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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF IREM-3, A NEW ACTIVATING MEMBER OF THE CMRF/IREM FAMILY

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To be presented for obtaining the Universitat Pompeu Fabra PhD degree. This work has been developed under the supervision of Dr. Joan Sayós Ortega in the Departament de Ciències Experimentals i de la Salut at Universitat Pompeu Fabra and the Immunobiology Unit (CIBBIM-Nanomedicine) at Hospital Universitari Vall d'Hebrón.

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A mis padres

"Cuanto más se sabe, menos se asegura"

Proverbio

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Hay que ver como son las cosas. La parte la tesis que debería ser más sencilla de escribir es precisamente la que más cuesta. Se hace difícil reflejar cinco años de tu vida en unas pocas páginas. Será porque esta aventura es a la vez la de mucha gente. Será porque nos damos cuenta de cómo cambian las cosas. Será porque el recuerdo nos ablanda y se materializa. Será…

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SUMARY

In the last years, different multigenic families of immune receptors have been identified. In this context, we find the recently described IREM/CD300 family of immune receptors which maps in the 17q25.1 chromosomical region and is restricted in expression to the cells with a myeloid origin. The human locus is composed by two genes encoding for inhibitory receptors (IRp60/CD300a and IREM-1/CD300f), two genes encoding for activating receptors (IREM-2/CD300e and IREM-3/CD300b) and two genes encoding for receptors with features that make its classification uncertain (CMRF-35/CD300c and IREM-4/CD300d). In the present work we describe the molecular characterization of IREM-3/CD300b receptor, the dissection of the intracellular signaling pathways engaged by this molecule and the functional relationship between the different IREM/CD300 family members.

RESUMEN

En los últimos años hemos sido testigos de la identificación de múltiples familias de receptores inmunológicos. En este contexto encontramos la recientemente descrita familia IREM/CD300 localizada en la región cromosómica 17q25.1 y restringida en expresión a las células de origen mieloide. El locus humano se compone de dos genes codificantes para receptores con propiedades inhibidoras (IRp60/CD300a y IREM-1/CD300f), dos genes codificantes para receptores con naturaleza activadora (IREM-2/CD300e y IREM-3/CD300b) y dos genes codificantes para receptores con características que no permiten su inclusión en ninguno de los anteriores grupos (CMRF-35/CD300c and IREM-4/CD300d). El presente trabajo describimos la caracterización molecular del receptor IREM-3/CD300b, las vías de señalización intracelular utilizadas por esta molécula y la relación funcional existente entre los diferentes miembros pertenecientes a la familia IREM/CD300.

ABBREVIATIONS

BcR	B-cell Receptor
BMMC	Bone Marrow-derived Mast Cells
CBL	Casitas B-lineage Lymphoma
CBP	Csk-Binding Protein
CD	Cluster of Differentiation
CLM	CMRF35-Like Molecule
CSK	c-Src Tyrosine Kinase
DAP-10	DNAX Adaptor protein of 10kDa
DAP-12	DNAX Adaptor protein of 12kDa
DIgR	Dendritic cell-derived Ig-like Receptor
DOK	Downstream Of Kinase
FcR	Fc Receptor
GM-CSF	Granulocyte/Macrophage-Colony Stimulating Factor
Grb-2	Growth factor Receptor Bound-2
Ig	Immunoglobulin
Ig-SF	Immunoglobulin Superfamily
ILT	Immunoglobulin-Like Transcript
IREM	Immune Receptor Expressed on Myeloid cells
IRp60	Inhibitory Receptor Protein 60kDa
ITAM	Immune receptor Tyrosine-based Activating Motif
ITIM	Immune receptor Tyrosine-based Inhibitory Motif
KIR	Killer Immunoglobulin-Like Receptor
LAT	Linker for Activation of T cells
LMIR	Leukocyte Mono-Ig-like Receptor
LPS	Lipopolysaccharide
MAIR	Myeloid-Associated Ig-like Receptor
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
NFAT	Nuclear Factor of Activated T ells
NF-kB	Nuclear Factor-κB
NK	Natural Killer Cell
NKIR	NK Inhibitory Receptor

PAG	Phosphoprotein Associated with Glycosphingolipid-enriched microdomains
PBMC	Peripheral Blood Mononuclear Cells
PEP	Polyproline-Enriched Phosphatase
PH-domain	Pleckstrin Homology-domain
PI3K	Phosphatydil Inositol 3-kinase
PIgR	Polymeric Ig Receptor
PLCγ	Phospholipase C gamma
PTB	PhosphoTyrosine Binding
RANKL	Receptor Activator of NF-kB Ligand
RBL	Rat Basophilic Leukemia
SCF	Stem Cell Factor
SH2	Src Homology-2 domain
SHIP	Src Homology-2 domain-containing Inositol Phosphatase
SHP-1/2	Src Homology-2 domain-containing protein tyrosine Phosphatase 1/2
SIGLEC	Sialic Acid Binding Ig-like lectins
SLP-76	SH2-domain containing Leukocyte Protein 76
SOS	Son Of Sevenless
Syk	Spleen tyrosine Kinase
TcR	T-Cell Receptor
TREM	Triggering Receptor Expressed by Myeloid cells
ZAP-70	ζ-chain-Associated Protein 70

INTRODUCTION

1. LEUKOCYTE RECEPTORS IN THE IMMUNE SYSTEM, BONE AND BRAIN

The immune system has arisen in organisms to discriminate between the self and foreign. Its function is to launch responses that reestablish the normal status when encountering external or internal aggressions such as pathogens or tumor cells. The immune system of vertebrates is the most complex among all organisms and is organized in layered defenses of increasing specificity. However, even simple unicellular organisms, such as bacteria, possess enzyme systems that protect them against viral infections.

The first layer of defense is constituted by physical and chemical barriers that prevent pathogens from entering the organism. When a pathogen breaches these barriers, the innate immune system provides a non-lasting and non-specific but immediate response constituted by humoral and cellular mechanisms. The humoral mechanisms include secreted inflammatory mediators and proteins belonging to the complement system (1, 2). Contrary, the cellular mechanisms require the activation of effector cells, mainly granulocytes, macrophages and natural killer cells. These cells initiate a rapid response to control pathogen spreading and proliferation in the meantime the host develops a more specific response. Innate immune cells eliminate pathogens and tumoral or infected cells through phagocytosis, enzyme release or redirected lysis processes. The great deal of these responses are triggered by pattern recognition receptors on the surface of myeloid cells. These non-clonal receptors recognize components that are conserved among broad groups of pathogens called pathogen-associated molecular patterns (PAMPs). These receptors are also able to recognize alarm signals delivered by damaged, injured or stressed cells (3-5). NK (Natural Killer) cells, despite their lymphoid origin, also participate in the innate immunity, having special importance in the defense against viruses and tumor cells (6). Innate cells and their receptors are also important mediators in the activation of the adaptive immune response that represents the third and more sophisticated layer of defense of vertebrates (7). This response is based on the selection and expansion of particular T and B lymphocytes expressing clonal receptors on their cell surface (8). The high diversity of these receptors guaranties the recognition of any strange or non-self antigen. Consequently, the adaptive immune system is able to generate tailored responses to specific pathogens or abnormal cells. The ability to mount these responses is maintained by the host due to a memory effect that is retained after the pathogen exposure and elimination. This allows the adaptive immune system to trigger faster and stronger responses each time the antigen is encountered.

A proper immune response involves the balanced participation of all the components mentioned above. When one or more components of the immune system are inactive or do not reach a proper threshold of activation, immunodeficiencies occur. Consequently, malignancies and/or pathogens are dangerously widespread in the organism leading to specific diseases. Contrary, when the immune response is exacerbate or when once activated can not be returned to the normal status, autoimmune disorders or hypersensibilities/allergies appear (8).

Immunity is driven by positive and negative signals delivered by activating and inhibitory receptors on the surface of all cells within the immune system. These receptors are responsible for the correct transduction of information from the surrounding environment to the interior of the cell. The balance of signals generated by individual receptors on a single cell is what defines its state of activation which can range from anergy to hyperactivation.

Upon interaction with their ligands, some of these receptors act by recruiting cytoplasmic tyrosine kinases triggering phosphorylation events that lead to cell differentiation, growth and survival, adhesion, migration, phagocytosis, cytokine production and/or cytotoxicity. In contrast, other immune receptors negatively regulate the activating signals by restricting their duration and/or intensity through complex and highly controlled mechanisms. Inhibitory receptors are not only important for the termination of immune responses. They are crucial in the delivery of continuous "off" signals that warranty the tolerance to self and for preventing the excessive activation of immune cells even when activating signals should prevail. This is of special importance in cells involved in innate immunity responses, because natural-killer cells, myeloid cells, and other effector cells recognizing self do not undergo a deletion process during maturation (9).

Each cell expresses simultaneously different activating and inhibitory receptors, which confers complementary and alternative mechanisms to control activation in response to different ligands. Generally, activating and inhibitory receptors recognize different ligands, but in some cases activating and inhibitory receptors with high homology within their extracellular regions can bind the same ligand, although with different affinity (10).

Both, activating and inhibitory receptors are composed by a variable number of extracellular domains, a transmembrane region and a cytoplasmic tail. Structural features concerning the extracellular region determine the classification of these receptors into two morphological superfamilies: the immunoglobulin-like superfamily and the C-type lectin superfamily (11, 12).

Leukocyte receptors and many of the molecules involved in the early steps of their signal transduction pathways are present in cells with a myeloid origin but homing tissues different from the classically considered. This is the case for microglia and osteoclasts.

Microglial cells are the macrophages of the central nervous system, but they fulfill a great variety of tasks related not only with the immune response but with the preservation of the brain homeostasis as well. Microglial cells derive from bone marrow hematopoietic stem cells which upon achieving the stage of monocytes migrate to the brain, where they settle and further differentiate into microglia (13). Microglial cells are in charge of the clearance of damaged neurons, amyloid plaques and infectious agents. Like macrophages, microglia use phagocytosis to achieve this aim. However, these cells are also able to launch cytotoxic responses by means of two different pathways. First, by secreting glutamate and aspartate that specifically eliminate injured neurons through NMDA-receptor mediated processes, and second, by releasing large amounts of proteases and reactive oxygen species in a process that has been named respiratory burst. Additionally, they contribute to the immune response by acting as antigen presenting cells to the infiltrating T lymphocytes that migrate to the brain during an inflammatory process. The microglial homeostatic effects comprise functions such as the induction of apoptosis in specific subpopulations of developing neurons, the control of synaptogenesis, the synthesis of neurotrophic factors, the regulation of synaptic transmission and the promotion of endothelial cell proliferation for vascular maintenance. Moreover, for a correct function of the nervous system, microglial cells need to establish fine communication networks with other cells within the glia, such as oligodendrocytes and astrocytes (14-16).

Osteoclasts are one of the cell types in the body involved in controlling the amount of bone tissue. In particular, these cells are in charge of removing bone by degrading its mineralized matrix in a process known as bone resorption (17). Osteoclasts are derived from promyeloid precursors in the bone marrow when exposed to RANKL (Receptor Activatior of NF-κB Ligand) and M-CSF (Macrophage-Colony Stimulating Factor), two soluble proteins produced by the neighbouring stromal cells and osteoblasts. These cytokines elicit cell fusion of the promyeloid precursors to form giant multinucleated cells and the acquisition of enzymes such as cathepsins and matrix metalloproteases that are essential for osteoclasts function (18).

In consequence, the study of leukocyte receptors should be extended to other tissues beyond the immune system, particularly for those receptors restricted or highly represented in the myeloid lineage.

1.1 INHIBITORY RECEPTORS

Inhibitory receptors display long cytoplasmic regions with tyrosine-based motifs that serve as docking sites for signaling molecules. Near all of these tyrosines fit the consensus for ITIM motifs (Immune receptor Tyrosine-based Inhibitory Motif), whose canonical sequence is

V/I/LxYxxL/V, where x denotes any amino acid (19, 20). The requirement for a specific amino acid at position -2 relative to the tyrosine is unusual among recognition motifs. The inhibitory function of many receptors has been deduced on the basis of the presence of cytoplasmic ITIMs on their sequences. Quite recently, a stringent search for potential ITIM-bearing receptors in the human proteome identified 109 candidates, of which only 36 have been described as inhibitory receptors (21).

The ITIM was originally defined as a tyrosine-containing sequence in the cytoplasmic tail of FcγRIIb which caused inhibition of B-cell activation upon co-engagement with the BcR (B-cell Receptor) (22). Later, the functional characterization of an inhibitory KIR (Killer Ig-like Receptor) showed that the phosphotyrosines on the ITIM motifs were able to bind phosphatases that were responsible for the inhibitory effects observed (23). At the present, it is well established that after engagement of inhibitory receptors by their ligands, ITIM tyrosine residues become phosphorylated, often by a Src family tyrosine kinase, which provides a docking site for the SH2 domain-bearing phosphatases SHP-1, SHP-2 and SHIP (24). Once recruited, tyrosine phosphatases become activated and dephosphorylate key signaling mediators and as consequence downregulate signaling cascades.

Inhibitory receptors and inhibitory processes are not simple to rule and evidence that SHP and/or SHIP play a role in the function of ITIM-containing receptors function is still missing in most cases. A great number of inhibitory receptors display multiple ITIM motifs in their cytoplasmic region what increases the number and the combination of molecules that can bind simultaneously the receptor, and in some cases ITIM coexist with activating motifs (25, 26). Additionally the ITIM status of phosphorylation is variable and thus, receptor triggering does not always result in phosphorylation of all the tyrosine residues contained in these motifs. Some receptors, such as PIR-B or LAIR-1/CD305, have been found to be constitutively phosphorylated and associated to phosphatases (27, 28). But others, as shown for ILT2/CD85j (Immunoglobulin-like transcript), exhibit regulation of ITIM phosphorylation by tyrosine residues outside of ITIM sequences (29). Furthermore, some receptors with inhibitory function recruit phosphatases through phosphorylated tyrosines that are not within typical ITIM motifs. This is the case for TNF-R, TRAIL and the death receptor Fas which bind SHP-1, SHP-2, and SHIP molecules through a YxxL motif in their cytoplasmic tails (30).

Invariably, phosphatases recruited by inhibitory receptors block activating signals by interfering at the earlier steps of the activating response and shutting down all subsequent events. However, ITIM-mediated inhibition is only local and transient. It does not induce a cell-wide or sustained non-responsiveness, but abrogates activating signals when and where they occur (31).

The negative regulation of the immune function is mainly controlled by tyrosine phosphatases, but other molecules such as Csk (c-Src Tyrosine kinase), Cbl (Casitas B-lineage Lymphoma) or Dok (Downstream Of Kinase), provide complementary ways for interfering with cell activation.

1.1.1 SHP-1 and SHP-2

SHP-1 and SHP-2 (Src homology-2 domain-containing protein tyrosine phosphatase) are two related cytoplasmic protein tyrosine phosphatases. In addition to the catalytic subunit, both molecules contain two tandem amino-terminal SH2 domains (Fig. 1A). While SHP-1 is restricted to hematopoietic and epithelial cells, SHP-2 is expressed ubiquitously. Despite the high homology between both phosphatases, selective binding is often observed with ITIM-receptors. The functional significance of this selectivity is unclear, but structural data suggest that recruitment of SHP-1 has a different outcome on signaling than recruitment of SHP-2. It has been hypothesized that these differences in the negative signaling exerted by SHP-1 and SHP-2 are relevant enough for receptors with an inhibitory function.

The activity of SHP phosphatases is primarily regulated by conformational modifications. The binding of SHP phosphatases to inhibitory receptors induces the phosphorylation of two regulatory tyrosines at the C-terminus of the protein and a subsequent release to the cytoplasm. Each one of these tyrosines has the potential to form an intramolecular interaction with one of the two SH2 domains present in the phosphatase. But only when both tyrosine residues are engaged, a conformational change is promoted and the enzyme becomes fully active. The spacing between the C-terminal tyrosines in SHP-1 is too short to allow the simultaneous binding of both SH2 domains. SHP-1 dissociation results in two mutually exclusive forms of the phosphatase, one of which is catalytically inactive. As consequence, SHP-1 is thought to be more active when bound to the inhibitory receptor than when released to the cytoplasm (32). In contrast, the longer spacing between the C-terminal tyrosines of SHP-2 is compatible with intramolecular engagement of both tyrosines residues with the N-terminal tandem SH2 domains (33). Therefore, SHP-2 has the potential to efficiently dephosphorylate its substrates while it is no longer bound to the inhibitory receptor as it is always released in a fully catalytically active conformation. In fact, even a transient association of SHP-2 with a single phosphorylated ITIM results in the release of a completely active SHP-2 enzyme. Oppositely, SHP-1 phosphatase has been shown to require two phosphorylated ITIMs in the receptor to which it binds.

SHP-1 substrates include a great variety of proteins, being of special importance the ITAM-containing proteins, Src and Syk family of tyrosine kinases, PI3K (Phosphatydil Inositol 3-Kinase) and LAT (Linker for Activation of T cells) and SLP-76 (SH2-domain containing Leukocyte Protein 76) adaptor proteins. LAT and SLP-76 molecules work by recruiting downstream signaling molecules like PLCγ (Phospholipase Cγ), Grb-2 (Growth factor receptor bound-2), Vav, PI3K and Cbl, all of them involved in activating signal transduction pathways (34) (Fig. 1B). SHP-1 has also been shown to associate with paxillin, vimentin and actin in activated macrophages, thus involving these phosphatases in regulating the adhesion properties of myeloid cells (35).

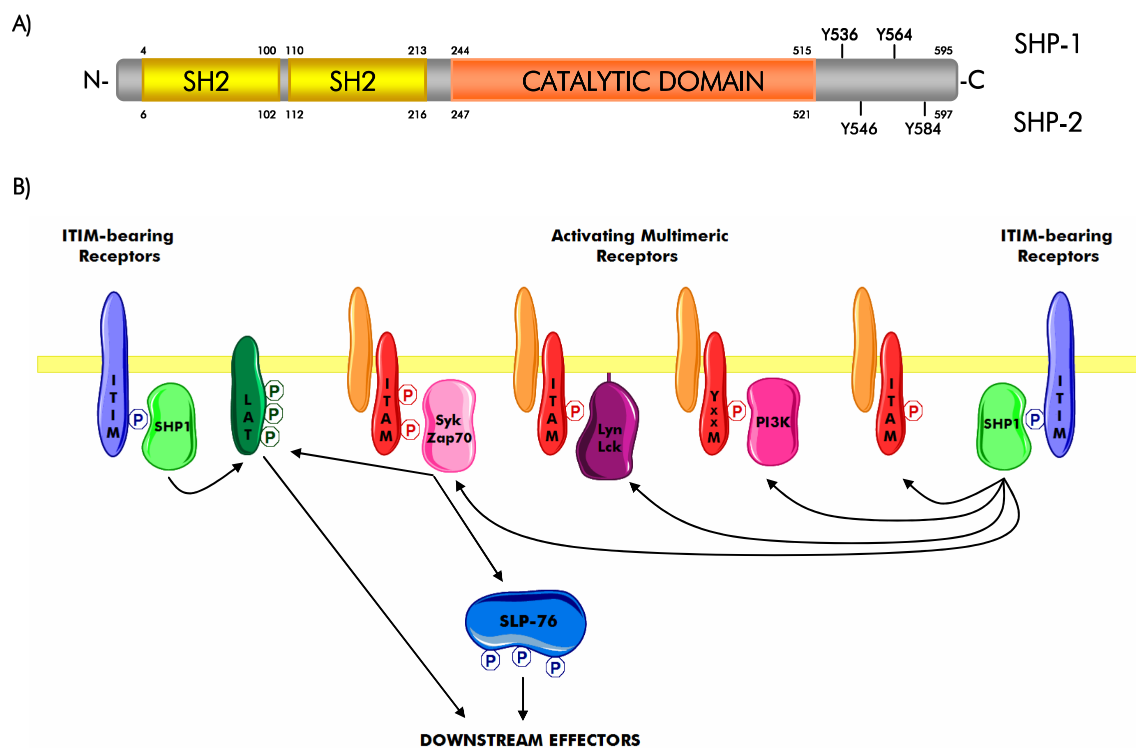

Figure 1: SHP-1/2 SH2-bearing phosphatases. (A) Domain organization of SHP-1 and SHP-2 phosphatases. Domains are shown in colored boxes and amino acid positions are noted. Regulatory tyrosines on the C-terminal region are shown. SH2: Src Homology-2 domain. **(B)** Model of operation for ITIM-bearing inhibitory receptors through SHP-1/2 molecules: upon receptor engagement and activation of Src family protein tyrosine kinases, the tyrosine residues in ITIMs are phosphorylated and recruit the tandem SH2 domains of the protein tyrosine phosphatases SHP-1 and SHP-2. These molecules are responsible of the dephosphorylation of key signaling molecules such as ITAM-bearing molecules, Src and Syk family tyrosine kinases and LAT and SLP-76 adaptors proteins.

A central role of SHP-1 as a negative regulator of signal transduction in the immune function, has been provided by the study of *motheaten* (*me*) and *viable motheaten* (me^{v}) mice, in which SHP-1 gene is spontaneously mutated (36). In both cases, the protein defect is caused

by a point mutation in one of the gene's splice site. *me* mice die prematurely, usually due to severe hemorrhagic pneumonitis. *me*^{v} mice succumb from a similar disease but at later age, probably as consequence of a low residual SHP-1 activity. Both strains exhibit severe abnormalities in multiple hematopoietic lineages (37). Of special relevance is the accumulation of myeloid-origin cells in tissues. Macrophage and granulocyte accumulation in lungs promotes severe pulmonary injuries that are thought to be the cause of death of these animals. Additionally, they develop a profound systemic autoimmune syndrome characterized by polyclonal B cell activation, hypergammaglobulinemia and production of autoantibodies.

SHP-2 role in inhibitory function is less well defined and in fact it has been shown to be often involved in activating signaling cascades (38). SHP-2 activating signals are thought to occur by means of increasing the half-life of activated Ras in the cell and by forming complexes with Grb-2 and Sos (Son Of Sevenless) molecules (39). In this context, SHP-2 is though to act as an adaptor protein by virtue of its tandem SH2 domains rather than as a catalytic enzyme (40).

Gain of function mutations in SHP-2 phosphatase have been identified in Noonan syndrome and various childhood leukemias (41, 42). These mutations cause hyperactivation of SHP-2 catalytic activity, drastically increasing the spreading and migration potential of various cell types, including fibroblasts, hematopoietic and endothelial cells with the concomitant development of aberrant angiogenic processes (43).

1.1.2 SHIP-1 and SHIP-2

SHIP phosphatases (Src homology-2 domain-containing inositol phosphatases) are present in all cells from the hematopoietic system, but SHIP-2 is expressed more ubiquitously and it can be found in most somatic tissues and cell types.

SHIP proteins are composed by a single N-terminal SH2 domain, a central lipid phosphatase domain and a long C-terminal region containing several motifs involved in protein-protein interactions (44)(Fig. 2A). SHIP-1 C-terminus bears two tyrosines contained within NPxY motifs, while SHIP-2 bears only one. These motifs, when tyrosine phosphorylated, provide putative binding sites for PTB (PhosphoTyrosine Binding)-containing proteins but also constitute the recruiting elements for SH2 domain-containing proteins. The C-terminus of SHIP-1/2 additionally contains several proline-rich regions that create docking sites for SH3 domain-bearing proteins. Due to these structural features, SHIP phosphatases function as adaptor proteins, linking cell membrane components to inner cytoplasmic signaling mediators. NPxY motifs have shown to be important in the interaction of SHIP-1 phosphatase with PI3K and the

Shc and Dok adaptor proteins, whereas proline-rich domains have been involved in Grb-2 and Src-kinase recruitment (45-48).

Different splicing and proteolytic variants have been identified for SHIP-1. Interestingly, the expression of these isoforms differs among cell types and stage of maturation suggesting unique roles for each of them (49).

SHIP-1 and SHIP-2 phosphatases are involved in the inhibition of immune receptor signaling. They operate orchestrating the phosphorylation status of membrane-associated lipids such as $PI(3,4,5)P_3$, $PI(3,4)P_2$ and $I(1,3,4,5)P_4$. SHIP proteins specifically dephosphorylate these lipids at the 5' position of the inositol ring. Phosphoinositides are implicated in membrane recruitment and activation of PH (Pleckstrin Homology)-domain containing effectors such as Btk kinases, PLCγ and the serine/threonine-specific protein kinase Akt (24). The effects on PLCγ are of special interest, because this enzyme leads to the production of IP_3 and DAG which stimulate calcium mobilization, as well as PKC (Protein Kinase C) and Ras-MAPK (Mitogen-Activated Protein Kinase) signaling pathways. Thus, recruitment of SHIP proteins by immune receptors and subsequent dephosphorylation of lipids, provides an efficient mechanism for inhibiting activating signals (Fig. 2B). Although expressed by all hematopoietic cells, SHIP-1 constitutes a potent negative regulator of B cells, mast cells and macrophages (44).

SHIP1-deficient mice are viable but exhibit a remarkable decrease in viability, primarily due to the infiltration of macrophages and neutrophils into the lungs. They also display abnormalities in the numbers and behavior of many hematopoietic-derived cells. Particularly, they have a significant increase in the percentage of mature myeloid lineage cells in peripheral lymphoid organs and a decrease in the percentage of lymphocytes. A similar effect is observed in bone marrow, suggesting that the distortions in the hematopoietic cell numbers occur throughout the maturation process of progenitors rather than in the proliferation of the mature cell compartment (50).

The role of SHIP-2 in the immune receptor signaling is poorly understood. However, through the creation of SHIP2-deficient mice, it has been established that SHIP-2 is not absolutely required for the immune cell development (51). This has been attributed to a functional compensation by SHIP-1. However, these mice develop increased insulin sensitivity, supporting the role of SHIP-2 as a negative regulator of cell signaling also outside the immune system.

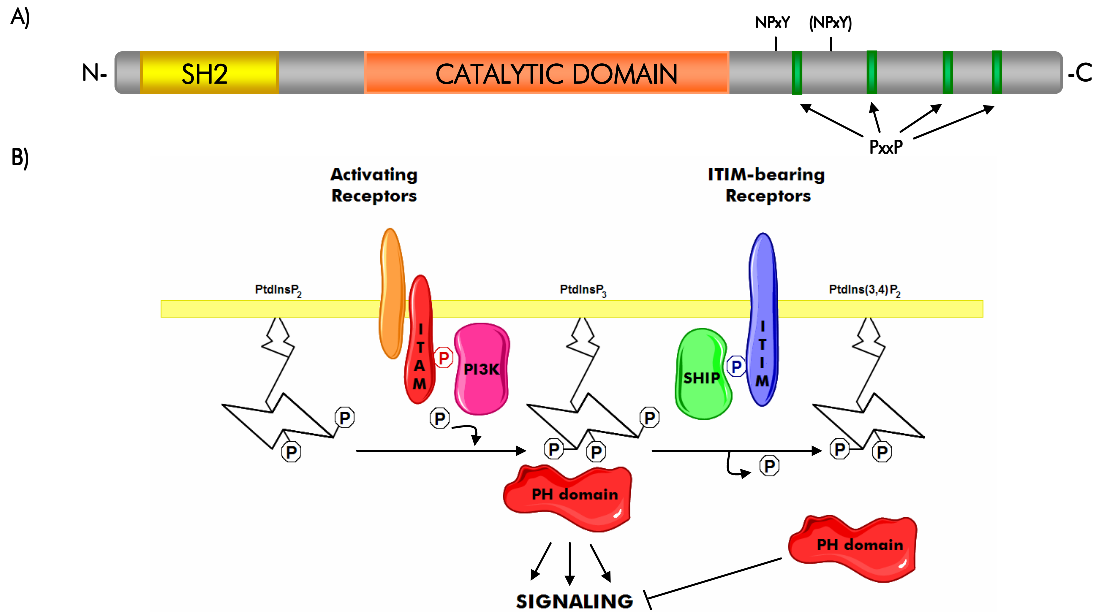

Figure 2: SHIP-1/2 phosphatase. (A) Domain organization of SHIP-1 and SHP-2 phosphatases. Domains are represented by colored boxes. The NPxY motif in brackets is absent in SHIP-2 molecule. SH2: Src Homology-2 domain. **(B)** Model of operation for ITIM-bearing inhibitory receptors through SHIP-1/2 phosphatases: ITIM motifs on inhibitory receptors recruit SHIP phosphatases that are able to dephosphorylate membrane bound inositol lipids. In absence of PIP3, PH-domains-bearing proteins, such Btk, Akt or PLCγ, are released from the plasma membrane and as consequence activating signaling pathways are blocked.

1.1.3 CSK

Csk (c-Src tyrosine Kinase) is a cytoplasmic protein tyrosine kinase expressed in all cell types but with a greater accumulation in hematopoietic cells. Most protein kinases in the cell phosphorylate a wide range of tyrosine-containing substrates, but Csk is known to be highly selective as it only phosphorylates proteins belonging to the Src family of kinases. Furthermore, it exclusively phosphorylates the inhibitory tyrosine contained within the C-terminal region of these molecules forcing them to assume a "closed" and inactive conformation. The basis for this selectivity is still unknown but it is likely that involves complex docking interactions dependent on the three-dimensional structural properties of both proteins (24).

Csk is composed by amino-terminal SH3 and SH2 domains and a carboxy-terminal catalytic subunit. This structure is very similar to Src kinases, but Csk is devoid of the myristoylation signal, the site of autophosphorylation at the catalytic unit and the carboxy-terminal inhibitory tyrosine (52)(Fig. 3A). As consequence the mechanisms regulating Csk function are totally different from those governing Src kinases.

Csk SH3 region interacts constitutively with the PEP phosphatase (Polyproline-Enriched Phosphatase). The Csk-PEP complex efficiently inactivates Src kinase-mediated signaling as a result of its dual ability to phosphorylate the inhibitory tyrosine of Src kinases and dephosphorylate their positive regulatory site (53).

Given that all Src tyrosine kinases are membrane-anchored molecules, Csk needs to translocate to the plasma membrane to develop its inhibitory function. This process is mediated by binding of Csk to membrane proteins and intracellular adaptors through its SH2 domain. The major Csk SH2 domain-binding protein is Cbp/PAG (Csk-Binding Protein/Phosphoprotein Associated with Glycosphingolipid-enriched microdomains), a lipid-raft-associated molecule with an important role in the initial steps of the TcR (T-cell Receptor) signaling (54). But Csk has also been found to bind caveolin-1, a molecule involved in extracellular matrix remodeling, and several focal adhesion-associated proteins such as paxillin, tensin and FAK (Focal Adhesion Kinase) (55-57). As consequence, Csk recruitment by these proteins restricts the duration of triggering signals and regulates cell–cell contacts and matrix adhesion processes (Fig. 3B). Additionally, Csk SH2 domain is able to bind tyrosine residues within ITIM and/or ITIM-like motifs present in the cytoplasmic region of inhibitory receptors. This is the case for CD85j/ILT2 and CD305/LAIR-I leukocyte receptors, which mediate their inhibitory function by combining the classical SHP/SHIP pathway with Csk signaling (58, 59).

There are inhibitory signals that have found to be sustained in basal conditions by Csk molecule. For instance, in resting T-cells, Csk is constantly targeted to the plasma membrane to inhibit Src kinases activity. Even in the absence of extracellular positive signals, Csk associates with Cbp/PAG in lipid rafts to maintain the activity of Lck and Fyn at the basal level. This tonic negative control is transiently relieved upon T cell activation and reacquired upon initiation of the intracellular signaling cascade to prevent excessive activation of these Src kinases (60, 61).

All together make Csk to be a potent inhibitor of immune receptor signaling, specially in T cells and macrophages. Concordantly, Csk knock out mice die at the early stages of embryonic development as a result of hyperactivation of Src kinases, which causes sever neural tube defects (62, 63).

A)

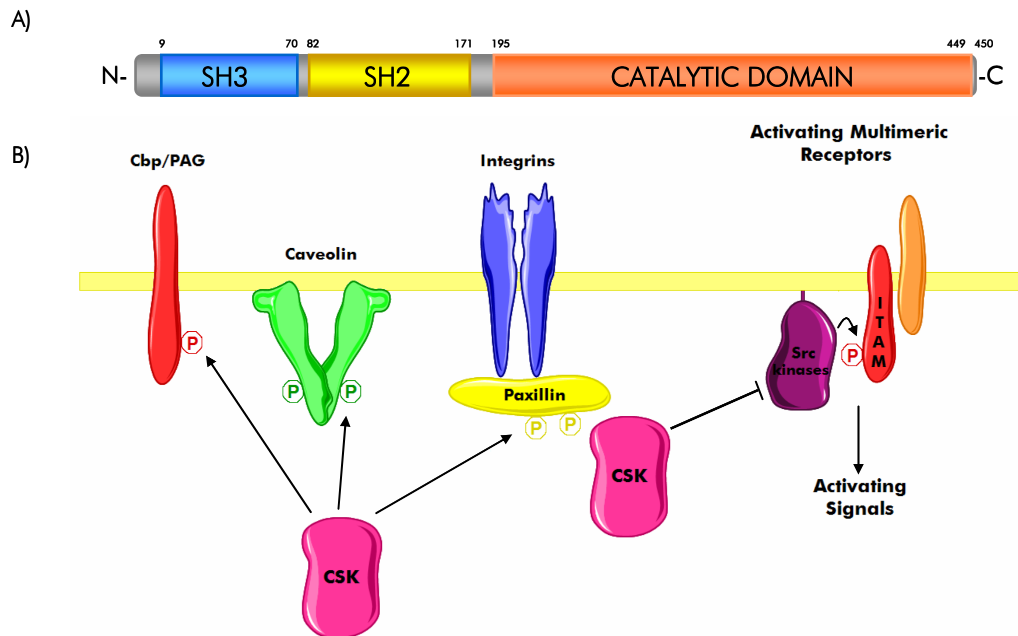

Figure 3: Csk kinase. (A) Domain organization of Csk kinase. Domains are represented by colored boxes and amino acid positions are noted. SH3: Src homology-3 domain, SH2: Src homology-2 domain. **(B)** Model for Csk inhibition: CSK is recruited to the plasma membrane by binding to specific tyrosine phosphorylation sites in transmembrane receptors and/or adaptor proteins. This allows Csk to become in close vicinity to Src family of tyrosine kinases which initiate the first triggering steps of cellular activation. By means of tyrosine phosphorylation, Csk induces a conformational change in Src kinases that blocks their catalytic activity and as consequence interrupts the propagation of activating signals.

1.1.4 CBL

Immune receptor signaling can also be inhibited by interfering with the function of critical mediators through selective protein degradation or steric hindrance. Cbl family of proteins (Casitas B-lineage lymphoma) are ubiquitin ligases that ubiquitinate and target several signaling molecules to the proteasome pathway (64). Src and Syk family of tyrosine kinases constitute the main substrates for Cbl catalytic activity. Moreover, Cbl proteins carry out important inhibitory functions modifying the interaction properties of key signaling components such as SLP-76, Vav, PI3K, Grb-2 and PLCγ (65).

Structurally, Cbl proteins consist of an N-terminal tyrosine-kinase-binding domain which mediates the binding to phosphotyrosine residues; a ring finger motif involved in the E3-ubiquitin ligase activity; a proline-rich region; and a C-terminal ubiquitin-associated domain (UBA) that overlaps with a leucine zipper motif (LZ). The tyrosine-kinase-binding domain (TKB) is in turn composed of three subdomains, a four-helix bundle (4H), two EF-hand folds and an SH2 domain (65)(Fig 4). It is well known that the interaction between Cbl molecules and Syk/Zap-70 kinases, takes place between the SH2 domain of Cbl and a conserved tyrosine

residue in the linker region of these kinases (24). The C-terminal proline-rich region facilitates most of the other associations observed for Cbl (66).

There are three mammalian Cbl proteins: c-Cbl, Cbl-b and Cbl-c. Their structure is highly conserved except in the C-terminal region which exhibits a variable length that determines the function of these proteins as adaptor molecules. c-Cbl and Cbl-b, but not Cbl-c, are found in immune cells (67).

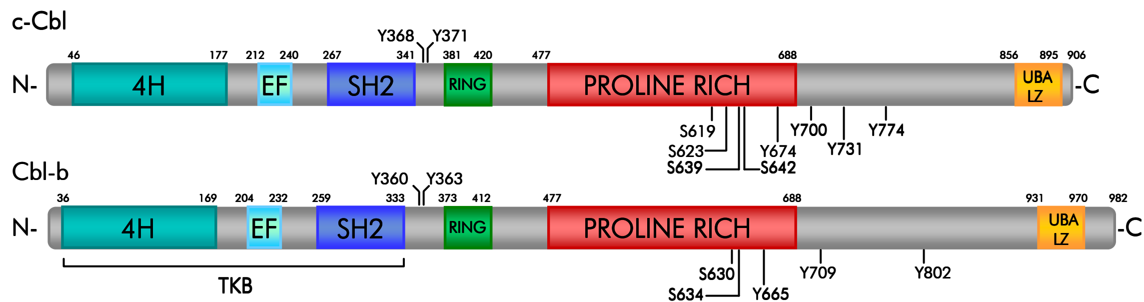

Figure 4: Cbl proteins domain organization. Only c-Cbl and Cbl-b are represented as per their expression on immune cells. The different domains are represented by colored boxes and the amino acid positions are noted. Regulatory residues are shown. 4H: 4 Helix-bundle, EF: EF-hand fold, SH2: Src Homology-2 domain, UBA/LZ: Ubiquitin-associated domain/leucine zipper.

Cbl proteins are primarily present in the cytoplasm but they can be targeted to the membrane and cytoskeleton. This is of particular importance for the inhibition of Src family of tyrosine kinases which are membrane-bound proteins. Thus, localization of Cbl proteins determines some of their biological effects. Translocation of Cbl proteins to the membrane has been shown to be regulated by tyrosine phosphorylation (68). Tyrosine regulatory sites are mainly localized within the linker that connects the ring domain with the TKB domain, but additional important tyrosine residues have been found spread all along the C-terminal region (Fig. 4). It has been shown that in response to stimulation of immune receptors, including TcR, BcR, FcR (Fc Receptor) and integrins, there is an enrichment of tyrosine phosphorylated Cbl in the plasma membrane (69). These tyrosine residues have also been found to be important for regulating the E3 activity, particularly in the Src kinase degradation function (70). It has been hypothesized that Cbl proteins are inactive until they encounter the kinase to be degraded. This occurs by autoinhibition mechanisms that the N-terminal region exerts on the E3 activity. The interaction between the enzyme and the activated kinase promotes the phosphorylation of the regulatory tyrosine residues within the linker region and concomitantly a structural rearrangement that switches on the ubiquitinase activity.

It is of note that ubiquitination also serves to trigger internalization and degradation of plasma membrane proteins in the lysosomes. Therefore, Cbl does not only promote

termination of signaling by degrading proteins but by inducing the endocytosis of activated receptors and their associated signaling complexes as well (71).

Inhibition of the immune signaling by Cbl acting as an adaptor protein, is mainly achieved by sequestering key signaling molecules involved in cellular activation events. For instance, Grb-2 binding to Cbl competes with the guanine-nucleotide-exchange factor Sos what blocks signaling through the MAPK pathway and inhibits cell proliferation (64).

Accordingly with the inhibitory role of these proteins, Cbl deficiencies promote the development of immunological disorders, particularly autoimmune diseases such as type I diabetes and systemic lupus (72-74). Moreover, Cbl knock out mice exhibit significant defects in the formation of the bone marrow cavity, manifesting the important role of antigen receptor signaling in hematopoietic cells homing tissues outside the immune system (66, 75).

1.1.5 DOK

The Dok (Downstream Of Kinase) family of adaptors molecules comprises five members. Dok-1, Dok-2 and Dok-3 are particularly abundant in hematopoietic cells but differ in their cellular distribution. Whereas Dok-1 accumulates in most hematopoietic cells, Dok-2 is present in T cells, mast cells, and macrophages, but not in B cells. Oppositely, Dok-3 abounds in B cells, mast cells and macrophages, but not in T cells. Dok-4 and Dok-5 proteins are found mainly in nonhematopoietic cells (24).

Dok molecules are composed by an N-terminal PH-domain, a central phosphotyrosine-binding domain (PTB) and a C-terminal region bearing several sites of tyrosine phosphorylation, that as seen form many proteins within the immune system, serve to regulate the function of these proteins (24)(Fig. 5). These tyrosines are rapidly phosphorylated by Src kinases upon immune receptor-mediated cellular activation. Dok proteins structure allow them to engage tyrosine phosphoryated proteins to inhibitory SH2-bearing molecules. This has been observed in response to a wide range of stimuli such as triggering of cytokine receptors, TcR, BcR and FcR. Among the negative regulators recruited by Dok proteins, are of special importance the previously described SHP-1, SHIP and Csk molecules, but also the Ras-GAP protein which interferes with ERK, JNK and p38 MAPK signaling pathways (76-81).

Some immune receptors displaying cytoplasmic tyrosine residues outside the ITIM consensus, such as CD200, have been found to mediate their inhibitory effects by recruiting Dok adaptor proteins (82).

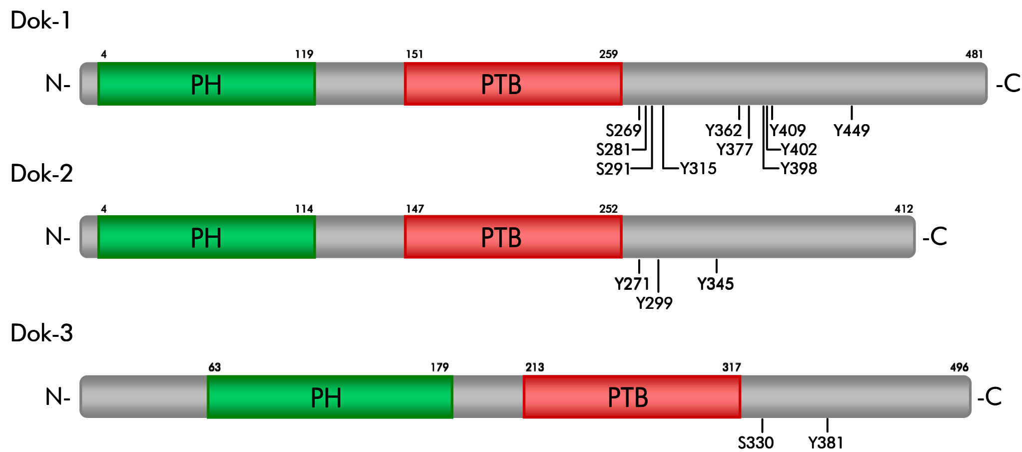

Figure 5: Dok proteins domain organization. Domains are represented by colored boxes. Amino acid positions for the different domains are noted and regulatory residues are shown. PH: pleckstrin-homology domain, PTB: phosphotyrosine-binding domain.

1.2 ACTIVATING RECEPTORS

Activation within the immune system takes place through cell surface oligomeric complexes. These complexes share an architecture similar to that of antigen and antibody receptors. Triggering receptors, which constitute the ligand recognition structure in the oligomer, display short cytoplasmic tails devoid of signaling motifs and are non-covalently coupled to specialized transmembrane signaling polypeptides (34). Contrary, these molecules have minimal extracellular binding domains and are not thought to have ligand-binding capacity on their own. The association between receptors and adaptor molecules occurs through a membrane-embedded salt bridge affecting a basic residue on the receptor (lysine or arginine) and an acidic residue on the adaptor protein (aspartic acid). Adaptors are able to mediate signal transduction by means of tyrosine-based modules present in their cytoplasmic tails. To date, two types of transmembrane adaptor proteins have been described: the ITAM-bearing adaptor proteins and the PI3K-associated adaptor DAP-10 (DNAX Adaptor protein of 10kDa) (6, 83). The first group is constituted by CD3ζ, FcRγ and DAP-12 (DNAX Adaptor protein of 12 kDa) molecules which harbor intracytoplasmic ITAM sequences (Immune receptor Tyrosine-based Activation Motif) which fit the canonical sequence YxxL/I$x_{6\text{-}8}$YxxL/I, where x denotes any amino acid. In contrast, DAP-10 displays an intracytoplasmic YxNM motif capable of recruiting the p85α regulatory subunit of the PI3K and the intracellular adaptor molecule Grb-2.

Upon aggregation of cell surface activating receptors, tyrosine kinases belonging to the Src family become activated leading to ITAM phosphorylation. This allows SH2 tandem protein

tyrosine kinases from the Syk family (Syk and ZAP-70) to be recruited to the cell membrane and become activated (83). Syk and ZAP-70 play major roles in early signaling events by phosphorylating crucial adaptor proteins including LAT (linker for activation of T cells), SLP-76 (SH2-containing leukocyte protein of 76 kDa) and BLNK (B-cell linker protein). The main function of these adaptors resides in their capacity to provide multiple tyrosine-phosphorylated docking sites for intracellular signaling molecules such as Grb-2, PLCγ, PI3K, Cbl, 3BP2 and Vav (34). The signaling through DAP-10 requires tyrosine phosphorylation of the YxNM motif by Src kinases, but subsequent events are independent of Syk family of kinases (84). DAP-10 signaling pathway includes PI3K, Grb-2, Rho family GTPases and PLCγ molecules (83). Both, ITAM-dependent signaling and DAP10-dependent signaling converge on a common pathway involving the activation of Rac, which sequentially leads to activation of the MAPK pathway constituted by PAK (p21-activated kinase), MEK (mitogen-activated or extracellular signal-regulated protein kinase) and ERK (extracellular signal-regulated kinase) kinases (Fig. 6). Both activating signaling pathways often lead to activation of transcription factors, including ELK-1(Ets Like Gene-1), AP1 (Activator Protein 1), NFAT (Nuclear Factor of Activated T cells) and NF-κB (Nuclear Factor- κB), which transcribe genes that encode for pro-inflammatory cytokines/chemokines and cell-surface molecules (85).

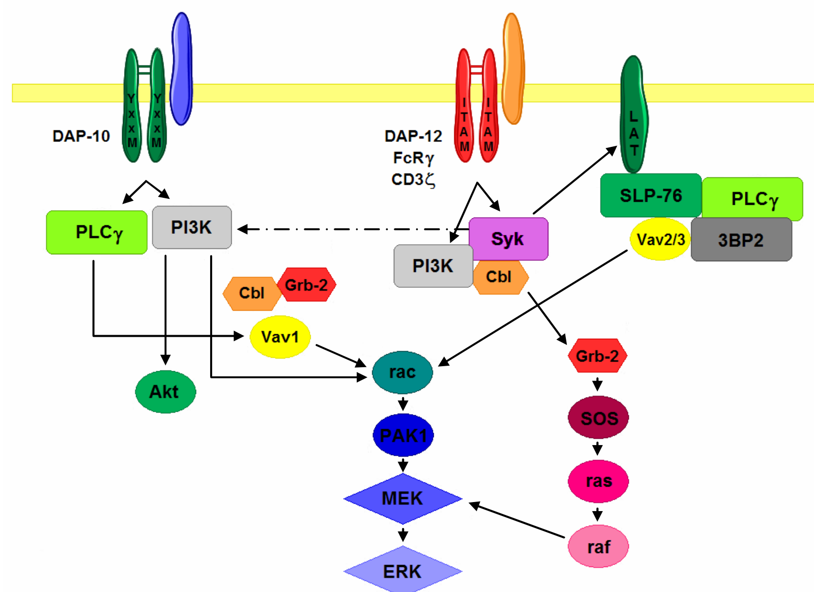

Figure 6. Signaling pathways triggered by activating immune receptors. Schematic representation of the signal transduction pathways initiated upon engagement of oligomeric activating receptors coupled to ITAM-bearing polypeptides (DAP-12, FcRγ and CD3ζ) and DAP-10.

Activating immune receptors belong to two evolutionary distinct protein superfamilies, the immunoglobulin-like and the C-type lectins, which differ not only in their primary sequence

and tertiary structure but also in the orientation of the transmembrane domains. The N-terminus is located in the extracellular space for the Ig-like family receptors (type I membrane proteins), while in the cytoplasm for C-type lectin receptors (type II membrane proteins). Despite this difference, the transmembrane domains of all these receptors carry a basic residue (lysine or arginine) which surprisingly is positioned at the same distance from the outer surface of the cell. This basic residue allows activating receptors to associate with signaling polypeptides bearing an acidic transmembrane residue, which invariably corresponds to an aspartic acid. Curiously, DAP-12 adaptor molecule has a strong preference for lysine over arginine and all receptors known to date to assemble with DAP-12 have a transmembrane lysine. Conversely, FcRγ has a preference for arginine over lysine and consistently, all receptors paired with this transmembrane adaptor protein bear an arginine within its transmembrane domain.

The charged residue on signaling polypeptides is located either close to the center (DAP-10 and DAP-12) or in the upper third of the transmembrane domain (CD3ζ and FcRγ), matching the location of the basic transmembrane residue on activating receptors (86-88)(Fig. 7). Adaptor polypeptides are all disulphide-linked dimers. Dimerization of these molecules has been hypothesized to occur to amplify activating signals, since clustering of tyrosine-based motifs constitutes an efficient way of increasing local concentrations of intracellular signaling molecules (89). Some of these polypeptides, such as CD3ζ, contain more than one tyrosine-signaling module within each chain what reinforces this theory. However, dimerization occurs differentially. CD3ζ and FcRγ form disulphide-linked dimers through a cysteine residue at transmembrane position 2, which is only one helical turn away form the aspartic acid required for the receptor assembly. Contrary, DAP-10 and DAP-12 dimerize through two disulphide bonds in the extracellular domain (90). These structural features have made the signaling dimers to be classified into two different groups.

Dimerization of transmembrane adaptors makes that the association with activating receptors results in the formation of a three-helix interface containing one basic and two acidic transmembrane residues. Both acidic residues within the adaptor dimer are critical for the assembly with activating receptors (90).

The ionizable residues within the transmembrane domains are sufficient to mediate the assembly between activating receptors and adaptor polypeptides. However, the remaining transmembrane residues are known to support dimer formation by establishing interhelical hydrogen bonds between heterologous strands (91). Receptor coupling is also believed to be conditioned by transmembrane surrounding residues (90). Even though the transmembrane aspartic acid is located at the same position in adaptors belonging to the same group, these adaptors do not interact with the same receptors. Thus, interactions among the three

transmembrane helices are thought to confer specificity to the receptor-adaptor complex formation. Although the particular contribution of these residues is largely unknown, it has been determined that the requirements they need to fulfill are a sufficient degree of hydrophobicity and an absence of steric hindrance (86).

The transmembrane basic and acidic residues also operate as quality control elements. Unassembled subunits are targeted for rapid endoplasmic-reticulum-associated degradation in a process that is directly dependent on the presence of unpaired polar transmembrane residues (92).

Transmembrane polypeptides are evolutionarily more ancient than activating receptors, because they are present in several orders of mammals, as well as in amphibians and bony fishes, while some activating receptors have been identified only in mammals (88). It is thought that the transmembrane arginine located in the N-terminal part of the transmembrane domain of FcRγ-interacting receptors arose independently from the transmembrane lysine of DAP12-interacting receptors which is located closer to the center of the transmembrane helix. Therefore, the assembly mechanisms have evolved separately but convergently.

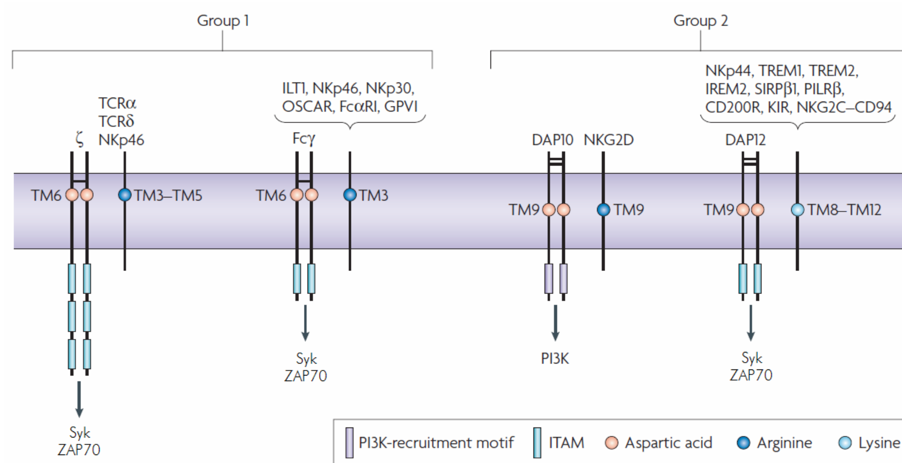

Fig 7. Charged residues within the transmembrane regions of CD3ζ, FcRγ, DAP-12, DAP-10 and their associated receptors. Schematic representation of receptor complexes with relative location of the charged residues within the transmembrane regions. The numbers noted indicate the distance in amino acids below the outer cell membrane. Taken from (87).

1.2.1 ITAM SEQUENCES AND ITAM-BEARING ADAPTOR PROTEINS

The ITAM was originally described as a common and conserved sequence in the cytoplasmic tail of the signaling chains associated to the T-cell, B-cell and certain types of Fc receptors (93). In fact, most of the knowledge concerning the transduction pathways initiated by engagement of ITAM-coupled receptors has been derived from data obtained from the

dissection of TcR, BcR and FcR signaling pathways. Most ITAMs are found in small adaptor proteins restricted to hematopoietic cells, but some molecules have integrated these motifs within their sequences. ITAMs have been observed on certain types of receptors, such as FcRIIA and Dectin-1 (94, 95). However, the ITAMs on these proteins do not match exactly the consensus sequence mentioned above, as a different spacing between and into the YxxL motifs occurs (96). ITAMs have also been observed on cytoplasmic proteins. This is the case for the actin-linking proteins ezrin, radixin and moesin, which are known to induce cytoskeletal reorganization by means of Syk signaling pathways upon engagement of leukocyte adhesion molecules such as PSGL-1 (P-Selectin Glycoprotein Ligand 1)(97). Additionally, some viral proteins have evolved to encode ITAM sequences as a mechanism of pathogenesis. These proteins are thought to disrupt the capacity of antigen receptors to signal normally in virus-infected cells and to promote cellular transformation and oncogenic phenotypes. ITAM-encoded viral proteins include the mouse mammary tumor virus-envelop (MMTV env gp52), Epstein-Barr virus-late membrane protein 2A (EBV LM2A), bovine leukemia virus gp30 (BLV gp30), NY hantavirus G1, simian immunodeficiency virus Nef protein and Kaposi's sarcoma herpes virus K1 transforming factor (98-103). Most of them are ligand-independent transmembrane/cytoplasmic proteins mediating sequestration of Src and Syk protein kinases.

Signaling though ITAMs activates the effector functions of immune cells. However, ITAM sequences have been shown recently to mediate inhibition under certain situations (104). ITAM inhibitory functions take place by direct engagement of inhibitory proteins such as SHP-1, SHP-2 and SHIP, but little is known about the precise mechanisms of their recruitment to ITAMs (105, 106). As for the ITIMs, it is likely that different mechanisms might be employed by individual ITAMs for the recruitment of specific phosphatases. At the present, there are two models explaining how the triggering of inhibitory ITAMs might occur. The first model is based on suboptimal receptor engagement. It has been hypothesized that partial receptor engagement by low affinity ligands may lead to weak activation of Src family kinases, which in turn would fail to fully phosphorylate both tyrosine residues within ITAM sequences. In this scenario, it is conceivable that monophosphorylated ITAM sequences interact preferentially with an SH2 domain contained in SHP-1, SHP-2 and/or SHIP phosphatases, than with the dual SH2 domains of Syk family of kinases (Fig. 8A). This model nicely explains the dual effects observed for IgA. Monomeric IgA is considered a discrete housekeeper of the immune system with multiple anti-inflammatory functions, whereas IgA forming immune complexes mediate inflammatory responses. The IgA receptor FcαRI, which is associated with the ITAM-containing FcRγ chain, inhibits activating responses by FcγR or FcεRI when bound to soluble IgA. The low avidity interaction of FcαRI with monomeric IgA, induces weak ITAM phosphorylation,

recruitment of SHP-1 and as consequence inactivation of the early signaling mediators Syk, LAT and ERK. Conversely, sustained aggregation of FcαRI by multimeric IgA stimulates cell activation by recruiting high amounts of Syk and aborting SHP-1 binding (107).

The second model explaining ITAMs inhibitory nature aroused from the observation that the cytoplasmic tail of CD3α can associate with acidic lipids on the inner face of the plasma cell membrane (108). When associated to lipids, ITAM-containing adaptors are highly resistant to phosphorylation by Src kinases. However, upon receptor clustering and reorganization of the lipid composition, ITAM-bearing polypeptides are released from the membrane becoming fully available for Src tyrosine kinases. Based on this fact, it has been hypothesized that ITAM-associated receptors may have different threshold for membrane release and activation of adaptor molecules. This way, low levels of receptor activation would lead to partial release of ITAM-bearing polypeptides and therefore the initiation of inhibitory signaling cascades. Higher levels of activation would conduce to fully release of ITAM-bearing adaptor proteins from the membrane and further efficient tyrosine phosphorylation (Fig. 8B).

Additionally, some ITAM motifs, such as the one in DAP-12 adaptor molecule, encode consensus ITIM sequences within. This ITAM motifs have been named closet ITIMs(96). Although DAP-12 has been described to mediate inhibition by means of different receptors, whether the tyrosines on this adaptor molecule are able to recruit SH2-bearing inhibitory phosphatases is unknown (109).

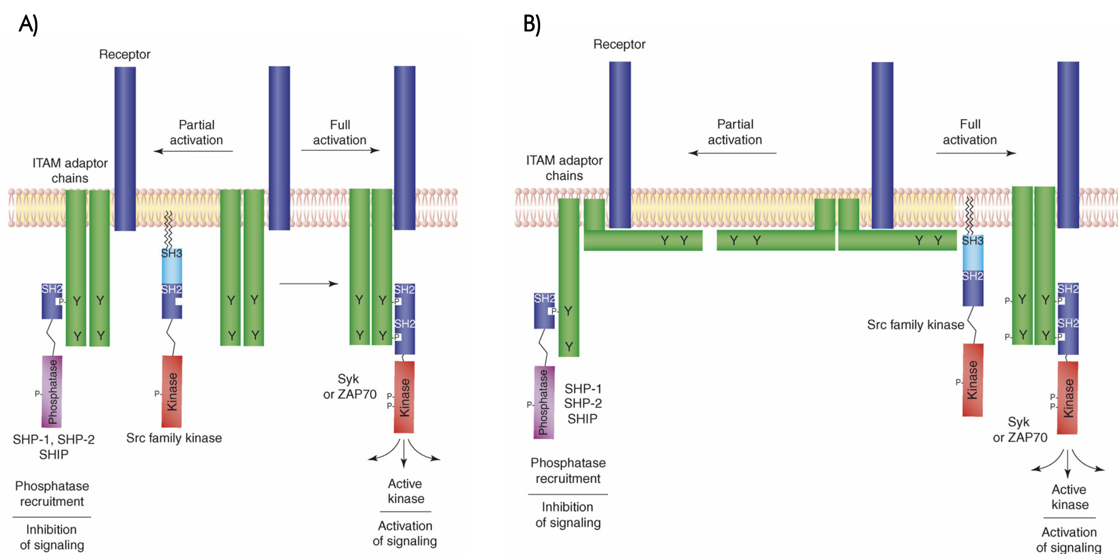

Figure 8. Models for explaining the activating/inhibitory signaling duality of certain ITAM-bearing adaptors. (A) Model based on ligand binding affinities: partial or incomplete engagement of activating receptors induces monophosphorylation of ITAM-bearing adaptors and further recruitment of SH2-containing phosphatases. **(B)** Model based on lipid binding and membrane organization: receptor clustering promotes ITAM-bearing adaptors release from the membrane enabling tyrosine phosphorylation of the cytoplasmic tails by Src family kinases. Partial release of the membrane favors monophosphorylation of ITAM motifs and recruitment of SHP-1, SHP-2 and SHIP phosphatases. From (89).

ITAMs are known to be present in a great variety of adaptor polypeptides that include CD3δ, CD3ε, CD3γ, CD3ζ, FcRγ, DAP-12, Igα and Igβ (110). The expression pattern of all of them is restricted to cells with a hematopoietic origin, but not all the cell types within the immune system express the same ITAM-containing adaptor proteins (Table 1). T cells express the CD3δ, ε, γ subunits and the TcR ζ chain (111). B cells express the Igα and Igβ chains of the BcR, as well as the FcRγ chain (112). Myeloid and NK cells express FcRγ chain and DAP-12 adaptor molecules (113, 114).

Name	Expression Pattern
CD3δ	T cells
CD3ε	T cells and activated NK cells
CD3γ	T cells
CD3ζ	T cells and NK cells
FcRγ	B cells, NK cells, dendritic cells, monocytes, macrophages, mast cells, osteoclasts a small subset of T cells
DAP-12	NK cells, dendritic cells, monocytes, macrophages, mast cells, osteoclasts, microglia, granulocytes and a small subset of B and T cells
Igα	B cells
Igβ	B cells

Table 1. ITAM-containing adaptor proteins and expression pattern within the hematopoietic lineage. Modified from(110).

Igα, Igβ, CD3δ, CD3ε and CD3γ do not interact with cell surface receptors others than the BcR and TcR respectively (8). These adaptor polypeptides present features slightly different from those mentioned previously. First they present larger extracellular domains adopting a structure that resembles the Ig-fold. Nevertheless, this domain does not posses ligand-binding capacity. And second, they associate forming disulfide-bonded heterodimers. Selectivity in the association of CD3 chains has been observed. CD3ε couples with both CD3δ and CD3γ, but no association has been detected between CD3δ and CD3γ. Contrary, CD3ζ, FcRγ and DAP-12 initiate signaling events of a great deal of leukocyte receptors and are expressed as disulfide-bonded homodimers (115). Interestingly, murine CD16 has been shown to associate in NK cells with CD3ζ/FcRγ heterodimers which compete with FcRγ homodimers and negatively regulate CD16-mediated activation (116).

Due to the IREM/CD300 myeloid pattern of expression we have focused our attention in DAP-12 and FcRγ adaptor molecules.

DAP-12

DAP-12 (DNAX Adaptor protein of 12kDa) was originally referred to as either KARAP (Killer Cell Activating Receptor Protein), TYROBP (Tyrosine Kinase Binding Protein), p13 or pp16. This protein was first identified as a disulfide-linked homodimer that could be tyrosine phosphorylated upon crosslinking of the activating KIR molecule KIR2DS2 (117). The cloning of the molecule was carried out through database search for molecules bearing homology with the human ITAM-containing gene products (118).

DAP-12 is expressed by all NK cells and myeloid cells (including osteoclasts and microglia), and it has also been detected in small subsets of T and B cells with an activated phenotype (110, 119). It has been shown to associate with several activating immune receptors contributing to multiple biological functions. DAP-12 was first found associated with the activating isoforms of the KIR family, and later with the activating members of the mouse Ly49 family of MHC (Major Histocompatibility Complex)-recognition proteins and the CD94-NKG2C complex (Natural-Killer Group 2, member C) (120, 121). To date, more than 20 receptor molecules have been shown to associate with DAP-12, especially in myeloid cells (Table 2).

Protein	Species	Ligand	Expression (predominant cells)
SIRP-β1	Human	?	Myeloid cells
SIRP-β1	Mouse	?	Myeloid cells
PILR-B	Human	Sialylated O-linked sugars	Myeloid cells
PILR-B	Mouse	CD99, sialylated O-linked	Myeloid cells, NK
CD200R3	Mouse	CD200	Myeloid cells, mast cells
CD200R4	Mouse	CD200	Myeloid cells, NK
MDL-1	Human	Dengue virus	Myeloid cells (macrophages)
MDL-1	Mouse	?	Myeloid cells (macrophages)
TREM-1	Human	?	Myeloid cells (monocytes, neutrophils)
TREM-1	Mouse	?	Myeloid cells
TREM-2	Human	?	Myeloid cells (dendritic cells, microglial cells)
TREM-2	Mouse	Anionic molecules	Myeloid cells (microglial cells, macrophage
TREM-3	Mouse	?	Myeloid cells
TREM-4	Mouse	?	Myeloid cells (spleen macrophages)
CD300b	Human	?	Myeloid cells
CD300b	Mouse	?	Myeloid cells (mast cells, granulocytes, macrophages, dendritic cells)
CD300e	Human	?	Myeloid cells (monocytes)
CD300d	Mouse	?	Myeloid cells (macrophage subset), B cells (marginal zone)
Siglec-H	Mouse	?	Myeloid cells (plasmacytoid dendritic cells)
Siglec-14	Human	A2-8-linked oligo Neu5A	Myeloid cells
Siglec-15	Human	Neu5Ac2-6GalNAca; Neu5Ac(alpha)2-3Galβ1-4Glc	Myeloid cells
Siglec-16	Human	?	Myeloid cells
Siglec-16	Mouse	?	Myeloid cells

Table 2. DAP12-associated receptors in myeloid cells. Adapted from (88) and (109).

The cytoplasmic tail of DAP-12 contains an ITAM acting as a docking site for Syk kinase. This in turn has been shown in NK cells to promote recruitment and activation of PI3K, PLCγ and ERK pathways (122). DAP12-deficient mice revealed lack of function for activating NK cell receptors, accumulation of dendritic cells in peripheral tissues and impairment of Th1 responses which is in agreement with the activating role of this adaptor polypeptide (123, 124). However, recent studies have demonstrated a role for DAP-12 adaptor molecule in the negative regulation of signaling pathways (109). Studies in macrophages have shown that DAP-12 signals are able to downregulate inflammatory responses following Toll-like receptor signaling. Additionally, DAP12-deficient macrophages and dendritic cells were found to produce higher concentration of inflammatory cytokines in response to pathogenic stimuli and showed increased ERK-1/2 phosphorylation (125-128). The activating TREM-2 molecule has been identified as a specific receptor underlying inhibition of TLR-mediated responses in macrophages (129). From these studies and others, it has been established that DAP12-mediated inhibition is likely to occur through of PI3K and PLCγ rather than SHP-1/SHIP phosphatases, which have never been found associated to DAP-12 cytoplasmatic ITAMs (Fig. 9). PI3K activates Akt, which is able to inhibit both MAPK and NF-κB activity induced by LPS (Lipopolysaccharide) (130). PLCγ in turn, can inhibit the recruitment of TIRAP/Mal and MyD88 adaptor proteins by means of reducing the amount of PtdIns(4,5)P_2, which are essential for the association of these molecules to the TLR-complex (131). Cbl has also been proposed as a key molecule in mediating DAP-12 inhibitory effects. Because Cbl is an E3 ubiquitin ligase, it might function in a negative-feedback loop in which DAP-12 ligation activates Cbl, which subsequently ubiquitylates and degradates Syk kinase (109).

A rare human pathology called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) or Nasu-Hakola disease has been shown to be caused by mutations disrupting DAP-12 gene (132). PLOSL is a recessively inherited disease characterized by a combination of psychotic symptoms rapidly progressing to presenile dementia and lipidic-bone cysts restricted to wrists and ankles. The first symptoms to be developed are the ones affecting the bone tissue. Bone fractures and fragility related to the presence of cysts and osteoporosis usually appear when the patients are between the second and third decade of life. Afterwards, appear the nervous system symptoms, which include the lost of primitive reflexes and a severe presenile dementia that leads to the premature death of the patient at 40-50 years of age. Strikingly, patients with PLOSL have no defects in cell-mediated immunity, evidencing a remarkable capacity of the human immune system to compensate the inactive DAP12-mediated activating pathways.

Dementia is thought to occur as a consequence of demyelination, which has been observed in brain histological sections of PLOSL patients and DAP-12 deficient mice (132-134). However, whether DAP-12 is expressed in oligodendrocytes, the cells involved in the myelin formation, has not been fully addressed and data generated by *ex vivo* culture of these cells are controversial. But dementia has also been attributed to the impaired synaptic function caused by synapse degeneration and accumulation of synaptic vesicles (135). As DAP-12 is detected in microglia but not in neurons or astrocytes, the adaptor molecule is thought to alter microglial physiology and subsequently the synaptic function and plasticity through a novel microglia-neuron interaction. It is of note that PLOSL disease has also been shown to occur by mutations in the TREM-2 gene (136). It is surprisingly how mutations in two different units of a multisubunit receptor complex result in an identical human disease phenotype.

DAP-12 molecule is also expressed in normal osteoclasts, the cells specialized in bone resorption (17). DAP12-deficient bone marrow precursors do not differentiate *in vitro* into mature osteoclasts. Consistently, DAP12-deficient mice develop severe ostopetrosis, also known as marble bone disease, which is characterized by an increased bone formation (133, 134). PLOSL patients osteclast differentiation is also dramatically affected (137).

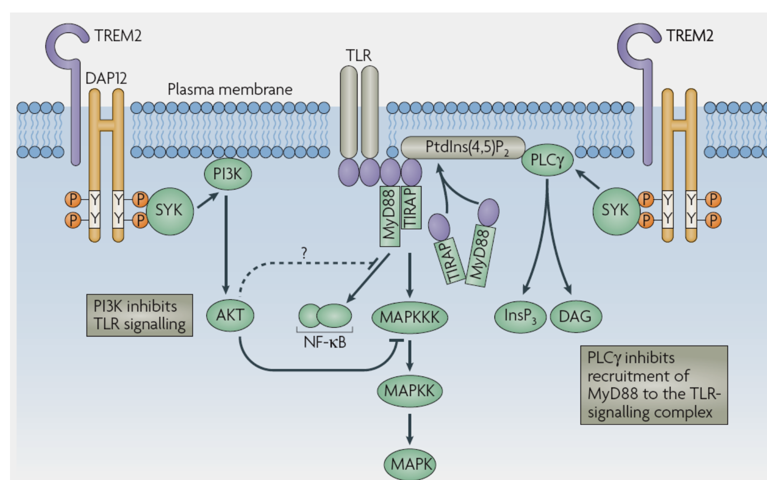

Fig 9. DAP-12 mediated inhibition of pathogenic stimuli. PI3K-mediated activation of Akt can inhibit TLR-responses by blocking the function of MAPK pathway. Additionally, PLCγ inhibits TIRAP/Mal and MyD88 adaptors recruitment to TLR-complexes. Taken from (109).

FcRγ

The FcRγ chain was originally described as part of the receptors for immunoglobulins (FcR). By binding to the antibody Fc-portion, FcR stimulate the effector functions of innate immune system, but they are also important for modulating adaptive responses by regulating B

and dendritic cell activation (138). Fc receptors are not only expressed in cells from the innate immune system such as NK cells, mast cells, neutrophils, monocytes and macrophages but in endothelial cells, mesangial cells and osteoclasts as well. Activating FcR function as part of multisubunit complexes and mediate multiple effects including phagocytosis, granule release and induction of killing (8). Only the α chain is required for the specific recognition of the Ig. The other chains are required for transport to the cell surface and for signal transduction when the Ig is bound to the receptor. Signal transduction by most FcRs is mediated by the γ chain, which contains an ITAM motif within its cytoplasmic tail able to recruit Syk tyrosine kinase (139).

Activating receptors in NK cells associating with FcRγ include NKp46, which has been implicated in mediating tumor cell lysis, and KIR2DLY (10, 140). Among myeloid cells, PIR-A, DCAR (DC immuno-Activating Receptor), OSCAR (Osteoclast-specific Activating Receptor) and ILT/CD85 receptors (Ig-Like Transcripts) interact with FcRγ to mediate their signaling (141-144).

FcRγ chain is required for the function of the activating Fc receptors in myeloid cells. Concordantly, macrophages from FcRγ-deficient mice lack the ability to phagocytate particles despite the normal binding of immune complexes. Defects in NK cell-mediated antibody-dependent cytotoxicity and mast cell-mediated allergic responses are also evident in these animals (113). DAP-12 appears to be primarily responsible for osteoclast differentiation, however FcRγ might sustain alternative pathways in the osteoclast development as DAP-12$^{-/-}$ FcR$\gamma^{-/-}$ mice exhibit a much more severe osteopetrosis when compared with DAP-12$^{-/-}$ mice (145).

1.2.2 DAP-10

DAP-10 was identified by its similarity to DAP-12 in the transmembrane region (117, 146). The gene encoding this adaptor molecule is adjacent to DAP-12 in the genome, although in the opposite transcriptional orientation. This organization has also been observed in zebrafish, indicating that DAP-10 and DAP-12 have been preserved as a pair for millions of years and that they probably arose by duplication from a common ancient gene (88).

DAP-10 is broadly expressed in the innate immune system, and its distribution is largely overlapping with DAP-12. However, DAP-10 is expressed in CD8$^+$ T cells, which rarely express DAP-12 (146).

DAP-10 bears a YxNM motif in its short cytoplasmic domain, which upon tyrosine phosphorylation allows the binding of the p85 regulatory subunit of PI3K and a Grb2-Vav1-Sos complex (147). As these two binding sites overlap, a single DAP-10 chain can bind either p85 or Grb-2, but not both at the same time. Mutagenesis studies have demonstrated that the tyrosine residue is essential for all the signaling through DAP-10, whereas mutation of the

asparagine residue, which ablates Grb-2 binding, or the methionine residue, which disrupts association with PI3K, results in different effects (148). DAP-10 YxNM motif is similar to the one present in CD28 molecule, which provides costimulatory signals to the TCR/CD3 complex in T cells (149). This has made DAP-10 to be considered as a costimulatory molecule rather than a pure stimulatory molecule.

Unlike the ITAM-containing receptors, DAP-10 complexes do not require Syk family kinases and LAT for signaling (84). DAP-10 is thought to be phosphorylated by Src family kinases as PP2, a chemical inhibitor of this family of kinases, totally prevents the signaling induced through the NKG2D-DAP10 complex (84). However, it has been recently proposed that DAP-10 is coupled with the IL-15 receptor and that Jak3 (Janus kinase 3), recruited and activated by this receptor, is also responsible for the phosphorylation of the YxNM motif (150).

NKG2D was the first receptor identified to associate with DAP-10, but several other receptors originally identified by their ability to associate with DAP-12, also pair with DAP-10 adaptor molecule (151). This is the case for mouse Ly49D, Ly49H, SIRP-β1, IREM-2/CD300e and Siglec-15 (152-154)(our unpublished data). Nevertheless, the association of these receptors with DAP-12 is more efficient, suggesting a preference for this adaptor molecule.

DAP10-deficient mice lack $CD4^{+}$ T-regulatory cells and as consequence exhibit a hyper-responsiveness phenotype of NKT cells, what enhances melanoma rejection (155). Contrary, $CD8^{+}$ T cells from these animals were found to lack NKG2D expression and were incapable of mounting tumor-specific responses (156). These differences suggest distinct roles for this molecule and their associated receptors in autoimmunity and cancer, and also manifest that not all DAP-10 mediated functions can be overcome by DAP-12 signaling.

1.2.3 SRC TYROSINE KINASES

The Src family of kinases comprises nine identified members: c-Src, c-Yes, c-Fgr, Fyn, Lck, Hck, Lyn, Blk, and Yrk. These enzymes display a wide range of expression patterns, but a subset of them is selectively expressed in the hematopoietic system. c-Src, c-Yes and presumably Yrk, are contained in most cells and therefore postulated to mediate general functions such as cytoskeleton organization and progression through mitosis. Contrary, c-Fgr, Fyn, Lck, Hck, Lyn and Blk, because of their restricted expression pattern, are thought to play specialized roles in hematopoietic cell physiology (157)(Table 3).

Name	Protein Product(s)	Expression Pattern
c-src	$p60^{c\text{-}src}$	Ubiquitous (high in platelets)
c-yes	$p62^{c\text{-}yes}$	Ubiquitous
c-fgr	$p58^{c\text{-}fgr}$	Granulocytes, monocytes, macrophages
fyn	$p59^{fynB}$	Ubiquitous (high in brain)
	$p59^{fynT}$	Most hematopoietic cells (high in T cells)
lck	$p56^{lck}$	T cells, natural killer cells, peripheral B cells
hck	$p56^{hck}$, $p59^{hck}$	Granulocytes, monocytes, macrophages, platelets
lyn	$p53^{lyn}$, $p56^{lynB}$	B cells, mast cells, monocytes, macrophages, platelets
btk	$p55^{blkB}$	B cells
yrk	$p60^{yrk}$	Ubiquitous (?)

Table 3. Src-related genes and their expression patterns. Taken from (157).

Src kinases activation has been linked to many cell-surface signaling events, including not only immune cell receptors but also growth factor, cytokine and G-protein-coupled receptors, as well as integrins and other cell adhesion molecules (158). Consequently, Src kinases represent critical molecules for the integration and transmission of diverse signals in a broad array of cellular contexts. Loss of Src kinases regulation has been linked to a variety of diseases, including several types of cancer, neurodegenerative diseases and epilepsy (159, 160).

Src-related enzymes exhibit a highly conserved primary sequence and the same N- to C-terminal structural arrangement: (1) membrane anchoring-domain, (2) unique domain, (3) SH3 domain, (4) SH2 domain, (5) kinase domain and (6) C-terminal tail (161, 162)(Fig. 10). The membrane anchoring-domain allows these molecules to be targeted to the inner cell membrane where they develop their catalytic activity. This domain comprises a myristoylation site, several sites of palmitoylation as well as a cluster of basic residues capable of interacting with acidic phospholipids in the membrane. Src kinases have been found to be preferentially associated to lipid rafts regions, which are characterized by its rigid and ordered structure (163). It is of note that some of these lipid modifications on Src kinases are reversible, resulting in pools of kinases differing in the cellular location. The unique domain is the region in which Src kinases display the highest sequence divergence. This domain does not have an apparent role, although for Lck, this region has been shown to mediate interactions with specific substrates and regulators (161). The SH3 domain interacts with polyproline ligands involved in signal amplification, while the SH2 domain has the ability to interact with tyrosine phosphorylated proteins. Src-family SH2 domains have been shown to bind preferentially to pYEEI motifs (164). SH3 and SH2 domains are linked together by a stretch of sequence called SH3–SH2 connector. The SH2 and kinase domains are linked by the SH2-kinase linker, which forms a polyproline helical structure.

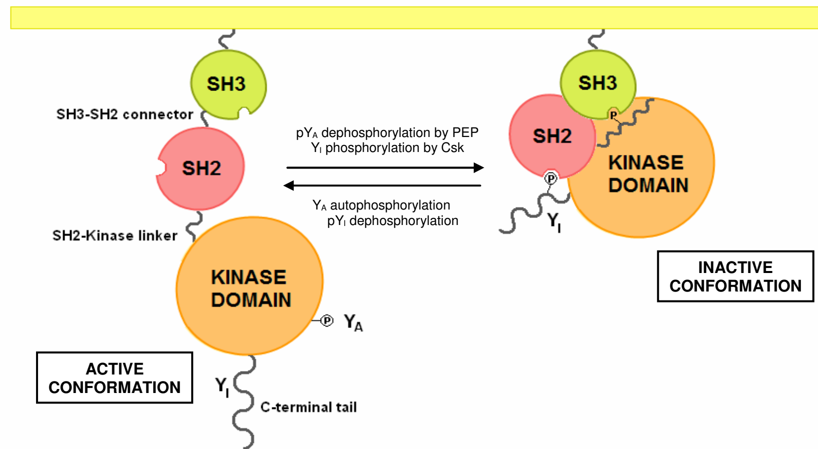

Fig 10. Domain organization and catalytic regulation of Src family kinases. The close or inactive conformation involves the interaction of the C-terminal tyrosine residue with the molecule's SH2 domain and the contact of a polyproline rich region on the SH2-Kinase linker with the molecule's SH3 domain. Y_I stands for the inhibitory tyrosine in the C-terminal tail and Y_A for the autophosphorylatory tyrosine in the kinase catalytic domain. Molecules involved in the phosphorylation and dephospholytarion events are noted. SH2 and SH3: Src Homology-2/3 domains.

The catalytic activity of Src kinases is regulated principally by tyrosine phosphorylation. Phosphorylation of the tyrosine within the C-terminal tail by Csk kinase causes a strong repression of the enzymatic activity, while its dephosphorylation leads to activation of catalytic functions. PEP, SHP-1 and CD45 have all been shown to be able to dephosphorylate this C-terminal site within various cell systems (53, 165, 166). The mechanism by which C-terminal tyrosine inhibits the catalytic function involves the intramolecular association between the phosphorylated C-terminus tyrosine and the SH2 domain of the enzyme. However, in the inactive state additional intra-molecular contacts have been observed such as the binding of the SH2-kinase linker to the SH3 and kinase domains (162)(Fig. 10). Autophosphorylation of a consensus tyrosine in the kinase domain enhances the catalytic activity (52, 167).

The most studied immune receptors in terms of Src kinase signaling are the antigen receptors on B and T lymphocytes (BcR and TcR), the receptor for IgE (FcεRI) on the surface of mast cells, basophils and eosinophils, and the receptors for IgG (FcγRI, FcγRIIA, FcγRIII) on the surface of macrophages, monocytes, myeloid, NK and mast cells. Engagement of antigen receptors is well known to induce the immediate activation of the Src kinases Lyn/Fyn/Blk forr the BcR signaling, and Lck/Fyn for the TcR signaling. In contrast, myeloid cells primarily activate Hck, Fgr and Lyn kinases in response to receptor crosslinking (163). Studies in knock out mice have pointed out a crucial role for these kinases in the development of the different hematopoietic lineages. For instance, Lck and Fyn are essential for T-cell development and function, while Lyn is required for B-lineage development/maturation and function of myeloid

cells (168-171). Others, such as Src are not required for cell viability but cover other functions such as bone formation, which if impaired are incompatible with the organism life progression (172). Nevertheless, due to the high degree of similarity and the overlapped expression patterns, the lost of some kinases can be compensated by other related kinases. This is the case for Blk, Yes and Hck kinases which if deleted individually do not impair any biological process (173-177).

1.2.4 SYK TYROSINE KINASES

Syk (Spleen Tyrosine Kinase) family of tyrosine kinases is another class of non-receptor tyrosine kinases expressed exclusively in cells from the immune system. The family is composed by two members: Syk and ZAP-70 (ζ-chain associated Protein 70). ZAP-70 is restricted to T and NK cells whereas Syk is present in a broader panel of cells including B cells, mast cells, myeloid cells, platelets, a subset of γδ T cells and immature thymocytes (178).

Syk kinases are composed by two tandem N-terminal SH2 domains and a C-terminal kinase domain (Fig. 11). The N-terminal SH2 domain binds to the C-terminal YxxL motif of the ITAM, whereas the C-terminal SH2 domain binds to the N-terminal YxxL sequence. SH2 domains are separated from the catalytic domain by means of a linker region, designated interdomain B, containing multiple tyrosine residues that when phosphorylated, act as docking sites for proteins such as PLCγ, Vav and Cbl, which are substrates for Syk and ZAP-70 kinases (179). Beyond the catalytic domain there are two extra tyrosine residues that serve as autoregulatory motifs, since when mutated give rise to gain-of-function mutants (180). The exact mechanisms by which these C-terminal tyrosines mediate their inhibitory effect are not known, but structural rearrangements such as the ones described for Src kinases have not been described to date. This inhibition is thought to involve binding of phosphatases to keep these tyrosines under a non-phosphorylated state. The catalytic activity of Syk tyrosine kinases is also regulated by phosphorylation events affecting two tyrosines residues lying off the enzymatic domain. In the non-activated state these tyrosines occupy the catalytic center of the molecule preventing the binding of ATP and substrates. Phosphorylation of one or both of the tyrosines, either by another kinase or by autophosphorylation, induces a conformational change that moves the loop out of the catalytic site allowing the binding of ATP and/or substrate (181). Phosphorylation of these tyrosines is often carried out by Src family kinases, but as mentioned autophosphorylation occurs, especially for Syk kinase. Contrary, ZAP-70 frequently requires the stimulatory input from Src kinases to acquire the fully functional state (182).

Syk kinase has been more extensively studied than ZAP-70. Syk has the ability to interact with the ITAMs of Igα CD3γ, CD3ε, FcεRIβ and FcεRIγ, what makes this molecule to be involved in the signal transduction pathways of a large number of receptors in different hematopoietic cell types. Syk has been found to be one of the major players controlling the development of B-lineage cells, as its association with the B-cell antigen-receptor complex in immature and mature B lymphocytes promotes the clonal expansion and further maturation of this cells (183, 184). Contrary, T-cell development is mainly controlled by ZAP-70, which has been found essential in the positive and negative selection events of T cells in the thymus (185, 186). Syk also governs multiple functions in myeloid cells. For instance, the release of inflammatory mediators in the form of secretory granules, cytokines and arachidonic acid metabolites by mast cells upon binding of antigen-IgE complexes is controlled by this kinase. Syk also associates with the tyrosine phosphorylated FcRγ chain of FcγRI and FcγRIII (CD16), which induce phagocytosis of antibody-antigen complexes and release of oxygen metabolites (187, 188).

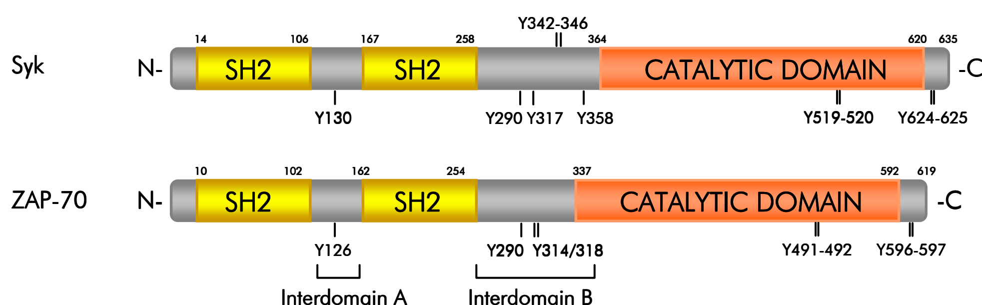

Fig 11. Domain organization of Syk family of kinases. Domains are represented by colored boxes. Amino acid positions for the different domains are noted and regulatory tyrosines are shown. SH2: Src Homology-2 domain.

2. FAMILIES OF IMMUNE RECEPTORS IN MYELOID CELLS

Myeloid cells count on receptors with differential functional aims: pattern recognition receptors for pathogens and stimulation of the innate immune responses; receptors for phagocytosis; receptors for nucleotides, oxidized lipids and proteins; receptors for the migration into tissues; cytokine and chemokine receptors to respond to humoral responses; accessory molecules for the T-cell activation; complement and Fc receptors for the uptake of pathogens and clearance of foreign antigens and receptors that modulate the myeloid cell function (189, 190).

Most immune receptors belong to multigenic families including both, activating and inhibitory members that are organized in clusters in the genome. Inhibitory and activating receptors are similar in their extracellular regions, but differ in the transmembrane and

cytoplasmic domains which confer opposite signaling properties. Regarding the extracellular region, receptors can be classified into two different groups: the Ig-like superfamily and the C-type lectin superfamily. We will focus in the description of some receptor families regulating the function of myeloid cells that belong to the Ig-like superfamily. Only the families exhibiting higher homology with IREM/CD300 receptors have been reviewed.

2.1 TREMs (Triggering Receptors Expressed by Myeloid cells)

The TREM family of receptors clusters in close vicinity to the MHC class II region. Human genes are located in chromosome 6p21 while the murine ones are found in the syntenic chromosome 17B3. The human TREM family is composed by an inhibitory (TLT-1, Trem-Like Transcript-1) and two activating receptors (TREM-1 and TREM-2) of the immunoglobulin superfamily, while the murine one comprises an inhibitory (Tlt-1) and three activating receptors (Trem-1, Trem-3 and Tlt-4) (85, 191, 192)(Fig. 12). The closest TREMs relative is NKp44, an activating NK cell receptor encoded in the vicinity of the TREM gene cluster. More distant relatives of these proteins include the CD300 family members and PIgR (Polymeric immunoglobulin receptors) (193).

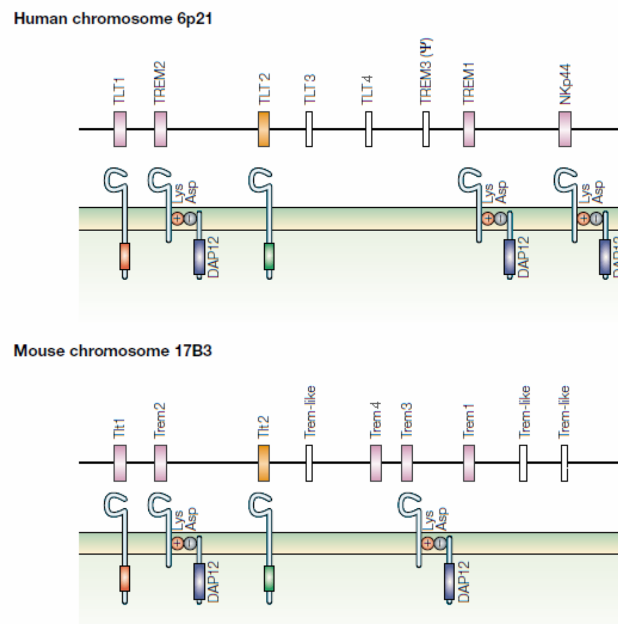

Figure 12. Organization of the human and mouse TREM gene cluster. Genes for TREMs and Nkp44 are represented by purple boxes. Genes encoding for ITIM-containing inhibitory receptors are represented by orange boxes. Green boxes represent cytoplasmic tyrosine-based motifs in new contexts. TREM-like immunoglobulin domains annotated in the human and mouse Genome Projects are represented by white boxes. TREM3(Ψ) in humans is a pseudogene and is also represented by a white box. Models of the encoded molecules are shown below the genes. Modified from (85).

TLT-1 presents two cytoplasmic ITIM motifs and a polyproline rich sequence. There is some controversy regarding the signaling delivered by this receptor. Initial experiments demonstrated TLT-1 ability to recruit SHP-1 phosphatase, a result consistent with the receptor being an inhibitory molecule (194). However, crosslinking of TLT-1 with FcεR results in increased calcium flux, indicating that TLT-1 can lead to activation (195). The fist ITIM motif on TLT-1 effectively recruits SHP-2 phosphatase, which itself is able to mediate either activation or inhibition depending on the experimental conditions. Whether the proline-rich sequence recruits and SH3-containing molecule triggering cell activation is currently unknown.

TREM-1, TREM-2 and TREM-3 display transmembrane lysine residues allowing the association of these receptors with DAP-12 adaptor molecule (196-199). Although this pathway canonically leads to cell activation, recent data have shown that in some context DAP-12 signaling downstream of TREM-2 has inhibitory effects on myeloid cells (126).

Expression of TREM receptors is restricted to cells of the myeloid lineage, including microglia and osteoclasts (200). In humans, TREM-1 is expressed by circulating and tissue resident neutrophils and monocytes/macrophages. TREM-1 expression is upregulated in human cells after sepsis, strongly suggesting a role for this molecule in inflammation (201). Engagement of this receptor in granulocytes and monocytes promotes the release of proinflammatory cytokines, including IL-1, IL-8 and MPO (Myeloperoxidase), MCP-1, (Monocyte Chemoattractant Protein 1), MCP-3, MIPα (Macrophage Inflammatory Protein α) and TNF-α. In monocytes, TREM-1 activating effect is enhanced when LPS is used as a co-stimulus, indicating a role in amplification of the TLR-mediated responses (202). Myeloid cells regulate the initial steps of the adaptive immune responses. In this line, TREM-1 has been shown to elicit T cell proliferation and stimulate the production of chemokines recruiting these lymphocytes (203).

TREM-2 is expressed by dendritic cells, osteoclasts and microglia and also signals through DAP-12 adaptor molecule (200). However, TREM-2 ligation does not promote translocation of NF-κB, showing clear differences between TREM-2 and TREM-1 signaling (197). TREM-2 crosslinking on immature dendritic cells induces upregulation of molecules involved in T cell-costimulation such as CD86, CD40 and MHC class II. TREM-2 stimulation in microglia results in an increase in ERK-1/2 phosphorylation and CCR7, but does not affect the expression of CD86 and MHC class II, suggesting a signaling for TREM-2 cell type-specific. TREM-2 in the central nervous system has been related with tissue homeostasis as per its role in the phagocytosis of apoptotic neurons (204). TREM-2 deficiency causes a disease known as Nasu-Hakola or PLOS, characterized by neurodegenerative pathology as well as for the

presence of multiple bone cysts (136). This phenotype is consistent with the expression and function of TREM-2 in microglial cells and osteoclasts.

To date, the nature of TREM ligands remains elusive. However, there are evidences suggesting that the ligand for TREM-1 may be expressed on human platelets (205). TREM-2 has been reported to bind both gram-positive and gram-negative bacteria indicating its ligand could be polyanionic bacterial products (206). Consistently with this, very recently TREM-2 has been shown to promote phagocytosis of bacteria (207).

2.2 SIRPs (Signal Regulatory Proteins)

SIRPs constitute a family of structurally related surface receptors expressed on the surface of leukocytes and especially on myeloid cells. The SIRP family members, also known as CD172, include paired activating and inhibitory molecules (208). SIRP gene products are classified according to their structural and functional properties. This way, the term SIRPα is used for transmembrane members with typical ITIM cytoplasmic motifs, SIRPβ is used for transmembrane members with a positively charged residue (typically a lysine) in the transmembrane domain, SIRPγ is used for transmembrane members lacking the two above properties, and SIRPδ is used for members lacking putative transmembrane regions and therefore soluble (209). The extracellular region of all of them consists of a variable number of Ig-like domains from the V-set or C1-set (Fig. 13).

The genes of the five identified SIRPs in humans, SIRPα, SIRPβ1, SIRPβ2, SIRPγ, SIRPδ and the SIRPβ3p pseudogene, are clustered in chromosome 20p13.

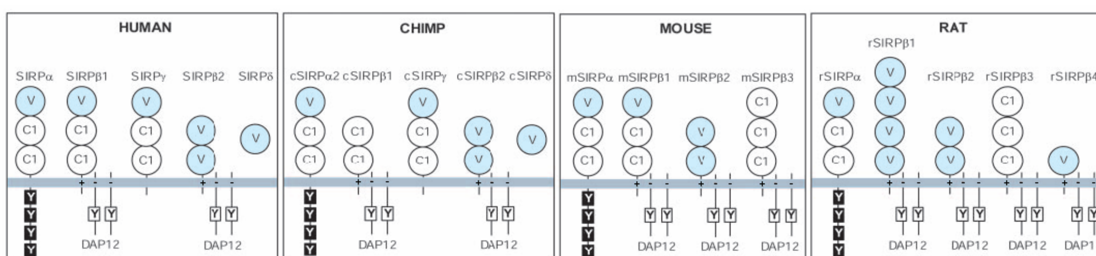

Figure 13. SIRP molecules in mammals. The existence of some SIRP proteins, including human SIRPα, SIRPβ1 and SIRPγ, rat SIRPα, mouse SIRPα, SIRPβ1, and SIRPβ2, is supported by direct biochemical evidence, whereas the others is based on prediction. The blue and white circles represent the V- and C1-set Ig domains, respectively. The black squared Y symbol represents tyrosine residues within ITIM motifs. The white squared Y symbol represents ITAM-contained tyrosine residues. Adapted from (208).

SIRPs are predominantly expressed by myeloid cells including macrophages, granulocytes, myeloid dendritic cells, mast cells and precursors including hematopoietic stem cells. SIRPα has been additionally found in the central nervous system and particularly in neurons (210).

SIRPα contains four cytoplasmic tyrosine residues in an ITIM context. Consistently, it has been shown to interact with SHP-1 and SHP-2 phosphatases, but also with SCAP2 (Src-family Associated Phosphoprotein 2), FYB (Fyn-Binding protein) and Grb-2 (210-212). SIRPα has been involved in the negative regulation of host cell phagocytosis and clearance processes, but also in the positive regulation of inflammatory mediator production (213).

Among SIRPβ proteins, murine SIRPβ1 has been the only one to be characterized functionally. Its transmembrane lysine residue permits DAP-12 recruitment which in turn promotes Syk and MAPK phosphorylation. SIRPβ1 induces phagocytosis by macrophages and migration of neutrophils (214, 215).

SIRPγ, unlike the other SIRP proteins, is expressed by T and NK cells. Despite its short cytoplasmic domain and the lack or charged transmembrane residues it is able to mediate cell-cell adhesion and support APC-T cell contact, enhancing antigen presentation and the subsequent T cell proliferation and cytokine secretion (216).

CD47, also known as IAP (Integrin-Associated Protein), has been identified as the ligand for SIRPα and SIRPγ (217, 218). CD47 is a broadly expressed transmembrane glycoprotein with a single extracellular Ig domain and five transmembrane spanning regions. The binding between both molecules occurs through the SIRP N-terminal V-like domain. CD47 has the ability to signal through G-coupled proteins, thus the interaction of SIRP with CD47 delivers a two-way signal transduction (219). SIRPα has also been shown to bind to soluble ligands such as surfactant proteins A and D (220). These proteins belong to the collectin family of proteins, containing globular lectin domains coupled to collagen-like tails and arranged as multimers. To date, no ligands have been found for SIRPβ.

2.3 CD200R

The human CD200 gene cluster is located on chromosome 3q12–13 and contains CD200R/OX2R and its ligand CD200/OX2 (221, 222). Phylogenetic analyses have turned out these genes to have evolved by duplication processes. Both molecules consist of two extracellular Ig-like domains, a transmembrane region and a cytoplasmic tail. Nevertheless, the receptor has a longer cytoplasmatic tail, which contains three tyrosine residues. One of them (Y297) is in the context for NPxY sequences which has been shown to be a binding site for proteins carrying PTB domains. These residues become phosphorylated by Src kinases upon

receptor triggering what allows the recruitment of Dok-1 and Dok-2 adaptor proteins and the subsequent binding of RasGAP and SHIP molecules. This causes the inhibition of the phosphoinositide and MAPK pathways, respectively (82, 223). Thus, the CD200R is an inhibitory receptor triggering myeloid cell inhibitory pathways distinctively from the more typical ITIM-bearing receptors. Particularly, CD200R is a potent regulator of mast cell function by restricting the duration and intensity of degranulation and cytokine secretion responses (224).

There is a third CD200 gene in humans, called CD200R1a, which contains a short cytoplasmic region and a charged residue in the transmembrane domain which is thought to pair with the signal transduction adaptor DAP-12 by homology with its murine counterparts mCD200RLa and mCD200RLb (222). Unfortunately, the interaction in humans has not been demonstrated.

CD200R expression is largely restricted to monocyte/macrophage/dendritic cells lineages, whereas its ligand, CD200, has a broader expression pattern including thymocytes, B cells, activated T cells, neurons, endothelial cells and macrophages at the onset of fusion processes (225). As the interaction between CD200 and its receptor occurs at the cell surface, the signal will only occur when the cells come into productive contacts. This combination of a broadly distributed ligand and a restricted receptor provides points of control for myeloid cells in a variety of tissues, including bone where CD200-CD200R interaction modulates macrophage fusion and differentiation in osteoclasts (226, 227).

CD200-defidient mice exhibit multiple defects in the myeloid compartment. Macrophage lineage cells, including brain microglia, present an activated phenotype and are more numerous (228). Despite this, osteoclasts are reduced in these animals and consequently there is an accumulation of bone mass (227).

CD200 homologues are found in a wide range of evolutionary diverse viruses. Most of these products are expressed in the lytic phases of the virus lifecycle what responds to the need to downregulate the myeloid cell activity during virion production (189). This validates the inhibitory role of CD200R receptor in the myeloid cell biology.

2.4 IREM/CD300 FAMILY

The IREM/CD300 family of immune receptors is the more recently described family within the Ig-like superfamily, and its genes are clustered in the genome as observed for many other immune receptors. The term IREM stands for Immune Receptor Expressed by Myeloid cells, and was assigned due to the myeloid-restricted expression pattern observed for the

receptors within the family. Nevertheless, in the last Leukocyte Differentiation Antigen Workshop both, the human and mouse IREM/CD300 receptors, were renamed as CD300 (229).

Human cluster of receptors is located in chromosome 17 (17q25.1) and spans approximately 250 kb (Fig. 14). The locus is composed by two genes encoding for inhibitory receptors (IRp60/CD300a and IREM-1/CD300f), two genes encoding for activating receptors (IREM-2/CD300e and IREM-3/CD300b) and two genes encoding for receptors with features that make its classification uncertain (CMRF-35/CD300c and IREM-4/CD300d). If observed in detail, two sets of specular genes can be established based on the structure of the encoded proteins. This suggests that the family has evolved by gene duplication and inversion.

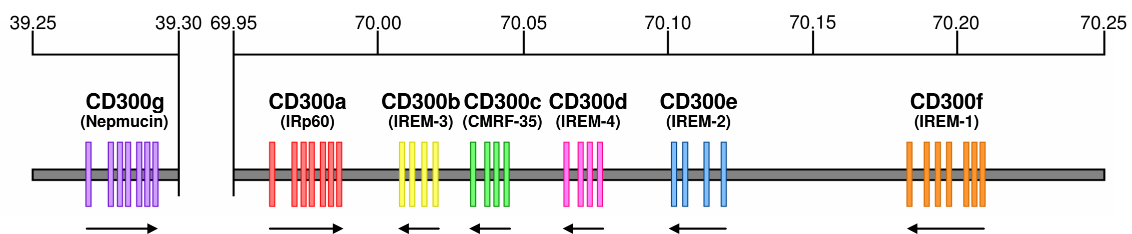

Figure 14. Genomic organization of the IREM/CD300 locus at human chromosomal region 17q25.1. Distances along the chromosome are represented in Mb. Gene orientation is indicated with arrows. Exons of each gene are represented by colored boxes. CD and traditional nomenclature is shown for each gene.

Some kb upstream in the chromosome (17q21), there is a seventh gene encoding for a receptor named nepmucin/CD300g. Despite the homology of CD300g immunoglobulin domain with the rest of CD300 members, it presents features such a mucin-like domain and a pattern of expression restricted to endothelial cells that converts this receptor in a distant member of the family (230-232). For this reason, neither the human nor the murine receptor will be described in detail in this thesis. Additionally, a pseudogene called CMRF-35J has been identified 3.2 kb upstream to the CMRF-35/CD300c gene. CMRF-35J presents a high degree of sequence similarity with CMRF-35/CD300c (88%) and an equivalent intron-exon display. However, it lacks the signal peptide sequence driving the expression of cell surface proteins and presents an insertion in the Ig-fold region that creates a premature stop codon in the putative translated sequence (233).

Our laboratory has cloned and characterized the four human IREM members: IREM-1/CD300f, IREM-2/CD300e, IREM-3/CD300b (presented in this thesis) and IREM-4/CD300d.

The human IREM/CD300 receptors have their murine orthologs in the CLM/CD300L (CMRF35-Like Molecules/CD300-Like) gene cluster. It contains nine members and is located in mouse chromosome 11 (11E2), which is syntenic to human chromosome 17 (25)(Fig. 15). Murine CD300g/CLM-9, as its human counterpart, is located some kb upstream from the

locus (between 11D and 11E1 regions). CLM/CD300 cluster is much more complex and diverse than the human locus, suggesting different evolutionary rates between both species. Only human inhibitory receptors and CD300g have unequivocal ortholog genes within the murine locus. For the rest of the members there is a misbalance caused by the different number of composing genes. Moreover, for the activating and the negatively charged receptors, a different ortholog can be assigned if choosing functional criteria based on structure or sequence homology. For instance, CLM-7 is the murine ortholog of CD300b based on sequence similarity, but it lacks the cytoplasmic tyrosine based motif which can be identified in CLM-4 and CLM-6 molecules. This fact makes CD300 nomenclature to be inappropriate for the murine locus.

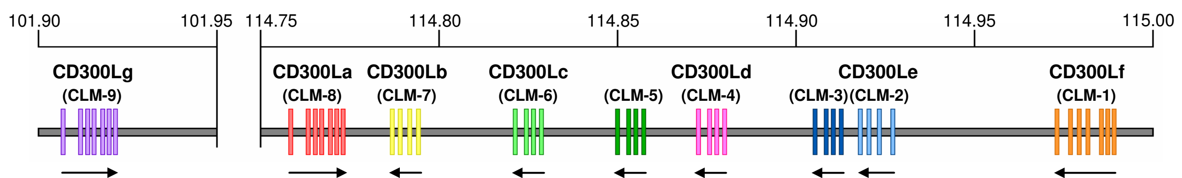

Figure 15. Genomic organization of the CLM/CD300 locus at mouse chromosomal region 11E2. Distances along the chromosome are represented in Mb. Gene orientation is indicated with arrows. Exons of each gene are represented by colored boxes. CD and traditional nomenclature is shown for each gene.

All members within the human and murine family are type I-transmembrane proteins sharing the same domain architecture (from N-terminus to C-terminus): a signal peptide, a single immunoglobulin domain, a membrane-proximal region, a transmembrane domain and a cytoplasmic tail.

Most of the receptors within the Ig-like superfamily are structures relatively complex as per the presence of multiple extracellular domains. However, there is a small group of simpler structures in which IREM/CD300 receptors would be included. The immunoglobulin domain of IREM/CD300 receptors is from the V-type, as determined by the long spacing between the two conserved cysteine residues (sufficient to accommodate the nine antiparallel β strands), and the arginine and aspartic acid residues forming the salt bridge between β strands D and F (involved in the stabilization of the Ig-fold). IREM/CD300 members display a second pair of cysteine residues, also present in the closely related TREM-2 and NKp44 receptors, which has been proposed as a mark for a discrete group of receptors evolving from a common ancestral V-type domain (234). Most IREM/CD300 receptors bear one or two *N*-glycosilation sites within the Ig domain. Only IREM-3/CD300b within the human receptors and CLM-1/CD300Lf, CLM-3, CLM-5 and CLM-9/CD300Lg within their murine counterparts, lack *N*-glycosylation acceptor sites.

The membrane-proximal region of IREM/CD300 receptors contains a high proportion of serine, threonine and proline residues. This predicts the molecules to adopt an extended conformation by comparison with the hinge-like sequences of the T cell surface antigen CD8. It has been shown that this structure raises the Ig-domain 10nm from the membrane, enhancing solvent exposure of the extracellular region (235). None of the IREM/CD300 receptors has found to dimerize/multimerize extracellularly through disulfide bridges between the immunoglobulin domains or the membrane-proximal regions.

The transmembrane and cytoplasmic regions vary among IREM/CD300 receptors. Inhibitory members display neutral transmembrane regions but long cytoplasmic tails with tyrosine-based signaling motifs, whereas activating receptors bear a positively charged amino acid residue within the transmembrane domain in combination with a short cytoplasmic tail. Oppositely to all known immune receptors, CMRF-35/CD300c and IREM-4/CD300d present a negatively charged residue in the transmembrane domain. As mentioned previously, the presence of positive charges on receptors predicts their association with transmembrane adaptor proteins such as the ITAM-bearing adaptors or the PI3K-associated adaptor DAP-10. But the functional meaning of receptors bearing a negative charge within their transmembrane area remains unknown, since no transmembrane adaptor proteins with opposed electrostatic charges have been described to date.

Regarding sequence homology, IREM/CD300 members where originally related to the Fc receptors for polymeric IgA and IgM, which controls the trafficking of these immunoglobulins through the glandular epithelium present in gut, liver and lactant mammary gland (236). Even though the ligands for IREM/CD300 receptors remain unknown, none of these receptors are able to bind immunoglobulins as seen for PIgRs, or MHC molecules as described for KIRs and ILTs. At the present, seems more appropriate to relate IREM/CD300 members to TREM family of receptors and NKp44.

A detailed description of the characterized human and mouse IREM/CD300 receptors appear as follows. CD300g has been omitted for the reasons mentioned above. A chronological criterion has been chosen for the description of the human receptors, as identification of the first receptors conditioned the identification of the followings. For the murine receptors, the order of appearance is based on the CD nomenclature, but to avoid leaving orphan murine receptors the CLM term has been used instead. Data regarding the cloning and characterization of all human and mouse IREM/CD300 sequences are resumed in the appendices (see Tables 1 and 2).

2.4.1 HUMAN IREM/CD300 RECEPTORS

CMRF-35/CD300c

CMRF-35/CD300c (also known as CMRF-35A) was identified in 1992, being the first member of the family to be cloned and one of the first Ig-like receptors to be identified. Cloning of KIRs, ILTs and TREMs occurred later on time (196, 237-239). Despite this, very little is known about CMRF-35/CD300c receptor.

The sequence encoding for the receptor was obtained by identification of the antigen recognized by a mAb (CMRF-35) raised in mice against a mixture of purified human PBMC (Peripheral Blood Mononuclear Cells) and leukemic large granular lymphocytes. The transcript, identified by expression cloning, encoded for a 224 aa type-I transmembrane protein displaying a single Ig-V-type extracellular domain followed by a membrane-proximal region, a transmembrane domain with a glutamic acid in a central position and a cytoplasmic tail consisting on 18 aa (240). The combination of a short cytoplasmic tail with a negatively charged residue on the transmembrane region, make its classification uncertain structurally but also functionally because, as noted before, no transmembrane adaptor proteins bearing acidic residues on the transmembrane region have been identified. This thesis introduces some insights about CMRF-35/CD300c functionality.

The gene encoding for CMRF-35/CD300c spans a region of approximately 4.5 kb and contains four exons: exon 1 encodes the 5′ untranslated region and the protein's signal peptide; exon 2 encodes the Ig domain; exon 3 encodes the membrane-proximal region; and exon 4 encodes the transmembrane region, the entire cytoplasmic tail and the 3′ untranslated region (233).

Poor and not very reliable information is available about CMRF-35/CD300c expression, as the CMRF-35 mAb that allowed the identification of the molecule crossreacts with others members of the family. In fact, it was the discrepancy among the expression data obtained through RT-PCR and flow cytometry, what allowed the identification of CMRF35-H9 (IRp60)(241). CMRF-35/CD300c transcript seems to be specifically expressed in all PBMC purified populations: $CD14^{+}$ myeloid cells, $CD3^{+}$ T lymphocytes ($CD8^{+}>>CD4^{+}$), $CD19^{+}$ B lymphocytes, $CD16^{+}$ NK cells and $CD15^{+}$ granulocytes. Consistent with this, THP-1, HL-60, U937 and NB4 myeloid cell lines and, Mann and WT49 B lymphoid cell lines, resulted positive for CMRF-35/CD300c transcript detection. Regarding protein expression, a polyclonal antibody raised against the cytoplasmic region of the molecule to avoid crossreactivity, showed $CD3^{+}$ T cells and $CD14^{+}$ myeloid cells to be positive for CMRF-35/CD300c receptor (241).

No other populations and subpopulations have been assayed. Erythrocytes and platelets did not show any staining as per the original CMRF-35 mAb (242).

IRp60/CD300a

IRp60/CD300a is an inhibitory member of the family. It was cloned independently by two groups following different strategies. The first report found in the literature is from the same authors that identified CMRF-35/CD300c. As mentioned before, it was the discrepancy among CMRF-35/CD300c expression data what favored the cloning of IRp60/CD300a (referred as CMRF35-H9 by these authors). CMRF35-H9 sequence was obtained from the eritroleukemic HEL cell line that stained positive for CMRF-35 mAb but was negative for CMRF-35/CD300c PCR amplification (241). One year after, Moretta's group reported IRp60 sequence from a polyclonal NK cell population (243). In this case, a functional criterion was followed to identify the receptor: a specific mAb raised in mice against an NK cell clone was able to inhibit the cytotoxicity triggered by NKp receptors. The antibody was found to recognize a molecule encoding for a sequence that they called IRp60 (Inhibitory Receptor protein 60). Both sequences exhibit a 98% amino acid identity. The slightly differences are probably due to the different source of DNA used by the authors.

IRp60/CD300a is a 299 aa-long type I-transmembrane glycoprotein composed by a single V-type Ig domain highly homologous to the CMRF-35/CD300c one (only 18 aa differ among both molecules), a membrane-proximal region with thirteen putative O-glycosylation sites, a transmembrane region and a long cytoplasmic tail bearing four tyrosine residues. Three of them are in the context for ITIM motifs (Y231, 255 and 267) and the last one, Y293, is within a tyrosine-based motif. The molecule has been shown to become efficiently N- and O-glycosilated in its extracellular portion and tyrosine-phosphorylated after pervanadate treatment in polyclonal NK cells (243).

The gene encoding for IRp60/CD300a spans a 12 kb region and contains seven exons. Exon 1 encodes the 5' untranslated region and the protein's signal peptide; exon 2 encodes the entire Ig domain; exon 3 encodes the membrane-proximal region; exon 4 encodes the transmembrane domain and exons 5, 6 and 7 encode the cytoplasmic tail. Additionally exon 7 contains the 3' untranslated region (244).

IRp60/CD300a is expressed by NK cells, monocytes, neutrophils and a fraction of $CD3^+$ T cells (243, 245). Additionally it has been found in circulating and tissue resident mast cells and eosinophils (246, 247). Its expression is downmodulated in CBMC (Cord Blood-derived Mast Cells) by eosinophil-derived cytotoxic proteins, such as MBP (Human Eosinophil-

derived Major Basic Protein) and EDN (Human eosinophil-derived Neurotoxin), which are accumulated in the cytoplasm of eosinophils and released upon inflammatory signals during the course of allergic processes (246). On the other hand, IRp60/CD300a is rapidly upregulated by LPS or GM-CSF (Granulocyte/Macrophage-Colony-Stimulating Factor)-stimulated neutrophils by an increase in the translocation of presynthesized receptor (245). These modulatory effects are of special interest in the context of pathologies that course with allergic chronic-inflammation, where a tight interaction between mast cells and granulocytes are known to occur.

In NK cells, IRp60/CD300a is able to recruit both SHP-1 and SHP-2 upon pervanadate treatment which is consistent with its role in inhibiting NKps-mediated cytotoxicity in this cells but also in CTL (Cytotoxic T-Lymphocytes)(243).

IRp60/CD300a inhibits in mast cells β-hexosaminidase and tryptase release, IL-4 secretion and SCF (Stem Cell Factor)-mediated differentiation and survival. The receptor was found to recruit in CBMC SHP-1 and SHIP upon ITIM-phosphorylation, thus the effects observed are caused as consequence of inhibited calcium flux responses and reduced Syk, LAT, ERK and p38 phosphorylation status (246, 248, 249).

In blood-purified eosinophils, IRp60/CD300a inhibits eotaxin-induced transmigration and the anti-apoptotic effect of IL-5 and GM-CSF. It also reduces the secretion of TNF-α, IL-β1, IL-4 and IFN-γ upon eosinophil activation (247). The receptor was found associated to SHP-1 (but not SHP-2), what led to a diminished phosphorylation of JAK2, p38 and ERK-1/2 signal transduction mediators. As these effects are calcium-independent, SHIP association is unlikely to occur in these cells.

IRp60/CD300a inhibits ROS (Reactive Oxygen Species) production upon ITAM-initiated inflammatory responses in neutrophils. Inhibition of calcium mobilization was also observed on these cells (245).

IREM-1/CD300f

IREM-1/CD300f sequence was obtained as a result of a yeast three hybrid screening to identify molecules within a PBMC library that could interact with the SH2 domains of SHP-1 phosphatase in a phosphotyrosine dependent manner (250). Another group reported the same sequence as IgSF13/NKIR (NK Inhibitory Receptor). This sequence was raised from a massive sequencing process of a dendritic cell cDNA library (251).

IREM-1/CD300f is a type I-transmembrane glycoprotein composed of a single V-type Ig domain, a membrane-proximal region, a transmembrane domain and cytoplasmic domain

with five tyrosine residues. Y205 and 249 are in the context for ITIM motifs, while Y236 and Y263 constitute docking sites for p85α, the PI3K regulatory subunit. Y236 is in fact a mixed motif, as it is also in the consensus for Grb-2 recruitment. The last tyrosine, Y284, is within a tyrosine-based motif, as observed for IRp60/CD300a (26, 250).

The gene spans 19 kb and is composed by nine exons that can give rise to four different splicing variants but only a single mature protein has been detected. Exon 1 and exon 2 code for different signal peptides, thus the gene presents two different transcriptional starts. Nevertheless, only the signal peptide encoded by exon 1 matches a valid leader sequence. Exon 5 is naturally skipped in the mature transcript. Then, exon 1 encodes the 5′ untranslated region and the signal peptide, exon 3 encodes the Ig domain, exon 4 encodes the membrane-proximal region, exon 6 encodes the transmembrane domain and exons 7, 8 and 9 encode the whole cytoplasmic and the 3′ untranslated region (only exon 9).

IREM-1/CD300f has been detected at different levels of intensity in all myeloid-derived cells but is completely absent in the lymphoid compartment. IREM-1/CD300f is expressed at high levels by resting and LPS-activated peripheral blood monocytes. The receptor is maintained in classically-activated macrophages (M1) and slightly downregulated in alternatively-activated macrophages (M2). In contrast, its expression is markedly downregulated in iMDDC (immature Monocyte-Derived Dendritic Cells), and is not reexpressed after maturation with LPS (mMDDC). Dull levels or IREM-1/CD300f have been observed in basophiles, eosinophils, neutrophils and mast cells (250)(unpublished data). It is of note that freshly isolated myeloid dendritic cells ($CD33^{+++}/DR^{++}$) express IREM-1/CD300f also at lower levels, what allowed IgSF13 identification.

IREM-1/CD300f resembles structurally inhibitory receptors but possesses dual functional features. It is able to deliver both, inhibitory and activating signals. Inhibition occurs, through recruitment of SHP-1 phosphatase. Tyrosine 205 is essential for SHP-1 binding but Y249 and Y284 are also needed for IREM-1 inhibitory function evidenced by functional experiments on the RBL-2H3 cell line (Rat Basophilic Leukemia). Activation has been observed when ITIM motifs and the distal tyrosine-based motif are disabled. IREM-1/CD300f activation is mediated by PI3K. Both, Y236 and Y263 are able to bind individually p85α, but the first has a stronger effect on recruitment. Additionally, IREM-1/CD300f is able to interact with Grb-2, presumably though Y263. In myeloid cell lines, IREM-1/CD300f cytoplasmic tail is able to bind simultaneously SHP-1 and p85α but not SHP-2 and/or SHIP. In this scenario, p85α phosphorylation was found to be regulated by SHP-1 recruitment (26, 250).

IREM-2/CD300e

IREM-2/CD300e is the only classical activating receptor within the IREM/CD300 family. Its identification was possible upon the discovery of IREM-1/CD300f sequence to which is 56% homologous in the extracellular Ig-like domain.

IREM-2/CD300e displays additionally to the V-type immunoglobulin domain and the stem region, a positively charged transmembrane domain and a short cytoplasmic tail.

The gene encoding for the receptor, which spans 11kb, is organized in four exons. As seen for other receptors, exon 1 encodes the 5' untranslated region and the signal peptide, exon 2 encodes for the immunoglobulin-like domain, exon 3 encodes both, the proximal-membrane and transmembrane domains and exon 4 encodes the cytoplasmic tail and the 3' untranslated region.

IREM-2/CD300e expression is restricted to myeloid-origin cells, in particular to monocytes and myeloid dendritic cells. Analysis of bone marrow samples have determined that its acquisition throughout the monocytic lineage occurs late in the differentiation process, since no detection of the molecule is observed until myeloid precursors acquire high levels of CD14 and CD64 antigens. Oppositely to the rest of IREM/CD300 members, IREM-2/C300e has not been detected in any of the myeloid cells lines tested (252). Moreover, IREM-2/CD300e expression is not maintained by *in vitro*-derived dendritic cells (252, 253).

This receptor is able to interact in transiently transfected COS-7 cells with two different transmembrane adaptor proteins, DAP-12 and DAP-10, but no information is available about IREM-2/CD300e interactions in resting and/or activated monocytes (252)(unpublished data). Despite this, the activating capacity of IREM-2/CD300e has been confirmed, as blood-purified monocytes are able to induce the secretion of TNFα upon triggering of the receptor. This effect has been linked to the capacity of IREM-2/CD300e to modify the intracellular calcium concentration levels (252).

IREM-4/CD300d

IREM-4/CD300d has not been characterized in the literature, but we have been able to generate some information based on its cloning and sequence analysis.

IREM-4/CD300d gene spans 13 kb and is composed of four exons. No alternatively spliced transcripts have been observed. Exon 1 encodes the 5' untranslated region and the signal peptide. Exon 2 encodes the Ig-like domain that is highly homologous to IREM-1, specifically they share a 73% of identity and a 79% of similarity based on amino acid

sequence. Exon 3 encodes for the membrane-proximal region, which also is predicted to be highly glycosylated, and for the transmembrane domain, that bears a glutamic acid in the center. Based on this, it is probable that IREM-4/CD300d uses the same signal transduction mechanisms than CMRF-35. And finally, exon 4 encodes for the cytoplasmic tail, that is composed only by 6 amino acids, and for the 3' untranslated region.

Despite the high degree of homology between IREM-1/CD300f and IREM-4/CD300d, the monoclonal antibodies raised in our group to asses IREM-1/CD300f expression do not crossreact with IREM-4/CD300d. Antibodies against IREM-4/CD300d have not been generated yet, thus the expression of IREM-4/CD300d remains unknown. Nevertheless, its expression is most likely to be also restricted to the myeloid compartment. Purified blood monocytes are positive for the presence of IREM-4/CD300d transcript, as this was the source we used for the cloning of the receptor.

2.4.2 MURINE CLM/CD300 RECEPTORS

CLM-8

CLM-8, also known as MAIR-I (Myeloid-Associated Ig-like Receptor), LMIR-1 (Leukocyte Mono-Ig-like Receptor) and CD300La, is one of the inhibitory receptors of the murine CLM/CD300 family. The original sequence, MAIR-I, was raised from a PCR-based subtractive hybridization assay over fetal liver cDNA to compare transcript expression between wild-type and PU.1$^{-/-}$ mice, which exhibit a defect of myeloid cells (254). Two variants of the same gene, differing in the presence or absence of four amino acids at the membrane-proximal regions, were identified and called MAIR-Ib and MAIR-Ia respectively. LMIR-1 sequence, which corresponds to MAIR-Ib form, was obtained from a screening of IgE-stimulated BMMC (Bone Marrow-derived Mast Cells) (255).

The gene, spanning approximately 15 kb, is composed of 7 exons codifying for the 5' untranslated region and signal peptide, the Ig-like domain, the membrane-proximal region, the transmembrane domain and the cytoplasmic tail (spread over exons 5, 6 and 7) respectively. The use of two different splicing acceptor sites at exon 3 results in two transcript variants differing in the presence or absence of two amino acids in the membrane-proximal region (254).

CLM-8 is present in myeloid cells such as peripheral blood monocytes, neutrophils, eosinophils and basophils, peritoneal and spleen macrophages, spleen dendritic cells, bone marrow-granulocytes and mast cells, but also in a subset of B cells present in the spleen marginal zone. CLM-8 expression is observed upon NK activation with IL-12 (254, 256).

CLM-8 has the prototypic structure for an inhibitory receptor. Additionally to the extracellular and the neutral transmembrane domain, CLM-8 presents a long cytoplasmic tail with five tyrosine residues. Two of them, Y258 and Y270, are in the context for ITIMs, while Y233 is a mixed motif fitting the consensus for Grb-2 recruitment and for internalization tyrosine-based sorting motifs. The last tyrosine residue, Y313, localized one amino acid prior to the end of the sequence is too distal to be included in any tyrosine-based motif. CLM-8 ITIM motifs are able to recruit under pervanadate treatment in transfected cell lines, all three tyrosine-phosphatases: SHP-1, SHP-2 and SHIP (255, 257). But the same experiment performed in BMMC, lead to different results. For some authors CLM-8 retains this capacity, while for others this receptor is only capable to recruit SHIP phosphatase (254, 255). Studies with CLM-8 mutant forms have shown that Y274 is essential and sufficient for phosphatase recruitment and the subsequent inhibitory effects, while Y262 needs the cooperative effect of the other tyrosines to develop its role. In eosinophils, downstream mediators affected by phosphatase recruitment include Syk, ERK-1/2 and PLCγ (256). Tyrosine 237 was proved in peritoneal macrophages to effectively mediate the endocytosis of the receptor (254).

As its human counterpart, murine CLM-8 is able to inhibit mast cell IgE-dependent serotonin and β-hexosaminidase release (254, 257).

CLM-8 role has been extensively studied in anaphylactic and allergic models due to its expression in mast cells and eosinophils, where it is particularly high. Stimulation of CLM-8 in a mice model for experimental asthma, led to a dramatic decrease of infiltrating eosinophils both in the peribronquial and perialveolar space, mucus-secreting goblet cells as well as a decrease in the secretion of Th2 cytokines and mast cells released-mediators such as IL-4, IL-5, IL-13, tryptase and eotaxin-2 (248, 256). Comparably, in two models of passive cutaneous anaphylaxis induced by IgE- and SCF-sensibilization, triggering of CLM-8 impaired mast cell degranulation and consequently the anaphylactic reaction (248, 249). Contrary, models of experimental allergic peritonitis in which CLM-8 signaling was suppressed by antagonistic antibodies, coursed with exacerbated inflammatory signals (246).

CLM-7

CLM-7 was originally cloned in our laboratory as mIREM-3, but other groups have also reported its sequence as LMIR-5 and CD300Lb. mIREM-3 and LMIR-5 are the same sequence, although some punctual amino acid substitutions can be distinguished among them. Most likely the changes observed constitute polymorphisms derived of the use of different mouse

strains. CLM-7 is five amino acids shorter due to a nucleotide deletion at the end of the sequence that changes the reading frame and induces the use of a premature stop codon.

CLM-7 protein exhibits, additionally to the Ig-like domain and the stem, a transmembrane region bearing a lysine residue and a short cytoplasmic tail that, oppositely to its human counterpart, is devoid of any signaling motif (258).

CLM-7 gene spans around 12 kb and is composed of five exons. Exon 3 is skipped in the transcript that gives rise to the complete and mature form of the protein. Three alternative splicing variants of the gene have been detected, but none of them encodes a full length protein due to the presence of premature stop codons occurring by insertion of exon 3 and/or use of alternative splice sites in exon 2 (unpublished data). Thus in the productive transcript, the signal peptide and the 5′ untranslated regions are encoded by exon 1, the immunoglobulin domain is encoded by exon 2, the membrane-proximal and transmembrane regions are both encoded by exon 4, and the cytoplasmic tail is encoded by exon 5.

The receptor reproduces the myeloid-restricted pattern observed for most of the members of IREM/CLM/CD300 family. CLM-7 has been found in spleen and peritoneal macrophages, granulocytes and spleen dendritic cells. It is to mention that among granulocytes, those resident in the bone marrow and as consequence immature, expressed higher levels that those in peripheral blood. Moreover, CLM-7 was expressed by mast and all *in vitro* derived bone marrow populations (259). CLM-7 expression levels are controlled by transmembrane adaptor proteins to which the receptor associates, DAP-12 and DAP-10. Recruitment of endogenous DAP-12 has been observed in BMMC transduced with LMIR-5 (259). In our hands, CLM-7 was also able to recruit DAP-10 and FcRγ in COS-7 transfected cells (unpublished data). Whether DAP-10 associates endogenously with CLM-7 is currently unknown but DAP10$^{-/-}$ granulocytes and BMMC display lower levels of the receptor suggesting a role of this adaptor en the CLM-7 signaling.

Triggering of the receptor in BMMC and FLMC (Fetal Liver Mast Cells) is able to induce cytokine and chemokine production (IL-6, TNFα and MCP-1), release of histamine granules, increase of adhesion to substrate and also promote cell survival in an IL3-free environment. These effects are known to be dependent on transmembrane adaptor phosphorylation by Syk kinase that in turn is responsible for ERK-1/2, p38 and Akt phosphorylation. A major role for DAP-12 versus DAP-10 has been observed in the CLM-7 activating signaling pathways (259). This is in accordance with DAP-10 coactivating function rather than activating.

CLM-4

Multiple groups have identified and characterized this receptor (CD300Ld) following different approaches and mouse strains. This has led to slightly different variants that can be considered polymorphisms from a single gene. As a result, multiple names for the same receptor are currently used: DIgR1 (Dendritic cell-derived Ig-like Receptor), MAIR-II, LMIR-2 and CLM-4 (listed by appearance in the literature). DIgR1 sequence was raised from a search in the murine EST database with human CMRF-35/CD300c and IRp60/CD300a sequences to which is 45% and 53% identical in the Ig-domain (260). MAIR-II was found by screening a cDNA library from peritoneal macrophages with MAIR-I Ig domain (CLM-8)(254). LMIR-2 also came out from a database search, but this time the LMIR-1 form (CLM-8 too) was used as bait (255). CLM-4, as all CLM family members, was found by genomic database search to identify genes in close vicinity to CLM-1 (25).

CLM-4 protein exhibits additionally to the Ig and membrane-proximal domains, a transmembrane region with a positively charged lysine and a short cytoplasmic tail with a tyrosine-based motif in the context for Grb-2 recruitment. This motif has not been explored. CLM-4 structure is more similar to human CD300b than to CD300d, which as described previously bears a negatively charged residue and no signaling motifs within the intracellular region.

The gene, that extends over 5 kb in the chromosome, is composed of four exons codifying for the 5′ untranslated region and signal peptide, the Ig-like domain, the membrane-proximal and transmembrane regions, and the cytoplasmic tail respectively. The use of two different splicing acceptor sites at exon 3 results in two transcript variants differing in the presence or absence of two amino acids in the membrane-proximal region (254).

CLM-4 is only expressed by peritoneal, spleen and bone marrow-derived macrophages and a subset of B cells specifically the resident in the marginal zone of the spleen. LPS exposure has shown to increase CLM-4 expression in these B cells (254).

According to the presence of a lysine in the transmembrane domain, CLM-4 is able to bind to DAP-12 adaptor protein in resting and LPS-stimulated B cells from the spleen and the peritoneum (254, 261). CLM-4 on peritoneal and bone marrow-derived macrophages is able to bind not only DAP-12 but FcRγ as well (261). This property is not shared by spleen macrophages, what supports the selectivity in molecular associations within the cells in the immune system. It is important to mention that in these cells, CLM-4 does not interact with DAP-12/FcRγ heterodimers. Other authors extend CLM-4 association to DAP-10 adaptor

protein, but this observation has only been performed in transiently transfected COS-1 cells (255). Whether this interaction occurs in real cells or not remains unknown.

CLM-4 mediates activating signals in peritoneal and spleen macrophages, leading to TNFα, IL-6 and MCP-1 cytokine and chemokine secretion. This effect is a consequence of increased ERK phosphorylation levels (254, 261).

CLM-1

CLM-1, known as CD300Lf, was the first identified CLM/CD300 moleucle. Its cloning gave raise to the identification of the whole locus. CLM-1 sequence was obtained from a search on the mouse EST database using the immunoglobulin domain of mouse TREM-2 and TREM-3 as baits (25). Afterwards, other groups have reported and characterized the same sequence by performing independent database searches. This has given rise to a multiplicity of names for CLM-1: DIgR2, MAIR-Va/b and LMIR-3 (262-264). CLM-1/LMIR-3/MAIR-Va is seven amino acids longer that DIgR2/MAIR-Vb. This is a consequence of the use of alternative acceptor splicing site in the boundary of intron/exon 3.

CLM-1 molecule has the archetypal structure for an inhibitory receptor as it displays a long cytoplasmic tail with multiple tyrosines in the context for the recruitment of SH2-bearing molecules. Particularly, CLM-1 displays two tyrosines that fall into consensus for ITIM motifs, a third one that lies in a PI3K docking site and a distal one that lies in a consensus motif for SAP recruitment (SLAM-associated Protein). Although it has not been reported, CLM-1 possess an additional tyrosine in a Grb-2 motif that has been shown to be active in its human counterpart (26). This mixed combination of inhibitory and activating motifs makes the molecule to be able to perform multiple functions depending on the tyrosine phosphorylation state and the availability of signaling mediators.

The receptor is broadly expressed in hematopoietic cells. Macrophages, B cells and granulocytes as well as a subset of T cells on the spleen stain positive for CLM-1 expression. The receptor is upregulated in granulocytes upon stimulation with LPS and maturation with G-CSF (264, 265).

CLM-1 is able to recruit SHP-1 phosphatase upon tyrosine phosphorylation, but not SHP-2 and/or SHIP (25, 262). Functionally, the receptor inhibits myeloid differentiation into fully active osteoclasts in response to RANKL and TGFβ. Indeed, CLM-1 expression decreases upon normal transition of myeloid cells into osteoclasts. However, implication of SHP-1 in CLM-1 bone remodeling processes has not been demonstrated. The receptor seems also to

regulate dendritic cell priming and proliferation of T cells. In particular, it has been proposed as an inhibitor of dendritic cell-initiated antigen-specific Th1 and CTL responses (262).

CLM-1 triggering is able to induce apoptosis in several transfected cell types. The nature of the apoptotic effect has not been elucidated yet, but nor caspases neither endoplasmic reticulum stress are the mechanisms by which the receptor mediates this effect. Moreover, even thought the mechanism is dependent on the receptor's cytoplasmic tail, none of the tyrosine-based motifs seems crutial for the induction of the apoptotic processes (265).

CLM-5

This molecule is known as CLM-5, MAIR-IV and LMIR-4 as its identification and cloning was performed by three independent groups. All the sequences came out from database searches although using different baits (25, 264, 266). As observed for other murine CLM/CD300 receptors, CLM-5 extracellular region displays several amino acidic substitutions on the extracellular region depending on the mouse strain. Despite not being included in the CD nomenclature its structure is very similar to human CMRF-35/CD300c and IREM-4/CD300d, as it displays a negatively charged residue in the transmembrane domain in combination with a short cytoplasmic tail.

The receptor is expressed by tissue and bone marrow macrophages, monocytes, spleen dendritic cells and by mature/immature granulocytes. In certain cells types, such as macrophages and granulocytes, CLM-5 has been shown to be upregulated upon stimulation with proinflammatory and maturing agents. Oppositely, its expression in granulocytes diminishes upon stimulation with LPS (264, 266).

CLM-5 coprecipitates with FcRγ in transfected cell lines and the association has been shown to take place endogenously in macrophages (266). There is no information about the interaction mechanism used by two negatively charged transmembrane proteins.

Consistent with the role of an activating receptor, CLM-5 stimulation elicits phosphorylation of key signaling molecules such as p38, ERK-1/2, JNK and Akt (264, 267). In bone marrow-transduced mast cells, CLM-5 induces cell survival in absence of IL-3, histamine release and IL-6 and TNFα cytokine secretion (264). The effect on cytokine release has also been observed in peritoneal macrophages and neutrophils (266). In addition, CLM-5 amplifies the activating signals delivered by engagement of FcεR in mast cells and TLR4 either in mast cells and granulocytes (264). These effects are dramatically impaired upon the use of inhibitors of Src kinase inhibitors or cell types derived from FcRγ, Lyn or Syk knock out mice (264). As

consequence, it has been hypothetized that CLM-5 triggering leads to FcRγ recruitment and ITAM phosphorylation first Lyn kinase and subsequently by Syk kinase.

AIMS

1. Cloning and molecular characterization of IREM-3/CD300b, a novel human Ig-like immune receptor belonging to the IREM/CD300 family.

2. Analysis of the signal transduction pathways mediated by IREM-3/CD300b receptor.

3. Molecular and functional characterization of IREM/CD300 immune complexes.

RESULTS

Article 1

Molecular and functional characterization of CD300b, a new activating immunoglobulin receptor able to transduce signals through two different pathways

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Molecular and functional characterization of CD300b, a new activating immunoglobulin receptor able to transduce signals through two different pathways[1]

Running Title: Cloning of CD300b

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Keywords: Cell activation, Monocytes/Macrophages, Signal transduction, Cell Surface Molecules, Human

ABSTRACT

Here we describe the characterization of human CD300b, a novel member of the CMRF-35/IREM multigene family of immune receptors. IREM-3 cDNA was cloned from a PHA-activated PBMC library and RT-PCR revealed the gene to be expressed preferentially in cells of myeloid origin. The CD300b cDNA open reading frame encodes a 201-amino acid type I protein composed of a single extracellular Ig V-type domain followed by a transmembrane region containing a positively charged residue (lysine), which is a common feature among receptors that associate with activating adaptor proteins. Indeed, CD300b was able to associate with DAP-12 and deliver different activating signals through this ITAM-based adaptor. Unusually for an activating receptor, the 29-amino acid cytoplasmic tail of CD300b contains a tyrosine-based motif that upon c-Fyn phosphorylation in yeast became a docking site for the intracellular signaling mediator Grb-2. Moreover, in the absence of DAP-12, CD300b was able to activate NFAT/AP-1-dependent transcriptional activity in RBL-2H3 cells. This activity could be abolished only by mutating both the cytoplasmic tyrosine and the transmembrane lysine. Our data suggest the existence of an unidentified molecule capable of interacting with CD300b through a charged residue of the transmembrane region and allowing receptor signaling independently of DAP-12. Therefore, CD300b defines a non-classical immunoglobulin receptor able to trigger signals by coupling distinct mediators and thus initiating different signaling pathways.

INTRODUCTION

Immune function is regulated by a balance between positive and negative signals delivered by activating and inhibitory receptors on the surface of leukocytes. These receptors are responsible for the correct transduction of information from the exterior to the interior of the cell and elicit a response that can range from reactivity to quiescence depending on the stimulus (1). Inhibitory receptors display long cytoplasmic tails presenting a variable number of ITIMs (Immune receptor Tyrosine-based Inhibitory Motif). Upon ligand binding, ITIMs are tyrosine-phosphorylated by Src kinases and become docking sites for SH2-containing phosphatases, such as SHIP and SHP-1. Dephosphorylation of key signaling molecules will lead to inhibition of cellular activation (2, 3). In contrast, classical activating receptors display both a short cytoplasmic tail having no known signaling motifs, and a positively charged residue in the transmembrane region that allows the receptor to associate with transmembrane adaptor proteins. To date, two types of transmembrane adaptor proteins have

been described: the ITAM-bearing adaptors (Immune receptor Tyrosine-based Activating Motif), including DAP-12/KARAP, FcεRIγ and CD3ζ; and the PI3Kinase-associated adaptor DAP-10. Engagement of activating receptors promotes tyrosine phosphorylation of transmembrane adaptors and subsequent recruitment of either protein tyrosine kinases containing Src homology 2 (SH2) domains, such as Syk or ZAP-70, or PI3Kinase that would contribute to activation of AKT and PLC-γ pathways. Activating signaling events will lead to secretion of cell mediators, migration, proliferation and/or differentiation, among other processes (4-8).

We recently identified IREM-1 and IREM-2, members of the CMRF-35/IREM family (CD300) of immunoglobulin receptors (9, 10). The genes of this family are clustered in a region on chromosome 17 (17q25.1) spanning approximately 250 kb, and code for both activating and inhibitory receptors. IREM-1 and IRp60 have been shown to recruit SHP-1/2 phosphatases and deliver inhibitory signals (9, 11). Oppositely, IREM-2 has been found to associate with DAP-12 adaptor and therefore deliver activating signals (10). The first identified member, CMRF-35, has a short cytoplasmic tail and a transmembrane region containing a negatively charged residue, structural features that make its classification uncertain (12).

In the present study, we report the identification and functional characterization of a novel Ig-like receptor belonging to the CMRF-35/IREM family. CD300b, expressed mainly on myeloid cells, is a non-classical activating receptor able to deliver signals not only by associating with the transmembrane adaptor protein DAP-12, but also by recruiting Grb-2 through a tyrosine-based motif present in the receptor's cytoplasmic tail.

MATERIALS AND METHODS

Cells and antibodies

Human T (Jurkat, Molt-4, T-All 103/102), B (Ramos, Daudi, RPMI-8866), NK (YT, NKL) and myelomonocytic cell lines (U937, THP-1, HL60, MonoMac6), and KU812, P815 and Raw264.7 cell lines were maintained in RPMI 1640/L-Glutamine medium supplemented with 10% heat-inactivated FBS, 25mM Hepes, 2mM glutamine, 100IU/mL penicillin and 100μg/mL streptomycin. T-All 103/102 and NKL culture media were additionally supplemented with 100U/mL of hIL-2 (BD Pharmingen, NJ, USA). COS-7 and RBL-2H3 cells were grown in DMEM containing 10% heat-inactivated FBS, 2mM glutamine, 1mM sodium pyruvate, 100IU/mL penicillin and 100μg/mL streptomycin. Human PBMC were obtained

from heparinized venous blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Human NK cells were isolated and cultured as described (13).

Anti-HA.11 mAb was from Covance (Berkeley, CA, USA). Mouse anti-phosphotyrosine cocktail coupled to HRP was obtained from Zymed (San Francisco, CA, USA). Anti-FLAG M2 mAb and anti-DNP SPE7 mAb were from Sigma (St. Louis, MO, USA). Anti-Grb2 mAb was from BD Tranduction Laboratories, and anti-Grb2 (C-23) pAb was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated goat anti-mouse antibody was from Amersham Pharmacia Biotech (Little Chalfont, UK). Streptavidin-HRP was purchased from Roche (Penzberg, Germany). Anti-rat high-affinity IgE receptor mAb was obtained from BD Pharmingen. Polyclonal rabbit anti-mouse FITC was from DAKO (Glostrup, Denmark). Z199 mAb, used as an isotypic control, and anti-HA12CA5 mAb were described previously (9, 14).

DNA reagents

Mammalian and yeast expression vectors pMES/hFyn (15), pFLAG-CMV2/FcεRIγ (10), pSRα/CD3ζ (10) and pACT2/Grb-2 (16) were described previously. Sequence encoding CD300b lacking the signal peptide was amplified by PCR using oligos 1 and 2 (Table I) and cloned into the BglII/SalI sites of the pDisplay vector. pDisplay/CD300b substitution mutants were generated with sets of mutagenic oligos according to the instructions of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). pDisplay/CD300b Y188F and pDisplay/CD300b K158L mutants were obtained by annealing oligos 3-4 and 5-6, respectively, with the pDisplay/CD300b construct. The pDisplay/CD300b K158L Y188F double mutant was created by annealing oligos 5-6 with the pDisplay/CD300b Y188F construct. Presence of the introduced mutations was confirmed by DNA sequencing with an ABI PRISM Big Dyes Terminator Cycle Sequencing Ready reaction kit. Two deletion mutants of CD300b affecting the cytoplasmic tail were obtained by PCR and cloned into the BglII/SalI sites of pDisplay. CD300b Δ1 (del 178-201aa) was amplified using oligos 1-7, and CD300b Δ2 (del 188-201aa) was amplified using oligos 1-8. pCDNA3/FLAG-DAP10 was obtained by subcloning FLAG-DAP10 of pMxNeo into the BamHI/EcoRI sites of pCDNA3 (17). A pCDNA3-FLAG vector was generated by cloning CD8 signal peptide followed by a FLAG-epitope into the HindIII site of pCDNA3. Sequences encoding DAP-12 and CD3ζ transmembrane adaptor proteins without their signal peptides were amplified by PCR and cloned into the BamHI/EcoRI and EcoRI/XhoI sites, respectively, of pCDNA3-FLAG. DAP-12 was amplified from pJFE14-SRα/DAP-12 using oligos 9-10 and CD3ζ was amplified from pSRα/CD3ζ using oligos 11-12 (10). A pBabePuro-HA expression vector was generated by

cloning the Igkappa signal peptide followed by an HA-epitope into the BamHI/EcoRI sites of pBabePuro. CD300b wt, CD300b Y188F, CD300b K158L and CD300b K158L Y188F were cloned into the EcoRI/SalI sites of pBabePuro-HA vector by PCR amplification from the pDisplay constructs using oligos 13-2. For the three-hybrid system assay, sequence encoding the CD300b cytoplasmic tail, obtained using oligos 14-2, was cloned into the EcoRI/SalI sites of pBridge/c-$Fyn_{420Y\text{-}F,531Y\text{-}F,176R\text{-}Q}$ MCSI cassette described previously (15). The pBridge/CD300b Cyto/c-Fyn vector was digested with BglII endonuclease and religated in order to obtain a pBridge/CD300bCyto/c-Fyn catalytic mutant construct.

Cloning strategy of CD300b and mIREM-3

A PCR strategy was used to amplify the full-length CD300b sequence from a PHA-activated PBMC cDNA library. Oligos 15-16 in Table I, mapping to the 5' and 3' untranslated regions of CD300b, were used for this purpose. PCR conditions were as follows: 94ºC 5min; 25 cycles of 94ºC 1min, 55ºC 30sec, 72ºC 2min; and 72ºC 10min. mIREM-3 full-length sequence was amplified from Raw264.7 cDNA using oligos 17-18, which also map to the 5' and 3' untranslated regions of mIREM-3. PCR conditions were as follows: 94ºC 5min; 35 cycles of 94ºC 1min, 55ºC 30sec, 72ºC 1min; and 72ºC 10min. DNA products were resolved in a 1% agarose gel and visualized by ethidium bromide staining. Expected size fragments were cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced for confirmation using the universal T7 primer as described above.

RT-PCR

RNA extracted with Trizol reagent (Gibco, Carlsbad, CA, USA) from human polyclonal NK cells and hematopoietic cell lines was retrotranscribed using a ProtoScript™ First Strand cDNA Synthesis Kit (NewEngland Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. CD300b amplification from human cell lines with oligos 15-16 was performed using 2μg of cDNA as template. The conditions used were: 94ºC 10min; 40 cycles of 94ºC 30sec, 60ºC 30sec, 72ºC 1min; and 72ºC 10min. CD300b amplification from human isolated blood populations was performed using 10ng of commercial cDNA ($polyA^{+}$ RNA origin) (Clontech, Mountain View, CA, USA) or 2.5μg of cDNA from polyclonal NK cells (total RNA origin). The conditions used were: 94ºC 10min; 35 cycles of 94ºC 30sec, 60ºC 30sec, 72ºC 1min; and 72º 10min. PCR products were cloned into pCR2.1-TOPO vector and sequenced for confirmation. GADPH amplification was carried out in parallel with oligos 19-

20. Detection of CD300b whole transcript in human and fetal tissues was performed using a Human Rapid-Scan™ Gene Expression Panel (Origene Technologies, Rockville, MD, USA) following the manufacturer's recommendations. β-actin amplification was used as an internal control. FastStart Taq DNA Polymerase (Roche) was used for all PCRs.

1	Sense	5'-CCTAGATCTCAAGGCCCAGAGTCTGTGAGA-3'	BglII
2	Antisense	5'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'	SalI
3	Sense	5'-GGGGAACAGCCTATCT**T**CATGAACTTCTCCGAAC-3'	-
4	Antisense	5'-GTTCGGAGAAGTTCATG**A** AGATAGGCTGTTCCCC-3'	-
5	Sense	5'-CTCCTGGTATTTGTG**TT**GGTGCCCATCTTGCTCA-3'	-
6	Antisense	5'-TGAGCAAGATGGGCACC**AA**CACAAATACCAGGAG-3'	-
7	Antisense	5'-GCCGTCGACCTACCTCTGAGACCCCTTCAA-3'	SalI
8	Antisense	5'-GCCGTCGACCTAGATAGGCTGTTCCCCTGG-3'	SalI
9	Sense	5'-CTGGGATCCCAGGCCCAGAGCGATTGC-3'	BamHI
10	Antisense	5'-CTGGAATTCTCATTTGTAATACGGCCT-3'	EcoRI
11	Sense	5'-CTGGAATTCGGCAGAGCTTTGGCCTGCTG-3'	EcoRI
12	Antisense	5'-CTGCTCGAGTTAGCGAGGGGGCAGGGCCT-3'	XhoI
13	Sense	5'-GCCGAATTCCAAGGCCCAGAGTCT-3'	EcoRI
14	Sense	5'-GCCGAATTCAAGGGGTCTCAGAGGGTC-3'	EcoRI
15	Sense	5'-AGAGTGCATCTGGGATCTGC-3'	-
16	Antisense	5'-CCGAGTCTCTTCTGGAAACG-3'	-
17	Sense	5'-TGTTCATCAAGGAGTGCA-3'	-
18	Antisense	5'-TGCTTATCCATCAGTCTA-3'	-
19	Sense	5'-CTCCGGGTCTTTGCAGTCGTATGG-3'	-
20	Antisense	5'-AGGCAGGAGCGCAGGGTTAGTCAC-3'	-

Table I: Oligos used in this study. Nucleotide changes introduced in the sequence for changing the desired amino acid are shown in bold letters. Restriction sites used for cloning are underlined.

Cell transfections

COS-7 cells (10^6) were transiently transfected using the DEAE-Dextran method (18) and lysed for the immunoprecipitation assay after 48h. For the generation of RBL-2H3 stable transfectants, 20×10^6 cells were electroporated in the presence of 20μg of linearized construct at 280V and 950μF in a Gene Pulser Electroporator (Bio-Rad, Hercules, CA, USA). Transfectants were selected and maintained in culture with 1mg/mL G418 (Gibco). RBL-2H3 double transfectants were generated sequentially and maintained with 1μg/mL Puromycin (Sigma) in addition to G418. COS-7 cells (10^5) for flow cytometry analysis were transfected with Fugene 6 reagent (Roche) according to the manufacturer's instructions.

Flow cytometry

Cell-surface expression of the desired molecules was checked by flow cytometry with the corresponding antibodies following standard techniques (19). Analysis was performed using a FACScan instrument and CellQuest™ software (BD Pharmingen).

Immunoprecipitation and Western Blotting

Cells were lysed at 4°C for 15min using 1% Tx-100 containing buffer described previously (15). Cell lysates were clarified by centrifugation at 14,000 g for 15 min at 4°C and the crude lysates were pre-cleared for 1 h at 4°C using 20μL of protein G-Sepharose beads (Amersham Biosciences) and 5μg of mouse or rabbit IgG (Sigma). Two additional pre-clearings were carried out for 30min at 4°C with 20μL of protein G-Sepharose beads. For immunoprecipitations, pre-cleared lysates were incubated with 30μL of protein G-Sepharose beads and 1μg of antibody for 3 hours at 4°C. Proteins in the crude lysates and immunoprecipitates were separated by SDS-PAGE and transferred onto PVDF filters (Millipore, Bedford, MA, USA). Filters were blocked for 1 hour with 5% skim-milk or 3% bovine serum albumin (Sigma) and then probed with the indicated antibodies. Bound antibodies were detected using enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Luciferase Assays

RBL-2H3 transfectants were transiently electroporated with a luciferase reporter plasmid (pT81Luc) containing three tandem copies of the distal NFAT/AP-1 site of the murine IL-2 promoter (20) (0.5μg/10^6cells) and a TK renilla construct (Promega, Madison, WI, USA) (0.1μg/10^6 cells). 24h post-transfection, 1.5×10^6 cells were stimulated for 7h with 5μg/mL of anti-HA12CA5 or a negative isotypic antibody using the murine mastocytoma P815 line as presenting cell (1×10^6). Plastic-coated anti-DNP IgE (5μg/mL) was used as positive control for stimulation and the P815 cell line in complete RPMI medium was used as negative control. Post-nuclear lysates were obtained as described (20) and luciferase activity was measured according to the Dual Luciferase Reporter Kit manual (Promega).

Hexosaminidase release assays

5×10^5 RBL-2H3 cells resuspended in 50μL of Tyrodes buffer (21) were stimulated for 1h at 37°C by plastic coated antibodies (5μg/mL). Supernatants (20μL) were collected and incubated for an additional hour at 37°C with 1mM 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.05M citrate buffer, pH 4.5 (50μL) (Sigma). Reactions were stopped by adding 0.2M glycine buffer, pH 10.7 (150μL), and the optical density was measured at 405nm. For determining the maximum degree of hexosaminidase release, cells were lysed with Tx-100 (1%) prior to incubation with the substrate.

Three-hybrid system assay

To characterize the interaction between the cytoplasmic tail of CD300b and Grb-2, a three-hybrid system assay was carried out by co-transforming CG1945 yeast with pGAD424, either alone or containing Grb-2, and pBridge/c-$Fyn_{420,\ 531Y\text{-}F,\ 176R\text{-}Q}$ or pBridge/c-Fyn catalytic mutant containing the cytoplasmic tail of CD300b. Transformants were plated on SD medium lacking tryptophan, leucine, and methionine. Clones were tested by β-galactosidase liquid culture assay using ONPG as a substrate as described (15).

RESULTS

Cloning and sequence analysis of CD300b and its murine ortholog mIREM-3

Following a three-hybrid system strategy in yeast, we recently described a novel cell surface inhibitory receptor termed IREM-1/CD300f (AY303545) (9). This immune receptor is a member of a multigene family of activating/inhibitory receptors located on human chromosome region 17q25.1 which includes the previously identified molecules CMRF-35/CD300c and CMRF35-H/IRp60/CD300a (11, 12, 22-24). With the aim of identifying new members of the same family, we searched for sequences having homology to IREM-1 and CMRF-35 in the Ensembl genome database[3]. This yielded a cDNA coding for the activating receptor IREM-2/CD300e (AF395839) (10). In the process we identified a second cDNA sequence that shared homology not only with IREM-1 and CMRF-35 but also with IREM-2, that we termed IREM-3. The sequence was found in the database as TREM-5 (AF427618, AY359025, BC028091) and recently named CD300b (25). We designed primers for amplification of the predicted cDNA from a PHA-activated PBMC cDNA library. The obtained 732-bp fragment contains an open reading frame of 606bp that encodes a protein of 201aa with a predicted molecular mass of 22.7 kDa (AY646929) (Fig 1A). Sequence analysis revealed CD300b to be a type I transmembrane protein driven by a signal peptide 17 aa in length (SignalP 3.0 server[4]). The extracellular region of CD300b displays a single Ig V-type domain followed by a 29-aa membrane-proximal region containing a high proportion of proline, serine and threonine residues that would confer on the molecule an extended open conformation (26). Additionally, the Ig domain stem presents many potential O-glycosylation sites. The transmembrane domain, which has a positively charged residue (lysine) in a central position, is followed by a short cytoplasmic tail of 29 residues in which a tyrosine-based motif can be distinguished (Fig 1A).

The CD300b gene is located at chromosomal region 17q25.1. Through alignment of cDNA and genomic sequences we determined the organization of the CD300b gene (Fig 1B). The GT-AC rule was used to define intron-exon boundaries (27). The CD300b gene spans ~10kb and contains four exons: exon 1 encodes the 5' untranslated region and the protein's signal peptide; exon 2 encodes the Ig domain; exon 3 encodes the membrane-proximal and transmembrane regions; and exon 4 encodes the entire cytoplasmic tail.

Alignment of residues of the extracellular Ig domain of CD300b with sequences of the CMRF-35/IREM family and other closely related immune receptors showed high levels of homology among the V-type Ig folds: 77% with IREM-1, 57% with IREM-2, 58% with IRp60, 60% with CMRF-35 and 44 with NKp44 (Fig 2A). A lower, although significant, degree of protein sequence homology was detected between the Ig domains of CD300b and TREM-1 and TREM-2 (41% and 42% respectively, alignments not shown). Noteworthy is the conservation of not only the pair of cysteine residues describing the Ig-V type fold (Cys_{36} and Cys_{104}) but also the second pair of cysteines also in the Ig domain (Cys_{50} and Cys_{58}). It has been proposed that the additional disulfide bond created by the second cysteine pair could define a discrete evolutionary group of receptors stemming from duplication of a common ancestral V-type Ig domain gene (28).

To identify mouse orthologs of CD300b, we used the human CD300b cDNA sequence to search the Ensembl mouse genome database. We identified a cDNA sequence highly homologous but not identical to CML-7, a member of a recently described family of murine myeloid receptors (29). Amplification of the mIREM-3 full-length transcript was performed using cDNA from the murine myelomonocytic cell line Raw264.7 as template. From a complex pattern of bands we cloned a cDNA fragment 684bp long containing an open reading frame of 630bp. The cDNA codes for a 209-aa protein with a predicted molecular weight of 23.7 kDa that we termed mIREM-3/CD300b (AY996128). Alignment of the cDNA to the mouse genome sequence located the mIREM-3 gene to chromosome 11, which is considered syntenic to human chromosome 17. We cloned four transcript variants of the gene, but, with the exception of mIREM-3, they all specified incomplete mRNAs (data not shown). The human and murine forms of CD300b share 73% amino acid identity (Fig 2B). It is striking that the tyrosine-based motif of the cytoplasmic tail is lost from the murine molecule while the conserved charged amino acid of the transmembrane domain is retained.

CD300b is expressed mainly in myelomonocytic cells

To determine the distribution of CD300b transcript, we performed RT-PCR on human hematopoietic cell lines, PBMC, and purified blood populations. CD300b transcript was found abundantly in all human myelomonocytic cell lines tested (U937, THP-1, MM6 and HL60), and weakly in both the NK cell line NKL and the B cell line RPMI-8866 (Fig 3A). However, when assessing the presence of the transcript in human hematopoietic isolated populations, CD300b was found exclusively in peripheral blood monocytes (Fig 3B). No specific cDNA amplification was observed in activated NK and B cells. To ensure that the detected transcripts did indeed correspond to CD300b, the PCR products were cloned and sequenced. Based on these data, it seems that expression of CD300b is restricted to myeloid lineages, as we observed previously for IREM-1 and IREM-2 (9, 10).

Next, we determined the distribution of CD300b transcript in human tissues. A wide set of tissues was positive for the presence of CD300b (Fig 3C). We were able to amplify CD300b from colon and lung, where mature differentiated myeloid cells can be found, and from placenta, bone marrow and fetal liver that constitute the main reservoirs for myeloid precursors (30). A small amount of transcript was amplified from spleen. In the future, CD300b protein distribution will be assessed with monoclonal antibodies in order to correlate transcript detection with cell-surface expression of the molecule.

Biochemical characterization of CD300b

COS-7 cells were transiently transfected with CD300b tagged with an HA-epitope. Forty-eight hours post-transfection the cells were lysed and subjected to anti-HA immunoprecipitation and SDS-PAGE analysis under both reducing and non-reducing conditions. Despite CD300b having a predicted molecular weight of 22.7 kDa, the molecule appeared as three discrete bands ranging in size from 26 to 32 kDa (Fig 4A), suggesting the occurrence of post-translational modifications to the mature protein. The equivalent electrophoretic mobilities observed under reducing and non-reducing conditions indicates that the receptor was not forming dimers/multimers through disulfide bridges (Fig 4A). The lack of asparagine residues in an N-glycosylation context suggests the possibility that the different bands may correspond to O-glycosylated and/or Ser/Thr-phosphorylated forms of the molecule. Treatment of the immunoprecipitates with a cocktail containing the most common O-glycosidases or with alkaline phosphatase had no effect on the number and size of bands (data not shown). Expression of the molecule without its cytoplasmic tail led to a reduction in

molecular weight of the bands but did not alter the triplet pattern (Fig 4B), indicating that whatever the modification may be, it affects the extracellular portion of the molecule. Similar results were obtained when the molecule was expressed in RBL-2H3 cells (data not shown).

CD300b associates with DAP-12/KARAP and becomes tyrosine phosphorylated in transfected COS-7 cells

The presence of a positively charged amino acid in the transmembrane region of an immune receptor strongly suggests binding to a signaling transmembrane adaptor molecule (5, 6). Considering that CD300b has a lysine residue in its transmembrane portion, we wanted to test whether the molecule was able to associate with any ITAM-bearing adaptors such as DAP-12/KARAP, FcεRIγ and CD3ζ. For this purpose, COS-7 cells were transiently transfected with CD300b-HA and the different ITAM-bearing adaptor molecules in both the presence and absence of c-Fyn kinase. CD300b was able to interact with DAP-12 only (Fig 5A). Furthermore, western blot analysis with anti-phosphotyrosine mAb revealed phosphorylation of the CD300b receptor. A low level of phosphorylation of CD300b was observed in the presence of the c-Fyn kinase alone and none when the kinase was absent, but it increased dramatically when c-Fyn was co-expressed with DAP-12 (Fig 5A). Some receptors such as SIRPβ1 and murine NKG2D are able to couple not only DAP-12 but also the PI3Kinase-associated adaptor DAP-10 (31, 32). We tested CD300b for its ability to recruit FLAG-tagged DAP-10. CD300b did not associate with DAP-10 nor did it become efficiently tyrosine-phosphorylated (data not shown).

It has been suggested that DAP-12 acts not only as a signaling molecule but also as a chaperone for certain immune receptors, preventing their retention in the ER and intracellular degradation by forming stable receptor-DAP-12 complexes (33, 34). To determine whether the presence of DAP-12 was necessary for CD300b surface expression, COS-7 cells were transfected with CD300b-HA in presence or absence of DAP12-FLAG. Surface expression of CD300b was evaluated after 48 hours by flow cytometry with anti-HA mAb. CD300b was detected on the cell surface independently of DAP-12 expression, indicating that CD300b does not require its adaptor-counterpart for membrane localization (Fig 5B). Nevertheless, CD300b expression was enhanced in the presence of DAP-12 (25.87±7.64% ME=39.22±2.34 vs 41.08±6.66% ME=76.60±4.37) (Fig 5B). Oppositely, DAP-12 expression was not modified by co-expression of the receptor (data not shown). Increased expression of CD300b was not detected when the receptor was co-transfected with any other

transmembrane adaptor protein and it was abolished when a CD300b mutant affecting transmembrane lysine 158 (K158L) was used (Fig 5B and data not shown).

Analysis of CD300b/DAP-12 complex formation and CD300b phosphorylation

The association between an activating immune receptor and its adaptor counterpart relies on the formation of a membrane-embedded salt bridge between a basic residue on the receptor (lysine or arginine) and an acidic residue on the adaptor protein (aspartate) (5, 6). As CD300b phosphorylation was occurring only in the presence of both c-Fyn and DAP-12, we addressed whether tyrosine phosphorylation was required for association of CD300b and DAP-12. For this purpose, we generated a set of HA-tagged CD300b mutants affecting the transmembrane lysine (K158L), the cytoplasmic tyrosine (Y188F) or both residues. The CD300b Lys mutant cotransfected into COS-7 cells with c-Fyn kinase and DAP-12 was unable to recruit the adaptor molecule but, unexpectedly, it retained the ability to become tyrosine-phosphorylated (Fig 5C). Therefore, in this system, tyrosine-phosphorylation of CD300b depends on the expression of DAP-12 but does not require its binding. The CD300b Tyr mutant recruited DAP-12 as efficiently as the wild-type form (WT), indicating that CD300b tyrosine phosphorylation is not needed for recruitment of the adaptor (Fig 5C). As expected, the CD300b double mutant could neither recruit DAP-12 nor become phosphorylated in the presence of c-Fyn kinase (Fig 5C). Association between CD300b and DAP-12 was maintained in absence of the c-Fyn kinase, indicating that phosphorylation of the adaptor was not essential for the formation of the complex (data not shown). Immunoprecipitation of DAP-12 resulted in co-immunoprecipitation of the three bands corresponding to CD300b, indicating that modification to the extracellular portion of the immune receptor does not affect its ability to recruit adaptor (data not shown).

CD300b engagement is able to activate transcriptional activity in the absence of DAP-12

Signal propagation to the intracellular compartment by activating receptors requires their association with transmembrane adaptor proteins (5-7). CD300b, unlike other activating immune receptors described to date, has a tyrosine-based motif in its cytoplasmic tail that could potentially deliver positive signals independently of DAP-12. In order to evaluate the activating capacity of the receptor, we stably expressed CD300b-HA on the surface of the RBL-2H3 cell line, in which DAP-12 is absent (31) (Fig 6A). The cell line was transiently transfected with a vector containing three copies of the NFAT/AP-1 response element fused to

the firefly luciferase gene. Transfected cells were stimulated with anti-HA mAb that would mimic CD300b natural ligand, as shown previously for IREM-2 (10). An increase in promoter activity, comparable to the one delivered by FcεRI stimulation, was achieved when the cells were stimulated with anti-HA mAb (Fig 6B). To determine the importance of the tyrosine-based motif in activating the 3×NFAT/AP-1 reporter gene, we generated an RBL-2H3 stable transfectant expressing the CD300b Tyr mutant form of the molecule. Substitution of the tyrosine residue caused a marked decrease in the activating capacity of the receptor but did not abolish it (Fig 6C). To address the possibility that other residues of CD300b were important for recruitment of signaling molecules, we generated a wider set of RBL-2H3 CD300b-HA stably transfected mutants (Fig 6A). The set included the previously described substitution mutants CD300b K158L and CD300b K158L+Y188F, and two deletion forms affecting the cytoplasmic tail: CD300b Δ1 (del 178-201) in which the whole cytoplasmic tail was removed, and CD300b Δ2 (del 188-201) in which the amino acids preceding the tyrosine-based motif were maintained. The poly-proline motif in the truncated cytoplasmic tail of CD300b Δ2 resembled a potential docking site for SH3 domain-containing molecules. However, both deletion mutants showed an activating capacity similar to the CD300b Y188F mutant, suggesting there are no other motifs in the cytoplasmic region able to contribute to reporter activation. Surprisingly, the CD300b K158L mutant caused a dramatic reduction in reporter activity, thus also establishing a role for the receptor's transmembrane region in signaling in the absence of DAP-12 (Fig 6C). Complete abrogation of reporter activity was achieved only when both residues, K158 and Y188, were mutated simultaneously (Fig 6C). No differences were observed among transfectants in the activating capacity and cell surface levels of FcεRI (≥99% expression, data not shown).

CD300b tyrosine does not mediate transcriptional activity is the presence of DAP-12

To explore the stimulatory function of CD300b in the presence of DAP-12, we stably expressed both molecules at the cell surface of the RBL-2H3 cells, which do not express either protein endogenously (Fig 7A). NFAT/AP-1-dependent transcriptional activity was enhanced more efficiently in the presence of DAP-12 (Fig 7B). To test whether the observed increase could be due to the convergence of two pathways initiated by CD300b, we performed a comparative analysis among RBL-2H3 cells expressing on the cell surface DAP-12 and mutants of CD300b altered at K158, Y188 or at both residues (Fig 7A). Mutation of the cytoplasmic tyrosine did not reduce transcriptional activity, indicating that the signal observed was generated solely from the association of CD300b with DAP-12 (Fig 7C). Additionally, we

can conclude that the signal induced by the receptor-adaptor complex was stronger than that produced by the receptor alone (Fig 7B). Consistent with these interpretations, mutation of the transmembrane lysine of CD300b, alone or in combination with mutation of the tyrosine residue, completely eliminated NFAT/AP-1-mediated transcriptional activity (Fig 7C).

CD300b induces hexosaminidase granule release only in the presence of DAP-12

NFAT/AP-1-dependent transcription was activated in RBL-2H3 cells by engagement of CD300b in the presence and absence of DAP-12 adaptor protein. We decided to look for additional pathways activated by CD300b either on its own or in association with DAP-12. Mast cells, when encountering a proper stimulus, are able to secrete proteases stored in cytoplasmic secretory granules (35). For instance, RBL-2H3 cells accumulate β-hexosaminidase protease whose release can be induced upon FcεRI crosslinking (36). Stimulation of RBL-2H3 transfectants expressing CD300b alone failed to elicit a hexosaminidase exocytotic response, whereas co-expression of CD300b and DAP-12 resulted in a level of hexosaminidase release comparable to that induced by IgE crosslinking (Fig 8A). Expression of a mutant form of CD300b in which the transmembrane lysine was mutated to a non-charged amino acid resulted in a reduction in protease release (Fig 8B), confirming the dependence of this process on DAP-12. In contrast, CD300b carrying a substitution of the key cytoplasmic tyrosine elicited levels of hexosaminidase release comparable to WT CD300b (Fig 8B), indicating that signaling through CD300b's tyrosine-based motif is not involved in the exocytotic pathway.

CD300b cytoplasmic tail recruits Grb-2 adaptor molecule in the presence of the Src-kinase c-Fyn

The cytoplasmic CD300b tyrosine-based motif matches the binding consensus sequence for the Grb-2 adaptor molecule (YxN) (37). To test for a phosphotyrosine-dependent interaction between CD300b and Grb-2, we performed a Gal4 three-hybrid system assay in the presence of either c-Fyn kinase or a c-Fyn kinase mutant lacking part of the catalytic domain. Interaction of CD300b and Grb-2 was detected only when c-Fyn kinase was fully active (Fig 9A). No interaction was observed when CG1945 yeast were cotransfected with CD300b and other molecules containing SH2/SH3 domains, such as Csk or PI3Kinase (p85alpha subunit), in the presence of c-Fyn (data not shown). The interaction between both molecules was also tested in a mammalian system by transfecting COS-7 cells

with the receptor in the presence and absence of the c-Fyn kinase. Endogenous Grb-2 was found to be bound to CD300b only when the kinase was co-expressed in the cells (Fig 9B).

DISCUSSION

With the identification and cloning of immune receptors, new forms of activating and inhibitory receptors that defy traditional classification are being recognized. Here, we describe the molecular cloning and characterization of a novel activating immune receptor belonging to the CMRF-35/IREM family. CD300b has a basic residue in its transmembrane domain and a functional tyrosine-based motif in its short cytoplasmic tail (Fig 1A). This structural arrangement confers on the molecule the ability to signal through two independent pathways: one through formation of a complex with an ITAM-bearing adaptor, and the other through the tyrosine residue in its cytoplasmic tail. No precedents are found in which an activating immunoglobulin receptor displays motifs involved in delivering activating signals. The closest similarity can be established with NKp44, an activating receptor expressed exclusively on activated NK cells. One of the three known NKp44 isoforms produced by alternative splicing contains both a lysine residue in its transmembrane region and a tyrosine-based sequence in its short cytoplasmic tail (AJ225109) that matches the ITIM consensus sequence (38, 39). Although able to be tyrosine-phosphorylated, the ITIM on NKp44 does not recruit SHP-1/2 or SHIP phosphatases and, as a consequence, it has no inhibitory function. (40).

CD300b by itself acts as a functional receptor in RBL-2H3 cells which endogenously lack DAP-12 (31). Stimulation of CD300b through the HA-epitope promoted NFAT/AP-1-dependent transcriptional activity (Fig 6B). The signal generated was transduced by the tyrosine-based motif on the receptor's cytoplasmic tail but a cooperative effect involving the molecule's transmembrane domain was needed (Fig 7C). We hypothesize that in RBL-2H3 and in the absence of DAP-12, CD300b cytoplasmic phosphorylation requires the formation of a complex with an unknown molecule. This molecule would interact with CD300b through the positively charged residue in its transmembrane domain, similar to how ITAM-based adaptors or DAP-10 bind cell surface receptors. This could explain why substitution of the lysine residue of CD300b eliminated the transcriptional activity (Fig 7C). The function of this unknown molecule, in the context of CD300b signaling, would be to recruit the kinase(s) responsible for the receptor's tyrosine phosphorylation. It is noteworthy that the RBL-2H3 cell line expresses FcεRIγ and DAP-10 adaptors, but neither was found to be associated with CD300b when assessed in COS-7 cells (31, 41). The proposed complex, when coupled to the receptor, seems able to indirectly activate NFAT/AP-1-dependent transcription. This may

explain why CD300b tyrosine or deletion mutants still exhibit a low-level activation of the reporter gene (Fig 7C). Future studies on this system will focus on phosphorylation status of the receptor under basal conditions and upon stimulation, and how the process is influenced by the integrity of its transmembrane region. In addition, efforts will move towards identifying the postulated coupling molecule.

The tyrosine-based motif of CD300b matches the consensus sequence for the Grb-2 (Growth factor receptor-bound protein 2) docking site (YxN) (37). As predicted, Grb-2 was able to interact in yeast and mammalian cells with the cytoplasmic tail of CD300b in a phosphotyrosine-dependent manner (Fig. 9). Grb-2 is a ubiquitous adaptor protein consisting of an SH3 and two SH2 domains lacking catalytic activity of its own. However, Grb-2 is able to link cell-surface receptors to downstream signaling molecules (42). Upon receptor crosslinking, Grb-2 translocates from the cytosol to the cell surface where it binds phosphorylated tyrosines on receptors via its SH2 domains. Simultaneously, Grb-2 is thought to use its SH3 domain to recruit other signaling molecules that would mediate a change in the activation status of the cell (43). For instance, Grb-2 contributes to the activation of the Ras/MAPK signal transduction pathway in response to extracellular signals (16). This could explain the CD300b-mediated transcriptional activation observed in our system of study. It will be necessary to map the signaling events conduced by Grb-2 upon CD300b crosslinking and determine their relevance to the function of myeloid cells.

Despite the presence of the tyrosine residue in the cytoplasmic tail, CD300b is able to act as a classical immune receptor and associate with an ITAM-bearing adaptor molecule (Fig 5A). Interaction with DAP-12 occurred independently of the tyrosine phosphorylation status of both the receptor and the adaptor (Fig 5C and data not shown). The association was confirmed to be functional in the RBL-2H3 cell expression system. Release of preformed intracellular mediators was promoted upon receptor stimulation through its association with DAP-12 adaptor. No hexosaminidase release was observed in the absence of the adaptor molecule or when its recruitment was disrupted (Fig 8). CD300b/DAP-12 complex was also able to activate NFAT/AP-1-dependent transcriptional activity (Fig 7B). The observed signal was generated exclusively through DAP-12, and not as a combined effect of signals initiated by the ITAM-bearing adaptor and CD300b cytoplasmic tail (Fig 7C). These data support the hypothesis of a complex recruited by the CD300b transmembrane region that is necessary for signaling through the receptor's cytoplasmic tail. Even so, we cannot discount the possibility that both pathways could act simultaneously and in a complementary manner in other processes such as cytokine release, migration, cell proliferation and/or differentiation (44). For example, our biochemical experiments in COS-7 cells show how CD300b presents a

curious dependence on DAP-12 in terms of cell surface expression and tyrosine phosphorylation (Fig 5B and 5C). In fact, the presence of DAP-12 enhanced more efficiently the phosphorylation of CD300b, even when no interaction with the adaptor through the transmembrane lysine took place (Fig 5C). These data suggest that in COS-7 cells, DAP-12 is able to modulate the level of tyrosine phosphorylation of CD300b indirectly through its interaction with other molecules, such as a second set of kinases. Another possibility is that the interaction between CD300b and DAP-12 does not rely exclusively on the transmembrane lysine. For instance, it has been demonstrated that the SH2 domain of the SAP adaptor molecule binds the c-Fyn SH3 domain and directly couples this kinase to the cytoplasmic tail of the immune receptor CD150, promoting CD150 tyrosine phosphorylation (45). New experiments are needed to fully understand the regulation of CD300b phosphorylation.

To understand the function of CD300b it will be necessary to focus our studies on those cells in which both the receptor and DAP-12 are naturally expressed. DAP-12 has been shown to be expressed and functional in NK and myeloid cells (46, 47). CD300b expression at the mRNA level is restricted to monocytes and myelomonocytic cell lines, similar to what has been described for IREM-1 and IREM-2 (Fig 3A and 3B). In addition, data on the Gene Expression Server[5] for TREM-5/CD300b revealed the presence of the transcript in dendritic cells, which could help to explain some of the transcripts detected in the tissue scan panel (Fig 3C). Nevertheless, these data will need to be confirmed using specific monoclonal antibodies. Several DAP12-associated activating myeloid Ig-like receptors, such as PIRL-β (48), SIRP-β (49), IREM-2 (10) and TREMs (50) have recently been described. Therefore, the existence of a DAP-12-free cell membrane in which CD300b would signal exclusively through its cytoplasmic tail seems unlikely. We think that CD300b signaling pathways are more likely to be controlled by engagement of the immune receptor with different ligands, compartmentalization of the molecule at the cell surface, and/or changes in CD300b affinity for extracellular, intracellular and transmembrane coupling molecules.

Based on the results obtained, comparative functional analyses between human and murine CD300b molecules would be very interesting. Despite the high degree of homology between the two receptors, murine CD300b lacks the tyrosine-based motif of the cytoplasmic tail (Fig 2B). The murine form will be essential for addressing the relevance of transmembrane associations in activating immune receptors by mechanisms distinct from the recruitment of classical adaptor proteins.

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FOOTNOTES

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[3]Ensembl BLAST Server at http://www.ensembl.org

[4]SignalP 3.0 Server at http://www.cbs.dtu.dk/services/SignalP

[5]Gene expression Server at http://symatlas.gnf.org/SymAtlas

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FIGURES

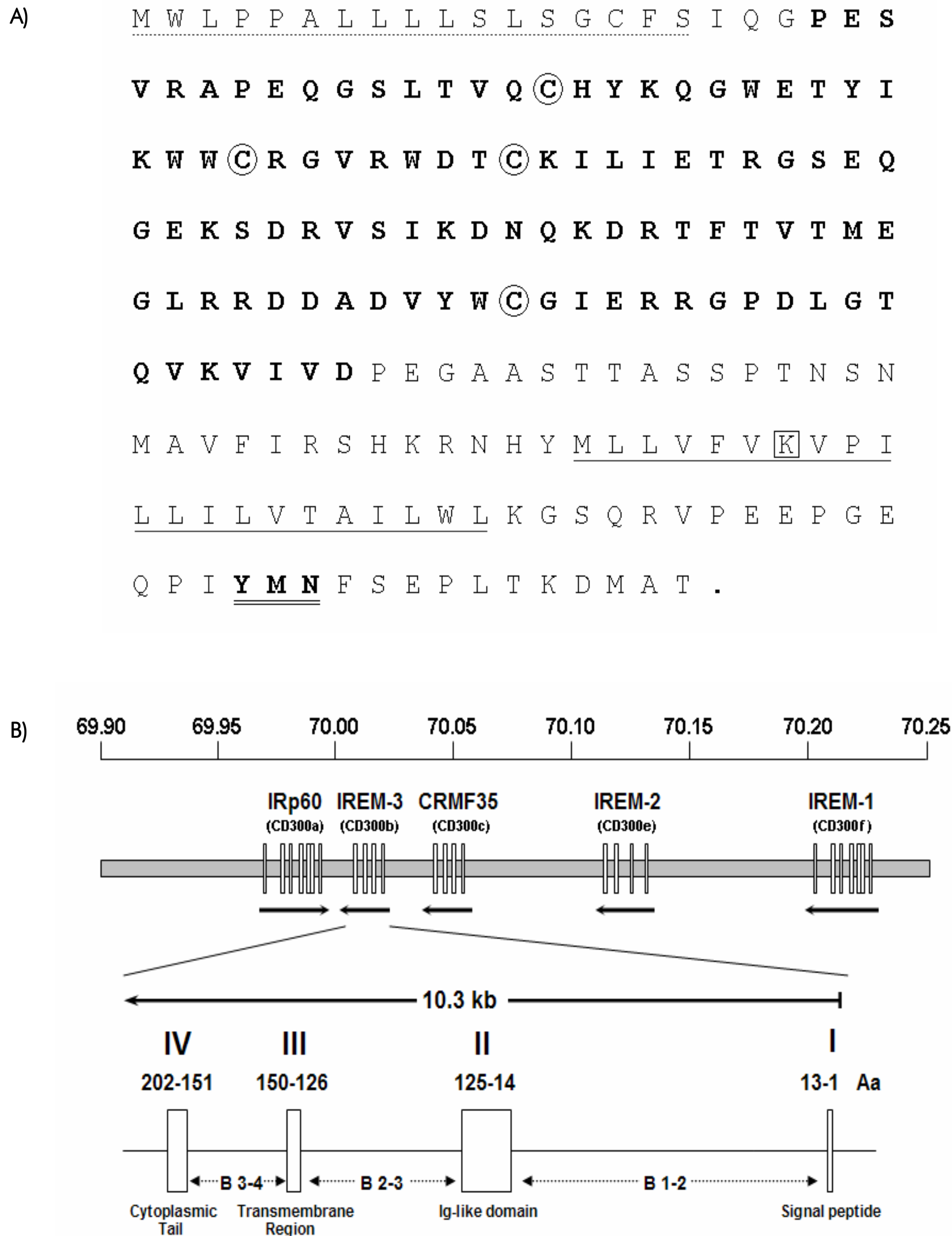

Figure 1. A) Predicted amino acid sequence of CD300b (AY646929). The putative signal peptide is underlined (dotted line). The Ig-like domain is marked in bold and the transmembrane domain is underlined (single line). Cysteine residues involved in the Ig-like domain fold are circled and the transmembrane charged lysine residue is boxed. The tyrosine-based motif located in the cytoplasmic tail is in bold and underlined (double-line). **B) CD300b gene organization.** Schematic organization of the CMRF-35/IREM locus at chromosomal region 17q25.1. CD300b genomic organization is shown below. Exons are represented by boxes (respective lengths in coding amino acids and domain architecture are shown); introns are represented by connecting lines.

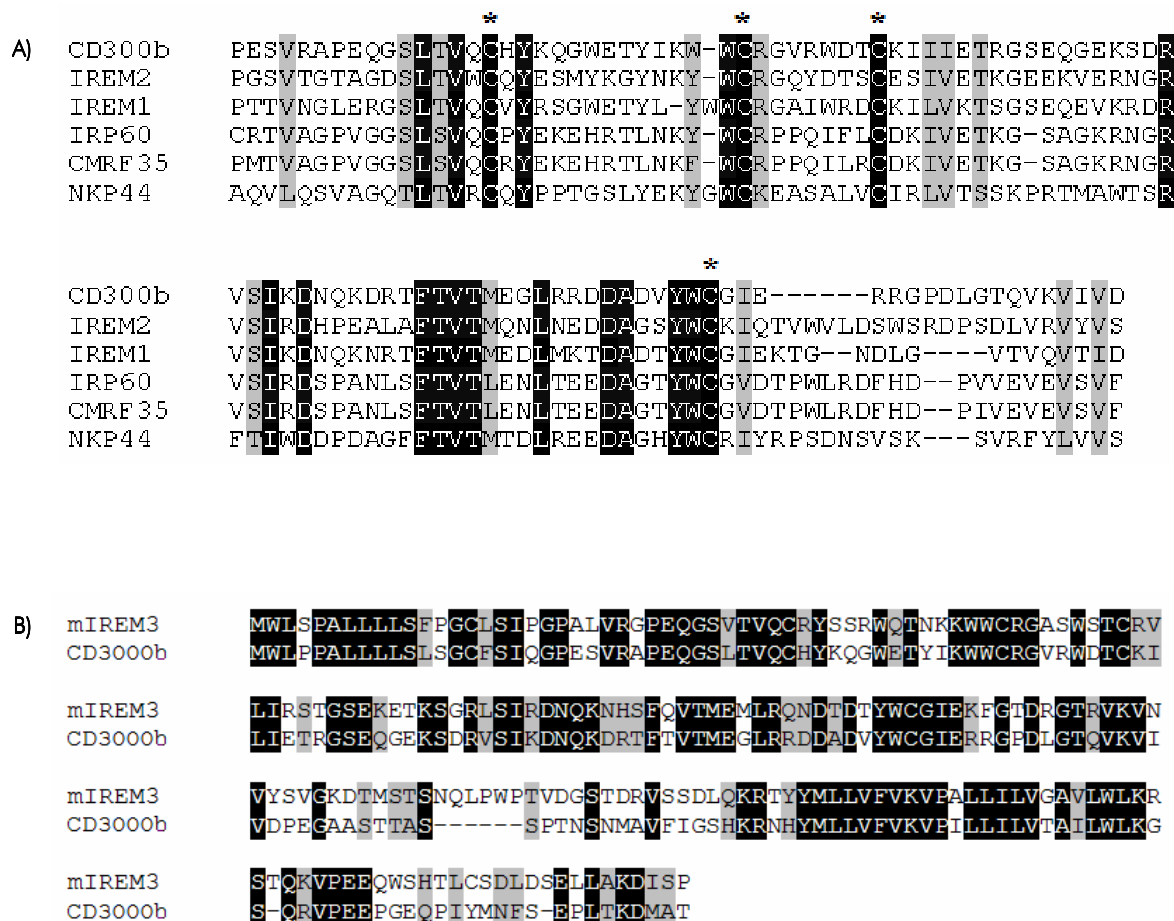

Figure 2. CD300b protein homologies. A) Sequence alignment of the CD300b Ig-like domain with Ig domains from closely related immune receptors (IREM-1, IREM-2, CMRF35, CMRF35-H/IRp60 and NKp44). Identical residues are shown on a black background and similar residues on a gray background. Conserved cysteine residues are marked with asterisks. **B)** Sequence alignment of CD300b with its murine ortholog, mIREM-3 (AY996128). The entire molecule is represented. Identical amino acids are shown on a black background and conservative changes on a gray background.

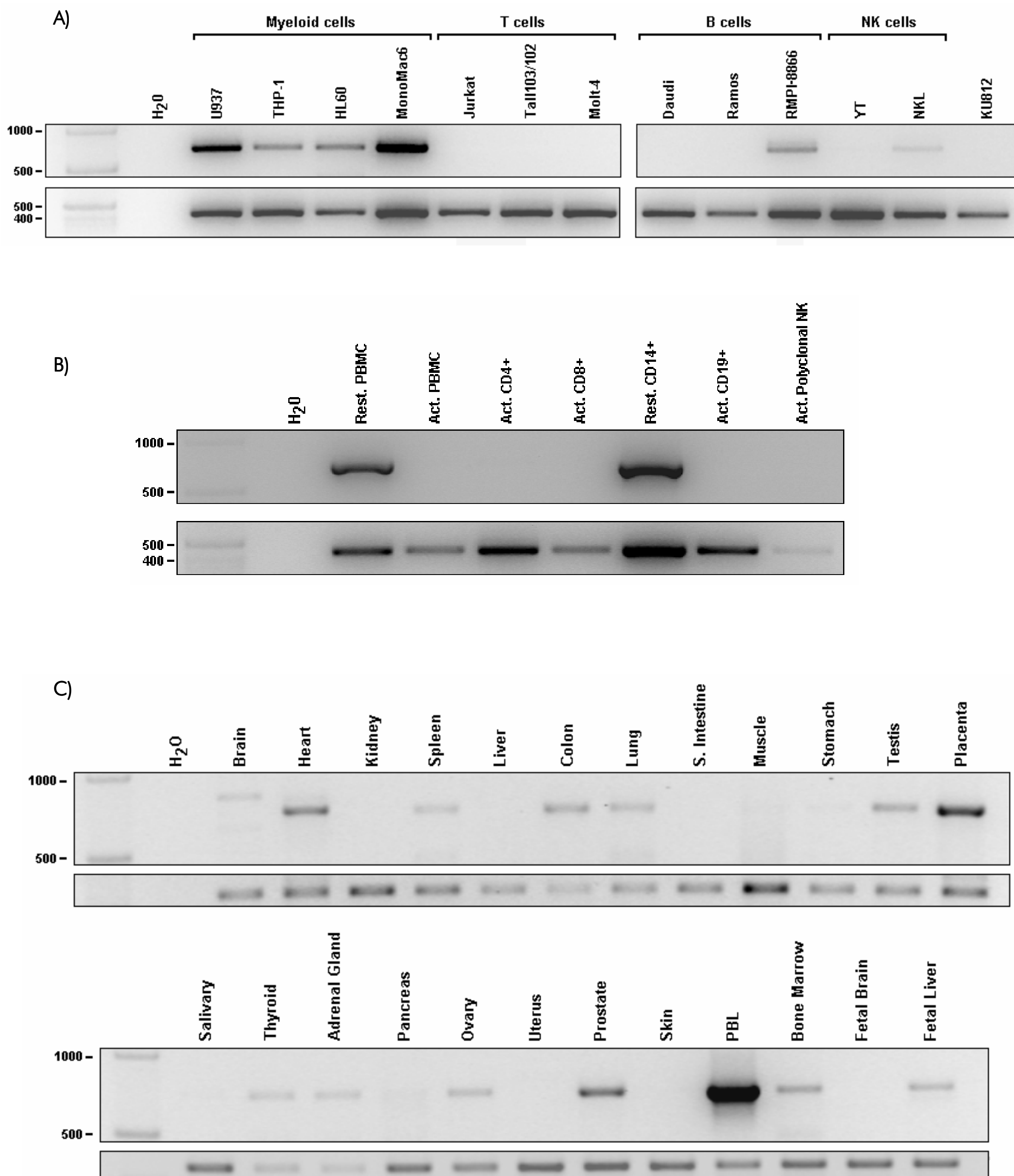

Fig 3. CD300b mRNA is expressed mainly in human myelomonocytic cells. RT-PCRs using RNA extracted from human hematopoietic cell lines **(A)**, fresh PBMC and purified blood populations **(B)** and adult and fetal tissues **(C)** were performed to amplify full-length cDNAs from CD300b transcript (upper panels). RT-PCRs of GADPH (A and B) or β-actin (C) were performed as amplification controls (lower panels). DNA size markers are shown on the left (bp).

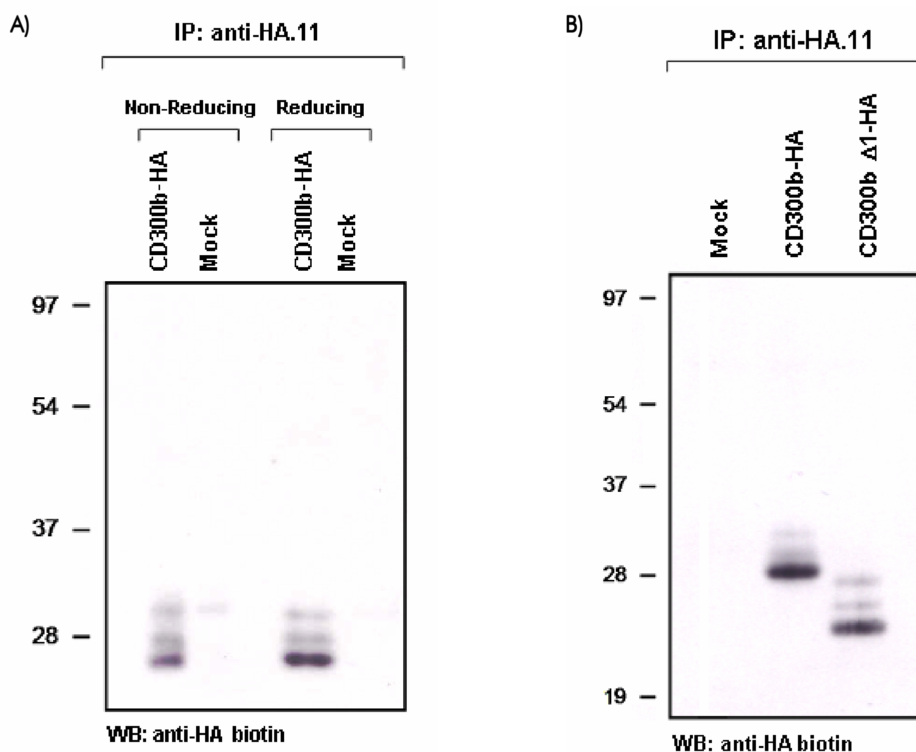

Fig 4. Biochemical characterization of CD300b. **A)** COS-7 cells transfected with an empty vector or with a CD300b-HA construct were subjected to immunoprecipitation using anti-HA.11 mAb. Samples were analyzed by 11% SDS-PAGE under reducing and non-reducing conditions. Proteins were transferred to a PVDF membrane and probed with anti-HA12CA5 mAb coupled to biotin and developed with streptavidin-HRP. **B)** HA-tagged CD300b WT and Δ1 mutant were expressed in COS-7 cells and resolved under reducing conditions as described.

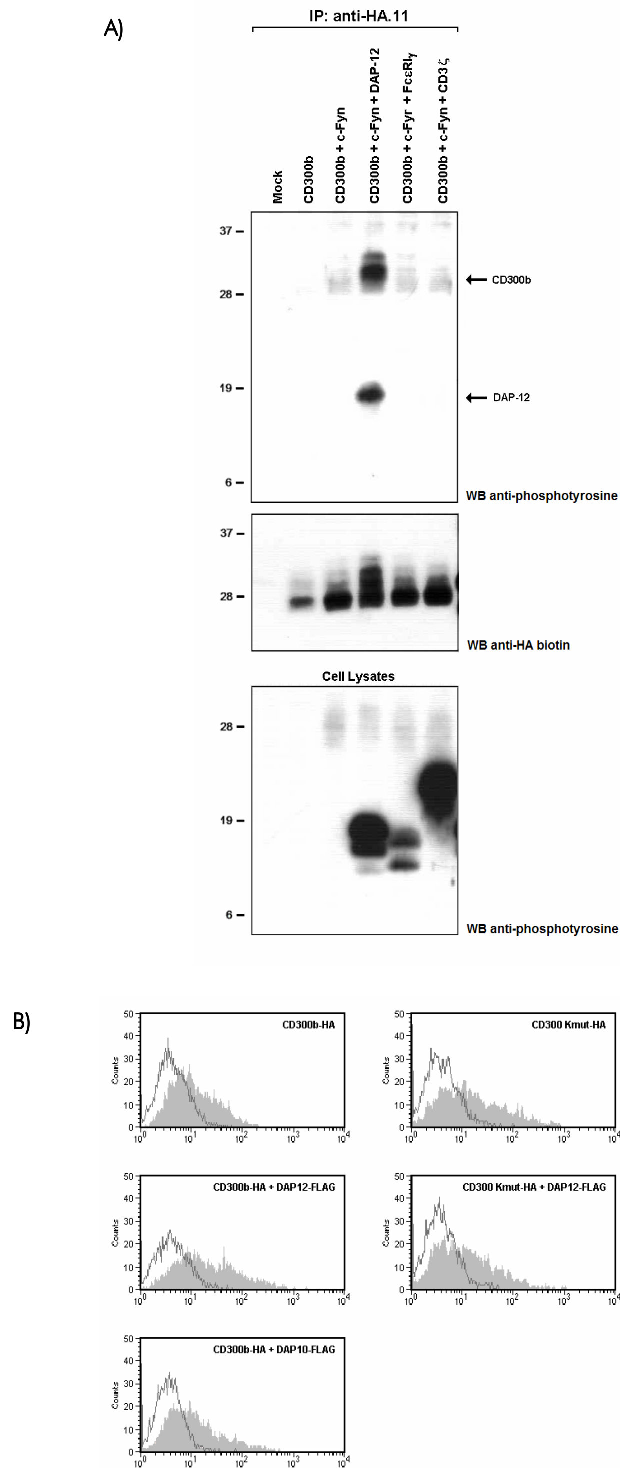

Fig 5. CD300b phosphorylation and association with DAP-12. A) COS-7 cells were transiently transfected using the DEAE-Dextran method with HA-tagged CD300b WT alone or in combination with c-Fyn, DAP-12, FcεRIγ or CD3ζ. Cell lysates were immunoprecipitated with anti-HA.11 mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to a PVDF filter and probed with the indicated antibodies. **B)** COS-7 cells were transiently transfected using Fugene 6 reagent with HA-tagged CD300b WT or K mutant in combination with FLAG-tagged adaptor molecules. Surface expression was monitored by flow cytometry using HA12CA5 mAb for the receptor (gray histogram), FLAG M2 mAb for the adaptors (not shown) and an isotypic mAb as a negative control (white histogram). The results show a representative experiment of three performed.

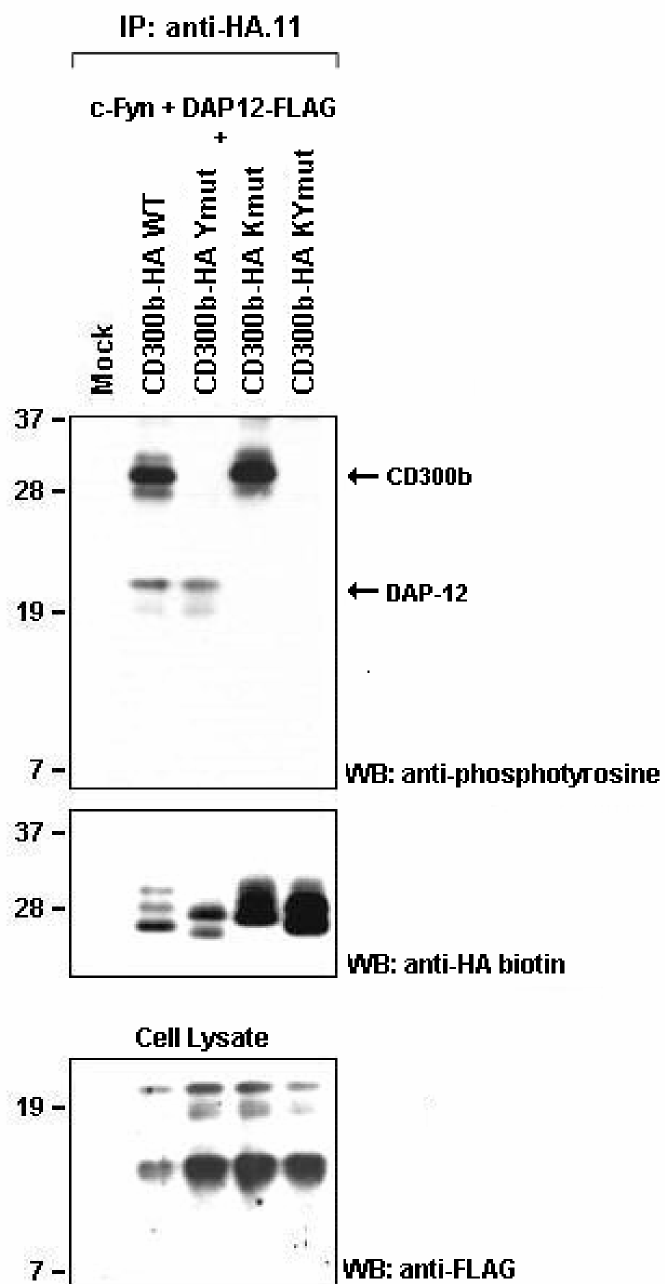

Fig 5. CD300b phosphorylation and association with DAP-12. C) COS-7 cells were transiently transfected using the DEAE-Dextran method with HA-tagged CD300b WT or mutant forms in combination with c-Fyn and DAP12-Flag. Cell lysates were immunoprecipitated with anti-HA.11 mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to a PVDF filter and probed with the indicated antibodies.

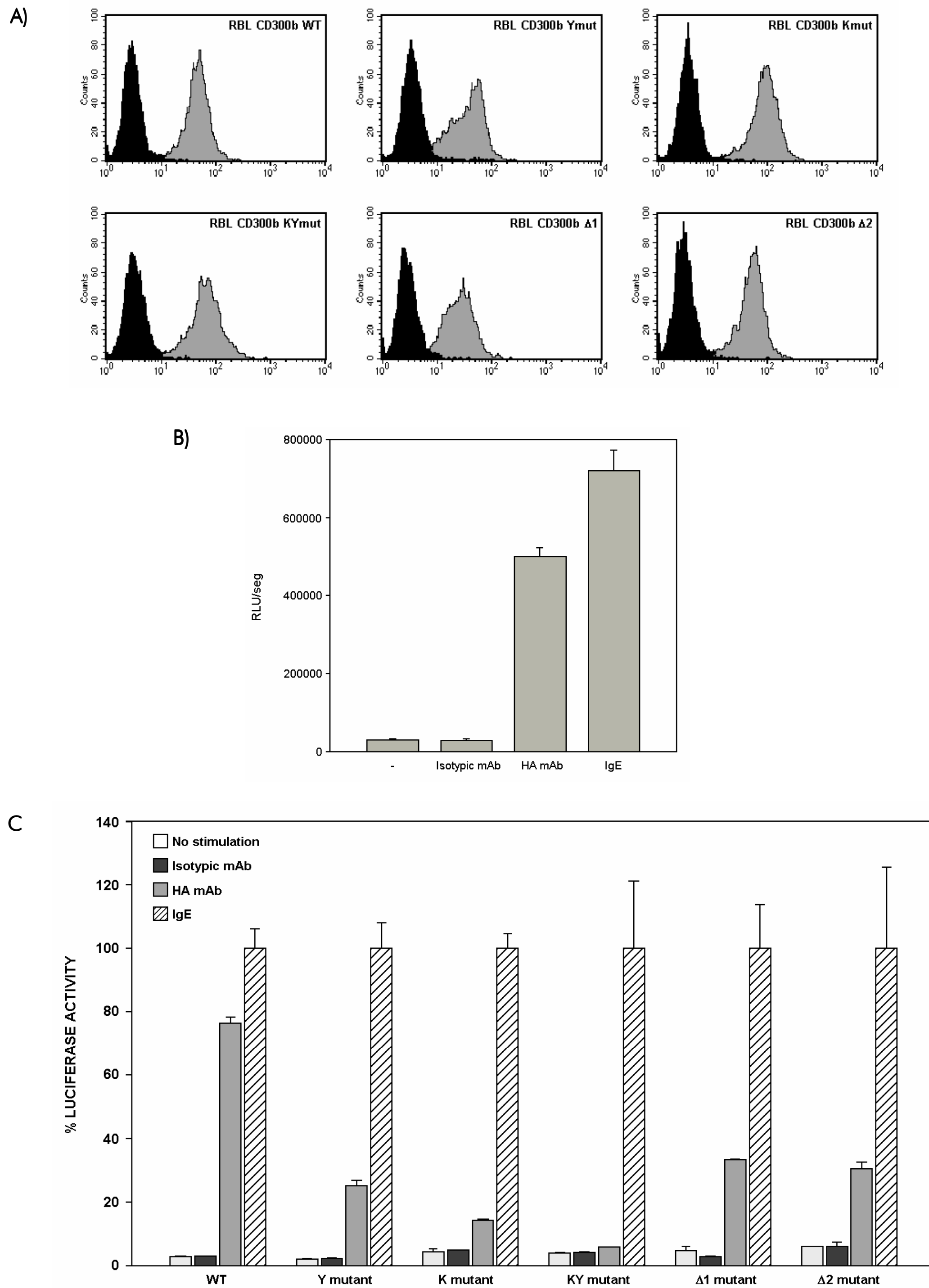

Fig 6. CD300b delivers an activating signal in the absence of DAP-12. A) RBL cells were stably transfected with HA-tagged forms of CD300b. Cell surface expression of CD300b molecules was checked by flow cytometry using anti-HA12CA5 mAb (gray histogram) or an isotypic mAb as a negative control (black histogram). **B, C)** RBL transfectants were transiently transfected with 3×NFAT/AP1-Luciferase and TK-Renilla plasmids. Luciferase activity was measured after stimulation for 7h with the indicated antibodies. Absolute luciferase units are shown in **B**. Data in **C** are normalized and expressed as a percentage of luciferase activity considering IgE stimulation as the top threshold of activation. Duplicates were performed for all the stimulations. The results are representative of three and two independent experiments respectively.

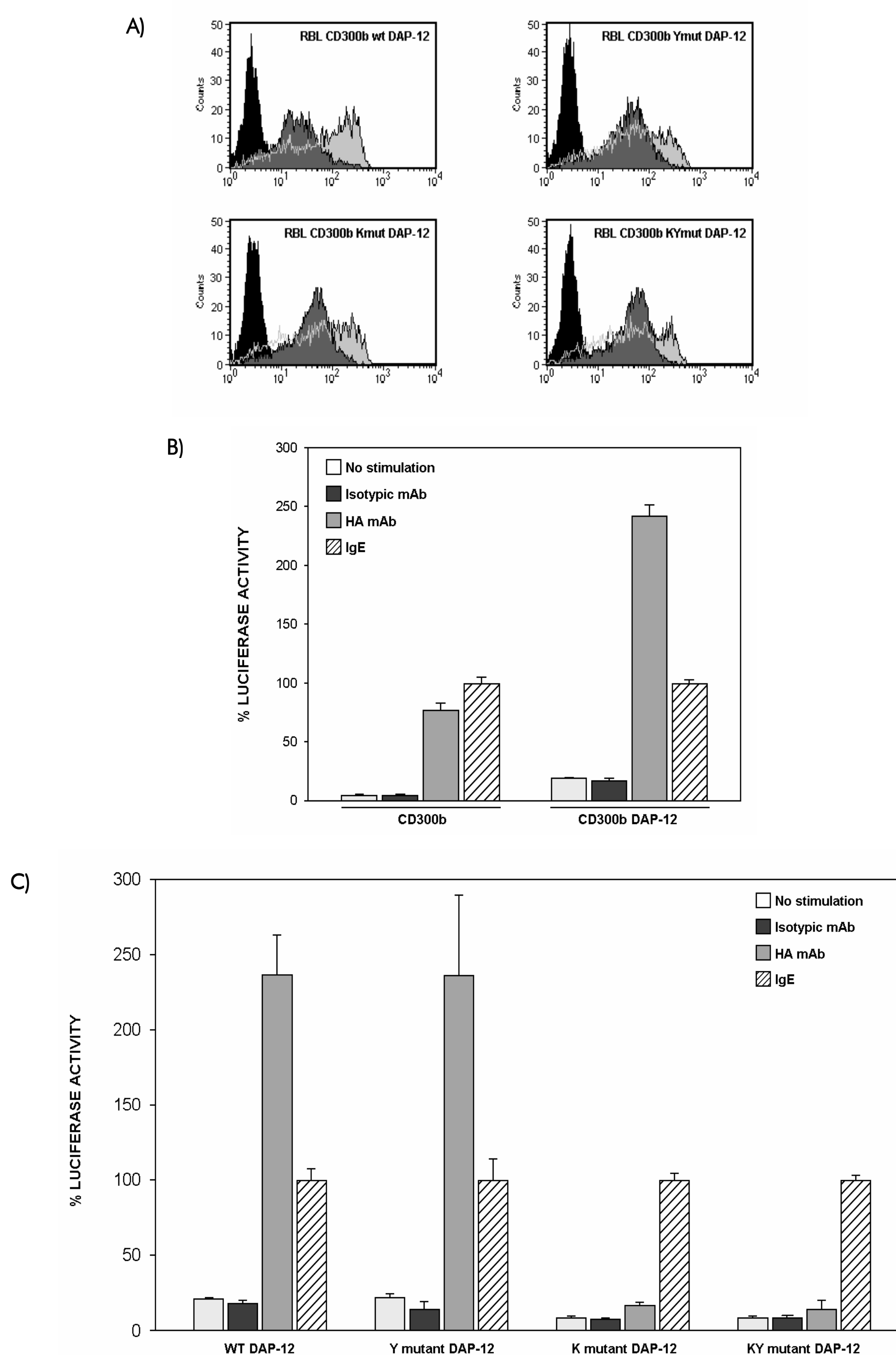

Fig 7. DAP-12 disrupts the transcriptional activation generated by CD300b cytoplasmic tail. A) RBL cells were first stably transfected with DAP12-FLAG and then with HA-tagged CD300b WT or mutants. Cell surface expression was checked by flow cytometry using anti-FLAG M2 mAb for DAP-12 (light gray histogram) and anti-HA12CA5 mAb for CD300b molecules (dark gray histogram). Isotypic mAb was used as a negative control (black histogram). **B, C)** RBL double transfectants were transiently transfected with 3×NFAT-AP1-Luciferase and TK-Renilla plasmids. Luciferase activity was measured after stimulation for 7h with the indicated antibodies. Results are normalized and expressed as a percentage of luciferase activity considering IgE stimulation as the top threshold of activation. Duplicates were performed for all stimulations. The results are representative of three independent experiments.

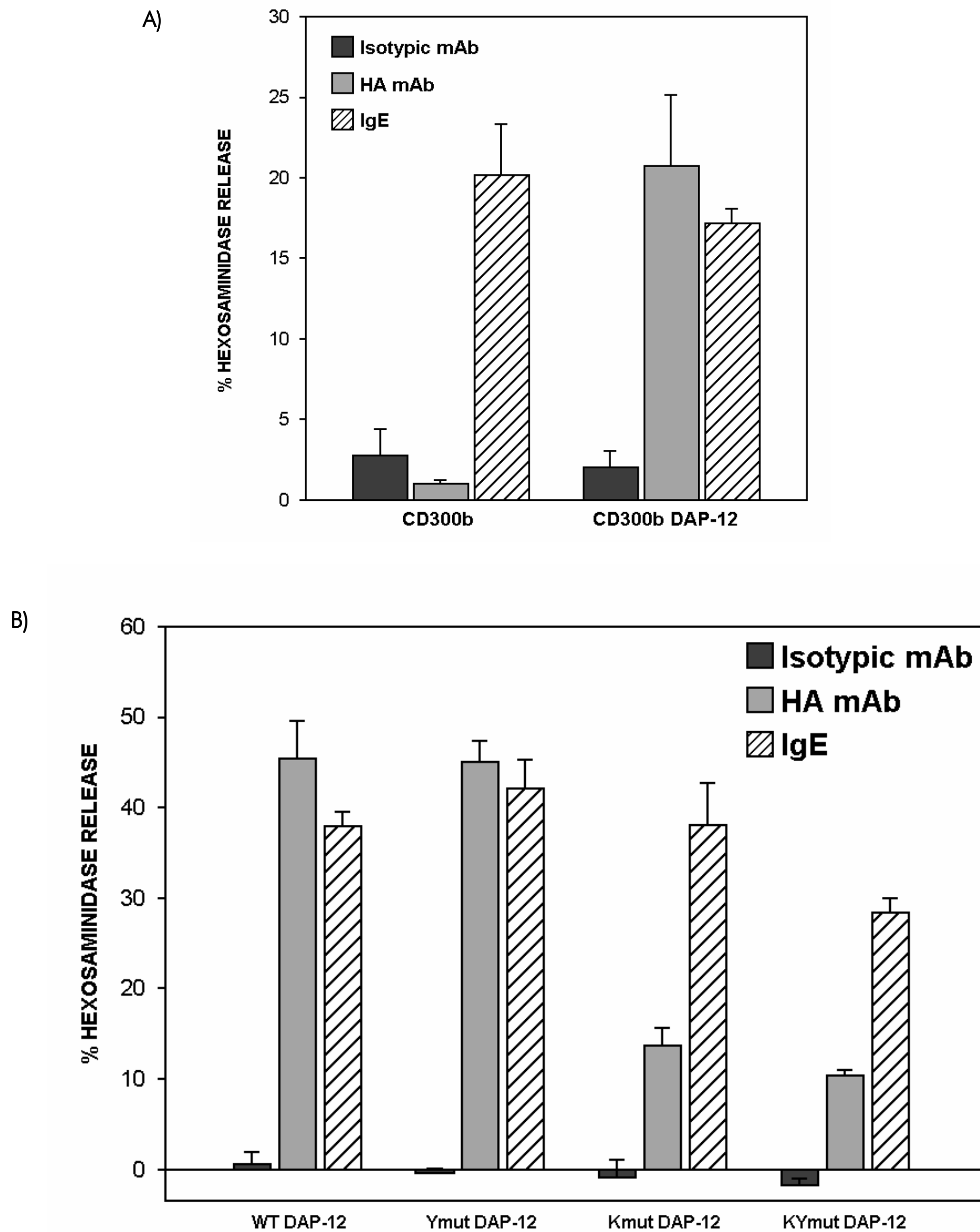

Fig 8. Hexosaminidase release is mediated by CD300b through its association with DAP-12. A, B) RBL cells expressing CD300b-HA alone or in combination with DAP-12 were stimulated with the indicated antibodies. Percentage of hexosaminidase release was assessed by incubating the supernatant with 4-nitrophenyl N-acetyl-β-D-glucosaminide substrate. Results are expressed as a percentage of specific hexosaminidase release: (abs test- abs spont)/(abs max – abs spont) × 100, where abs spont is the spontaneous release obtained with cells incubated in absence of Abs and abs max is the maximum release obtained with cells lysed using Tx-100. Spontaneous and maximum release did not differ markedly between transfectants. Each assay was set up in triplicate. The results are representative of three independent experiments.

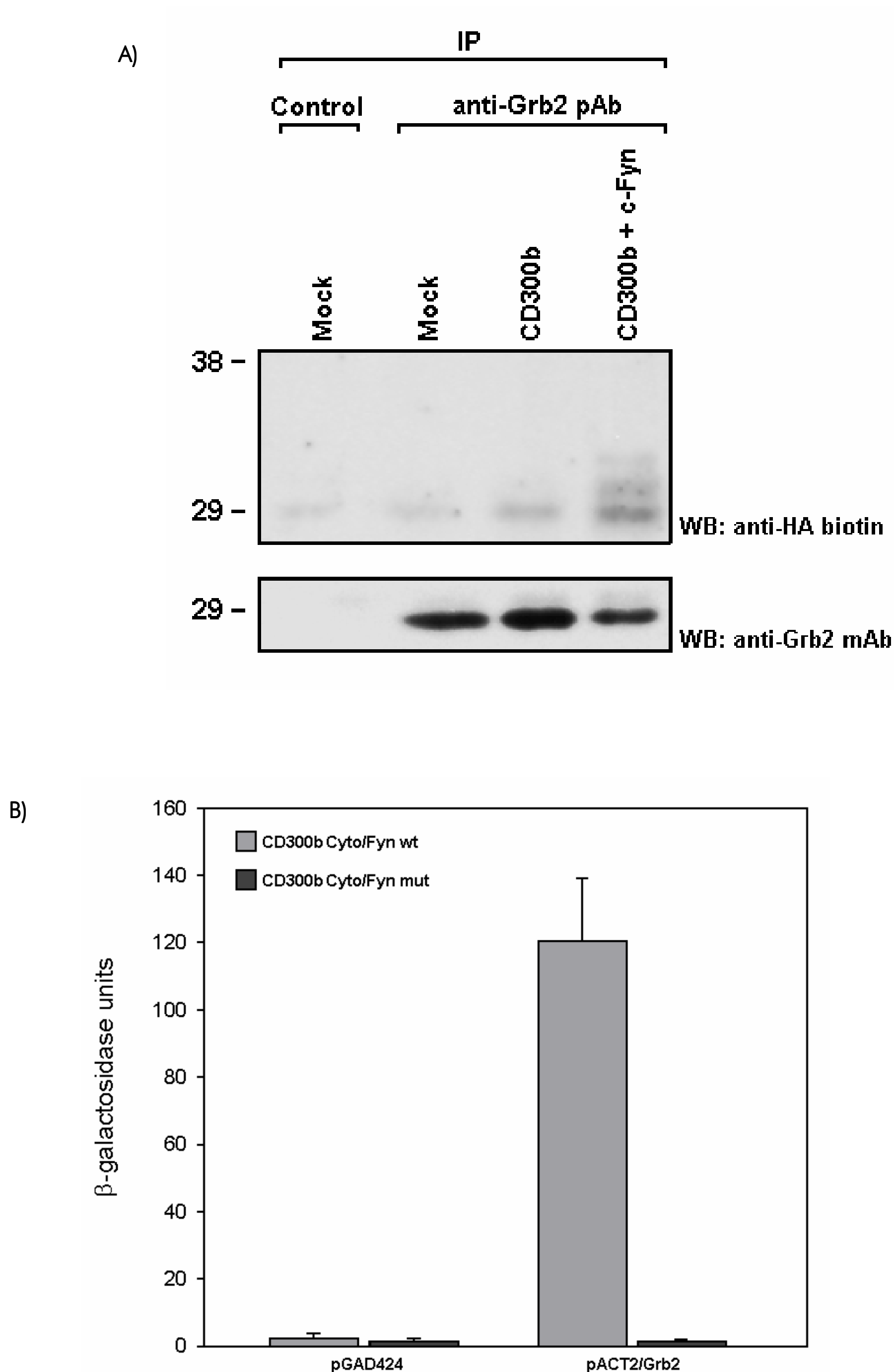

Fig 9. CD300b cytoplasmic tail recruits Grb-2 in a phosphotyrosine-dependent way. A) Three-hybrid system in yeast. Liquid culture assay with ONPG as substrate to measure β-galactosidase activity was used to score the interaction of Grb-2 with CD300b cytoplasmic tail in the presence of c-Fyn WT or a c-Fyn catalytic mutant. For each construct three independent colonies were tested in the galactosidase assay. The results are representative of three independent experiments. **B)** COS-7 cells were transiently transfected with CD300b-HA alone or with c-Fyn kinase. Endogenous Grb-2 was immunoprecipitated. Western blots were carried out with the indicated antibodies.

Article 2

The immune receptors CD300 form heterocomplexes by interacting through their immunoglobulin domains

Submitted

The immune receptors CD300 form heterocomplexes by interacting through their immunoglobulin domains[1]

Running Title: CD300 receptors association

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Keywords: Cell surface molecules, monocytes/macrophages, cell activation, signal transduction

ABSTRACT

The human CD300 family of immune receptors includes activating and inhibitory receptors, as well as receptors of uncertain function. In the present work we show that CD300c, a molecule bearing a negative charge within its transmembrane domain, is able to deliver activating signals in the transfected RBL-2H3 cell line. CD300c activating function depends on the presence of a glutamic acid in its transmembrane domain, because its substitution by a hydrofobic residue totally blocks CD300c mediated function. As far as there are no ITAM-bearing adaptors with positively charged transmembrane domains that could explain CD300c function, we analyzed the possibility that CD300c signaling could be mediated through its interaction with CD300b, an activating receptor from the same family displaying a complementary transmembrane positive charge and a functional cytoplasmic tyrosine-based motif. The presence of CD300b enhanced the activating signaling delivered by engagement of CD300c, strongly suggesting a functional association between both receptors. Moreover, CD300c and CD300b coprecipitate in COS-7 cells, demonstrating a physical direct interaction between them. Surprisingly, the association was not dependent on the charges within the receptors' transmembrane regions, but on the extracellular immunoglobulin domains. In addition, we demonstrated that all CD300 family members are able to interact with each other, even with themselves. These results suggest the existence of complexes of CD300 receptors on the surface of myeloid cells. The proportion of activating/inhibitory receptors present in such complexes could represent a new mechanism for a finely tuned regulation of cell activation.

INTRODUCTION

The immune system function is controlled by a precise balance between positive and negative signals mediated by receptors on the surface of leukocytes. Structure strongly determines the functional properties of immune receptors. In this regard, inhibitory receptors deliver intracellular signals directly due to the presence of ITIMs within their long cytoplasmic tails. Upon receptor-ligand interaction, ITIMs become tyrosine phosphorylated and thereafter recruit Src homology 2 (SH2) bearing phosphatases such as SHP-1/2 and SHIP. This, in turn, starts a cascade of intracellular signaling events promoting cell inhibition (1, 2). On the contrary, activating receptors need to associate with specialized signal transduction transmembrane polypeptides because of the lack of functional intracellular signaling units within their sequences. This association takes places at the transmembrane region and is

dependent on the formation of a non-covalently bond between oppositely charged amino acid residues within the receptor and the adaptor polypeptide. Ligand engagement of activating receptors results in the phosphorylation of tyrosine-based motifs present in the cytoplasmic tails of the associated adaptor molecules. This phosphorylation is needed for the recruitment of protein tyrosine kinases that will stimulate a series of intracellular events inducing differentiation, growth and survival, adhesion, migration, phagocytosis, cytokine production and/or cytotoxicity (3, 4). DAP-12 (DNAX-activating protein of 12 kDa), FcRγ and CD3ζ polypeptides contain ITAMs for the recruitment of Syk and ZAP-70 protein tyrosine kinases, while DAP-10 displays a YxxM motif that serves as a docking site for PI3-Kinase (5, 6).

The CD300 leukocyte surface molecules are encoded by a cluster of genes on human chromosome 17q25.1 (7). The CD300 family includes six molecules with the ability to deliver activating or inhibitory signals to the cells on which they are expressed, that are mainly from the myeloid origin. CD300a/IRp60 and CD300f/IREM-1 act as inhibitory receptors whereas CD300b/IREM-3 and CD300e/IREM-2 trigger activating signals (8-13). It is worth mentioning that CD300f presents a putative functional duality, as it has been recently shown to deliver activating signals though the recruitment of PI3-Kinase (14). CD300c/CMRF-35 and CD300d/IREM-4 function remains uncertain as consequence of their particular structural properties (15). Both receptors combine a transmembrane region containing a negatively charged residue with a short cytoplasmic tail devoid of known signaling motifs.

We previously described that CD300b is a non-classical activating receptor given its ability to signal independently of DAP-12 adaptor protein (12). This property is conferred by a tyrosine-based motif present in the receptor's cytoplasmic tail which recruits Growth factor receptor-bound protein 2 (Grb-2). However, CD300b signaling in absence of DAP-12 seemed to be dependent on an unidentified molecule responsible for the receptor's tyrosine phosphorylation. In the present work, we have tried to identify that molecule, which according to our previous data had to bind CD300b by means of its transmembrane region. Our results indicate that CD300b interacts with CD300c, but in a transmembrane-independent fashion. The association between these receptors relies on the extracellular region of both molecules and enhances the original functional activity of CD300c. Complex formation can be extended to the rest of CD300 family members, which not only interact with CD300c but with any of the members in the family, including themselves. Thus, the capacity of CD300 receptors to form complexes suggests a new mechanism to precisely control immune signaling. The formation of activating and/or inhibitory immune receptor complexes may represent an effective way to control the intensity and duration of immune signals.

MATERIALS AND METHODS

Cells and antibodies

P815 cells were maintained in RPMI 1640/L-Glutamine medium supplemented with 10% heat inactivated FBS, 25mM Hepes, 2mM glutamine, 100IU/ml penicillin and 100μg/ml streptomycin. COS-7 and RBL-2H3 cells were grown in DMEM containing 10% heat inactivated FBS, 2mM glutamine, 1mM sodium pyruvate, 100IU/ml penicillin and 100μg/ml streptomycin. Anti-HA.11 mAb was from Covance. Anti-FLAG M2 mAb, anti-DNP SPE7 mAb, mouse IgG Purified Immunoglobulin and sheep anti-mouse IgG were from Sigma-Aldrich. Streptavidin-HRP was purchased from Roche. Anti-Rat High affinity IgE Receptor mAb was obtained from BD Pharmingen. Polyclonal Rabbit anti-Mouse FITC was from DAKO. Anti-CD3 SPV-T3b was purchased from Abcam. Anti-HA12CA5 mAb and anti-Myc9E10 mAb were described before (11, 16). Polyclonal antibody against a peptide mapping in the cytoplasmic tail of CD300c (SSRSRQNWPKGENQ) was raised in rabbit (Sigma-Aldrich).

DNA Reagents

A nested PCR strategy was used to amplify and clone CD300c molecule into pDisplay mammalian expression vector. In the first PCR round the full-length CD300c sequence was amplified from a human spleen cDNA library using oligos 1-2 shown in Table I, mapping to the 5′ and 3′ untranslated regions of the receptor. The PCR product was used as template for amplification of CD300c sequence lacking the signal peptide using oligos 3 and 4 and cloned into BglII/SalI sites of the pDisplay vector. pDisplay/CD300c E191V substitution mutant was generated with mutagenic oligos 5 and 6 according to the instructions of the QuickChange Site-Directed Mutagenesis kit (Stratagene). Presence of the introduced mutation was confirmed by DNA sequencing with an ABI PRISM Big Dyes Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems). Two deletion mutants of CD300c affecting the cytoplasmic tail or the immunoglobulin domain were obtained by PCR and cloned into the BglII/SalI sites of pDisplay. CD300c Δcyto (del 209-224 aa) and ΔIg (del 1-132 aa) were amplified using oligos 3-7 and 8-4 respectively. A pBabePuro-2×Myc expression was generated by cloning the Igκ signal peptide followed by two Myc epitopes in tandem into the BamHI/EcoRI sites of pBabePuro. CD300c WT, CD300c E191V and CD300c Δcyto were cloned into the EcoRI/SalI sites of pBabePuro-2×Myc vector by PCR amplification from the pDisplay constructs using oligos 4/7-9 (oligo 4 for CD300c WT and E191V mutant, and

oligo 7 for CD300c Δcyto). CD300b wild-type (WT), CD300b Y188F, CD300b K158L and CD300b Δ1 (del 178-201aa) cloned into pDisplay vector were described before(12). CD300b WT and CD300b K158L were subcloned into the BamHI/EcoRI sites of pcDNA3-FLAG vector by PCR amplification from the pDisplay constructs using oligos 10-11. CD300b Δ1 was subcloned using the same strategy but using the BamHI/XhoI sites of pcDNA3-FLAG and oligos 10-12. An immunoglobulin domain deletion mutant of CD300b (del 1-122) was amplified by PCR using oligos 13-11 and cloned into the BamHI/EcoRI sites of pcDNA3-FLAG. Sequences encoding for CD300f and CD300e without their signal peptides were amplified by PCR and cloned into the BamHI/XhoI and EcoRI/XhoI sites of pcDNA3-FLAG, respectively. CD300f was amplified from pDisplay/CD300f using oligos 14-15 and CD300e was amplified using oligos 16-17 from pDisplay/CD300e (11, 13). A nested PCR strategy similar to that described for CD300c was used for cloning CD300a, TREM-1 and CD28 into pcDNA3-FLAG vector. The first PCR round was conducted with oligos mapping the 5′ and 3′ untranslated regions of the genes and the second round with oligos coupled to restriction sites for their cloning into the BamHI/XhoI sites of pCDNA3-FLAG. CD300a was amplified from a PHA-activated PBMC cDNA library (Clontech) using the sets of oligos 18-19 and 20-21. TREM1 was amplified from purified human monocytes cDNA using the sets of oligos 22-23 and 24-25. CD28 was amplified from PBMC cDNA using the sets of oligos 26-27 and 28-29.

1	Sense	5′ - TGCTGGGAGGAGACTACA - 3′	-
2	Antisense	5′ - GGAGGCTCACAAAGGATT - 3′	-
3	Sense	5′ - CCTAGATCTACCGTGGCGGGCCCCGTGGGG - 3′	*Blg*II
4	Antisense	5′ - GCCGTCGACCTACTGGTTCTCACCCTTGGG - 3′	*Sal*I
5	Sense	5′ - CTCCTGGTCCTCTTGG**T**GCTGCCCCTGCTCCTG - 3′	-
6	Antisense	5′ – CAGGAGCAGGGGCAGC**A**CCAAGAGGACCAG - 3′	-
7	Antisense	5′ - GCCGTCGACCTAAGGTCTGTTCACCCA - 3′	*Sal*I
8	Sense	5′ - CCTAGATCTCCGGCCGGGACGACCACA - 3′	*Blg*II
9	Sense	5′ CTGGAATTCACCGTGGCGGGCCCCGTG - 3′	*Eco*RI
10	Sense	5′ - CTGGGATCCCAAGGCCCAGAGTCTGTG - 3′	*Bam*HI
11	Antisense	5′ - CTGGAATTCCTAAGTGGCCATGTCTTT - 3′	*Eco*RI
12	Antisense	5′ - GCCGTCGACCTACCTCTGAGACCCCTTCAA - 3′	*Sal*I
13	Sense	5′ - CTGGGATCCCCAGAGGGAGCGGCTTCC - 3′	*Bam*HI
14	Sense	5′ - CCTAGATCTGGCTACTCCATTGCCACTCAA - 3′	*Blg*II
15	Antisense	5′- GCCGTCGACCTAAGGCCTGCTGATGGTGCTGTATTC - 3′	*Sal*I
16	Sense	5′ - CCTGAATTCTTTCAGGCTGTTTGTCTCTG - 3′	*Eco*RI
17	Antisense	5′ - GGACTCGAGCTATCTTCCAGGAGGAGC - 3′	*Xho*I
18	Sense	5′ - GCACCAAGAAAAGCAGAA - 3′	-
19	Antisense	5′ - GGCAGGACAAAAGCCTAT - 3′	-
20	Sense	5′ - CCTAGATCTAGCAAATGCAGGACCGTGGCG - 3′	*Blg*II
21	Antisense	5′ - GCCGTCGACCTATGTCTTCCTTATCACACT - 3′	*Sal*I
22	Sense	5′ - GCTGGTGCACAGGAAGGATG - 3′	-
23	Antisense	5′ - GGCTGGAAGTCAGAGGACATT - 3′	-
24	Sense	5′ - CCTAGATCTGCAACTAAATTAACTGAG - 3′	*Blg*II
25	Antisense	5′ - GCCGTCGACCTAGGGTACAAATGACCT - 3′	*Sal*I
26	Sense	5′ - TTCAGTTCCCCTCACACTTCGGGT - 3′	-
27	Antisense	5′ - TGGCGGTCATTTCCTATCCAGAGC - 3′	-
28	Sense	5′ - CCTAGATCTATTTTGGTGAAGCAGTCG - 3′	*Blg*II
29	Antisense	5′ - GCCGTCGACTCAGGAGCGATAGGCTGC - 3′	*Sal*I

Table 1: Oligos used in this study. Nucleotide changes introduced in the sequence for amino acid substitution are shown in bold letters. Restriction sites used for cloning are underlined and specified on right.

Cell transfections

COS-7 cells (10^5) were transiently transfected with Fugene® 6 Transfection Reagent (Roche) according to the manufacturer's instructions. For the generation of RBL-2H3 stable transfectants, 20×10^6 cells were electroporated in the presence of 20μg of linearized construct at 250V, 960μF and 100Ω in a Gene Pulser Electroporator (Bio-Rad). Transfectants were selected and maintained in culture with the appropriate selection antibiotic. G418 (Invitrogen Life Technologies) and Puromycin (Sigma-Aldrich) were used at 1mg/ml and 1μg/ml respectively. Positive cells were further selected by immunostaining with the appropriate antibodies and sorting with magnetic Dynabeads® M-450 coated with Sheep anti-Mouse IgG (Dynal).

Flow cytometry

Cell surface expression of the desired molecules was tested by indirect immunofluorescence following standard techniques (17). Analysis was performed using a FACScalibur instrument and CellQuest software (BD Pharmingen).

Immunoprecipitation and Western blot analysis

Cells were lysed at 4°C for 20 min using 1% Triton X-100 (Tx-100) -containing buffer described previously (18). Cell lysates were clarified by centrifugation at 16,000 × g for 15 min at 4°. Crude lysates were precleared for 1 h at 4°C using 20μL of IgG Sepharose 6 Fast Flow (Amersham Biosciences). Two additional preclearings were conducted for 30 min at 4°C. For immunoprecipitations, precleared lysates were incubated with 30μL of Protein G-Sepharose beads (Amersham Biosciences) and 1μg of Ab for 3h at 4°C. Proteins in the crude lysates (2%) and immunoprecipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) filters (Millipore). Filters were blocked with 5% skim-milk and then probed with the indicated Abs at appropriate dilutions. Bound Abs were detected using West Pico Supersignal kit (Pierce).

Luciferase assays

RBL-2H3 transfectants were transiently electroporated with a luciferase reporter plasmid (pT81Luc) containing three tandem copies of the distal NFAT/AP-1 site of the murine

IL-2 promoter (19) (0.5μg/10^6 cells) and a TK Renilla construct (Promega) (0.1μg/10^6 cells). Twenty-four hours post transfection, 1.5×10^6 cells were stimulated for 7h with the indicated antibodies using the murine mastocytoma P815 cell line as presenting cell (1×10^6). Plastic-coated anti-DNP IgE (5μg/ml) was used as positive control for RBL-2H3 cells stimulation and the P815 cell line cultured in supplemented RPMI 1640/L-glutamine medium alone was used as negative control. Postnuclear lysates were obtained as described (19) and luciferase activity was measured according to the Dual Luciferase Report kit manual (Promega) using a FB12 Luminometer (Berthold).

β-hexosaminidase release assays

5×10^5 RBL-2H3 transfectant cells resuspended in 50μL of Tyrode's buffer (20) were stimulated in 96-well plates for 1h at 37°C and 5% CO_2 by plastic-coated Abs (5μg/ml) previously cross-linked with sheep anti-mouse (5μg/ml) (Sigma). 20μL of supernatants were transferred to a new plate and incubated for an additional hour at 37°C and 5% CO_2 with 50μL of 1mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich) in 0.05M citrate buffer (pH 4.5). Reactions were stopped and color was developed by adding 100μL of 0.2M glycine buffer (pH 10.7). OD was measured at 405nm. Results were expressed as a percentage of specific hexosaminidase release: (Abs test – Abs spont)/(Abs max – Abs spont) $\times$ 100, where Abs spont is the spontaneous release obtained with cells incubated in absence of Abs and Abs max is the maximum release obtained with cells lysed using Tx-100 (1%) prior to the collection of the supernatants.

RESULTS

CD300c molecule is a functional triggering receptor in RBL-2H3 cells

CD300c is a type I transmembrane protein that belongs to the CD300 locus located at chromosomal region 17q25.1. Despite being the first member of the family to be cloned, little information is known about this immune receptor. CD300c displays a short cytoplasmic tail in combination with a transmembrane region bearing a negatively charged residue. These features and the lack of experimental data regarding its function make its classification uncertain (15). Additionally, CD300c cell surface expression pattern has not been elucidated in detail. Monoclonal antibodies against CD300c strongly cross react with CD300a as consequence of the high degree of extracellular homology that exists between them (21, 22).

We wanted to address whether CD300c is a functional immunoglobulin receptor and characterize the nature of the signal triggered upon its engagement. We have extensively used the RBL-2H3 mast cell line as per its inducible exocytotic response of preformed cytoplasmic secretory granules and reporter-dependent transcriptional activity upon receptor crosslinking (12, 14). We stably expressed CD300c-2×Myc on the surface of RBL-2H3 cells (Fig. 1A). The cell line was transiently transfected with a luciferase reporter gene under the control of a NFAT/AP-1-dependent promoter and stimulated with anti-Myc mAb. Engagement of the receptor elicited an increase in promoter activity comparable to that delivered by FcεRI stimulation (Fig 1B). CD300c, unlike other activating immune receptors, has a negatively charged residue in its transmembrane region but to date there are no reports in the literature describing transmembrane adaptor proteins with a complementary positive charge. To more precisely analyze the signals generated by CD300c we determined whether the transmembrane charge, the cytoplasmic region or both, were responsible for the receptor's signal transduction. We generated two RBL-2H3 stable transfectants expressing CD300c-2×Myc mutants affecting the transmembrane glutamic acid (E191V) or the cytoplasmic tail (ΔCyto) (Fig. 1A). The CD300c ΔCyto mutant mediated the same transcriptional activity as the wild type, suggesting that there are no signaling motifs within the cytoplasmic region of CD300c able to contribute to the activation of the reporter (Fig. 1B). The substitution of the negative transmembrane charge by a hydrophobic amino acid led a dramatic reduction in the reporter activity, thus establishing a role for the receptor's transmembrane region (Fig. 1B). Equivalent results were obtained when assessing β-hexosaminidase granule release upon CD300c stimulation (Fig. 1C). It is of note that no differences were observed among transfectants in the activating capacity and cell surface levels of FcεRI (data not shown).

CD300c associates with CD300b in transfected COS-7 cells

CD300b receptor is a non-classical activating receptor able to deliver signals by associating with the transmembrane adaptor protein DAP-12 and by recruiting Grb-2. The recruitment of these adaptor molecules are two independent events. CD300b binds DAP-12 through a lysine residue within the transmembrane domain, while Grb-2 is recruited by means of a tyrosine-based motif present in the receptor's cytoplasmic tail (12). Previous results made us hypothesize about the existence of a complex recruited by CD300b transmembrane region necessary for the signaling in absence of DAP-12 (12). The presence of oppositely charged amino acid residues embedded in the transmembrane regions of CD300b and CD300c, together with the fact that the rat CD300c orthologous could be expressed in RBL-2H3 cells

suggested the binding between both receptors. To test this possibility, COS-7 cells were transiently transfected with CD300c-HA in the presence and absence of CD300b-FLAG. Both molecules were found to be associated (Fig. 2). Furthermore, Western blot analysis revealed that the complex formation led to an uneven recovery of CD300c in the immunoprecipitates. Systematically, only one of the three electrophoretic bands described for CD300c was expressed when cotransfected with CD300b (Fig. 2).

CD300c/CD300b association relies on Ig domains

In order to verify that the association between both receptors was dependent on the formation of a membrane-embedded salt bridge involving the acidic residue on CD300c and the basic residue on CD300b, we generated substitution mutants affecting these key residues (HA-tagged CD300c E191V and FLAG-tagged CD300b K158L). COS-7 cells were cotransfected following all possible combinations between wild type and mutant forms of the receptors. Surprisingly, CD300c/CD300b complex formation was not disrupted by substitution of transmembrane residues, neither individual nor in combination (Fig. 3A).

To further map the interaction between both immune receptors, a wider set of mutants were assessed in the cotransfection experiments. We conducted cytoplasmic tail deletions in order to create HA-CD300c Δcyto and FLAG-CD300b Δcyto mutant forms for co-expression in COS-7 cells with the wild type forms. Intracellular association between both molecules was ruled out given that none of the cytoplasmic mutants could disrupt the CD300c/CD300b complex formation (Fig. 3B).

Next, we tested whether CD300c and CD300b were interacting through their extracellular portions, and more specifically through the Ig domain. COS-7 cells were transfected with epitope-tagged forms of the receptors in which the Ig domain was removed from the extracellular region. The membrane-proximal portion, also known as the stem region, was maintained. Immunoglobulin domains were shown to be essential for the interaction between CD300b and CD300c as their removal led to the disruption of the complex (Fig. 4C).

CD300 family members, polymeric Ig receptors (pIgR), NKp44, CLM-1 and TLT-1 display in their Ig domains not only the pair of cysteine residues describing the Ig-V-type fold but also, a second pair of cystein residues that has been proposed as a differential trade for a discrete evolutionary group of receptors stemming from the ancestral V-type Ig domain (23). Crystallization of CD300f extracellular domain has shown that the second disulphide bond is important for the stabilization of a β-hairpin involved in the creation of a solvent-exposed

cavity suitable for ligand accommodation (24, 25). According to this, we wanted to evaluate the role of the second disulphide bridge in the formation of the CD300c/CD300b complex. The association between both receptors was not affected when a CD300b receptor carrying a substitution in Cys50 was used in the COS-7 cotransfection assays instead of the wild type form (Fig. 3D).

CD300b enhances CD300c-mediated transcriptional activity

To analyze whether the association between CD300c and CD300b modified their individual functional properties, we performed transcriptional activity assays comparing the activation induced by single and double RBL-2H3 stable transfectants upon individual crosslinking of the receptors (Fig. 4A). CD300b-HA and CD300c-2×Myc transfectants lead to similar activation of the NFAT/AP-1 reporter gene, whereas CD300b-HA/CD300c-2×Myc transfectant exhibited a 2-fold increase in the transcriptional activity when stimulated through the CD300c receptor (Fig. 4B). No change was observed when the double transfectant was stimulated through CD300b receptor (Fig. 4B). The activating capacity and cell surface levels of FcεRI did not change as per the double transfection (data not shown). Therefore, the increase in the positive signal generated by the complex seemed to be propagated through CD300b cytoplasmic tail that, as described previously, displays a functional tyrosine-based motif. Hexosaminidase granule release in the CD300b/CD300c transfectant did not exhibit the same increase when stimulated under the same experimental conditions (Fig. 4C). This fact supported our hypothesis of CD300b as a signaling enhancer of the complex. CD300b receptor is not able to elicit exocytotic responses in absence of DAP-12, an adaptor molecule that is not endogenously expressed by RBL-2H3 cells (12).

CD300c is able to interact in COS-7 cells with all CD300 family members but not with other Ig-like immune receptors

As the interaction observed between CD300c and CD300b was taking place through the extracellular domains, we wanted to address whether CD300c was capable to associate with other CD300 members. COS-7 cells were transiently transfected with CD300c-HA and FLAG-tagged CD300a, CD300e and CD300f. All CD300 members were able to associate with CD300c (Fig. 5). Next, we evaluated whether the interaction observed could be extended to other immunoglobulin receptors or was instead a particularity of CD300 family. We tested the interaction between CD300c and TREM-1, which is closely related to CD300 receptors

regarding homology among the V-type Ig fold and cell surface distribution; and CD28 that despite evolving differentially in terms of expression pattern shares with CD300 family members very similar protein structural features (35, 36). Neither TREM-1 nor CD28 were able to interact with CD300c (Fig. 6). Consequently, we can conclude that the interactions observed occur specifically in immune receptors belonging to the CD300 family.

It is of note the high variability affecting CD300c electrophoretic pattern. The cotransfection of CD300c with receptors to which it does not associate results in an electrophoretic pattern for CD300c comparable to that obtained when the molecule is transfected alone (Fig. 2 and Fig. 5). Contrary, when CD300c is coexpressed with CD300 receptors, to which it positively binds, the electrophoretic pattern of the molecule changes, being different for each of the combinations tested.

All CD300 receptors are able to interact between them

According to our data, it was possible that individually all the members of the CD300 family exhibited the same interacting capacity than CD300c. In order to check our hypothesis, we cotransfected in COS-7 cells the CD300 receptors identified in the laboratory (CD300b, CD300e and CD300f) following all possible combinations. Coprecipitations occurred independently of the combination of receptors used, indicating that most probably all the members belonging to the family are able to interact between them (Fig. 7).

DISCUSSION

Although CD300c receptor was the first member of the CD300 family to be identified, there is no information about the signaling pathways in which it could be involved (15). In this paper we have shown that CD300c molecule was able to induce triggering signals in RBL-2H3 cells. Its stimulation through epitopes promoted NFAT/AP-1-dependent transcriptional activity and release of β-hexosaminidase granules. These results were similar to those described for the activating receptor CD300b, but not identical (12). In the case of CD300b, it was necessary to cotransfect RBL-2H3 cells with the ITAM-bearing adaptor DAP-12 in order to induce release of β-hexosaminidase granules through engagement of the receptor, while CD300c was able to induce cell degranulation in the absence of DAP-12. CD300c cytoplasmic tail did not contribute to the signaling despite it displays a very high proportion of polar amino acids that could mediate the recruitment of intracellular signaling mediators through electrostatic interactions. This data, together with mutagenesis studies showing that

the functional properties of the receptor relied on the transmembrane arginine residue, strongly suggested the existence of an unknown ITAM/YxxM-bearing-like adaptor displaying a positive charge in its transmembrane domain. To date there are no experimental evidences of transmembrane polypeptides fitting these structural requirements that could develop functions similar to those exhibited by DAP-12, FcRγ, CD3ζ and DAP-10 (26). Bioinformatic tools have not been productive either, as there are no reports in the literature in which EST databases have been screened to identify genes that could match this new type of transmembrane adaptors. CLM-5, also known as MAIR-IV and LMIR-4, can be considered the structural murine ortholog of CD300c. It has been shown that this receptor is able to recruit FcRγ adaptor protein in transfected cell lines and peritoneal macrophages (27-29). However, the interaction has not been mapped and although it seems to be direct, it is unlikely that involves the residues on the transmembrane region as both molecules bear negatively charged amino acids on it. In any case, considering that RBL-2H3 cells endogenously express FcRγ, we checked out the possible association between these two molecules. In our hands, experiments of coprecipitation in transfected COS-7 cells did not show any association between CD300c and FcRγ (data not show). It is noteworthy that these experimental conditions were suitable for detecting coprecipitation of CD300b and CD300e with DAP-12 and DAP-10 adaptor molecules (12, 13). This could indicate that these molecules do not bind each other directly, or alternatively that the interaction between them is weaker, and as consequence easily disruptible, than the provided by the formation of salt bridge between oppositely charged amino acids. Future plans include knocking down the expression of FcRγ in RBL-2H3 cells in order to confirm if CD300c signaling is at least, mediated by FcRγ polypeptide.

In any case, the presence of a transmembrane negatively charged amino acid indispensable for CD300c function still suggested the existence of a positively charged mediator. The CD300b activating receptor from the CD300 family displays a lysine residue embedded in its transmembrane domain, and atypically for an activating receptor, a cytoplasmic tyrosine-based motif. We have previously shown that this motif is phosphorylated by Src kinases and constitute the docking site for Grb-2 adaptor protein, what allows CD300b receptor to signal independently of DAP-12 molecule. However, CD300b cytoplasmic tyrosine-phosphorylation seems to require the formation of a complex with an unknown molecule. It is remarkable that other groups have also implicated an unidentified adaptor in the CD300b-mediated signaling pathway (30). As CD300b signaling in a DAP12-free environment was dependent on the positively charge residue in the receptor's transmembrane domain, we hypothesized that the rat orthologous of CD300c present in RBL-2H3 cells could be that unknown molecule. CD300c was found to associate with CD300b in COS-7

transfected cells. Unexpectedly, the association of both molecules occurred extracellularly by means of the immunoglobulin domains instead of the transmembrane charged residues. The immunoglobulin fold is present in a wide range of molecules that includes not only cell surface antigen receptors but also coreceptors and costimulatory molecules, antigen presenting structures, cell adhesion molecules and certain types of cytokine receptors. Most of the cell surface antigen receptors are clustered in the genome. This is the case for the ILT, KIR, NKp, SIRP or TREM receptors (31-35). Ig-like domains from receptors belonging to the same family present a high degree of homology among them, but sometimes the similarity is also maintained between receptors belonging to different families. This is likely a consequence of duplication processes driving the appearance of the different families along evolution. For instance, CD300 receptors are highly homologous to TREM and Nkp44 receptors (35, 37). Despite the conservation concerning the Ig-fold, CD300c was able to complex exclusively with CD300 family receptors. CD300c did not bind to TREM-1 and neither to CD28, which is more distant to these receptors because of its expression in T cells but shares equivalent structural features with them (35, 36). The ability to interact with CD300 family members was observed not only for CD300c but with all the CD300 members cloned in the laboratory. Thus, interactions combining any two CD300 members are possible. Even the interaction of CD300 members with themselves was detected (data not shown). The capacity of these receptors to form homo and heterodimers/multimers was not detected before due to the fact that complex formation does not require the establishment of disulfide bridges.

The Ig-fold consists on a sandwich-like structure formed by two sheets of antiparallel β-strands. Interactions between hydrophobic amino acids on the inner side of the sandwich and a highly conserved disulfide bond formed between cysteine residues in the B and F strands, stabilize the Ig-fold (38). We can not perform studies with deletion mutants affecting different parts of the immunoglobulin domain to more precisely map the interactions observed. This would lead to an improper positioning of β-sheets and degradation of the proteins through the proteasome pathway. Our first attempt to define the residues involved in CD300 receptors interaction was the substitution of the cystein residues forming the second disulfide bridge present in the Ig fold of these molecules. This trait is considered a differential mark of a discrete group of immune receptors which include not only the CD300 receptors but also NKp44 and TREM-2 (23). Crystallization of CD300f receptor revealed these cystein residues to be within a loop that defines the bottom of a protruding body extending from the main immunoglobulin body (24, 25). In addition, these residues are not present in TREM-1 and CD28 which failed in recruiting CD300c. However mutation of Cys50 in CD300b sequence did not prevent the complex formation with CD300c. In the future we will

concentrate in the mutation of other residues lying off the same cavity and non-conserved between CD300 family members and TREM-1 receptor.

Nevertheless it is likely that the interactions observed require not only the lateral chains of certain amino acids but also the posttranslational modifications that can be coupled to them. CD300b does not bear N-glycans in its immunoglobulin domain but associates with CD300 members, including itself (12)(data not shown). And O-glycosilation is predicted to occur mostly to the serine and threonine residues within the membrane-proximal portion of these receptors, which was maintained in the ΔIg mutant forms that failed to form CD300 complexes. As consequence, the putative modifications promoting the binding of these receptors should be others than N- and O-glycosilations.

From all the interactions detected biochemically we have focused on CD300b-CD300c at a functional level. We have observed an increased signaling capacity of CD300c when complexed with CD300b. If human CD300c is confirmed to signal through FcRγ polypeptide in RBL-2H3 cells, then it is likely that the effect observed in the double transfectant is a consequence of FcRγ recruitment and a concomitant increase in CD300b phosphorylation rate. Our hypothesis is that upon CD300c crosslinking in the CD300b-CD300c transfectant, CD300c would lead FcRγ recruitment and subsequent ITAM phosphorylation by Lyn tyrosine kinase which is abundantly expressed in RBL-2H3 cells. This in turn, would induce Syk to bind the FcRγ phosphorylated ITAMs. In this environment, in which tyrosine kinases accumulate at the inner cell membrane, CD300b cytoplasmic tyrosine-based motif would be more easily phosphorylated and consequently an increase in the activating capacity of the complex by means of CD300c would be observed. If this is like we propose and taking into account that CD300 family members are able to interact between them, then it is feasible that their association in complexes constitutes an additional way to regulate the signals triggered by immune receptors. The formation of activating receptor complexes would be useful for maintain the stimulating signals along time and/or increase their potency, while the formation of mixed activating and inhibitory complexes would facilitate their termination or attenuation. At this point it would be necessary to determine whether the association between the different CD300 receptors takes place constitutively or if it occurs only upon initiation of the signaling. In this regard, our results seem to point out that some of the associations could be taking place before the molecules reach the cell surface.

Another issue that should be considered in the future is how these associations modify the ligand binding properties of individual receptors. The Ig-like domain is the molecule's ligand recognition unit, but as the association between CD300 receptors relies also within the same domain, as a result the formation of complexes can mask ligand-binding epitopes at the

same time that creates new ones. Thus it would be important to determine whether CD300 receptors are able to bind different ligands depending on their inclusion into complexes or not.

FOOTNOTES

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FIGURES

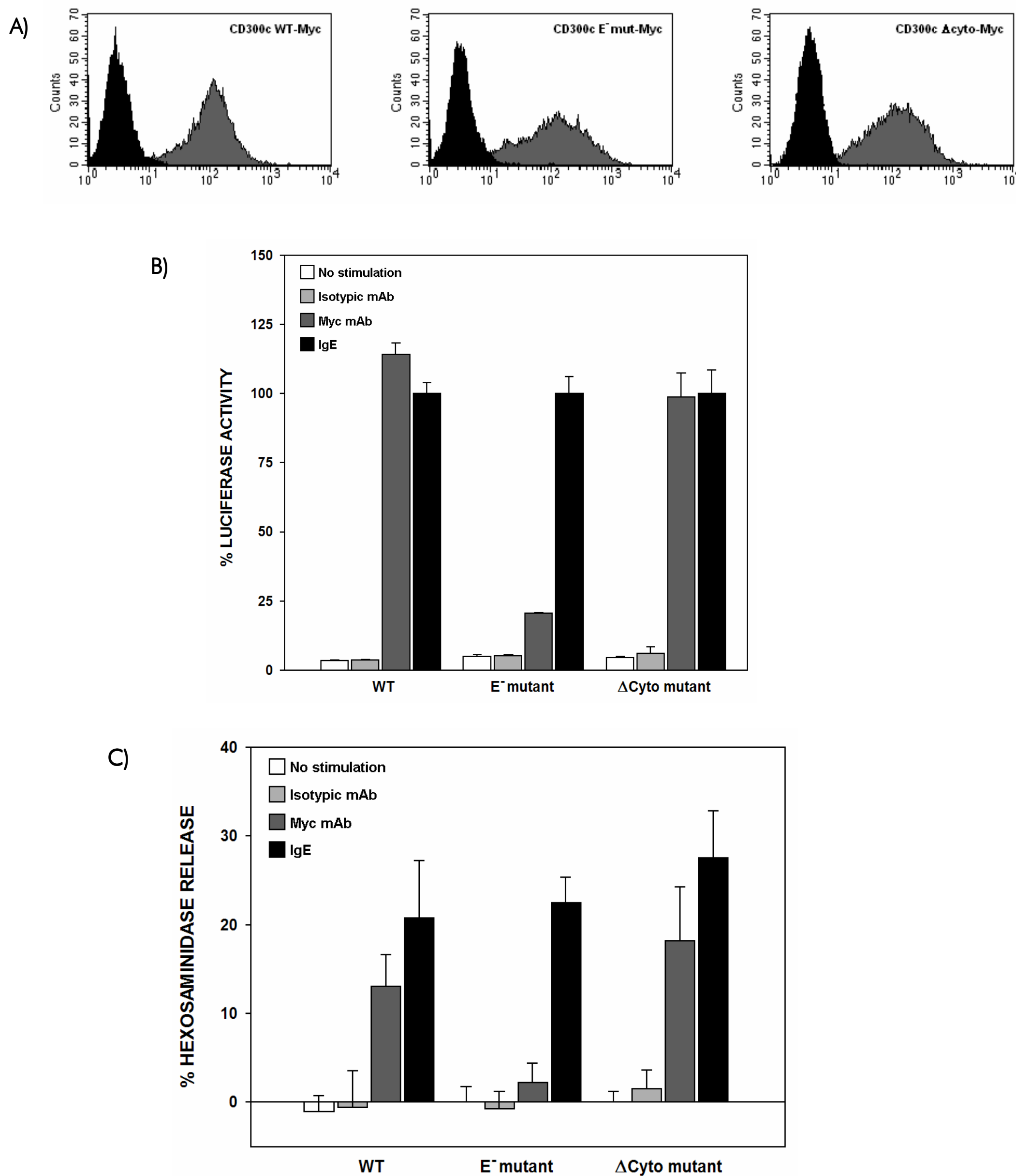


Fig 1. CD300c delivers activating signals in RBL-2H3 cells. A) RBL cells were stably transfected with 2×Myc-tagged forms of CD300c. Cell surface expression of CD300c molecules was checked by flow cytometry using anti-Myc9E10 mAb (gray histogram) or an isotypic mAb as a negative control (black histogram). **B)** RBL transfectants were transiently transfected with 3×NFAT/AP1-Luciferase and TK-Renilla plasmids. Luciferase activity was measured after stimulation for 7h with the indicated antibodies. Data are normalized and expressed as a percentage of luciferase activity considering IgE stimulation as the top threshold of activation. Duplicates were performed for all the stimulations. The results are representative of three independent experiments respectively. **C)** RBL transfectants were stimulated with the indicated antibodies. Percentage of hexosaminidase release was assessed by incubating the supernatant with 4-nitrophenyl N-acetyl-β-D-glucosaminide substrate. Data were normalized as described in Material and methods. Each assay was set up in triplicate. The result is a mean of three independent experiments.

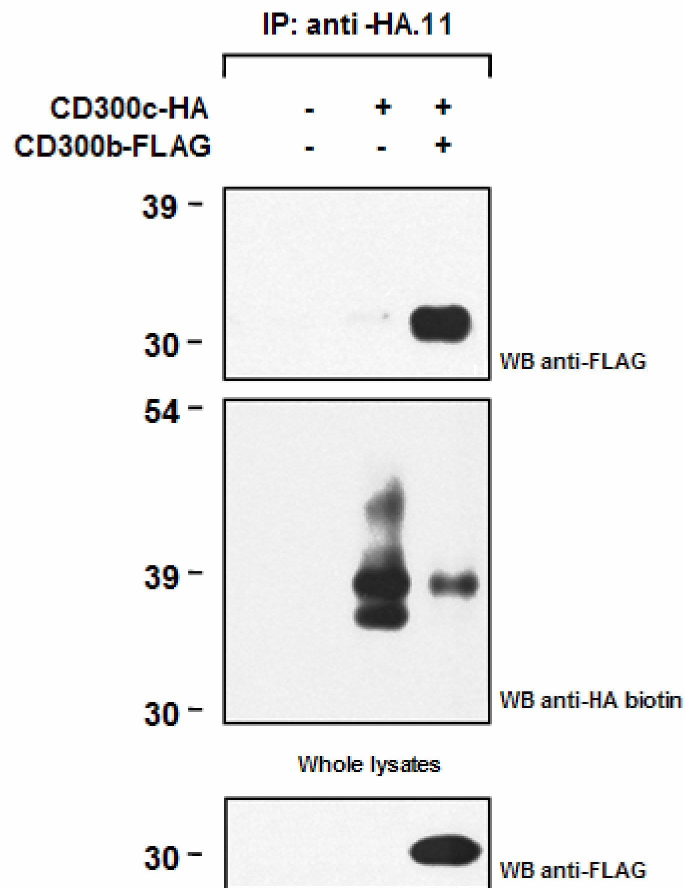


Fig 2. CD300c associates with CD300b in transfected COS-7 cells. COS-7 cells were transiently transfected with HA-tagged CD300c alone or in combination with Flag-tagged CD300b. Cell lysates were immunoprecipitated with anti-HA.11 mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to a PVDF filter and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

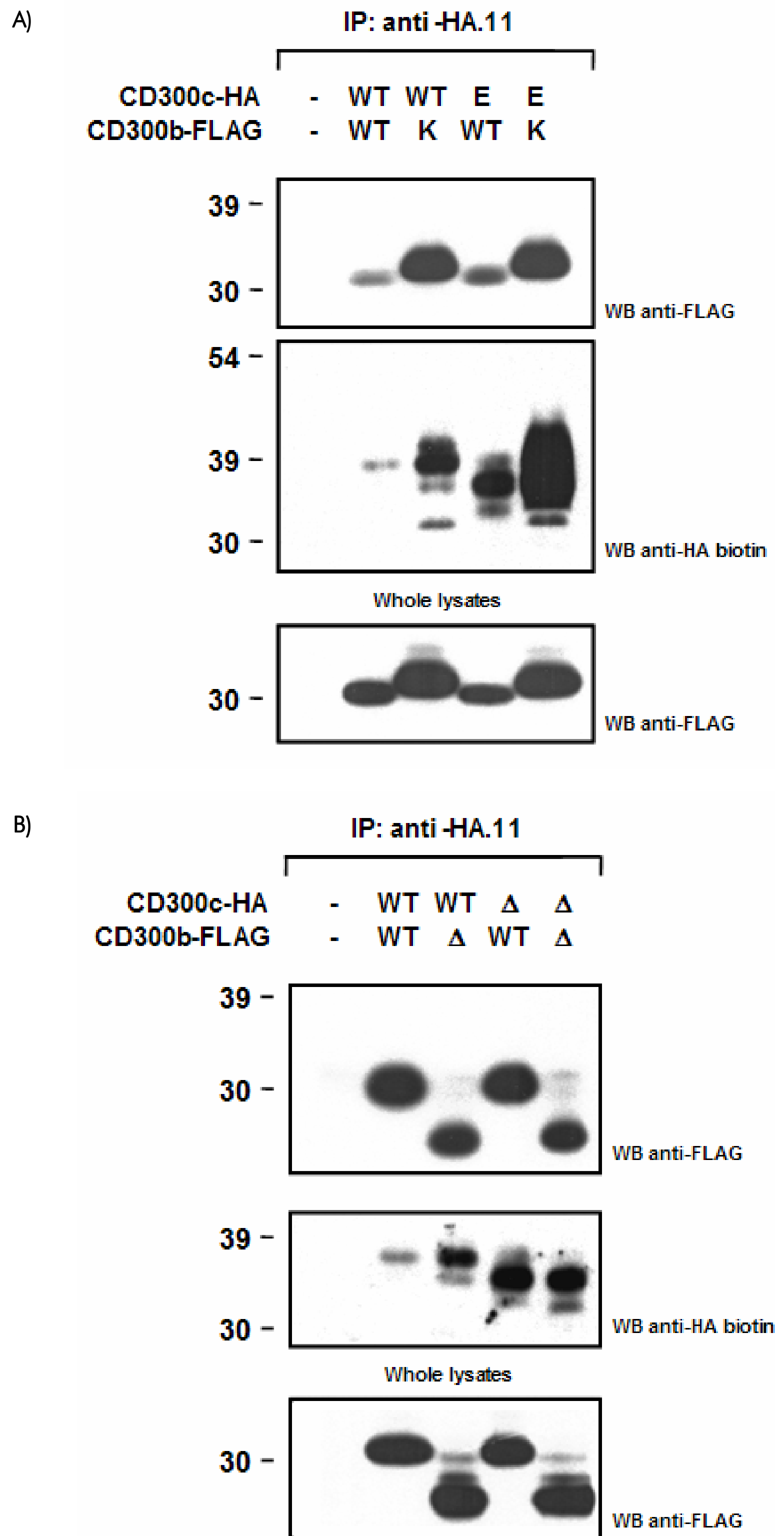
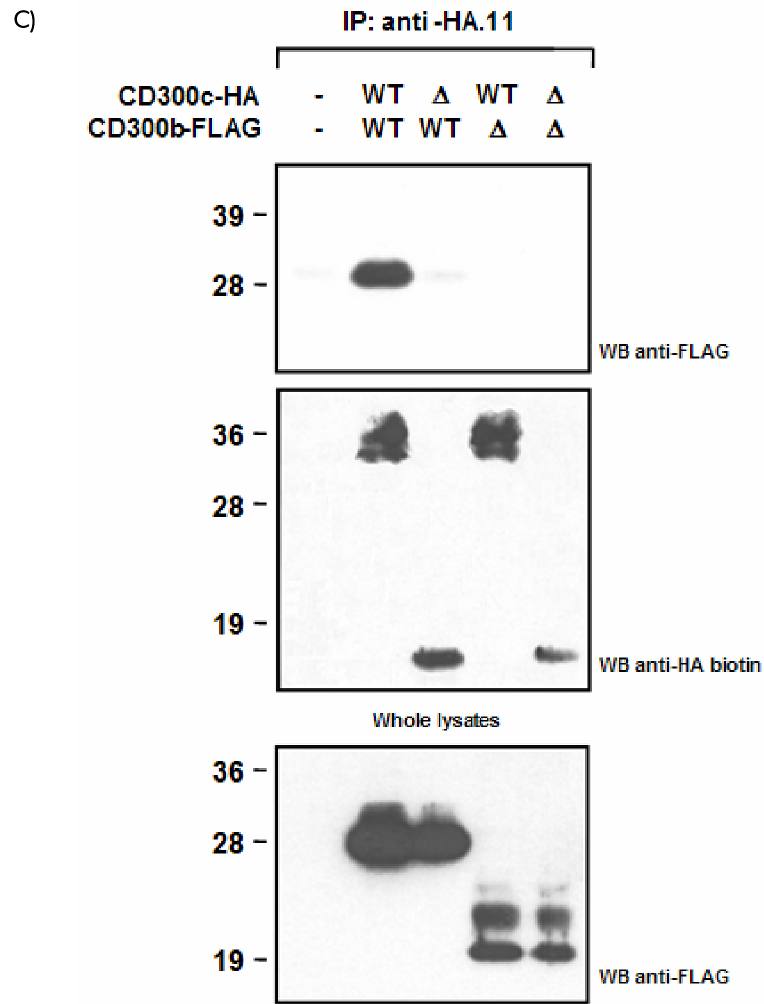


Fig 3. CD300c and CD300b interact through their immunoglobulin-like domains. COS-7 cells were transiently transfected with HA-tagged CD300c in combination with Flag-tagged CD300b. Wild type forms were tested against transmembrane substitution mutants **(A)**, intracellular deletion mutants **(B)**, extracellular deletion mutants **(C)** or cysteine substitution mutants **(D)** in order to map the interaction between both molecules. CD300c was immunoprecipitated with anti-HA.11 in all cases. Western blots were conducted with the indicated Abs. Whole cell lysates (2%) were included as controls.

C)



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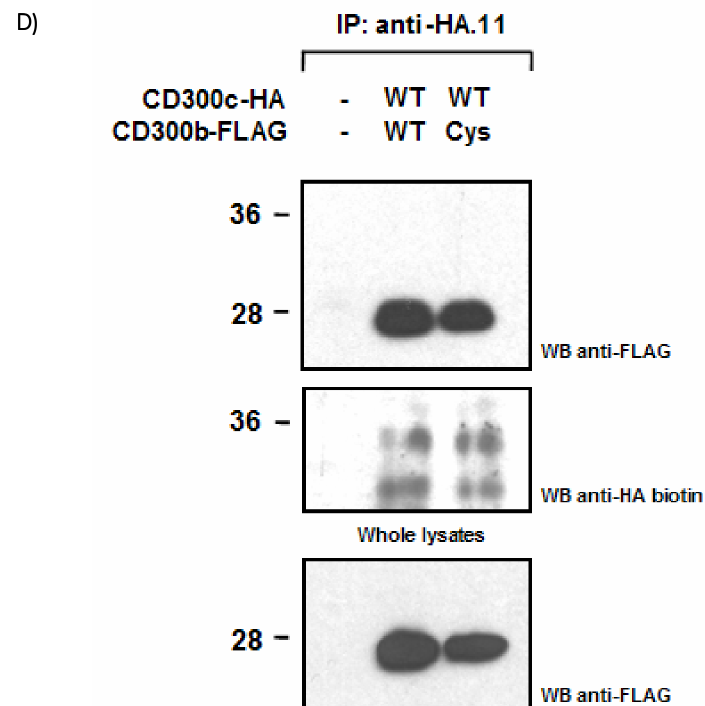

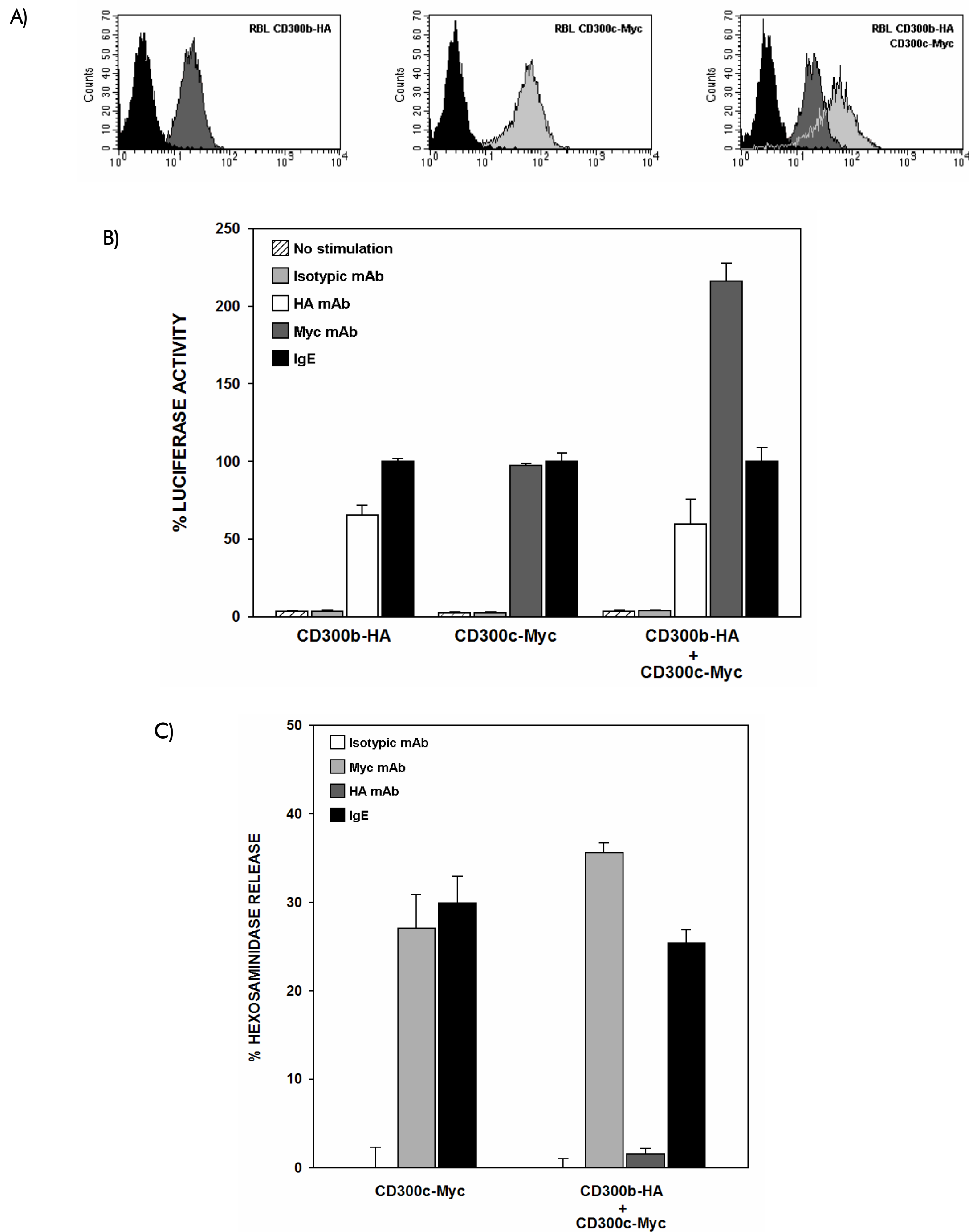

Fig 4. CD300c acts as a modifier of CD300b signaling. A) RBL-2H3 cells stably expressing CD300b-HA on the cell surface were transfected with CD300c-2×Myc. Cell surface expression of the different transfectants was checked by flow cytometry using anti-HA12CA5 mAb for CD300b (dark grey histogram) and anti-Myc9E10 for CD300c (light grey historgram). Isotypic mAb was used as negative control (black histogram). **B)** RBL-2H3 simple and double transfectants were transiently transfected with 3×NFAT/AP1-Luciferase and TK-Renilla plasmids. Luciferase activity was measured after stimulation for 7h with the indicated antibodies. Data are normalized as described before. Duplicates were performed for all stimulations. **C)** RBL-2H3 transfectants were stimulated with the indicated Abs for assessing hexosaminidase release as described. Data are expressed as a percentage of specific release. Each stimulation point was set up in triplicate. All the results are representative of three independent experiments.

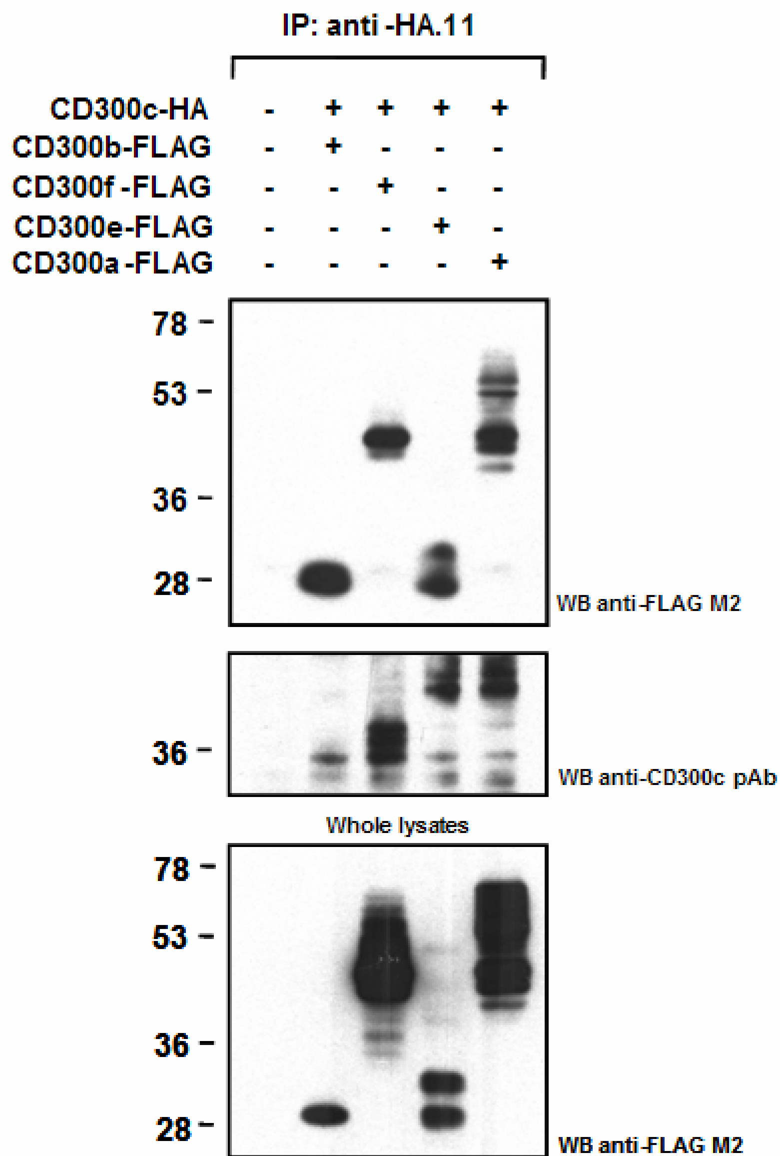

Fig. 5. CD300c is able to interact with all CD300 family members. CD300c-HA was transiently transfected in COS-7 cells in combination with the different CD300 receptors tagged with a Flag-epitope (CD300a, CD300b, CD300e and CD300f). Cell lysates were immunoprecipitated with anti-HA.11. Samples were analyzed by SDS-PAGE, transferred to PVDF filters and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

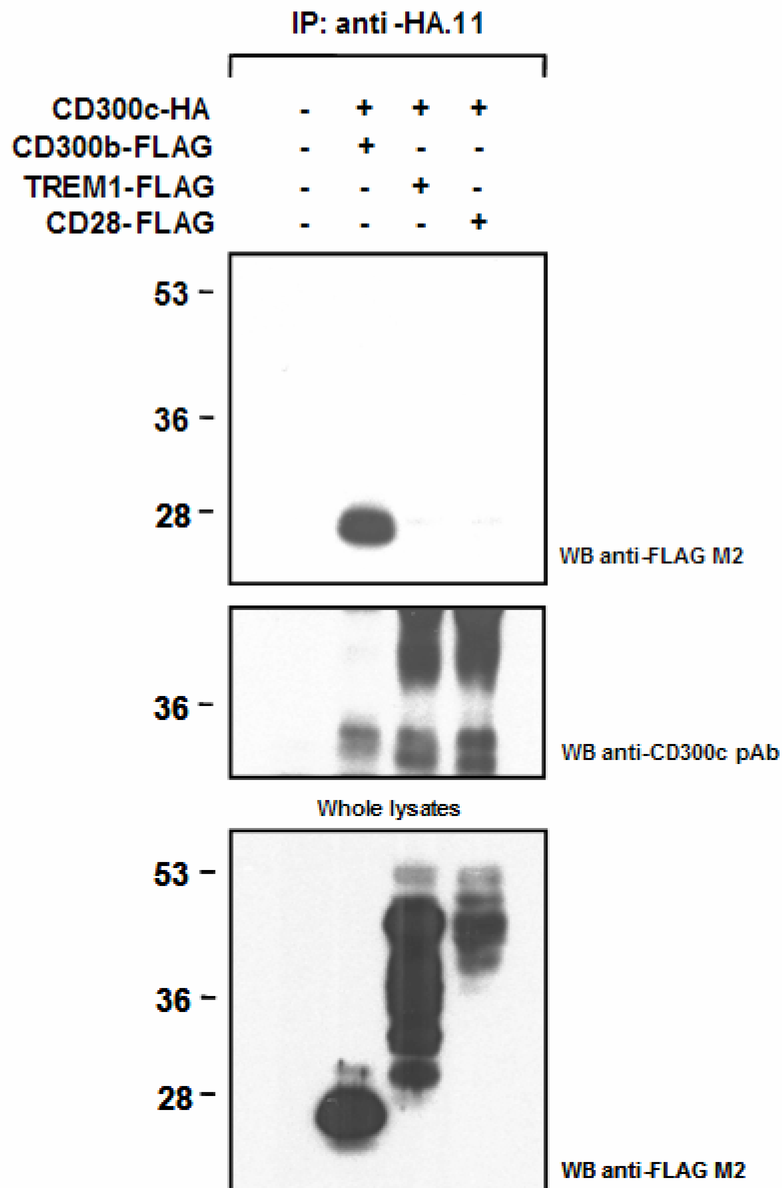

Fig. 6. CD300c establishes interactions specifically with CD300 family members. CD300c does not interact with Ig-like receptors closely related to CD300 family. CD300c-HA was transiently transfected in COS-7 cells in combination with Flag-tagged TREM-1 and CD28 immunoglobulin receptors. Cotransfection with CD300b was used as positive control. Immunoprecipitation was carried out with anti-HA.11. Proteins were analyzed by SDS-PAGE, transferred to PVDF filters and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

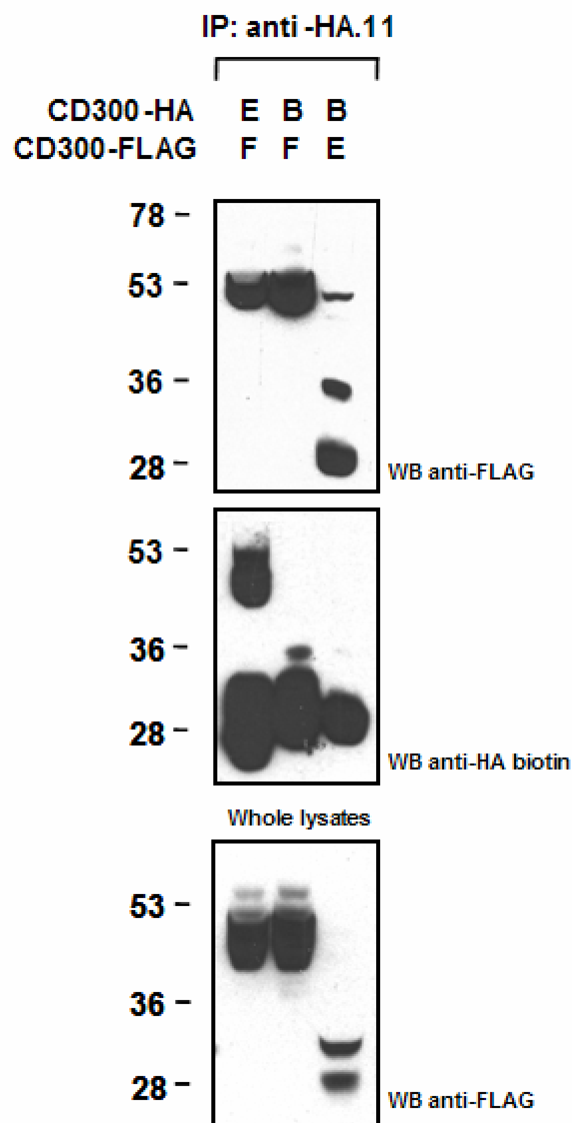

Fig. 7. Interaction among CD300 family members occurs in COS-7 cells. CD300b, CD300e and CD300f (HA- or Flag-tagged) were transiently cotransfected in COS-7 cells following all possible combinations. Immunoprecipitations were performed against HA-tagged receptors with anti-HA.11. Coprecipitation was assessed by proving the filters with the indicated antibodies. Whole cell lysates (2%) were included as controls.

APPENDICES

1. TABLES FOR IREM/CLM/CD300 RECEPTORS

Table 1: Human IREM/CD300 receptors

CD nomenclature	Alternative name	DNA accession number	Protein accession number	Crystal structure (PDB)	Cloning paper	Equivalent/Similar to
CD300a	CMRF35-H9	AF020314	AAD01646	-	(241)	IRp60
	IRp60	AJ238323	CAB66145	-	(243)	CMRF35-H9
CD300b	IREM-3	AY646929	AAV69612	-	(258)	TREM-5
CD300c	CMRF-35A	X66171	CAA46948	-	(240)	-
CD300d	IREM-4	EF137868	ABM30606	-	-	-
CD300e	IREM-2	AF395839	AAP42154	-	(252)	-
CD300f	IREM-1	AY303545	AAP57942	2NMS	(250)	IgSF13/NKIR
	IREM-1 Sv1	AF375480	AAP42152	-	(250)	-
	IREM-1 Sv2	AF375481	AAP42153	-	(250)	-
	IREM-1 Sv3	AAH28199		-	(250)	-
	IgSF13/NKIR	AF251706	AAM19099	-	(251)	IREM-1
CD300g	Nepmucin-α1	AF427619	AAN86134	-	(230)	TREM-4α
	Nepmucin-α2	AF427620	AAN86135	-	(230)	TREM-4β
	Nepmucin-δ	AY358364	AAQ88730	-	(230)	-
	Nepmucin-γ	-	-	-	(230)	-

Table 2: Mouse CLM/CD300 receptors

CD nomenclature	Alternative name	DNA accession number	Protein accession number	Crystal structure (PDB)	Cloning paper	Equivalent/Similar to	Mouse strain
CD300a	CLM-8	AY457054	AAR27945	-	(25)	MAIR-Ib/LMIR-1	C57BL/6
	LMIR-1	AB095675	BAC80268	-	(255)	MAIR-Ib/CLM-8	CBA/J
	MAIR-Ia	AB091765	BAC77074	-	(254)	-	C57BL/6
	MAIR-Ib	AB091766	BAC77075	-	(254)	CLM-8/LMIR-1	C57BL/6
CD300b	CLM-7	AY457053	AAR27944	-	(25)	mIREM-3	C57BL/6
	mIREM-3	AY996128	AAY56360		(258)	CLM-7	Balb/C
CD300c	CLM-6	AY457052	AAR27943	-	(25)	-	C57BL/6
CD300d	CLM-4	AY457050	AAR27941	-	(25)	MAIR-IIa DIgR1/LMIR-2	C57BL/6
	DIgR1	AY048685	AAL12222	-	(260)	MAIR-IIa CLM-4/LMIR-2	C57BL/6
	LMIR-2	AB098476	BAC80269	-	(255)	MAIR-IIa CLM-4/DIgR1	CBA/J
	MAIR-IIa	AB091767	BAC77076	-	(254)	CLM-4 DIgR1/LMIR-2	C57BL/6
	MAIR-IIb	AB091768	BAC77077	-	(254)	-	C57BL/6
CD300e	CLM-2	AY457048	AAR27939	-	(25)	-	C57BL/6
CD300f	CLM-1	AY457047	AAR27938	1ZOX	(25)	MAIR-Va/LMIR-3	C57BL/6
	DIgR2	AY214460	AAO59487	-	(260)	MAIR-Vb	C57BL/6
	MAIR-Va	-	-	-	-	CLM-1	C57BL/6
	MAIR-Vb	-	-	-	-	DIgR2	C57BL/6
	LMIR-3	AB292061	BAF46248	-	(264)	MAIR-Va/CLM-1	CBA/J
CD300g	CLM-9	AY457055	AAR27946	-	(25)	Nepmucin Iso D	C57BL/6
	Nepmucin Iso A	AB243063	BAE96046	-	(231)	-	
	Nepmucin Iso B	AB243064	BAE96047	-	(231)	-	
	Nepmucin Iso C	AB243065	BAE96048	-	(231)	-	
	Nepmucin Iso D	AB243066	BAE96049	-	(231)	CLM-9	
?	CLM-3	AY457049	AAR27940	-	(25)	-	C57BL/6
?	CLM-5	AY457051	AAR27942	-	(25)	LMIR-4/MAIR-IV	C57BL/6
	MAIR-IV	-	-	-	-	CLM-5/LMIR-4	C57BL/6
	LMIR-4	AB292062	BAF46249	-	(264)	CLM-5/MAIR-IV	CBA/J

2. GENERATION OF IREM3-mIgG2a FUSION PROTEIN AND anti-IREM3/CD300b mAb

Generation of IREM3-mIgG2a fusion protein

Multiple strategies for the production of monoclonal antibodies against a desired protein are available nowadays. We resorted to the use of purified chimerical proteins as immunizing agents. Specifically, IREM-3/CD300b extracellular domain was fused to the constant region of mouse Ig2Ga immunoglobulin. IREM-3 extracellular region was amplified by PCR using 5′-CCG AAG CTT G GGC TGT TTC TCC ATC CAA GGC-3′ and 5′-CTG GGA TCC AAG TTC CTC TTG TGG GAG CCG AT-3′ primers, and cloned into the *Bam*HI/*Hind*III sites of pSEC/mIgG2a vector described before (268). This construction was used to transiently transfect HEK293T cells using calcium transfection method (269). Twenty-four hours after transfection DMEM medium was removed and substituted by CHO protein-free medium (Sigma). Supernatants were collected after three days and immunoprecipitated with protein A-Sepharose CL-4B beads (Amersham Biosciences) for 2h at 4°C. Samples were run on SDS-PAGE under non-reducing conditions and transferred to polyvinylidene difluoride (PVDF) filters (Millipore). Detection of IREM3-mIgG2a protein was performed using specific antibodies recognizing mouse IgG2a-HPR (Zymed). No crossreaction was obtained when proving the filters with anti-mouse kappa-biotin (Southern Biotechnology) discarding the visualized product to be serum remaining immunoglobulins (data not shown).

Next, we generated a cell line that stably expressed and secreted IREM-3/mIgG2a protein. CHO-K1 cells (7.5×10^6 cells) were electroporated in the presence of 20μg of linearized pSEC/IREM3-mIgG2a at 250V, 960μF and 100Ω in a Gene Pulser Electroporator (Bio-Rad). Transfectants were selected and maintained in culture with 250μg/mL of zeocin (Invitrogen). Production levels of IREM3-mIgG2a in the CHO-K1 polyclones were assessed by ELISA and western blot. The clones secreting higher rates of fusion protein were selected for production of the protein at a bigger scale. IREM3-mIgG2a protein was purified through affinity chromatography with protein A-Sepharose CL-4B. Fusion protein was eluted with 0.1M Glycine, 1M NaCl pH 2.5 and reconstituted with HEPES buffer pH 9. Solubilization buffer was changed to PBS using Vivaspin Concentrator columns 30,000 Mw (Vivascience). Purified protein was run on SDS-PAGE under non-reducing conditions and visualized for purity by GelCode Blue Stain (Pierce) and western blot with anti-mouse IgG2a-HRP linked Ab (Fig. 2A).

Generation of monoclonal antibodies against IREM-3/CD300b

For the obtention of monoclonal antibodies against IREM-3/CD300b three immunization, fusion and screening procedures were carried out.

Fusion 1

Six-week-old female BALB/c mice were immunized with the purified IREM3-mIgG2a protein. Immunization protocol was the following:

- Day 0: Intraperitoneal administration with 10μg of purified IREM3/mIgG2a protein in PBS 1x with complete Freund's adjuvant (Sigma) 1:1;
- Day 27 and 54: Intraperitoneal administration with 10μg of purified IREM3/mIgG2a protein in PBS 1x with incomplete Freund's adjuvant (Sigma) 1:1;
- Day 81: Intravenous administration of 2μg of purified IREM3/mIgG2a protein in PBS 1x.

Twenty-four hours later mice were bleeded and serum was checked for recognition of both IREM3-mIgG2a fusion protein (ELISA) and IREM-3 whole molecule in the surface of stably transfected cells (FACS).

Three days after the last immunization animals were sacrificed and mouse spleen cells were isolated and fused with mouse SP2 myeloma cells following standard methods (270). Hybridoma cells were cultured in RPMI medium in the presence of HAT selection antibiotic (Gibco). HAT component has removed and substituted by HT (Gibco) seven days after the establishment of the culture to eliminate toxicity and allow cell growing of selected hybridomas. Six days after hybridoma supernatants were tested. Hybridoma supernatants were screened by ELISA using IREM3/mIgG2a protein. 96 well-plates were coated o/n at 4°C with purified IREM3-mIgG2a fusion protein in carbonate/bicarbonate buffer pH 9. Wells were washed twice with PBS-Tween 0.05% and blocked for 1h at 37°C with BSA 2% in PBS. Wells were washed twice again and incubated with hybridoma supernatants diluted 1/3 in PBS-BSA 2%. After 2h of incubation at room temperature, four washes were performed. Samples were incubated for 30min at room temperature with goat anti-mouse κ chain specific-biotin (Southern Biotechnology) in PBS-BSA 2%. Samples were washed twice and incubated for 30min at room temperature with Streptavidin-HRP (Roche) in PBS. ELISA was developed with TMB substrate reagent (BD Bioscience) and stopped by addition of 1M orto-phosphoric acid solution (Merk). Optical density was determined at 450 nm.

ELISA positive supernatants were tested for the recognition of RBL-2H3, RBL-2H3 IREM3-HA and the myeloid cell lines U937, THP-1, HL60 and MonoMac6 (258).

In the fusion procedure we established 960 wells. After HAT selection 871 wells experimented cell growth (90.7% fusion efficiency) and were tested by ELISA. 129 wells were positive and further tested for recognition of RBL-2H3 IREM3-HA transfectant vs RBL-2H3 parental cell line. None of them specifically recognized RBL-2H3 IREM3-HA cells.

Fusion 2

Thinking that IREM-3/CD300b immunoglobulin domain on the fusion protein could be improperly folded upon acid elution, we decide to use a murine B cell line stably expressing IREM3-HA on the membrane as immunogen. 300.19 cells (8×10^6) were stably transfected with 10μg of linealized pDisplay/IREM3-HA construct by electroporation at 250V, 950μF and 100Ω in a Gene Pulser Electroporator (Bio-Rad). Selection and maintenance of the transfected cell lines was performed by addition of G418 (Gibco) at 1.2mg/mL to the culture media. Surface expression of IREM3-HA was determined by flow cytometry (Fig. 2B). Immunized mice from the first fusion procedure received an intraperitoneal immunization with 10×10^6 300.19/IREM3-HA cells in PBS 1x. Twenty-eight days after, mice received an intravenous boost consisting on 2×10^6 300.19/IREM3-HA cells and 2μg IREM3-mIgG2a fusion protein in PBS 1x. Fusion and screening process were carried out as described above. This time, two modifications were introduced in the screening procedure. First, we used a mixture of anti-mouse κ and λ chain specific-biotin for detecting the antibodies bound to IREM3-mIgG2a fusion protein in the ELISA assays. Second, ELISA positive supernatants were tested by immunofluorescence on RBL-2H3 cells expressing IREM-3/CD300b molecule fused to a FLAG-epitope to avoid interference of HA-tag present on 300.19/IREM3-HA cells (Fig. 2B).

This time, 1440 wells were established upon the fusion procedure. 1409 wells survived to the HAT selection (97.8% fusion efficiency) and could be tested by ELISA. 310 supernatants were further tested for RBL-2H3 and RBL-2H3 IREM3-FLAG recognition. No specific antibodies against IREM-3/CD300b were obtained.

Fusion 3

Six-week-old female BALB/c mice were immunized with the purified IREM3-mIgG2a protein and the 300.19/IREM3-HA cells. Immunization protocol was what follows:

- Day 0: Intraperitoneal administration with 20μg of purified IREM3/mIgG2a protein in PBS 1x with complete Freund's adjuvant 1:1;

- Day 27: Intraperitoneal administration with 10μg of purified IREM3/mIgG2a protein + 15×10^6 300.19/IREM3-HA cells in PBS 1x;
- Day 54: Intraperitoneal administration with 10μg of purified IREM3/mIgG2a protein + 10×10^6 300.19/IREM3-HA cells in PBS 1x;
- Day 81: Intraperitoneal administration with 2μg of purified IREM3/mIgG2a protein + 1×10^6 300.19/IREM3-HA cells in PBS 1x and intravenous administration with 5μg of purified IREM3/mIgG2a protein + 2×10^6 300.19/IREM3-HA cells in PBS 1x.

Fusion process was carried out as described above. Screening of the supernatants was performed by immunofluorescence staining against RBL-2H3 IREM3-FLAG cells. 1488 wells were established upon the fusion procedure. 1438 wells survived to the HAT selection (99.3% fusion efficiency) and were tested by FACS. 30 supernatants were positive for RBL-2H3 IREM3-FLAG cells and were further checked for recognition of RBL-2H3, U937 (myeloid), Jurkat (lymphoid-T) cell lines. Binding of these supernatants to COS-7 cells transiently transfected with IREM3-HA molecule was also performed. No specific antibodies against IREM-3/CD300b were obtained.

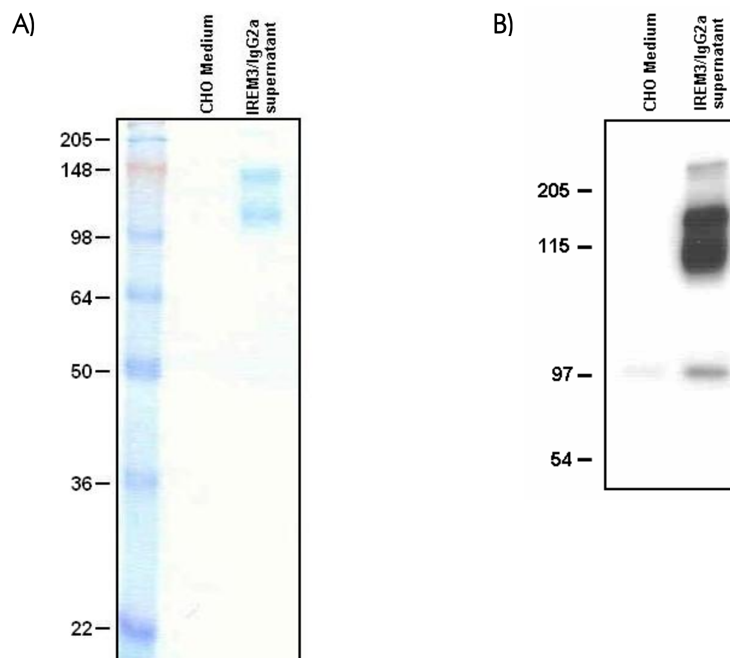

Figure 2A. IREM3-mIgG2a fusion protein analysis. Supernatants from CHO-K1 cells stably expressing and secreting IREM3-mIgG2a fusion protein were subjected to a protein A-Sepharose column purification. Purified and reconstituted protein products were run on 10% SDS-PAGE gels under non-reducing conditions and assessed for purity by Comassie Blue Staining **(A)** and Western blot with anti-mouse IgG2a-HRP linked Ab **(B)**.

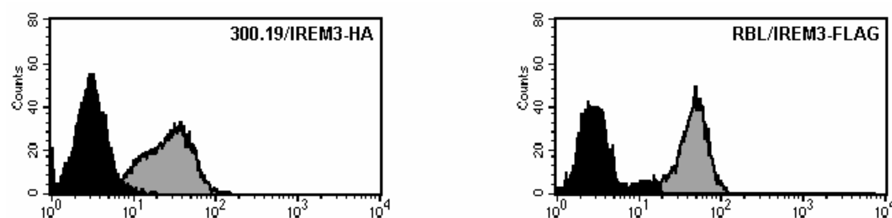

Figure 2B. IREM-3/CD300b surface expression. 300.19 and RBL-2H3 cells were stably transfected with IREM3 HA or FLAG-tagged. Cell surface expression of IREM3/CD300b molecules was checked by flow cytometry using anti-HA12CA5 or anti-FLAG M2 mAb (gray histograms) or an isotypic mached mAb as a negative control (black histogram)

3. GENERATION OF anti-IREM3/CD300b polyclonal serums

Two twelve-week-old New Zealand White rabbits were immunized with a purified peptide mapping in the cytoplasmic tail of IREM-3/CD300b (sequence CMAVFIGSHKRNHYM). The peptide was coupled to KLH (Keyhole Limpet Hemocyanin), a high molecular weight mass protein widely used as a carrier protein in antibody production because of the excellent immunogenicity that confers to the attached antigens. The immunization and serum collection protocol was what follows:

- Day 0: Subcutaneous administration of 200μg KLH-peptide in complete Freund's adjuvant;
- Day 14, 28 and 42: Subcutaneous administration of 100μg KLH-peptide in incomplete Freund's adjuvant;
- Day 49: First bleed collection;
- Day 56: Subcutaneous administration of 100μg KLH-peptide in incomplete Freund's adjuvant;
- Day 63: Second bleed collection;
- Day 70: Subcutaneous administration of 100μg KLH-peptide in incomplete Freund's adjuvant;
- Day 77: Final bleed collection.

4. MACROPHAGE DIFFERENTIATION AND RT-PCR

PBMCs were purified from human blood by standard gradient density centrifugation using Lymphoprep™ (Atom). For isolation of monocytes, PBMCs were resuspended at a final concentration of 10×10^6 cells/mL in RPMI 1640 with 10% FCS and allowed to adhere to plastic for 1h at 37ºC and 5% CO_2. Non-adherent cells where discarded. For differentiation studies, 3×10^6 isolated monocyte-enriched cell population were plated in 6 well plates and cultured for 5 days in the presence of GM-CSF (20ng/mL) to differentiate monocytes into naïve macrophages. Cells were further treated for 48h with the following cytokines to obtain activated macrophage from the different subtypes: M1 macrophages: LPS (100ng/mL) + IFNγ (500u/mL); M2a macrophages: IL-4 (20ng/mL) or IL-13 (20ng/mL); M2c macrophages: IL-10 (20ng/mL). All cytokines were purchased to Peprotech.

Monocyte-to-macrophage differentiation was assessed by flow cytometry using phycoerythrin (PE)-labeled IgG mAb specific for CD14, CD1a and CD83 (BD Biosciences)(data not shown).

RNA from monocytes and macrophages was extracted with Trizol reagent (Gibco), treated with DNAseI Amplification Grade (Invitrogen) and retrotranscribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was performed using TaqMan Gene Expression Assay for CD300b gene (Hs00380072_m1, Applied Biosystems). 18S amplification control was used for cycle normalization. Data were analyzed using 7500 SDS Software (Applied Biosystems). Expression of IREM-3/CD300b in macrophages was expressed as fold of induction/reduction considering monocytes as the normalizing cell type (Table 3).

Monocytes		M1 macrophages		M2a macrophages (IL-4 activation)		M2a macrophages (IL-13 activation)		M2c macrophages	
Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
1	0.14-10.96	0.169	0.04-0.55	13.80	0.17-34.67	14.12	2.68-47.85	4.168	0.99-11.21

Table 3. IREM-3/CD300b transcript expression in monocyte *versus* macrophage populations. Monocytes (n=10), Macrophage populations (n=18).

5. LUCIFERASE ASSAYS IN JURKAT T CELLS

Jurkat T cells were stably transfected with wild type IREM3-HA or a mutant form affecting cytoplasmic tyrosine Y188 (Fig. 4A). Jurkat transfectants were transiently electroporated with a luciferase reporter plasmid (pT81Luc) containing three tandem copies of the distal NFAT/AP-1 site of the murine IL-2 promoter (271) ($0.5\mu g/10^6$ cells) and a TK Renilla construct (Promega) ($0.1\mu g/10^6$ cells). Twenty-four hours post transfection, 1.5×10^6 cells were stimulated for 7h with the indicated antibodies using the murine mastocytoma P815 cell line as presenting cell (1×10^6). Anti-CD3 T3b was used as positive control for Jurkat stimulation and the P815 cell line cultured in supplemented RPMI 1640/L-glutamine medium alone was used as negative control. Postnuclear lysates were obtained as described (271) and luciferase activity was measured according to the Dual Luciferase Report kit manual (Promega) using a FB12 Luminometer (Berthold). Data were normalized and expressed as a percentage of luciferase activity considering TcR stimulation as the top threshold of activation (Fig. 4B)

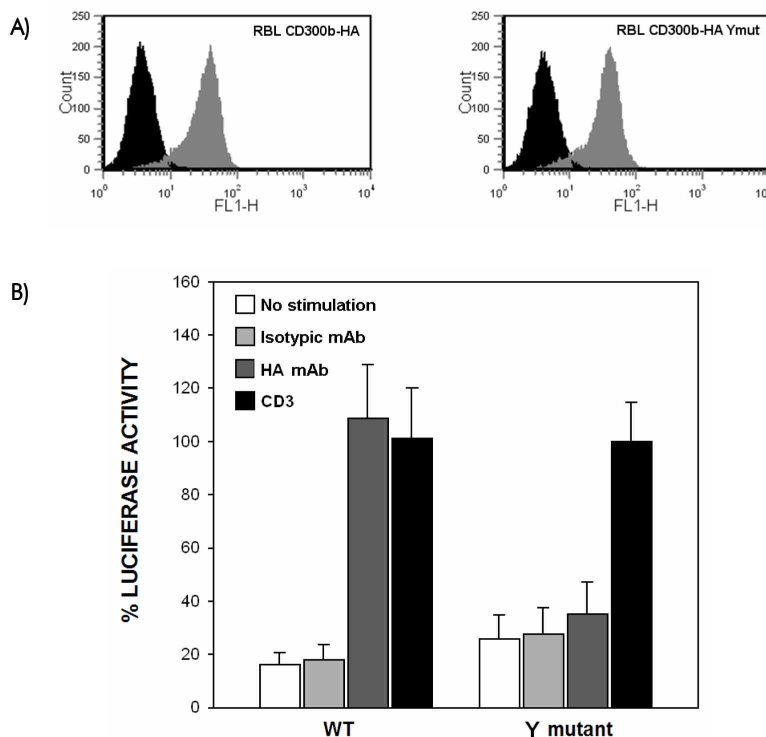

Figure 4. IREM-3/CD300b is able to deliver activating signals in the absence of extracellular and transmembrane interacting partners. Monocytes (n=10), **A)** Jurkat T cells were transfected with IREM3/CD300b-HA wild type or IREM3/CD300b Y188F-HA. Cell surface expression was checked by flow cytometry using anti-HA12CA5 mAb (dark grey histogram). Isotypic mAb was used as negative control (black histogram). **B)** Jurkat transfectants were transiently transfected with 3×NFAT/AP1-Luciferase and TK-Renilla plasmids. Luciferase activity was measured after stimulation for 7h with the indicated antibodies. Data are normalized as described. Triplicates were performed for all stimulations.

DISCUSSION

Eight years ago it was carried out in the laboratory a three hybrid screening in yeast to identify new molecules interacting with the SH2 domains of the protein phosphatase SHP-1 in a phosphotyrosine-depending manner. The specific aim of the screening was the cloning of inhibitory receptors belonging to novel immune receptor families. At that time, families of receptors such as the TREMs and SIGLECs (Sialic Acid Binding Ig-like lectins) were coming up in the literature, confirming that still could be clusters of non-identified leukocyte receptors (85, 272). Moreover, the annotation of the human genome was not completed and as consequence the tools for *in silico* identification of new molecules were not as developed as they are at the present. The screening resulted in several clones coding for a sequence compatible with the cytoplasmic portion of a putative novel inhibitory receptor. The complete sequence, termed IREM-1/CD300f (Immune Receptor Expressed by Myeloid cells) was predicted, cloned and characterized. IREM-1/CD300f gene was found to map in close vicinity with the previously described molecules CMRF-35/CD300c and IRp60/CD300a, and the encoded protein shared with them a high degree of sequence homology, especially regarding the extracellular portion.

In order to identify new members belonging to the same family we performed a search on the Ensemble genome database with IREM-1/CD300f and CMRF-35/CD300c sequences. This strategy resulted in the identification of a cDNA coding for the activating receptor IREM-2/CD300e. In the search process we identified two additional cDNAs that shared homology not only with IREM-1/CD300f and CMRF-35/CD300c but also with IREM-2/CD300e. They were termed IREM-3/CD300b and IREM-4/CD300d. This thesis has been focused in the cloning and characterization at molecular and functional levels of IREM-3/CD300b receptor. However, the results obtained during this process forced us to broaden the framework of study and to work simultaneously with all the members in the family.

IREM/CD300 gene family is located in human chromosome 17q25.1 and includes six clustered genes encoding for different inhibitory and activating immune receptors. This locus has been found to be genetically associated to psoriasis, a chronic inflammatory disease of the skin caused by a primary infiltration of proinflammatory cells both, in the dermis and epidermis, and a secondary epidermal cell proliferation (273). This locus, named PSORS2, has also been associated genetically to other inflammatory diseases such as atopic dermatitis and rheumatoid arthritis (274, 275). These traits suggested a possible role for the members of this family in the induction, control and suppression of inflammatory responses. The murine ortholog locus, called CLM (CMRF35-like molecules), was described lately in the syntenic chromosome 11 and consists on nine members (25). There is not a good correlation between the human and mouse orthologs as consequence of duplication processes in mice

affecting primarily CD300 activating receptors. In addition, in the cases in which clear orthologs can be established, important features such as the presence of signaling motifs are not conserved between human and mice proteins. For instance, CLM-1, the mouse ortholog of IREM-1/CD300f, does not display any docking site for the PI3-Kinase regulatory subunit p85α, and mIREM-3/CD300Lb is devoid of the cytoplasmic Grb-2 binding site. Consequently, the generation of knock out mice models for inferring the functionality of human CD300 family members is inappropriate.

IREM-3/CD300b expression pattern was broadly assessed at the mRNA level. As observed for other CD300 family members, IREM-3/CD300b expression is restricted to the myeloid lineage. Its transcript is only found in freshly isolated $CD14^+$ blood cells and in myeloid-derived cell lines (U937, THP-1, HL60 and Monomac6). Consistently, tissues such as spleen, colon and lung where mature and differentiated myeloid cells can be found, were positive for IREM-3/CD300b transcript. The same was observed for tissues containing high proportion of myeloid precursors such as placenta, bone marrow and fetal liver.

Real time PCR experiments were performed to track IREM-3/CD300b expression along the maturation process of monocytes into macrophages. Macrophages play an important role in the host defense but also in homeostatic processes such as the clearance of senescent cells and tissue remodeling/repair after injury or infection. Most of the tissue-resident macrophages in the normal adult are derived from circulating bone marrow–derived monocytes. Expression studies of surface receptors and differentiation antigens have shown that tissue macrophages are markedly heterogeneous. The acquisition of very different phenotypes reflects the functional specialization within particular microenvironments (190). In response to metabolic and proinflammatory signals, additional monocytes are recruited to local sites with the objective to increase the number of effector cells. These monocytes differentiate into mature cells but acquire a phenotype different from the one presented by resident macrophages, what increases the heterogeneity mentioned above (276). Although it is clear that monocytes are the precursors of macrophages, the differentiation pathways driving this phenomenon are poorly understood. However, great efforts have been made to try to reproduce *in vitro* the distinct phenotypes and physiological activities observed in these cells. Two types of macrophages with opposing roles can be derived experimentally from monocytes: the classically activated macrophages (or M1) and the alternatively activated macrophages (or M2). M1 macrophages are the so-called "killer macrophages" due to their potent microbicidal properties, especially against intracellular pathogens. They are induced upon exposure of monocytes to IFN-γ, TNF-α or TLR ligands. The acquisition of this phenotype results in an increased production of pro-inflammatory cytokines (IL-1β, IL-6, IL-12,

IL-18, TNF-α, CCL3, CXL9 and CXCL10), iNOS and ROS, and in an increased expression of MHC class II and antigen presenting molecules. These cells are supposed to emulate those macrophages generated under the action of NK cells and Th1-type T lymphocytes (277). Contrary, M2 macrophages support Th2-associated effector functions. Beyond infection, M2 macrophages play a role in the resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis. As consequence, these cells have been proposed as important mediators of humoral immunity and tissue repair (278). M2 macrophages can be further subdivided into M2a, M2b and M2c types. M2a macrophages are elicited upon monocyte exposure to IL-4 or IL-13, while M2b are obtained upon stimulation with immune complexes in combination with IL-1β or the ligands for TLRs, CD40 or CD44. M2c macrophages mimic what has been called the deactivated state of macrophages, which is characterized by anti-inflammatory cytokine production and reduced MHC class II expression. These cells can be achieved by monocyte culture with IL-10, TGF-β or lipidic mediators (PGE_2), or by ligation of inhibitory receptors like CD200 or CD172a (276, 279).

Downregulation of IREM-3/CD300b at mRNA level was observed upon differentiation of monocytes into M1 macrophages by stimulation with LPS and IFNγ. Contrary, the transcript was markedly upregulated when generating M2 macrophages, especially when raising M2a subtype both with IL-4 or IL-13 (see appendices for details). A wide range of myeloid functions have been shown to be modulated by surface receptors. Classically, macrophage immune recognition has been focused in the study of scavenger receptors, TLRs and C-type lectins. They mostly recognize pathogen and apoptotic cell components and function by promoting the initiation of clearance mechanisms. However, an important role is also developed by members of the immunoglobulin superfamily such as TREMs, SIGLECs, TLT, CD200R or CD172α (190). The role of IREM-3/CD300b in the regulation of macrophage function will be addressed in the future.

The distribution of cell surface molecules in macrophage populations is of special interest in tumor immunology. Important differences have been observed between M1 and M2 activated-macrophages in terms of tumor support. While M1 macrophages have been shown to exert anti-tumor activity by killing tumor cells and eliciting tissue disruptive reactions, M2 macrophages promote tumor progression by enhancing cell growth and formation of new blood vessels (280). A prominent component of solid tumors is represented by non-tumoral cells, including stromal cells and leukocytes. Among the latter, tumor-associated macrophages (TAM) are the major component. Many observations indicate that these macrophages present M2-features leading to tumor progression. In fact, clinical studies have found a marked correlation between the macrophage content of tumors and patient

prognosis. Consequently, molecules skewing the macrophage phenotype into M2-type constitute potential targets for anti-cancer therapies (281, 282). At the present, we are starting a new research line in the laboratory focused in characterizing the role of IREM-3/CD300b, and the rest of CD300 receptors, in the function of macrophages infiltrated in colorectal cancer tumors. Very recently it has been reported that the epithelial proliferation at the intestinal cripts is coincident with the infiltration of TREM-2 expressing macrophages and a peak of secretion of IL-4 and IL-13 cytokines (283). It is feasible that a similar effect could be observed for IREM-3/CD300b considering the upregulation of its transcript in M2a macrophages and the high degree of similarities with TREM receptors.

The assessment of mRNA distribution provides valuable information about the expression pattern of a certain molecule. But given the existence of multiple mechanisms for the control of protein expression at a posttranscriptional level, transcript detection is not synonymous of protein expression. For this reason, we wanted to complement the distribution studies by assessing IREM-3/CD300b protein expression. We tried to generate monoclonal antibodies in mice recognizing the receptor's extracellular portion, but none of the three different attempts was successful (see appendices for details). Initially, we attributed the failure of the process to the high degree of homology between IREM-3/CD300b and its murine ortholog (76% in the Ig-domain and 73% considering the whole molecule)(258). Consistent with this, the serums from boosted animals never scored high values when tested for the recognition of IREM-3/CD300b fusion protein by ELISA. The obtainment of antibodies against IREM-1/CD300f molecule was also a long and difficult process. It was necessary to perform five fusions to raise UP-D1 and UP-D2 monoclonal antibodies (250). In contrast, for IREM-2/CD300e receptor the procedure worked perfectly well and UP-H1 and UP-H2 antibodies were obtained after a single immunization protocol (252). IREM-2/CD300e molecule does not have a clear ortholog in the murine CLM cluster. CLM-2, CLM-4 and CLM-6 are all homologous to IREM-2/CD300e receptor (25). However, to simplify the nomenclature only CLM-2 has been officially named CD300e. Oppositely, both IREM-1/CD300f and IREM-3/CD300b have clear murine counterparts regarding sequence homology, CLM-1 and CLM-7 respectively, and share with them a higher percentage of identity and similarity than the observed for IREM-2/CD300e and any of its three orthologs (77% for IREM-1/CD300f, 73% for IREM-3/CD300b and 50% for IREM-2/CD300e). In consequence, IREM-2/CD300e was likely much more antigenic than IREM-1/CD300f and IREM-3/CD300b. At the present, considering that CD300 molecules are able to interact between them, and most probably with receptors from the same family but in other organisms, we think the failure in obtaining IREM-3/CD300b monoclonal antibodies was not only due to the antigenic properties of molecule,

but also to the hybridoma screening process in which RBL-2H3 transfectants were used. In the mean time we find out the best strategy to generate specific monoclonal antibodies recognizing IREM-3/CD300b extracellular domain, we have developed a polyclonal serum recognizing specifically the receptor's cytoplasmatic tail (see appendices for details). This tool is being very useful in the biochemical experiments but unfortunately does not allow us to stimulate leukocyte populations, cell lines and/or transfectants.

Originally, we considered IREM/CD300 family of receptors structurally and functionally related to those in the human leukocyte receptor complex (LCR). This complex, localized in chromosome 19, contains at least 26 genes that include two clusters of immunoglobulin-like transcript (ILT)/leukocyte immunoglobulin-like receptor (LIR)/monocyte-macrophage inhibitory receptor (MIR) loci, a cluster of killer cell inhibitory receptor (KIR) genes, two leukocyte-associated immunoglobulin-like receptor genes (LAIR), as well as the Fc receptor for IgA and the natural cytotoxicity receptor I loci (284). However, the ligand binding properties of IREM/CD300 receptors are distant from these receptors, as none of the members has found to bind MHC class molecules or immunoglobulins. Lately, due to myeloid restricted pattern of expression and sequence identity, IREM/CD300 molecules were considered more closely related to TREM receptors (85). But at the present, considering the particular structural features of some IREM/CD300 receptors, the dual signaling capacities presented by others, and the ability of all of them to form complexes, we think IREM/CD300 family can not be compared with any of the already reported families of immune receptors.

IREM-3/CD300b is a type I transmembrane protein composed by a single V-type Ig domain in the extracellular portion, a transmembrane region and a cytoplasmic tail. Consistent with the structure for a typical activating receptor, IREM-3/CD300b displays a positively charged residue (lysine) embedded in the transmembrane region and short cytoplasmic tail. However, IREM-3/CD300b intracellular region is not devoid of signaling motifs as expected for activating receptors. Indeed, it contains a tyrosine-based motif in the context for Grb-2 recruitment. These particular features made us consider IREM-3/CD300b as a non-classical activating receptor, and direct all the studies to identify the different signaling mechanisms by which the molecule could mediate cell activation.

Despite our data supporting the aggregation of IREM/CD300 receptors in complexes at the cell surface, it is possible to approach them as "conventional" and individual receptors in terms of intracellular and transmembrane signaling. IREM-3/CD300b was described as an activating receptor able to induce triggering signals by means of DAP-12 and Grb-2 recruitment (258). We have demonstrated that the association with DAP-12 takes place at the transmembrane region and relies on the formation of a salt bridge between a lysine in IREM-

3/CD300b and an aspartic acid in DAP-12. Contrary, Grb-2 recruitment occurs by means of a tyrosine-based motif present in IREM-3/CD300b cytoplasmic tail and as consequence, is dependent on phosphorylation events exerted by Src kinases. This capacity to bind different mediators allows IREM-3/CD300b receptor to signal through multiple signaling pathways. We have shown that IREM-3/CD300b stably transfected in RBL-2H3 mediates some signals exclusively through the association to DAP-12, such as the exocytotic response, while others, can be elicited by several pathways although differing in strength. In this line, IREM-3/CD300b is able to induce transcriptional activation both, in the presence and absence of DAP-12 adaptor molecule. IREM-3/CD300b transcriptional activity in the absence of DAP-12 polypeptide is mediated by the receptor's cytoplasmatic tyrosine, but its phosphorylation under these conditions seems to be dependent on the receptor's transmembrane region. We have not detected IREM-3/CD300b association with DAP-10 or FcRγ transmembrane adaptors, which are endogenously expressed by RBL-2H3 cells. Other authors state that IREM-3/CD300b binds DAP-10 molecule additionally to DAP-12 (259). In our opinion this is unlikely as per many reasons. First, if IREM-3/CD300b transcriptional activity in the absence of DAP-12 was dependent on DAP-10 it should not be taking place directly, as DAP-10 is a co-stimulatory molecule rarely signaling without preceding events led by other triggering receptors. And second, if DAP-10 was the molecule involved in enhancing IREM-3/CD300b phosphorylation in absence of DAP-12, when knocking down DAP-10 expression, the receptor's signaling should be abolished and this did not occurred (259). It is remarkable that even though transmembrane adaptor proteins form dimers, the capacity of heterodimerization has never been observed (86). Thus the effects observed for IREM-3/CD300b are not consequence of signaling through DAP10/DAP12 heterodimers.

In the absence of transmembrane and/or extracellular associations, IREM-3/CD300b receptor still keeps signaling properties. The receptor is capable to mediate transcriptional activation in Jurkat cells which do not express endogenously IREM/CD300 family members and neither DAP-12, DAP-10 or FcRγ transmembrane adaptors. Concordantly, substitution of the tyrosine in IREM-3/CD300b cytoplasmic Grb2-recruiting motif led to complete abrogation of the receptor's signaling (see appendices for details).

All together, these results suggest IREM-3/CD300b receptor could be functioning in at least three multiple ways: on its own by means of its tyrosine-based motif; associated to DAP-12 adaptor molecule without using its tyrosine-based motif; and associated to DAP-12 adaptor molecule and using simultaneously the tyrosine-based motif. Although we have not find a read out fitting the last scenario, we know it is likely to occur because immunoprecipitating Grb-2 molecule we have been able to recover IREM-3/CD300b

associated with DAP-12 adaptor (unpublished data). The association to different signaling molecules may vary within the cell type but also within the cell location, reflecting different physiological requirements. For instance, MAIR-II (murine CD300d) binds both DAP-12 and FcRγ in bone marrow-derived and peritoneal macrophages, but in splenic macrophages, the same receptor binds only DAP-12 adaptor molecule (261). Physiologically, peritoneal macrophages require faster activation that those on the spleen, thus it is reasonable that under these conditions a receptor that is suited to bind to two different adaptor proteins couples both of them to ensure an immediate threshold of activation. It is conceivable that IREM-3/CD300b could function similarly to provide rapid and strong activating signals by engaging simultaneously Grb-2 and DAP-12 pathways.

Nevertheless, IREM-3/CD300b signaling increases in complexity considering that it is able to establish lateral interactions with IREM/CD300 family members. We have focused our studies in the signaling exerted by the CMRF-35/IREM-3 complex in RBL-2H3 cells. According to our experiments, the complex formation enhances CMRF-35/CD300c transcriptional activity by means of IREM-3/CD300b. We think this occurs as consequence of FcRγ recruitment by CMRF-35/CD300c which leads a subsequent increase in the tyrosine phosphorylation state of IREM-3/CD300b cytoplasmic tail. The interaction between a transmembrane negatively charged receptor and FcRγ polypeptide has been reported in the literature. CLM-5, also known as MAIR-IV and LMIR-4, is the murine structural ortholog of CMRF-35/CD300c. This molecule has been shown to bind FcRγ in transfected cell lines but also in purified blood neutrophils (266, 267). Unfortunately, the interaction between them has not been mapped. As mentioned in the manuscript, the interaction of FcRγ with CMRF-35/CD300c, if occurring, might take place in a different way than the one with the classical activating receptors, because although being direct, it can not be detected using equivalent experimental conditions. We have been able to visualize the interaction in transiently transfected COS-7 cells by using detergents that preserve weaker interactions (unpublished data). We are working in the mapping of the interaction, to see if it correlates with the functional properties exhibited by CMRF-35/CD300c receptor in RBL-2H3 cells in which the transmembrane negative charge was found to be essential. Some authors sustain that FcRγ adaptor molecule contains a positively charged residue (arginine) distally in the transmembrane region (285). It is worth mentioning that the inclusion of this residue in the transmembrane domain varies depending on the prediction server used and most often, the arginine is not considered to be contained in this domain. As we can not obviate this fact, we will check whether the interaction between CMRF-35/CD300c and FcRγ occurs through these oppositely charged residues. As these amino acids are placed more distantly than usual, the

interaction between both molecules would be sterically more difficult and as a result harder to maintain, what would explain the opposed results obtained with different detergents. Independently of the motifs involved in the interaction, FcRγ seems to play a role in CMRF-35/CD300c signaling, as silencing of the adaptor protein impaired the transcriptional activity mediated by the receptor (unpublished data). IREM-3/CMRF-35 complex formation induces a positive signal higher than the one induced by the individual receptors but, whether this occurs when using a different combination of IREM/CD300 activating receptors is unknown. In the future it will be necessary to carry out functional experiments combining different IREM/CD300 inhibitory receptors but also activating with inhibitory receptors.

Although IREM-3/CD300b has a 22.7 kDa predicted molecular mass, the protein appeared as a discrete pattern of three bands ranging from 26 to 32kDa. This data suggested the existence of posttranslational modifications affecting the receptor's primary sequence. Glycosylation is a common modification in cell surface molecules. It has been shown to affect protein folding, localization, trafficking, antigenicity and ligand binding properties (286). IREM-3/CD300b, oppositely to the rest of IREM/CD300 receptors, does not possess asparagine residues in an N-glycosylation context (Asn-X-Ser/Thr, being X any amino acid except proline). Thus it could be possible that the different bands observed for IREM-3/CD300b corresponded to O-glycosylated forms of the molecule. Although there are not specific acceptor motifs for O-glycosylation, it is well known that this modification mainly occurs in serine and threonine residues localized in proline rich areas. According to this, IREM-3/CD300b membrane-proximal region contains many serine and threonine residues suitable for O-glycosylation. The treatment of cell lysates from IREM3-HA transfected COS-7 cells with a cocktail of enzymes containing a wide range of O-glycosydases (sialidase, β-1,4 galactosidase, β-acetil N-glucosaminidase and hexosaminidase) did not modified IREM-3/CD300b electrophoretic pattern (data not shown). O-glycans are built up in a stepwise-fashion by addition of sugars one at a time to a growing chain. The diversity of glycotransferases is as big as the number of O-sugars with different structure. Thus, it is possible that the glycosydases used in our experiments, despite being the more common, were not the suitable ones for removing the putative O-sugars attached to IREM-3/CD300b polypeptidic chain. We have not conducted more experiments in this direction because we think IREM-3/CD300b bears modifications other than O-glycosylation. Our western blots for IREM-3/CD300b show a pattern of bands that is not consistent with a step-wise process like O-glycosylation. When solubilizing the cell content we are not only recovering the final O-glycosylated form of a particular protein but also the intermediate species. This fact is reflected in the electrophoretic pattern and as consequence, O-glycosylated proteins are often

visualized as smears rather than as a collection of sharp individual bands that is what we observe for IREM-3/CD300b.

Other types of glycosylation can occur in the extracellular portion of cell surface proteins. This is the case for C-mannosylation that consists in the addition of α-manopyranosyl groups to thryptophan residues in a Trp-X-X-Trp/Cys context (287). IREM-3/CD300b bears one of these motifs within its immunoglobulin domain. However, deletion of this region resulted in a global reduction of the molecular weight of the protein but not in the disappearance of any of the three bands.

Intracellular modifications were also considered. IREM-3/CD300b presents intracellular serine and threonine residues that could be target sites for serine/threonine kinases, but both alkaline phosphatase treatment and removal of the cytoplasmic tail of the receptor failed in modifying the observed pattern of bands (data not shown).

The combinations of all these studies have allowed us restrict the area in which these modifications take place: the membrane-proximal region. Nevertheless, we believe that modifications with low impact in the final molecular weight of the protein occur simultaneously within the Ig-fold. We are very interested in identifying these modifications because of its putative role in both, the aggregation of CD300 molecules and the interaction of CD300 immune receptors with their physiological ligands.

Our data strongly support the idea that the IREM/CD300 Ig domains are able to establish two different kinds of interactions: first, a trans-interaction with their ligands, and second, a cis-interaction with other CD300 molecules. The nature of IREM/CD300 ligands, including IREM-3/CD300b, remains to be determined. However, multiple predictions have been made regarding this issue. IRp60 ligand has been hypothesized to be present in bone marrow as this receptor acts an important regulator of mast cell differentiation (249). One of the ligands for CLM-1, the murine ortholog of IREM-1/CD300f, is thought to be expressed by the Raw264 myeloid cell line as a transmembrane protein or a soluble factor, as the receptor is able to inhibit osteoclast differentiation independently of the receptor crosslinking (25). A different group has stated that this ligand, or a different one, could also be present in T cells as per the staining observed in $CD3^{+}$ cells (predominantly $CD4^{+}$) with a fusion protein containing CLM-1 extracellular domain. Moreover, the use of this fusion protein efficiently blocked the interaction between dendritic cells (where CLM-1 is endogenously expressed) and T cells, affecting the proliferative capacity of the later. Same effects where observed when silencing CLM-1 expression in dendritic cells, ruling out an antagonistic effect of the fusion protein on T cells (262). In the laboratory we have detected some binding of IREM-1/CD300f

fusion protein to lymphoid tumor cell lines such as the Jurkat or HUT-78, what supports this observation (unpublished data).

In order to approach IREM-3/CD300b ligand identification, we have generated an IREM3-mIgG2a fusion protein to screen by immunofluorescence several hematopoietic cell lines. We have detected a strong binding of the chimera to monocytes from freshly isolated PBMC (data not shown). In the next months we will work out in the identification of the molecule(s) to which IREM-3/CD300b is binding by means of expression cloning techniques. We have already generated a cDNA expression library in pCDNA3 vector from monocytes staining positive for IREM-3/CD300b fusion protein. The identification of IREM-3/CD300b ligand(s) would be crucial in the elucidation of the physiological relevance of this molecule in the regulation of myeloid cell biology.

As IREM/CD300 receptors have been found to heterodimerize/heteromultimerize on the cell surface, it would be necessary to address whether the ligands for individual receptors are able to act on complexes or if receptor clustering promotes the formation of new recognition surfaces and as consequence, binding of alternative ligands. If occurring the first scenario, it is likely that the ligand affinities vary depending on the complex receptor composition. This could have important functional implications, as partial or incomplete receptor crosslinking has been shown to invert the signaling nature of certain receptors. This is the mechanism by which ITAM-associated activating immune receptors are thought to promote inhibitory signals (89, 96).

The most unexpected results in our work are related with the capability of IREM/CD300 receptors to form heterocomplexes. Our first hypothesis when detecting IREM-3/CD300b binding to CMRF-35/CD300c was that both molecules where able to interact through the positive and negative charges present within their transmembrane domains. In this scenario, these molecules could only interact laterally to form cis-interacting heterodimers. But once discarded, because of the interaction of both molecules through the immunoglobulin domain, the coprecipitation of receptors could be explained by either cis or trans-interactions. To test the possibility that IREM-3/CD300b and CMRF-35/CD300c bound each other head-to-head as a receptor-ligand pair we analyzed the binding of soluble IREM3-mIgG2a fusion protein to CHO-K1 cells stably transfected with CMRF-35/CD300c. No staining was detected in this experiment, indicating that most probably the CD300 molecules form functional complexes on the surface of myeloid cells (data not shown). Moreover, this observation is supported by the functional assays conducted in the CMRF-35/IREM-3 double RBL-2H3 stable transfectant in which activation was dependent on receptor crosslinking. In any case, further

experiments are being currently performed to demonstrate without any doubt the presence of the described complexes on the cell surface.

There are several examples in the literature describing lateral associations between immunoglobulin-containing molecules. Some of these interactions have been mapped and are known to occur by means of disulfide bridges in the proximal transmembrane regions. This is the case for homodimeric structures such as CD28 and CTLA-4 and heterodimeric complexes including the α and β chains of the CD8 coreceptor and the Igα/Igβ molecules from the B-cell receptor (8, 149, 288, 289). Disulfide bridge interactions have shown to be assisted by carbohydrates or loop contacts. For instance, one of the immunoglobulin domains of the TcRα subunit displays sugar chains establishing several hydrogen bonds with the β subunit to enhance the binding between both molecules (290). At the same time, some loop regions within the Ig-fold of these α and β chains have been suggested to make important contacts with CD3$\varepsilon\gamma$ and $\varepsilon\delta$ subunits (87). However, in other cases such as in the CD4 homodimerization, the integrins assembly or the MHC class I and II complex formation, the interactions have not been elucidated in detail (8, 291-293).

The ability of IREM/CD300 receptors to associate through the immunoglobulin domains could be interpreted as a retained feature from the polymeric immunoglobulin receptors (PIgR) which mediate the transepithelial IgA/IgM trafficking. IREM/CD300 receptors were originally related to PIgR based on sequence similarity (240). These receptors are composed by five tandem V-type Ig-like domains but only the more distal one, V_1, is highly homologous to the IREM/CD300 extracellular domain. PIgR binding to polymeric Ig occurs through a non-covalent bond between the V_1 and C_H2 immunoglobulin domains, respectively (294). The region comprised between residues 15 and 37 on V_1 domain was found to be responsible for the binding, but this region, mapping in the b and c β-strands of the Ig-fold, is highly constant between IREM/CD300 receptors and TREM-1, which was devoid of the associating capability. This made us to focus in a different region that additionally is much more attractive due to its putative implication in ligand recognition. IREM-1/CD300f crystallization identified a protruding body from the main immunoglobulin β-sandwich which defines a groove with hydrophobic features (295, 296). This structure was also observed in NKp44, TREM-1 (human and mouse), CLM-1 and PIgRs crystals, but the amino acidic composition of the cavity is markedly different between IREM/CD300 family members and these other closely related receptors (234, 297-299). The most variable residues are located in the loop limiting the cavity from the bottom, and in the F-strand, that although forming part of the main body has the lateral chains of its amino acids exposed to the groove. Future experiments would determine the relevance of these residues in the establishment of

IREM/CD300 complex formation. It would also be important to identify residues within this area susceptible of post-translational modification. An IRp60/CD300a three-dimensional structure has been recently obtained by SAXS (Small-angle X-rays scattering). Although this technique is not as resolutive as crystallization, it provides reliable structural information of molecules in solution and under physiological conditions. The group performing these experiments used an IRp60/CD300a immunoglobulin domain produced in bacteria which was found to be monomeric in gel filtration assays (300). Considering that the only difference between procariotic and eucariotic cells, in terms of protein production, is the way in which they introduce and edit posttranslational modifications, then it is likely that IREM/CD300 complex formation as discussed previously could also be conditioned by protein modification (301).

There are multiple implications regarding the presence and formation of IREM/CD300 complexes on the surface of myeloid cells. Our data indicates that all the members within the family are competent to form heterocomplexes and that all combinations are possible. But this does not mean that all heterocomplexes have to be present on the cell surface of these cells. To start with, there are some combinations that will never take place due to the differential myeloid expression pattern affecting IREM/CD300 family members. For instance IREM-1/IREM-2 heterocomplexes if occurring, could only be observed in freshly isolated blood monocytes and myeloid dendritic cells. The interactions of IREM-1/CD300f in granulocytes, where it is also expressed, should be with IREM/CD300 members different from IREM-2/CD300e, as this receptor would never be found in those cell types. Moreover, the expression of IREM/CD300 family members can be modulated upon stimulation and/or differentiation processes. Thus, interactions taking place in a particular scenario could be disrupted as consequence of a change in the surrounding environment. But also the other way around, interactions not occurring under certain conditions could be favored upon an environmental change. Continuing with the example of IREM-1/IREM-2 heterocomplexes, it is likely that the interaction between both molecules in monocytes could be easily disrupted upon stimulation with IL-4, as IREM-2/CD300e is drastically downregulated from the cell surface under these conditions.

The presence or absence of IREM/CD300 family members along cell development and maturation is probably controlled at transcriptional level. Transcription of some genes has been shown to be dependent on the availability of cell lineage specific transcription factors (302). Genes that in turn, are able to condition global processes such as monocyte-to-macrophage differentiation or macrophage polarization (303). In this line, CMRF-35/CD300c promoter bears response elements for myeloid-specific transcription factors such as MZF-1 (Myeloid Zinc Finger-1)(233, 244)).

Contrary, rapid changes in the cell surface expression are more consistent with a variation in the rate of receptor uptake from the membrane or translocation from preformed granules. For instance, IRp60/CD300a is rapidly upregulated on human neutrophils in response to pro-inflammatory agents and it occurs in a protein synthesis-independent manner (245). A similar effect was observed for MAIR-II (murine CD300d), which increased its surface expression both in macrophages and splenic B cells upon LPS exposure (254, 261).

It is noteworthy that the upregulation and downregulation of adaptor and signaling mediators can also modulate the receptors/complexes signaling properties. For instance, LPS-stimulation leads an increase in the expression of MAIR-II but also upregulates FcRγ. In certain types of macrophages MAIR-II is able to bind both DAP-12 and FcRγ adaptor molecules (261). But clearly, LPS-exposure of these cells would misbalance MAIR-II signaling towards FcRγ-mediated pathways.

Another issue to be considered is that although two or more receptors are expressed in the same cell and its expression covaries in the same way, they may not be forming complexes because of being restricted to different membrane microdomains. The plasma membrane is heterogeneous and is organized into lipid raft and non–lipid raft domains. The firsts are enriched in lipids such as cholesterol and sphingomyelin that contribute to the formation of rigid and organized structures fusing upon certain stimuli. By providing an additional level of compartmentalization, lipid rafts have been proposed to control many signaling and trafficking events (304, 305). In fact, several proteins involved in the earliest steps of cell activation are polarized in terms of membrane association. Src family kinases have high affinity for lipid rafts domains, while CD45, SHP-1 and SHP-2 phosphatases are often found in less organized lipid regions (306). This lead to think that lipid rafts orchestrate cell signaling by separating positive signaling molecules (kinases) from negative signaling molecules (phosphatases) on the plasma membrane. Compartmentalization of the immune receptors to which these signaling mediators bind also occurs. In this line, IREM/CD300 receptors could be restricted to different compartments for preventing their association. Alternatively, they could be targeted to the same lipid compartments allowing complex formation either constitutively or under conditions promoting fusion of equivalent membrane microdomains.

As the signaling of all IREM/CD300 receptors is dependent in some extent on tyrosine phosphorylation, it would be necessary to address whether when forming complexes processivity occurs. For instance, it has been described that upon ligand engagement, CD19 Y513 is the first residue in the cytoplasmic tail of the molecule to become phosphorylated by the Lyn kinase, despite the receptor displays other tyrosines in an equivalent context. But only

Y513 allows Lyn recruitment by means of its SH2 domain and subsequent phosphorylation of Y482, which is essential for the kinase transphosphorylation, autophosphorylation and signal amplification process (307). Thus, when interacting two or more different IREM/CD300 activating receptors it would be necessary to establish the order in which the intracellular signaling events take place, but also to address whether the order determines the final strength of the output.

To sum up, this thesis has contributed to the knowledge of IREM/CD300 family of immune receptors by characterizing one of its members, IREM-3/CD300b. But more importantly, it has led to the identification of what could constitute a new mechanism for finely balance and control the immune signaling in myeloid cells by means of receptor complex formation.

CONCLUSIONS

1. IREM-3/CD300b is a novel activating immunoglobulin like receptor belonging to the IREM/CD300 family. Structurally, IREM-3/CD300b is composed by a single V-type Ig domain, a transmembrane region bearing a positively charged lysine and a cytoplasmic tail with a tyrosine-based motif in a YxN context.

2. IREM-3/CD300b expression at the mRNA level is restricted to the myeloid compartment. Its putative ligand, although not yet identified, is expressed in the same compartment.

3. IREM-3/CD300b associates with Grb-2, DAP-12 and IREM/CD300 family members by means of its different domains. Grb-2 adaptor molecule is recruited by IREM-3/CD300b cytoplasmic tyrosine-based motif, DAP-12 polypeptide association depends on the transmembrane charged lysine residue and complex formation with IREM/CD300 receptors relies on the lateral interaction of immunoglobulin domains.

4. IREM-3/CD300b engagement promotes cell activation through alternative signaling pathways depending on the availability of these signal transduction partners. One of them is mediated by the association with the ITAM-bearing adaptor DAP-12. A second and alternative pathway is triggered by the recruitment of Grb-2 and is dependent on an unidentified molecule interacting with IREM-3/CD300b through the positive charge within the transmembrane region.

5. IREM/CD300 receptors signaling is modulated by the establishment of cell surface complexes with themselves. The establishment these complexes is likely to constitute a new mechanism for finely balance myeloid cell function.

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