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CLONING AND CHARACTERIZATION OF A NOVEL INHIBITORY RECEPTOR EXPRESSED BY MYELOID CELLS

PhD Thesis

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A Huguis

“The truth is rarely pure, and never simple”

Oscar Wilde in “The impostance of being Ernest”

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Abbreviations

BM	Bone marrow
CD	Cluster of differentiation
CLM	CMRF35-like molecule
DAP10	DNAX adaptor protein of 10 kDa
DAP12	DNAX adaptor protein of 12 kDa
DC	Dendritic Cells
FcR	Fc Receptor
GM-CSF	Granulocyte/Macrophage colony stimulating factor
Grb-2	Growth factor receptor bound 2
IFN	Interferon
Ig-SF	Immunoglobulin-like superfamily
ILT	Immunoglobulin-like Transcripts
IL	Interleukin
iMDDC	immature monocyte-derived dendritic cell
mMDDC	mature monocyte-derived dendritic cell
KIR	Killer Immunoglobuline-like Receptor
MAb	Monoclonal antibody
MCSF	Macrophage colony stimulating factor
MHC	Major Histocompatibility Complex
NK	Natural Killer cell
PBMCs	Peripheral blood mononuclear cells
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-kinase
SAP	SLAM-associated protein

SHIP	*Src* homology SH2 domain-containing inositol phosphatase
SH2	*Src* homology domain 2
SHP	*Src* homology domain 2 phosphatase
SIGLEC	Sialic acid Immunoglobuline Lectin
SIRP	Signal Regulatory Protein
TNF	Tumor Necrosis Factor

I. INTRODUCTION

INTRODUCTION

I.I. Leucocyte receptors

A proper immune response involves the balanced participation of several cell types including T and B lymphocytes, NK cells, and myeloid cells such as macrophages, dendritic and mast cells. Immune cells are stimulated due to interactions between a variety of immunoreceptors and their ligands. When faced to infection, the immune system relies on a complex input of signals from its various receptors to direct an appropriate response (1). After productive interaction with their ligands, some of these receptors work by recruiting cytoplasmic tyrosine kinases, which trigger phosphorylation signals that lead to cell activation. On the contrary, other immunoreceptors negatively regulate, through complex and highly controlled mechanisms, the activating signals restricting the duration and/or intensity of cell activation. It is of note that the same type of leucocytes can simultaneously express different activating and/or inhibitory receptors, providing to the cell alternative/complementary mechanisms of control (2). Such mechanisms are particularly important for cells involved in innate immune responses. Furthermore, even when activating signals prevail, inhibitory receptors are still required for preventing excessive activation of immune cells and facilitate the termination of immune responses (3).

Genetic evidence indicates that the inhibitory processes are crucial for a normal immune response and homeostasis. Alterations in the components of the inhibitory machinery can result in autoimmune diseases, inflammatory disorders or lymphoid malignancies (2). Different immunoreceptors are summarized in Table 1.

Table 1. Immunoreceptors from innate immune cells			
Receptors	**Distribution**	**Ligands**	**Family**
Activating and Inhibitory receptors			
FcγR	Monocytes, macrophages, basophils, mastocytes, B cells and NK cells	IgG	Ig-SF
ILT/LILR	Myeloid cells, NK cells, B and T lymphocytes	MHC class I	Ig-SF
CMRF-35	Myeloid cells, B and NK cells, subpopulations of T lymphocytes	unknown	Ig-SF
KIRs	NK cells and T cells subpopulations	MHC class I	Ig-SF
SIRPs	Macrophages, dendritic cells and non immune cells	CD47	Ig-SF
CD94/NKG2	NK cells and T cells subpopulations	HLA-E	Lectin like
TREMs	Myeloid cells and platelets	Anionic ligands	Ig-SF
Inhibitory receptors			
LAIR1	Lymphocytes and myeloid cells	unknown	Ig-SF
SIGLECs	Leucocytes	sialic acid	Ig-SF
CD66a/CEACAM1	Neutrophils, activated T and NK cells, epithelial and endothelial cells	CD66, galectin-3	Ig-SF
KLRG1	Mastocytes, basophils, T and NK lymphocytes	unknown	lectin
Activating receptors			
NKGD2	Myeloid cells, NK cells, subpopulations of T lymphocytes	MICA/B	lectin
NCRs	NK cells	unknown	Ig-SF
MDL-1	Monocytes, macrophages	unknown	lectin

I.II. Inhibitory and activating receptors in innate immunity

A first line of defense is provided by the coordinated action of phagocytes and NK cells that act controlling pathogen proliferation and invasion until the adaptive response of specific T and B cells is developed (4). Indeed, a rapid response is generated due to the fact that phagocytes and NK cells that do not require to undergo clonal expansion can enter and defend the tissue almost as soon as it becomes infected. These effector cells can eliminate pathogens through several mechanisms ranging from phagocytosis and secretion of cytokines to further recruitment of other cells. The different reactions can even lead to elimination of the infection without participation of an adaptive response and without manifestation of disease (5).

Innate immune cell function is regulated by an array of surface receptors with either activating or inhibitory capacity (6). In the case of myeloid cells, including macrophages, the extracellular portion of surface receptors mediates the interaction with natural and/or altered self components of the host as well as with a range of microorganisms. Surface immunoreceptors participate in the regulation of cell differentiation, growth and survival, adhesion, migration, phagocytosis and cytotoxicity through the recognition of a wide range of endogenous and exogenous ligands (7).

I.II.1. Activating receptors

Cell activation within the immune system occurs through multisubunit surface receptors expressed often associated with transmembrane adaptor proteins. Most triggering receptors bear a charged amino acid residue in their transmembrane domain. An electrostatic interaction occurs between the positively charged amino acid of the

receptor with a negatively charged residue in the adaptor protein (8). Adaptors are able to mediate signal transduction through one or more motifs present in their cytoplasmic tails called ITAMs from **I**mmunoreceptor **T**yrosine/based **A**ctivating **M**otif, that have a consensus sequence that consists of YxxL/x_7/YxxL (where x denotes any amino acid). In this context, engagement of activating receptors lacking canonical cytoplasmic signaling motifs, results in a rapid protein tyrosine phosphorylation that is essential for receptor function (9-12). ITAMs were first identified in the immune system, where they are required for the expression and signaling of several activating immunoreceptors (13, 14). Polypeptides bearing cytoplasmic ITAMs domains constitute a family of signaling molecules, including DAP12 (DNAX activating protein of 12 kDa) (15, 16), FcRγ (17), and TCRζ (18).

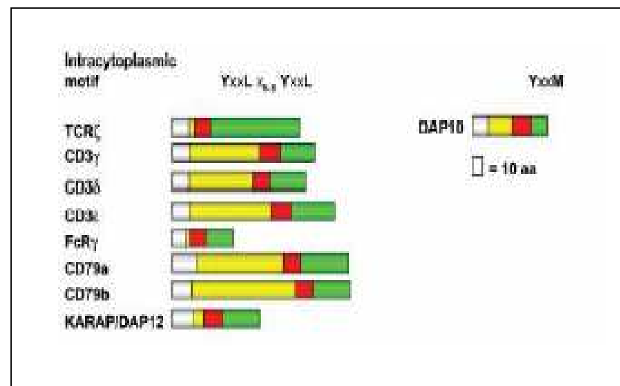

Figure 1. Family of ITAM-bearing adaptor molecules and DAP10. White regions correspond to the leader peptides, yellow regions to the extracellular domains, red regions to the transmembrane domains and green regions to the cytoplasmic domains. Cytoplasmic ITAM sequences (top left), which allow PTK recruitment, or the YxxM sequence (top right), recruiting phosphatidyl inositol 3 kinase (PI3K). Taken from Tomasello et al.(14).

After engagement of the triggering receptors associated to ITAM-bearing adaptors, ITAMs become phosphorylated by Src family kinases, and this allows the recruitment of the tandem Src homology domain 2 (SH2) containing protein tyrosines kinases Syk and ZAP70 (12, 19, 20). ZAP70 is restricted to T cells and NK cells, whereas Syk is present in B cells, mast cells, myeloid cells and platelets, as well as a subset of γδ T cells and immature thymocytes. Syk and ZAP 70 are regulated by tyrosine phosphorylation. When activated, Syk family kinases phosphorylate downstream mediators including adaptors such as LAT, SLP-76 and Blnk. They also cooperate with Src family kinases to phosphorylate PLCγ and Vav. Finally, members of the Btk family of kinases, i.e. Btk, Itk and Tec are also involved in the activating ITAMs mediated signaling (2). Some activating receptors can associate with another adaptor protein termed DAP10 (DNAX activating protein of 10 kDa) (21). It displays an YxxM motif instead of an ITAM, allowing recruitment and activation of the p85 regulatory subunit of PI3 kinase.

The term phosphoinositide 3-kinase (PI3K), refers to a family of enzymes that phosphorylate D-*myo*-phophatidylinositol (PtdIns) or its derivative in the 3-hydroxil inositol group. The 3-phosphoinositides act as membrane targeting signals to recruit selected proteins, and regulate functions as vesicle trafficking, cytoskeletal reorganization and signal transduction. In nearly all cases studied, modification of PI3K function or its abrogation leads to a profound effect on cellular responses (22). PI3K are categorized as class I, II or III depending on the subunit structure, regulation and substrate selectivity (23-25). Class I PI3Ks enzymes are expressed mostly by cells of the immune system (26), and are the only ones that transform PtdIns $(4,5)P_2$ in PIP_3, a crucial molecule that acts as a second messenger. Class I PI3K are heterodimers composed by a catalytic subunit called p110 because of its molecular weight, and a

regulatory subunit called p85. The class IA enzyme can be composed of one of the three isoforms of p110 (p110α, p110β and p110γ) encoded by different genes. In the case of class IB isoform it is composed by the p110γ catalytic subunit and the regulatory p101. There are also 5 regulatory subunits (p85α, p85β, p55α, p55γ, p50α), encoded by three genes, that as alternative splicing generate p85α, p55α and p50α proteins. Most immune receptors activate class IA and/or class IB PI3Ks, leading to the production of PIP_3.

The adaptor/regulatory subunit p85 is a modular protein with several functional motifs. It has two SH2 domains, which mediate binding to phosphorylated tyrosines that are in the context of YxxM sequences. p85 also has an SH3 domain and a BCR homology domain (BH) that is related to a GTP-activating (GAP) domain. This module binds to GTP-bound forms of RAC and CDC42. In this way, the subunit might generate signals independently of the catalytic p110 subunit, and act as an adaptor instead of a regulatory subunit (27).

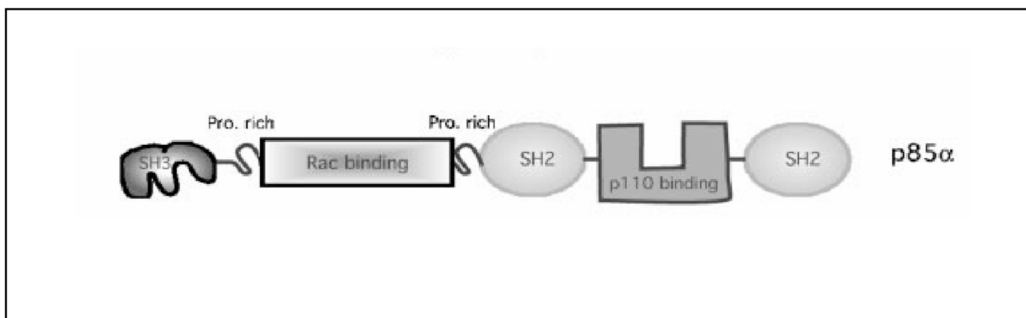

Figure 2. Structure of the p85α regulatory subunit of PI3 kinase. Taken from Deane et. al. (22)

For class IA PI3Ks, the best understood activation mechanism involves the binding of two SH2 domains to pYxxM. This interaction is triggered by receptors that

have either intrinsic or associated tyrosine kinase activity, bringing PI3K to membrane-associated complexes and allowing interactions leading to further activation. For example GTP-bound Ras binds to class IA catalytic subunits only after activation of PI3K in the context of a pYxxM-SH2 interaction (28).

I.II.2. Inhibitory receptors

Inhibitory receptors interacting with their cognate ligands deliver continuous "off" signals to cells of the innate immune system that warrant tolerance to self. The common feature that identifies inhibitory receptors is the ability to negatively modulate signals initiated by other cell surface receptors. The inhibitory receptor molecular hallmark is the presence in its cytoplasmic region of tyrosine-based motifs called ITIMs from **I**mmunoreceptor **T**yrosine/based **I**nhibitory **M**otif, whose consensus sequence is V/IxYxxL/V (29, 30). The inhibitory function is mediated only upon clustering with activating receptors on the cell surface. Actually, the ITIM motif was first elucidated in the Fcγ RIII receptor that after cross-linking with the BCR inhibits the BCR-evoked induction of intracellular calcium mobilization and cell activation [Thomas, 1995 # 1171]. In some cases, the activating and inhibitory receptors recognize different ligands, whereas in others, closely related pairs of inhibitory and activating molecules (with similar extracellular domains) can bind the same ligand (1). After engagement of inhibitory receptors by the ligand, ITIMs tyrosine residues, become phosphorylated usually by a Src family kinase and the resulting phosphotyrosine residues constitute docking sites for SH2 (*src* homolgy type II) domain containing cytoplasmic phosphatases such as SHP-1, SHP-2 or SHIP (31, 32).

Once recruited, tyrosine phosphatases become activated and dephosphorylate key signaling mediators and as a consequence down regulate signaling cascades. This

inhibition occurs at the earliest steps of the activating response, shutting down all subsequent events, including calcium mobilization, cytokine release, transcriptional activation and cellular proliferation (1). SHP-1 and SHP-2 are cytoplasmic phosphotyrosine phosphatases. Using different experimental approaches, SHP-1 was shown to act at different levels in the signaling cascade. For instance, it was reported that it can dephosphorylate Src kinases, ITAM-bearing molecules, Syk kinases, adaptors such as SLP-76, and enzymes like Vav and PI3K. Moreover, there is evidence that SHP-1 can also dephosphorylate ITIMs providing a putative mechanism of auto regulation (2, 33-35).

SHIP-1 and SHIP-2 are able to dephosphorylate membrane bound inositol phosphates (PIP_3). In the absence of PIP_3, Pleckstrin Homology (PH) domains-bearing proteins, like Btk or PLCγ are released from the membrane, and a sustained calcium signal is blocked by preventing the influx of extracellular calcium (36) (29).

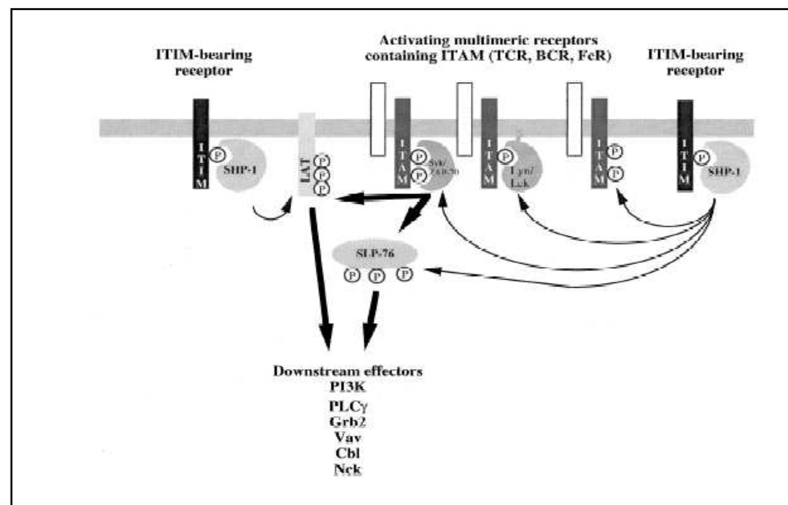

Figure 3. Model of operation for ITIM-bearing receptors. Upon receptor engagement, ITIMs are tyrosine phosphorylated and recruit protein tyrosine phosphatases, (i.e. SHP-1). Substrates of SHP-1 may include a variety of tyrosine phosphorylated proteins, such as ITAM, Src-family tyrosine kinases, Syk family tyrosine kinases, adaptor proteins LAT and SLP-76. Dephosphorylation of these signaling components leads to the early extinction of the activating signaling pathways. Taken from Tomasello et al. (37).

I.III. SHP-1

I.III.1. Structural and biochemical features of SHP-1

The crystal structure of SH2 domain-containing protein tyrosine phosphatases has provided insight into the mechanism by which phosphorylated ITIMs transduce inhibitory signals. SHP-1 is a cytoplasmic protein tyrosine phosphatase containing two

adjacent SH2 domains followed by a phosphatase domain and a carboxy-terminal region that contains two conserved tyrosine phosphorylation sites (38).

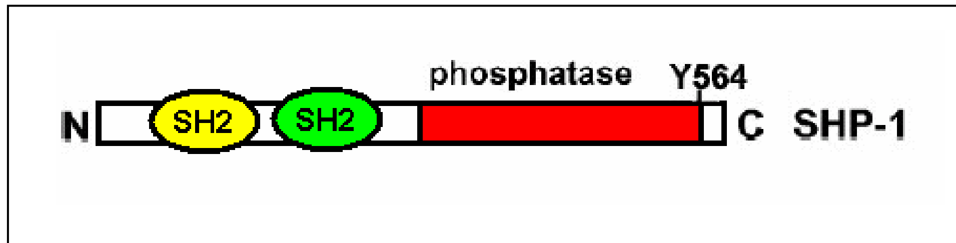

Figure 3. Structure of SHP-1 phosphatase implicated in the negative regulation of immunoreceptor signaling

Even though both SH2 domains of SHP-1 can bind phospho-ITIMs, the more amino terminal SH2 domain likely plays an auto regulatory role. The N-terminal SH2 domain is necessary and sufficient for auto inhibition through an intramolecular association with the phosphatase domain, so truncation of the C-terminal SH2 domain has little effect on the enzyme activity. On the contrary, removal of the N-terminal domain results in strong activation of the enzyme. It has been proposed that the N-terminal SH2 domain acts both as a regulatory and recruitment domain, whereas the C-terminal SH2 has just a role in recruitment (39). It was shown that N terminal SH2 domain (N-SH2) of SHP-1, and also SHP-2 (based on its crystallographic data (40)), mediates basal repression due to direct interaction between the N-SH2 and the catalytic domain. Thus, binding of the enzyme to phosphorylated ITIM peptides via N-SH2 relieves this interaction leading to a 10 fold increase in SHP-1 activity (41).

I.III.2. SHP-1 deficiency

A significant clue regarding the role of SHP-1 in the immune function, was provided by the study of the *motheaten* (*me)* and *viable motheaten* (me^v) mice, in which the SHP-1 gene is mutated (42-44).

The recessive *motheaten* (*me)* and *viable motheaten* (me^v) allelic variants result in the most deleterious abnormalities in hematopoiesis and dysregulation of the immune system that are known to be caused by a single gene mutation. In these mice, *shp-1* gene called *Hcph* from hematopoietic cell phosphatase, displays a point mutation at a splice site that results either in an absence or a severe reduction in SHP-1 activity.

A major immunopathological abnormality observed in *me/me* and me^v/me^v mice is the overgrowth of macrophages and granulocytes in lungs, skin and limbs. This accumulation of macrophages and neutrophils in lungs results in pneumonitis, while inflammation in the limbs is associated with arthritic changes in the joints. Impaired immunological function was evidenced in *me/me* mice by reduced proliferative responses to T- and B-cell mitogens, absence of T cytotoxic responses and severely reduced NK cell function.

Table 2. Immune and hematopoietic abnormalities in motheaten and viable motheaten mice.

Cell population	Phenotype
B cells	Polyclonal B cell activation, CD5 expression, accumulation of plasmacytoid Mott cells.
T cells	Dysfunctional, early thymic involution, increased thymocyte proliferation following T cell receptor stimulation.
Macrophages	Accumulation in lungs and elsewhere
Neutrophils	Accumulation in skin, lungs and blood.
Natural Killer cells	Decreased activity.
Langerhans cells	Marked depletion of epidermal dendritic cells.
Red blood cells	Hemolytic anemia.
Mast cells	Increased numbers of dermal mast cells.
Bone marrow stromal cells	Failure to support hematopoiesis.
Stem cells	Development of disease in bone/marrow chimeras.

In addition to severe immunodeficiency, *me/me* mice develop systemic autoimmunity, which is characterized by polyclonal B cell activation accompanied by hypergammaglobulinemia, production of auto antibodies of widespread inflammatory lesions, including severe pneumonitis. The proximate cause of death of *me/me* mice appears to be pulmonary injury, with accumulation of alveolar macrophages and neutrophils in the lower respiratory tract (45).

In summary, the central role of SHP-1 as a negative regulator of signal transduction is reflected by the pleiotropic effects of the *me/me* and me^v/me^v mutations in mice.

I.III.3. SHP-1 mediated regulation in myeloid cells

Protein tyrosines phosphatases, and particularly SHP-1, are key regulators of myeloid cell function, as demonstrated in the severe inflammation observed in the SHP-1 deficient *motheaten* mice. These mice display, as discussed above, an expansion of the myelomonocytic cell population as wells as an accumulation of alveolar macrophages leading to lethal hemorrhagic interstitial pneumonitis. These data show a central role for SHP-1 in the suppression of signaling cascades that control myeloid cell growth, survival and activation. Myeloid cells express several ITIM-bearing receptors belonging to different families including ILT, SIRPs, TREM or CMRF-35. Engagement of these receptors leads to phosphorylation of ITIMs with subsequent recruitment and activation of SHP-1. SHP-1 substrates after activation include for example FcγRI and Syk in monocytes. After co-engagement of FcγRI and the MHC class I binding receptors ILT-2 or ILT-4, SHP-1 is recruited to ITIMs, dephosphorylating FcγRI and Syk (46).

Moreover, far beyond the ITIMs, SHP-1 has shown to associate with paxillin, vimentin, and filamentous actin in CSF-1 activated macrophages (47) and to interact with p85, dephosphorylating it and, consequently down regulating PI3 kinase activity (48). These data also provide some clues about the involvement of SHP-1 in the regulation of the adhesion properties of myeloid cells. In addition, SHP-1 has been demonstrated to interact in myeloid cells with various signaling mediators such as Grb-2, Cbl, STAT3, STAT5, Shc and Vav, and therefore appears to participate in regulating different signaling pathways (33).

I.IV. Families of immunoreceptors that modulate the function of myeloid cells

Most immunoreceptors belong to multigenic families including both activating and inhibitory members that are organized in clusters in the genome. One feature of these receptor families is that they comprise inhibitory and activating isoforms that are similar in their extracellular regions, whereas the transmembrane and cytoplasmic domains are different and confer diverse signaling properties. Regarding the extracellular region, receptors can be classified into different groups belonging either to the Ig superfamily or to the C-type lectin superfamily (49). We will focus here in the description of the most important receptors expressed in myeloid cells belonging to the Ig superfamily.

I.IV.1. Immunoglobulin-like Superfamily receptors

I.IV.1.1. ILT/MIR/LIR/CD85

This family includes at least ten distinct receptors that are characterized by the presence of either two or four homologous extracellular C-2-type Ig-like domains, including activating and inhibitory molecules. Immunoglobulin-like Transcripts (ILT) genes map on human chromosome 19q13.4 in the region termed Leukocyte Receptor Complex (LCR). Apart from ILTs, this region encodes for several families of related Ig superfamily proteins such as KIRs (Killer Immunoglogulin-like Receptors) (5), the Fc receptor for IgA (50), LAIR (Leukocyte Associated Inhibitory Receptor) (51) and NKp46 (52).

Inhibitory ILTs (i.e. ILT2, ILT3, ILT4, ILT5 and LIR8) have between two and four cytoplasmic ITIMs (see figure 4). Upon receptor engagement, ITIMs become phosphorylated, and constitute docking sites for SHP-1 (53).

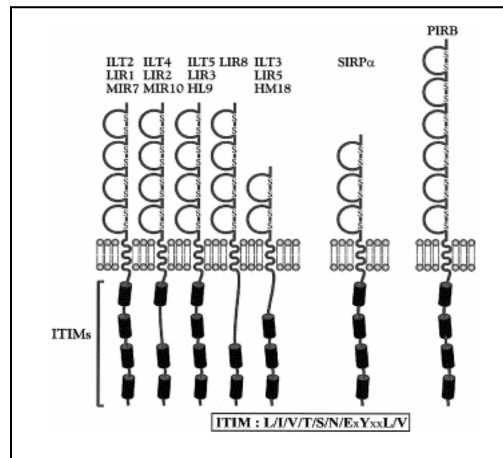

Figure 4. Inhibitory molecules of the ILT/LIR/MIR, SIRP, and PIR receptor families.

Other ILTs (i.e. ILT1, ILT7, ILT8 and LIR6a), display a short cytoplasmic tail with no signaling motifs and a positive charged arginine residue in the transmembrane domain (Figure 5). These receptors associate with the adapter protein FcRγ (gamma chain of Fc receptors) that contains a negatively charged residue in the transmembrane region, and a cytoplasmic ITAM that delivers the positive signal. Interestingly, ILT6 corresponds to a putative soluble receptor lacking transmembrane and cytoplasmic regions.

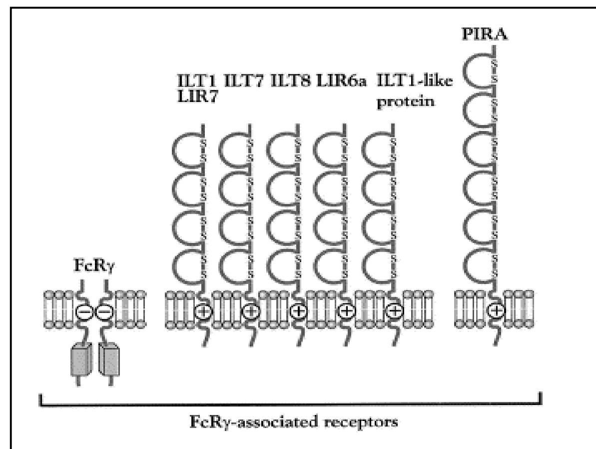

Figure 5. Activating molecules of the ILT/LIR/MIR, SIRP, and PIR receptor families.

Most cells of the innate and adaptive immune systems express at least one member of the ILT family. Even though ILTs are preferentially expressed on the surface of myelomonocytic cells like macrophages and dendritic cells (DC), ILT2 is expressed by all peripheral B cells and subsets of NK cells, while T lymphocytes express ILT1, ILT2, or ILT5 (54). Within the myeloid compartment, peripheral blood monocytes express all ILTs, while granulocytes express only ILT1 and ILT5. Peripheral blood DCs express ILT1 and/or ILT3, and depending on their expression DCs can be subdivided in two subsets (55). ILT1+/ILT3+ cells include both immature CD1a positive and mature DCs, whereas ILT1-/ILT3+ cells correspond to plasmacytoid DCs, that represent a unique cell type specialized in the production of type I interferon (55, 56).

ILT2 and ILT4 broadly bind classical and non-classical MHC class I molecules, preventing activation against normal cells (54, 57, 58). In addition, ILT2 also binds a cytomegalovirus-class I homolog called UL18 (59, 60). The nature of ligands for other members of the ILT family remains to be discovered.

ILT homologues in mice are known as PIR (Paired Immunoglobulin-like Receptors). Among them, PIRB displays cytoplasmic ITIMs, that have been observed to be constitutively phosphorylated, suggesting the interaction with an endogenous ligand, possibly MHC class I (61). PIRB associates to SHP-1 and is able to deliver negative signals. PIRs are expressed by myeloid cells, granulocytes and B cells (7). Deletion of the PIRB gene leads to impaired DC maturation, increased Th2 response, and enhanced graft-versus-host disease. Other PIR, like PIRA associates with Fcγ chain.

I.IV.1.2. Triggering Receptors Expressed by Myeloid Cells (TREMs)

The TREM family of receptors comprises in humans at least an inhibitory and two activating receptors. There is in mice a third receptor of unknown function that in humans corresponds to a pseudogene [Colonna, 2003 # 778]. As shown in figure 6, TREM genes cluster in human chromosome 6p21 and mouse chromosome 17, closely linked to the MHC class II region and to the NKp44 gene in humans. The TREM gene cluster includes a gene encoding the TREM like transcript (TLT) that presents a cytoplasmic ITIM and has been shown to recruit SHP-1 (62).

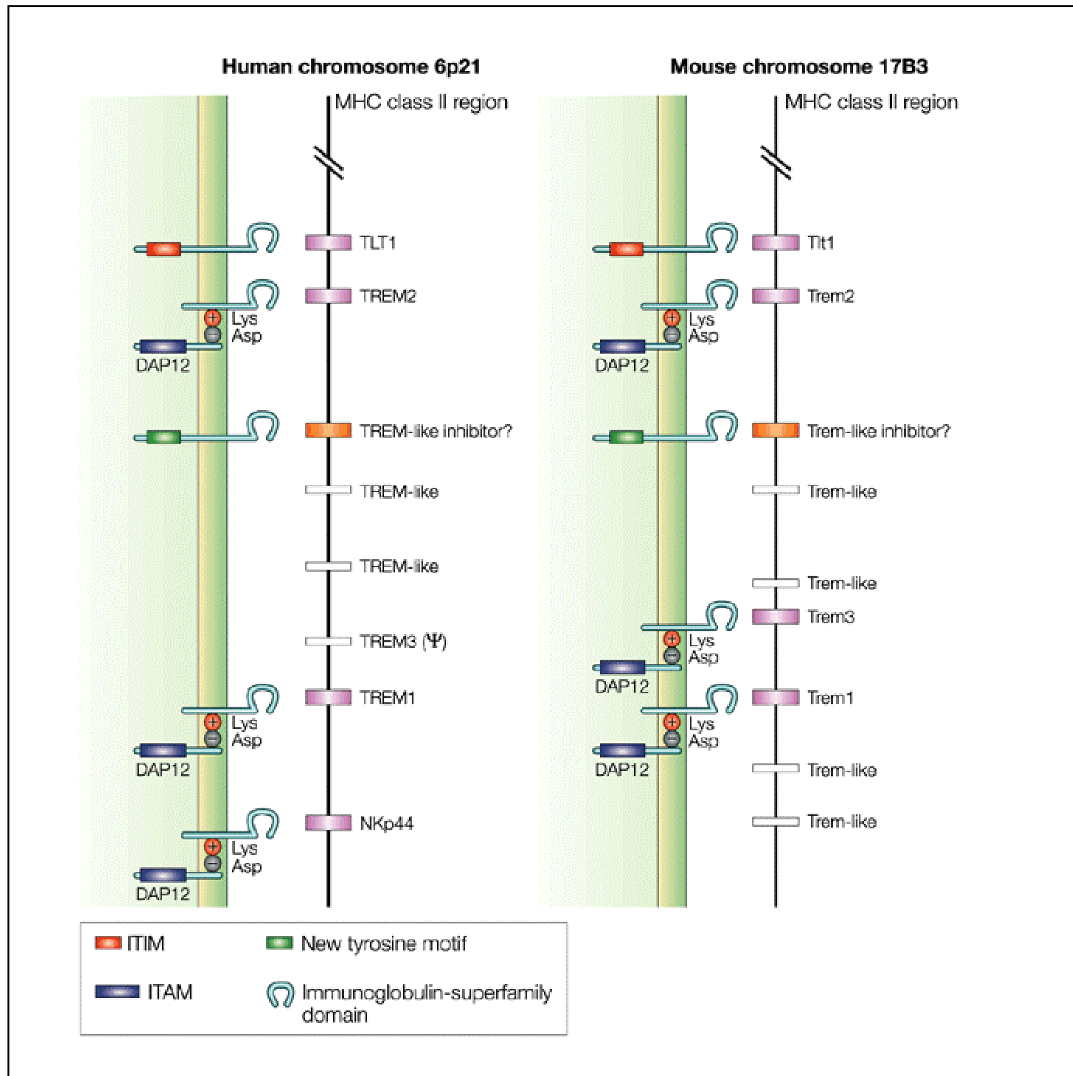

Figure 3. Organization of human and mouse TREM gene cluster. (Taken from Nature Immunology Reviews (4)).

TREM-1 and TREM-2, are homologous to the activating NK cell receptor NKp44. Though more distantly related, the CMRF35 family (63-65) and the receptor for polymeric immunoglobulin (pIgR) display also a significant homology with TREMs but, TREMs, NKp44 or CMRF35 molecules do not bind immunoglobulin.

Human TREM-1 and TREM-2 are glycoproteins that consist of a single V-type Ig-like domain, a transmembrane region with a charged lysine residue and a short cytoplasmic tail. Both receptors associate to the adaptor protein DAP12 for signaling (66, 67). DAP12 ITAMs become phosphorylated after TREMs engagement, providing a docking site for the tyrosine kinases ζ-chain-associated protein 70 (ZAP 70) and spleen tyrosine kinase (Syk). Adaptor complexes that contain c-Cbl and Grb2 (growth factor receptor binding protein 2) are then recruited and phosphorylated by these kinases, which leads to phosphatidylinositol 3 kinase (PI3K), phospholipase Cγ1 and extracellular signal regulated kinase (ERK) activation (68). As a result of these signals, intracellular Ca^{2+} mobilization, rearrangement of the actin cytoskeleton, and activation of transcription of several genes is generated. In mice, both TREM-2 and TREM-3 have been shown to associate to endogenous DAP12 in transfected macrophage cell lines, promoting cellular activation and nitric oxide release (69, 70). A soluble form of TREM-1 (TREM-1sv1) has been described, that could be secreted and modulate TREM-1 mediated activation and signaling.

Expression of TREM receptors is restricted to cells of the myeloid lineage, including microglia and osteoclasts (4). In humans, TREM-1 is expressed by neutrophils and a subset of monocytes/macrophages. In tissues TREM-1 is expressed by alveolar macrophages in the lung. In addition, TREM-1 is highly expressed by infiltrating neutrophils in human skin and lymph nodes infected by bacteria and fungi; TREM-1 expression is also upregulated in human cells after sepsis (71). The expression pattern of TREM-1 strongly suggests a role for this molecule in inflammation. Engagement of this receptor with specific monoclonal antibodies in both granulocytes and monocytes promotes the release of proinflammatory cytokines, including interleukin 8, MCP1, (Monocyte Chemoattractant Protein 1), MCP3 and MIPα

(Macrophage Inflammatory Protein α). In monocytes, crosslinking of TREM-1 induces the production of TNFα and IL-1β, and the effect is enhanced when LPS is used as a co-stimulus, indicating a role in amplification of the Toll-like receptor mediated responses (66). This hypothesis has been evaluated *in vivo* in an animal model of LPS induced septic shock. Blocking TREM-1 by treating mice with a soluble protein containing the TREM-1 extracellular domain fused to a human Ig Fc, reduced animal death (72). TREM-1 stimulates the production of chemokines that recruit lymphocytes, indicating that TREM-1 may constitute a bridge between innate and adaptive immune responses (73). Given that TREM-1 is considered an amplifier of inflammation in response to LPS and bacteria, it constitutes an attractive therapeutic target in inflammatory states like sepsis. In this regard, dynamics of surface and soluble TREM-1 expression during endotoxemia in humans have also been investigated. This study revealed that granulocyte TREM-1 expression was high at baseline but was immediately down-regulated upon LPS exposure along with an increase in soluble TREM-1 (71). Monocytes displayed a gradual up-regulation of TREM-1 upon stimulation with LPS both *in vivo* and *in vitro.* The LPS-induced changes in TREM-1 surface expression were not a result of increased TNF-α or IL-10 production (72). Studies with different inhibitors supported a role for PI3K in LPS-induced up-regulation of TREM-1 on monocytes.

TREM-2, is expressed by dendritic cells (DCs), osteoclasts and microglia. Human TREM-2 is expressed by immature monocyte derived dendritic cells (iMDDC), differentiated *in vitro* from monocytes treated with IL-4 and GM-CSF (67). Engagement of TREM-2 with monoclonal antibodies in DCs has been shown to induce their incomplete maturation, in which the expression of CCR7, MHC class II and the co-stimulatory molecule CD86 are upregulated (67). This partial activation of DC could be

due to triggering of protein tyrosine kinases, ERK and Ca2+ pathways mediated by TREM2 through DAP12 signaling. TREM-2 deficiency causes a disease known as Nasu-Hakola or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy dementia, characterized by neurodegenerative pathology as well as for the presence of multiple bone cysts. The phenotype is very similarto that developed by DAP12 mutation (74), and is consistent with the expression and function of TREM-2 in osteoclast and microglial cells,

TREM-2 has been reported to bind both Gram-positive and Gram-negative bacteria, and this interaction can be blocked by purified anionic bacterial products like LPS or lipoteicoic acid. In addition, TREM-2 is also able to bind cell surface molecules present in human astrocytoma cell lines (75).

I.IV.1.3. Signal Regulatory Proteins (SIRPs)

Another set of receptors expressed in the immune system, especially on myeloid cells, is the SIRP or CD172 family that includes also paired activating and inhibitory molecules (76, 77). The genes of the five identified SIRPs in humans, SIRPα, SIRPβ1, SIRPβ2, SIRPγ, SIRPδ and a pseudogene SIRPβ3p, are clustered in chromosome 20p13. SIRPα, SIRPβ1 and SIRPγ are closer among them and appear to form a subgroup, whereas the other genes are more divergent (78).

SIRPα, SIRPβ1 and SIRPγ are characterized in humans for having an extracellular region with three Ig-like domains, a single V-set followed by two C1-set Ig-like domains. SIRPα contains four cytoplasmic tyrosine residues in an ITIM context, and has been shown to interact with SHP-1 and SHP-2 (79). SIRPβ2 appears to encode a cell surface receptor composed of two extracellular Ig V type domains, and like

SIRPβ1, has a positively charged residue in the transmembrane region, predicting the association with DAP12. SIRPδ encodes a putative soluble molecule with a single V-type Ig-domain, and analysis of ESTs evidence that it could be expressed by sperm cells (78).

SIRPs are predominantly expressed by myeloid cells including macrophages, granulocytes, myeloid dendritic cells, mast cells and precursors including hematopoietic stem cells. Outside the immune system SIRPα is expressed by neurons in the central nervous system. SIRPβ1 is also expressed by monocytes, granulocytes and DCs.

CD47 has been identified as the ligand of the SIRPα and SIRPγ. It is a broadly expressed transmembrane glycoprotein with a single extracellular Ig domain and five transmembrane spanning regions. Binding to the receptor occurs through SIRP NH2-terminal V-like domain. CD47 has also the ability of signaling and thus its interaction with SIRP gives the possibility of a two- way signal transduction (80).

I.IV.1.4. CD200R

CD200R or OX2R is an Ig-like molecule identified as the countereceptor of CD200 to which it is genetically related. CD200R consists of two Ig-like domains and a transmembrane region. It presents three cytoplasmic tyrosine residues that can mediate an inhibitory function (81). CD200R may associate with SH2-containing inositol phosphatase (SHIP), consistent with its apparent role in the downregulation of myeloid cell activity.

CD200R expression is largely restricted to monocyte/macrophage/DCs lineages, whereas its ligand, CD200, has a broad expression pattern that includes thymocytes, B cells, activated T cells, neurons and endothelial cells but not resting macrophages. Thus,

a model has been developed in which CD200 expressed by a variety of cell types regulates the activation and function of myeloid cells, via its interaction with CD200R.

I.IV.1.5. SIGLECs

Siglecs (**S**ialic **A**cid-binding **I**mmunoglobulin **S**uperfamily **lec**tins) are a family of cell surface molecules of the IgSF that are characterized for having an N-terminal V-set Ig domain that mediates binding to sialic acid, and a variable number of C-type Ig domains. Members of the Siglec family are divided in two subgroups comprising the CD33-related Siglecs (Siglec 3, and Siglec-5 to Siglec-11), and the CD22, sialoadhesin (Siglec 1) and myelin associated glycoprotein (MAG, Siglec 4) group. Genes of this subgroup have been mapped to human chromosome 19q13.4, spanning a genomic region of approximately 506.2 kb and is located 43.2 kb telomeric to the adjacent kallikrein gene locus (82).

Siglecs are thought to play an important role as regulators of innate immunity, as they display at least one conserved ITIM and an ITIM-like motif in their cytoplasmic tail, that enables the eight CD33- related Siglecs to bind SHP-1 and/or SHP-2 (83). A wide number of effects have been described regarding the function of these molecules by engaging them with specific monoclonal antibodies, including the induction of apoptosis in eosinophils and neutrophils (84), inhibition of proliferation of acute myeloid leukemia (AML) cells (85, 86), augmentation of neutrophil respiratory burst (87), and release of cytokines from monocytes (88). However, a number of questions remain opened regarding the precise Siglecs mediated signaling. For example, it has been shown that Siglec-5 functions as a inhibitory receptor in the absence of ITIM phosphorylation (89). In the mouse an activating member, Siglec H, has been recently

described. This molecule is present in mouse plasmacytoid dendritic cells, and associates to DAP12 through a lysine residue in its transmembrane region (90). CD22 is a well-characterized inhibitory receptor that regulates B-cell receptor (BCR) signaling. B cells from CD22 deficient mice show enhanced and prolonged Ca^{2+} influx after BCR stimulation in comparison with normal B cells. Knock out cells exhibit an activated phenotype and an enhanced response to LPS (91). On the other hand, sialoadhesin or Siglec-1 lacks cytoplasmatic ITIMs and may have a role in cellular trafficking and host defense functions (83), whereas MAG has been implicated in the process of myelination (92). All CD33-related Siglecs are expressed on cells from the innate immune system, including monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells and dendritic cells (93, 94). Sialoadhesin is expressed by macrophages, CD22 is present in B cells and MAG is expressed by non-immune cells including oligodendrocytes and Schwann cells (92). The main characteristic of Siglecs is their ability to bind molecules containing sialic acid. Sialic acids may represent broadly expressed “self” ligands that interact with inhibitory Siglec preventing self reactivity (95). However, not all receptors have the same specificity. Unlike ape CD33-related siglecs, human CD33-related siglecs bind N-glycolylneuraminic acid (Neu5Gc) as well as its precursor N-acetylneuraminic acid (Neu5Ac). These data favor the hypothesis that CD33-related Siglecs function primarily as self-recognition receptors for endogenous sialylated ligands, that, rather than pathogen-associated sialic acids directly influence the evolution of these receptors. Yet, it is not excluded that they could also be involved in recognizing bacterial pathogens expressing sialic acids, also from the Neu5Ac form (96). In addition, it is also conceivable that Siglecs may interact with endogenous cell surface proteins in a sialic acid independent manner (97, 98).

I.IV.1.6. CMRF35/IREM/CD300 family

A recently characterized family of IgSF is the CMRF-35/IREM/CD300 family (99). The genes of this family are organized in a cluster in the human chromosome region 17q25.1 spanning a region of 250kb (see figure 7) and encode for both inhibitory ITIM-bearing molecules and adaptor-associated activating receptors (65). The CLM gene cluster , the murine ortholog of CD300, has nine members and is located in mouse chromosome 11, which is syntenic to human chromosome 17 (100).

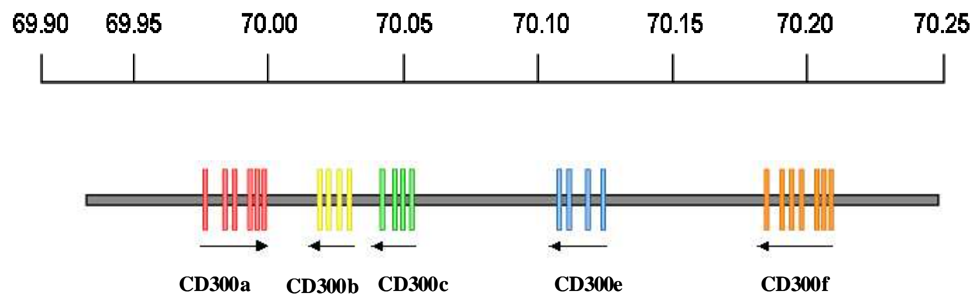

Figure 4. Genomic organization of the CMRF35/CD300/IREM cluster in human chromosome 17q.25

(CD300a) is an inhibitory member of the CMRF35 family that displays two ITIMs that after phosphorylation recruit both SHP-1 and SHP-2. This receptor is able to inhibit NK mediated cytotoxicity (101), mast cell degranulation (102) and eosinophil eotaxin-dependent transmigration in a calcium independent fashion, as well as the antiapoptotic effect of IL-5 and GM-CSF. Additionally crosslinking of IRp60 in

eosinophils, inhibited IL-5-mediated JAK2 phosphorylation, and also IL-5/GM-CSF mediated ERK 1/2 and p38 phosphorylation (103).

By contrast CD300e/IREM-2 is an activating receptor that associates with the adaptor DAP12 on myeloid cells through a positive charged lysine in the transmembrane region. After engagement with specific monoclonal antibodies, IREM-2 is able to stimulate TNFα production by human monocytes, as well as to induce transcriptional activity of an NFAT (nuclear factor of activated T cells) dependent reporter in RBL transfected cells (104). CMRF-35 (CD300c) was the first member of the family to be cloned. It presents a short cytoplasmic tail that does not display any known signaling motif and includes a negatively charged glutamic acid residue in the transmembrane region. It has been hypothesized that this negatively charged residue could allow its electrostatic interaction with other transmembrane molecules (105). Regarding their expression pattern, IREM-2 appears restricted to myeloid cells, whereas IRp60 has a broad distribution in the immune system being present in NK cells, myeloid cells and some T cell subsets. There is no reliable information about CD300c expression, as the available CMRF35 mAb is cross-reactive with other members of the family (i.e.IRp60). The work presented in this thesis, has been devoted to the characterization of another inhibitory member of this family (CD300f or IREM-1).

II. AIMS

AIMS

1) Cloning and characterization of a novel human SHP-1 binding receptor (IREM-1).

2) Generation of mAbs specific for IREM-1 and analysis of its distribution in hematopoietic cells.

3) Molecular analysis of IREM-1-mediated signaling.

III. ARTICLE 1

Running Title: Immune receptor expressed on myeloid cells

IREM-1 IS A NOVEL INHIBITORY RECEPTOR EXPRESSED BY MYELOID CELLS

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Keywords: Inhibitory receptor, myeloid cell, SHP-1, Ig superfamily

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SUMMARY

Using a three-hybrid strategy we have identified a novel cell surface molecule, which interacts with the SH2 domains of SHP-1, termed "immune receptor expressed on myeloid cells 1" (IREM-1). The full-length cDNA, encodes for a polypeptide of 290 amino acids, that presents an extracellular single V-type Ig domain, a transmembrane region and a cytoplasmic tail with five tyrosine residues, two of which are in the context of immunoreceptor tyrosine-based inhibitory motif (ITIM). Moreover, cDNA encoding for three other splicing forms of IREM-1, named IREM-1 Sv1, Sv2 and Sv3 were cloned by RT-PCR. The gene encoding for IREM-1 contains 9 exons, is located in human chromosome 17 (17q25.1) and is homologous to previously identified molecules termed CMRF-35 and IRp60. RT-PCR, northern blot and FACS analysis with specific monoclonal antibodies indicated that IREM-1 is expressed on monocytes, granulocytes, and myeloid leukemia cell lines. Western blot analysis confirmed the recruitment of SHP-1 to IREM-1 and demonstrated that phosphotyrosine residue 205 is the main docking site for this interaction. Finally, cross-linking of IREM-1 results in the inhibition of FcRε induced activation. Our results indicate that IREM-1 is a novel inhibitory receptor of the Ig-superfamily in myeloid cells.

INTRODUCTION

Leukocyte functions are regulated by a balance between positive and negative signals delivered by activating and inhibitory receptors. Inhibitory receptors play an essential role maintaining a threshold of activation in the immune system, that is overcome by triggering signals in response to pathological stimuli. These mechanisms allow leukocytes to discriminate between normal cells and pathogenic agents, tumors and grafts. There is experimental evidence supporting that a dysfunction of inhibitory receptors may contribute to the development of chronic autoimmune diseases [1-3].

A group of inhibitory receptors belongs to the Immunoglobulin superfamily (Ig-SF) whereas others are lectin-like molecules. The first group includes several multigenic families of receptors (CD33-like subgroup of Siglecs, KIRs, ILTs, FCAR and LAIRs) that are encoded within the leukocyte receptor complex on chromosome 19 (19q13.4), as well as other gene families (i.e. SIRPs, PILRs and TREMs) located in different chromosomes [3, 4]. Lectin-like inhibitory receptors comprise CD72, NKG2A and members of the Ly49 family [5, 6]. Some inhibitory receptors recognize well-defined endogenous molecules (i.e. MHC class I, IgG Fc, sialic acid, CD47), whereas for others the nature of their ligands remains unknown.

The molecular basis for the functional activity of immune inhibitory receptors is the presence of cytoplasmic ITIMs [3]. Upon receptor-ligand interaction ITIMs become tyrosine phosphorylated, recruiting and activating SH2-containing phosphatases, like SHP-1 and SHIP, which mediate the inhibitory signal. The Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) is mainly expressed in the cytoplasm of hematopoietic cells [7]. It contains two SH2 domains, one catalytic phosphatase

domain and two sites for tyrosine phosphorylation at the C–terminus region. Upon recruitment by the inhibitory receptor, SHP-1 becomes activated and dephosphorylates key elements of the activation pathway in lymphoid and myeloid cells, thus inhibiting cell activation. SHP-1 controls the activating signaling cascade at its proximal steps, shutting down subsequent events [8].

By using a three-hybrid strategy in yeast we cloned a novel member of the Ig superfamily preferentially expressed by myeloid cells that contains cytoplasmic ITIMs and is capable of delivering negative signals. This "immune receptor expressed on myeloid cells-1" (IREM-1) is a member of a multigenic family located in human chromosome 17 (17q25.1) that includes previously identified molecules termed CMRF-35 and CMRF35-H/IRp60 [9-11].

RESULTS

Cloning of a novel "immune receptor expressed on myeloid cells-1" (IREM-1)

Our goal was to identify molecules that could interact with the SH2 domains of the tyrosine phosphatase SHP-1 in a phosphotyrosine dependent manner. To this end, we used a three hybrid system in yeast to screen a library of PHA activated peripheral blood mononuclear cells (Clontech) using the SHP-1 SH2 domains as bait in the presence of the Src-kinase c-fyn $_{420,\ 531Y\text{-}F,\ 176R\text{-}Q}$ mutant. The three-hybrid screening ($8x10^6$ independent clones) produced more than 200 positive clones, selected for their ability to grow in a media lacking histidine and for their β-galactosidase activity [12]. Five of these clones encoded for a polypeptide of 93 amino acids that contained five tyrosines, two of them in the context of ITIM motifs. By searching human cDNA´s databases we found partial homology of our sequence with a putative inhibitory receptor called CMRF-35H (Database accession numbers AF176991) (Fig 1-B). Blast search was carried out on the Ensembl genome database (Ensembl BLAST Server at www.ensembl.org) and a predicted cDNA containing that sequence at the 3' region was found. We next designed primers and cloned two different cDNA using activated PBMCs RNA as template in a two round nested PCR strategy (see methods). We cloned two cDNA fragments, one (IREM-1 splice variant 1) with an open reading frame of 882 bp (AF375480), and a second (IREM-1 splice variant 2) with an open reading frame of 474 bp (AF375481). IREM-1 Sv1 sequence is predicted to be translated into a type I glycoprotein with 293 amino acids (Fig 1).

A)

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aacggggacctgtctgaagagaagATGCCCCTGCTGACACTCTACCTGCTCCTCTTCTGGCTCTCAGGC...
                        M  P  L  L  T  L  Y  L  L  L  F  W  L  S  G ...
cagaaccactagaaatcattaggaccaccaggacagcctctccacagccatctgATGTGGCTGCCTCAGCTC
                                                      M  W  L  P  Q  L
GACCTCATGAGGGTCATCAGTGCTAAGAGTCAAGGCTACTCCATTGCCACTCAAATCACCGGTCCAACAACA
 D  L  M  R  V  I  S  A  K  S  Q  G  Y  S  I  A  T  Q  I  T  G  P  T  T
GTGAATGGCTTGGAGCGGGGCTCCTTGACCGTGCAGTGTGTTTACAGATCAGGCTGGGAGACCTACTTGAAG
 V  N  G  L  E  R  G  S  L  T  V  Q  C  V  Y  R  S  G  W  E  T  Y  L  K
TGGTGGTGTCGAGGAGCTATTTGGCGTGACTGCAAGATCCTTGTTAAAACCAGTGGGTCAGAGCAGGAGGTG
 W  W  C  R  G  A  I  W  R  D  C  K  I  L  V  K  T  S  G  S  E  Q  E  V
AAGAGGGACCGGGTGTCCATCAAGGACAATCAGAAAAACCGCACGTTCACTGTGACCATGGAGGATCTCATG
 K  R  D  R  V  S  I  K  D  N  Q  K  N  R  T  F  T  V  T  M  E  D  L  M
AAAACTGATGCTGACACTTACTGGTGTGGAATTGAGAAAACTGGAAATGACCTTGGGGTCACAGTTCAAGTG
 K  T  D  A  D  T  Y  W  C  G  I  E  K  T  G  N  D  L  G  V  T  V  Q  V
ACCATTGACCCAGCACCAGTCACCCAAGAAGAAATTAGCAGCTCCCCAACTCTGACCGGCCACCACTTGGAC
 T  I  D  P  A  P  V  T  Q  E  E  I  S  S  S  P  T  L  T  G  H  H  L  D
AACAGGCACAAGCTCTTGAAGCTCAGTGTCCTCTTGCCCCTCATCTTCACCATATTGTTGCTGCTTTTGGTG
 N  R  H  K  L  L  K  L  S  V  L  L  P  L  I  F  T  I  L  L  L  L  L  V
GCCGCCTCACTCTTGGCTTGGAGGATGATGAAGTACCAGCAGAAAGCAGCCGGGATGTCCCCAGAGCAGGTA
 A  A  S  L  L  A  W  R  M  M  K  Y  Q  Q  K  A  A  G  M  S  P  E  Q  V
CTGCAGCCCTTGGAGGGCGACCTCTGCTATGCAGACCTGACCCTGCAGCAGGCCGGAACCTCCCCGCGAAAG
 L  Q  P  L  E  G  D  L  C  Y  A  D  L  T  L  Q  Q  A  G  T  S  P  R  K
GCTACCACGAAGCTTTCCTCTGCCCAGGTTGACCAGGTGGAAGTGGAATATGTCACCATGGCTTCCTTGCCG
 A  T  T  K  L  S  S  A  Q  V  D  Q  V  E  V  E  Y  V  T  M  A  S  L  P
AAGGAGGACATTTCCTATGCATCTCTGACCTTGGGTGCTGAGGATCAGGAACCGACCTACTGCAACATGGGC
 K  E  D  I  S  Y  A  S  L  T  L  G  A  E  D  Q  E  P  T  Y  C  N  M  G
CACCTCAGTAGCCACCTCCCCGGCAGGGGCCCTGAGGAGCCCACGGAATACAGCACCATCAGCAGGCCTTAG
 H  L  S  S  H  L  P  G  R  G  P  E  E  P  T  E  Y  S  T  I  S  R  P  .
cctgcactccaggctccttcttggaccccaggctgtgagcacactcctgcctcatcgac
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Figure 1. (A) Nucleotide and predicted amino acid sequences of IREM-1 (AY303545) and IREM-1 Sv1 (AF375480). The nucleotide sequences of IREM-1 and IREM-1 Sv1 containing an open reading frame of 873 bp and 882 bp are shown in upper case. The 5' untranslated region of both isoforms are shown in lower case. The predicted amino acid sequences are shown above the nucleotide sequence. The putative signal peptides are dotted underlined (IREM-1) and double underlined (IREM-1 Sv1). Ig-like domain is in bold type, the transmembrane domain is single underlined and the consensus ITIM-like sequences are in bold type and single underlined. N-glycosylation site is boxed and cysteines involved in the Ig-like fold are circled.

B)

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IREM-1      ------MPLLTLYLLLFWLSGYSIATQITGPTTVNGLERGSLTVQCVYRSGWETYLKWWC
IRp60       ----MWLPW---ALLLLWVPGC---FALSKCRTVAGPVGGSLSVQCPYEKEHRTLNKYWC
CMRF35      MTARAWASWRSSALLLLLVPGY---FPLSHPMTVAGPVGGSLSVQCRYEKEHRTLNKFWC

IREM-1      RGAIWRDCKILVKTSGSEQEVKRDRVSIKDNQKNRTFTVTMEDLMKTDADTYWCGIEKTG
IRp60       RPPQIFLCDKIVETKGSAGK-RNGRVSIRDSPANLSFTVTLENLTEEDAGTYWCGVDTPW
CMRF35      RPPQILRCDKIVETKGSAGK-RNGRVSIRDSPANLSFTVTLENLTEEDAGTYWCGVDTPW

IREM-1      N----DLGVTVQVTIDPAPVTQEETSSSPTLTG------------------------HHL
IRp60       LRDFHDPVVEVEVSVFPASTSMTPASITAAKTSTITTAFPPVSSTTLFAVGATHSASIQE
CMRF35      LRDFHDPIVEVEVSVFPAGTTTASSPQSSMGTSGPPTKLPVHTWPSVTRKDSPEPSPHPG

IREM-1      DNRHKLLKLSVLLPLIFTILLLLLVAASLLAWRMMKYQQKAAG---MSPEQVLQPLEGDL
IRp60       ETEEVVNSQLPLLLSLLALLLLLLVGASLLAWRMFQKWIKAGDHSELSQNPKQAATQSEL
CMRF35      SLFSNVRFLLLVLLELPLLLSMLGAVLWVNRPQRSSRSRQNWPKGENQ

IREM-1      CYADLTLQLAGTSPQKATTKLSSAQVDQVEVEYVTMASLPKEDISYASLTLGAEDQEPTY
IRp60       HYANLELLMWPLQEK-------PAPPREVEVEYSTVAS-PREELHYASVVFDS-------

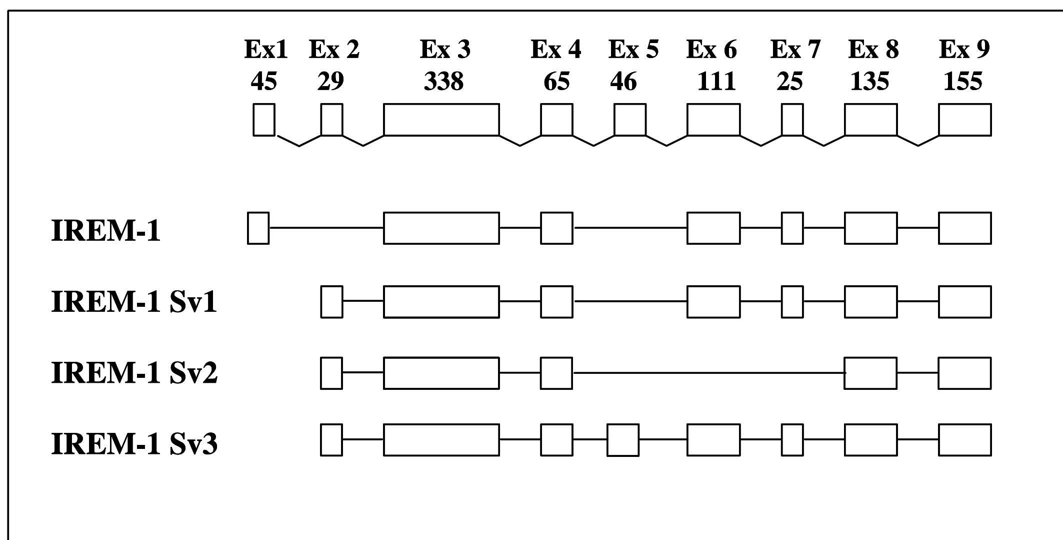
IREM-1      CNMGHLSSHLPGRGPEEPTEYSTISRP
IRp60       -NTNRIAAQRP-REEEPDSDYSVIRKT
```

Figure 1. (B) Protein sequence alignment of IREM-1 with CMRF-35 and CMRF-35H/IRp60. Identical amino acids are shown on black background and similar residues are in gray. Alignment was done for the three proteins using IREM-1 amino acids 1-142 (extracellular domain), and only for IREM-1 and CMRF-35H/IRp60 using IREM-1 amino acids 143-290 (transmembrane and cytoplasmic tail).

This molecule displays a single V-Ig domain at the N-terminus, a transmembrane fragment and a cytoplasmic tail including five tyrosine residues (Fig 1-A). IREM-1 Sv2 is a splicing variant that lacks exon 6 and 7, thus lacking the transmembrane domain (Fig 2). By screening the human expressed sequence tag (EST) database with the complete IREM-1 Sv1 cDNA (www.ncbi.nlm.nih.gov/BLAST/) we found a number of EST clones containing both isoforms Sv1 and Sv2, and a new isoform (AAH28199) that we called Sv3 with the insertion of an additional exon (exon 5). We cloned by RT-PCR

a cDNA fragment of 502 bp that contains exon 5 and a subsequent frame shift. Interestingly, we found sequences (AF251706, BQ073166) in which the 5' UT untranslated and the first translated nucleotides were different to those found in IREM Sv1 and Sv2. Through alignment of cDNA's and EST sequences with the genomic sequence we determined the genomic organization of the IREM gene (Fig 2-A). We realized that there were two different transcriptional starts at exon 1 and exon 2. With new primers we amplified another IREM-1 isoform from activated PBMCs derived cDNA (accession number AY303545). This isoform has an open reading frame of 873 bp that encodes for a 290 amino acid polypeptide almost identical to IREM-1 Sv1, but with a different leader peptide. In fact, when we analyzed both putative signal peptides using the program SignalP V2.0 (www.cbs.dtu.dk) only that present in IREM-1 fitted with a valid signal peptide sequence. In summary, we here describe a putative inhibitory receptor with two different transcriptional starts (IREM-1 and IREM-1 Sv1), and two truncated forms that present a V-type Ig domain but do not display any transmembrane region (IREM-1 Sv2 and Sv3) (Fig 2-B).

A)



B)

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IREM-1         --MPLLTLYLLLFWLS-GYSIATQITGPTTVNGLERGSLTVQCVYRSGWETYLKWWCRGA
IREM-1 Sv1     MWLPQLDLMRVISAKSQGYSIATQITGPTTVNGLERGSLTVQCVYRSGWETYLKWWCRGA
IREM-1 Sv2     MWLPQLDLMRVISAKSQGYSIATQITGPTTVNGLERGSLTVQCVYRSGWETYLKWWCRGA
IREM-1 Sv3     MWLPQLDLMRVISAKSQGYSIATQITGPTTVNGLERGSLTVQCVYRSGWETYLKWWCRGA

IREM-1         IWRDCKILVKTSGSEQEVKRDRVSIKDNQKNRTFTVTMEDLMKTDADTYWCGIEKTGNDL
IREM-1 Sv1     IWRDCKILVKTSGSEQEVKRDRVSIKDNQKNRTFTVTMEDLMKTDADTYWCGIEKTGNDL
IREM-1 Sv2     IWRDCKILVKTSGSEQEVKRNRVSIKDNQKNRTFTVTMEDLMKTDADTYWCGIEKTGNDL
IREM-1 Sv3     IWRDCKILVKTSGSEQEVKRDRVSIKDNQKNRTFTVTMEDLMKTDADTYWCGIEKTGNDL

IREM-1         GVTVQVTIDPAPVTQEETSSSPTLTGHHLDNRHKLLKLSVLLPLIFTILLLLLVAASLLA
IREM-1 Sv1     GVTVQVTIDPAPVTQEEISSSPTLTGHHLDNRHKLLKLSVLLPLIFTILLLLLVAASLLA
IREM-1 Sv2     GVTVQVTIDPAPVTQEETSSSPTLTGHHLDNRYCSPWRATSAMQT
IREM-1 Sv3     GVTVQVTIDPAPVTQEETSSSPTLTGHHLDNSSRDVPRAGTAAPGGRPLLCRPDPAAGRN

IREM-1         WRMMKYQQKAAGMSPEQVLQPLEGDLCYADLTLQLAGTSPRKATTKLSSAQVDQVEVEYV
IREM-1 Sv1     WRMMKYQQKAAGMSPEQVLQPLEGDLCYADLTLQQAGTSPRKATTKLSSAQVDQVEVEYV
IREM-1 Sv3     LPAKGYHEAFLCPG

IREM-1         TMASLPKEDISYASLTLGAEDQEPTYCNMGRLSSHLPGRGPEEPTEYSTISRP
IREM-1 Sv1     TMASLPKEDISYASLTLGAEDQEPTYCNMGHLSSHLPGRGPEEPTEYSTISRP
```

Figure 2. (A) Genomic organization and splicing pattern of IREM-1. A diagram representing the four splice variants of IREM-1 is shown. Exons are represented by solid boxes with lengths mentioned in base pairs and introns by the connecting lines.

(**B**) **Predicted amino acid sequence of IREM-1 splice variants.** Comparison of the predicted amino acid sequence of IREM-1 (AY303545), IREM-1 Sv1 (AF375480), IREM-1 Sv2 (AF375481) and IREM-1 Sv3 (AAH28199). Identical amino acids are shown on black background.

IREM-1 is expressed by myeloid cells

We analyzed by RT-PCR the expression pattern of IREM-1 mRNA. The expected size band (985 bp) was amplified from non activated PBMCs as well as from PHA activated PBMCs from different donors (data not shown). To more accurately address the distribution of IREM-1, we evaluated by RT-PCR purified T lymphocytes, NK cells and monocytes. Transcripts were detected in isolated peripheral blood monocytes, whereas polyclonal NK and T cell populations were negative for the presence of IREM-1 mRNA (Fig 3-A). Northern blot analysis with total RNA extracted from several hemopoietic cell lines was also performed in order to asses the expression pattern of IREM-1. A specific 356 bp probe encoding for the cytoplasmic portion of IREM-1 was used to hybridize the membrane. Specific hybridization with a single band of 1.5 kb was detected in the U937 human monocytic cells, as well as in the HL60 promyelocytic leukemia cell line, but not in T or B lineage cell lines (Fig 3-B). We developed specific anti-IREM-1 mAbs UP-D1 and UP-D2 to accurately study this molecule. These antibodies did not cross-react with IREM-2 (Accession number AF395839), another member of the family (Aguilar et al, submitted) expressed only by monocytic cells (Fig 4A). In accordance with the mRNA detection results, double color FACS analysis of different PBMCs populations revealed that IREM-1 was detectable on the surface of all $CD14^+$ monocytes and $CD15^+$ granulocytes (Fig. 4B-4C), whereas no expression was detected on $CD19^+$ B lymphocytes, the $CD56^+$ population including NK cells and $CD3^+$ T lymphocytes (Fig. 4B).

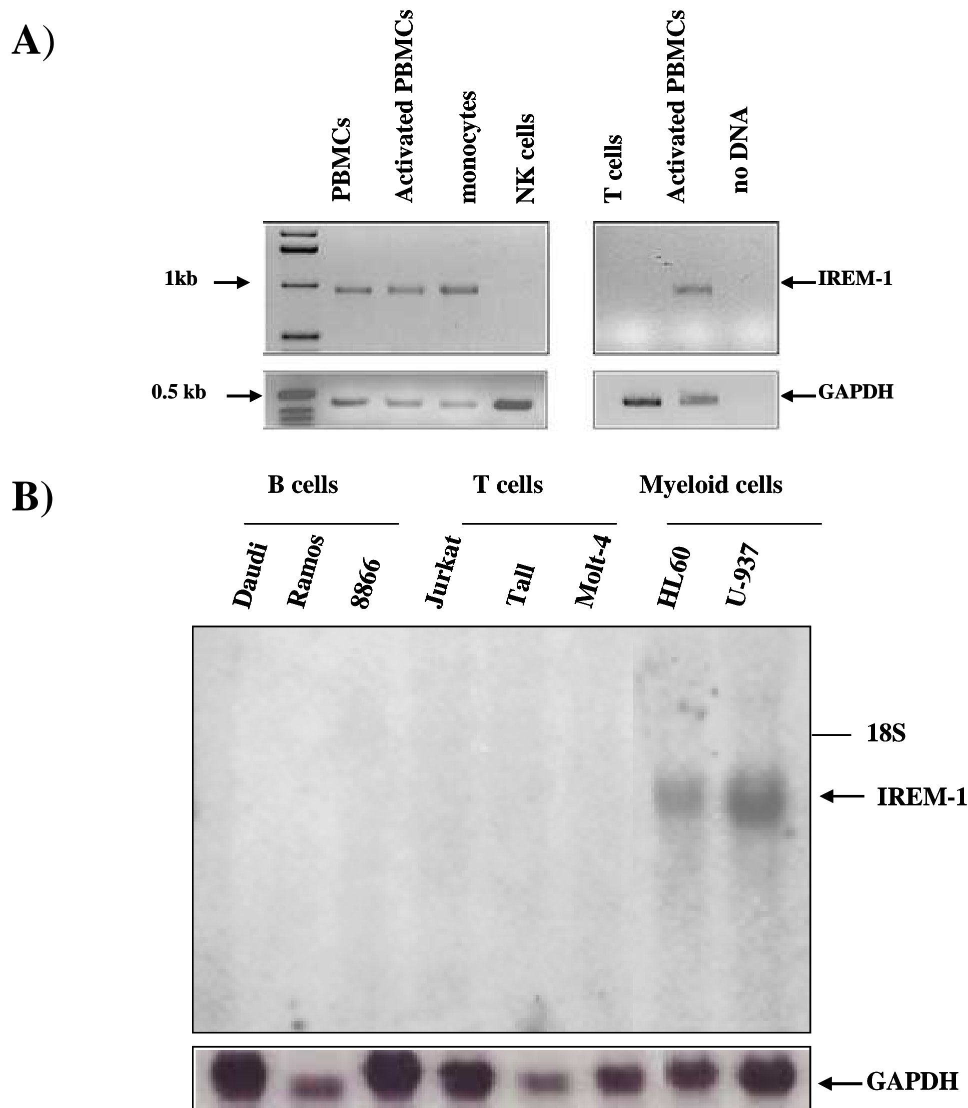


Figure 3. IREM-1 mRNA is expressed of human monocytes and myeloid cell lines.

(A) RT-PCR using RNA from human fresh PBMCs, PHA activated PBMCs, isolated blood monocytes and polyclonal NK and T cells was performed to amplify full length IREM-1. cDNA products were resolved on 1% agarose gel. Molecular weight markers are shown on the left. **(B)** Northern blot analysis of total RNA from several hematopoietic cell lines was carried out with a ^{32}P-radiolabelled probe encoding for the cytoplasmic region of IREM-1. GAPDH ^{32}P-radiolabelled probe was used as loading control. The positions of 28S and 18S ribosomal subunits are indicated.

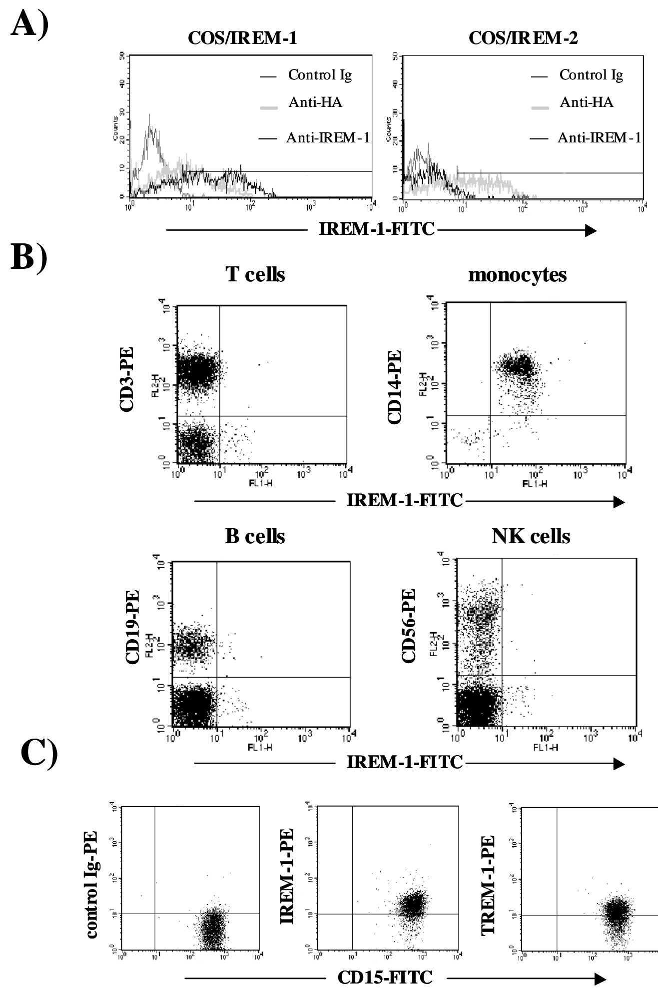


Figure 4. IREM-1 is expressed on the surface of human myeloid cells. (A) COS-7 cells were transfected with HA-IREM-1 or HA-IREM-2 and stained by indirect immunofluorescence with the UP-D1 anti IREM-1 mAb, an anti-HA mAb or an isotypic control Ig, and a FITC-labeled goat anti-mouse Ig. **(B)** PBMC were sequentially stained with UP-D1 anti-IREM-1 mAb and FITC-labeled goat anti-mouse Ig, followed by incubation with PE-conjugated anti-CD3, anti-CD19, anti-CD56 or anti-CD14 mAbs. **(C)** Dextran-enriched granulocytes were sequentially stained by indirect immunofluorescence with anti-IREM-1, anti-TREM-1 mAb or an isotype control Ig and a PE-conjugated goat anti-mouse Ig, followed by incubation with an FITC-conjugated anti-CD15 mAb. In FACS analysis cells were gated according to the FLS/90ºLS distribution.

Expression of IREM-1 was maintained after culturing monocytes either alone or with LPS for 72hs. By contrast, surface expression of the molecule was markedly downregulated in monocyte-derived immature dendritic cells (Fig. 5) and was not re-expressed after inducing their maturation by LPS treatment that was monitored by the induction of CD83 expression (data not shown).

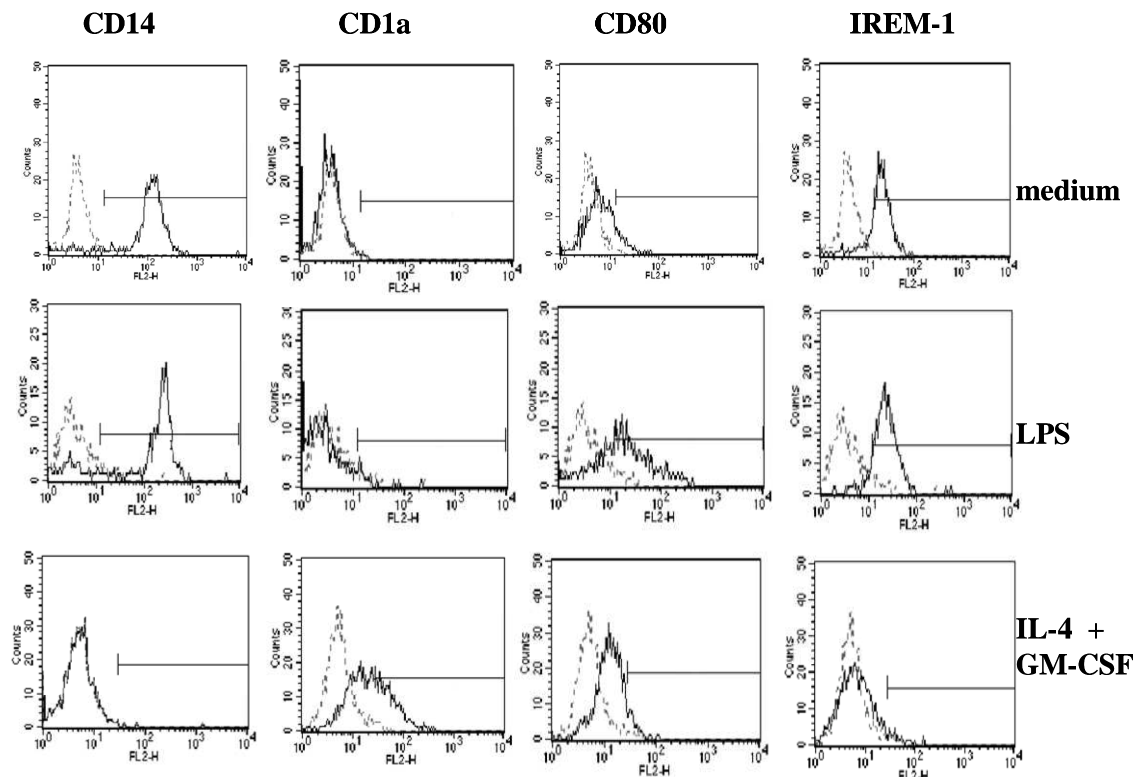


Figure 5. IREM-1 expression is downregulated in monocyte-derived dendritic cells. Surface expression of IREM-1 was analyzed on monocytes cultured for 72 h either alone or stimulated with LPS (1 µg/ml)**,** as well as in differentiated monocyte-derived immature dendritic cells. FACS analysis was carried out after labeling with anti-IREM-1 mAb and a secondary PE-conjugated goat anti-mouse Ig antiserum; in parallel samples were stained with PE-conjugated anti-CD80, CD14 and CD1a. Background fluorescence with a control Ig is indicated by a dashed line.

To further study IREM-1, immunoprecipitation of biotin-labeled U937 monocytic cell line using anti-IREM-1 UP-D1 MAb was carried out. When resolved by SDS-PAGE, IREM-1 showed two discrete bands of approximately 53 and 59 kD both under reducing or non- reducing conditions, showing that this molecule does not oligomerize at the cell surface through disulfide bonds (Fig 6).

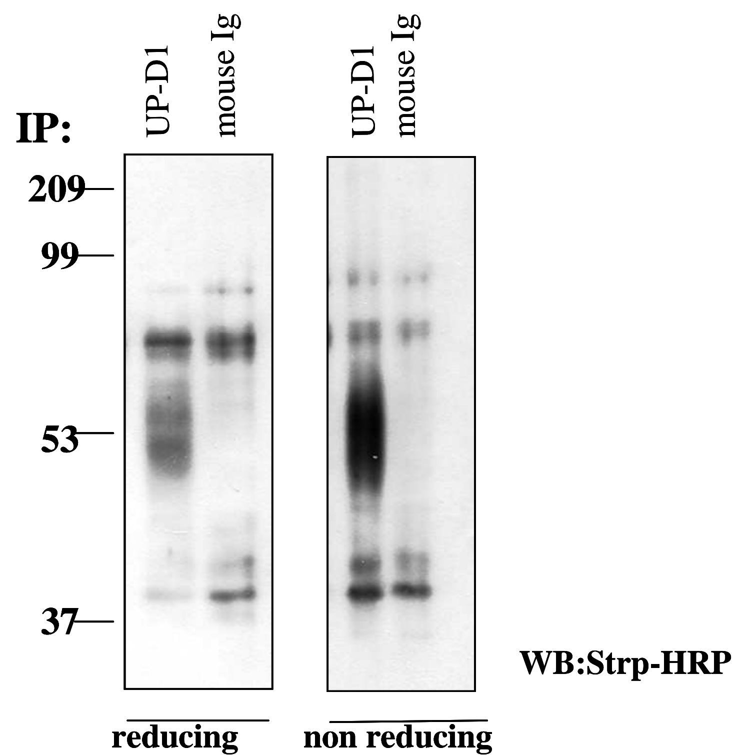


Figure 6. Immunoprecipitation of IREM-1. Lysates from biotin-labeled U-937 cells were immunoprecipitated with the UP-D1 anti-IREM-1 mAb or an isotypic control. Samples were analyzed by SDS-PAGE using 10% polyacrylamide gels both under reducing and non-reducing conditions. Western blot was developed with Streptavidin-HRP.

IREM-1 interaction with SHP-1 is dependent on tyrosine phosphorylation of the receptor

In order to analyze whether the association between IREM-1 and SHP-1 was indeed dependent on phosphorylation, we co-transfected the CG1945 yeast strain with pGAD10/IREM-1 and pBridge, pBridge/SHP-1, pBridge/c-fyn and pBridge/SHP-1/c-fyn to perform a three hybrid system assay. Transformants were grown in SD media lacking Trp, Leu and Met. Association between IREM-1 and SHP-1 was only detected as β-galactosidase activity when the kinase c-fyn was present (Fig 6-A). These data suggested that tyrosine phosphorylation within the cytoplasmic tail of IREM-1 was required for the interaction.

To confirm the interaction between SHP-1 and IREM-1, we used the U-937 monocytic cell line that expresses both IREM-1 and SHP-1. Cells were treated or not with sodium pervanadate to induce tyrosine phosphorylation, lysed and immunoprecipitated with UP-D1 mAb or an isotypic Ig negative control. Western blot analysis of the precipitates with anti-phosphotyrosine antibodies showed that IREM-1 was phosphorylated only after pervanadate treatment. Re-blotting of the membrane with anti SHP-1 antibodies revealed that this phosphatase was co-precipitated only when IREM-1 was tyrosine phosphorylated (Fig 6B). These results confirmed the interaction between IREM-1 and SHP-1 and showed the capability of IREM-1 to recruit SHP-1.

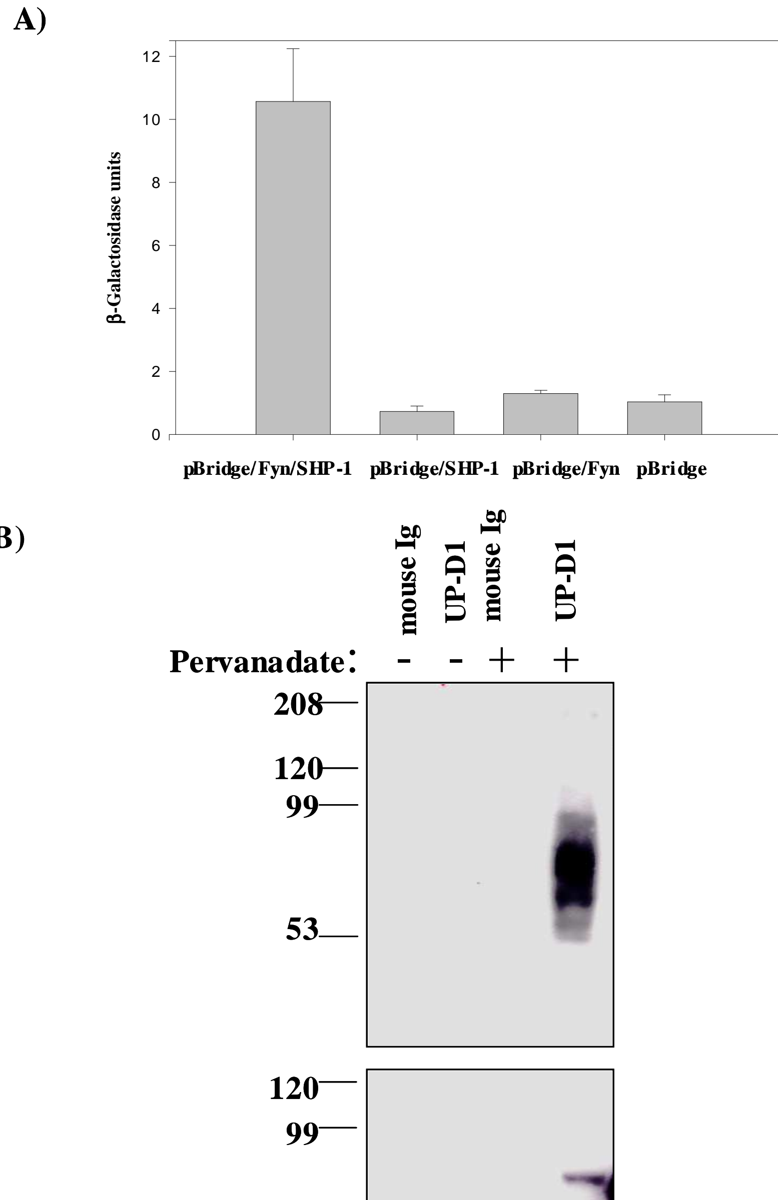


Figure 6. **SHP-1 recruitment by IREM-1 is dependent on tyrosine phosphorylation (A) IREM-1 interacts with SHP-1 in yeast only in the presence of c-fyn**. The interaction of SHP-1 with the cytoplasmic tail of IREM-1 in the presence or absence of Fyn $_{420Y\text{-}F,\ 531Y\text{-}F,\ 176R\text{-}Q}$ took place in yeast cells and was measured in a β-galactosidase assay using ONPG as substrate. For each construct, at least three independent colonies were tested in the β-galactosidase assay. **(B) Phosphorylated IREM-1 associates to SHP-1 in monocytic cells**. U-937 cells were treated (+) or not (-) with sodium pervanadate and IREM-1 immunoprecipitated with UP-D1 mAb or an isotypic control.

After SDS-PAGE, proteins were transferred to PVDF membranes and blotted with anti-phosphotyrosine and anti-SHP-1 antibodies. Molecular weight markers are indicated on the left.

IREM-1 tyrosine residue 205 is required for SHP-1 recruitment.

In order to verify that phosphorylation of putative ITIM motifs within IREM-1 is required for and mediates the recruitment of SHP-1 via its SH2 domains, we generated a number of IREM-1 tyrosine to phenylalanine substitution mutants. Two tyrosine residues, Tyr 205 and Tyr 249 match with the strict ITIM consensus sequence, whereas a third residue, tyrosine 284, is in the context of an ITIM-like motif. COS-7 cells were transfected with wild type IREM-1 or the different mutants together with SHP-1 and the c-fyn kinase. Cell lysates were immunoprecipitated with anti-HA antibodies and samples were analyzed by western blot with anti-phosphotyrosine, biotinylated anti-HA and anti-SHP-1. As shown in figure 8, SHP-1 failed to interact with IREM-1 when tyrosine 205 was mutated, which suggests that this tyrosine is essential for the recruitment of the phosphatase. On the other hand, mutation of both tyrosines 249 and 284 did not interfere with SHP-1 recruitment, indicating that these residues are not relevant for this interaction. Taken together, our results support that SHP-1 binding to IREM-1 is dependent on the phosphorylation of tyrosine residue 205.

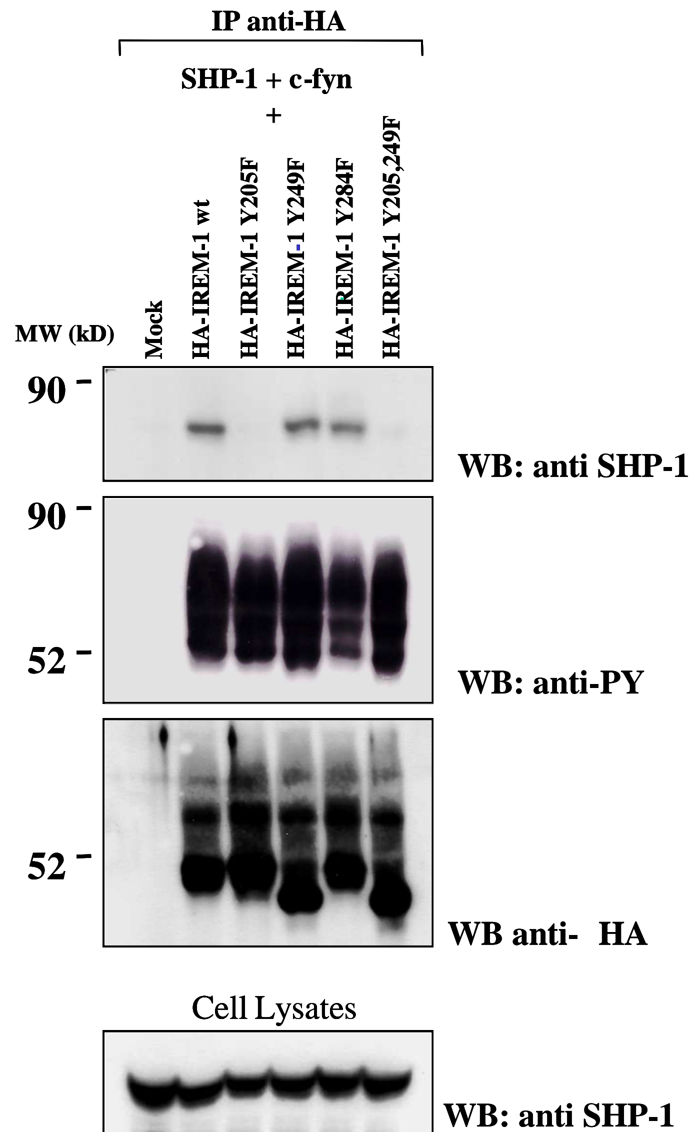
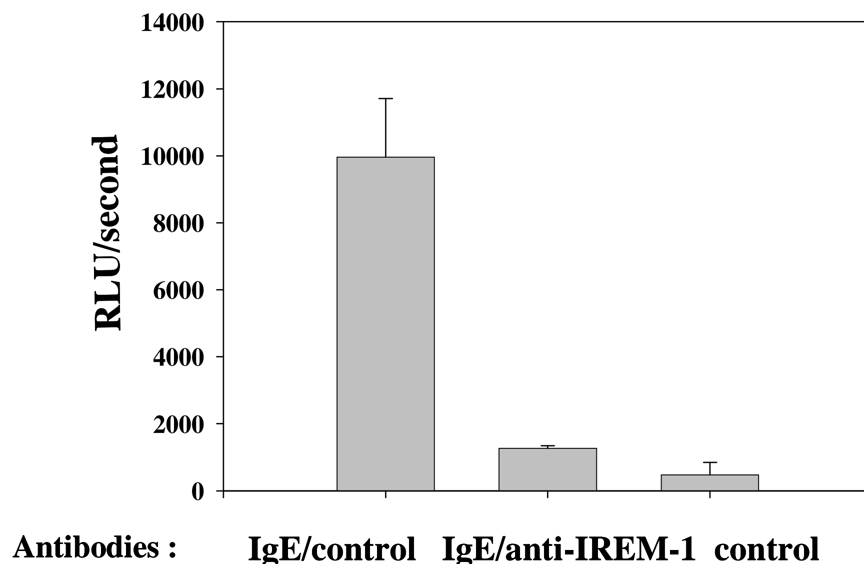


Figure 8. IREM-1 tyrosine residue 205 is required for SHP-1 recruitment. COS-7 cells were transfected with SHP-1 and c-fyn, and the indicated HA-IREM-1 constructs. Transfected cells were lysed and HA-IREM-1 proteins were immunoprecipitated with 1 μg of anti-HA monoclonal antibody. Proteins were transferred to PVDF membranes and western blotted with anti-SHP-1, anti-phosphotyrosine-HRP and biotinylated anti-HA. Molecular weight markers are indicated on the left.

IREM-1 engagement delivers an inhibitory signal

Taking into account the association between IREM-1 and SHP-1, we addressed the putative inhibitory role of this molecule. To this end, we used the RBL cell line stably transfected with IREM-1. RBL cells can be activated through its FcεR with IgE and inhibitory receptors have been shown to down regulate this activating pathway by recruiting SHP-1 [3, 13]. Cells were transfected with plasmids encoding the luciferase reporter gene under the control of NFAT- or NFkB-dependent promoters. Cells were stimulated through the FcεR by cross-linking with an anti-DNP IgE mAb in the presence of the UP-D2 anti-IREM-1 mAb or an isotypic Ig control. Cross linking of the FcεR induced cell activation as revealed by the increase of luciferase activity (Fig 8A and 8B);, the activity of NFAT and NFkB transcription factors was dramatically reduced when both FcεR and IREM-1 were simultaneously engaged. These data confirmed that IREM-1 is able to function as an inhibitory receptor in myeloid cells.



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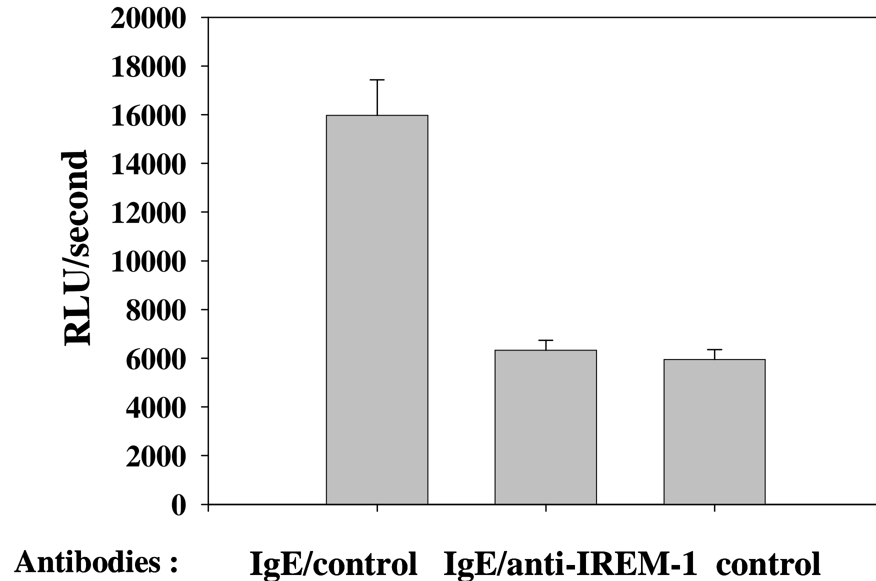

Figure 9. **IREM-1 delivers an inhibitory signal.** A luciferase reporter assay was developed to directly verify IREM-1 mediated inhibition in RBL/IREM-1 cells. Cells were transiently transfected with a plasmid carrying the luciferase reporter gene under the control of three tandem copies of a NFAT/AP-1 site (3xNFAT-Luc) **(A)**, or under the control of two copies of a consensus NF-κB site (2xNF-κB-Luc) **(B)**. Subsequently, cells were stimulated with anti-DNP IgE mAb, in the presence or absence of an anti-IREM-1 mAb or a control isotypic-matched Ig; in every case mAbs were adsorbed onto plastic culture plates. Luciferase activity was measured after stimulation with the indicated antibodies, normalized to TK-renilla for each point, and expressed as RLU/second. These results are representative of three independent experiments.

DISCUSSION

The aim of our work was to develop a comprehensive search for molecules that interact with SHP-1 and thus could mediate an inhibitory role in the immune response. Herein we describe a novel inhibitory receptor that belongs to the Ig superfamily, termed IREM-1. Upon engagement by specific ligands, ITIM-containing immunoreceptors act by recruiting phosphatases (i.e. SHP-1) after phosphorylation of cytoplasmic tyrosine residues. The inhibitory signal downregulates cytokine release, cytotoxic activity and cell proliferation [3].

Most of these regulatory molecules belong to families of proteins that also include activating members; moreover, their genes are often organized in clusters (i.e. ILTs, KIRs) [4, 14, 15]. Unlike inhibitory molecules, the activating counterparts lack signaling motifs and present a charged residue in the transmembrane region that allows their association to ITAM-bearing adapters that deliver the activating signal.

Sequence analysis of IREM-1 revealed this molecule as a member of the CMRF-35 family of receptors described elsewhere [9, 11]. This family is encoded in a region of human chromosome 17 (17q24-q25) and, as in other inhibitory receptor families, the CMRF-35 cluster includes ITIM-bearing molecules as well as short-tailed proteins [16, 17]. Members of this gene family have been shown to be expressed on cells of different hematopoietic lineages such as T cell subsets, B lymphocytes, NK cells, monocytes, macrophages and granulocytes [18]. An inhibitory member of the family (CMRF-35H or IRp60) is expressed by several human hematopoietic cell types including myelomonocytic cells, NK cells, as well as T lymphocytes [10, 11]. This receptor

delivers a negative signal after engagement by mAbs, inhibiting human NK cells cytolytic activity. In the present report, we show that IREM-1 mRNA was detected in both fresh and PHA activated PBMCs, as well as monocytes and a number of myeloid cell lines (i.e. HL-60 or U-937) . Double color flow cytometry analysis by using the UP-D1 and UP-D2 anti-IREM-1 mAbs confirmed that IREM-1 is expressed by all blood $CD14^{+}$ monocytes as well as by $CD15^{+}$ granulocytes. Remarkably, IREM-1 expression was maintained after treatment of monocytes with LPS, whereas it was downregulated after *in vitro* differentiation to dendritic cells. These results support that IREM-1 is mainly expressed by cells of the myeloid lineage. A putative IREM-1 murine orthologue, CLM-1, has been recently shown to be also expressed mainly by myeloid cells [19]. A myeloid-restricted expression pattern has been observed for a number of immunoreceptors such as TREM-1 [4] and PILRα [20, 21], some ILTs [22, 23], and IREM-2, another member of the same family (Aguilar et al, submitted), indicating a possible role for these molecules in the regulation of the innate immune response. IREM-1 and IREM-2 are the only known members of the CMRF35 family with a myeloid restricted expression pattern; it is of note that UP-D1 and UP-D2 mAbs do not cross-react with IREM-2.

Biochemical studies showed that IREM-1 is a monomeric type I transmembrane receptor. The IREM-1 polypeptide backbone has a predicted size of 32 kD, whereas immunoprecipitated IREM-1 appeared as two discrete bands of 53 kD and 59 kD (Fig. 5). The fact that a similar pattern was obtained in both transfectants (Fig. 7) and cells constitutively expressing IREM-1 suggests that its heterogeneous electrophoretic mobility is likely due to different levels of N- and/or O-linked glycosylation . IREM-1 was cloned for its ability to bind SHP-1 and, consistent with the presence of

intracellular ITIMs, co-immunoprecipitation and western blot analysis confirmed that IREM-1 can recruit the SHP-1 phosphatase in a phosphorylation dependent manner in myeloid cells. The best candidates for phosphorylation by Src kinases and SHP-1 recruitment, are Y205 and Y249, matching with the ITIM (I/L/VxYxxL/V) consensus sequence described for other inhibitory receptors [24]. Mutagenesis of ITIM tyrosine residues to phenylalanine supported that tyrosine residue 205 was the main docking site for SHP-1. A model for SHP-1 activation has been proposed, in which the amino-terminal SH2 domain of SHP-1 serves both, as a regulatory domain and as a high affinity recruiting unit, whereas the C-terminal SH2 domain merely acts as a secondary recruiting unit [25]. According to this model, tyrosine 205 would be responsible for recruiting the amino-terminal SH2 domain of SHP-1, whilst the second tyrosine residue in the context of an ITIM (Y249), might be able to recruit the C-terminal SH2 domain, to fully activate SHP-1. This interaction has been described for other inhibitory receptors in which tyrosine phosphorylation by a Src kinase generates docking sites for phosphatases having Src homology 2 domains (SH2) (i.e. SHP-1).

It has been shown that SHP-1-mediated inhibition targets early stages of the activation response, shutting down subsequent events such as induction of gene transcription [3, 26]. The earliest events upon ligation of triggering immunoreceptors are the activation of Src kinases and the subsequent recruitment and activation of other kinases (i.e. ZAP-70 or Syk) [27]. These events lead to calcium mobilization and activation of different transcription factors including Nuclear Factors of Activated T cells (NFATs) or NFκB. Whereas NFATs activate the transcription of a large number of genes during an effective immune response [28], NFκB regulates the inducible expression of a wide range of proinflammatory mediators such as cytokines, chemokines, and leukocyte

adhesion molecules [29]. We used IREM-1 transfected RBL as a myeloid cell model to evaluate the possible inhibitory function of IREM-1. Our data show that engagement of IREM-1 prevented gene transcription induced by signals delivered through an activating receptor (i.e. FcεR). This result is in line with previous reports showing that another member of the CMRF-35 family, the IRp60 receptor, delivers a negative signal [10].

Taken together, our data suggest that IREM-1 may be involved in functional regulation of myeloid cells. It is conceivable that together with other inhibitory molecules this receptor may contribute to keep a threshold of activation, counterbalancing the function of other triggering receptors of the same [16] or other families expressed on myeloid cells (i.e. FcR, TREMs, ILTs). In this regard, studies linking this gene family to psoriasis susceptibility [16] suggest that these receptors may have an important immunoregulatory role controlling inflammation. The identification of the IREM-1 physiological ligand, becomes an essential step to establish the biological relevance of this molecule in the regulation of myeloid cell functions.

MATERIALS AND METHODS

Cells and antibodies.

Human T (Jurkat, Molt-4), B (Ramos, Daudi and RPMI-8866) and myeloid cell lines (U937 and HL60) were grown in RPMI 1640/Glutamax medium supplemented with 10% heat–inactivated fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin (growing medium). COS-7 and rat basophilic leukaemia (RBL) cells were grown in DMEM with 10% heat–inactivated fetal calf serum, 2mM glutamine, 1mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin. The T γδ cell line T-ALL 103/2 was kindly provided by Dr. D. Santoli. (The Wistar Institute, Philadelphia, USA). PBMCs were obtained from heparinized venous blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Recovered PBMCs were washed with PBS and activated with 2 μg/ml phytohemagglutinin (PHA) and further expanded in IL-2 (200 U/ml) containing growing medium. Blood monocytes were isolated from PBMCs by adhesion to plastic for 1 hour at 37 ºC [22] ; in some experiments monocytes were cultured either alone or stimulated for 72 h with 1 μg/ml LPS (Sigma, St Louis, MO, USA). Immature dendritic cells were obtained by treatment of monocytes with 25ng/ml IL-4 (R&D Systems, MN, USA) plus 100ng/ml GM-CSF (Leucomax, Novartis) for 6 days and treated with LPS for additional 48 hours to achieve maturation. PE-conjugated anti-CD3, anti-CD19, anti-CD56, anti-CD14, anti-CD1a, anti-CD80 CD83 were from Becton Dickinson (Becton Dickinson, San Jose, CA, USA). Polyclonal activated NK and T cell populations were obtained from PBMCs as previously described [30]. Granulocytes were separated from heparinized blood of healthy donors by 3%-dextran 500 (Amersham) sedimentation, followed by hypotonic lysis of erythocytes. Anti-HA monoclonal antibody 12CA5 was described before [12].

Anti-SHP-1 rabbit serum was obtained from Santa Cruz (Santa Cruz Biotechnology, Inc.,CA, USA). Anti-DNP IgE mouse antibody was purchased by Sigma. Phosphotyrosine-specific monoclonal antibody directly conjugated to horseradish peroxidase (PY-7E1, PY-1B2, PY20) was from Zymed Laboratories, Inc., CA, USA. The anti-TREM1 mAb was kindly provided by Dr. M. Colonna (Washington University School of Medicine, St Louis, USA).

DNA reagents

Yeast expression constructs pBridge/c-fyn$_{420,\ 531Y\text{-}F,\ 176R\text{-}Q}$/SHP-1, pBridge/c-fyn$_{420,\ 531Y\text{-}F,\ 176R\text{-}Q}$ and pBridge/SHP-1 and mammalian expression vectors encoding for human SHP-1 an c-fyn have been described [12]. For expression in COS-7 cells, IREM-1 (without signal peptide) was amplified by PCR and cloned into the BglII/SalI sites of pDisplay (Invitrogen Corp., CA, USA) with primers: 5' CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3' and: 5' GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GTA 3'. Tyr to Phe substitutions of wild type IREM-1 were generated by site-directed mutagenesis by PCR with primers containing the desired change. IREM–1 Y205F was generated by amplification of two fragments with the pair of primers: 5´CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3´ and 5´GGT CAG GTC TGC AAA GCA GAG GTC GCC CTC 3´, and 5´GGC GAC CTC TGC TTT GCA GAC CTG ACC CTG 3´and 5´GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GTA 3´and annealed at overlapping ends. After filling and amplification DNA was cloned in pDisplay vector. IREM Y249F was generated as described with the primers :5´CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3´ and 5´ GGT CAG AGA TGC AAA GGA AAT GTC CTC CTT 3´, and 5´ GAG GAC ATT TCC TTT GCA TCT CTG ACC TTG 3´ and 5´GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT

GTA 3´. IREM-1 Y284F was generated in one step PCR with the primers 5' CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3' and: 5' GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GAA TTC 3´. IREM-1 Y205F/Y249F double mutant was generated from the single mutant construct of IREM-1 Y249F by amplification of two DNA fragments with the pair of primers used previously to insert the Y205F mutation; all mutations were verified by sequencing **.**

Three hybrid-system assay

The three-hybrid screening was carried out transforming sequentially CG1945 yeast strain with pBridge $\text{Fyn}_{420,\ 531\text{Y-F},\ 176\text{R-Q}}$ containing SHP-1 first and then with 1 mg of the PHA activated PBMC cDNA library (BD Biosciences, Clontech, Palo Alto, CA, USA) as described [12]. To analyze IREM-1 interaction with SHP-1 three-hybrid assays were carried out by co-transforming CG1945 yeast strain with pGAD10/IREM-1 and pBridge, or pBridge/SHP-1, or pBridge/c-$\text{fyn}_{420,\ 531\text{Y-F},\ 176\text{R-Q}}$ or pBridge/c-$\text{fyn}_{420,\ 531\text{Y-F},\ 176\text{R-Q}}$/SHP-1. Transformants were plated in SD media supplemented with a -Trp, -Leu, -Met dropout. Clones were tested by β-galactosidase liquid culture assay using ONPG as a substrate as described before [31].

RT-PCR and Cloning strategy

Retro transcription of RNA from PBMCs, T cells, monocytes and NK cells was performed by incubating 1 µg of RNA with Oligo dT primer and AMV-retrotranscriptase enzyme (Promega Corp., Madison, WI, USA) for 1 h at 42 ºC and reaction was stopped heating sample 2 minutes at 95ºC. PCR reaction to amplify IREM-1 was developed with 5µg cDNA as template, using the pair of primers: 5´ AAC GGG GAC CTG TCT GAA G 3´and 5´GTC GAT GAG GCA GGA GTG TGC TCA CAG

3´. PCR conditions used were 94ºC 10 min, 94ºC 30 sec, 63ºC 30 sec, 72 ºC 1min (40 cycles), 72 ºC 10 min, in the presence of FastStart Taq DNA Polymerase (Roche). Nested PCR to clone IREM-1 Sv1 and Sv2 and a cDNA fragment of IREM-1 Sv3 was done using 1 µg of PHA activated PBMCs cDNA as template. First PCR round was performed with the primers 5´ CCA GAA CCA CTA GAA ATC ATT AGG ACC 3´and 5´ GTC GAT GAG GCA GGA GTG TGC TCA CAG 3´, mapping in the 5´UTR and the 3´UTR respectively. Second PCR round was done using the first PCR as template with the primers 5´ CCT AGA TCT ATG AGG GTC ATC AGT GCT AAG AGT 3´and 5´ GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GTA 3´. A cDNA fragment of IREM-1 Sv3 was cloned in a second PCR round with the primers: 5´ACA ATC AGA AAA ACC GCA CGT 3´ and 5´ TGC TGA TGG TGC TGT ATT CCG 3´. In both rounds PCR conditions were: 94ºC for 3 min, and 94ºC for 1 min, 65ºC for 1,5 min and 72 ºC for 3 min (30 cycles). PCR products were resolved in 1% agarose gels and visualized by ethidium bromide staining. Expected size fragments were cloned into a pCR2.1 cloning vector (Invitrogen) and sequenced with ABI PRISM Bigs Dyes Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, USA) and universal T7 primer.

Northern blotting

Total RNA was extracted from cells with Trizol reagent (GIBCO BRL) according to the manufacturer’s instructions. 10 µg of RNA/lane were formamide/formaldehyde denatured, resolved in an 1% denaturing agarose gel and transferred onto nylon filter (Nytran Super Change, Schleider and Schuell), as detailed elsewhere [32]. Probe was obtained by PCR amplifying of the cytoplasmic portion of IREM-1 (primers: 5´CCT CTG CTA TGC AGA CCT GAC CCT GCA 3´and 5´ GAC GTT TAG AAA ACC

CCA ACT CCT AG 3´ and labeled with [α-^{32} P] dCTP using the Random Primed DNA Labeling Kit (Roche). Filter was pre-hybridized and hybridized under standard conditions. Filters were subjected to auto radiography for 10 days at -80 ºC (Kodak films, Rochester, NY, USA).

Cell Transfection

COS-7 (10^7) cells were transiently transfected with DEAE-Dextran method and then lysed for immunoprecipitation assays as described before [12]. RBL cells were stably transfected with 20 μg of pDisplay/IREM-1 construct by electroporation at 280V and 950μF in a Gene Pulser Electroporator (BioRad, CA, USA) and selection with 1 mg/ml of G-418 (Invitrogen). For luciferase assays, luciferase reporter plasmid (0.5 μg/10^6 cells) 3XNFAT-luc [33] and 2XκB-luc [34], and TK renilla (0.1 μg/10^6 cells) (Promega) plasmid were transiently transfected in RBL/IREM-1 cells by electroporation (10^6 cells/point). Cells were lysed and luciferase activity measured according with the manufacturer's instructions (Promega).

Production of IREM-IgG2a fusion protein and anti-IREM-1 specific monoclonal antibodies

IREM-1 extracellular domain was amplified by PCR with the pair of primers 5´CGA AGC TTC GGC TAC TCC ATT GCC ACT CAA 3´/3´CTG GGA TCC TG GGT CTG CAT AGC AGA GGT CGC 5´ and cloned in the cassette pSec/IgG2a [35] between BamHI and HindIII restriction sites. CHO cells were stably transfected with this construct, positives cells were selected with 300 μg/mL of Zeocyn (Clontech). The chimeric protein was purified from the supernatant using a Protein A column, and collected fractions were verified by Coomasie blue staining and western blot. BALB/c

mice were immunized with IREM-IgG2a fusion protein following standard methods [36]. Hybridoma supernatants were screened by ELISA using IREM-IgG2a fusion protein and positive samples were further selected by FACS analysis of Jurkat/HA-IREM-1-transfected cells.

Flow cytometry analysis

IREM-1 expression on PBMCs was tested with UP-D1 and UP-D2 mAbs anti-IREM-1 mAbs by immunofluorescence and flow cytometry analysis (FACScan Becton Dickinson & Co, Mountain View, CA, USA) as previously described [13]. For two-color staining studies phycoerythrin (PE)-labeled anti-CD3, anti-CD14, anti-CD56 and anti-CD19 (Becton Dickinson) were used. FITC –labeled anti-CD15 was purchased from DAKO. CellQuest software (Becton Dickinson) was used for data analysis.

Pervanadate treatment, immunoprecipitation and western blotting

U-937 cells were biotinylated using EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) following manufacturer's instructions. When indicated, cells were incubated with 1 mM of sodium pervanadate for 15 min at 37ºC , lysed and immunoprecipitated as described [12]. Horseradish peroxidase conjugated secondary antibodies were from Amersham Pharmacia Biotech, and Streptavidin-HRP was from Roche. Blots were developed with West Pico Supersignal kit (Pierce, Rockford, IL, USA) and visualized on Hyperfilm (Amersham Pharmacia Biotech).

Luciferase assays

RBL/IREM-1 cells were transfected with a luciferase reporter plasmid and cultured in growing medium for 24 hours. Transfected cells were aliquoted in 12-well plates

(Corning, NY, USA), and stimulated with 10 μg/ml of plastic adsorbed purified anti-IREM-1 mAb or an Ig isotypic control, and 5 μg/ml of anti-DNP IgE (Sigma). Cells were lysed with 5X lysis buffer (Promega) 6 hours after stimulation. Dual Luciferase reporter kit (Promega) was used.

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IV. ARTICLE 2

The IREM-1 (CD300f) inhibitory receptor associates with the p85α subunit of phosphoinositide 3-kinase [1]

Running Title: IREM-1 recruits PI3K

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Keywords: Myeloid cells, PI3K , SHP-1, Human, leukocyte receptor.

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ABSTRACT

The IREM-1 (CD300f) inhibitory receptor displays five cytoplasmic tyrosine residues, two of them (Y205 and Y249) fit with immunoreceptor tyrosine-based inhibition motifs (ITIM), whereas Y236 and Y263 constitute putative binding sites for phosphoinositide 3-kinase (PI3K). In the present study, immunoprecipitation analysis revealed that both the p85α subunit of PI3K and SHP-1 could be recruited by IREM-1, in transfected cells as well as in the U937 monocytic leukemia cells which constitutively express the receptor. By assaying the ability of different IREM-1 mutants to regulate the secretion of β-hexosaminidase induced via FcRεI in rat basophilic leukemia (RBL) cells, both Y205 and Y249 appeared crucial for IREM-1-mediated inhibition. Remarkably, engagement of an IREM-1 mutant ($Y_{205,249,284}F$), that did not recruit SHP-1 and lost its inhibitory function, induced RBL cell degranulation. This effect was dependent on the recruitment of PI3K, requiring the integrity of Y236 and Y263, and was blocked by PI3K inhibitors (i.e. wortmannin and LY-294002). All together, these data reveal a putative functional duality of the IREM-1 myeloid cell receptor.

V. ANNEX

MATERIALS AND METHODS

Cells and cytokines

PBMCs were purified from human blood by standard gradient density centrifugation (Lymphoprep™, Axis-Shield PoC AS, Oslo, Norway). For isolation of monocytes, PBMCs were resuspended at a final concentration of $2x10^6$/ml in RPMI 1640 with 10% FCS and allowed to adhere to plastic for 60 minutes at 37ºC washing of non-adherent cells with PBS. Alternatively, monocytes were purified by magnetic sorting using CD14 microbeads (Milteny Biotech, Bergisch Gladbach, Germany), as described (106).

For differentiation studies, isolated monocyte-enriched cell populations were plated at $2x10^6$ cells/ml in 6 well plates, and then cultured for 6 days either in medium alone or in the presence of IL-4 (25ng/ml) (R&D Systems, MN, USA) and GM-CSF (100ng/ml) (Leucomax, Novartis) to induce differentiation to iMDDC; cells were further treated with LPS 1μg/ml for 48 hours to achieve maturation. Alternatively, cells were treated with GM-CSF alone to differentiate into naïve macrophages. Classical macrophages were obtained by treatment of naïve macrophages with INFγ (500 units/ml) for 48 hours, and alternatively activated macrophages were obtained by treatment of naïve macrophages with 1000U/ml IL-4 for 48 hours (107). The former studies were done in collaboration with Dr. Angel Corbí from CIB, CSIC, Madrid. For subsequent analysis, cells were detached with PBS 5mM EDTA at room temperature.

FACS analysis and antibodies

Anti-myc, anti-IREM-1 (UP-D1 and UP-D2), phycoerythrin (PE)-labeled IgG mAbs specific for CD86, CD80, CD14, CD1a and isotype matched control antibodies (Becton Dickinson) were used for FCM analysis. Single and multicolor stainings were performed as previously described (108, 109) (106), and samples were analysed either

in a FACScan, FACSCalibur flow cytometer with CellQuest software (Becton Dickinson Biosciences-BDB-, San Jose, CA), and EPICS-XL flow cytometer (Coulter Científica, Madrid, Spain), respectively.

Further analysis of IREM-1 expression were performed in bone marrow (BM) and peripheral blood (PB) samples from 6 adult healthy donors (6 BM and 4 PB samples) using a multicolor staining direct immunofluorescence method (collaboration of Drs A.Orfao and A. García Montero, Universidad de Salamanca). In these studies cell populations were identified according to previously described methods (109) as follows. Populations identified in BM samples: mature B cells , T/NK cells, basophils, eosinophils, lymphoplasmocytoid dendritic cells ($CD123^{+++}$, $HLA\text{-}DR^{+}$), mast cells ($CD117^{+++}$, $CD45^{+}$), $CD34^{+}$ and $CD34^{-}$ B cell precursors, myeloid precursors, nucleated erythroid cells, $CD16^{+}$ monocytic dendritic cells, monocytic cells . Populations analyzed in peripheral blood samples were: B cells, T/NK cells, basophils, eosinophils, neutrophils (SSC^{high}, $CD16^{+}$, $CD14^{-/dim}$), lymphoplasmocytoid dendritic cells ($CD123^{+++}$, $HLA\text{-}DR^{+}$, $CD16^{-}$, $CD33^{+}$), $CD16^{+}$monocyte/dendritic cells ($CD123^{dim}$, $HLA\text{-}DR^{++}$, $CD16^{+}$, $CD33^{+}$), myeloid dendritic cells ($CD33^{+++}$, $HLA\text{-}DR^{++}$) and monocytes ($CD14^{++}$, $CD33^{++}$).

RESULTS

IREM-1 expression along myeloid differentiation

Analysis of normal bone marrow samples with UP-D2 mAb indicated that IREM-1 is weakly expressed in CD34+ progenitor cells (Fig.1, Table I).

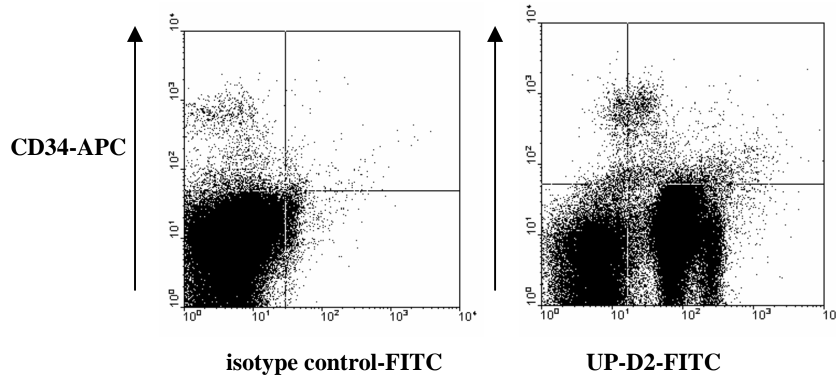

Figure 1. IREM-1 expression in bone marrow-derived CD34+ cells. Staining and FACS analysis of bone marrow cells was carried out with CD34-APC and IREM-1-FITC mAbs.

IREM-1 expression was also present in CD34+ B cell precursors becoming undetectable in CD34- CD45+ B cells, as well as in plasma cells. A weak staining with anti IREM-1 mAb (UP-D2) was observed in CD34+CD117+ precursors.

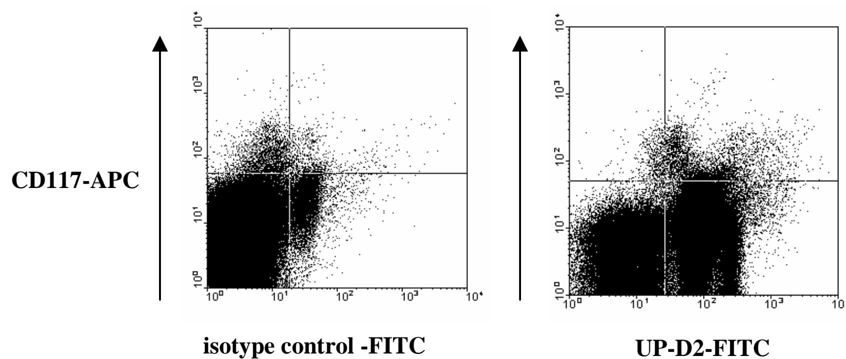

Figure 2. IREM-1 expression in bone marrow-derived CD117+ cells. Staining and FACS analysis was carried out with the CD117-APC and IREM-1-FITC mAbs.

IREM-1 expression is upregulated during myeloid differentiation to the monocytic lineage. As compared to the weak staining intensity of $CD34^+$ precursors, IREM-1 expression was increased in $CD64^+CD14^-$ precursors, being gradually upregulated as cells acquired CD14 and reaching maximal levels in mature monocytes and $CD16^+$ myelomonocytic cells.

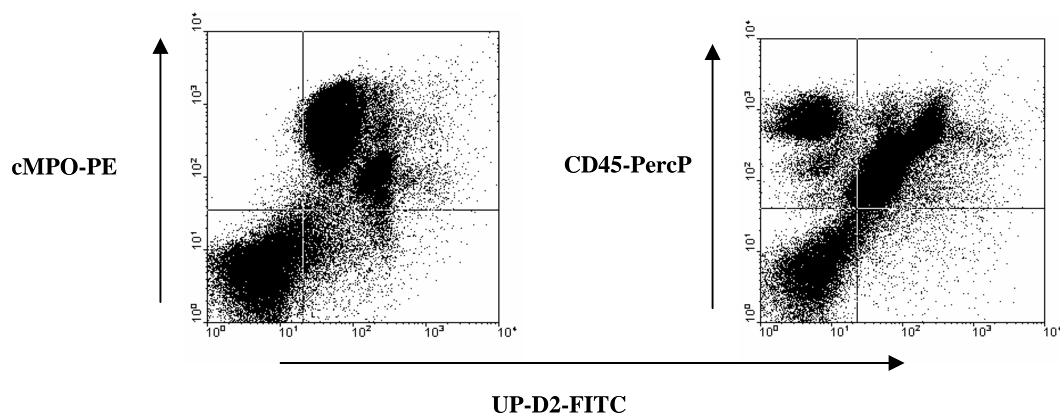

Figure 3. IREM-1 expression in neutrophils Cells were stained with UP-D2-FITC, CD45 PerCP and cMPO-PE mAbs .

Along maturation of the neutrophil lineage, IREM-1 expression also varied. A weak staining was observed in myeloblasts that increased in the transitional stages (CD34-CD117-/+), to promyelocyte CD34-CD117-SSC^{high}, and decreased in the stages of metamyelocyte (MFI 90-100) and mature neutrophil.

Table 1: IREM-1 expression in bone marrow-derived cell populations

Cell Population	*#1*	*#2*	*#3*
CD34+ precursors	+	+	+
B lymphocytes	–	–	–
Plasma cells	–	–	–
T lymphocytes	–	–	–
NK cells	–	–	–
Eosinophils	++	++	++
Mastocytes	+	+	+
Basophils	+	+	+
Lymphoplasmacytoid dendritic cells (CD123^{+++}/DR^{++})	+	+	+
Myelomonocytic CD16^{+} cells	++	++	+++
Myeloid dendritic cells CD33^{+++}/DR^{++}	++	++	++
Neutrophils	++	++	++
Monocytes	++	++	++
CD117+ erythroid precursors	+	+	+

Flow cytometry analysis of IREM-1 expression was carried out in bone marrow samples from different donors (n=3). Cell populations were defined as described in Methods. Relative staining intensity is represented. We arbitrarily express intensity as : + (MFI <75), ++ (MFI between 100-200) and +++ (MFI>240)

IREM-1 expression in peripheral blood leucocytes

Different peripheral blood cell populations were analyzed by flow cytometrry to study IREM-1 expression using the UP-D2 mAb. IREM-1 was comparably detected at the highest levels in mature monocytes, including $CD14^{+}$ and $CD16^{+}$ cells. By contrast, it was expressed at lower intensity by $CD33^{++}$ dendritic cells, being undetectable in lymphoplasmacytoid dendritic cells. The dull IREM-1 expression in basophils and neutrophils observed in BM samples was also confirmed. Altogether the data further supported that IREM-1 is restricted to the myeloid lineage.

Table II. IREM-1 expression in peripheral blood leucocytes

Cell population	*#1*	*#2*	*#3*	*#4*
B Lymphocytes	–	–	–	–
T Lymphocytes	–	–	–	–
NK cells	–	–	–	–
Eosinophils	+	+	+	+
Basophils	N.T.	+ MFI 49	+ MFI 45	+ MFI 56
Lymphopasmacytoid dendritic cells $CD123^{+++}/DR^{++}$	–	–	–	–
Myelomonocytic $CD16^{+}$ cells	++ MFI 231	+++ MFI 313	+++ MFI 248	N.T.
Myeloid dendritic cells $CD33^{+++}/DR^{++}$	+ MFI 77	++ MFI 134	++ MFI 165	++ MFI 129
Neutrophils	+ MFI 48	+ MFI 58	+ MFI 75	+ MFI 67
Monocytes	++ MFI 173	+++ MFI 244	+++ MFI 257	+++ MFI 247

Flow cytometry analysis of IREM-1 expression was carried out in peripheral blood samples from different donors (n=4). Cell populations were defined as described in Methods. Relative staining intensity is represented; numbers in brackets correspond to the mean fluorescence intensity (MFI). We expressed the staining intensity as arbitrary units as follows: + (MFI <75), ++ (MFI between 100-200) and +++ (MFI>240).

IREM-1 expression in monocyte-derived macrophages and dendritic cells

IREM-1 was expressed at high levels by peripheral blood monocytes, being maintained during differentiation to classical activated monocyte-derived macrophages and slightly downregulated in alternatively activated macrophages (Figure 4).

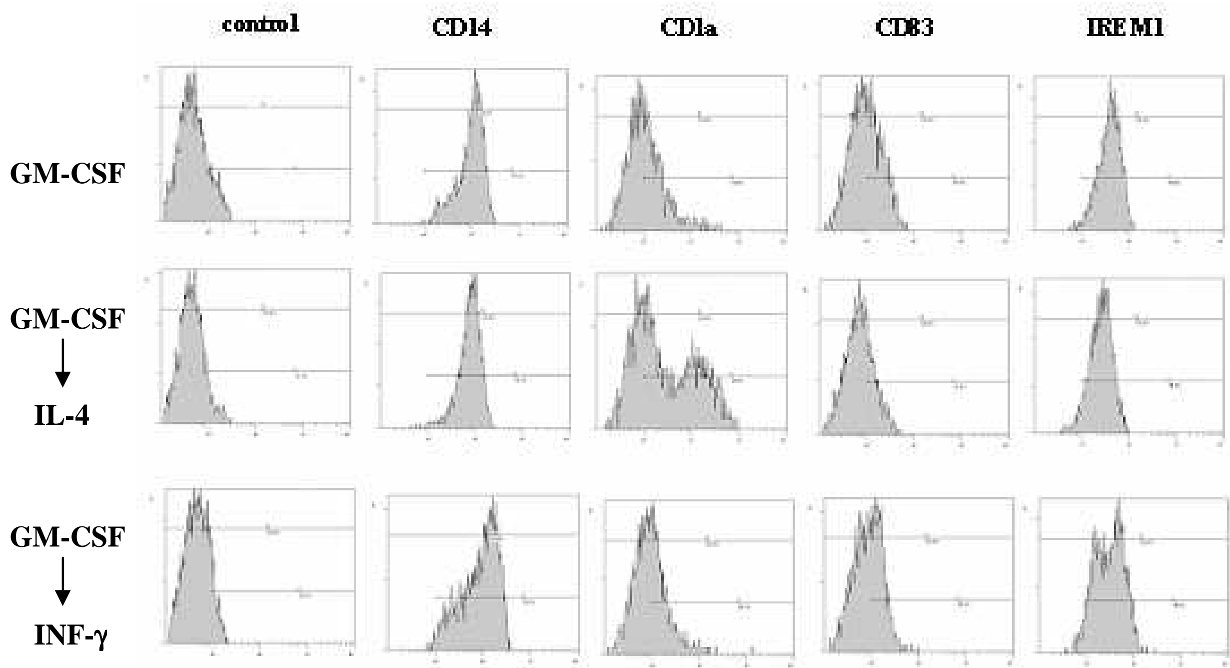

Figure 4. IREM1 expression in monocyte-derived macrophages. Macrophages were differentiated with different cytokines from peripheral blood monocytes as described in Methods. Flow cytometry analysis was performed with the indicated mAbs.

By contrast, IREM-1 expression was markedly downregulated in monocyte-derived immature dendritic cells (Fig. 5), and was not re-expressed after inducing their maturation by LPS treatment, which was monitored by the induction of CD86 expression.

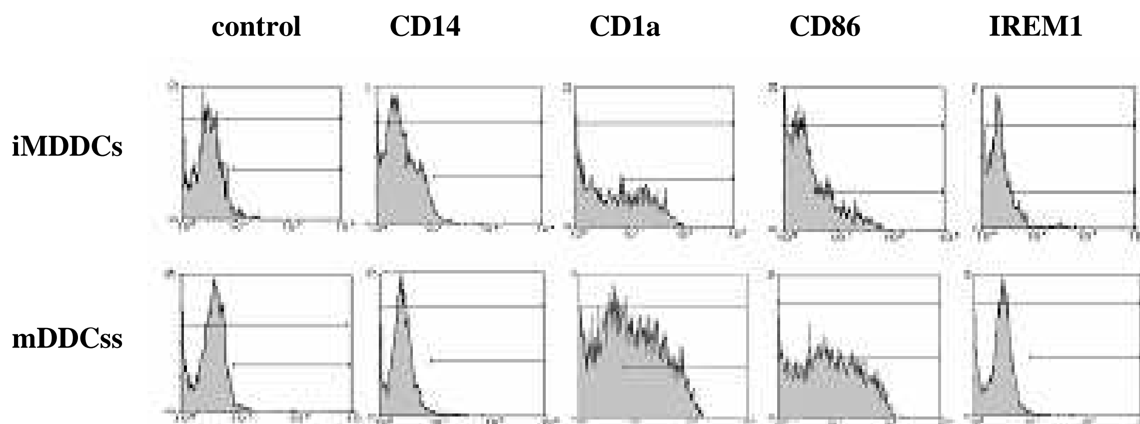

Figure 5. IREM1 expression in monocyte derived-dendritic cells (MDDC). Dendritic cells were differentiated from peripheral blood monocytes as described in Methods. Flow cytometry analysis was performed with the indicated mAbs.

Taken together, our data indicate that IREM1 is a myeloid-restricted molecule, that is expressed at variable intensity levels by different cell populations. IREM-1 was undetectable in all lymphoid lineages and was regulated during differentiation. Studies performed in collaboration with Dr. Angel Corbí, confirmed at the RNA level the results obtained for the expression of IREM-1 protein along *in vitro* differentiation of macrophages and dendritic cells. Further studies are required in order to asses IREM-1 expression in different human tissues, given the marked phenotypic and functional heterogeneity of macrophages and dendritic cells. In addition, further molecular studies

are required to confirm the expression and function of IREM-1 in cells stained at low intensity with the specific mAb, particularly CD34+ bone marrow progenitor cells, eosinophils, basophils and mast cells.

VI. DISCUSSION

DISCUSSION

A search for molecules interacting with the SHP-1 phosphatase depending on tyrosine phosphorylation was developed in our laboratory by Friederike Kitzig and Joan Sayós, using a three hybrid system in yeast to screen a PHA activated PBMCs library using the SH2 domains of SHP-1 as a bait. As a result, a sequence compatible with the cytoplasmic portion of a putative novel inhibitory receptor was found. With that material as a start point, our goal was the cloning and characterization of the full length molecule. This thesis describes a novel inhibitory receptor that belongs to the Ig superfamily, and that was termed Immune Receptor Expressed by Myeloid cells-1 (IREM-1). This receptor was predicted to be a type I transmembrane glycoprotein with a single V-type Ig domain, a transmembrane region, and a long cytoplasmic tail with five tyrosines in the context of several signaling motifs. The Ig-domain displays a single N-glycosylation site (NxS/T, being x any aminoacid except proline) at asparagine 87, and treatment of IREM-1 with N-glycosidase changed its electhophoretic mobility confirming such post-translational modification. Moreover, treatment of IREM-1 with an enzyme cocktail containing sialidase, β-1, 4 galactosidade, β-acetil N-glucosaminidase and hexosaminidase revealed O-glycosylation of the receptor.

IREM-1 showed high homology with several immunoreceptors and appeared related to a poorly characterized family called CMRF35 (65), recently renamed as CD300L (99). CMRF35 was the first member of this family that was cloned and gave name to the family (63). The CMRF35/CD300L gene family is located in human chromosome 17q.25, and includes at least 5 clustered genes encoding for different inhibitory and activating immunoreceptors.

The IREM-1 Ig domain shares 27% aminoacid identity (59 % homology) with the DAP12-associated activating member CD300e/IREM-2 (104), 34% identity (55%

homology) with the inhibitory CD300a/IRp60/CMRF-35H and 37% identity (57% homology) with CMRF35, a molecule of unknown function that bears a negative charge in its transmembrane region. This locus has been proposed to be linked to psoriasis susceptibility in humans, suggesting a possible role for the members of this family in inflammation or inflammatory diseases (65). The predicted allelic polymorphisms described so far in GeneBank, suggest that IREM-1 is a quite conserved molecule, as compared with other immune gene families such as ILTs or KIRs (110-112).

The murine ortholog locus was more recently described in the syntenic chromosome 11, and it consists of 9 members of the family called CLM (CMRF35 like molecules) (100). The first member of this family called CLM-1 was found by searching the GenBank EST database for sequences related to TREM-2 Ig domain (31% identity). The cytoplasmic domain of CLM-1 contains multiple tyrosine residues, including two that correspond to consensus ITIM motifs, a third that lies in a YxxM motif potentially associating to phosphatidylinositol 3'-kinase, and an ITIM-like motif (TxYxxI). Actually, CLM-1 shares 57% aminoacid identity (77% homology) with IREM-1 and represents its murine ortholog. Further searching revealed eight putative genes with homologous extracellular domains closely related to CLM-1.

CLM-2, CLM-4, and CLM-6 correspond to putative activating receptors as they display a transmembrane lysine residue, which allows their association with adaptor proteins such as DAP12. CLM-5 contains a short cytoplasmic domain and a negatively charged glutamic acid residue in its transmembrane domain, like human CMRF-35A, which may allow heterodimerization with another receptor or association with an adaptor molecule. CLM-8 might be ortholog to the human inhibitory CMRF-35H/IRp60 receptor. Even though the cytoplasmic tyrosine residues of CLM-8 cytoplasmic tails are conserved, it is unpredictable whether it functions as an inhibitory molecule, as

important adjacent residues have been modified. CLM-9 contains a single tyrosine residue that is not in the context of any known consensus motif for recruitment of signaling proteins. Only the inhibitory receptor CLM-1 has been fully characterized, and shown to be selectively expressed by myeloid cells. Moreover, it has been suggested to play a role in the inhibition of monocyte differentiation to osteoclasts in an experimental model using the murine macrophage RAW cell line (100). It is of note that the murine gene family has nine members whereas just five genes are present in human CD300L family which suggests a rapid evolution of this locus, as proposed for other gene families involved in innate immunity (83) .

Like its human counterparts, CLM molecules have a single V-type Ig extracellular domain, which appears to share homology with other immune receptors such as TREM-2 and NKp44 (100, 113). The amino acid sequence of CLM-1 shows conservation of the Ig domain cysteines and the adjoining consensus residues, as well as of two internal Ig domain cysteine residues that are shared by TREM-2, NKp44, and CMRF-35 receptors (114). Sequence alignment of the Ig-domains of the human proteins revealed that they have a sequence identity of approximately 30% that may point to related functions. As mentioned, it is of note that NKp44, TREM-2 (but not TREM-1) and IREM molecules contain a second pair of extracellular cysteine residues, topologically located as the NKp44 second disulfide bridge (Cys37-Cys45), additional to the one required for conforming the Ig-domain. It has been hypothesized that despite the weak sequence similarity, both the Ig V-type domain and the 37-45 disulfide bridge signature are shared by all the above mentioned receptors, and suggests that they might constitute a discrete evolutionary group stemming from a common ancestral V-type Ig domain gene. With respect to the above considerations, it should be pointed out that NKp44 and TREM2 are encoded in human chromosome 6p21.1, whereas the CMRF35 molecules

including IREM-1 are encoded in human chromosome 17q25.2. (114). It is also conceivable that this particular structure shared by IREM-1 with TREM-2 and NKp44, is involved in receptor-ligand interactions, and that these receptors could form a subgroup of innate immune receptors with similar binding properties and/or related ligands, as proposed for Siglecs (115).

Our first data showed that IREM-1 expression is restricted to the myeloid lineage at the mRNA and protein level. A wide range of cell functions related to differentiation, growth and survival, adhesion, migration, phagocytosis, activation, and cytotoxicity are modulated in myeloid cells by surface inhibitory receptors (116), suggesting that IREM-1 would also participate in the regulation of this cell lineage. In order to study the expression of IREM-1, we generated two specific mAbs (UP-D1 and UP-D2), and analyzed in detail IREM-1 surface expression both in peripheral blood and bone marrow samples in collaboration with Drs. Alberto Orfao and Andrés García-Montero (University of Salamanca).

IREM-1 is detectable in CD34+ bone marrow precursors, and its expression varies during hematopoietic differentiation. Whether IREM-1 is expressed by the pool of uncommitted CD34+ cells, or is present in the earliest stages of CD34+ myeloid-committed population remains to be determined. Information about normal maturation pathways of different myeloid cell lineages (109), especially for those less represented in the bone marrow is still limited. To the date, there is not a single specific marker that would identify commitment of hematopoietic precursors into any of the myeloid lineages (109, 117). CD117 is considered, together with CD13 and CD33, the earliest antigen to be detected during differentiation of hematopoietic precursors into myeloid cells (117-119). IREM-1 is weakly but detectably expressed by immature CD117+ cells and its expression increases as cells differentiate into the myeloid compartment and is

maintained in all mature myeloid cell types. At present, cytoplasmic expression of myeloperoxidase (MPO), lisozyme, and tryptase are considered as the most characteristic markers of myeloid cells, but they are restricted to a few myeloid lineages (120-122). According to this, MPO and lisozyme reactivity is found in granulomonocytic precursors whereas tryptase reactivity is linked to maturation into mast cells and basophils (109). Finally, CD14 and CD15 are expressed at the surface of mature monocytes and neutrophils respectively, even though when these markers are co-expressed during maturation into both myeloid lineages (117). IREM-1 has been detected at different intensity levels in all myeloid lineages namely monocytes, granulocytes, basophils, mast cells and eosinophils. Cells expressing IREM-1 at the highest intensity correspond to peripheral CD14+ monocytes and CD16+ myeloid dendritic cells.

Monocyte recruitment to tissues is elicited by metabolic and pro-inflammatory immune stimuli. After recruitment, differentiation into macrophages and DCs occurs, thus contributing to host defense, and tissue remodeling and repair (123). Although it is clear that monocytes are precursors of both macrophages and DCs, these differentiation pathways are still poorly studied *in vivo.* Dendritic cells can be differentiated *in vitro* by treatment of blood monocytes with IL-4 and GM-CSF (124, 125). In fact, the generation of DC from blood monocytes depends critically on IL-4, based on its capacity to inhibit macrophage colony formation (126). It has been previously demonstrated that IL-4 can regulate the expression of myeloid surface molecules, such as CD209/DC-SIGN (DC-specific ICAM3 grabbing non integrin). DC-SIGN is expressed only by a small population of blood CD14+ cells, and is strongly up-regulated after treatment of the cells with IL-4. (127). On the contrary, other molecules like TREM-1 display a different pattern, being expressed by human blood monocytes and neutrophils but absent on

dendritic cells (66). *In vivo*, not all types of macrophages display comparably expression of TREM-1. For instance, macrophages from lymph nodes or tonsils (>80%), that are subjected to repeated episodes of inflammation; express TREM-1 on the cell surface. By contrast, in the intestinal mucosa, the resident macrophage population contains only few TREM-1-expressing macrophages (<10%), as confirmed by FACS analysis and immunostainings of frozen tissue sections (128). The other member of that family, TREM-2 is expressed by monocyte-derived dendritic cells. DC maturation induced by LPS, TNFα, CD40L-expressing cells, IL-1ß, CpG oligonucleotides, or aggregated IgG, led to complete downregulation of TREM-2 (67).

Remarkably, IREM-1 expression was downregulated both in immature and mature monocyte-derived dendritic cells (iMDDC or mMDDC), differentiated *in vitro* by treatment of human blood monocytes with IL-4 and GM-CSF. By contrast, no downregulation of IREM-1 was observed after treatment with GM-CSF alone (naïve macrophages), GM-CSF plus LPS or IFNγ, or just LPS. Treatment of monocytes with these stimuli results in a phenotype that corresponds to the so-called M1 or classical activated "killer macrophages". The M1 macrophages are characterized by having high iNOS levels and producing large amounts of NO (129, 130). M1 macrophages are generated under the action of INFγ produced by Th1-type T-lymphocytes and NK cells. This cytokine is essential for the production of antimicrobial products by macrophages, as well as for the up-regulation of MHC class II molecules (131). On the other hand, M2 "healer macrophages" (also known as "alternative macrophages") are differentiated by the mediators of Th2-type responses like IL-4 or IL-13. These cells have been proposed to have a role in humoral immunity and repair (107., 132). Downregulation of IREM-1 both at the mRNA and protein level was observed after differentiation of monocytes into M2 "alternative macrophages", upon treatment of naïve macrophages with IL-4. It

is conceivable, that some inhibition of IREM-1 expression could be mediated in a direct or indirect way by IL-4 as was also suggested by downregulation in *in vitro* differentiated MDDCs. The presence of IREM-1 in hematopoietic progenitors opens the question as to whether this receptor might play a role in regulation of myeloid differentiation. It has been reported that engagement of other ITIM bearing receptors i.e. CD33 inhibits the differentiation of monocytes and CD34+ progenitors into dendritic cells (133). The possibility that IREM-1 might have a role in regulating myeloid differentiation should be addressed in the future.

Taken together, the data show that IREM-1 is widely expressed within the myeloid compartment at different stages of differentiation and its expression can be regulated by cytokines.

IREM-1 was first identified by its ability to recruit the SHP-1 phosphatase in a tyrosine phosphorylation dependent manner. In the U937 monocytic cell line, SHP-1 associated to IREM-1 after treatment of cells with sodium pervanadate. We demonstrated that IREM-1 is able, after engagement with a monoclonal antibody, to deliver a negative signal over the ITAM-bearing FcεRI in a heterologous system (i.e. RBL-2H3 cells). Yet, the activating receptor(s) that could physiologically be inhibited by IREM-1 remain to be defined. IREM-1 was shown to be weakly expressed both by human basophils and mast cells, and it is conceivable that inhibition mediated by this receptor could have some relevance in the regulation of allergic inflammatory responses driven by FcεRI. In this regard, IRp60/CD300a, the other inhibitory member of the family, was recently shown to mediate inhibition of IgE-dependent degranulation in human mast cells. Engagement of IRp60 with immune-complexes also inhibited stem-cell factor induced survival, involving a mechanism dependent on tyrosine phosphorylation, phosphatase recruitment and decrease of calcium influx (102). Cross-

linking of IRp60 on the surface of human eosinophils inhibited eotaxin-dependent transmigration of eosinophils in a calcium independent fashion, as well as the antiapoptotic effect of IL-5 and GM-CSF. Additionally, IRp60 crosslinking inhibited IL-5 mediated JAK2 phosphorylation, and also IL-5/GM-CSF mediated ERK 1/2 and p38 phosphorylation (103). In addition, it was previously demonstrated that IRp60 is able to recruit SHP-1 but not SHP-2 (101).

There is evidence that a number of ITIM-bearing receptors regulate the activation of cells that participate in the allergic responses *in vivo*. For example in the PIR-B null mouse, in which B cells are hyperresponsive to BCR-mediated activation, there is an augmented Th2 polarization in response to immunization and increased generation of IgE (134). It is conceivable that other ITAM-bearing receptors from myeloid cells such as FcRs, ILT, TREMs, or other IREMs could also be targets for IREM-1-mediated inhibition.

We mapped the interaction of IREM-1 with SHP-1 and found that a single tyrosine residue (Y205) appears to be the main docking site for this phosphatase. Different models for SHP-1 binding to ITIMs have been reported. In the case of the CD85j (ILT2) receptor, both biochemical and functional analyses revealed tyrosines 644 (SIYATL) and 614 (VTYAQL) as the SHP-1 docking sites required for ILT2 inhibitory function, and removal of both tyrosines was required to abolish SHP-1 binding (135). On the contrary, for Siglecs-7 and –9 that bind SHP-1 and SHP-2, site-directed mutagenesis revealed that only the membrane-proximal tyrosine motif is in both cases necessary and sufficient for the inhibitory function. However, the mutation of proximal or distal ITIM alone, or in combination, completely abrogated SHP-1 recruitment, whereas SHP-2 binding showed to be mediated only by the proximal motif (136). Negative signaling and SHP-1 activation was also shown for the inhibitory

receptor Siglec-5, even in the absence of tyrosine phosphorylation. Tyrosines to alanine mutants of siglec-5 ITIMs, but not in tyrosine to phenylalanine mutants, are still able to deliver inhibitory signals. These findings establish that Siglec-5 can be classified as an inhibitory receptor with the potential to mediate SHP-1 and/or SHP-2-dependent signaling in the absence of tyrosine phosphorylation; yet the physiological relevance of this finding is unclear. In the presence of tyrosine phosphorylation, the membrane proximal motif is dominant over the distal motif for interactions with protein tyrosine phosphatases (89).

IREM-1 Y205, which appears to be crucial for SHP-1 binding, constitutes the membrane proximal ITIM. However, mutation of this residue to phenylalanine was insufficient to abrogate the inhibitory potential of IREM-1. To further elucidate the role of individual tyrosines in IREM-1 function, we performed IgE-dependent degranulation experiments using the RBL-2H3 cell line, expressing wild type or tyrosine to phenylalanine mutants. IREM-1 displays five cytoplasmic tyrosine motifs, all of them in the context of different signaling consensus sequences. Y205 and Y249 match with ITIMs (1), whereas Y236 and Y263 constitute YxxM canonical motifs that could mediate interaction with the SH2 domains of the p85 regulatory subunit of PI3 kinase (137). Coincidentally, Y263 also fits to the YxN motif for association with the SH2 domain of Grb2 (growth factor receptor-bound protein 2) (137). Finally, Y284 could represent an ITIM-like motif or an endocytosis signal (Yxxϕ), where ϕ is an amino acid with a bulky hydrophobic side chain (138). This motif, also present in some CD33-related Siglecs like Siglec-10 (139), has been erroneously proposed as a possible binding site for Slam Associated Protein (SAP). Actually, the SAP binding motif has been described as TIYxx(V/I) (140). However, IREM-1 presents instead of the isoleucine residue at –1 position, a negatively charged aspartic acid residue which

disrupts this motif. Consistently, we did not detect SAP association to IREM-1 using a three hybrid system assay in yeast.

It is conceivable, that IREM-1 may play different roles in the physiology of myeloid cells, depending upon the stimulus and selective phosphorylation of cytoplasmic tyrosine residues. We found that both Y205 and Y249 participate in IREM-1 mediated inhibition, since single mutation of any of them abolished its inhibitory function. Actually, to fully abrogate IREM-1-mediated negative regulation of FcεRI, elimination of all Y205, Y249 and Y284 was required. These data suggest that Y284 may also participate in the inhibitory function of IREM-1. A possible role for this tyrosine residue may be the regulation of the state of phosphorylation of other tyrosines in the cytoplasmic tail of IREM-1, as it has been described for other inhibitory receptors. For instance, upon ligand engagement, CD19 becomes phosphorylated in residue Y513 by the Src kinase Lyn. Subsequently, Lyn binds phosphorylated Y513 through its SH2 domain and phosphorylates residue Y482. This residue binds a second Lyn molecule, which leads to Lyn transphosphorylation and autophosphorylation. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification (141). It cannot be ruled out that other molecules might also contribute to IREM-1 inhibitory function. In this regard, it has been described that IREM-1 interacts with the inositol-phosphatase SHIP-1 both in transfected cells and in U-937 myelomonocytic cells (142). Despite published data, thus far we have been unable to co-precipitate IREM-1 and SHIP-1 and to confirm the possible contribution of this interaction to IREM-1 function. In addition, other molecules might also contribute to the inhibitory activity of IREM-1. In this regard, inhibitory receptors LAIR-1 and CD85j have been shown to interact with Csk, that could negatively regulate the Src kinase activity involved in ITIM phosphorylation and SHP recruitment (143, 144).

Studies are in progress to evaluate the possible recruitment of other mediators (i.e Grb-2).

As discussed above, the IREM-1 $Y_{205,249,284}F$ triple mutant that lost the inhibitory function still displayed cytoplasmic Y236 and Y263, both of which are in the context of the p85 recruiting motif YxxM. We demonstrated that IREM-1 is able to associate to p85, in a phosphotyrosine dependent-fashion. Both YxxM motifs are able to bind this molecule, and impairment of the interaction requires mutation of Y236 and Y263. The IREM-1 murine ortholog, CLM-1, conserves all the tyrosine residues present in IREM-1. Nonetheless, due to changes of adjacent aminoacids, the binding site for p85 and Grb-2 generated by Y263 (distal YxxM) in IREM-1 only constitutes a Grb-2 site (YxN) in CLM-1, as the methionine residue in position +3 has changed to threonine; moreover, CLM-1 did not interact with p85 in myeloid cells treated with sodium pervanadate (100). It remains to be determined whether IREM-1 and CLM-1 interact with Grb-2, and whether the adapter may compete with p85 for binding to the distal YxxM in IREM-1.

The pharmacological inhibitors of PI3 kinase, wortmannin and LY-294002 have been broadly used to analyze the implication of this enzyme activity in a number of biological processes (23). We observed that concentrations of both inhibitors known to block selectively PI3 kinase activity, were able to suppress IREM-1 mediated degranulation induced by engagement of IREM-1 $Y_{205,\ 249,\ 284}F$ triple mutant expressed in RBL cells.

Our results point out to the possibility that IREM-1 could deliver both activating and inhibitory signals. In this regard, it has been reported that CTLA-4 functions as a negative regulator in T cells able to bind SHP-2, but may also associate to p85 under certain conditions (145). Other receptors such as 2B4 also have the capacity of

delivering both negative and positive signals. In fact, 2B4 is able to bind SHP-1, SHP-2 or Csk as negative mediators, as well as p85 and SAP that trigger cell activation (146). It is conceivable, that different conditions could drive the response to inhibition, through recruitment to phosphatases, or to activation through the PI3 kinase pathway. The balance between activating or inhibitory signals can depend on the availability of the different participating mediators, i.e. tyrosine kinases, phosphatases and PI3K. Differential compartmentalization of signaling mediators might preferentially lead to activation of one or the other pathway. In addition, optimal signaling through the PI 3-kinase pathway depends on a critical molecular balance between the regulatory p85α and catalytic p110 subunits (147). IREM-1 could shift this balance through the recruitment of p85 to its cytoplasmic tail, without phosphorylation and activation of the catalytic subunit. Is also conceivable that qualitative/quantitative variations in the avidity of the interaction of IREM-1 with its ligand(s) and/or the co-participation of other signaling events could lead to the activation of diverse sets of kinases, promoting different phosphorylation states of the receptor.

The presence of IREM-1 in CD34+ progenitors and its capacity of triggering PI3kinase dependent pathways suggest that this receptor may have a role beyond the negative regulation of inflammatory processes in mature myeloid cells. The fact that PI3 kinase is a key element in the regulation of apoptosis and cell survival through activation of Akt/PKB pathway (148) suggest a putative role for IREM-1 in regulating differentiation and cell survival from immature progenitors to mature myeloid cells.

A key issue to understand the biological role of IREM-1, is the identification of its ligand(s). Inhibitory receptors are believed to recognize self ligands in order to prevent autoimmunity (29, 111, 149, 150). Some receptors (i.e. ILTs or KIRs) recognize MHC class I molecules regulating innate immune responses (151, 152). Siglecs

recognize widely distributed sialic acid residues on cell surface molecules (115). On the other hand, CEACAM-1 expressed by activated NK cells, T cells and neutrophils, is able to interact with itself as well as with other endogenous ligands (i.e.galectin-3) (153), and with pathogen derived molecules (i.e. meningococcal virulence-associated Opa proteins) (154). CEACAM-1 has been shown to deliver an HLA class I–independent inhibitory signal in NK cells (155).

The nature of IREM-1 ligand(s), remains to be determined. In order to approach this issue, we generated an IREM-1- mIgG2a fusion protein (108) to screen by immunofluorescence several hematopoietic cell lines, as well as transfectants expressing different class I HLA molecules. Preliminary data revealed that IREM-1, unlike other myeloid receptors such as ILT2 and ILT4, does not bind class I HLA molecules. On the other hand, we detected some binding of IREM-1 fusion protein to lymphoid tumor cell lines including the Jurkat T cell leukemia and the HLA class I-negative 721.221 B cell line. Myeloid cell lines (i.e. U-937, HL-60 or THP-1) were not stained by the IREM-1 fusion protein. As a complementary approach to search for the ligand, a chimerical protein consisting of IREM-1 extracellular and transmembrane domains fused to the TCR ζ-chain was generated and transfected into RBL cells. No activation was observed upon incubation of the IREM-1-ζ-chain transfectant with Jurkat or 721.221 cells using a reporter assay. The possibility that lack of activation may be due to the requirement of other complementary receptor-ligand interactions missing in the heterologous RBL system cannot be ruled out. CLM-1-dependent inhibition of osteoclastogenesis that occurs just by transfection of the molecule in mouse macrophage RAW cell line, without any other stimulation of the receptor strongly suggests the presence of an endogenous ligand in myeloid cells (100).

The identification of IREM-1 ligand(s) appears crucial in order to elucidate the physiological relevance of this molecule in the regulation of myeloid cell biology.

VII. CONCLUSIONS

CONCLUSIONS

1) IREM-1 is a novel Ig-like receptor belonging to the CMRF-35/CD300 family that associates to SHP-1.

2) IREM-1 expression is restricted to the myeloid cell lineage, and is detected in monocytes, and granulocytes, as well as in CD34+ hematopoietic precursors.

3) IREM-1 expression is lost in immature monocyte-derived dendritic cells (iMDDC) differentiated *in vitro* with IL-4 and GM-CSF, as well as in LPS-induced mature monocyte-derived dendritic cells (mMDDC).

4) IREM-1 expressed in RBL cells inhibits FcεR-dependent degranulation and activation of gene transcription.

5) The ITIM formed by Y205 constitutes the main docking site for SHP-1.

6) ITIMs formed by both Y205 and Y249 co-participate in the inhibitory function of IREM-1.

7) IREM-1 binds the p85α subunit of PI3K involving two YxxM motifs constituted by Y236 and Y263.

8) Engagement of an IREM-1 mutant binding p85α but not SHP-1 induced secretion in RBL cells. This response was blocked by PI3K inhibitors, thus revealing a putative functional duality of IREM-1.

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