B and the samples were analyzed for the distribution of NKG2D, DAP10 and pVav by western blotting.

3.2. Proteomic analysis of DRMs upon NKG2D activation

In the last years proteomic approaches (qualitative and quantitative) have contributed to a better understanding of the proteins involved in membrane rafts, clustering and signaling. Several investigations have addressed the protein composition of lipid rafts in different cell lines and primary cells (Foster and Chan, 2007). However, the characterization of the raft proteome in NK cells has not been fully studied. Therefore, as an initial approach, a qualitative raft proteomic study was carried out to identify which proteins are recruited to rafts upon NKG2D activation.

Proteins of the low density DRM fractions were precipitated and separated by SDS-PAGE. After coomassie staining, 10 bands were excised for subsequent in-gel digestion and analysis by LC-MS/MS. A selection of the most relevant proteins identified in three independent experiments under control conditions and after NKG2D crosslinking is shown in Table IV.2, indicating the molecular weight, score, number of queries matched, and the percentage of the identified protein. Annexin A2, a protein that participates in cytoskeleton remodeling (Gerke *et al.*, 2005), and actin-binding proteins, including cofilin, profilin and plastin-2 were detected. Rho GDI proteins, which regulate the GDP/GTP exchange reaction of the Rho GTPases (Ridley, 2001) have also been identified. The presence of some of the proteins was confirmed by western blot (Figure IV.21).

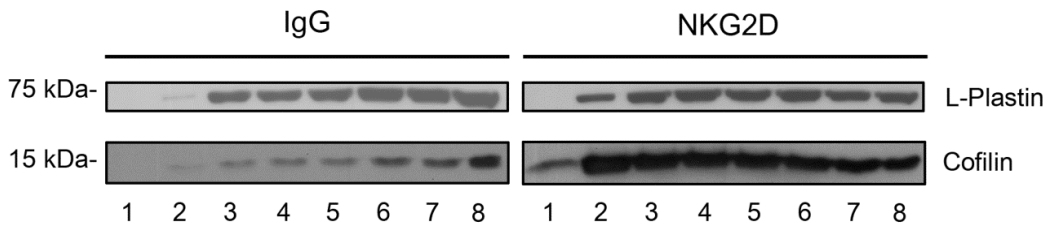


Figure IV.21. Western blot analysis of sucrose density gradient fractions. L-plastin and cofilin were identified by mass spectrometry and detected by western blotting.

IgG

Code	Protein name	Molecular weight	Score	Queries matched	% protein
P60709	Actin, cytoplasmic 1	41.737	325	11	34
P23528	Cofilin-1	18.502	59	2	18
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36.053	199	7	22
P13796	Plastin-2	70.289	124	4	12
Q71U36	Tubulin alpha-1A chain	50.136	265	11	37
P07347	Tubulin beta chain	49.671	332	13	35

NKG2D

Code	Protein name	Molecular weight	Score	Queries matched	% protein
P60709	Actin, cytoplasmic 1	41.737	472	13	37
P07355	Annexin A2	38.604	62	3	10
P27797	Calreticulin	48.182	105	4	14
P23528	Cofilin-1	18.502	373	8	42
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36.053	214	8	28
P13796	Plastin-2	70.289	620	21	37
P07737	Profilin-1	15.054	74	2	20
P52565	Rho GDP-dissociation inhibitor 1	23.207	42	2	11
P52566	Rho GDP-dissociation inhibitor 2	22.988	50	2	19
Q71U36	Tubulin alpha-1A chain	50.136	265	11	37
P07347	Tubulin beta chain	49.671	332	13	35

Table IV.2. Identified proteins in DRMs isolated by sucrose gradient ultracentrifugation.

Proteins related to the actin cytoskeleton comprise the biggest category of proteins identified in the DRM fractions, which suggests that the cytoskeleton may be important for organizing membrane domains upon NKG2D activation. Therefore, to investigate the rafts associated with the cytoskeleton further, raft fractionation experiments involving β-OG selective solubility were performed. Octyl-glucoside is a nonionic detergent that

efficiently dissolve DRMs but does not disrupt protein associations with the cytoskeleton (Miettinen and Jalkanen, 1994). NKL cells were stimulated and lysed and rafts were solubilized with 0.2% Triton X-100 plus 60 mM β-OG at 37ºC. Proteins of the isolated fractions were separated by SDS-PAGE and then digested as indicated above. A selection of the proteins identified in the raft fractions of three independent experiments is shown in Table IV.3. Some of them were previously found in the DRM fractions, *i.e.* annexin A2, cofilin, profilin and plastin-2. In addition, other proteins that regulate cytoskeleton dynamics were found. Moesin is a member of the ERM protein family, and ERM proteins, as previously mentioned, act as linkers between the cytoskeleton and the plasma membrane. The protein IQGAP1 is a key regulator of the cytoskeleton involved in the formation of actin filament structures, such as lamellipodia and membrane ruffling, and binds to activated Cdc42 (Brandt and Grosse, 2007). Alpha-actinin 4 and coronin 1A are actin-binding proteins that are also crucial in a variety of intracellular structures, including those required in cell locomotion. The Rho GTPase RhoA was also identified. Some of the proteins were detected by western blot, as in DRM proteomic analysis (Figure IV.22).

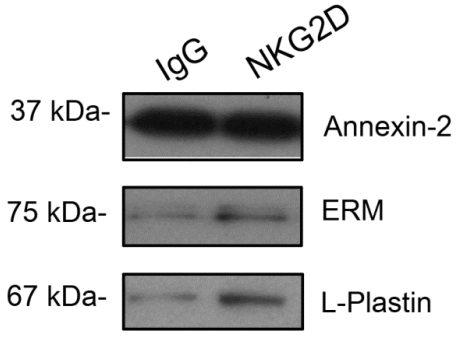


Figure IV.22. Western blot analysis of DRM fractions isolated by β-OG selective solubility. Annexin-2, ERM proteins and L-plastin were identified by mass spectrometry and detected by western blotting.

IgG

Code	Protein name	Molecular weight	Score	Queries matched	% protein
P60709	Annexin A2	38.604	285	12	41
P23528	Coronin-1A	51.026	117	5	16
P04406	Galectin-1	14.716	68	1	11
P13796	Plastin-2	70.289	178	8	16
P07737	Profilin-1	15.054	93	3	31
P07347	Tubulin beta chain	49.671	88	8	20
P08670	Vimentin	53.652	216	40	56

NKG2D

Code	Protein name	Molecular weight	Score	Queries matched	% protein
O43707	Alpha-actinin-4	104.854	233	13	21
P07355	Annexin A2	38.604	399	16	49
P23528	Cofilin-1	18.502	129	5	38
P31146	Coronin-1A	51.026	160	9	20
P04406	Galectin-1	14.716	73	2	18
P26038	Moesin	67.820	108	6	13
P13796	Plastin-2	70.289	466	19	38
P07737	Profilin-1	15.054	206	6	42
P46940	Ras GTPase-activating-like protein IQGAP1	189.252	187	7	10
P61586	Transforming protein RhoA	21.768	61	2	11
O75083	WD repeat-containing protein 1	66.194	66	6	18

Table IV.3. Proteins identified in DRM fractions isolated by β-OG selective solubility.

3.3. L-plastin is required for NKG2D-DAP10 recruitment to lipid rafts

The proteomic analyses of DRMs and raft fractions isolated by β-OG selective solubility revealed that many proteins involved in the actin cytoskeleton rearrangement are actively recruited into lipid rafts upon NKG2D activation. The cytoskeleton helps establish membrane rafts, favoring raft-

associated signaling events (Chichili and Rodgers, 2009). Thus, it was next explored whether any of these proteins might be involved in NKG2D recruitment to rafts. Plastin-2 was the preferred option, since it was identified in DRM isolation experiments and in rafts isolated by ß-OG selective solubility. Plastin-2 (also known as L-plastin or LPL) belongs to a subclass of actin-binding proteins called actin-bundling proteins. L-plastin is an isoform that is exclusively expressed in leukocytes (Delanote *et al.*, 2005) that is required for several immune functions, such as integrin function in neutrophils, T cell activation, and B and T cell chemotaxis (Morley, 2012).

NKL cells were transfected with L-plastin siRNA (Figure IV.23A) and raft fractionation experiments by ß-OG selective solubility upon NKG2D activation were performed. Raft fractions were analyzed by western blot to detect NKG2D, DAP10 and pVav. As shown in Figure IV.23B, the amounts of the NKG2D-DAP10 complex and phosphorylated form of Vav recruited to DRM fractions following receptor activation decreased in cells transfected with L-plastin siRNA relative to control. These results suggest a role for L-plastin in the clustering of NKG2D receptor into rafts.

3.4. Role of L-plastin in NK cell migration

It has recently been reported that L-plastin regulates CXCL12-induced T cell polarization and migration (Freeley *et al.*, 2012). Therefore, a possible role for L-plastin in NKG2D-mediated NK cell chemotaxis was investigated. To this end, L-plastin expression was knocked down in human primary NK cells isolated from peripheral blood using siRNA (Figure IV.24A). Cells were activated with plate-bound IgG and anti-NKG2D antibodies and chemotaxis assays toward a CXCL12 gradient were then performed (Figure IV.24B). NKG2D activation inhibited NK cell chemotaxis in cells transfected with control siRNA (*$p<0.05$), consistent with the results described in section 1.1

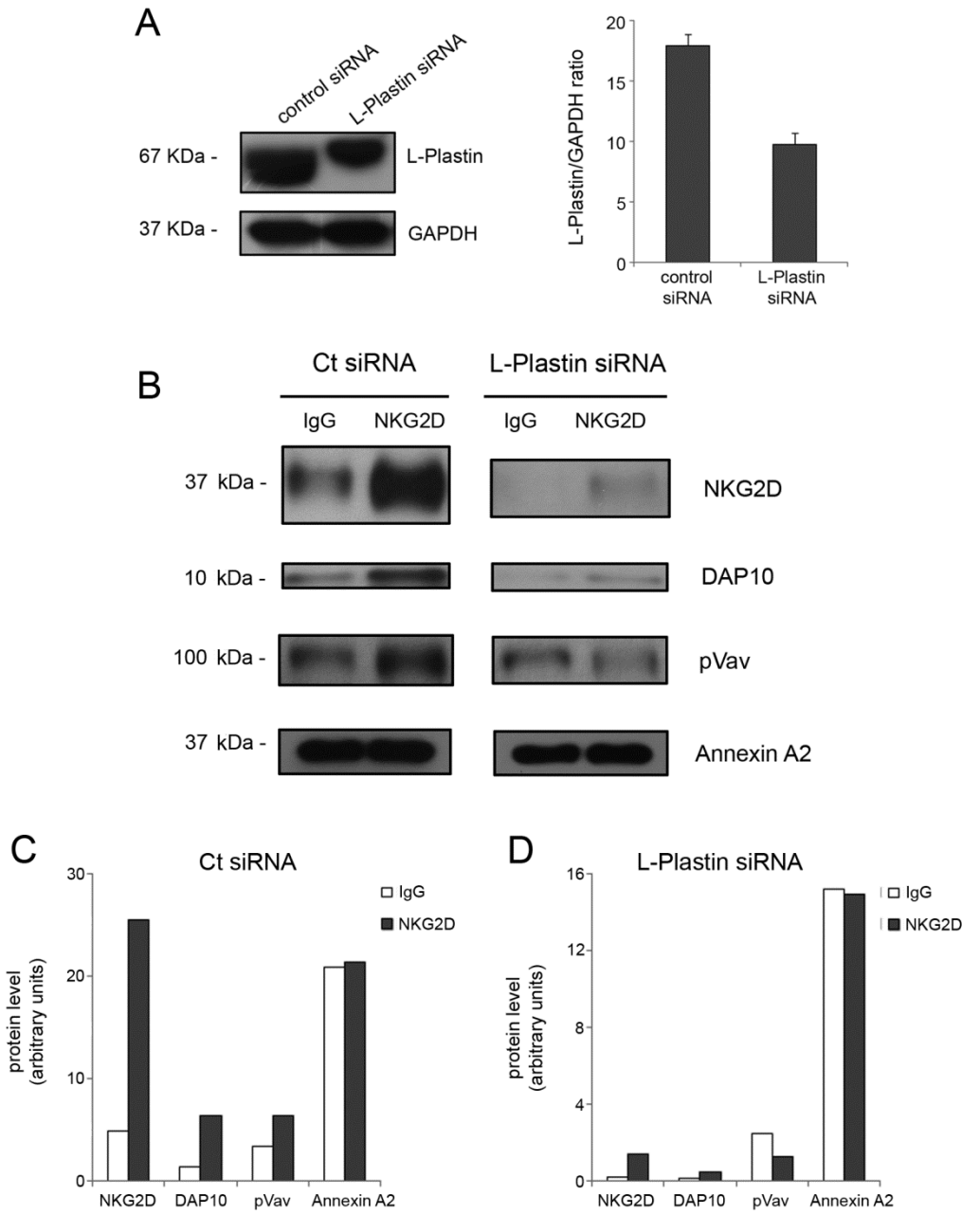


Figure IV.23. L-plastin in NKG2D recruitment to rafts. (A) NKL cells were transfected with control or L-plastin siRNA. **(B)** Twenty-four hours after transfection, DRM fractionation by ß-OG selective solubility upon NKG2D activation was performed. DRM fractions were subjected to SDS-PAGE and NKG2D, DAP10, pVav and Annexin A2 were detected by western blotting. Protein levels were quantified by densitometry for each condition in control siRNA **(C)** and L-Plastin siRNA **(D)** transfected cells. The blots and the graphs shown are from one out of three independent experiments.

(35.82% of inhibition). Conversely, activation of NKG2D failed to reduce migration rates in cells transfected with L-plastin siRNA in comparison with IgG-treated cells (19.37% of inhibition). In addition, the reduction of L-plastin expression impaired the migratory ability of control-treated cells compared with that found in control siRNA-transfected cells (*p<0.05).

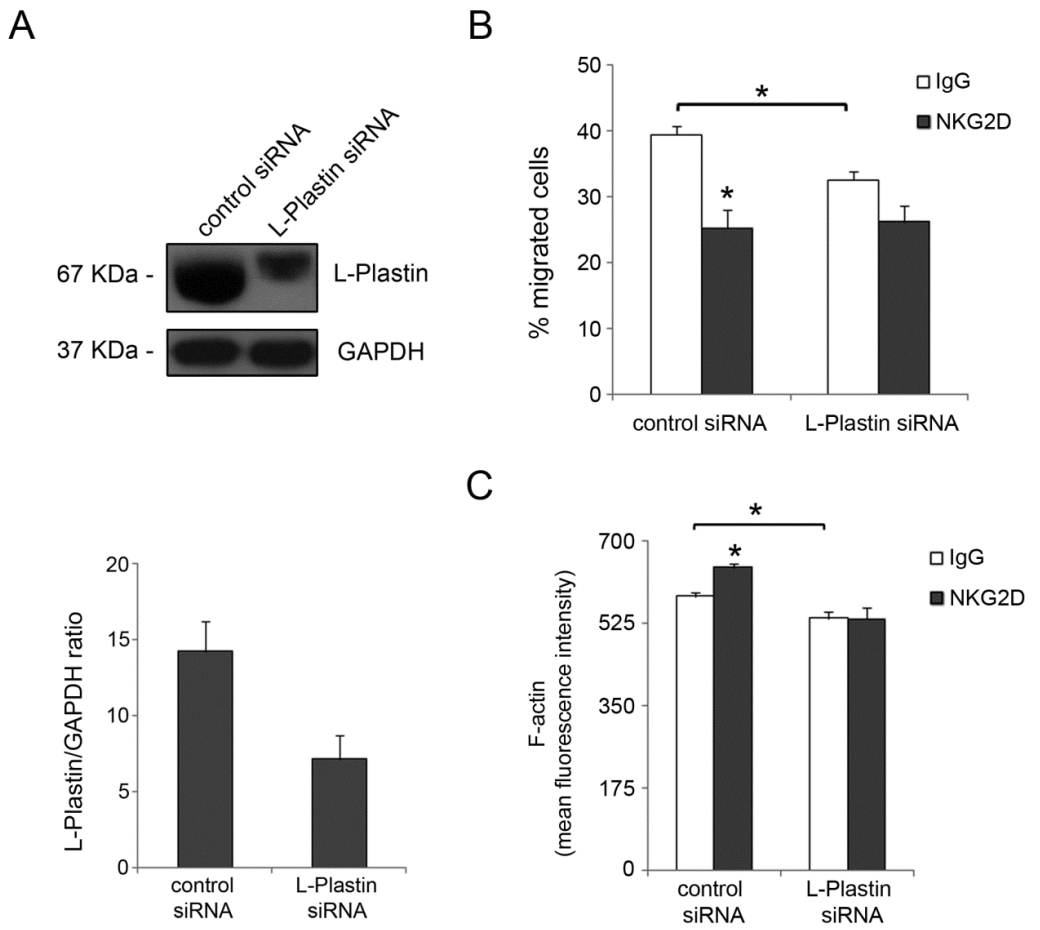


Figure IV.24. Role of L-plastin in NKG2D-mediated cell migration. (A) Human NK cells were transfected with siRNA targeting L-plastin or a siRNA control. Cells were lysed and L-plastin expression levels were analyzed by western blotting. Anti-GAPDH blots were included as protein loading controls. **(B)** Twenty four hours after transfection, cells were incubated with anti-NKG2D or IgG and their ability to migrate was assayed. Data shown are the mean + SEM of two replicates pooled from three independent experiments (*p<0.05). **(C)** F-actin content was measured in human NK cells transfected with siRNA for L-plastin. Cells were incubated with anti-NKG2D or IgG, then fixed, permeabilized, stained with phalloidin-TRITC, and the mean fluorescence intensity was determined. Results indicate the mean of three independent experiments + SEM.

Given that binding of L-plastin to actin has been proposed as stabilizing and protecting F-actin from depolymerization (Lebart *et al.*, 2004), levels of F-actin were measured by flow cytometry in NK cells transfected with L-plastin siRNA (Figure IV.24C). An increase in F-actin content upon NKG2D crosslinking was observed in cells transfected with control siRNA (*$p<0.05$). Conversely, F-actin content decreased upon NKG2D activation in cells transfected with L-plastin siRNA although it did not reach statistical significance. Knockdown of L-plastin resulted in a significant decrease in the mean fluorescence intensity of F-actin in IgG-treated cells (*$p<0.05$).

Altogether, these results showed that the actin cytoskeleton participates in NKG2D recruitment to lipid rafts. Specifically, L-plastin is involved in the clustering of the receptor upon activation and plays a role in NKG2D-mediated inhibition of NK cell chemotaxis.

4. CELL MIGRATION OF LYMPHOCYTE SUBSETS IN PATIENTS WITH MULTIPLE SCLEROSIS

The majority of studies have focused on the adaptive immune system to elucidate its role in the pathogenesis of MS (Fletcher *et al.*, 2010). The potential involvement of other immune cells is, however, still controversial, especially due to the lack of data regarding their presence on inflammatory infiltrates in MS brain samples. NK cells have been proposed to promote or prevent the progression of MS. In EAE, heat shock protein 70-peptide complexes (Hsp70-pc) have been shown to induce NK cell-dependent tolerance, which involves a reduction in T cell reactivity to PLP (Galazka *et al.*, 2006). This immunoregulatory function depends on the expression of H60, a murine NKG2D ligand, since blocking its expression abolishes Hsp70-pc-induced tolerance (Galazka *et al.*, 2007). In addition, the expression of NKG2DL (MICA/B) on oligodendrocytes in active MS lesions and close to activated macrophages have been observed; disruption of the NKG2D-NKG2DL interaction using a blocking anti-NKG2D Ab impairs NK-mediated killing of human primary oligodendrocytes (Saikali *et al.*, 2007). Other report describes that NK cells have the capacity of killing resting microglia via NKG2D and NKp46, thus limiting antigen presentation by microglial cells during neuroinflammation (Lünemann *et al.*, 2008), but additional mechanisms in which NKG2D could be involved in MS remain unknown.

NKG2D activation inhibits NK cell and CD8+ T cell migration. Transendothelial leukocyte migration into the CNS is promoted under inflammatory conditions in MS, since levels of adhesion molecules and chemokines expression are increased in cerebral endothelial cells. Therefore, it was considered of interest to explore the migratory ability toward a CXCL12 gradient of different lymphocyte subsets (CD4+ T cells, CD8+ T cells, NK cells

and B cells). To this end, a cohort of untreated RRMS patients in the remitting phase (n=11) or during relapse (n=7), patients with CIS (n=7) and sex- and age-matched healthy donors was recruited as indicated in Material and Methods (section 15.1), and transwell migration assays combined with 6-colour flow cytometry were performed. This approach allows the simultaneous study of the four lymphocyte subsets. It was previously necessary to set up the flow cytometry technique to analyze simultaneously the six parameters that define the four lymphocyte subpopulations of interest. Flow cytometry is normally used to analyze whole blood, purified cells or cell lines which are washed once or more than once after fluorochrome staining in order to remove unbound antibodies and cell debris. Since the initial numbers of cells, as well as the number of migrated cells are essential data in the study, the washing step was not feasible in this approach. To set up the flow cytometry analysis, compensation adjustments as well as the gating strategy were defined as described in Material and Methods (section 15.5).

4.1. Lymphocyte subsets are not altered in patients with MS or CIS

The analysis of the initial frequencies of CD4+ and CD8+ T cells, NK cells and B lymphocytes was performed to explore any substantial difference between patients with remitting RRMS (rem RRMS), during relapse (rel RRMS) or CIS and the control group. The percentages were calculated relative to total number of lymphocytes (defined as CD45+ cells in the flow cytometry analysis). No significant differences were found in any of the three groups of patients compared with healthy controls (Table IV.4).

rem RRMS			
	Control	**Patient**	**p-value**
%CD4+	37.66 ± 2.48	37.39 ± 1.45	0.928
%CD8+	22.39 ± 1.88	24.93 ± 3.12	0.495
%CD16/56+	7.00 ± 1.42	5.24 ± 0.75	0.285
%CD19+	1.35 ± 0.16	1.99 ± 0.40	0.152
rel RRMS			
	Control	**Patient**	**p-value**
%CD4+	37.27 ± 3.12	39.49 ± 3.38	0.638
%CD8+	28.49 ± 2.93	25.18 ± 3.02	0.446
%CD16/56+	2.84 ± 0.56	3.47 ± 0.91	0.569
%CD19+	1.25 ± 0.24	2.21 ± 0.62	0.177
CIS			
	Control	**Patient**	**p-value**
%CD4+	38.17 ± 2.04	34.94 ± 1.51	0.227
%CD8+	22.42 ± 2.25	22.41 ± 2.59	0.998
%CD16/56+	4.53 ± 0.98	3.68 ± 0.51	0.458
%CD19+	1.79 ± 0.32	2.09 ± 0.30	0.521

Table IV.4. Frequencies of the four lymphocyte subsets in healthy donors and patients. Remitting RRMS (n=11), relapse RRMS (n=7) and CIS (n=7). Data shown are the mean ± SEM and the p-values obtained in ANOVA analysis. No significant differences were found between the groups in the different subpopulations.

4.2. Chemotaxis of lymphocyte subsets in MS and CIS patients

In MS, disruption of the BBB promotes the entry of the cells of the immune system into the CNS. Thus, leukocyte migration and the consequent interaction between leukocytes and cerebral endothelial cells play a pivotal role in the pathogenesis of MS. CXCL12-induced migration of peripheral blood lymphocytes (CD45+) was analyzed in RRMS patients in the remitting phase, during relapse and CIS and their respective control groups to rule out that the disease is affecting, in general, the migratory ability of the cells (Figure IV.25).

Approximately, 22-26% of PBLs migrated across the fibronectin layer, and migration rates were similar in healthy donors and patients (Table IV.5).

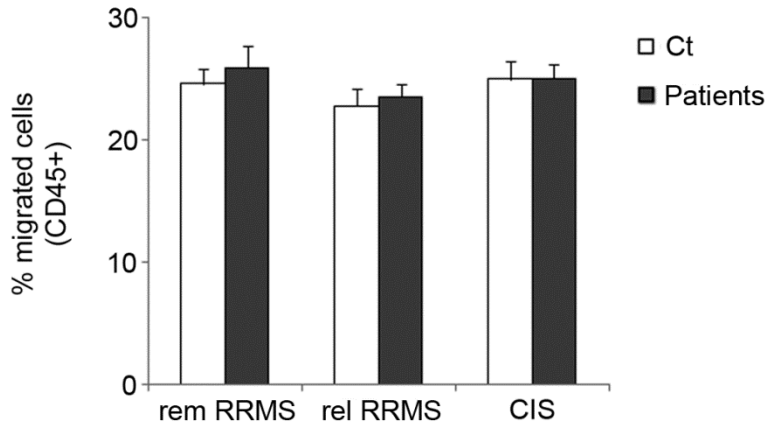


Figure IV.25. Migration rates of total lymphocytes (CD45+) in healthy controls and patients. Graphs show the mean + SEM of two replicates pooled from 11 (remitting RRMS), or 7 (relapse RRMS and CIS) independent experiments.

%CD45	Control	Patients	p-value
rem RRMS (n=11)	24.64 ± 1.16	25.95 ± 1.76	0.531
rel RRMS (n=7)	22.80 ± 1.47	23.58 ± 1.03	0.681
CIS (n=7)	25.02 ± 1.48	25.08 ± 1.13	0.963

Table IV.5. Percentages of migrated cells in the different groups of patients and in the control groups. Data shown are the mean ± SEM and the p-values obtained in GLM analysis.

Initial PBL and migrated cells from MS patients and healthy controls were subjected to 6-color flow cytometry analysis to identify the different subsets according to the gating strategy described in Methods (section 15.5) (Figure IV.26). Then the percentage of migrated cells of CD4+ T cells, CD8+ T cells, CD16/56+ and CD19+ cells was estimated relative to the initial number of cells of each population (Figure IV.27). The chemotaxis of CD4+ T cells, CD8+ T cells and B cells is not affected in any of the three groups of patients in comparison with healthy donors. In contrast, the migration rates of NK cells

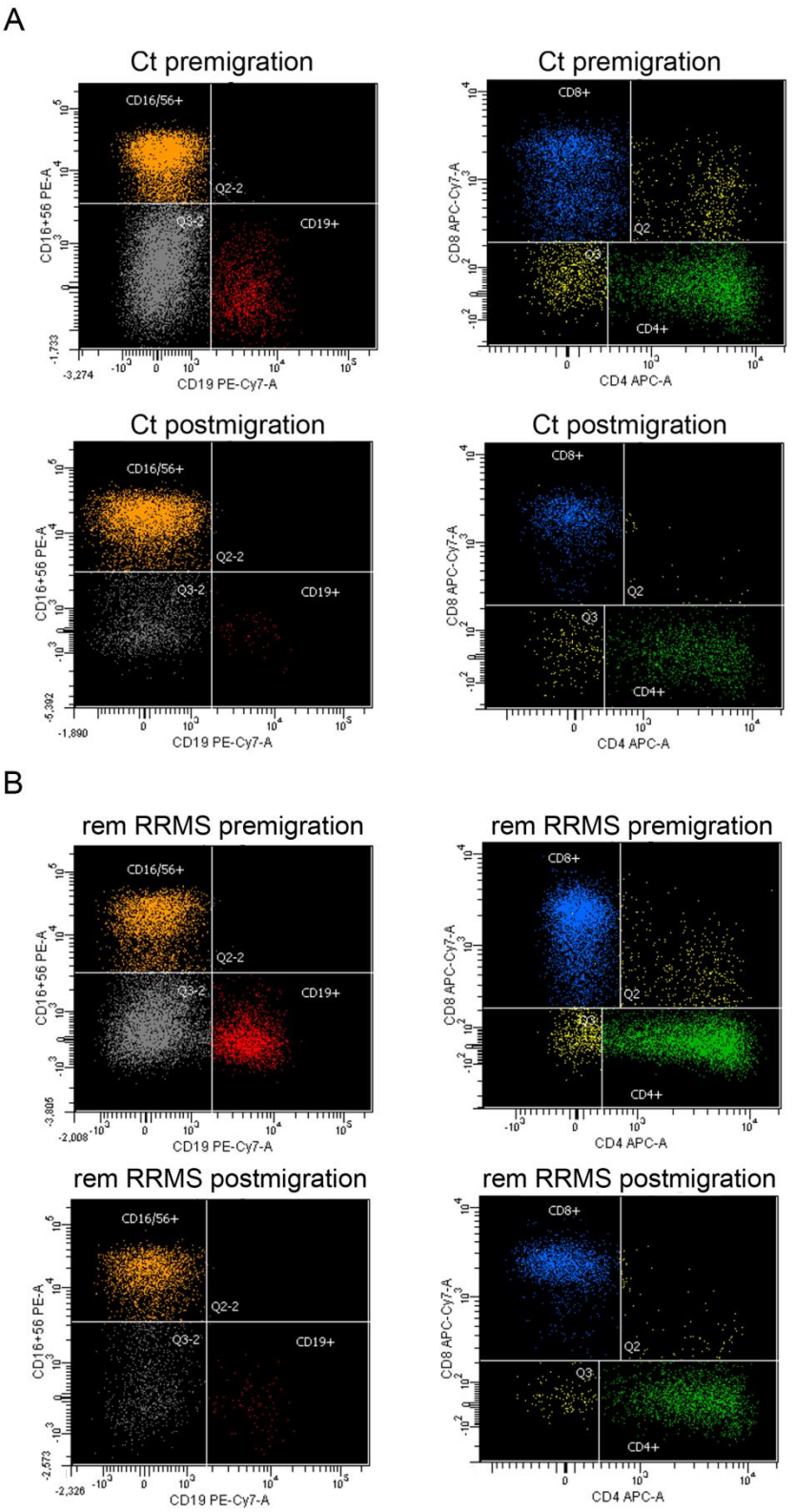


Figure IV.26. Representative panel of the analysis of the cell subsets. **(A)** represents a healthy donor and **(B)** shows a MS patient. The upper panels of A and B show the initial subpopulation (premigration), whereas the lower panels are the migrated cell subsets.

obtained in remitting MS and CIS patients were significantly increased compared with their respective control groups (p=0.013 and p=0.004, respectively). Nevertheless, in MS patients during relapse no significant differences were found in the chemotaxis of NK cells (Table IV.6).

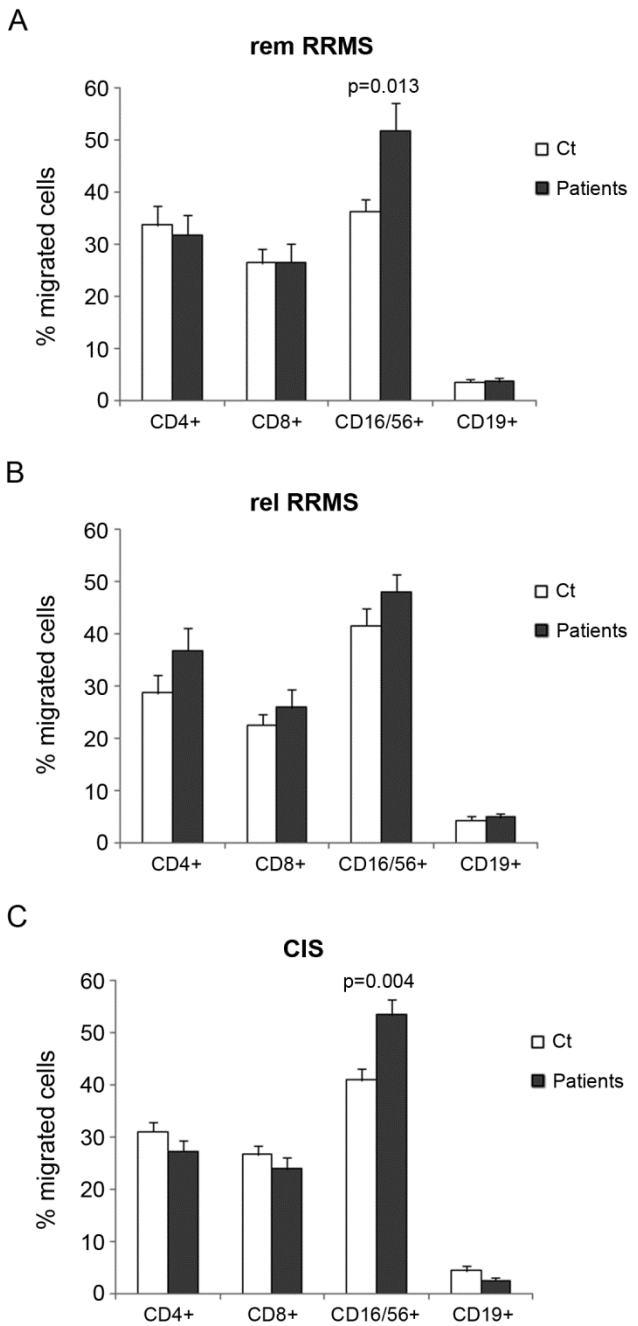


Figure IV.27. Migration rates of the different cell subtypes. The rates were estimated relative to the initial number of cells of each population. Graphs shown represent the mean + SEM of two replicates pooled from 11 (remitting RRMS), or 7 (relapse RRMS and CIS) independent experiments. Data were analyzed using GLM.

rem RRMS			
	Control	**Patient**	**p-value**
%CD4+	33.76 ± 3.77	31.92 ± 3.68	0.690
%CD8+	26.49 ± 2.73	26.66 ± 3.63	0.970
%CD16/56+	36.41 ± 2.20	51.93 ± 5.21	**0.013**
%CD19+	3.58 ± 0.46	3.73 ± 0.68	0.813
rel RRMS			
	Control	**Patient**	**p-value**
%CD4+	28.77 ± 3.42	36.93 ± 4.21	0.218
%CD8+	22.65 ± 2.05	25.94 ± 3.52	0.477
%CD16/56+	41.68 ± 3.16	48.16 ± 3.39	0.137
%CD19+	4.31 ± 0.89	4.95 ± 0.65	0.472
CIS			
	Control	**Patient**	**p-value**
%CD4+	31.14 ± 1.73	27.31 ± 2.05	0.280
%CD8+	26.75 ± 1.79	24.01 ± 2.09	0.241
%CD16/56+	41.00 ± 2.27	53.49 ± 3.07	**0.004**
%CD19+	4.48 ± 0.84	2.61 ± 0.44	0.148

Table IV.6. Percentages of migrated cells for each subpopulation in patients and healthy donors. Data shown are the mean ± SEM and the p-values obtained in GLM analysis.

4.3. The frequency of NK CXCR4+ cells is increased in remitting MS and CIS patients

In order to gain insight into the mechanism responsible for the differences observed in the migratory ability of NK cells in MS, the possibility that the levels of the CXCL12 receptor or the integrin CD49d are modified in circulating lymphocytes was explored. A second cohort of remitting MS patients (n=17), patients with CIS (n=9), and healthy controls (n=18) was recruited as indicated in Material and Methods (section 15.1). Blood samples were collected and the frequency of CD4+, CD8+ and NK cells expressing CD49 and CXCR4, as well as their surface levels, were analyzed by flow

cytometry. First, it was checked that there were no differences in the percentages of total leukocytes (CD45+), CD4+ and CD8+ (gated on CD3+), and NK cells (gated on CD3-) between the patients and the healthy donors (Figure IV. 28). The frequency of total leukocytes and the cell subsets did not vary in the patients in comparison with the control group (Table IV. 7). Thus, it may be ruled out that possible differences in CXCR4 or CD49d expression were due to significant alterations in the cell populations.

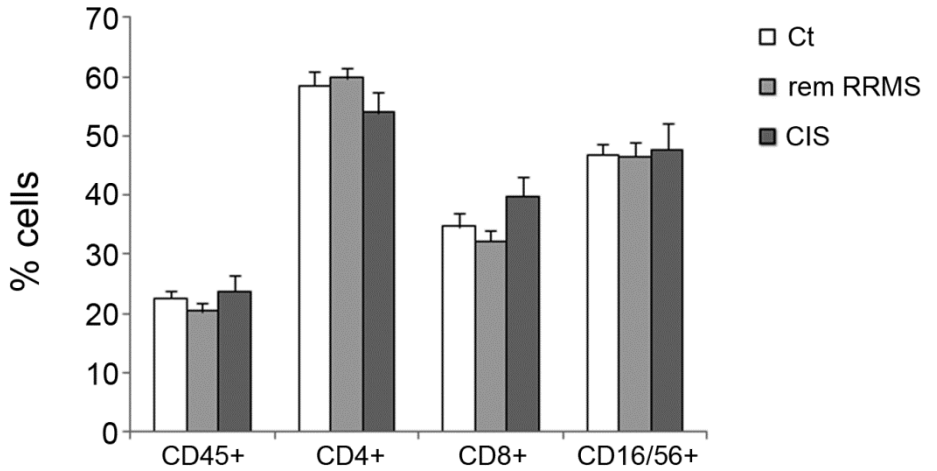


Figure IV.28. Frequencies of total leukocytes (CD45+), CD4+, CD8+ and CD16/56+ cells in patients and healthy donors. MS patients in the remitting phase (n=17); patients with CIS (n=9); healthy donors (n=18). The graph shows the mean + SEM.

	Ct (n=18)	rem RRMS (n=17)	CIS (n=9)	p-value
%CD45	22.52 ± 1.16	20.37 ± 1.37	23.70 ± 2.72	0.422
%CD4	58.42 ± 2.37	59.73 ± 1.82	53.99 ± 3.41	0.362
%CD8	34.71 ± 2.37	32.16 ± 1.76	39.87 ± 3.29	0.151
%CD16/56	46.76 ± 1.73	46.54 ± 2.53	47.73 ± 4.55	0.975

Table IV.7. Percentages of the different cell populations in patients and healthy donors. Data shown are the mean ± SEM and the p-values obtained in Welch test of equality of means.

The frequency of cells positive for CD49d staining in MS and CIS patients was similar to that found in circulating lymphocytes of healthy donors (Figure IV.29A). Similar results were obtained when the levels of CD49d on the surface of CD8+, CD4+ and CD16/56+ were analyzed, as the median fluorescence intensity showed no differences between the groups (Figure IV.29B).

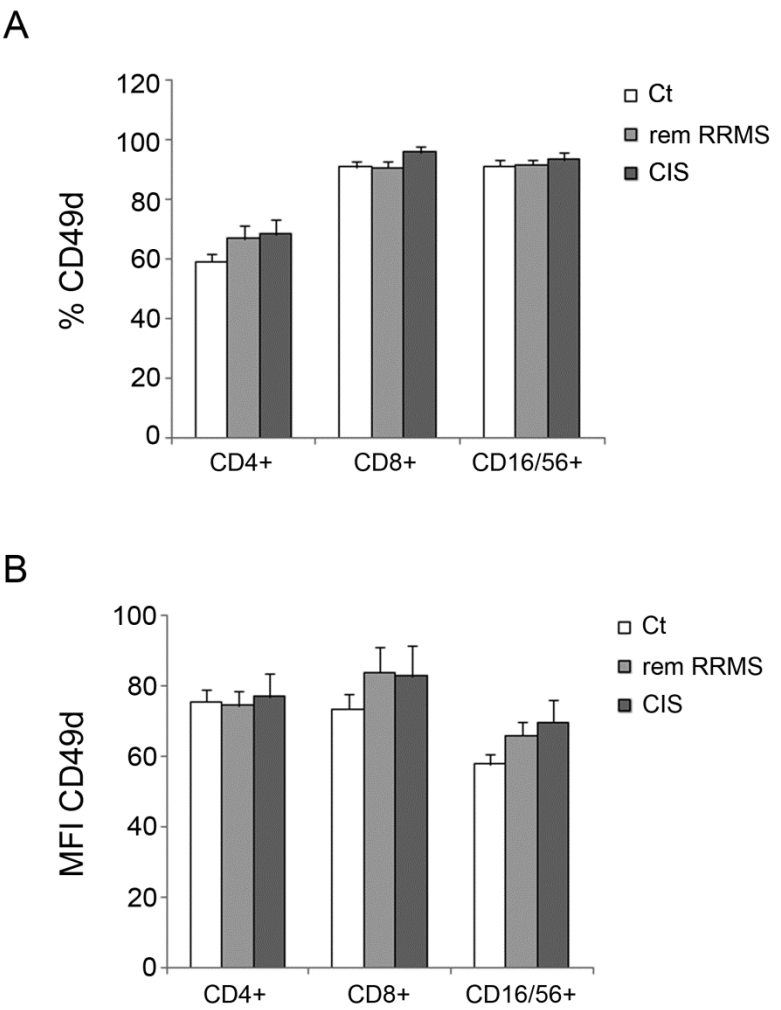


Figure IV.29. CD49d in MS and CIS patients. (A) Percentage of the different cell subsets expressing CD49d and its surface levels **(B)** in healthy donors (n=19), MS patients in the remitting phase (n=15) and CIS patients (n=9; in CD16/56+ n=8).

Regarding CXCR4, the frequency of NK cells expressing this receptor in RRMS patients and CIS patients was significantly higher than in healthy controls (Figure IV.30A), whilst the surface levels indicated by the MFI remained similar (Figure IV.30B). Representative dot plots of NK cells expressing CXCR4 in a healthy subject and in a MS patient are shown in Figure IV.30C. No differences were observed in the other leukocyte subpopulations.

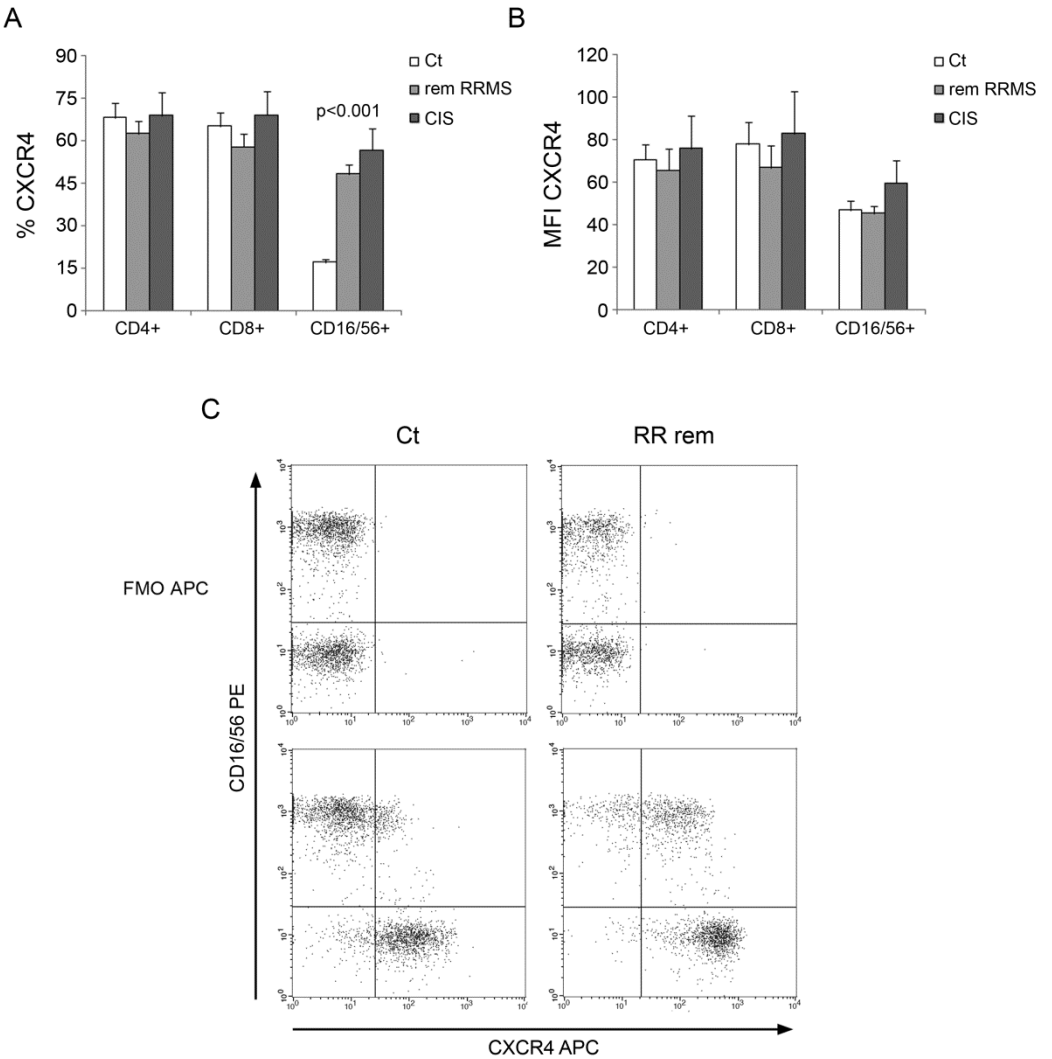


Figure IV.30. CXCR4 in MS and CIS patients. (A) Percentages of the different cell subsets expressing CXCR4 and its surface levels **(B)** in healthy donors (n=19), MS patients in the remitting phase (n=19; in CD4+ and CD8+ n=17) and CIS patients (n=8). **(C)** Representative dot plot showing CXCR4 expression by gating CD45+CD3- cells in a MS patient and a healthy donor.

	Ct	rem RRMS	CIS	p-value
CD4+				
%CD49d	59.18 ± 2.82	66.93 ± 4.19	68.55 ± 5.05	0.178
MFI CD49d	75.68 ± 3.48	74.67 ± 4.04	77.19 ± 6.32	0.946
%CXCR4	68.22 ± 5.25	62.55 ± 4.36	68.91 ± 8.41	0.630
MFI CXCR4	70.70 ± 7.35	65.72 ± 10.23	76.03 ± 15.34	0.851
CD8+				
%CD49d	90.96 ± 1.73	90.75 ± 2.03	96.09 ± 1.59	0.063
MFI CD49d	73.51 ± 4.50	83.99 ± 7.06	82.88 ± 8.61	0.394
%CXCR4	65.20 ± 4.90	57.99 ± 4.47	69.11 ± 8.26	0.408
MFI CXCR4	78.06 ± 10.33	67.07 ± 10.29	82.91 ± 19.87	0.683
CD16/56+				
%CD49d	91.34 ± 1.96	91.65 ± 1.72	93.54 ± 2.46	0.770
MFI CD49d	57.99 ± 2.58	65.96 ± 3.82	69.88 ± 6.21	0.120
%CXCR4	17.16 ± 1.06	48.45 ± 3.27	56.78 ± 7.53	**<0.001**
MFI CXCR4	46.88 ± 4.30	45.34 ± 3.54	59.82 ± 10.71	0.469

Table IV.8. Percentages of cells expressing CD49d and CXCR4 and their surface levels in patients and healthy donors. Data shown are the mean ± SEM and the p-values obtained in Welch test of equality of means.

Therefore, the increase observed in NK cell migration rates in RRMS patients in the remitting phase and in CIS patients correlates with an increase in frequency of NK cells expressing CXCR4 in PBMC. Given that MICA/B is detected in MS lesions (Saikali *et al.*, 2007), engagement of NKG2D on this CXCR4+ NK-cell subset via binding of these ligands could play a role in the pathogenesis of the disease.

V. DISCUSSION

1. ROLE OF NKG2D RECEPTOR IN CELL MIGRATION

The NKG2D recognition system plays an important role in the immune responses. However, a sustained expression of NKG2DL on tumor cells, the release of the ligands into their soluble form, as well as their secretion via microvesicles, are able to downregulate NKG2D surface levels and impair NK cell and CD8+ T cell effector functions. These mechanisms are known to contribute to the evasion of the immune surveillance system.

The aim of this study was to explore the role of NKG2D in the regulation of other processes that may be involved in the immune response or evasion, such as cell migration, since it is essential for an appropriate recruitment of cells of the immune system to the target tissues during an immune response.

1.1. NKG2D in NK cell migration

NKG2D initiated NK cell function is regulated by a balance of activating and inhibitory signals on the NK cell, which eventually determines the contact with the target cell (Lanier, 2005). NKG2D triggering by anti-NKG2D antibodies or MICA, a natural ligand of this receptor, promotes a stop signal that may be reverted when inhibitory signals dominate (Culley *et al.* 2009). NKG2D activation impaired CXCL12-induced chemotaxis of human NK cells stimulated with IL-2 for 48 h, whereas no effect was observed in nonstimulated cells. These results suggest that the regulation of cell migration by NKG2D signaling takes place in the context of an immune response and were reproduced when the IL-2-dependent NKL cell line was employed. The ability to migrate of NK cells also decreased when NKG2D was stimulated with ULBP-2, a natural ligand of this receptor. This regulation may be disrupted

when inhibitory signals dominate since co-ligation of the inhibitory receptor NKG2A could abrogate NKG2D-mediated inhibition of cell chemotaxis (Figure V.1).

Taken together, these observations are not only consistent with the stop signal induced by NKG2D-NKG2DL interaction described by Culley *et al.* (2009), but also show that the effect of NKG2D stimulation on NK cell migration is maintained after removal of NKG2D stimulus.

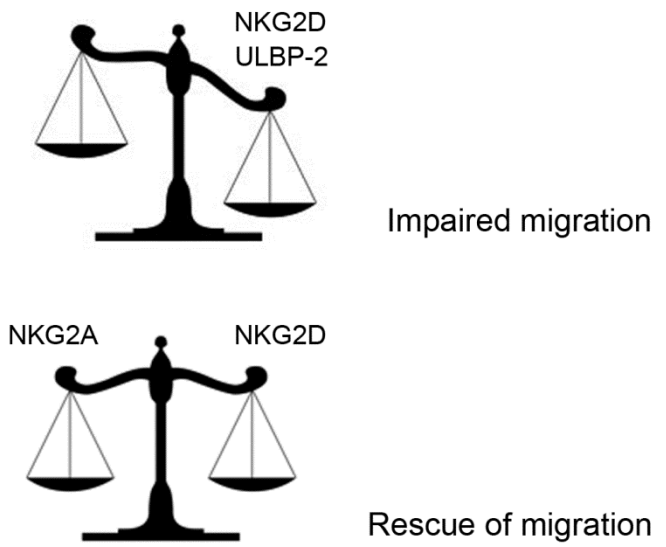


Figure V.1. Activating and inhibitory signals in the regulation of NKG2D-mediated NK cell migration. While NKG2D activation through anti-NKG2D or ULBP-2 inhibit cell migration, co-ligation of an inhibitory receptor (NKG2A) partially rescues cell migration rates.

NKG2D activation did not alter the surface levels of the chemokine receptor CXCR4, the expression of CD11a and CD49d on the cell surface remained unchanged, and cells adhered normally to fibronectin, indicating that the differences observed are not due to a defect on chemokine engagement or an increase in cell adhesion. Immune cell responses involve a complex regulation of the cytoskeleton dynamics to migrate to the tissues and contact the target cell, among other functions (Vicente-Manzanares and Sánchez-

Madrid, 2004). The F-actin content measured by flow cytometry showed an increase upon antibody cross-linking, and less cells with a polarized morphology were observed in NKG2D-treated cells, which points to a role for the Rho GTPase family of proteins.

Rho GTPases play an important role in NK activity as they regulate cell polarization, adhesion, and vesicle trafficking of the cytotoxic granules towards the target cell (Wittman and Waterman-Storer, 2001; Ridley, 2001). In this study, the activity of the best characterized Rho GTPases (Rac1, RhoA and Cdc42) in response to NKG2D activation was analyzed in pull-down assays. Rac1 and Cdc42 were activated by NKG2D ligation, while RhoA activity decreased. The antagonistic activity of Rac and Rho has been previously described in fibroblast cells (Sander *et al.*, 1999) and in cell types of the immune system, such as T cells (Cernuda-Morollón *et al.*, 2010) and neutrophils (Xu et al, 2003). A balance between Rac and Rho activities is necessary for migratory polarity, with Rac regulating actin polymerization and leading edge formation, while RhoA activity is responsible for rear end organization. Cdc42 activity was increased compared with the control condition. This Rho GTPase regulates the MTOC localization in the IS (Stowers *et al.*, 1995) and together with Rac1 controls cytoskeletal reorganization at the leading edge of the migrating cell and is essential for the establishment of cell polarity (Etienne-Manneville and Hall, 2002). It has been described that constitutively activated mutants of RhoA, Rac1, and Cdc42 impair T cell migration. Noteworthy is that mutant of Cdc42 has more of an inhibitory effect on CXCL12-mediated migration (del Pozo *et al.*, 1999).

Given that Rac1 and Cdc42 promote actin polymerization at the leading edge of the migrating cell and that NKG2D activation correlates with an increase in the F-actin content, the role of these proteins in NKG2D-mediated migration was further explored. The pretreatment with the Rac inhibitor NSC23766 had no effect in the migration rates upon NKG2D activation,

although an increase in cell motility of control-treated cells was found. Thus, despite the fact that inhibition of Rac is not sufficient to rescue NK cell migration, certain levels of active Rac are necessary for an appropriate polarization and subsequent migration. In contrast, administration of wiskostatin, a selective chemical inhibitor of N-WASp, partially rescued NK cell chemotaxis upon NKG2D activation. These results suggest a prominent role of Cdc42 on the regulation of NK cell migration in response to NKG2D activation.

Cdc42 interacts with both N-WASp and WASp effectors. The major findings of the involvement of their interaction are described in lymphocytes of patients suffering the Wiskott-Aldrich syndrome (WAS). WASp function is crucial in T cell chemotaxis (Haddad *et al.*, 2001; Snapper *et al.*, 2005; Gallego *et al.*, 2005) and is required to correctly form the IS in NK cells, as mutations in WASp impaired NK activity (Orange *et al.*, 2002). However, implication of N-WASp in NKG2D-mediated signaling and NK cell motility has not been explored. Knocking down N-WASp expression with siRNA partially reverted NKG2D effect on cell migration, consistent with the wiskostatin effect. Regarding WASp, the inhibition of its expression led to a defect in NK cell motility in IgG-treated cells as described previously (Stabile *et al.*, 2010) and NKG2D activation failed to decrease migration rates. These results indicate that both proteins N-WASp and WASp act downstream of Cdc42 for the regulation of NK cell migration. However, the possibility of a defective NKG2D-induced response or chemokine-induced inside-out signaling in WASp knocked-down cells cannot be ruled out, as this protein is necessary for the IS formation and it has been shown that its function is not compensated by N-WASp in NK cells (Gismondi *et al.*, 2004; Orange *et al.*, 2007).

Cell migration involves important changes in the actin cytoskeleton and microtubules network. NK cells treated with wiskostatin or transfected with N-WASp-specific siRNA showed no significant decrease in F-actin upon NKG2D

crosslinking compared to control cells or control siRNA transfected cells, respectively. A recent work describes that IL-2 may promote actin polymerization via the induction of WAVE2 in a WASp-independent manner (Orange *et al.*, 2011). WAVE proteins, which are other WASp family members, mediate cytoskeletal effects of active Rac (Bompard and Caron, 2004). Thus, different pathways regulate cytoskeletal rearrangement and may compensate for the WASp defect. In addition to actin dynamics, the Cdc42/WASp pathway regulates accumulation of raft patches at the cell surface in different cell lines (Golub and Caroni, 2005). These domains act as microtubule stabilizers through IQGAP1 association and promote sustained directional motility. In NK cells, Cdc42, WASp, and Cdc42-interacting protein-4 (CIP4) regulate MTOC positioning upon cell activation, and WASp deficiency results in reduced F-actin accumulation and MTOC polarization (Banerjee *et al.*, 2007). Therefore a balance between both actin and microtubules dynamics is required for cell polarization and migration. It is plausible that over-activation of Cdc42 and its downstream effectors may alter the balance between the microtubules' growth and catastrophe and F-actin accumulation inhibiting cell migration.

In summary, both N-WASp and WASp participate in the inhibition of NK cell chemotaxis in response to NKG2D activation, but this effect is not dependent on the regulation of F-actin dynamics. Recognition of the NKG2DL is beneficial in immune responses against tumors and viral infections facilitating the formation of the IS. However, NKG2DL secreted via microvesicles may interact with the receptor promoting an alternative mechanism of immune evasion impairing NK cells to migrate to the target tissue (Figure V.2).

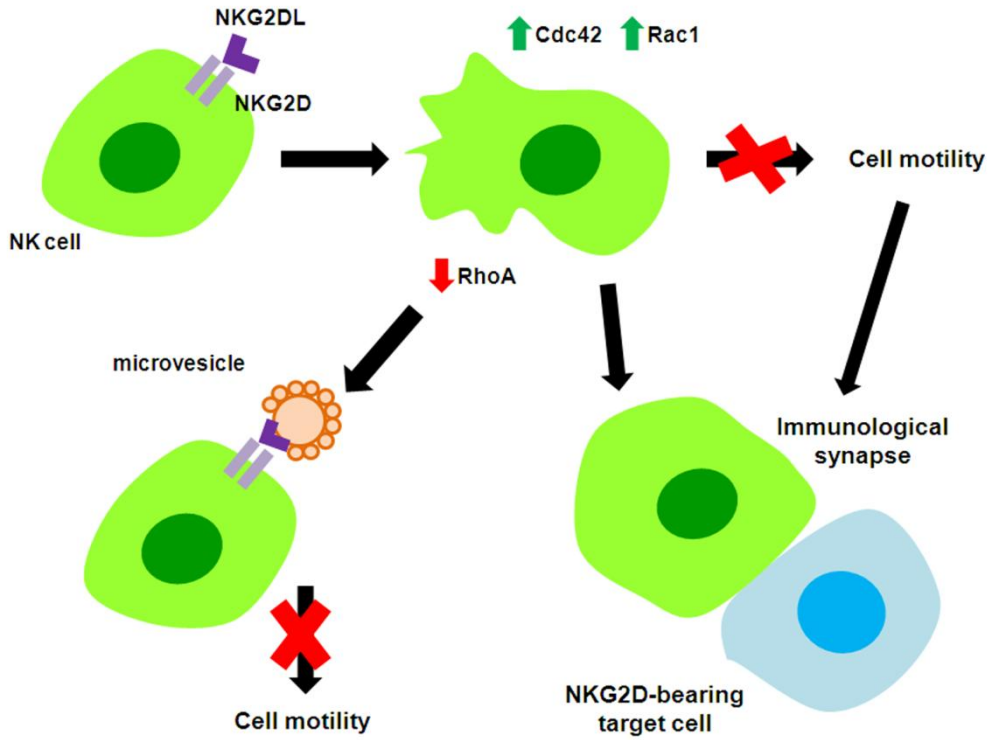


Figure V.2. Role of NKG2D in NK cell migration. Activation of NKG2D regulates the activity of the Rho GTPases Rac1, RhoA and Cdc42, and inhibits the migratory ability of NK cells. On one hand, this mechanism may favor the formation of an IS. On the other hand, microvesicles bearing NKG2DL may interact and impair NK cell motility contributing to the immune evasion.

1.2. NKG2D in CD8+ T cell migration

CD28 is the prototypical T cell costimulatory molecule, and its function is well established in T cells. NKG2D and CD28 recruit the p85 subunit of PI3K to propagate their signals through the YXXM signaling motif. For this reason, it has been postulated that NKG2D could act as a costimulatory molecule in CD8+ T cells, although the role of this receptor in T cell costimulation has not been fully explored. Since NKG2D activation regulates NK cell chemotaxis, it was then explored whether it could be involved in CD8+ T cell migration upon costimulation in comparison with CD28.

The migratory ability of T lymphocytes is essential in immunity. Under physiological conditions, T cells continuously recirculate through lymph nodes scanning the surface of antigen presenting cells. Cell motility is also required to reach the sites of inflammation, which in turn depends on the interaction with chemokines or the extracellular matrix. T cells require two signals to get fully activated. The first signal is provided through the TcR/CD3 complex. Engagement of the TcR triggers signaling pathways that lead to changes in actin cytoskeleton dynamics in order to deliver a stop signal that allows the interaction with an antigen presenting cell and the formation of an immunological synapse. The signals delivered by costimulatory molecules (e.g. CD2 or CD28) sustain TcR signaling and eventually allows T cells to develop an effective immune response. In addition, there is a complex interplay between CXCR4 and TcR signaling (Kumar *et al.*, 2006; Patrussi *et al.*, 2007). TcR triggering has been shown to reduce the migratory response to a CXCL12 gradient (Peacock and Jirik, 1999). CXCR4 is recruited to the IS during T cell—APC contact and is proposed to favor this interaction and enhance T cell activation (Molon *et al.*, 2005). The stop signal delivered by the TcR may compete with chemokine-mediated go signals to control the establishment of the IS and T cell activation (Dustin, 2004).

It is well known that TcR engagement inhibits T cell migration (Dustin *et al.*, 1997; Dustin, 2008; Cernuda-Morollón *et al.*, 2010). Consistent with these studies, CD3 activation impaired CD8+ T cell chemotaxis. Costimulation of the TcR through CD28 and NKG2D further reduced migration rates. Neither activation of CD28 nor activation of NKG2D alone was sufficient to impair CD8+ T cell chemotaxis. The decrease in migration rates was not attributable to an enhanced adhesion to fibronectin. The percentage of adhered cells upon CD3/NKG2D activation was reduced compared with the other experimental conditions. However, this difference is not great enough to consider that this could explain the impairment in the migratory ability of these cells (51.49% *vs.*

57.38% under control conditions). When cells were not stimulated with IL-2, NKG2D- and CD28-mediated costimulation did not efficiently inhibit cell migration, suggesting that regulation of CD8+ T cell chemotaxis through the two costimulatory receptors takes place in the context of an immune response similar to that observed in NK cells.

Since Rho GTPases were found to be implicated in NKG2D-mediated inhibition of chemotaxis in NK cells, the activity of Rac1 and Cdc42 was studied in CD8+ T cells upon CD3/CD28 and CD3/NKG2D activation. Rac1 is activated upon T cell receptor activation and costimulation via CD28 and NKG2D. However, CD3/NKG2D, but not CD3/CD28, additionally activated Cdc42, suggesting that the activity of this Rho GTPase is essentially regulated by the NKG2D receptor. CD28 ligation alone has been shown to activate Cdc42 (Salazar-Fontana *et al.*, 2003), which does not correlate with the results found in this work. Cdc42 activation assays were carried out in different cell types, as Salazar-Fontana *et al.* employed the Jurkat cell line and resting CD4+ T cells, and this could explain the lack of concordance.

Cdc42 can signal through its specific effectors, WASp and N-WASp (Rohatgi *et al.*, 2000; Higgs and Pollard, 2000), which regulate actin polymerization by activating the Arp2/3 complex (Millard *et al.*, 2004). Deficiency of WASp has been shown to impair cell signaling and actin rearrangement in WAS patients (Bouma *et al.*, 2009). As mentioned above, WASp-deficient leukocytes exhibit decreased chemotaxis toward a CXCL12 gradient (Snapper *et al.*, 2005) and the interaction of Cdc42-WASp is important for CXCL12-induced cell motility (Haddad *et al.*, 2001). This work explored in more detail a possible function of N-WASp in T cell migration and in CD3/NKG2D-mediated signaling. The partial depletion of N-WASp by siRNA decreased the motility of control-treated cells, suggesting that certain levels of the protein are required under basal conditions for their random migration ability or chemotactic response. However, knockdown of N-WASp did not alter

CD3- or CD3/CD28-mediated T cell chemotaxis. These results point to additional mechanisms that may be activated downstream CD3, which are absent under basal conditions and that are compensating this partial lack of N-WASp, as it occurs upon CD3/CD28 activation. Conversely, knockdown of N-WASp impaired CD3/NKG2D-mediated inhibition of CD8+ T cell chemotaxis, since migration rates are significantly higher than those found in control siRNA transfected cells. Despite the expression of the homolog WASp is not affected under these conditions, N-WASp is required for CD3/NKG2D-mediated cell migration, which suggests a role for the Cdc42/N-WASp axis in CD3/NKG2D signaling.

To gain insight into the different signaling pathways triggered by both costimulatory molecules, the phosphorylation of proteins involved in TcR signaling and cytoskeleton dynamics using the N-WASp inhibitor wiskostatin was analyzed. It is worth noting the results concerning the actin-binding protein cofilin. Previous studies have reported that T cell costimulation activates cofilin by dephosphorylation, promoting its actin-severing function (Lee *et al.*, 2000). Costimulation via NKG2D also reduced the phosphorylation levels of cofilin. These results may be related to the decrease observed in F-actin content. Disruption of Cdc42 binding through the inhibition of N-WASp further reduced cofilin phosphorylation upon CD28 costimulation and in CD3/NKG2D-stimulated CD8+ T cells. However, this decrease was specially marked upon CD3/NKG2D activation which suggests similar but not identical pathways for the regulation of cofilin activity. Cofilin function is regulated by several mechanisms such as pH change, Slingshot and Chronophin phosphatases, as well as by LIM kinase (LIMK) protein phosphorylation (Bamburg, 1999; Huang *et al.*, 2006). Rho GTPases are known to modulate actin dynamics through LIMK1- and LIMK2-mediated inactivation of cofilin (Yang *et al.*, 1998; Sumi *et al.*, 1999). In human T lymphocytes, serine

phosphatases protein phosphatases 1 (PP1) and 2A (PP2A) dephosphorylate and activate cofilin, thus promoting enhanced polymerization and depolymerization of actin filaments (Ambach *et al.*, 2000). Moreover, CD28 costimulation regulates cofilin activity through the GTPase Ras and its effectors MEK and PI3K (Wabnitz *et al.*, 2006). The activity of cofilin has been found to be essential in the formation of the IS, as well as in the induction of T lymphocyte proliferation and cytokine production (Eibert *et al.*, 2004). Since N-WASp depletion using siRNA partially rescued CD3/NKG2D-mediated cell migration and pretreatment with wiskostatin dramatically decreased levels of phosphorylated cofilin this protein could also be involved in cell chemotaxis, which is other actin-dependent process. Nevertheless, the decrease in cofilin phosphorylation is not clearly correlated with F-actin content determined in knockdown experiments. This observation may be due to a compensatory mechanism consisting of other members of the WASp family proteins and cortactin, which activate the Arp2/3 complex (Weaver *et al.*, 2003). For instance, Rac1, which is activated upon costimulation via CD28 and NKG2D, associates with WAVE proteins, which regulate actin polymerization at the leading edge of the migrating cell (Miki *et al.*, 1998; Miki *et al.*, 2000). Rac1 activation is also required for cortactin localization to the cortical actin network within lamellipodia (Head *et al.*, 2003).

In summary, this study provides new insights into the role of NKG2D as a costimulatory molecule in the migratory ability of human CD8+ T cells, finding similarities and differences with CD28. Costimulation via NKG2D preferentially activates the Cdc42/N-WASp axis, in contrast to CD28-mediated costimulation, and N-WASp is required for CD3/NKG2D inhibition of CD8+ T cell migration. Moreover, CD3/NKG2D differentially regulates actin cytoskeleton dynamics through N-WASp and the actin-binding protein cofilin. Thus, NKG2D costimulation, analogous to the behavior of the costimulatory

molecule CD28, enhances TCR signaling in a similar but not identical manner (Figure V.3).

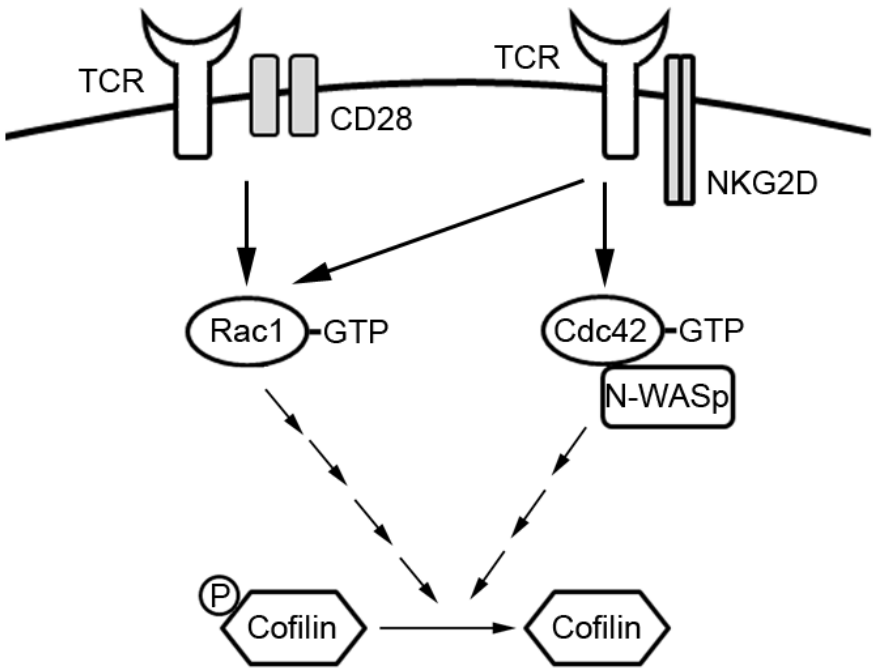


Figure V.3. Schematic model depicting the proposed signaling pathways triggered by CD3/CD28 and CD3/NKG2D. CD28 and NKG2D-mediated costimulation have a similar effect on CD8+ T cell chemotaxis but act through different signaling pathways. Whilst CD28-mediated costimulation leads to Rac1 activation, costimulation via NKG2D additionally induces Cdc42 activation. Both signaling cascades converge at cofilin since CD3/CD28 and CD3/NKG2D activation promotes cofilin dephosphorylation. This regulation is more marked upon NKG2D-mediated costimulation, as inhibition of N-WASp further decreased cofilin phosphorylation after CD3/NKG2D engagement but not upon CD3/CD28 activation.

The present project has addressed the role that NKG2D plays in the chemotaxis of the main cell subsets that express this receptor. The set of results and mechanisms discussed above may account not only for NKG2D-induced stop signal responsible for NK- or CD8+ T cell-target cell interaction, but could also participate in tumor evasion through the interaction with NKG2DL present on the surface of microvesicles.

2. ROLE OF LIPID RAFTS IN NKG2D-MEDIATED SIGNALING

Lipid rafts are microdomains of the plasma membrane highly enriched in cholesterol and glycosphingolipids that function as platforms for signal transduction. In addition, the association of the actin cytoskeleton with membrane rafts is essential for their structure and function and also maintains the clustering of the raft-associated proteins (Chichili and Rodgers, 2009).

In NK cells, the recruitment of activating receptors into membrane rafts is required to ensure their correct organization at the site of target cell contact and thereby NK cell effector functions. Prevention of this recruitment by inhibitory receptors is known to impair NK cell activation (Lou *et al.*, 2000; Fasset *et al.*, 2001; Watzl and Long, 2003; Riteau *et al.*, 2003; Sanni *et al.*, 2004; Masilamani *et al.*, 2006). In this project, the recruitment of the activating receptor NKG2D into membrane rafts and its potential involvement in NK cell migration were explored.

2.1. Analysis of the raft proteome in NK cells upon NKG2D activation

As outlined above, although several studies have investigated how activating receptors in NK cells are recruited into membrane rafts for NK cell activity, this process has not been fully elucidated regarding the NKG2D receptor. To address this issue, sucrose gradient experiments were performed to isolate DRMs upon NKG2D activation. This receptor is known to be recruited upon activation (Endt *et al.*, 2007). Consistent with this report, NKG2D was identified in DRMs. Furthermore, the adaptor protein DAP10 and the phosphorylated form of Vav were identified. Vav activates downstream

NKG2D and is essential for raft clustering (Lou *et al.*, 2000; Villalba *et al.*, 2001). To understand the overall dynamic changes of proteins within lipid rafts upon NKG2D activation in NK cells, the lipid raft proteome was analyzed using LC-MS/MS. Several proteins involved in the actin cytoskeleton dynamics were identified, as previously shown by other proteomic analyses in a variety of cell types (von Haller *et al.*, 2001; Nebl *et al.*, 2002; Bini *et al.*, 2003; Yanagida *et al.*, 2007; Lin *et al.*, 2010). Thus, these results indicate the existence of interactions between the actin cytoskeleton and membrane rafts.

To analyze these interactions further, raft fractionation by ß-OG selective solubility experiments were performed in order to dissolve DRMs without disrupting the protein associations with the cytoskeleton. A qualitative proteomic approach was carried out as in the DRM fractions. Additional proteins that support a role of the actin cytoskeleton in NKG2D-recruitment to rafts were found. Moreover, some of the proteins identified are closely related to Rho GTPases, as Rho GDI proteins or IQGAP1, which interacts with Rac1 and Cdc42 and controls actin polymerization through the activation of N-WASp (Le Clainche *et al.*, 2007).

2.2. L-plastin in NKG2D clustering and NK cell migration

A close interaction between rafts and the actin cytoskeleton is necessary for T cell activation (Bunnell *et al.*, 2003; Samstag *et al.*, 2003; Gomez and Billadeau, 2008). In NK cells, the recruitment of the activating receptor 2B4 into membrane rafts depends on actin polymerization and inhibitory signals may disrupt this recruitment and modulate NK cell activation (Watzl and Long, 2003). The possibility that the cytoskeletal proteins identified in our analysis could be actively involved in NKG2D recruitment into rafts was then explored. Specifically, the protein L-plastin was investigated since it was identified by both proteomic approaches. This actin-bundling protein regulates several

processes in a wide variety of cells of the immune system. In T cells, L-plastin promotes leukocyte integrin-mediated adhesion (Jones *et al.*, 1998) and is involved in the formation of the immunological synapse (Wang *et al.*, 2010; Wabnitz *et al.*, 2010). However, the requirement of L-plastin in NK cell functions remains unclear. L-plastin depletion by siRNA impaired NKG2D-DAP10 and pVav recruitment to lipid rafts, as observed in the raft fractions isolated by ß-OG. Therefore, these results suggest a role for this protein in activation-induced recruitment of the NKG2D receptor to rafts.

L-plastin has been shown to regulate CXCL12-induced chemotaxis of T lymphocytes (Freeley *et al.*, 2012). In addition, some of the proteins identified are directly related to cell motility and, moreover, to the Rho GTPases previously studied in NKG2D-mediated migration. It was therefore evaluated whether a defective NKG2D recruitment due to L-plastin depletion might affect NKG2D-mediated NK cell migration. Knockdown of L-plastin led to a defective NK cell motility under basal conditions. Moreover, the inhibition of NK cell chemotaxis upon NKG2D activation was abrogated in cells transfected with L-plastin siRNA. The activation of L-plastin by phosphorylation positively regulates its F-actin binding activity (Janji *et al.*, 2006), as well as F-actin localization, and is required for polarization and migration of chemokine-stimulated T lymphocytes (Freeley *et al.*, 2012). Knockdown of L-plastin correlated with a decrease in the F-actin levels in control-treated NK cells. NKG2D cross-linking promoted an increase in the F-actin levels, as previously found when studying the role of NKG2D in NK cell migration. This effect is, however, abrogated after silencing L-plastin expression. These results are consistent with the protective role of L-plastin preventing F-actin depolymerization or reducing the actin filament turn-over (Lebart *et al.*, 2004; Al Tanoury *et al.*, 2010).

In summary, the present project has studied the raft proteome following NKG2D activation by qualitative proteomics, which has contributed to identifying the actin-bundling protein L-plastin as an important player in the clustering of the activating receptor NKG2D into lipid rafts and in NKG2D-mediated inhibition of NK cell chemotaxis (Figure V.4)

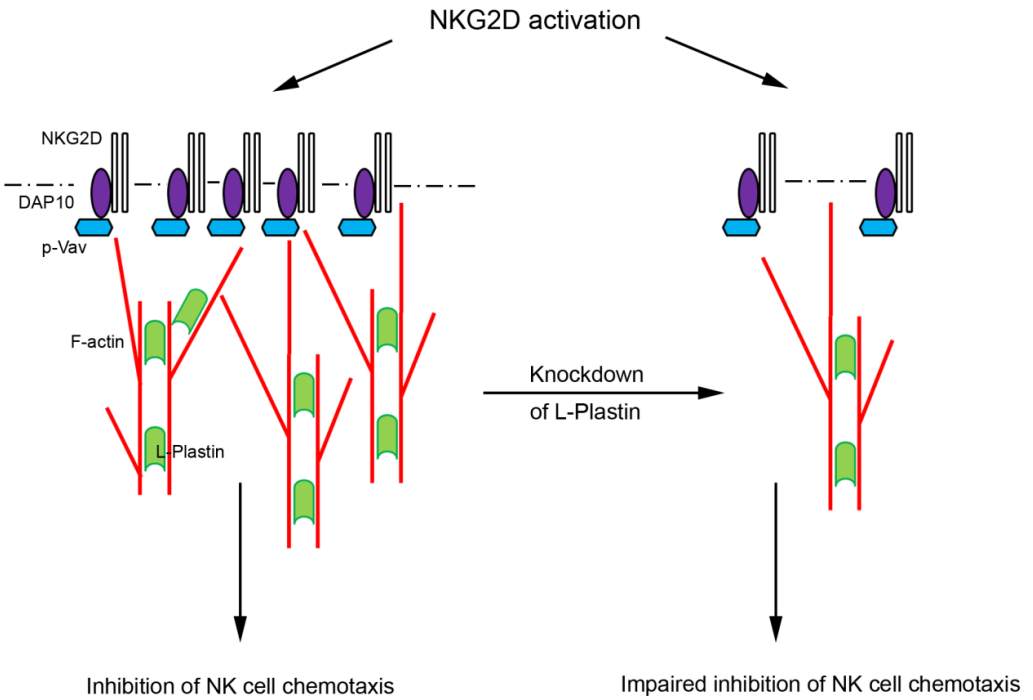


Figure V.4. L-plastin involvement in NKG2D clustering and NK cell migration. L-plastin contributes to NKG2D-DAP10 complex clustering into rafts upon NKG2D-activation and, thus, to NKG2D-mediated inhibition of NK cell chemotaxis. Depletion of L-plastin using siRNA impairs the recruitment of the receptor into lipid rafts and affects the inhibition of NK cell chemotaxis.

3. LYMPHOCYTE MIGRATION IN MULTIPLE SCLEROSIS

Multiple sclerosis is a neurodegenerative disorder mediated by the immune system. Cells of the immune system are reactive against several myelin components, which causes chronic demyelination, axonal loss, and gliosis (Lucchinetti *et al.*, 2005). To date, three hypotheses have been postulated to elucidate the etiology of MS: viral, immunological, and vascular. However, not one of them is able to fully explain the origin of the disease (Alexander *et al.*, 2010). Most of the studies have focused on the characterization of the mechanisms that mediate the loss of tolerance to myelin. Nevertheless, several research groups have gained insight into the role of the BBB in the pathogenesis of MS in the last years (Engelhardt, 2006). Given that MS has been traditionally considered as pathology mediated by CD4+ T lymphocytes (Kasper and Shoemaker, 2010), the analysis of lymphocyte migration has focused on T cells (Prat *et al.*, 1999; Prat *et al.*, 2002). However, analysis of perivascular infiltrates in patients with MS (post-mortem) (Lucchinetti *et al.*, 2000), as well as in the animal model (experimental autoimmune encephalomyelitis, EAE) (Matsumoto *et al.*, 1998), have revealed a potential role of additional cell types.

Migration of mononuclear cells across the BBB is considered to play a crucial role in the pathogenesis of MS (Compston and Coles, 2008). In this project, the simultaneous analysis of the migratory capacity of four different lymphocyte subsets from peripheral blood (CD4+ and CD8+ T cells, B cells and NK cells) was investigated in homogeneous groups of untreated patients with RRMS in the remitting phase, during relapse and in patients with CIS.

Migration experiments were performed on fibronectin-coated transwells and toward a CXCL12 gradient. Fibronectin is a major component of the basement membrane that surrounds cerebral endothelium and its expression in MS lesions is significantly increased in comparison with normal adult CNS

(Sobel and Mitchell, 1989). Its receptor on leukocyte surface VLA4 (alpha4/beta1, or CD49d/29 integrin) plays a prominent role on MS progression through its interaction with VCAM-1 at the endothelial surface, and it is a target of a monoclonal therapy in MS (Polman *et al.*, 2006; Bielekova and Beker, 2010). Regarding CXCL12, this chemokine and its receptor CXCR4 are constitutively expressed in the CNS, where they regulate several processes (Li and Ransohoff, 2008). Elevated CXCL12 levels were found in active and inactive chronic MS lesions as well as in CSF (Krumbholz *et al.*, 2006). Levels of this chemokine are also increased on astrocytes in active MS lesions (Calderón *et al.*, 2006). Moreover, it has been described that CXCL12 redistributes toward the luminal side on the parenchymal endothelial surface in MS. This relocalization was associated with CXCR4 activation in infiltrating leukocytes (McCandless *et al.*, 2008).

3.1. NK cell migration in the context of multiple sclerosis

The simultaneous analysis of the migratory ability of the four lymphocyte subsets by flow cytometry showed that chemotaxis of CD4+, CD8+, and B cells was similar in the three groups of patients and their respective control groups. However, NK cell chemotaxis was significantly increased in MS patients in the remitting phase and in CIS patients in comparison with the control groups. In contrast, the migration rate of NK cells in relapsing MS was similar to that found in healthy donors. Previous studies have explored the migratory ability of total T lymphocytes (*i.e.* CD3+ cells) through fibronectin and in an *in vitro* BBB model (Prat *et al.*, 1999; Prat *et al.*, 2002). In these reports, an increased migration rate of T lymphocytes is observed. However, it cannot be ruled out that this difference may be due to other cell subsets, such as NKT cells, which express CD3 on their cell surface. Dressel *et al.* (2007) investigated migration of CD4+ and CD8+ T cells isolated

by magnetic bead sorting, finding that the motility of CD4+ T cells was higher in MS patients. The methodological differences make no comparison to the results obtained in this project. Additionally, the potential role of chemokines in the regulation of leukocyte extravasation into CNS was not taken into account. The effect of some drugs employed in the treatment of MS in lymphocyte migration has also been addressed. Kopadze *et al.* (2006 and 2009) analyzed CD4+, CD8+, CD19+ and CD14+ migration before and after *in vitro* administration of mitoxantrone and cladribine. These treatments showed an inhibitory effect on monocytes (CD14+) and CD4+ and CD8+ T cells.

The levels of the integrin CD49d and the chemokine receptor CXCR4 were analyzed in CD4+ and CD8+ T cells and in NK cells in order to gain further insight into mechanisms involved in the migratory ability of peripheral blood lymphocytes from MS patients and patients with CIS. This approach was not feasible in a second cohort of MS patients during relapse; due to clinical management, patients received corticosteroids as soon as the relapse was diagnosed. The percentage of cells expressing CD49d and the surface levels indicated by the median fluorescence intensity were similar in patients and healthy controls. The frequency of NK cells expressing CXCR4, but not its surface levels, was markedly higher in remitting MS and CIS patients than in healthy controls, which correlates with higher NK migration rates. The role of chemokines and chemokine receptors in the pathogenesis of MS has been investigated in several studies (Sørensen *et al.*, 1999; Misu *et al.*, 2001; Uzawa *et al.*, 2010). An increase in the percentage of CD14+ cells expressing CXCR4 was found in SPMS patients compared with RRMS and PPMS patients and healthy controls, but not in CD4+, CD8+, or CD19+ cells (Martínez-Cáceres *et al.*, 2002). The surface expression of this receptor was not evaluated in NK cells. The expression of different chemokine receptors has also been studied during pregnancy in MS patients. CXCR4 was increased in CD4+ and CD8+ T cells, which may be related with anti-inflammatory responses (López *et al.*, 2006).

Given the increased percentage of CXCR4 in NK cells found in this study, the fact that in remitting MS and CIS patients the migratory ability of peripheral blood NK cells is potentially greater may contribute to a better response regulating the adaptive immune system.

The involvement of NK cells in autoimmune diseases remains unclear, with different studies reporting promoting or preventing roles (Schleinitz *et al.*, 2010). In the case of MS, the implication of NK cells in the pathophysiology of the disease has been investigated for several years. Nevertheless, whether they play a beneficial or detrimental role is still not well understood, as opposing effects were observed in the different experimental models. Deletion of NK cells in a MOG35-55-induced mild form of EAE leads to production of Th1 cytokines, T cell proliferation and disease aggravation (Zhang *et al.*, 1997). In a different experimental approach through MBP injection, antibody-mediated depletion of NK cells increased disease exacerbation (Matsumoto *et al.*, 1998). An increased EAE-related mortality in CX3CR1 KO mice, a selective mediator of NK cell recruitment to the CNS, has been described (Huang *et al.*, 2006). In addition, NK cells in the CNS may suppress inflammatory Th17 responses in EAE. On the contrary, NK cell depletion in a MOG-induced EAE reduced the clinical disease inducing changes in T cells and DC cell responses (Winkler-Pickett *et al.*, 2008). Studies in humans support a more beneficial role of NK cells in MS. NK cells express high levels of CD95 and may downregulate IFN-ɔ secretion in memory T cells in the remitting phase of MS (Takahashi *et al.*, 2001 and 2004), which suggest a role in maintaining the remission state of the disease. Certain subpopulations of NK cells have been found to play a regulatory role in MS, specifically the $CD56^{bright}$ NK cell subset. An expansion of this population is observed in MS patients with a combined treatment with IFN-ɓ and daclizumab, which correlates with a reduction in brain inflammation and secretion of anti-inflammatory cytokines (Bielekova *et al.*, 2006). Other studies have confirmed an increase in the proportion of $CD56^{bright}$ cells in

response to IFN-β treatment correlating with a positive clinical response (Saraste *et al.*, 2007; Vandenbark *et al.*, 2009; Martínez-Rodríguez *et al.*, 2011). Additionally, the ability of $CD56^{bright}$ NK cells to produce IFN-γ upon *in vitro* IL-2 stimulation is impaired in cells isolated from untreated RRMS patients (Lünemann *et al.*, 2011). Amelioration of MS during pregnancy has also been correlated with an expansion of this NK cell subset with simultaneous cessation of disease activity (Airas *et al.*, 2008). In this project, NK cells were identified as CD3-CD16+CD56+ in order to study all the subpopulations present in peripheral blood, although it would be of interest for future investigations to analyze the $CD56^{bright}$ subset and the expression of CXCR4.

The ligands for NKG2D MICA/B are expressed on the surface of oligodendrocytes in active MS lesions (Saikali *et al.*, 2007), which has been therefore proposed as a mechanism that enhances the interaction between the immune system and CNS. Activation of NKG2D receptor induces a stop signal (Culley *et al.*, 2009) and the results presented in this project demonstrate that NKG2D inhibits NK cell migration. Thus, engagement of NKG2D with its ligands might promote a stop signal in NK cells within the CNS, favoring their cytotoxic activity.

It is worth noting that the approach employed in this project represents a novelty since the handling of the samples has been minimized and none of the patients recruited for this study had received immunomodulatory or immunosuppressive treatment and had taken steroids for at least three months. Altogether, the results obtained suggest that a NK cell response is induced in the onset of MS, increasing their migratory ability and facilitating the regulation of auto-reactive T cells or activated microglia in the CNS.

VI. CONCLUSIONS

CONCLUSIONS

1.- Activation of NKG2D inhibits NK cell chemotaxis and regulates the activity of the Rho GTPases Rac1, RhoA and Cdc42.

2.- N-WASp and WASp proteins participate in the inhibition of NK cell migration in response to NKG2D engagement, and this effect is independent of the regulation of F-actin dynamics.

3.- NKG2D-mediated costimulation inhibits CD8+ T cell chemotaxis.

4.- Costimulation via NKG2D preferentially activates Cdc42/N-WASp axis in CD8+ T cells and differentially regulates actin cytoskeleton dynamics through N-WASP and cofilin, in contrast to CD28-mediated costimulation.

5.- The NKG2D-DAP10 complex is recruited to membrane rafts upon receptor activation.

6.- L-plastin is required for NKG2D recruitment into membrane rafts and for the regulation of NKG2D-mediated NK cell chemotaxis.

7.- Chemotaxis of NK cells is increased in patients with remitting MS and CIS, but not in MS patients during relapse.

8.- The percentage of NK cells expressing the chemokine receptor CXCR4 is increased in patients with remitting MS and CIS.

CONCLUSIONES

1.- La activación de NKG2D inhibe la quimiotaxis de las células NK y regula la actividad de las Rho GTPasas Rac1, RhoA y Cdc42.

2.- Las proteínas N-WASp y WASp participan en la inhibición de la migración de las células NK en respuesta a la activación de NKG2D, siendo este efecto independiente de la regulación de la dinámica de F-actina.

3.- La coestimulación mediada por NKG2D inhibe la quimiotaxis de las células T CD8+.

4.- La coestimulación vía NKG2D activa preferentemente el eje Cdc42/N-WASp y regula de manera diferente la dinámica del citoesqueleto de actina a través de N-WASp y cofilina en comparación con la coestimulación mediada por CD28.

5.- El complejo NKG2D-DAP10 es reclutado en las balsas lípidicas tras la activación del receptor.

6.- La L-plastina es necesaria para el reclutamiento de NKG2D a las balsas lipídicas y para la regulación de la quimiotaxis de células NK mediada por NKG2D.

7.- La quimiotaxis de las células NK se encuentra incrementada en pacientes con esclerosis múltiple en remisión y pacientes con síndrome clínicamente aislado, pero no en pacientes en brote.

8.- El porcentaje de células NK que expresan el receptor de quimiocina CXCR4 está incrementado en pacientes con esclerosis múltiple en la fase de remisión y en pacientes con síndrome clínicamente aislado.

VII. REFERENCES

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VIII. APPENDIX

Publications derived from this thesis:

Serrano-Pertierra, E., Cernuda-Morollón, E. and López-Larrea, C. Wiskott-Aldrich syndrome protein (WASp) and N-WASp are involved in the regulation of NK-cell migration upon NKG2D activation. 2012. *Eur. J. Immunol.* 42: 2142–2151.

Participation in congress:

2009:

September, 13–16. Berlin. *2nd European Congress of Immunology.*

Poster: 'Regulation of NK cell migration by NKG2D activation.' Serrano-Pertierra, E., Cernuda-Morollón E., López-Larrea C.

2010:

June, 23–26. San Sebastián. *XXXV Congress of Spanish Society of Immunology*.

Oral communication: 'Regulación de la migración de células NK por la activación de NKG2D.' Serrano Pertierra, E., Cernuda-Morollón, E., López-Larrea, C.

2012:

September, 5–8. Glasgow. 3rd European Congress of Immunology.

Oral communication: 'Role of Wiskott-Aldrich syndrome proteins (WASp) in NKG2D-mediated natural killer cell migration.' Serrano Pertierra, E., Cernuda-Morollón,E.,López-Larrea,C.

2013:

August, 22–27. Milan. *15th International Congress of Immunology.*

Poster: 'CD28 and NKG2D-mediated costimulation regulates CD8+ T cell chemotaxis through different signaling pathways.' Serrano-Pertierra, E., Cernuda-Morollón, E., López-Larrea, C.