Molecular and functional characterization of the immunoreceptors CD300d and CD300f

Caracterització molecular i funcional dels immunoreceptors CD300d I CD300f

Emma Comas Casellas

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE IMMUNORECEPTORS CD300d and CD300f

CARACTERITZACIÓ MOLECULAR I FUNCTIONAL DELS IMMUNORECEPTORS CD300d I CD300f

Programa de Doctorat en Biomedicina

Memòria presentada per Emma Comas Casellas per optar al grau de Doctora per la Universitat de Barcelona.

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“seria bonic si, per a cada mar que ens espera, hi hagués un riu per a nosaltres”

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Content

Abbreviations

Ab: antibody
AD: activating domain
ADCC: antibody-dependent cellular cytotoxicity
Ala, (A): alanine
AML: acute myeloid leukemia
APC: antigen-presenting cell
aPL: antiphospholipid antibodies
Arg, (R): arginine
Asp, (D): aspartic acid
BAFF: B cell activating factor
BD: binding domain
BMMCs: bone marrow-derived mast cells
cCHO: complete Chinese hamster ovary medium
CCR3: CC chemokine receptor 3
CD: celiac disease
CD: cluster of designation
CDC: complement-dependent cytotoxicity
cDMEM: complete Dulbecco's modified eagle's medium
CHO*:* Chinese hamster ovary medium
CLM: CMRF-like molecules
CNS: central nervous system
Co-IP: co-immunoprecipitation
cRPMI: complete Roswell park memorial institute medium
DAG: diacylglycerol
DAMPs: damage associated molecular patterns
DAP12: DNAX-activating protein 12
DCs: dendritic cells
DMEM: Dulbecco's modified eagle's medium
EAE: experimental autoimmune encephalomyelitis
EDN: human eosinophil-derived neurotoxin
ELISA: enzyme-linked immunosorbant assay
EPOR: erythropoietin receptor
ER: endoplasmic reticulum
ERKs: extracellular regulated kinases
FAK: focal adhesion kinase

FBS: fetal bovine serum

FcεRIγ, FcRγ: IgE-binding subunit gamma

FcεRIα: IgE-binding subunit alpha

FcεRIβ: IgE-binding subunit beta

Fc: crytallizable fragment

FITC: Fluorescein isothicyanate

GFP: Green fluorescent protein

Glu, (E): glutamic acid

GM-CSF: granulocyte-macrophages colony-stimulating factor

GPCRs: G-protein coupled receptors

HA: hemagglutinin

His, (H): histidine

hVps: human vacuolar protein sorting

Ig: immunoglobulin

IgA: immunoglobulin isotype A

IgC: immunoglobulin constant

IgE: immunoglobulin isotype E

IgG: immunoglobulin isotype G

IgM: immunoglobulin isotype M

IgSF: immunoglobulin superfamily

IgV: immunoglobulin variable

INF-γ: interferon-gamma

IP: immunoprecipitation

IREM: immune receptor expressed on myeloid cells

Irp60: inhibitory receptor protein 60

ITAMs: immunoreceptor tyrosine-based activating motifs

ITIMs: immunoreceptor tyrosine based inhibitory motifs

JNK: Jun N-terminal kinase

LB media: Luria Broth media

LB: lysis buffer

Leu, (L): leucine

LMIR: leukocyte mono-Ig-like receptor

LPS: lipopolysaccharide

mAb: monoclonal antibody

MAIR: myeloid-associated Ig-like receptors

MAPK: mitogen-activated protein kinase

MBP: Human Eosinophil-derived Major Basic Protein

MC: mast cell

MDIR: modular domain immune type receptors

MDSCs: myeloid-derived suppressor cells
Met, (M): methionine
MMPs: matrix metalloproteinases
MS: multiple sclerosis
NF-κB: nuclear factor-kappa B
NFAT: nuclear factor for activation of T cells
NK cells: natural killer cells
O/N: over night
ORF: open reading frame
pAb: polyclonal Antibody
PAMs: pathogen associated molecular patterns
PBMCs: peripheral blood mononuclear cells
PC: phosphatidylcoline
pDC: plasmacytoid dendritic cell
PE: phosphatidylethanolamine
PFA: paraphormaldehyde
Phe, (F): phenylalanine
PI: propidium iodide
PI3K: phosphatidylinositol-3 kinase
PIP3: phosphatidylinositol-3,4,5-trisphosphate
PKC: protein kinase C
PLCγ: phospholipase C gamma
PMA: phorbol 12-myristate 13-acetate
PPMS: primary progressive multiple sclerosis
PRLR: prolactin receptor
PRRs: pattern recognition receptors
PS: phosphatidylserine
PTB: phosphotyrosine binding
PtdIns: phosphoinositide lipids
PTP: protein tyrosine phosphatases
pTyr: phosphorylated tyrosines
PVDF: polyvinylidene difluoride
RA: rheumatoid arthritis
RPMI: Roswell park memorial institute medium
RRMS: relapsing remitting multiple sclerosis
RT: room temperature
RTKs: tyrosine kinase receptors
RT-PCR: real time PCR
SAP: antiphospholipid syndrome
SCF: stem cell factor

SDS-PAGE: polyacrylamide electrophoresis gel containing sodium dodecyl sulfate

Ser, (S): serine

SFKs: Src family kinases

SH2: Src homology 2

SHIP: SH2-containing inositol polyphosphate 5-phosphatase

shRNA: small hairpin RNA or short hairpin RNA

SLP: SH2 domain-containing leukocyte protein

SN: supernatant

SOCS-1: Suppressor of cytokine signaling–1

SP: sphingomyelin

TAM: tumor-associated macrophages

TCR: T-cell receptor

Th1 cells: type 1 T cells

Th2 cells: type 2 helper T cells

TIM: T-cell immunoglobulin and mucin

TLR: Toll-like receptor

TNFα: tumor necrosis factor alpha

Treg: regulatory T

Trp, (W): tryptophan

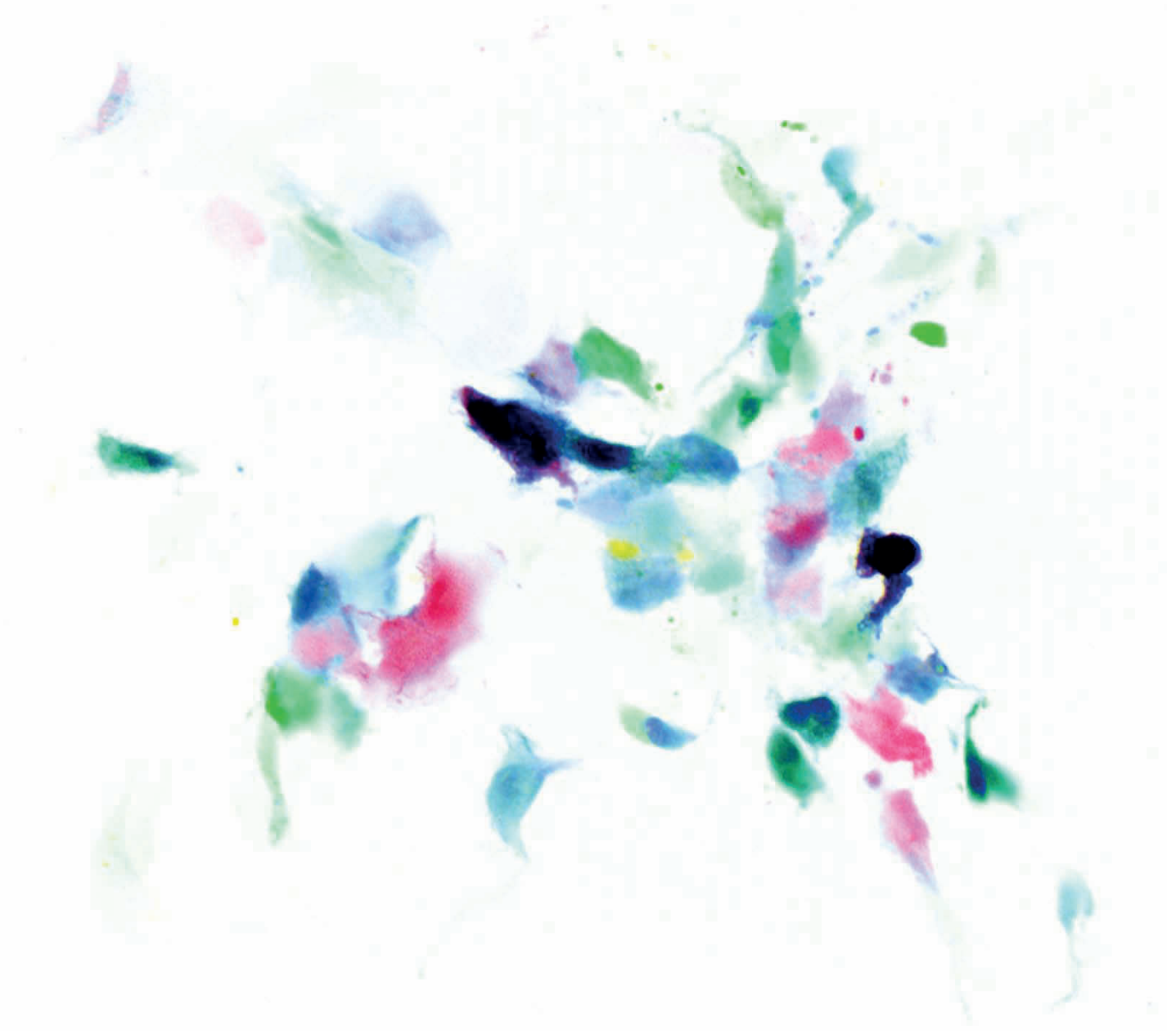
UAS: upstream activation sequence

UTR: untranslated regions

Val, (V): valine

VEGF: vascular endothelial growth factor

Introduction



IMMUNE SYSTEM

The immune system is a network of cells, and organs that work together to defend the body against exogenous attacks coming from bacteria, viruses, fungi, protozoa, and not microbial substance like allergens, irritants and toxic compounds among others. In simple terms, immune system can be organized in layers of increasing specificity, including mechanical, chemical and biological barriers. Physical and chemical barriers prevent pathogens from entering the organisms. However, if a pathogen breaches these barriers, the innate immune system provides an immediate, but poor-specific response. If pathogens successfully evade the innate response, a stronger third layer of protection, the adaptive immune system is activated [1].

It is indispensable that the immune system can differentiate the body's own cells and substance from the non-selves. Furthermore, within the own once, this system has to be able to discriminate between healthy signals and those produced by stressed, damaged or otherwise malfunctioning cells, which can do harmful effects [2].

Innate immunity

Innate immunity is an evolutionarily ancient part of the host defense mechanisms. During evolution, the innate immune system appeared before the adaptive immune system [3] and the same innate immunity molecular modules are found in plants and animals, meaning that it arose before the split into these two kingdoms [4].

Innate immunity operates through the intertwined action of several cell types, each playing non-redundant, and in most cases not fully understood functions during homeostasis and disease. In a general way, by using antimicrobial peptides, phagocytes and alternative complement pathway, the innate system controls the replication of the pathogens [2].

Among the cells that bear innate immune recognition receptors are not only macrophages but also: dendritic cells (DCs), mast cells, neutrophils, eosinophils, and natural killer cells (NK) [5]. The origin, location and relation between the different cell types from the immune system are summarized in (**Figure 1**).

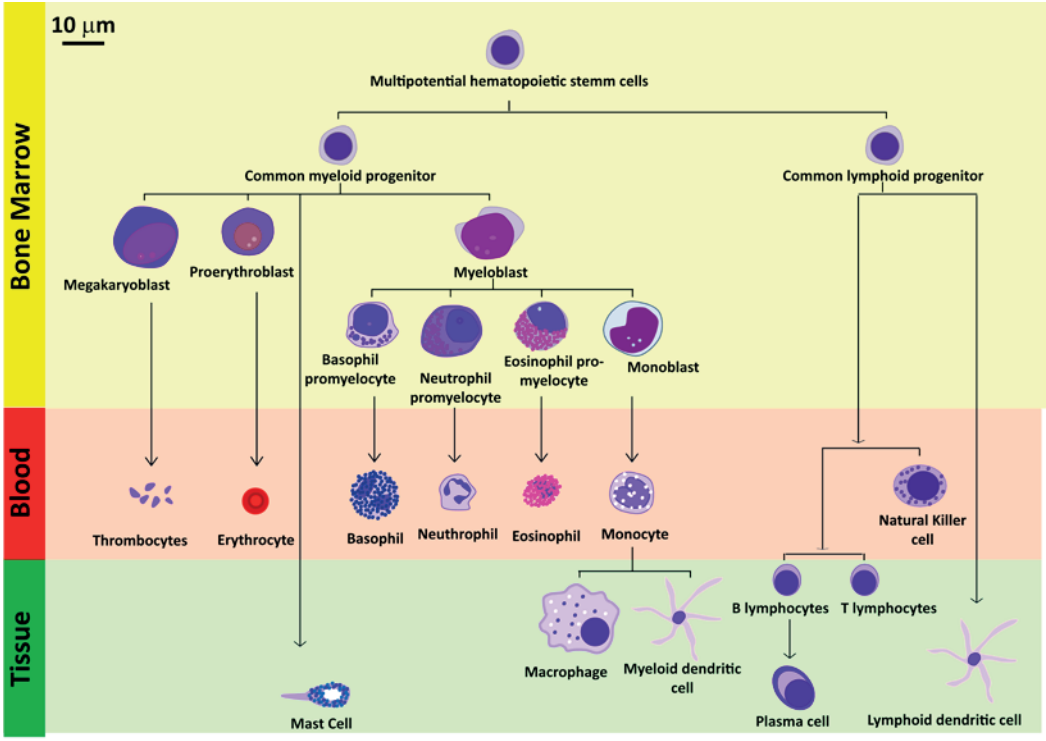


Figure 1: Hematopoiesis

Formerly, it was though that innate immunity recognized infective microbial in a non-specifically manner; however, in the mid-1990s it was proved the existence of specific receptors that have evolved to detect components of foreign pathogens. Those receptors were called pattern recognition receptors (PRRs) and are encoded in the germ-line, which means that the specificity of each receptor is genetically predetermined, allowing the action of natural selection on them but imposing a limit in the number or variety of the receptor in the organism. Using PRRs, macrophages serve as sentinel cells for the immune response.

On the other hand, the molecules recognized by PRRs are called pathogen associated molecular patterns (PAMs), which include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such bacteria, viruses, parasites and fungi. Apart from PAMs there are other type of molecules recognized by PRRs called damage associated molecular patterns (DAMPs), which come from the own body and include nuclear or cytosolic proteins expressed during cellular stress, to mention some [5].

Because microbial pathogens are extremely heterogeneous with a much higher rate of mutation than their hosts, the innate immune response uses two strategies:

- *Pattern-recognition strategy*, which is based on the recognition of PAMs and DAMPs through PRRs leading to an inflammatory response [6].
- *Missing self strategy*, which is based on the detection of molecular features (host gene products, host specific protein or lipid modifications) that are unique to the host and absent from the pathogens or any other foreign entity. This strategy involves the MHC class I molecules and its recognition by NK cells. Thus, the inhibitory killer immunoglobulin receptor (iKIR) of NK cells transduce a negative signal after recognizing the self-MHC class I molecule, that permits a resting state of the immune system. However, the absence or incomplete expression of a set of host MHC class I molecules will be sufficient to render a diseased or infected host cell susceptible to NK cell attack [7].

Adaptive immunity

Adaptive immunity is a potent defense against microbial infection and highly sophisticated system evolved in early vertebrates. It allows for a specific, strong immune response as well as immunological memory, where each pathogen is remembered by a signature antigen. The adaptive immune response is antigen-specific and requires the recognition of specific non-self antigens during a process called antigen presentation. The ability to mount these personalized responses is maintained in the body by memory cells [8].

The Toll-like receptor (TLR) family is the best characterized class of pattern recognition receptors (PRRs) in mammalian species. TLRs sense microbial infection and engage multiple mechanisms that control the initiation of not only innate immunity but also adaptive immunity responses [9]. Specifically, the generation of adaptive immunity begins in the peripheral tissues with DCs identifying microbial antigens though TLR. Subsequently, DCs mature, lose their capacity for endocytosis and migrate into the draining lymph nodes. This migration is mediated by TLR-induced downregulation of inflammatory chemokine receptors and upregulation of the receptors for lymphoid chemokines [6]. Once in the lymph nodes, DCs present microorganism-derived peptide antigens expressed on the cell surface with MHC class II antigen to naive T cells, initiating an antigen-specific adaptive immune response [10 11]. Subsequently, DCs provide the naive T cell with two signals required for their activation. The first signal is the antigen-specific signal received as a result of binding of the T cell receptor to peptide presented by the MHC molecule. The second signal is provided by costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86), which are expressed by the DCs and trigger CD28 expressed on naive T cells. Depending on the density of the peptides presented, types of costimulatory molecules expressed and cytokines secreted by the DCs, naive CD4+ T cells differentiate into either Th1 or Th2 cells. Th1 subset of CD4+ T cells secrete cytokines usually associated with inflammation, such as interferon γ (IFN-γ) and

tumor necrosis factor (TNF) and induce cell-mediated immune responses. The Th2 subset produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and is associated with humoral-type immune responses [12]. Primary activation of cytotoxic CD8+ T cells requires similar signals from the DCs as CD4+ T cells, whereas memory CD8+ T cell responses require CD4+ T cell help [13] [14].

Noteworthy, type I interferon family of cytokines are also essential in diverse processes of adaptive immunity, such as promoting the proliferation of memory T cells and preventing T cell apoptosis. Also, IFN-γ secretion, from CD8+ T cells in mice [15] and CD4+ T cells in humans [16], stimulate the activation of macrophages. On the other hand, IFN-α/β enable B cells to undergo isotype switching and differentiation into plasma cells through the activation of DCs [17]. Both, IFN-α/β are critical activators of NK cells [18] and also induce DCs maturation following stimulation via CpG, poly(I:C), LPS treatment or viral infection [19] [20].

MYELOID CELLS

The two main populations in myeloid lineage are macrophages and DCs, which display many common cell surface receptors but have distinct functional activities.

Monocytes and Macrophages

Undifferentiated monocyte and macrophages migrate through several body compartments, including bone marrow, blood, lymphoid tissues, and all non-hematopoietic tissues. Resident macrophages are present in organs constitutively, in the absence of overt inflammation, and perform trophic as well as homeostatic roles in the removal of apoptotic cells, serving as sentinels of injury and infection. Tissue macrophages can replicate locally, but are terminally differentiated, turning over at different rates, depending on the stimulus and tissue environment [21]. However, the lifespan of macrophages varies from hours to years depending on the nature of the immune response [22]. Furthermore, to cover a range of different functional roles during inflammation and its resolution, the activation of macrophages can be affected by huge types of substances, like: cytokines, metabolites, plasma proteins, and microbial ligands present in the inflammatory milieu.

The development of monocytes into mature and fully activated macrophages can be artificially divided into four successive stages [21]:

Differentiation

Macrophages colony-stimulating factor (M-CSF) or granulocytes-macrophages colony-stimulating factor (GM-CSF) cytokines [23], retinoic acids [24] and oxidized low density lipoproteins [25] induce the differentiation of the cells, from monocytes to macrophages.

Priming

Priming means preparation for the activation, it is a pre-activated state induced mainly by INF-γ [26], interleukin 4 (IL-4) [27]or IL-13 [28] in natural conditions. *In vitro*, apart from using these cytokines, it is also possible to use a chemical compound called phorbol 12-myristate 13-acetate (PMA) [29], both in cell lines or primary myeloid cultures. This state is possible because these stimuli *per se* are not very strong, but influence the inflammatory potential of macrophages and their response to other stimulus.

Activation

In these phase, macrophages reach mature functional phenotype in response to microbial and opsonic stimulus such as antibody complexes.

Resolution

This state of the macrophage is only possible if the immune cell survives its inflammatory task and consists in deactivation, which permits not only a loss in the pro-inflammatory potential of the cell but also produce functional changes, allowing the cell to clear debris and express general repair functions. Some cytokines as IL-10 [30] and TGF-β [31] are involved in these changes, and also a multitude of anti-inflammatory mediators such as nucleotides, lipoxins [32] and glucocorticoids [33].

Functional classification of Macrophages and the regulation of their functions

The functional capability of macrophages is affected by its localization in the body. Thus the main population of macrophages is found in the circulation and in the spleen. Alternatively, tissue-resident macrophages include: osteoclasts (inside the bones), microglia (in the brain and the CNS), alveolar macrophages (in the lung), histiocytes (in the interstitial connective tissue) and Kupffer cells (in the liver) [22] [34]. Furthermore, it is important to mention that other myeloid cells apart from microglia can be found in the CNS, for example infiltrating perivascular macrophages, meningeal macrophages and choroid plexus macrophages [35].

Apart from the body localization, the function of the macrophages also depends on the way they get activated, regarding that feel a variety of functional phenotypes have been described:

Classically activated M1 macrophages

M1 polarized macrophages are part of the afferent and efferent branch of Th1 immune responses, which mediate host defense and antitumor immunity [36]. This polarization is instructed by different molecules, some of which are INF-γ produced by natural killer and T cells; and selected cytokines like GM-CSF secreted by macrophages, T cells, mast cells, endothelial cells and fibroblasts; or TNFα produced mainly by activated macrophages. Also some microbial products, like lipopolysaccharide (LPS) between others PAMs and DAMPs, initiate signaling cascades after being recognized by PRRs, leading to INF-γ release which synergy with natural killer and T cells activity to polarize macrophages toward the M1 phenotype [36]. M1 macrophages have the capacity to produce high amounts of inflammatory cytokines IL-12, IL-23 and few amount of anti-inflammatory cytokine IL-10. The production of huge quantity of reactive oxygen and nitrogen intermediates is also a well-known characteristic of this phenotype. Altogether promotes inflammation and permits the recruitment of neutrophils, facilitating the killing of microbial pathogens, intracellular parasites and tumorigenic or infected cells [37] [38] [39] (**Figure 2**).

Alternatively activated M2 macrophages

M2 polarized macrophages are part of the Th2 immune activity, which orchestrates encapsulation and containment of parasites, promotes tissue repair by remodeling and favors tumor progression by suppressing immune response [36] [39]. This M2 programmed

phenotype is facilitated mainly by the cytokines IL-4 and IL-13, which resulted from STAT6 transcription factor activation in CD4+ type 2 helper T cells (Th2 cells). In addition to Th2 cells, a variety of innate IL-4 and IL-13-producing cells, such as basophils, nuocytes and natural helper cells, also may contribute to M2 polarization [40 41 42]. Although, apart from IL-4 and IL-13; IL-10, IL-21, GM-CSF, IL-33, glucocorticoid hormones and unique transcription factors regulate the differentiation of M2 cells [43 44]. In the contrary to M1 macrophages, M2 macrophages have the capacity to produce low amounts of IL-12 or IL-23 and high amounts of IL-10; also exhibit high levels of scavenger, mannose, and galactose-type receptors. Regarding tissue repair, M2 macrophages are rapidly recruited to wounds after platelet degranulation, due the response of macrophages to platelet derived growth factor [45]. Once there, macrophages can secrete a wide variety of cytokines and chemokines, as well as matrix metalloproteinases and their tissue inhibitors, that regulate the recruitment of cells and deposition of extracellular matrix components at sites of tissue injury [46].

On the other hand, macrophages with a M2-like phenotype also regulate important metabolic functions [47], such as maintaining of the adipocyte function, insulin sensitivity and glucose tolerance, which prevents the development of diet-induced obesity [48 49]. Some studies indicated that during obesity progression adipose-associated macrophages switch form an M2-like phenotype to a classically activated M1-like cells with potent pro-inflammatory activity [47].

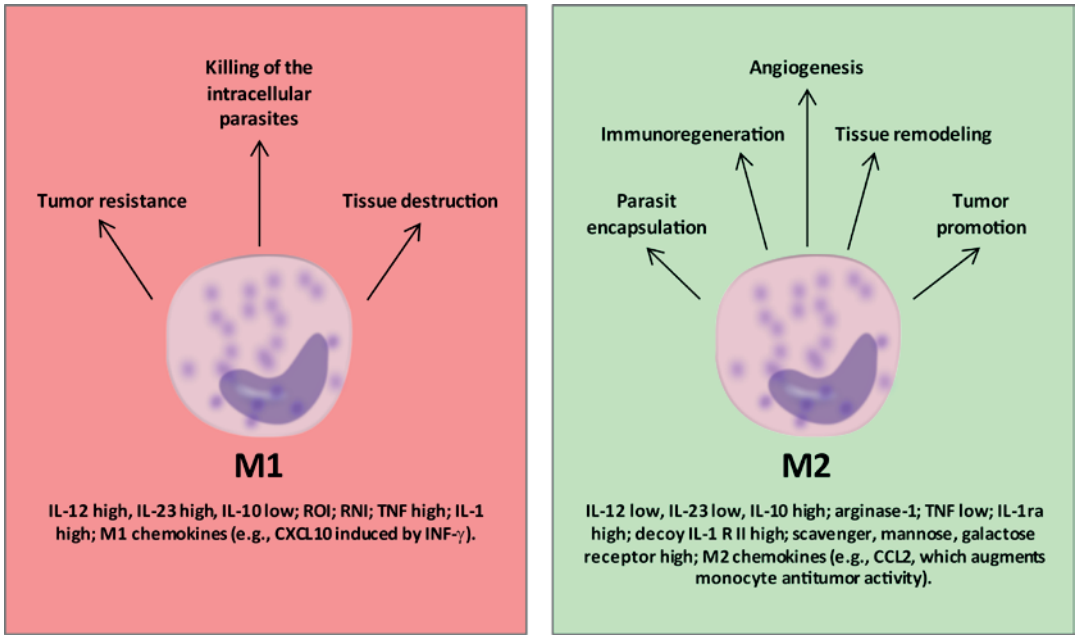


Figure 2: Key properties and functions of polarized macrophages to M1 and M2 phenotypes [39]. ROI and RNI indicate reactive oxygen and nitrogen intermediates.

Regulatory Macrophages

Regulatory macrophages population is defined by production of the immunosuppressive cytokines IL-10 and TGF-β1. This phenotype is similar to the suppressive M2 because its propensity to induce Th2 and regulatory T cells responses, but differs from the M2 because it is induced by Toll-like receptor (TLR) agonists in the presence of immunoglobulin G (IgG) immune complexes, apoptotic cells and prostaglandins. In terms of function, particularly adept at suppressing antimicrobial immunity and are poor antigen-presenting cells [50].

Tumor associated Macrophages

It is known that tumor growth is promoted by tumor-associated macrophages (TAM), a major leukocyte population present in tumors. Accordingly, in many but not all human tumors, a high frequency of infiltrating TAM is associated with poor prognosis because they suppress immunity against the tumor [51 52]. It has been reported that in established tumors, TAM have a skewed M2 phenotype, characterized by low production of IL-12 and high production of IL-10 [53].

In conclusion, distinct subpopulations of macrophages with unique functional abilities have been described, however it is thought that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations [36]. Thus, in general, macrophages exhibit two main and opposite phenotypes: they can induce host defense, antitumor immunity and inflammatory response or suppress these functions. This is why a fine regulation of its activation state is essential to maintain the homeostasis in all the body.

Immunologic abnormalities

Given the essential role of the innate immune system in regulating all aspects of immunity, it is conceivable that dysfunction of the components of innate immunity can contribute to diseases. Two general types of genetic alterations could lead to immunologic abnormalities; mutations that inactivate the receptors or signaling molecules involved in innate immune recognitions and mutations that render them constitutively active. The first type of mutations would be expected to result in various types of immunodeficiencies. The second type of mutations would trigger inflammatory reactions and could thus contribute to a wide variety of conditions with an inflammatory component, including asthma, allergy, arthritis, and autoimmunity [3].

MEMBRANE LEUKOCYTES RECEPTORS

Leukocyte membrane molecules enable cells to relay information from the external environment, via complex signaling pathways, to direct cellular processes that either maintain homeostasis or initiate a response. These membrane molecules include receptors for the recognition of pathogens (C-type lectins and scavenger receptors), receptors implicated in the phagocytosis of bacteria or apoptotic cells (scavenger receptors), receptors participating in the adhesion processes (integrins), receptors for cytokines and chemokines (Ig Superfamily receptors, tumor necrosis factor receptors, interleukin receptors), receptors involved in chemotaxis, receptors linked to antibody functions (Ig Superfamily receptors), among others [54].

The host's wellbeing relies on its ability to self-regulate the immune receptor responses using counterbalancing mechanisms to fine tune effector functions. The characterization of activating and inhibitory counterpart receptors on myeloid cells, as well as the identification of their physiological ligands, has provided important insights into the fundamental mechanisms of immunity and homeostasis.

Activating or inhibitory receptor isoforms are defined by residues within their transmembrane and cytoplasmic domains, while extracellular domains are responsible for ligand binding at the cell surface. Protein sequence motifs known as immunoreceptor tyrosine-based activating motifs (ITAMs) and immunoreceptor tyrosine based inhibitory motifs (ITIMs) are responsible for the activating or inhibitory signals transmitted by the myeloid receptors.

Activating receptors

Activating receptors have a short cytoplasmic tail and usually show a positively charged amino acid residue within their transmembrane region that allows their association with adapters (e.g., DNAX-activating protein 12 (DAP12), FcRγ, or CD3ζ) [55].

The canonical ITAM motif YxxL/Ix6-8YxxL/I (where x represents any amino acid) is now recognized to be present in the cytoplasmic domain of a huge number of receptors and transmembrane adapter proteins, where it serves as a critical link to downstream signaling cascades [56]. Upon receptor stimulation, the tyrosines embedded within the ITAM are phosphorylated by some member of the Src family kinases (SFKs), converting these residues in docking site for Src homology 2 (SH2) domain-containing proteins, primarily members of the Syk tyrosine kinase family and other adapter molecules [57]. Thus, the phosphorylation of ITAM's tyrosines is necessary and sufficient for the induction of downstream intracellular signals. Afterwards signal transduction cascade activates a number of well-known effector pathways

such us phospholipase Cγ (PLCγ), protein kinase C (PKC), phosphatidylinositol-3 kinase (PI3K), Ras, mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB, and nuclear factor for activation of T cells (NFAT) [55]. The final effectors regulate calcium mobilization, transcriptional activation, cytokine production, migration, proliferation and/or differentiation [58].

Activating mediators

Src family Kinases

The regulated activation of Src family nonreceptor tyrosine kinases (SFKs) by diverse growth factor, cytokine, adhesion, and antigen receptors is critical for generating an appropriate cellular response to a broad array of external stimuli [59] [60]. SFKs transduce signals for cell growth, differentiation, and survival; influence cellular adhesion, migration and invasion; and also regulate synaptic transmission [61]. Thus, loss of SFKs regulation has been linked to a variety of diseases, including several types of cancer [62] [63], neurodegenerative diseases [64] [65], epilepsy [66] , as well as HIV/AIDS [67].

The SFKs of the human genome includes eleven members [68], eight of which have been well-characterized in mammalian cells (Fyn, Lyn, Hck, c-Yes, Blk, Fgr, and Lck, in addition to c-Src itself). Most cell types express multiple SFKs. Some family members are ubiquitously expressed (e.g., c-Src, Yes, and Fyn), while others show more restricted patterns of expression (e.g., Hck and Fgr in myeloid leukocytes and Lck in T-lymphocytes) [69].

All the SFKs members share a conserved domain structure, consisting of consecutive SH3, SH2, tyrosine kinase SH1 domains and also an SH4 membrane-targeting region at their N-terminus, which is always myristoylated and sometimes palmitoylated [70] [71]. The SH4 region is followed by a unique domain of 50–70 residues, which is divergent among family members. A hallmark of SFKs is a short C-terminal tail, which bears an autoinhibitory phosphorylation site (Tyrosine 527 in Src) [72]. Like most protein kinases, SFKs members require phosphorylation within a segment of the kinase domain, termed the activation loop, for full catalytic activity. Contrary, the inactivating phosphorylation on Tyr 527 is carried out by the Src-specific kinase Csk [73]. Phosphorylation of the C-terminal tail promotes assembly of the SH2, SH3 and kinase domains into an autoinhibited conformation maintained by intimate interactions among these domains [74]. Schematic representation of the inactive and active conformations of Src family kinases is illustrated (**Figure 3**).

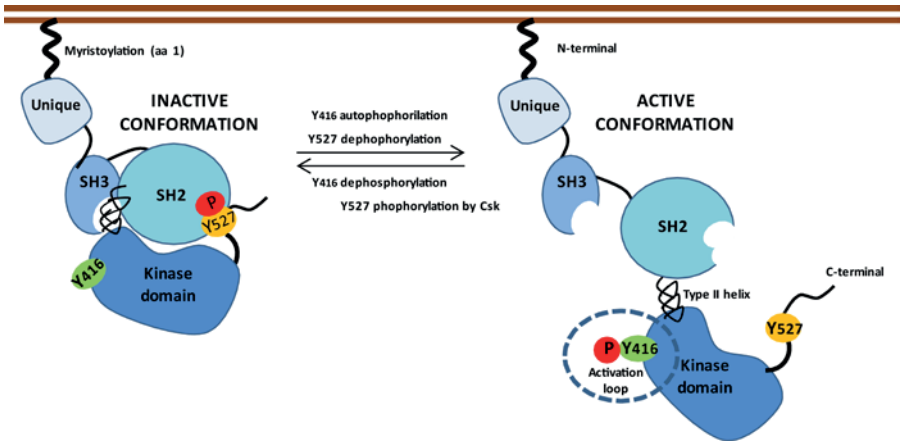


Figure 3: The inactive/active conformations of Src family kinases. The Src kinase architecture consists of four domains: the unique region, which varies among family members, followed by the SH3, SH2, and tyrosine kinase domains. The activating (Tyr 416) and autoinhibitory (Tyr 527) phosphorylation sites are indicated. The activation loop of the kinase domain contains the activating (Tyr 416). In the autoinhibited form of Src kinases, the SH2 domain binds the phosphorylated C-terminal tail, and the SH3 domain binds the linker segment between the SH2 and kinase domains, which forms a polyproline type II helix.

Syk kinase

Syk is a 72 kDa non-receptor tyrosine kinase that contains a C-terminal kinase domain and two N-terminal SRC homology 2 (SH2) domains that bind phosphorylated ITAMs. The linker region, between the SH2 domains and the kinase domain, was designated interdomain B and contains multiple tyrosines. These tyrosines, when phosphorylated, act as docking sites for proteins which might be substrates for Syk [75] . Other tyrosine residues beyond the catalytic domain in C-terminal region, when mutated to phenylalanine, give rise to gain of function [76]. Schematic representation of the inactive and active conformations of Syk is illustrated (**Figure 4**).

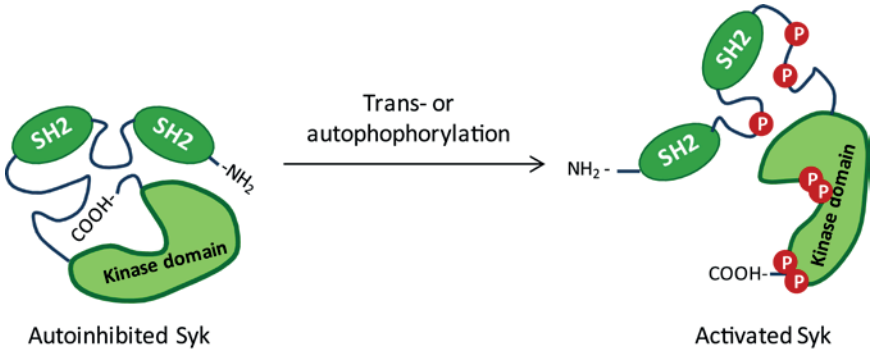


Figure 4: Suggested globular structure of autoinhibited (left) and activated (right) Syk with some phosphorylated tyrosines indicated [77].

Syk is most highly expressed by hematopoietic cells; however mammals also express a Syk homologue, Zap-70, which is mostly restricted to T cells and natural killer (NK) cells. Syk-related kinases are also found in invertebrates. Furthermore, whereas the activation of Zap-70 by immune receptors requires Src-family kinases, Syk can function in a Src-family kinase-independent manner. This difference might be due to the ability of Syk, but not Zap-70, to induce phosphorylation of ITAMs independent of Src-family kinases [78].

Several experiments illustrate the essential role of Syk in degranulation, leukotriene production and release of cytokines. NFAT activation and stimulation of the extracellular-signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) protein kinase pathways were also Syk dependent [77]. By contrast, Syk was not required for the phosphorylation of the ITAMs of the receptor or for the activation of Lyn. These results strongly support the model of immune receptor signaling that involves a sequential activation of kinases. The model proposes that engagement of the receptor leads to activation of a Src-family kinase, which phosphorylates the ITAMs of receptor- associated proteins. The phosphorylated ITAMs then bind a Syk- family kinase leading to its activation and the eventual propagation of signals to downstream pathways [79]. Furthermore, recent studies indicate several non-immune functions of ITAM-Syk signaling. Syk, which is activated downstream of DAP12 and FcRγ in an ITAM-dependent manner, is required for osteoclast development and function [80] [81], important for several platelet functions [82] and required for the separation of lymphatic vessels from the general circulation during vascular development [83].

Of the several intermediate molecules implicated in relaying Syk- or Zap-70-mediated downstream signaling, Vav family members, phospholipase Cγ (PLCγ) isoforms, the regulatory subunits of phosphatidylinositol-3 kinases (PI3Ks) and the SH2 domain-containing leukocyte protein (SLP) family members SLP76 and SLP65 can directly associate with Syk and/or Zap-70. In addition to the diverse roles of Syk in basic biological processes, it is also involved in the pathogenesis of several human diseases, including allergy, autoimmunity and various hematological malignancies [57].

PI3K

Eight mammalian isoforms of phosphoinositide 3-kinases (PI3Ks) exist and they are grouped into three classes on the basis of their substrate specificity and structure (class I, II and III). All isoenzymes possess the 'PI3K core', consisting of three domains: the C2, the helical and the catalytic domains [84]. In response to cell stimulation by growth factors and hormones, PI3Ks specifically catalyze the phosphorylation of the 3-position of the inositol ring of selected phosphoinositide lipids (PtdIns) [85]. The lipid products of PI3Ks can act as second messengers within the cell by activating several proteins through regulation of their intracellular localization or conformational changes. These proteins, in turn, control many intracellular functions such as cell proliferation, survival, migration, glucose homoeostasis and membrane trafficking [86]. Deregulation of PI3K-dependent cellular pathways is associated with several diseases, including cancer and diabetes [87] [86].

Class IA PI3Ks are heterodimers of one catalytic subunit (p110α, p110β or p110δ) and one regulatory subunit (p85) (**Figure 5**). The catalytic subunit contains the catalytic domain and a Ras-binding domain. The subunit p85 contain SH2 domains, which bind phosphorylated tyrosines (pTyr) in a specific amino acid sequence context. Class IA PI3Ks are mainly activated by tyrosine kinase receptors (RTKs) [84] and also by adapter molecules.

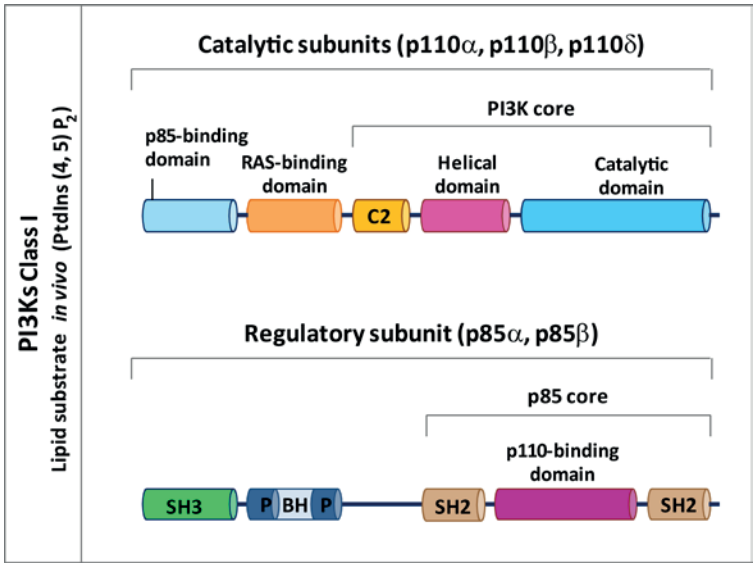


Figure 5: Schematic domain structure of the class IA PI3 kinases.

Whereas p110α and p110β are ubiquitously expressed, p110δ expression is low in most cells but highly enriched in leukocytes [88] [89] [90] and to a lesser extent in neurons [91].

Regarding the function of class IA PI3K in myeloid cells, it has been published that all the isoforms regulate Rac1 GTPase, whereas p110δ selectively regulates RhoA, the tumor suppressor gene PTEN and Akt activity in primary monocytes, after the stimulation through the colony-stimulating factor 1 receptor (CSF1R).Furthermore, p110δ is also involved in proliferation and chemotaxis in these cells [92]. In mast cells, it has been observed that the genetic or pharmacological inactivation of the p110δ isoform leads to defective stem cell factor (SCF) -mediated *in vitro* proliferation, adhesion and migration, and to impaired allergen-IgE induced degranulation and cytokine release; demonstrating the involvement of the p110δ isoform in allergenic responses [93]. In B and T cells, p110δ seems to be adapted to transmit antigen-receptor signaling [94] and to be important for the B cells homeostasis and function by regulating BCR-mediated calcium flux and activation of phospholipase Cγ2 [95]. Taking into account all the biological roles of p110δ, this isoform of PI3K is a good targets for the treatment of a range of autoimmune diseases such as Rheumatoid Arthritis, Systemic Lupus Erythematosus, Multiple Sclerosis or Psoriasis, reviewed in [96].

Class II PI3Ks were discovered on the basis of their sequence homology with class I and class III PI3Ks rather than in a functional context and their physiological roles are still poorly

understood. [84]. However, mammalian cell based studies have implicated class II PI3Ks in biological processes such as cell migration, glucose metabolism, exocytosis, smooth muscle cell contraction and apoptosis [86].

The class III PI3K human vacuolar protein sorting (hVps) 34 is a monomer which lacks the Ras-binding and the regulatory-subunit-binding domains and catalyzes specifically the synthesis of PtdIns3P. However, all known biological functions of Vps34 in mammals relate to the regulation of vesicle traffic, including autophagy, endocytosis and phagocytosis [97].

JNK

c-Jun N-terminal kinases (JNKs) are members of the mitogen-activated protein kinase (MAPK) family that includes the extracellular regulated kinases (ERKs) and p38 kinases. Three JNK genes (JNK1,-2, and -3) have been identified in humans; however, splice variants result in a total of 10 isoforms. JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 is restricted to the brain, heart, and testis [98] [99]. Mice lacking JNK1 or JNK2 exhibit deficits in T-helper (CD4+) cell function [100] [101], and double knockout animals are embryonic lethal [102]. The JNK3 knockout mouse exhibits resistance to kainic acid-induced apoptosis in the hippocampus and to subsequent seizures [103].

Regarding the cellular pathway, JNK is a serine threonine protein kinase that phosphorylates c-Jun [104], a component of the transcription factor activator protein-1 (AP-1) [105]. In complex with other DNA binding proteins, AP-1 regulates the transcription of numerous genes including cytokines [e.g., IFN- IL-2, and tumor necrosis factor (TNF)] [106], growth factors [e.g., vascular endothelial growth factor (VEGF)] [107], immunoglobulins (e.g., light chain) [108], inflammatory enzymes (e.g., COX-2) [109], and matrix metalloproteinases (e.g., MMP-13) [110].

PKC

Protein kinases C (PKCs) are a family of serine/threonine kinases, which mediates several signal transduction pathways and have been shown to regulate sets of biological functions as diverse as cell growth, differentiation, apoptosis, transformation and tumorigenicity [111] [112].

All PKCs have a common general structure with 2 principal modules: a NH2-terminal regulatory domain that contains the membrane- targeting motifs and a COOH-terminal catalytic domain that binds ATP and substrates. According to differences in the binding capability of their regulatory domain, the ten presently known members of the PKC family have been grouped into 3 classes: the classical PKCs (α, β1, β2, and γ) which are regulated by calcium, diacylglycerol (DAG) and phospholipids, the novel PKCs (δ, ε, and θ) which are regulated by DAG and phospholipids but lack the calcium binding domain, and the atypical subgroup (ζ, λ or i, the mouse ortholog of λ in humans) which are insensible to both calcium and DAG [113].

Adapter molecules

Grb2

Grb2 is an adapter protein ubiquitously expressed in all embryonic and adult tissues[114]. Grb2 has a single SH2 domain that binds preferentially to pYXNX motifs (where pY is a phosphorylated tyrosine, N is asparagine and X any residue) [115], which are found on a number of activated tyrosine kinase receptors (RTKs). The SH2 domain is flanked by two SH3 domains that bind proline and arginine-rich motifs in downstream effectors [116], as consequence Grb2 provides a critical link between cell surface growth factor receptors and Ras signaling pathway.

Apart from participating in a variety of RTK signaling pathways [117] (e.g., hepatocyte growth factor receptor, platelet derived growth factor receptor, etc.) Grb2 is implicated in nonreceptor tyrosine kinases, such as focal adhesion kinase (FAK) and Bcr/Abl, as well as substrates of tyrosine kinases. As such, it has been implicated in the oncogenesis of several important human malignancies. In addition to this function, research over the last decade has revealed other fundamental roles for Grb2 in cell motility and angiogenesis processes that also contribute to tumor growth, invasiveness and metastasis [114]

Grb2 is also involved in SHP-1 phosphatase inhibitory activity, on cytokine receptors as prolactin receptor (PRLR) and erythropoietin receptor (EPOR), by recruiting the suppressor of cytokine signaling–1 (SOCS-1). Grb2/SOCS-1 complex down-regulates Jak/Stat activation pathway [118]. In addition, Grb2 has demonstrated its importance in viruses entry and infection [119].

FcRγand DAP12

FcRγ and DAP12 are ITAM-containing signaling transmembrane adapter molecules. As mentioned before ITAM intracellular signaling pathway represents perhaps the dominant mechanism by which immune cells respond to their environment. Usually these adapter molecules coupled to the ligand binding receptor through charged amino acid interactions within the transmembrane regions of each protein (commonly aspartic acid in the ITAM-containing subunit and lysine or arginine in the receptor). All ITAM signaling adapters, including DAP12 and FcRγ, are small (15 – 20 kDa) proteins with short extracellular regions that contain a cysteine residue to allow the molecules to form homodimers though disulfide bonds. When the ITAM motif within the cytoplasmic region is dually phosphorylated provides a high affinity binding site for Zap-70 and Syk kinases (**Figure 6**).

The ITAM sequences of DAP12 and FcRγ are remarkably conserved through evolution, and related orthologs of these proteins are found in all vertebrates, demonstrating their central role in immune cell signaling. DAP12 and FcRγ are found in most cells of the innate immune system, including neutrophils, macrophages, various dendritic cell (DC) types, basophils, eosinophils, mast cells and NK cells. [120].

Originally, both DAP12 and FcRγ were defined as pairing with immunoreceptors that induce cellular activation. For example, DAP12 mediates NK cell activation through Ly49D and Ly49H in the mouse [121] or the CD94/NKG2C complex in the human [122], whereas FcRγ mediates macrophage, neutrophil and mast cell activation following engagement of their Fcγ or Fcε receptors [123]. However, the repertoire of immunoreceptors to which these adapters pair, to mediate innate immune cell activation has grown considerably. Thus, FcRγ has been demonstrated to mediate signaling through immunoreceptors such as Mincle [124], Dectin-2 [125], and CLM5 (also called MAIR-IV) [126]. The spectrum of DAP12-associated receptors is even larger, including many immunoreceptors such as TREM-2 [127], MDL-1 [128] and PDC-TREM [129]. The ligands for some, but not all, of these immunoreceptors are known, and consist of proteins or carbohydrates structures present on many pathogenic organisms, as well as endogenous cellular macromolecules[130].

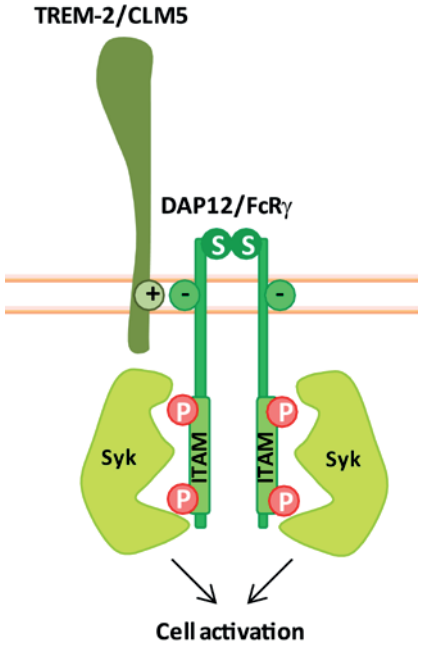


Figure 6: Adapter proteins DAP12 and FcRγ associate with transmembrane receptors to initiate activating signal transduction. TREM-2 associates with DAP12 via complementary charged amino acid residues in the transmembrane domains. Similarly, CLM5 pairs with the FcRγ chain. Upon stimulation of the extracellular domain of the receptors, DAP12 or FcRγ are tyrosine phosphorylated, likely by Src family kinases, and the Syk tyrosine kinase is recruited to the phosphotyrosines via the SH2 domains of the kinase. Activation of Syk leads to initiation of a number of intracellular signaling cascades.

Furthermore, recently it has been described the involvement of DAP12 and FcRγ in signaling pathways mediated by receptors that do not directly associate with these signaling adapters using positively charged amino acids in their transmembrane regions, these receptors use to be called non-immunoreceptors. Examples of non-immunoreceptors in myeloid cells that rely on ITAM adapters for signaling include integrins, growth factor receptors and antigen presenting MHC proteins. Thus, FcRγ is able to mediate signaling of non-immunoreceptors such as the IL-3 receptor [131] or receptors involved in mycobacterial sensing [132], and DAP12 is involved in the signaling of the c-Fms receptor [133]. In many cases, signaling from non-immunoreceptors requires both DAP12 and FcRγ, as loss of function is only seen in cells derived from mice lacking both adapters [134 135 136].

Perhaps even more remarkable, it is now well established that DAP12 and FcRγ are implicated in the inhibitory functions of innate immune receptors as TREM-2, Siglec-H or FcαRI as well [130 137].

DAP10

DAP10 is a transmembrane adapter molecule which was identified by its similarity to DAP12 in the transmembrane region [138]. Remarkably, the gene encoding DAP10 (HCST) is adjacent to DAP12 in the genome but in the opposite transcriptional orientation. DAP10-encoding genes have been identified in mammals, amphibians and fish [139 140]. DAP10 has a minimal extracellular region and has a conserved cysteine to create a disulfide-bonded homodimer. The distribution of DAP10 covers essentially all myeloid cells and NK cells in mice and humans. One significant difference compared with DAP12, is the constitutively expression of DAP10 in CD8+ T cells. The signaling function of DAP10 is distinct from DAP12, in that the only signaling motif in the short cytoplasmic domain of DAP10 is a YINM sequence, which when phosphorylated is able to bind either the p85 subunit of PI3K (through YXXM) or the adapter Grb2 (through YXNX) [141] (**Figure 7**) .

The biological roles of DAP10 and DAP12 are distinct; the activation through DAP12-associated receptors potently induces the production of cytokines, whereas DAP10 is much less efficient at this activity. The ability of DAP10 to activate the PI3K-Akt pathway suggests a role in cell survival, although this has not been evaluated.

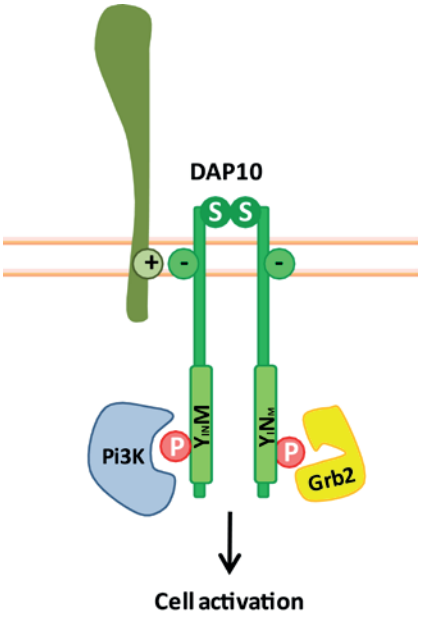


Figure 7: Adapter protein DAP10 associates with transmembrane receptors via complementary charged amino acid residues in the transmembrane domain of each protein, to initiate activating signal transduction. Upon stimulation of the extracellular domain of the receptor, DAP10 is tyrosine phosphorylated, and the Pi3 kinase or Grb2 are recruited to the phosphotyrosines initiating intracellular signaling cascades.

Human NKG2D was the first receptor identified to associate with DAP10 [138], and the receptor complex is a hexamer, composed of one NKG2D homodimer assembled with two DAP10 homodimers [142]. Several other receptors originally identified by their ability to

associate with DAP12 also appear capable of pairing with DAP10 by co-transfection and co-immunoprecipitation studies, for example, Ly49H, Ly49D, Sirp-b1, Siglec-15, and CD300lb. These interactions are likely due to the similarity in the transmembrane domain of both adapter molecules, however these associations with DAP10 have not yet been shown in primary cells [143].

Inhibitory receptors

Inhibitory receptors display a long cytoplasmic tail characterized by the presence of the immunoreceptor tyrosine-based inhibitory motif (ITIM) [144]. The prototype 6–amino acid ITIM sequence is (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/ Val), where X denotes any amino acid. Ligand induced clustering of these inhibitory receptors results in tyrosine phosphorylation, often by a Src family kinase, which provides a docking site for the recruitment of cytoplasmic phosphatases having a SH2 domain [145]. Two classes of SH2- containing inhibitory signaling effector molecules have been identified: the tyrosine phosphatase SHP-1 and the inositol phosphatase SHIP [146] [147]. After tyrosine phosphatases become activated, dephosphorylate key signaling mediators of activation pathways, such as Syk, LAT, BLNK/SLP-76, Vav, PI3K, and cytoskeleton structures; consequently downregulating the signaling cascade.

The ITIM can also bind to another cytoplasmic tyrosine phosphatase, SHP-2. However, the role of this molecule in inhibitory function is less well defined and accumulating evidence suggests that it plays a positive role downstream to various receptors [148].

Inhibitory mediators

There are 107 genes codifying for protein tyrosine phosphatases (PTP) in the human genome. Among all the PTPs, 38 are considered as classical PTPs, characterized by the V/I-H-C-S-X-G motif within their catalytic domains and their strictly phosphotyrosine specificity. Classical PTPs are subdivided still further into the receptor-like PTPs (rPTPs) and the non-receptor-like, or cytosolic PTPs (nrPTPs). The receptor-like PTPs contain a membrane-spanning region, an extracellular region of varying size that in most cases contains structural domains and a cytosolic region that in the majority of cases contains two catalytic domains although only one of these is catalytically active. The majority of cytosolic PTPs have a multidomain structure in which accessory modules (e.g., PDZ, FERM, Pro-rich and SH2 domains) have been added to the catalytic domain. These accessory modules are presumed to perform important targeting functions within the cell, directing the catalytic domain to precise locations and protein complexes, where their substrates and regulatory proteins reside. Of all the active PTPs within the genome only two cytosolic PTPs (SHP-1 and SHP-2), contain tandem SH2 domains.[149].

SHP-1

SHP-1 is composed of a central catalytic domain, containing the characteristic protein tyrosine phosphatases signature motif VHCSAGIGRTG, two SH2 domains at their N-termini, and a C-terminus. At the basal state, the N-terminal SH2-domain is intramolecularly associated with the PTP domain, thereby repressing its activity. Upon binding of a tyrosine-phosphorylated peptide to the SH2 domains, this suppression is released leading to the activation of the phosphatase [150]. The C-terminus possesses potential tyrosine and serine phosphorylation sites, membrane lipid interaction sites, lipid raft localization sites and proline-rich domain interaction motives, which altogether are important for the localization and the activity regulation of the PTP [151] [149] (**Figure 8**).

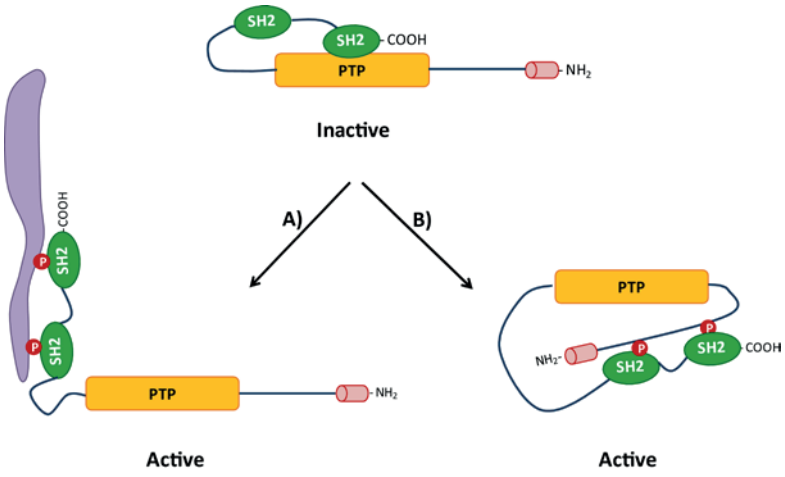


Figure 8: Regulation of SHP-1. The N-terminal SH2 domain of SHP-1 is inserted into the catalytic cleft, thereby repressing its activity. Two potential mechanisms of SHP-1 activation have been proposed. A) Binding of a phosphotyrosine peptide to SH2 domains disrupts the inativating interaction leading to an activation of the phosphatase. B) SHP-1 may be phosphorylated on tyrosines in its C-terminus; these phosphorylated residues engage the SH2 domains enabling enzyme activation.

SHP-1 expression is restricted mainly to hematopoietic and epithelial cells (breast, prostate, ovary, pancreas and thyroid, amongst others), and is widely accepted as a negative regulator of signaling events [152]. SHP-1 binds and dephosphorylates tyrosine kinase receptors, non-receptor cytosolic tyrosine kinases and/or downstream effectors regulating cellular signals such as ERKs, JNKs STAT, JAK2, NF-κB, c-SRC and PI3K-AKT [153]. Although, the precise function and targets of SHP-1 in non-hematopoietic cells are largely unknown, it has been observed that attenuates and/or terminates signal transduction pathways that involve cell proliferation, differentiation, survival, apoptosis and adhesion [154] [155] [156] [157]. Furthermore, SHP-1 regulates the glucose homeostasis through modulation of insulin signaling in liver and muscle, as well as hepatic insulin clearance [158], and is a negative regulator of bone resorption, affecting both the formation and the function of osteoclasts [159]. Finally, SHP-1 has been proposed to be a tumor suppressor gene candidate in

lymphoma, leukemia, and other cancers, because it functions as an antagonist to the growth-promoting and oncogenic potentials of tyrosine kinases [155].

SHIP

SH2-containing Inositol 5'Phosphatase (SHIP) was initially detected in the more mature cells of the blood, but it is now apparent that almost all cells of the bone marrow and blood express at least one form of this protein. The presence of SHIP mRNA has been detected at the earliest stages of hematopoietic cell development in mouse embryos [160], and protein expression has been observed in all blood cell lineages, to various degrees [161]. Furthermore, during differentiation, expression of various SHIP isoforms was observed in the human ML-1 myeloid leukemia cell line from an immature myeloid state to mature macrophages or granulocytes. The immature cells expressed a p110 SHIP isoform primarily (e.g., SHIPδ), whereas the mature cells expressed mostly SHIPα and SHIPβ. This expression pattern also was observed in murine bone marrow cells and mature macrophages derived from these bone marrow cells [162]. Furthermore, the level of SHIP expression in T lymphocytes was upregulated throughout development, while in B lymphocytes SHIP is expressed at a relatively constant level. These results indicate complex splicing events for SHIP expression during hematopoietic cell development, with potentially different functions for each isoform and cell lineage. Many reports have demonstrated that SHIP mRNA is expressed in the spermatids and localized to the cell membrane. Although the function of SHIP in spermatogenesis is not yet known [163].

SHIP contains several identifiable motifs, important for protein–protein interactions. The amino terminal SH2 domain was an early identifying characteristic of SHIP, and is vital in the interactions of SHIP with a large number of intracellular signaling proteins. The central amino acid portion of SHIP encodes an enzymatic activity for removal of phosphate from the 5' position of inositol polyphosphate. The carboxy-terminal domain encodes two NPXY motifs, upon tyrosine phosphorylation of these motifs, proteins containing a phosphotyrosine binding (PTB) domain are known to interact with SHIP at these sites [164]. Phosphorylation of the NPXY motifs may also serve as potential interaction sites for SH2 domain-containing proteins, depending on the three amino acids adjacent to the carboxyl side of the tyrosine. Finally, several PxxP motifs are present within the carboxyl terminus and may serve as binding sites for proteins containing SH3 domains. Together, these structural features describe a unique signaling protein, whose functional significance will be dependent upon the subsequent interactions, enzymatic activity and localization [147].

HUMAN CD300 FAMILY RECEPTORS

The cluster of differentiation (or cluster of designation), often abbreviated as CD, is a protocol used for the identification and investigation of cell surface molecules providing targets for immunophenotyping of cells. Physiologically, CD molecules can act in numerous ways, often acting as receptors of ligands important to the cell. A signal cascade is usually initiated, altering the behavior of the cell. Some CD proteins do not play a role in cell signaling, but have other functions, such as cell adhesion [165]. For humans exist more than 360 CD molecules [166].

The CD300 molecules are a family of leukocyte membrane regulatory molecules that modulate a diverse range of effector functions by coordinating the engagement of inhibitory and stimulatory family members to finally modulate immune responses, particularly on myeloid cell. [167].

The CD300 molecules form part of the immunoglobulin superfamily (IgSF). The IgSF is reported to be the largest superfamily of proteins in the human immune genome. At the structural level, this superfamily has Ig variable (IgV) and/or Ig constant (IgC) domains, which shares sequence similarities but has different folding patterns. All the Ig-like domains are composed of ∼100 amino acids, that share a common two-layered fold composed of two anti-parallel β-sheets. Nearly all Ig domains contain a conserved disulfide bond that links the back and front sheets. Of particular importance are the loops connecting specific strands, which correspond to complementarily-determining regions (CDR) and are important for the ligand recognition (**Figure 9**). Despite the common overall architecture, Ig domains can share as low as 15% sequence identity, and this variability results in a wide range of structural and organizational variations, that underlie the enormous diversity of biological function exhibited by members of the Ig superfamily [168].

Importantly in terms of evolution, CD300 molecules have the closest sequence similarity of any mammalian proteins to modular domain immune type receptors (MDIR), which are members of a multigene family of stimulatory and inhibitory Ig superfamily receptors from the clearnose skate (*Raja eglanteria*), a phylogenetically ancient vertebrate [169]. Noteworthy, the CD300 Ig-like fold has conserved an amino acid motif, YWCR, and two (instead of one) disulfide bonds, indicating strong evolutionary pressure to maintain CD300-like domains from ancient vertebrates onwards [170]. The CD300 molecules are also distant relatives of the Fc receptor for polymeric IgA and IgM, the TREM molecules and CD336 (NKp46), which also contain two disulfide bonds within their IgV domains, defining a novel group of Ig superfamily molecules with common ancestry [167].

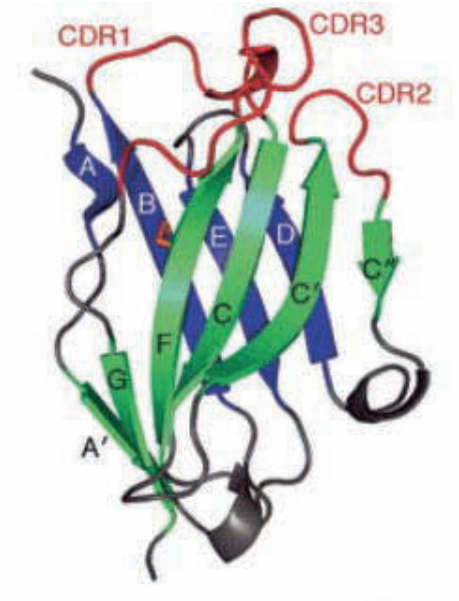


Figure 9: Structure of an IgV domain. Strands of the front (green) and back (blue) sheets of the heavy-chain IgV domain are shown and the conserved disulfide bond connecting front and back strands is colored in orange. The CDR loops are highlighted in red [168].

The individual CD300 proteins were named alphabetically: CD300A, -B(LB), -C, -D(LD), -E and -F(LF); relating to the order of encoding genes on human chromosome 17 (17q22-25) [171]. CD300g, the most distantly related CD300 molecule, is encoded by a gene mapped at some distance from the main complex, and shares a similar single IgV-like domain sequence but lacks the structural hallmarks suggestive of stimulatory or inhibitory potential [167]. A cluster of orthologous genes in mouse, numbered CMRF-like molecules (CLM) were mapped to the syntenic region on mouse chromosome 11D (CLM-1 to CLM-9) [172]. The human–mouse CD300 orthologous have been identified by their gene organization within the complex and by phylogenetic analysis[167]. However, they are not necessarily functional orthologous [173] [126] (**Figure 10**). Furthermore, within the mouse molecules, each member can differ in some amino acids according the mouse strain from which it has been cloned. Thus, Leukocyte Mono-Ig-like Receptors (LMIRs) derived from CBA/J strain and CMRF-35-like molecules (CLMs) also termed Myeloid-Associated Ig-like Receptors (MAIRs) from C57BL/6 strain. In practice LMIRs and CLMs are considered the same molecules.

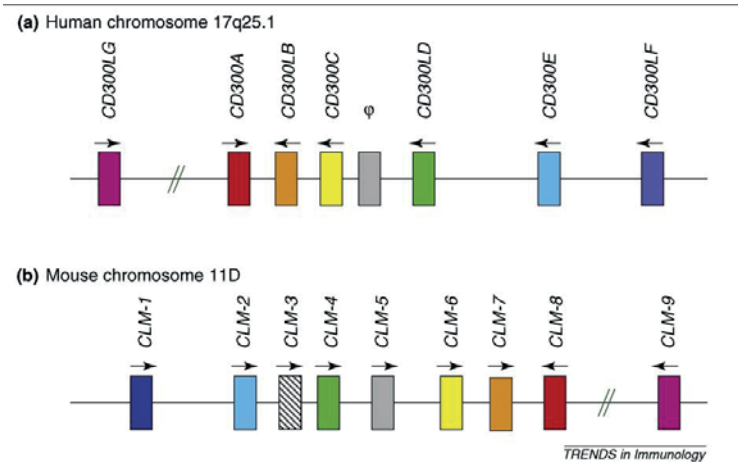


Figure 10: Schematic diagram showing the organization of (A) the human and (B) the mouse CD300 genes. Gene orthologous are shaded similarly. Arrow indicated direction of transcription [167].

General characteristics of CD300 receptors

All the CD300 molecules are type I transmembrane glycoproteins with a single IgV-like extracellular domain and an extended membrane proximal region that links the Ig and transmembrane domains and is rich in prolines, serines and threonines. The IgV domain structures of CD300a and CD300f have been confirmed by crystallization [170] [174]. CD300b, CD300c, and CD300e has the typical molecular structure of activating receptors with short cytoplasmic tails, usually devoid of signaling motifs, and transmembrane domains containing a charged amino acid residue, which enables association with other transmembrane molecules, such as adapter molecules. On the other hand the cytoplasmic domains of CD300a and CD300f contain tyrosine-based signaling motifs, such as immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which enable the direct initiation of the signaling cascade by interacting with signaling molecules as phosphatases, kinases and other peptides. A detail of the important motifs and residues within the transmembrane and cytoplasmic domains of the CD300 receptors, together with the related signaling molecules related with each receptor is shown (**Figure 11**), and will be extensively argued in the following pages.

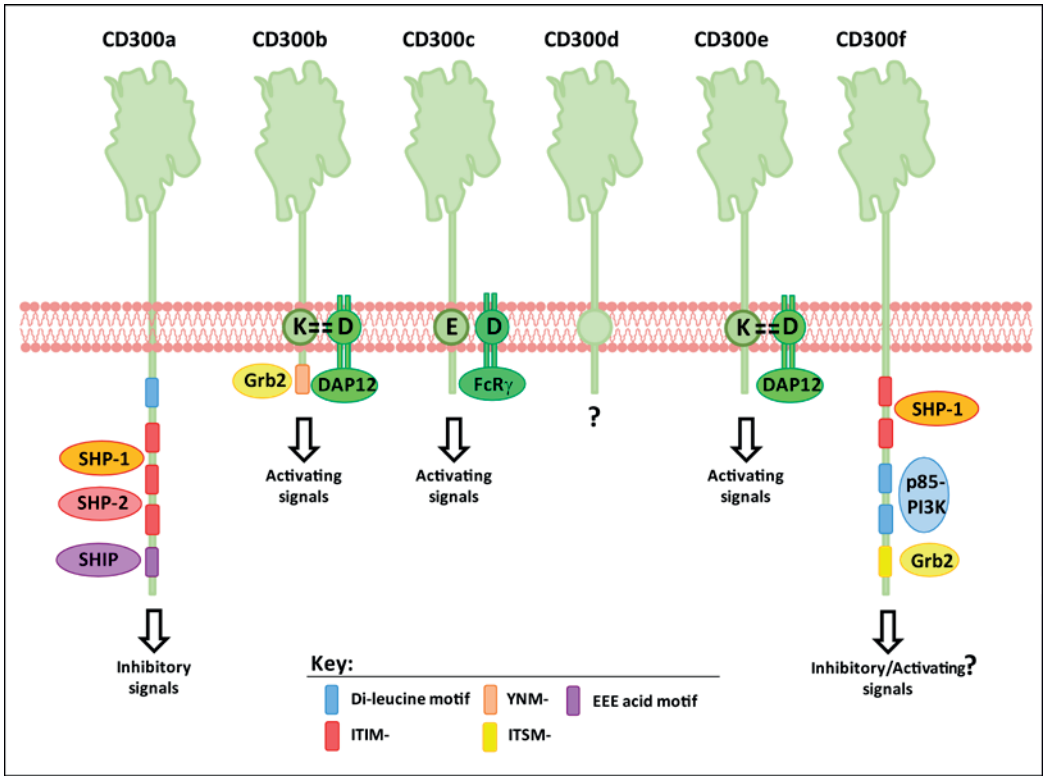


Figure 11: Schema of the individual CD300 molecules in human. The stimulatory members (CD300b, CD300c, CD300d and CD300e) have a single charged amino acid (E or K) in the transmembrane domain, and a short cytoplasmic tail; the inhibitory members (CD300a and CD300f) have long cytoplasmic regions that contain tyrosine-based signaling motifs. The potential signaling motifs in each molecule are indicated. Interactions with adapter molecules, phosphatases, kinases or other signaling molecules that have been described in the literature are indicated and also the nature of the signaling pathway downstream the receptors. The question marks indicate that downstream signaling is unknown at this stage.

Homo- and heterocomplexes formation between CD300 members

Initially it was proposed that CD300 molecules fitted the classical activating/inhibitory immunoreceptor model, as comparison of the extracellular domain sequences of inhibitory and stimulatory receptors identified pairs of molecules that probably bind similar ligands. For example, the inhibitor CD300a has high sequence identity with CD300c family partner [175] and the same was observed between the inhibitor CD300f and the activating receptor CD300b. However, now it is clear that the family presents a more complex behavior than expected, due to the ability of the members to bind each other through their Ig domains, independently of disulfide bridges, before their export to the plasma membrane [176]. It is proposed that the interaction may relay in the presence of certain metal ions, as seen for other receptors as TIMs (T-cell Ig and mucin) [177]. Furthermore, the integration of CD300 molecules in complexes modifies the signaling properties of individual receptors allowing synergies, at the same time as agonistic and/or antagonistic processes. For example, the

stimulation of CD300c or CD300b in transfected cells lead to a similar activation of the NFAT/AP-1 reporter gene, whereas cells transfected with both receptors exhibit a 2-fold increase in transcriptional activity when stimulated through the CD300c (**Figure 12**) [176]. Thus the formation of the complex between the members of the CD300 family, suggested a new mechanism by which CD300 complexes could regulate the activation of myeloid cells, upon interaction with their natural ligands.

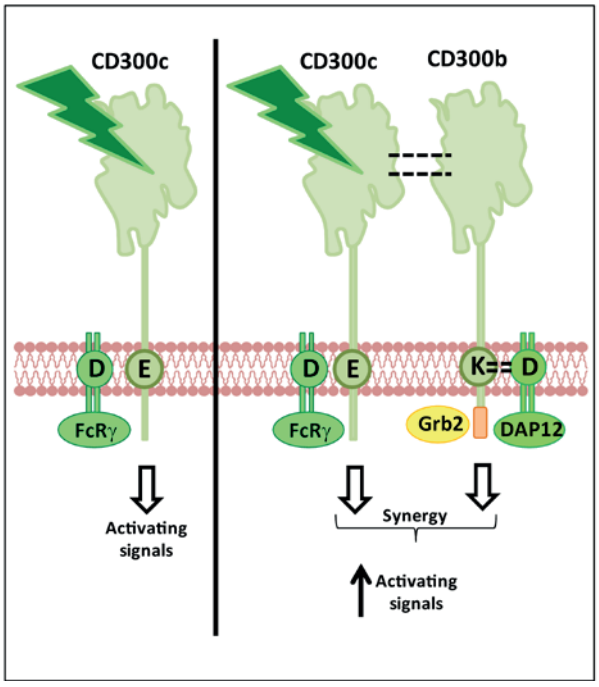


Figure 12: Comparison of the signaling pathway between individual CD300c and the heterocomplex CD300c-CD300b. The stimulation activating response achieved when CD300c and CD300b forms a heterocomplex exhibits a 2-fold increase compared to the response through the individual CD300c.

Molecular characteristics, distribution and function of the CD300 receptors

CD300g

CD300g, also called nepmucin or CD300Lg, is a type I transmembrane protein and its Ig domain is high homolog with the rest of CD300 members. However there are multiple reasons that convert this receptor in a distant member of the family. CD300g is the only member containing an extracellular mucin-like domain. The cellular and tissue distribution of CD300Lg is restricted to endothelial cells [178] and its role in L-selectin dependent lymphocyte rolling and adhesion is totally unrelated with the activating/inhibitory capabilities of the CD300 immunoreceptors [179].

CD300a

CD300a, also called inhibitory receptor protein 60 (Irp60), is a type I transmembrane protein of 299 aa, characterized by a 17-aa signal peptide, a 161-aa extracellular region containing a single IgV domain, a 22-aa transmembrane portion and a 99-aa cytoplasmic tail. The stretch of amino acids connecting the ectodomain with the transmembrane region contains 13 putative O-linked glycosylation sites. In addition, in the extracellular region, CD300a displays two potential N-glycosylation sites. Importantly, the cytoplasmic portion of the receptor contains a "non-classical" motif (SDYSVI) and three motifs (LHYANL, VEYSTV, LHYASV) that fit the consensus sequence for classical ITIM [180]. The CD300a ITIM motifs has the capacity for SHP-1, SHP-2 and SHIP phosphatases recruitment [181]. However, recently it has been publish that just SHP-1 is utilized by CD300a for its inhibitory activity [182]. The CD300a cytoplasmic region also contains a di-leucine motif that is associated with endocytosis and delivery to lysosomes [180].

The CD300a mRNA is present in spleen, placenta, lung and liver; but not in heart, brain, skeletal muscle and pancreas. CD300a protein, is expressed on various immune cell types like T cells, B cells, NK cells and neutrophils [180 183].

Distinct functions of the CD300a receptor and variations on its surface expression would be commented in the next paragraphs and are summarized (**Table 1**) (**Table 2**).

The Ab-mediated cross-linking of CD300a on NK cells results in down-regulation of NK cytolytic activity *in vitro*. Furthermore, in human peripheral blood neutrophils, CD300a is able to inhibit ITAM mediated ROS production by inhibiting Ca^{2+} flux [184]. CD300a also exhibit inhibitory function interfering T-cell receptor (TCR)–mediated signaling [185]. Naïve $CD4^+$ T cells express low levels of CD300a, while high expression of CD300a is related with a new subtype of effector and memory $CD4^+$ T cells. $CD300a^{high}$ $CD4^+$ cells are able to respond to specific recall antigen proliferating and prompting CD25 expression and IL-2 production. However, following nonspecific activation these cells increase the production of IFN-γ and appear to be programmed to die rapidly inducing an apoptotic process, that may be designed to avoid inappropriate autoimmune activation [186]. Deeper analysis demonstrate

that after TCR stimulation, CD300a could be involved in the production of INF-γ via the T-box transcription factor Eomesoderminis in CD300a^{high} CD4+ cells, which will be associated to a Th1 cell poly-functional phenotype. In agreement, TGF-β1 factor, which is required for the development of regulatory T (Treg) and Th17 cells (both CD300a^{-}), exhibits a potent negative regulatory effect on CD300a surface expression [187]. CD300a has been also associate with a subpopulation of CD8+ T lymphocytes, related with effector functions and characterized by more cytotoxic phenotype than that of CD300a^{-} cells [188]. Concerning B cells, CD300a is mainly expressed in memory and plasmablasts/plasma cells, where negatively regulates the BCR signaling. Naïve B cells are mostly negative or express very low levels of the receptor. In agreement, TLR9 agonists, known to induce proliferation and differentiation of resting B cells into Ab-secreting cells, increase the expression of the CD300a receptor in the B cell surface [183].

On the contrary, in plasmacytoid dendritic cells (pDCs), INF-α secretion mediated by TRL7 and TRL9 signaling negatively regulate the expression of CD300a in an autologous manner. However, when CD300a is expressed in the pDCs surface, its stimulation inhibits the secretion of the inflammatory cytokines TNFα, linked to a reduction in HLA-DR (MHC class II molecule) surface expression and to an upregulation of INF-α. Altogether, suggest that the CD300a molecule plays an important role in balancing pDC IFN-α and TNFα production in response to TLR activation [189].

The surface expression of the CD300a receptor is also described in human mast cells, where receptor crosslinking inhibits IgE-induced degranulation through the abolishment of Ca^{2+} influx, and inhibits Stem Cell Factor (SCF) -mediated mast cells survival, through a mechanism involving tyrosine phosphorylation and phosphatase SHP-1 recruitment [190]. However, the activity of CD300a in mast cells is downregulated by eosinophil-derived cytotoxic proteins found in the allergic inflammatory milieu, as Human Eosinophil-derived Major Basic Protein (MBP) and Human eosinophil-derived Neurotoxin (EDN) [181]. Later it was observed the expression of CD300a in eosinophils themselves, where significantly decreased the ability to transmigrate in response to eotaxin and inhibits IL-5/GM-CSF-induced eosinophil survival and activation, through affecting JAK2 and MAP kinases phosphorylation [191]. In addition, it has been publish that human basophils constitutively express CD300a, where it seems to be closely involved in the inhibition of IgE-mediated anaphylactic degranulation, accordingly its expression is up-regulated in response to IgE-dependent cell activation [192]. Furthermore, it has been demonstrated that linking IgE with CD300a using a bispecific antibody (IE1^{H}) fragment leads to potent inhibition of allergic reactions. The antibody inhibited *in vitro* mediators release and completely blocked allergic responses in murine models of passive cutaneous anaphylaxis and experimental asthma, establishing the role of CD300a as a critical modulator of the allergic setting and indicate this molecule as a novel target for allergy therapy [193].

Regarding the ligands of CD300a, recently it has been publish that amino phospholipids phosphatidylserine (PS) and especially phosphatidylethanolamine (PE) are the natural ligands [194]. In normal cells, PS and PE reside in the inner leaflet of the plasma membrane, whereas

phosphatidylcoline (PC) and sphingomyelin (SP) are the main phospholipids of the outer leaflet. Contrary, from the early stages of apoptosis, cells suffer dynamic changes provoking loss of membrane phospholipid asymmetry, inducing the exposure of PS and PE in the outer leaflet of the plasma membrane [195] [196], being one of the mechanisms leading to phagocytic recognition of dead cells. The relevance and significance of the interaction between CD300a and PE is demonstrated by the role of the receptor in inhibiting, instead of promoting, the engulfment of late apoptotic cells by macrophages or L929 cell line [194]. This concept fits nicely with the fact that, essentially all immunologic responses are the result of tipping the balance between inhibition and activation. In agreement, CD300a negatively regulates mast cell inflammatory response to microbial infection. Concretely, the receptor suppresses the production of LPS-induced inflammatory cytokines and chemokines mediated by mast cells, provoking a defective recruitment of neutrophils to the infection [197].

CELL TYPE	CD300a FUNCTION
NK cells	- CD300a down-regulates the NK cytolytic activity *in vitro*
Neutrophils	- CD300a inhibits the ITAM mediated ROS production by inhibiting Ca^{2+} flux
T cells	- CD300a negatively regulates the TCR signaling - CD300a is involved the production of INF-γ in CD4+ T cells - CD300a increments the cytotoxic phenotype in CD8+ T cells
B cells	- CD300a negatively regulates the BCR signaling in memory and plasma B cells
Plasmacytoid dendritic cells (pDCs)	- CD300a inhibits the secretion of the inflammatory cytokine TNFα, linked to a reduction in HLA-DR (MHC class II molecule) surface expression and to an upregulation of INF-α
Mast cells	- CD300a inhibits the IgE-induced degranulation through the abolishment of Ca^{2+} influx - CD300a inhibits the inflammatory response to microbial infection, suppressing the production of LPS-induced inflammatory cytokines and chemokines - MBP and EDN inhibit CD300a function
Eosinophils	- CD300a decreases the ability to transmigrate in response to eotaxin - CD300a inhibits the IL-5/GM-CSF-induced eosinophil survival and activation
Basophils	- CD300a inhibits the IgE-mediated anaphylactic degranulation - CD300a inhibits the allergic reactions
Macrophages	- CD300a inhibits the engulfment of late apoptotic cells

Table 1: Summary of the CD300a functions in distinct cell types.

CELL TYPE	CD300a EXPRESSION and SUBSTANCES AFFECTING THE CD300a EXPRESSION
T cells	- Naïve $CD4^+$ T cells express low levels of CD300a - Effector and memory $CD4^+$ T cells express high levels of CD300a - TGF-β1 factor negatively regulated CD300a surface expression - Cytotoxic CD8+ T cells express high levels of CD300a
B cells	- Naïve B cells are mostly negative or express very low levels of the CD300a - Memory and plasma B cells express high levels of CD300a - TLR9 agonists increase the surface expression of CD300a
Plasmacytoid dendritic cells (pDCs)	- INF-α secretion mediated by TRL7 and TRL9 signaling negatively regulate the expression of CD300a
Basophils	- CD300a is constitutively express in basophils - CD300a expression is up-regulated in response to IgE-dependent cell activation

Table 2: Summary of the CD300a expression in distinct cell types.

CLM-8

CLM-8, also called LMIR1 or MAIR-I, has been proposed to be the mouse ortholog for CD300a, with a sequence homology around 44%. This receptor is expressed on the majority of the myeloid lineage cells, including bone marrow-derived cultured mast cells and primary macrophages, granulocytes and dendritic cells, and also in a subset of B cells, furthermore its expression is up-regulated on NK cells after stimulation with IL-12. Interestingly, in these cells after receptor crosslinking, the surface expression of the receptor is down-regulated by receptor internalization [198]. While the CD300a presents three ITIM motifs within its cytoplasmic tail, CLM-8 just shows one. However, the tyrosines contained in this motif (Y258, Y270) are sufficient for the recruitment of the phosphatases SHP-1 and SHIP after CLM-8 crosslinking. Also, co-ligation of CLM-8 with FcεRI is essential to inhibit mast cell IgE-mediated degranulation [199].

In agreement with the described implication of CD300a in allergy, neutralization of CLM-8 leads to an augmented response to allergen challenge [181]. In addition, the co-aggregation of CD300a with CC chemokine receptor 3 (CCR3), using a bispecific antibody fragment (LC1), inhibits mast cells (MCs) and eosinophil activation *in vitro*. While, i*n vivo* LC1 activity has been demonstrated to inhibit eosinophil signaling, by reducing ERK1/2 phosphorylation and abrogating PLCγ signaling, and inhibits eosinophil and MCs mediator release. Furthermore, in a mouse model of chronic established asthma, LC1 inhibits the eosinophil-derived production of TGF-β1 and reverse the lung inflammation and inhibits lung remodeling. Taken together, emphasizing a fundamental role of CLM-8 to suppress chronic allergic airway inflammation mediated by MCs and eosinophils [200].

CD300b

CD300b, also called immune receptor expressed on myeloid cells 3 (IREM3), is a type I transmembrane protein driven by a signal peptide 17-aa in length. The extracellular region of CD300b displays a single Ig V-type domain followed by a 29-aa membrane-proximal region which presents many potential O-glycosylation sites. The transmembrane domain has a positively charged residue (lysine, K158) in a central position, and is followed by a short cytoplasmic tail of 29 residues, in which a tyrosine-based motif can be distinguished.

The engagement of CD300b deliver positive signaling based on the interaction with DAP12. Furthermore the interaction with DAP12 favors CD300b surface expression. Although, in the absence of DAP12, CD300b is expressed and able to generate activating response in a process implicating the Grb2 adapter molecule together with the cytoplasmic tyrosine (Y188) of the receptor, which matches the consensus sequence for the Grb-2 docking site (YxN). Importantly, the transmembrane lysine (K158) of the CD300b is indispensible for the receptor activity in the absence of DAP12 [201].

The CD300b mRNA is expressed in placenta, spleen, lung and thymus but not bone marrow. CD300b protein, is expressed only on cells from myeloid origin [167]. In RBL-2H3 cells CD300b crosslinking induces hexosaminidase granule release only in the presence of DAP-12. However, Grb2 together with other unknown signaling molecule, interacting with the receptor through the transmembrane lysine, could explain the induction of Ras/MAPK signal transduction pathway in the absence of DAP12 [201].

Retinoic acid (RA) and phorbol 12-myristate 13-acetate (PMA) have been described to increase synergistically the expression of CD300b at both the mRNA level, by 40–60 fold, and protein level, in human THP-1 cells. RA is the active oxidized metabolite of vitamin A, and has long been recognized to play a role in immune regulation. RA acts as a regulator of gene transcription, diffuses through the cell membrane and then enters the nucleus where it binds to the nuclear receptor complex of RAR and RXR [202]. RA alone increases the gene transcription of CD300b up to 20 fold. On the other hand, PMA alone also induce the mRNA expression of CD300b, although less potently. The action of PMA on the CD300B gene transcription requires signaling via the MEK/ERK1/2 signaling pathway. Interestingly, CD300b protein level apparently can be increased by PMA, but not RA alone, suggesting that the CD300B transcripts induced by RA are “sterile” in that they accumulate in the cell but are not efficiently translated unless or until a second signal, for example delivered by PMA [203].

In vitro it has been suggested that T cell transmembrane, immunoglobulin, and mucin TIM-1 and TIM-4 are possible ligands for CD300b and its mouse ortholog CLM-7 [204].

CLM-7

CLM-7, also called LMIR5 or MAIR-VII, shares a 55% identity at overall amino acid sequences with CD300b and exhibits N-glycosylation modifications while the human receptor does not. Importantly, the putative tyrosine phosphorylation motif (YXN) in the short cytoplasmic tail of CD300b is absent in CLM-7, devoiding the mouse counterpart from the alternative activating pathway independent on DAP12. In terms of function, DAP12 adapter enhanced the expression of CLM-7 in the cell surface and is essential for its activating pathway [205].

The interaction between CLM-7 and TIM-1 involves Ig domains of CLM-7 and the FG loop of the Ig-like domain in TIM-1. Despite the finding that CLM-7 bind to TIM-1 or TIM-4 at close proximity to the PS-binding site, the interaction is not affecting the phagocytosis of apoptotic cells mediated by TIM receptors. In addition, interaction of CLM-7 with surface expressed TIM-1 or TIM-4, as well as with soluble variants, induced the CLM-7 mediated cytokines release in mast cells. Furthermore, endogenous CLM-7 interaction with TIM-1 induces the accumulation of neutrophils in the kidney of mouse model of kidney ischemia/reperfusion injury, a mouse *in vivo* model for TIM-1 induction. In agreement, CLM-7 deficiency ameliorates the renal tubular damage symptom of the mentioned mouse model. Altogether demonstrating the relevance of the interaction between CLM-7/CD300b with TIM receptors [204].

Regarding TIM family receptors, they play a critical role in regulating immune responses, including transplant tolerance, autoimmunity, the regulation of allergy and asthma, and the response to viral infections [206] [207] [208]. TIM genes encode type I cell-surface glycoproteins with common structural features including an N-terminal immunoglobulin (Ig)-like domain, a mucin domain with O-linked glycosylations and with N-linked glycosylations close to the membrane, a single transmembrane domain, and a cytoplasmic region with tyrosine phosphorylation motif(s), except in TIM-4. Importantly, it has been demonstrated that TIM-1, TIM-3, and TIM-4 are pattern recognition receptors specialized for recognition of phosphatidylserine (PS). Importantly within the immunoglobulin domain, a conserved pocket able to coordinate metal ions, as calcium, appears designed for the specific recognition of PS by TIMs. TIM1 is preferentially expressed on Th2 cells and functions as a potent costimulatory molecule for T-cell activation, while TIM-4 is exclusively expressed on antigen-presenting cells (APCs), where it mediates phagocytosis of apoptotic cells and plays an important role in maintaining tolerance. Together, TIM molecules provide a functional repertoire for recognition of apoptotic cells, which determines whether apoptotic cell recognition leads to immune activation or tolerance, depending on the TIM molecule engaged and the cell type on which it is expressed [209].

sCLM-7

Recently it has been publish the existence of a soluble variant for the CD300b/CLM-7 receptor (sCLM-7). This soluble form of the receptor is constitutively released by primary neutrophils of BM, as by RAW264.7 cells. Furthermore, LPS stimulation increased sCLM-7

and downregulated surface CLM-7 expression in neutrophils. A role for neutrophil-specific matrix metalloproteinases (MMPs) has been proved to participate in the proteolytic cleavage of the surface receptor. Interestingly, mimic forms of sCLM-7 caused cytokine production on peritoneal macrophages by interacting with an unidentified ligand other than TIM-1/4. At the same time CLM-7-/- mice, which fail to produce sCLM-7, exhibit lower levels of pro-inflammatory cytokine production in response to LPS and are more resistant to LPS- or peritonitis- induced septic death. Consequently, sCLM-7 is described as an inflammatory mediator linking the initial activation of LPS-stimulated neutrophils with lethal inflammation in sepsis [210].

CD300c

CD300c also called CMRF-35/CMRF35A is a type I transmembrane protein displaying a single Ig V-type domain followed by a membrane-proximal region. The transmembrane domain includes a charged residue, which instead of being a basic amino acid is an acidic residue (glutamic acid, E), and has demonstrated to be important for the association of CD300c with the adapter molecule FcRγ chain. The short cytoplasmic tail of CD300c has not known signaling motifs, thus the interaction with FcRγ is important for the receptor to deliver activating signals [176].

CD300c was the first member of the CD300 family receptor to be identified in 1992 by Jackson DG, *et.,* al. Even though, very little is known about its endogenous activity, in part due to the fact that the extracellular domains of CD300a and CD300c are highly homologous showing 80% identity, and with the available monoclonal antibodies it is impossible to distinguish CD300a from CD300c on the cell surface [211] . By using *in vitro* approaches, it has been characterized its activating capability trough the interaction with the adapter molecule FcRγ [176].

CD300c transcripts are present in high levels in the spleen and thymus. Regarding cell types, its detected in monocytes, NK cells, in some subsets of helper T cells and in some B lymphoblastic cell lines [167].

CLM-5

CLM-5, also called LMIR4 or MAIR-IV, appears to be the murine orthologous to the human CD300c receptor. Like CD300c, CLM-5 contains a short cytoplasmic domain and a negatively charged glutamate residue in its transmembrane domain, which has an unknown function, but which may allow association with adapter molecules [172]. CLM-5 has two different molecular masses, ~35 and ~25 kDa, representing the mature and glycosylated form of CLM-5, and the immature unglycosylated form. The glycosylation of CLM-5 is likely O-linked, considering no possible N-linked glycosylation sequence in the extracellular domain of CLM-5. Concerning its homology with other members of the family, the Ig-like domain of CLM-5 is 91% identical to that of CLM-1. The CLM-5 mRNA is expressed in the spleen,

trachea and the lung. Further expression analysis in hematopoietic cells revels that dendritic cells, macrophages and granulocytes primary cells express CLM-5 [212].

Deeper molecular analyses confirm that FcRγ adapter molecule associates with CLM-5 and its involved in the cell surface expression of the receptor, although the transmembrane residues of both molecules are not involved in the interaction. Regarding CLM-5 function, in monocytic/macrophages cell lines, after receptor crosslinking, itself and FcRγ are phosphorylated initiating an activating signaling pathway that involved MAPK and finally produce morphological changes [173]. In mast cells and neutrophils, CLM-5 aggregation induces the secretion of newly synthesized and preformed chemical mediators, in a pathway dependent on FcRγ adapter, Lyn and Syk kinases [212] [126]. Regarding receptor regulation it is observed that in mast cells, G-CSF and LPS increase the expression of CLM-5, while receptor crosslinking synergy with activating response induced by LPS via TLR4 receptor or induced by IgE plus antigen through FcεRI receptor. Interestingly, co-ligation of CLM-1 and CLM-5 dramatically abrogate the pro-inflammatory potential of CLM-5 [212].

CD300d

The sequence of the CD300d receptor, also called IREM4, was identified in 2006 in our lab. However, due its particular structural properties its function has remained uncertain, being one of the aims of this thesis.

CLM-4

CLM-4, also called MAIR-II or LMIR2, is proposed as the mouse ortholog for CD300d [167], though it is published to be the ortholog for CD300c also [198]. This confusion is due to the structural similarities between both human receptors. The homology between the Ig domain of CLM-4 with that of CD300c or CD300d is high, however when the global protein is analyzed, a remarkable difference is observed in the transmembrane region, where CLM-4 exhibit a positive charged residue instead of a negative one, characteristic of CD300c and CD300d.

Regarding CLM-4 expression and function, it is known to be expressed in a subsets of B cells and peritoneal and splenic macrophages [198]. In splenic macrophages, CLM-4 interacts exclusively with DAP12 adapter, to initiate an activating cascade leading to the secretion of TNFα. However, in peritoneal macrophages not only DAP12 if not also FcRγ adapter is involved in the cytokine release mediated by CLM-4. Also it was observed that LPS treatment induce up-regulation of CLM-4 surface expression mediated by FcRγ in peritoneal macrophages. Importantly, the lysine residue in the transmembrane region of CLM-4 is involved in the association with both DAP12 and the FcRγ chain adapters [213]. Controversially, in B cells CLM-4 inhibits proliferation mediated by BCR and TRL-9, through a mechanism involving recruitment of SHP-1 by DAP12 [214] .

CD300e

CD300e also termed IREM2, is a type I transmembrane protein, with a putative hydrophobic 12-aa signal peptide. The extracellular region contains a single IgV domain and a 44-aa linker, with a single potential N-glycosylation site. The transmembrane domain displays a charged residue (lysine, K) followed by a short cytoplasmic tail of 10-aa. The molecular mass of CD300e is 34KDa when resolved by SDS-PAGE and it is reduced to 31 KDa after N-deglycosylation. Importantly the presence of the lysine in its transmembrane region enables specific interaction with DAP12 adapter. Even this adapter is not required for the surface expression of the receptor in transfected cells, it seems important for its activating activity. Regarding CD300e distribution, the receptor is selectively expressed in nonactivated PBMCs as well as in PHA-activated PBMCs and in dendritic cells (DC), being down-regulated upon *in vitro* differentiation of monocytes [215].

Accordingly with its activating receptor structure, in monocytes the crosslinking of the CD300e induces Ca^{2+} mobilization, the production of ROS, the up-regulation of activating molecules as CD25, CD83, CD86 and the induction of pro-inflammatory chemokines and cytokines production (e.g., IL-8/CXCL8 and TNFα). In primary isolated dendritic cells, CD300e ligation triggers TNFα, IL-6, IL-8/CXCL8 and IL-10 production and up-regulates the co-stimulatory molecules CD40, CD83 and CD86. Finally all these processes facilitate the survival of monocytes and DC and enhanced the ability of DC to promote T-cell activation [216].

CD300f

CD300f also termed IREM1, is a type I transmembrane protein of 294 aa. Four different splicing variants have been observed for the CD300f receptor, two of them coding for entire molecules that differ in transcriptional starts (Irem-1 and Irem-1 Sv1). However, in fact, only the putative signaling peptide present in Irem-1 fitted with a valid signal peptide sequence. The other two splicing variants codify for putative truncated forms that present a IgV domain but do not display any transmembrane region (Irem-1 Sv2 and Sv3). The entire CD300f is characterized by an 18-aa signal peptide, a 141-aa extracellular region containing a single IgV domain, a 22-aa transmembrane portion and a 113-aa cytoplasmic tail. The CD300f polypeptide backbone has a predicted size of 32 kDa, whereas isolated CD300f appeared as two discrete bands of 53 kDa and 59 kDa, due to different levels of N- and/or O-linked glycosylation. CD300f is expressed in peripheral blood monocytes and granulocytes, as well as in different monocytic cell lines. Interestingly, the surface expression of the receptor is markedly down-regulated in monocyte-derived immature dendritic cells [217].

Importantly, the cytoplasmic portion of the receptor contains five tyrosine residues, from which Y205 (LCYADL) and Y249 (ISYASL) fit with the ITIM consensus structure and Y284 (TEYSTI) with an ITIM-like motif known as ITSM motifs. Of them, Y205 constitutes the main

docking site for SHP-1 recruitment, even though the three tyrosines participated in the inhibitory activity on FcεRI receptor (IgE receptor) mediated by CD300f receptor in myeloid cells. Surprisingly, in the cytoplasmic tail of the receptor, Y236 (YVTM) and Y263 (YCNM) fit with consensus binding motif for the p85 regulatory subunit of PI3K, and have been proved to be important for the recruitment of the kinase by CD300f. Furthermore, the Y263 fits with an YxN consensus sequence known to recruit the Grb2 adapter. Thus, initially both PI3K and Grb2 molecules were proposed to be involved in a putative activating function of the CD300f receptor, revealing a potential dual role for this receptor as an inhibitory and activating molecule on myeloid cells. [218].

Distinct functions of the CD300f receptor would be commented in the next paragraphs and are summarized (**Table 3**).

Deeper molecular studies in myeloid cells lines THP-1 and U937, regarding the inhibitory pathway of CD300f, demonstrate that the receptor crosslinking or the use of synthetic peptides representing the ITIM-like domains of CD300f, block both ERK- and PI3K- mediated B cell activating factor (BAFF) signaling pathway. Accordingly with previous publications, the inhibitory pathway is dependent on CD300f interaction with SHP-1 phosphatase. Concretely, CD300f crosslinking inhibits ERK kinase activity, preventing phosphorylation/degradation of IκB, impairing the expression of pro-inflammatory mediators such us IL-8 and MMP-9 mediated by BAFF. On the other hand, the CD300f stimulation reversed the BAFF-mediated inhibition of phagocytosis via PI3K activity [219].

Similar studies performed in THP-1 cell line demonstrated that CD300f stimulation blocks both MyD88- and TRIF- mediated TLR signaling pathways, also through activation of SHP-1, which prevents IKK activation, phosphorylation/degradation of IκB, and subsequent activation of NF-κB. It is not known how SHP-1 suppresses either MyD88- or TRIF-mediated cellular signaling [220]. However, several previous observations demonstrated the inhibitory function of SHP-1 in LPS signaling. For example, LPS-induced expression of pro-inflammatory mediators was inhibited by the over expression of SHP-1 in murine macrophages [221]. In addition, SHP-1 was shown to be involved in the inhibition of LPS signaling by leukocyte Ig-like receptors (LILRs) [222]. Later on, the phosphatase SHP-2 has also been demonstrated to play an important role in the inhibition of TRIF-mediated cellular signaling after CD300f crosslinking [223].

Recently, another interesting functional study supports the inhibitory effect of CD300f, and the potential therapeutic applications of the CD300f signaling pathway. In a rat model of acute brain damage, produced by injection of the glutamic acid analog NMDA, it was demonstrated that over expression of CD300f in the central nervous system (CNS), using the modular recombinant NLSCt vector, had a neuroprotective role. In the same study it was proposed the existence of putative ligands for CD300f in primary cultures of oligodendrocytes, neurons and some astrocytes, whereas no signal was detected in microglia. Referent to neurons, *in vitro* hippocampal and cortical neurons expressed the putative ligand(s), however *in vivo* analysis failed on demonstrating that result. It is

noteworthy, that in the areas of the brain affected by lesion an increase in the expression of the putative ligand(s) was detected. Regarding the expression of the receptor itself in the CNS, the rat ortholog for CD300f (rCD300f) was detected in primary cultures of microglia, oligodendrocytes and cortical or hippocampal neurons, but not in astrocytes, however *in vivo* it was impossible to detect the receptor in CNS and spleen cryosections [224]. Taken together, the fact that both, the endogenous CD300f receptor and its physiological ligand(s), were found in diverse types of cells in brain, suggests that the neuroprotective role of CD300f is the result of a complex network of cell-to-cell interactions.

On the contrary, activating functions of the receptor are highlight in a range of *in vitro*, *ex vivo* and *in vivo* functional studies performed using specificity antibodies against the CD300f receptor in acute myeloid leukemia (AML) blast, which was proved to express surface CD300f. The results indicated the ability of the anti-CD300f Abs to induce AML cell killing and delay in xenograft tumor growth, mediated by antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or direct apoptotic activities. Taken together, the results show that CD300f mAbs are potent and selective cytotoxic agents against CD300f-expressing cells and exhibit potential as targeted anticancer agents through a combination of effector mechanisms [225]. Thus, CD300f has potential not only as a novel therapeutic target for antibody-mediated immunotherapy, but also to serve as possible diagnostic marker in detection of minimal residual disease and relapse.

CELL TYPE/TISSUE	CD300f FUNCTION
Basophils	- CD300f inhibits FcεRI receptor (IgE receptor)
Monocytic cell lines (U937 and THP1)	- CD300f blocks both ERK- and PI3K- mediated B cell activating factor (BAFF) signaling pathway - CD300f blocks both MyD88- and TRIF- mediated TLR signaling pathways
CNS	- Over expression of CD300f in the central nervous system (CNS) has a neuroprotective role in a mouse model of acute brain damage
AML Blasts	- CD300f crosslinking induces killing of acute myeloid leukemia (AML) cells and delay the growth of xenograft tumors

Table 3: Summary of the CD300f functions in distinct cell types and tissues.

CLM-1

CLM-1, also termed MAIR-V or LMIR3, is proposed as the mouse ortholog for CD300f. Interestingly, the Ig domain of CLM-5 showed 91% identity and 97% similarity at the amino acid level with that of CLM-1 [173]. A significant difference is observed between CD300f and CLM-1 receptors, the cytoplasmic domain of CLM-1 contains four tyrosine residues instead of the five found in CD300f, however in CLM-1 as in the human receptor, two tyrosines are fall into consensus ITIM motifs, a third lies in a YxxM consensus motif that has been associated with activation signaling (via association with PI3K), and the fourth tyrosine lies

in a consensus motif for SLAM-associated protein binding (TxYxxI) known as ITSM motifs. The molecular weight of the mature CLM-1 is detected as a band of 60 KDa in a SDS-PAGE.

Initially using transfected monocyte cell line RAW, CLM-1 was found to be associated with SHP-1 after phosphorylation; while association with SHP-2, SHIP or PI3K was not detected in the same experimental conditions [172]. Even so, later on using the transfected Ba/F3 cell line (proB cell line) it was proved its capability to interact also with SHP-2 phosphatase, PI3K and Grb2 [226].

The CLM-1 mRNA was found to be expressed in the spleen, blood, peritoneal cavity and the less abundantly in lung. Further expression analysis in hematopoietic cells revealed expression in primary dendritic cells, macrophages and granulocytes, mast cells, and is barely detectable, if any, in lymphoid cells [173].

Distinct functions of the CLM-1 receptor would be commented in the next paragraphs and are summarized (**Table 4**).

The inhibitory function of CLM-1 was initially supported by its capability to block osteoclast progression at an intermediate to late stage of differentiation [172]. Furthermore, DIgR2, a proposed splicing variant of CLM-1 (96% identical), preferentially expressed by antigen presenting cells (APCs) inhibits DC-initiated T cell proliferation and Ag-specific T cell responses, both *in vitro* and *in vivo*, further indicating that T cells expressed a putative DIgR2/CLM-1-specific ligand [227].

In addition, it have been published that the cytokines (IL-6 and TNFα) production induced by crosslinking of the FcεRI receptor (IgE receptor) in bone marrow mononuclear cells (BMMCs), was impaired by co-stimulation with CLM-1. This inhibitory pathway was shown to attenuate ERK phosphorylation and to relay on both ITIM and ITSM motifs in the cytoplasmic region of the receptor [226]. On the contrary, in the same study a CLM-1 mutant, devoid of any cytoplasmic tyrosine residue, induces the production of a significant level of IL-6 in the transduced BMMCs, in a pathway dependent on Fc$R\gamma$ adapter. These results highlight the potential of CLM-1 to transmit an activating signal independent of its cytoplasmic tyrosine residues, as observed also for the CD300f human receptor in the RBL-2H3 cell system. Nevertheless, the engagement of CLM-1 in the presence of other stimulus demonstrated the involvement of cytoplasmic tyrosine residues of both, CLM-1 and Fc$R\gamma$ in the activating response. Thus, endogenous or transduced CLM-1 enhanced the IL-6 cytokine production in BMMCs co-stimulated with LPS (TLR4 agonist), while the receptor functions as an inhibitory receptor in BMMCs co-stimulated with other TLR agonists, such as zymosan, polyinosinic:polycytidylic acid (poly I:C), or CpG oligodeoxynucleotides (CpG-ODN), which are ligands for TLR2, TLR3 or TLR9, respectively [226].

Other functional analyses support the activating function of the receptor, by demonstrating that CLM-1 crosslinking induced cell death in peritoneal macrophages. Interestingly the results indicate that cytoplasmic residues other than the tyrosines from the ITIM, the ITSM or the consensus binding motif for the p85 regulatory subunit of PI3K play and essential role in induction of cell death. Furthermore, CLM-1-mediated cell death is independent of

caspases, of endoplasmic reticulum (ER) stress and of autophagy. Interestingly, no obvious morphological difference can be observed in the cytoplasm and nuclei of transduced cells, however cells show shrinkage, aggregation and lack of fine blebs on the cell surface, similar morphological feature observed in apoptotic transfecting cells induced by UV [228].

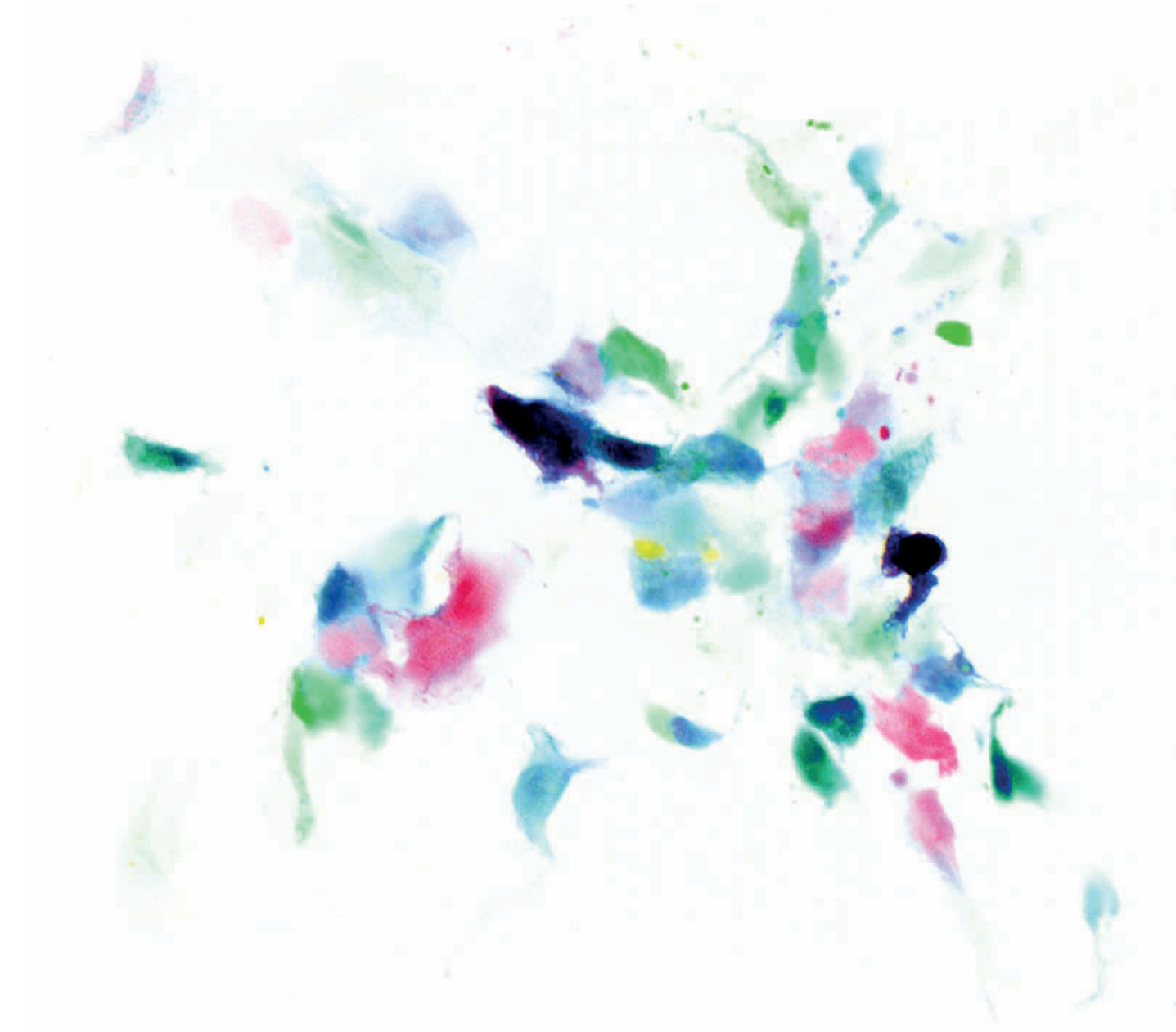
Controversially, *in vivo* assays using the MOG_{35-55}-induced experimental autoimmune encephalomyelitis (EAE) model, which corresponds to the mouse model for the preclinical stage of human Multiple Sclerosis disease, demonstrated that disease severity was significantly increased in mice lacking CLM-1. Leukocytes harvested from spinal cord of CLM-1 KO mice produced significantly elevated levels of nitric oxide and myeloid-specific pro-inflammatory cytokines as compared with WT mice, and the lack of CLM-1 exacerbated demyelination [229]. Altogether, terminate that CLM-1 has a neuroprotective role in autoimmune demyelination processes, supporting the inhibitory effect of the CLM-1 receptor.

Finally, in agreement with the previous studies demonstrating that both CLM-1 and CD300f can provide activation signals, more recent investigations demonstrated that upon receptor engagement, fibroblastic cells lines (L929 cells and NIH3T3 cells) transduced with CLM-1 could promote phagocytosis of apoptotic or activated lymphocyte, which express phosphatidylserine (PS) on the outer membrane. In the same study, ELISA assays using liposomes containing different phospholipids, demonstrated the specific interaction of CLM-1 and PS and the involvement of metal ions, like Ca^{2+}, in the binding. Importantly, the phagocytosis mediated by CLM-1 was suppressed by Annexin V pre-treatment, reinforcing the identification of PS as ligand for CLM-1 [230].

CELL TYPE/TISSUE	CLM-1 FUNCTION
Osteoclasts	- CLM-1 blocks osteoclast differentiation
BMMCs (Bone marrow mononuclear cell)	- Co-stimulation of CLM-1 impairs the production of the IL-6 and TNFα cytokines mediated by FcεRI receptor - Co-stimulation of a CLM-1 mutant, devoid of any cytoplasmic tyrosine residue, induces the production of a significant level of IL-6, in a pathway dependent on FcRγ adapter - Endogenous CLM-1 enhances the IL-6 cytokine production in BMMCs co-stimulated with LPS (TLR4 agonist) - Endogenous CLM-1 functions as an inhibitory receptor in BMMCs co-stimulated with TLR2, TLR3 or TLR9 agonists
Peritoneal macrophages	CLM-1-mediates cell death in a process independent of: caspases, endoplasmic reticulum (ER) stress or autophagy
CNS	- CLM-1 has a neuroprotective role in autoimmune demyelination processes occurring in the experimental autoimmune encephalomyelitis (EAE) model
Fibroblastic cells lines (L929 and NIH3T3)	- CLM-1 promotes phagocytosis of apoptotic or activated lymphocytes, which express phosphatidylserine (PS) on the outer membrane

Table 4: Summary of the CLM-1 functions in distinct cell types and tissues.

Methods



Mammal cells culture

Cell culture mediums composition

Complete Dulbecco's Modified Eagle's Medium (cDMEM)

Incomplete DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin antibiotics.

Complete Roswell Park Memorial Institute Medium (cRPMI)

Incomplete RMPI (variety 1640) supplemented with 10% heat-inactivated FBS, 1mM sodium Pyruvate, 100 U/ml penicillin, 25mM Hepes Buffer and 100 µg/ml streptomycin antibiotics.

Complete Chinese Hamster Ovary Medium (cCHO)

Incomplete CHO supplemented with 4mM L-glutamine.

Characteristics of the used cell lines

Cell Line	Origin	Morphology	Growth properties	Growth medium
COS-7	Monkey kidney SV40 transformed	Fibroblast-like	Adherent	cDMEM
RBL-2H3	Rat Basophilic leukemia	Fibroblast	Adherent	cDMEM
U937	Human histiocytic lymphoma	Monocytic	Suspension	cRPMI
THP-1	Human acute monocytic leukemia	Lymphoblast	Suspension	cRPMI + 0.05mM β–mercaptoethanol
MonoMac6 (MM6)	Human monoblastic leukemia	Monocytic	Suspension	cRPMI
HL-60	Human acute promyelocytic leukemia	Myeloblastic	Suspension	cRPMI
CHO-K1	Chinese hamster ovary	Epithelial-like	Adherent/suspension	cRPMI or cCHO
Hela	Human cervix adenocarcinoma	Epithelial	Adherent	cDMEM
Daudi	B lymphoblast	Lymphoblast	Suspension	cRPMI

Table 5: All the cells were maintained inside incubators at 37°C with 5% CO_2 atmosphere.

Obtainment and culture of primary monocytes

Peripheral blood mononuclear cells (PBMCs) were obtained from a leucopack blood preparations, from human normal donors, provided by the Banc de Sang i Teixits (Barcelona, Spain).

Each leucopack was diluted 1:3 with PBS (1X), and 35 ml of leucopack dilution was overlaid onto 15 ml of Ficoll-PaqueTM PLUS (GE Healthcare) in order to isolate PBMCs by density gradient centrifugation. Tubes were centrifuged at 1000x g, without break for, 30 min at room temperature. After centrifuging, the PBMCs layer was recovered and washed 3 times with PBS (1X). The resulting pellet was washed twice with PBS (1X), and cells were resuspended in incomplete RPMI medium and seeded at a density of $8x10^6$ cell/ml. After an incubation, of 2h at 37ºC, adherent cells (predominantly monocytes) were washed thoroughly with PBS (1X) and detached mechanically with cell scraper in PBS-EDTA 5mM buffer. After centrifuging, primary monocytes were resuspended in complete RPMI medium and seeded at a density of $2.5x10^5$cell/ml.

Reagents used to differentiate or stimulate the primary monocytes

Reagent	Using conditions	Differentiation	Company
GM-CSF	20 ng/ml (5 days) on primary monocytes	Pre-differentiation to macrophages, naïve macrophages	Peprotech
IFN-γ + LPS	25 ng/ml; 100 ng/ml (2 days) on pre-treated monocytes with GM-CSF	M1ca macrophages	Peprotech; Sigma-Aldrich (St. Louis, MO)
rIL-4	20 ng/ml (2 days)	M2a (IL-4) macrophages	Peprotech
rIL-13	20 ng/ml (2 days)	M2a (IL-13) macrophages	Peprotech
rIL-10	20 ng/ml (2 days)	M2c macrophages	Peprotech
PMA	25 ng/ml (1-2 days)	Pre-differentiation of myeloid cell lines	Calbiochem-Merk Millipore

Table 6: Specification for the use of biochemical compounds needed to stimulate or differentiate the cells.

Antibodies

Primary Antibodies and immunoglobulins

Antibody	Cross-reactivity	Host	Applications (Dilution)	Company
Anti-CD300f (UPD1 and UPD2)	Human	Mouse monoclonal	IP (1 μg/ml), Crosslinking (10 μg/ml)	Generated in the laboratory
UPD1-Biotin or UPD2-Biotin	Human	Mouse monoclonal	ELISA (1:1000)	Generated in the laboratory
Supernatant Anti-CD300f (UPD1 and UPD2)	Human	Mouse monoclonal	Cytometry (1:1)	Generated in the laboratory
Supernatant Anti-HA (12CA5)	Human	-	Cytometry (1:1)	Home made
Supernatant Anti-Myc (9E10)	Human	-	Cytometry (1:1)	Home made
Ascites Anti-HA.11 (16B12)	-	Mouse monoclonal	IP (1-2 μg/ml)	Covance
Anti-HA-Biotin (12CA5)	-	Mouse monoclonal	WB (1:5000)	Roche Applied Science (Mannheim)
Anti-FcRγ	Human, mouse, rat	Rabbit polyclonal	IP (1 μg/ml), WB (1-0,2 μg/ml)	Millipore (Billerica, MA)
Anti-FLAG (M2)	-	Mouse monoclonal	IP (1 μg/ml)	Sigma-Aldrich (St. Louis, MO)
Anti-FLAG (M2) - HRP	-	Mouse monoclonal	WB (0,2 μg/ml)	Sigma-Aldrich (St. Louis, MO)
Anti-Tubulin	Human, mouse, rat	Mouse monoclonal	WB (1:1000)	Sigma-Aldrich (St. Louis, MO)
Anti-DAP12 (FL-113)	Human, mouse, rat	Rabbit polyclonal	WB (1:2000)	Santa Cruz Biotechnology (Santa Cruz, California)
Anti-SHIP	Human, rat, mouse	Rabbit polyclonal	WB (1:500)	Santa Cruz Biotechnology (Santa Cruz, California)
Anti-GRP-78	Human	Rabbit polyclonal	Immunofluorescence (1:10)	Santa Cruz Biotechnology (Santa Cruz, California)
IgG Mouse	-		IP (1 μg/ml)	Sigma-Aldrich (St. Louis, MO)

Table 7: Primary antibodies and immunoglobulins used.

Secondary Antibodies and labeled molecules

Antibody / Molecule	Cross-reactivity	Host	Applications (Dilution)	Company
Streptavidin-POD	Biotin	-	WB (1:5000), ELISA (1:5000)	Roche Applied Science (Mannheim)
Anti-Rabbit-HRP	Ig Rabbit	Goat polyclonal	WB (1:20.000)	GE Healthcare
Anti-Mouse-FITC	Ig Mouse	Rabbit polyclonal	Cytometry (1:100)	DakoCytomation (Denmark)
Anti-Phosphotyrosine, Plus Cocktail-HRP	Phosphotyrosine	Mouse monoclonal	WB (1:50.000)	Invitrogen Corporation
Anti-Mouse	Ig Mouse	Goat polyclonal	Plate Crosslinking	Sigma-Aldrich (St. Louis, MO)
Anti-Rabbit- Alexa 594	Ig Rabbit	Goat polyclonal	Immunofluorescence (1:500)	Molecular Probes
DAPI	DNA	-	0,2 µg/ml	-

Table 8: Secondary antibodies and labeled molecules used.

DNA constructs and PCR reactions

The mammalian expression constructs needed were generated by PCR, in most of the cases under the general conditions: 94°C for 3 min and 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, using a proof reading *Pfu* DNA polymerase (Promega). PCR products were resolved in 1% agarose gels, visualized by ethidium bromide staining and further confirmed by DNA sequencing under Big Dye™ cycling conditions, on an Applied Biosystems 3730xl DNA Analyzer (Macrogen Inc.).

For the obtaining of chimerical molecules, formed by the fusion of two independent molecules, the PCR conditions were modified. It was needed to perform three reactions; the first was a conventional PCR, where each part of the chimerical molecule was generated separately; with the peculiarity of having a small complementation sequences between them, in one of their extremes, to allow the fusion. Next, these products were used to perform an annealing PCR without primers, using the following conditions: 94°C for 4min, 50°C for 2 min, and 72°C for 2 min. The annealing product was used as template for the final PCR reaction, following general conditions.

The CD300d molecule without signal peptide was subcloned from pcDNA3.1-V5-His TOPO into pDisplay and pCDNA3-FLAG expression vectors. Chimerical molecule CD300c/d (immunoglobulin domain, stem, and transmembrane region from CD300c and the cytoplasmic tail of CD300d), chimerical molecule CD300d/f (immunoglobulin domain and stem region of CD300d and the transmembrane and cytoplasmic tail of CD300f) and chimerical molecule CD300a/f (immunoglobulin domain and stem region from hCD300a, transmembrane and the cytoplasmic tail of CD300f) were generated and cloned into the pDisplay vector. CD300f ΔCyto (deletion from 1 to 178 aa) and Chimera CD300a/f ΔCyto (deletion from 1 to 202 aa) mutants molecules were generated by conventional PCR, using the wild type forms as template, and finally cloned into pDisplay vector. Full-length CD300c, CD300d, and CD300f were subcloned from pcDNA3.1-TOPO into the pEGFP-N3 vector. Details have been summarized (**Table 9**).

Other mammalian expression constructs used, has been described before pDisplay/ CD300a [176], -CD300b [201], -CD300c WT and ΔCyto (deletion from 209 to 224 aa)[176], -CD300e [215], and -CD300f [217]; pCDNA3- FLAG/CD300a, -CD300b, -CD300c, -CD300e, -CD300f, and -FcRγ [176]; pcDNA3-Flag/DAP12 and-DAP10 [201]; pBabePuro-2xMyc/CD300c [176]; pMES-fyn [231]; and pALTERMAX-mSyk Y323F-Myc was kindly provided by Dr. Hamid Band (University of Nebraska Medical Center, USA). The yeast expression constructs and libraries used for three hybrid assay has been described previously [217 231].

Mutagenesis

pDisplay/CD300d (R173S), -(E173A), and -(F168L/F170V); and pcDNA3-FLAG/FcRγ (D29A) substitution mutants were generated by PCR amplification with mutagenic oligonucleotides according to the instructions of the QuikChange site-directed mutagenesis kit (Stratagene). Details have been summarized (**Table 10**).

Molecule	Vector	Oligonucleotides sequence 5'-3'	Cloning sites	Template
CD300d FL	pCDNA3. 1/V5-His TOPO	Sense: TCACCAAGGAGAGGAGAGGA	-	Human monocyte cDNA
		Antisense: ACTCTCATCATCGGGCTGAC	-	
CD300d WT	pDisplay-HA	Sense: CCGAGATCTAAAATCACTGGTCCAACAACA	*Bgl*II	pCDNA3.1-CD300d FL
		Antisense: GCCGTCGACTCAAGACCTTCTTTGTGGTCT	*Sal*I	
Chimera CD300c-d	pDisplay-HA	Sense: CCTAGATCTACCGTGGCGGGCCCCGTGGGG	*Bgl*II	pDisplay-CD300c WT
		Antisense: GCCGTCGACTCAAGAGCGTCTCTGAGGTCTGTTCAC	*Sal*I	
Chimera CD300d-f	pDisplay-HA	Sense: TGGAATTCGGCTTGGGGATATCCACCATGG	-	pDisplay-CD300d WT
		Antisense: GGACACTGAGGAGCGGGGACCTGGTGAGGG	-	
		Sense: GTCCCCGCTCCTCAGTGTCCTCCTGCCCCT	-	pDisplay-CD300f WT
		Antisense: CAGATCCTCTTCTGAGATGAGTTTTTGTTC	-	
CD300d WT	pCDNA3-Flag	Sense: CCGAGATCTAAAATCACTGGTCCAACAACA	*Bgl*II (*Bam*HI)	pCDNA3.1-CD300d FL
		Antisense: GCCGTCGACTCAAGACCTTCTTTGTGGTCT	*Sal*I (*Xho*I)	
CD300c FL	pEGFP-N3	Sense: CCGGAATTCACAGGAATGACTGCCAGG	*Eco*RI	pCDNA3.1-CD300c FL
		Antisense: GCGGTCGACCTGGTTCTCACCCTT	*Sal*I	
CD300d FL	pEGFP-N3	Sense: CCGGAATTCGACAGGACCATGTGGCTGTCC	*Eco*RI	pCDNA3.1-CD300d FL
		Antisense: GCGGGATCCAGACCTTCTTTGTGGTCTGTT	*Bam*HI	
CD300f FL	pEGFP-N3	Sense: CCGGAATTCGAAGAGAAGATGCCCCTGCTG	*Eco*RI	pCDNA3.1-CD30f FL
		Antisense: GCGGGATCCAGGCCTGCTGATGGTGCTGTA	*Bam*HI	
Chimera CD300a-f	pDisplay-HA	Sense: CCTAGATCTAGCAAATGCAGGACCGTGGCG	*Bgl*II	pDisplay-HA/CD300a
		Antisense: GACACTGAGCTGTGAGTTCACCACCTCCTC	-	
		Sense: AACTCACAGCTCAGTGTCCTCCTGCCCCTC	-	pDisplay-HA/CD300f
		Antisense: GCCGTCGACCTAAGGCCTGCTGATGGTGCTGTATTC	*Sal*I	
CD300f ΔCyto	pDisplay-HA	Sense: CCTAGATCTGGCTACTCCATTGCCACTCAA	*Bgl*II	pDisplay-HA/CD300f
		Antisense: GGCAGATCTTTACGCCCAAGCCAAGAGTGAGGCGGC	*Bgl*II	
Chimera CD300a-f ΔCyto	pDisplay-HA	Sense: CCTAGATCTAGCAAATGCAGGACCGTGGCG	*Bgl*II	pDisplay-HA Chimera a-f
		Antisense: GGCAGATCTTTACGCCCAAGCCAAGAGTGAGGCGGC	*Bgl*II	

Table 9: Constructs generated for the study. Oligonucleotides used for product amplification and templates are listed. Restriction sites used for cloning are underlined. Compatible restriction sites in the vector when used are in parenthesis. *FL = Full length

Molecule	Vector	Oligonucleotides sequence 5'-3'	Cloning sites	Template
CD300d R193S	pDisplay-HA	Sense: CCGAGATCTAAAATCACTGGTCCAACAACA	*Bgl*II	pDisplay/ CD300d
		Antisense: GCCGTCGACTCAAGA**G**CTTCTTTGTGGTCT	*Sal*I	
CD300d E173A	pDisplay-HA	Sense: CTGTTCCTCCTGG**C**GCTGCCTCTGCTCCTG	*Bgl*II	pDisplay/ CD300d
		Antisense: CAGGAGCAGAGGCAGC**G**CCAGGAGGAACAG	*Sal*I	
CD300d F168L-F170V	pDisplay-HA	Sense: ACCCACTTCCTGTT**A**CTG**G**TCCTCCTGGAG	*Bgl*II	pDisplay/ CD300d
		Antisense: CTCCAGGAGGA**C**CAG**T**AACAGGAAGTGGGT	*Sal*I	
FcRγ D29A	pCDNA3-Flag	Sense: GCTCTGCTATATCCTGG**C**TGCCATCCTGTTTCTGT	*Bam*HI	pcDNA3-FLAG/FcRγ
		Antisense: ACAGAAACAGGATGGCA**G**CCAGGATATAGCAGAGC	*Eco*RI	

Table 10: Mutagenesis constructions generated for the study. Oligonucleotides used for product amplification and templates are listed. Nucleotides changes introduced in the sequence for amino acid substitutions are shown in bold letters. Restriction sites used for cloning are underlined.

Insertion of DNA fragments into expression vectors

Desired DNA fragments, obtained by PCR or enzymatic restriction digestion of an existing construction, were inserted into linearized plasmids with compatible extremes using the T4 DNA ligase (Invitrogen), following the manufacturer's instructions.

Three-hybrid assay in yeast

In order to identify new binding partners of the CD300f receptor, involved in its signaling pathway, the cytoplasmic tail of the receptor (Cyto CD300f) was cloned in the MCS I of pBridge Vector to be used as bait in a three-hybrid assay. This assay is known to enable the detection of protein-protein interaction and also permits to evaluate the effect of phosphorylation on the interaction, using simple phenotypic assays in yeast. The Yeast Protocol Handbook PT3024-1 (from Clontech) for Two-hybrid System was used. Protocols and the composition for the used buffers can be found in the following link:

(http://coli.usal.es/web/abydl/biblioteca/bibelectro.alu/documentos/protocolos/Yeast_protocols_Clontech.pdf)

The key to the two hybrid assay is that the function of some eukaryotic transcription factors, like GAL4, can be reconstituted when their activating and binding domains (AD and BD respectively), are sufficient close one to the other, allowing the transcription of reporter genes

under the control of upstream activation sequence (UAS), a sequence target for the GAL4 transcription factor.

Genetically modified yeast strain CG1945, lacking the biosynthesis of certain essential amino acids (tryptophan (Trp), leucine (Leu), methionine (Met) and histidine (His)) and presenting the expression of reporter genes His and β-galactosidase under the control of GAL4 transcription factor, was transformed with *Bait* and *Prey* plasmids sequentially (it is assumed that each cell is transformed with no more than a single plasmid at one time, and that a maximum of two plasmids can be introduced inside one yeast cell). In the case of the present study, *Bait* plasmid was a pBridge vector encoding for Trp (TRP1 gene) and also for the BD of the transcription factor GAL4, fuse to the Cyto CD300f sequence. On the other hand, the *Prey* plasmids were a library of pGAD10 vectors, encoding for Leu (LEU2 gene) and the (AD) of the transcription factor GAL4, fuse to different cDNAs from a library of PMA activated monocytes. Theoretically the library consisted of $2 \cdot 10^6$ independent clones (**Figure 13**).

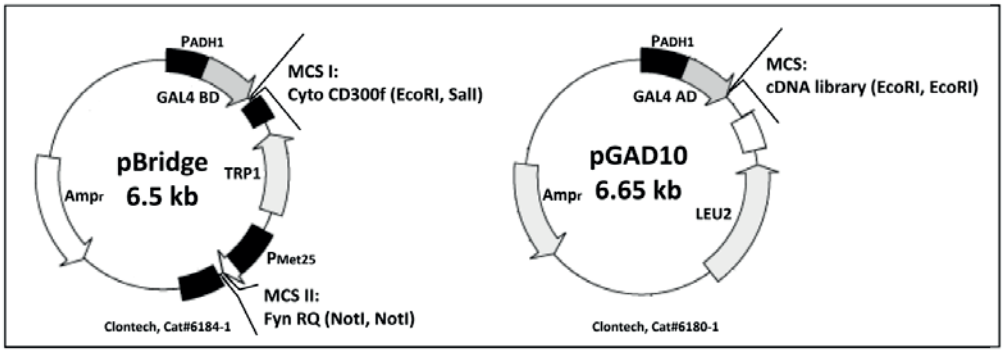


Figure 13: pBridge and pGAD10 vectors map. The molecules cloned into the MCS I from the pBridge vector and the molecules cloned into the MCS from the pGAD10 vector were constitutively expressed under the control of the promoter PADH1. The expression of the molecule cloned into the MCS II of the pBridge vector was a conditional promoter controlled by the presence or absence of methionine in the medium.

Yeast cells correctly co-transformed were positive selected by growing the cells into plates with restrictive medium, without Trp and Leu. Then, colonies were tested for the reporter genes expression. Just in the cases where the Cyto CD300f interacted with the protein encoded by a cDNA of the library, the GAL4 transcription factor was reconstituted inducing the expression of reporter gens (**Figure 14**).

For testing the expression of His, the yeast colonies were grown in restrictive medium plates without Trp, neither Leu nor His. Just cells transcribing His were able to grow on the plates. Then, in order to confirm these results, the expression of β-galactosidase was tested. Small pieces of Whatman paper were put in contact with each yeast colony and then soaked into X-gal solution, X-gal is a colorless substrate that becomes a blue product after been transformed by b-galactosidase. However, using both techniques it was unfeasible to determine the intensity of the interaction between the *Bait* and the *Prey*

molecules. In order to solve that, a third assay was performed involving the β-galactosidase activity. Each positive colony was grown in liquid restrictive medium, X-gal solution was added to the culture after yeast lysis, and β-galactosidase activity was measured, as much intense it was the blue, as much strong it was the interaction.

The effect of phosphorylation in the assay was controlled by a conditional methionine promoter, such that Fyn kinase was expressed in the absence of methionine. That allowed expression to be switched on/off by a simple change in the medium composition.

One by one the plasmidic cDNA from positive yeast clones was extracted, to transfect bacteria, which will increase the amount of the plasmid by cell division. Finally the plasmidic cDNA was purified from the bacteria culture, analyzed by restriction digestion and sequenced in order to identify for which protein is codifying.

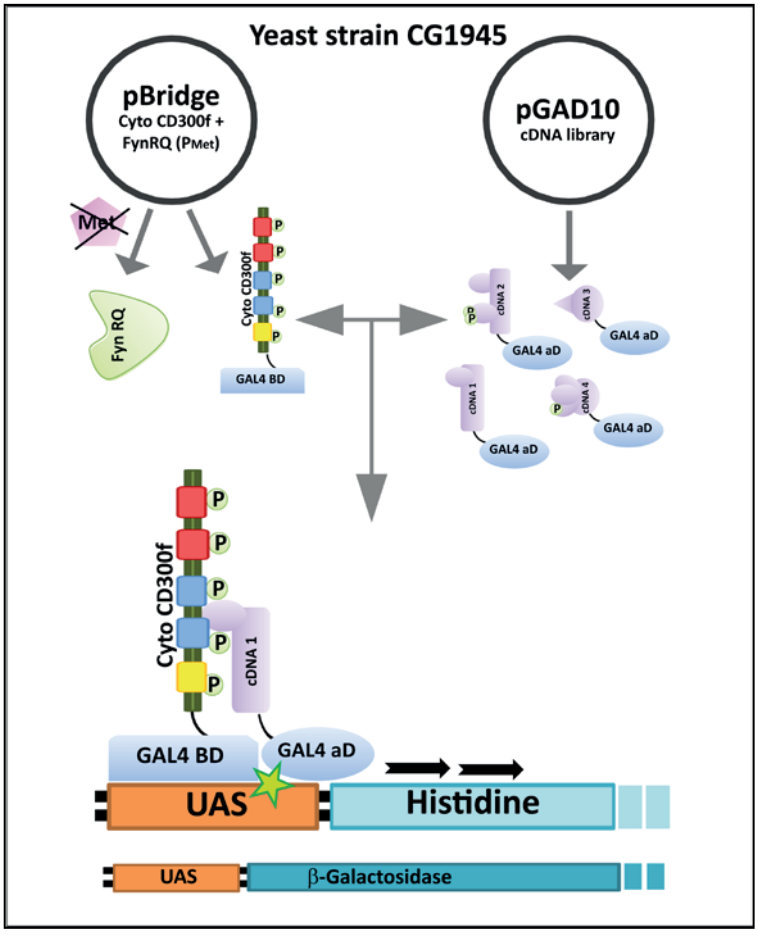


Figure 14: Illustration of the three hybrid system.

Quantitative Real Time -PCR

Total RNA from cell was extracted with TRIzol® reagent (Invitrogen), then treated with DNAse I amplification grade (Invitrogen), and retrotranscribed to cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturers' instructions.

The cDNA coming from U937 myeloid cell line, six hours after CD300f stimulation, was used to perform a quantitative real time PCR using Taqman Gene Expression Assay for IL-1β (Hs00174086_m1), IL-6 (Hs00174131_m1), IL-10 (Hs00174086_m1), COX-2 (Hs00153133_m1) and TNFα (Hs00174128_m1) (Applied Biosystems).

The cDNAs coming from Hela cells line, primary T Cells, primary B Cells, primary NK Cells, primary monocytes, primary Granulocytes, primary PBMCs and different subtypes of *in vitro* differentiated macrophages (M1, M2a, M2c) were used to perform quantitative real time PCRs using Taqman Gene Expression Assay for CD300d (E34) (Applied Biosystems).

In all the experiments 18s was used as endogenous control for cycle normalization (RT-PCR Pre-developed assay reagents 18S rRNA System) (Applied Biosystems).

Samples were run for 40 cycles (15 s 95°C, 1 min 60°C) in a 7500 Fast Real-Time PCR System (Applied Biosystems). All PCRs were set up in triplicates.

Transient transfection of DNA in COS-7 mammal cell line

COS-7 cell line growth in plates (10 cm diameter) ($6{\cdot}10^5$ cells/condition) were transfected with 6 μg of plasmidic DNA using LyoVec (Invivogen) according to the manufacturer's instructions. The correct expression of the transfected DNAs was checked by analyzing the cells by cytometry, by immunofluorescence or by WB of their lysates, 48 or 72 hours post-transfection.

Stable transfection of DNA in RBL-2h3 cell line

For the generation of RBL-2H3 stable transfectants, $20{\cdot}10^6$ cells/condition were resuspended in incomplete DMEM medium, transferred into a 4 mm gap electroporation cuvette and electroporated in the presence of 20 μg of linearized plasmidic DNA, at 200 ohms (Ω), 250 volts (V) and 960 micro–farads (μF), in a Gene Pulser electroporator (Bio-Rad). Electroporated cells were seeded in 4 plates (10 cm diameter) in complete DMEM medium to let them to recover for 24 hours. After, transfected cells were selected and maintained in culture with cDMEM plus selection antibiotic, for example: 1mg/ml of G418 (Invitrogen), or 1 μg/ml of Puromycin (Sigma-Aldrich).

Stable transduction of shRNA in U937 human cell line

U937 cells were infected with lentiviral shRNA particles targeting FcRγ or DAP12 (Santa Cruz Biotechnology Inc.) according to manufacturer's instructions. Scramble shRNA was also used as a negative control. Infected cells were selected and maintained in culture with cRPMI plus 0.25 μg/ml of Puromycin.

Inhibitors treatment of U937 human cell line

U937 cells were incubated with inhibitors or vehicles for 15 min at 37ºC prior to seed them in the Ab-coated-plates. JNK kinase inhibitor SP600125 and PKC inhibitor Bisindolylmaleimide (Bis I) were from Santa Cruz Biotechnology Inc. PKC inhibitor Gö6976 was from Sigma-Aldrich, PKC inhibitor Gö6983 was from TOCRIS and Syk inhibitor R406 was provided from AstraZeneca LP. All the chemicals were dissolved in DMSO except BisI that was reconstituted in distilled water. DMSO concentration did not exceed 0.1% in any of the cases.

Generation of competent bacteria cells for heat shock

There are a lot of protocols to obtain bacteria cells able to be transformed by heat shock. The protocol used in the laboratory is described afterwards and the buffers used are summarized (**Table 11**). Briefly, DH5α sub cloning efficiency bacteria cells (Invitrogen) were seeded on a LB-Agar (Luria Broth Base, Invitrogen, plus Agar) plate and incubated O/N at 37ºC. Next day one colony from the plate was expanded into 5 ml of LB liquid culture, O/N at 37ºC, with shaking. Third day early in the morning 40 μl of the O/N culture was expanded to 400 ml of SOB medium supplemented with 4ml of Mg solution 2M. From then, 3 nights of incubation at 18ºC were needed to allow the adequate growing of the bacteria. At the sixth day it was necessary to control the growth of the culture by measuring the OD at 600λ, until it arise a value between 0.4 and 0.6, indicating that the culture was in the exponential growing phase. At that point the cells were harvest 15 min, 4ºC at 1811g speed. The supernatant was removed and the pellet was resuspended in 140 ml of TB buffer. Then the cells needed to be incubated, 10 min at 4ºC, and to be harvest again. The supernatant was removed again to eliminate any trace of SOB medium and cells were resuspended in 32 ml of fresh TB buffer, then 2.6 ml of DMSO were added to the cells suspension. At that point it was very important to incubate the cells on ice for at least 10 min (O/N is better, because the competence increases with the time at 4ºC). Finally, bacteria could be aliquot (220 μl/eppendorff) and freeze into N_2 bath.

Buffers and Solutions	Components
Mg Solution 2M	For 10 ml: - 2.47 g $MgSO_4 \cdot 7H_2O$ (1M) - 2.03 g $MgCl_2 \cdot 6H_2O$ (1M) - Mili-Q H_2O until 10 ml - Filter to assure sterility (0.22 mm)
SOB Medium	For 400 ml: - 8 g Bacto tryptone - 2 g Bacto yeast extract - 0.8 ml NaCl 5M (2.92g in 10 ml of H_2O) - 0.5 ml KCl 2M (1.49g in 19 ml of H_2O) - Mili-Q H_2O until 400 ml - Autoclave - Add before using: - 4 ml of Mg Solution 2M
TB Buffer	For 500 ml: - 1.5 g PIPES (10 mM) - 1.1 g $CaCl_2$ (15 mM) - 9.3 g KCL (250 mM) - Mili-Q H_2O until 500 ml - Bring the solution to ph 6.7- 6.8 with NaOH 5M - Add 5.45 g $MnCl_2 \cdot 4H_2O$ (55mM) - Filter to assure sterility (0.22 mm)

Table 11: Composition of the buffers used for the obtaining of heat shock competent bacteria.

Heat shock Bacteria transformation and recovery of plasmidic DNA

Heat Shock Competent bacteria cells E.coli DH5α was transformed with plasmidic DNA, generated in the laboratory by genetic enginery, to increase the amount of it. Any construction, wandered to be transformed, have to fulfill two essential requirement: to carry a replication start sequence for bacteria (e.g., ColE1 origin, pUC origin), allowing the duplication of the plasmidic DNA at the same time as the bacteria replicate its own DNA before cell division, and to codify for some antibiotic resistance, in order to permit the selection of correctly transformed bacteria from the rest.

100 µl of competent bacteria were mixed with 1 µg of supercoiled plasmids or 10 µl of ligation reaction, incubated 30 min at 4ºC before heat shock (1min, at 42ºC) and then cooled down again for 2 min at 4ºC. Finally the bacteria were seeded in Petri plates containing LB medium (Luria Broth Base, Invitrogen) plus Agar and selective antibiotic, usually ampicillin at 100 µg/ml, or kanamycin at 50 µg/ml. Plates were incubated O/N at 37ºC, to allow the growth of bacteria colonies. In order to obtain big amounts of bacteria,

ten colonies were grown in medium with antibiotic O/N at 37ºC with shaking, 3 ml for Minipreps or 250 ml for Midipreps. Finally, the plasmidic DNA was extracted using the NucleoSpin® Plasmid or NucleoBond® Xtra Midi plus kids (Macherey-Nagel).

Immunoprecipitation

IP is a method that uses the antigen-antibody reaction principle to identify a protein from a mixture of compounds. The Key to the IP assay is the use of highly porous agarose beads that provide a solid-phase support where molecules can be attach in a specific way. This support present two advantages: first, a very high potential binding capacity, as virtually the entire sponge-like structure of the agarose particle (50 to 150 µm in size) is available for binding antibodies (which will in turn bind the target proteins); second, standard laboratory equipment is enough for all the aspects of the IP protocol.

Briefly, harvest cells were lysed for 30 min at 4ºC using different complete lysis buffer (LB) (**Table 12**). Usually, each confluent (10 cm diameter) plate of COS-7 or RBL 2H3 cells were lysed into 1 ml of complete LB and each $10 \cdot 10^6$ U937 cells were lysed into 250 µl of Complete LB. Cell lysates were clarified by centrifugation at 13.200 xg, for 15 min at 4ºC. Once the crude lysate was obtained, a little amount (30 µl) was saved at 4ºC to be used as control and the rest was process. Prior to the IP itself, a pre-cleaning procedure of the whole lysate was required, in order to remove potentially reactive components able to bind to the IP antibody or protein A/G beads in a non-specific way. The basic pre-cleaning procedure consisted in three rounds of incubation of the whole lysate with 20 µl of protein G-Shepharose beads (Amersham Bioscences) plus isotopic control Ab (usually 1 µg/ml of Mouse Ig), 30 min at 4ºC, discarding the beads in every round. Afterwards, pre-cleaned lysate was incubated with 30 µl of protein G-Shepharose plus 1 µg/ml of specific Ab for 3h at 4ºC. Then, the beads are cleaned x3 with incomplete LB in order to eliminate any compound retained between the agarose beads. Finally, the components of the bound immune complex (both antigen and Ab) are eluted from the agarose support by adding Sample Buffer (**Table 13**) and incubating the mixture for 5min at 100ºC. Immunoprecipitated proteins and the ones that are able to interact with them, called co-immunoprecipitated proteins, are further analyzed by SDS-PAGE and immunoblotting.

Types of LB	Detergent	Other compounds
Incomplete LB	-	For 1 l: - 4.8 g Hepes pH 7.5 (20 mM) - 8.8 g NaCl (150 mM) - 2.1 g NaF (50 mM) - 0.18 g Na_3VO_4 (1mM) - 0.008 g PAO (Phenylarsine oxide) (50 mM) - 1 mM EGTA - Mili-Q $_{H2}O$ until 1 l
Complete LB Chaps 1%	CHAPS	For 10 ml: - 10 ml of Incomplete LB - 0.1 g CHAPS - 0,1- 1 mM PMSF - Protease inhibitor cocktail
Complete LB Triton 1%	Triton x-100	For 10 ml: - 10 ml of Incomplete LB - 100 µl of Triton X-100 - 0,1- 1 mM PMSF - Protease inhibitor cocktail

Table 12: Composition of the lysis buffers.

SDS-PAGE and immunoblotting

The SDS-PAGE and immunoblotting assays were used to monitor the presence and quantity of proteins; also to observe posttranslational modifications or determine specific enzymatic activity.

Briefly, once the protein extracts were obtained and denaturalized, by mixing them with Sample buffer and incubating 5 min at 100ºC, they could be loaded into the SDS-PAGE. The gel was composed by two phases, 5% acrylamide Stacking gel above and 10 % to 12 % acrylamide Separating gel below. When voltage was applied along the gel (80 V during O/N), proteins migrated. Then, proteins from within the gel were moved onto a PVDF membrane (from Thermo Scientific) using electric current, the intensity of transference in miliamperes units was calculated (size of the gel in cm^2 x 0.8) and was applied for 3 hours. Afterwards the membrane could be kept at 4ºC in T-TBS (TBS-tween 1X buffer) until being incubated with the appropriate Abs.

For immunoblotting, a preblocking steep of the surface of the membrane was achieved by placing it in a dilution of 5% of milk in T-TBS, for 1 hour at RT (room temperature) under gentle agitation. Then the blocking was washed and the primary Ab, diluted in T-TBS, was added to the membrane and incubated for 1 hour at RT, under gentle agitation. Afterwards primary Ab was washed and the secondary Ab, diluted in T-TBS, was added and incubated

for 30 min, at the same conditions as before. The secondary Abs were always linked to horseradish peroxidase reporter enzyme, which was able to cleave the chemiluminescent agent SuperSignal West Pico (from Thermo Scientific). Finally sensitive photographic film (from Agfa Healthcare NV) was placed against the membrane, to obtain an image. The proportion of the signal depends on the amount of the stained protein present in the membrane. All the buffers and solutions used for that protocol are described (**Table 13**), (**Table 14**) and (**Table 15**).

Buffer and solutions for protein extracts preparation	Compounds
SDS 10%	For 100 ml: - 10 g SDS - Mili-Q H_2O until 100 ml
Sample buffer 2X	For 100 ml: - 10 ml Glycerol - 30 ml SDS 10% - 12.5 ml Tris-Upper (4X) - 1 ml Bromophenol Blue 0.5% - 5 ml β-Mercaptoethanol - Mili-Q H_2O until 100 ml

Table 13: Composition of the buffer and solutions used for protein extracts preparation.

Buffer and solutions for SDS-PAGE	Compounds
Tris-Upper solution (4X)	For 100 ml: - 6.06 g Tris (0.5 M) pH 6.8 - 4 ml SDS 10% - Mili-Q H_2O until 100 ml
Tris-Lower solution (4X)	For 100 ml: - 18.17 g Tris (1.5 M) pH 8.8 - 4 ml SDS 10% - Mili-Q H_2O until 100 ml
Separating gel 10-12%	For 30 ml: - 7.5 ml Tris-Lower pH 8.8 (4X) - 7.5- 10 ml Acry/Bis (40%) - 15- 13.5 ml H_2O - 300 µl APS (100mg/ml) - 15 µl TEMED
Stacking gel 5%	For 10 ml: - 2.5 ml Tris-Upper pH 6.8 (4X) - 1.25 ml Acry/Bis (40%) - 6.25 ml H_2O - 100 µl APS (100mg/ml) - 5 µl TEMED
Running Buffer (10X)	For 1L: - 30 g Tris - 5 g SDS - 14.5 g Glycine - Mili-Q H_2O until 1L
Transfer Buffer (1X)	For 1L: - 5.8 g Tris - 0.37 g SDS - 2.9 g Glycine - 200 ml Methanol - Mili-Q H_2O until 1L

Table 14: Composition of the buffers used in the SDS-PAGE assay.

Buffers for immunoblotting	Compounds
TBS-tween (T-TBS) (10X)	For 1L: - 24.1.g Tris - 80 g NaCl - 20 ml Tween - pH at 7.6 - Mili-Q H_2O until 1 L
Stripping Buffer (1X)	For 1L: - 3.75 g Glycine (50 mM) - 87.66 g NaCl (1.5 M) - pH at 2.4 - Mili-Q H_2O until 1 L

Table 15: Composition of the buffers used in the immunoblotting assay or Western-blotting (WB).

Flow Cytometry

The expression of molecules in the cell surface of different cells lines was tested by indirect immunofluorescence technique. A primary Ab against the desired molecule is incubated with the cells in PBS (1X), for 20 min at 4ºC. Then cells were washed with PBS (1X) and the secondary antibody labeled with some fluorochrome (usually FITC) was added diluted in PBS (1X) and incubated for 20 more minutes at 4ºC. Finally, cells were washed again and stained with PI at 1 μg/ml (Sigma-Aldrich (St. Louis, MO)), in order to discriminate living cells from dead ones. All the centrifugations were performed at 4ºC, for 10 s. at 8000 rpm.

In the case where the cells used express Fc receptors (like U937, THP-1, RBL-2H3, among others), they need a pre-blocking step previous to the primary Ab incubation, in order to avoid unspecific interaction of the Ab to the cell surface, throw the Fc of the immunoglobulin. The pre-blocking consisted of incubating the cells with an excess of human IgG immunoglobulin (at 30 μg/ml) for 30 min at 4ºC.

The concentration of the used antibodies is summarized (**Table 7**) and (**Table 8**). The samples were acquired on a FACSCalibur using the Cell Quest Software (BD Biosciences); afterwards the results were analyzed with the FCS Express 4 Image (De Novo Software).

Immunofluorescence

Cos-7 cells were seeded on 24 well/plates ($1.8 \cdot 10^4$ cells/well) containing glass coverslips, which was previously treated with 0.1% gelatin solution in H_2O for 10 min. Cells were cultured O/N to permit a good attachment to the substrate and the next morning transfected with LyoVec, using molecules labeled with GFP. Twenty four hours post-transfection, cells were washed two times with PBS (1X) for 5 min, fixed with 4% PFA in PBS

(1X) for 10 min and washed again. Then were permeabilized with 0.5% Triton X-100 in PBS (1X) for 5 min, washed two times more and stained with DAPI at 0.1 ng/ml for 1 min. Afterwards, coverslips were washed with PBS (1X) two times, rinsed with water, dried and placed in glass slides using Fluoromount TM Aqueous Mounting Medium (Sigma-Aldrich (St. Louis, MO)). Fluorescence images were captured using a FV Olympus confocal microscope.

ELISA assay for TNFα and IL-1β cytokines detection in conditioned medium

Twenty-four hours after cell stimulation, conditioned mediums from myeloid cell line U937 or primary monocyte cells were collected and clarified by centrifugation at 1000xg for 15 min at 4ºC. TNFα and IL-1β protein levels were quantified by ELISA according to manufacturer's instructions (BD Biosciences).

ELISA Assay for soluble CD300f detection in human fluids

To develop and (enzyme-linked immunosorbant assay) ELISA assay, able to detect the soluble variant of the CD300f receptor in human fluids, some molecular tools need to be generated.

Biotinylation of the antibody used for the detection step of the ELISA

First of all, it was necessary to biotinylate the Ab that want to be used as capturing Ab and detecting Ab for the assay. UPD1 or UPD2 mAb, were the candidates because their ability to detect CD300f at the extracellular domain. It is important to mention that, for the Biotin molecule to be able to interact with an Ab, it was essential that the Ab was dissolved in water or PBS (1X), any other salt added to the solution inhibits the biotinylation reaction.

For the biotinylation reaction itself it was necessary to incubate 1 molecule of Ab with 20 molecules of Biotin (EZ-Link™ Sulfo-NHS-Biotin from Pierce) during 2 hours at 4ºC. It was assumed that the molecular weight of the UPD1 and UPD2 Ab was 150.000 g/mol. After the incubation, in order to eliminate the excess of Biotin, it was necessary to perform a dialysis procedure, achieved introducing the mixture of Ab and Biotin inside a cassette (Slide-A-Lyzer® Dialysis Cassette Extra Strength from Thermo Scientific) and introducing the cassette in a recipient containing 2 liters of PBS (1X). The cassette was kept in the recipient O/N at 4ºC in agitation. Finally the content of the cassette was recovered and the concentration of the Ab was measured with the NanoDrop 2000 Spectrophotometer (Thermo Scientific).

To verify the correct biotinylation of the Ab, they were coated in an ELISA plate O/N and after a blocking steep (PBS (1X) with 10% FBS for 2 h) streptavidin-HRP conjugated was added. Then, streptavidin excess was washed and Substrate Solution was added to observe any HRP activity. Not biotinylated UPD1 and UPD2, or mIgG2a were used as negative controls.

CD300f-IgG2a fusion protein as positive control of the ELISA Assay

The recombinant protein CD300f-IgG2a fused the extracellular domain of the CD300f receptor to the constant part to the mouse IgG2a immunoglobulin. It was previously generated in the laboratory to be used like a primary antibody in cytometry assay, in order to detect the presence of putative ligands for the CD300f in the surface of myeloid cells. However that tool might be used also as positive control of the ELISA assay due its structure.

Production of the CD300f-IgG2a fusion protein

To obtain significant amount of the fusion protein a procedure involving cell transfection and protein purification was required. Briefly, $7.5 \cdot 10^6$ CHO-K1 cells were resuspended in incomplete RPMI 1640 medium, transferred into a 4 mm gap electroporation cuvette and stable transfected with 20 μg of the pSECTag/ IgCD300f-mIgG2a construction by electroporation at 100 Ω, 250 V and 960 μF in a Gene Pulser electroporator (Bio-Rad). Electroporated cells were resuspended in complete RPMI 1640 medium and seeded in 24 well plates at $1 \cdot 10^4$ cell/750 μl/well. Next day, transfected cells were selected and maintained in culture with cCHO culture medium plus 250 μg/ml of the Zeocin selection antibiotic (Invivogen). For all the clones that survive the selection with Zeocin the amount of fusion protein released in the supernatant was checked by ELISA assay.

The ELISA assay consisted of coating the ELISA plate O/N at 4ºC with a Sheep anti-Mouse (SAM) Ab at (5μg/ml) in Coating Buffer. Next morning, plate was washed twice with Wash Buffer and the Blocking Solution was added and incubated for 1 h at 37ºC. Then the Blocking was discarded and the samples, corresponding to the supernatant (SN) of the different clones, were added and incubated 2 h at RT. As negative control cCHO medium was used and for positive controls cCHO medium containing different amounts of mIgG2a were used. Afterwards, samples were discarded and the plate was washed twice with Wash Buffer before adding the detection Ab, Anti-Mouse IgG2a-HRP diluted 1:5000 in PBS (1X), which was incubated for 30 min at RT. Then the detection Ab was discarded, wells were washed twice with Washing Buffer and twice with PBS (1X). Afterwards, Substrate Solution was added and incubated until the positive controls exhibited a good amount of colored product, on that moment Stop Solution was added and the absorbance was measured at 450 nm. Buffers and Solution compositions are summarized (**Table 16**).

Once checked which clones produce significant amount of the fusion protein, the corresponding SNs were analyzed by SDS-PAGE and stained with Bio-Safe™ Comassie Stain (from Bio-Rad), to verify that the size of the protein matched with the predicted molecular weight of it. From all the positive clones, one was chose to be expanded in order to obtain huge amount of SN (around 1 or 2 liters).

Purification of the CD300f-IgG2a protein from the cells supernatant

The procedure used to purify the soluble protein from the SN was the following: the SN was centrifuged twice at 3000xg, for 10 min at 4ºC-10ºC, in order to eliminate any cell debris. Then 90 ml from the SN was mixed with 10 ml of PBS (10X), to neutralize the pH, and the SN was passed throw an affinity column formed of protein A resin, which had the property to interact with the heavy chain of Abs or immunoglobulins. Afterwards, the column was washed with 30 ml of PBS (1X) and the fusion protein was eluted with 3 ml of Elution Buffer in different aliquots (300 µl/ aliquot) containing 30 µl of Hepes Buffer. Subsequently, the amount of protein contained in each aliquot was analyzed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). The aliquot showing higher amounts of protein (usually 0.3-0.5 mg/ml) were frozen until being concentrated. Afterwards, all the aliquots were unified, and the solution, where the soluble protein was contained, was substituted for sterile PBS (1X) using Vivaspin 15 ultrafiltration device (from Sartorius Stedim Biotech), at the same time that the protein was concentrated to a volume allowing a final concentration around 1 mg/ml. Buffers and Solution compositions are summarized (**Table 17**).

Buffers and Solutions of the ELISA Assay	Compounds
Coating Buffer **(0.1 M Sodium Carbonate pH 9.5)** **Keep at 4ºC** **100µl/well**	For 1L: - 3.56 g Na_2CO_3 - 8,4g $NaHCO_3$ - pH at 9.5 - Mili-Q H_2O until 1 L
Blocking Buffer **200µl/well**	For ELISA Assay to detect the fusion protein IgCD300f-mIgG2a: - PBS (1X) with 2% BSA For ELISA Assay to detect the soluble CD300f receptor - PBS (1x) with 10% Filtrated FBS - pH 7.2 – 7.5
Washing Buffer **200µl/well**	0.05% Tween 20 in PBS (1X) pH at 7.2 – 7.4
Substrate Solution **100µl/well**	1:1 Mixture of Color Reagent A and Color Reagent B (Becton Dickinson) Prepare it at the moment of use
Stop Solution **50µl/well**	6N HCL

Table 16: Composition of the buffers and solutions used in the ELISA assays.

Buffers used in the protein purification	Compounds
Elution Buffer **(Sterile)** **Keep at 4ºC**	0.1M Glycine 1M NaCl pH 2.4
Hepes	2M Hepes pH 9

Table 17: Composition of the buffers used in the purification of the recombinant protein.

ELISA protocol for the detection of the soluble CD300f molecule in human serum

The ELISA assay consisted of coating the ELISA plate O/N at 4ºC with UPD1 mAb at (10µg/ml) in Coating Buffer. Next morning, plate was washed three times with Wash Buffer and the Blocking Solution was added and incubated for 1 h at RT. Then the Blocking was discarded and the samples, corresponding to the subject serums diluted 1:2 in PBS (1X), were added and incubated 2 h at RT. Afterwards, samples were discarded and the plate was washed three times with Wash Buffer before adding the biotinylated-UPD2 detection mAb, diluted in PBS (1X) at (1µg/ml), which was incubated for 1 h at RT. Subsequently the detection Ab was discarded and wells were washed five times with Washing Buffer. Then streptavidin-HRP diluted 1:5000 in PBS (1X) was added and incubated 1h at RT. Afterwards, streptavidin-HRP was discarded, wells were washed seven times with Washing Buffer and Substrate Solution was added and incubated until the positive controls exhibited a significant amount of colored product, on that moment Stop Solution was added and the absorbance was measured at 450 nm. Buffers and Solution compositions can be check in (**Table 16**).

Objectives

OBJECTIVE ONE

- To characterize the novel CD300d receptor at molecular and functional levels.

OBJECTIVE TWO

- To verify the proposed inhibitory/activating functional duality of the endogenous CD300f receptor. In addition, to concrete the molecular mediators behind the putative activating pathways.

OBJECTIVE THREE

- To develop an assay able to detect putative soluble variants of the CD300f receptor in human fluids, in order to prove its existence and to study its involvement in autoimmune diseases.

Results

CLONING AND CHARACTERIZATION OF CD300d

Cloning of human CD300d

With the aim of identifying new members of the CD300 family, the sequences of known human CD300 molecules were used to blast the Ensembl genome database (Ensembl BLAST Server at http://www.ensembl.org). The search resulted in a cDNA encoding for a novel putative CD300 receptor termed CD300d. Next, primers were designed to amplify the putative CD300d nucleotide sequence from cDNA obtained from human monocytes. The 644bp PCR product contained an open reading frame of 585 bp, encoding for a protein of 194 aa with a predicted molecular mass of 21.5 kDa (EF137868) (**Figure 15**).

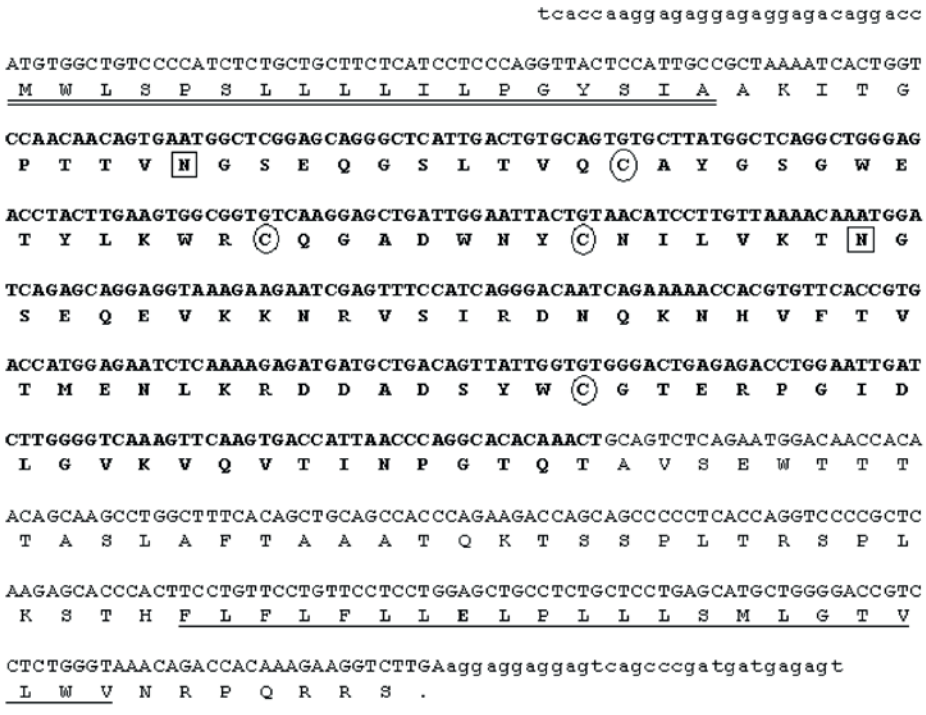


Figure 15: Schematic representation of nucleotide and amino acid sequence of CD300d receptor (EF137868). The nucleotide sequence of human CD300d containing an open reading frame (ORF) of 585bp is shown in upper case while the 5' and 3' untranslated regions (UTR) are shown in lower case. The corresponding amino acid sequence is represented below the nucleotide sequence. The putative signal peptide is double underlined, the immunoglobulin like domain (Ig-like domain) is in bold type and the transmembrane domain is single underlined. Potential N-glycosylation sites are boxed, cysteine residues involved in the Ig- like domain fold are circled and the transmembrane charged glutamic acid is bold.

Sequence analysis of human CD300d

Predicted CD300d protein

Sequence analysis identified CD300d as a type I transmembrane receptor. The receptor is driven by a signal peptide 18-aa in length. The extracellular region presents a single IgV-type domain, followed by a 41-aa membrane-proximal or stem region, the transmembrane domain and a very short cytoplasmic tail of 7-aa at the C-terminal end of the protein. The cytoplasmic domain did not show any known signaling motif (**Figure 15**).

The immunoglobulin-like domain of CD300d was stabilized by two disulphide bonds between cyteine residues: C39:C107, conserved in all the immunoglobulin-like receptors, and C53:C61, conserved in all the CD300 family receptors (**Figure 15**).

The transmembrane domain presented a negatively charged residue (glutamic acid, E173) in a central position, which might be important for the interaction with signaling molecules through the transmembrane region (**Figure 15**). Specific servers used for the analysis are detailed (**Table 18**).

SERVER	Predicted Structure	Amino acids
SignalP 3.0	Signal peptide	1 to 18
Specialized BLAST	Immunoglobulin domain	24 to 130
TMHMM 2.0	Transmembrane region	166 to 188

Table 18: CD300d sequence analysis. List of the servers used to predict the tertiary structure of CD300d from its amino acid sequence. The amino acids defining each structure are specified.

CD300d gene organization

The gene organization of CD300d was determined by aligning of the cDNA with the genomic sequences. The gene spanned a 12.5 kb region on chromosome 17 (position 17q25.1) and it was composed of four exons. The first exon encoded for the 5' untranslated region and the signal peptide, the Ig domain is encoded by exon 2, the membrane-proximal region was encoded by exon 3, the transmembrane domain and the cytoplasmic tail were encoded by exon 4 (**Figure 16**).

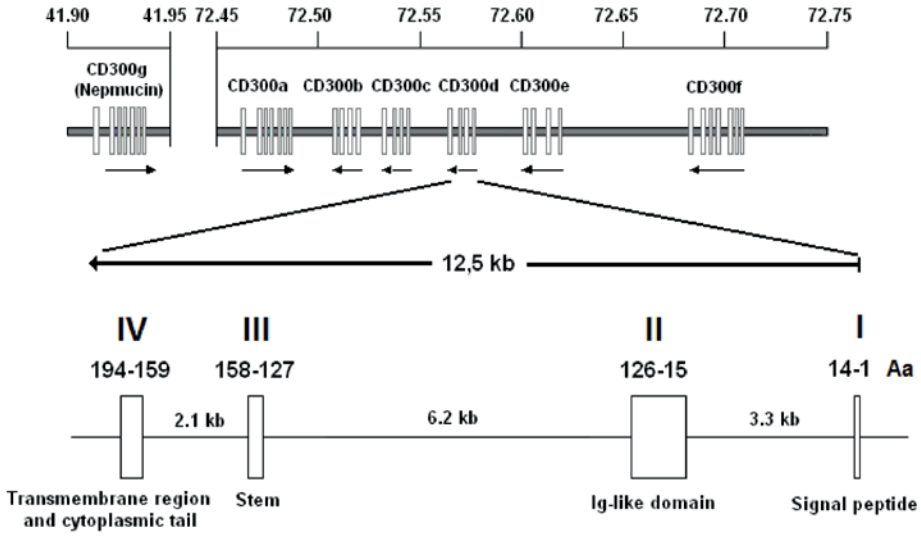


Figure 16: CD300d gene organization: Schematic organization of the CD300 locus at chromosomal region 17q.25.1. CD300d genomic organization is shown below. Exons are represented by boxes (respective amino acid lengths and domain architecture are shown); introns are represented by connecting lines.

Prediction of posttranslational modifications of CD300d

Specific posttranslational modifications such as glycosylation (N-glycosylation and O-glycosylation) are common in proteins that are expressed in the cell membrane or are released in the external media. The CBS Prediction Servers (http://www.cbs.dtu.dk/services) was used to identify putative posttranslational modifications in CD300d. As result it was observed that, the Ig domain contained two potential N-glycosylation sites, whereas the stem area displayed ten putative O-glycosylation sites (**Table 19**).

SERVER	Posttranslational Modification	Amino acids
NetNGlyc 1.0	N-glycosylation	N28, N68
NetOGlyc 3.1	O-glycosylation	T123, T130, T136, T137, T138, T139, T145, T149, T152, S141

Table 19: CD300d posttranslational modifications. List of the different servers used to predict the posttranslational modifications present in the CD300d and detail of the type of the modification and the exact amino acid affected.

Homology of CD300d with other CD300 receptors

The degree of homology of the extracellular Ig domain of CD300d with sequences of CD300 family members and closely related receptors (CD300Lg and TREM-1), was analyzed. The results of the alignment showed a high homology between the Ig domains of CD300d and CD300f, while other members of the family presented less but still significant homology. A lower degree of protein sequence homology was detected between the Ig domains of CD300d and other related proteins as CD300Lg and TREM-1 (**Figure 17** and **Table 20**). Interestingly, when the homologies among the exons encoding for the transmembrane and intracellular domains of CD300 molecules were analyzed, CD300c was the closest to CD300d. These data suggested that multiple gene duplications of CD300-related genes might have occurred along evolution, probably from a common ancestor with CD300Lg.

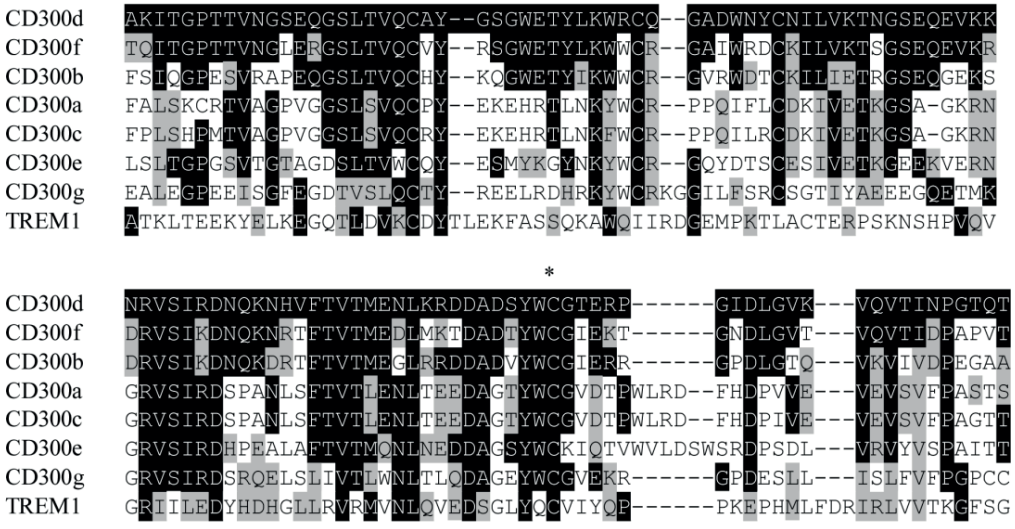


Figure 17: Alignment of the CD300d Ig-like domain with the Ig-domains from CD300 proteins and other related receptors. Identical residues are shown on black background, and similar residues are on grey background. Conserved cysteine residues are marked with asterisks.

Ig-domain Receptor	% Identity with CD300d Ig-domain
CD300a	39.2
CD300b	58.9
CD300c	41.1
CD300e	41.1
CD300f	71.4
CD300Lg	31.2
TREM-1	17.8

Table 20: Detail of the amino acids similarity (expressed by % of identity) between the CD300d Ig-like domain and the Ig-like domains of the rest of CD300 family molecules and the TREM-1 receptor.

Expression of CD300d in primary myeloid cell and cell lines

To determine the distribution of the CD300d transcript, a Real Time PCR assay was performed on PBMCs and purified blood populations as well as diverse hematopoietic cell lines. CD300d was found abundantly in PBMCs but exclusively in the myeloid compartment, including monocytes and granulocytes populations. No cDNA amplification was observed in T, B and NK lymphocytes (**Figure 18**).

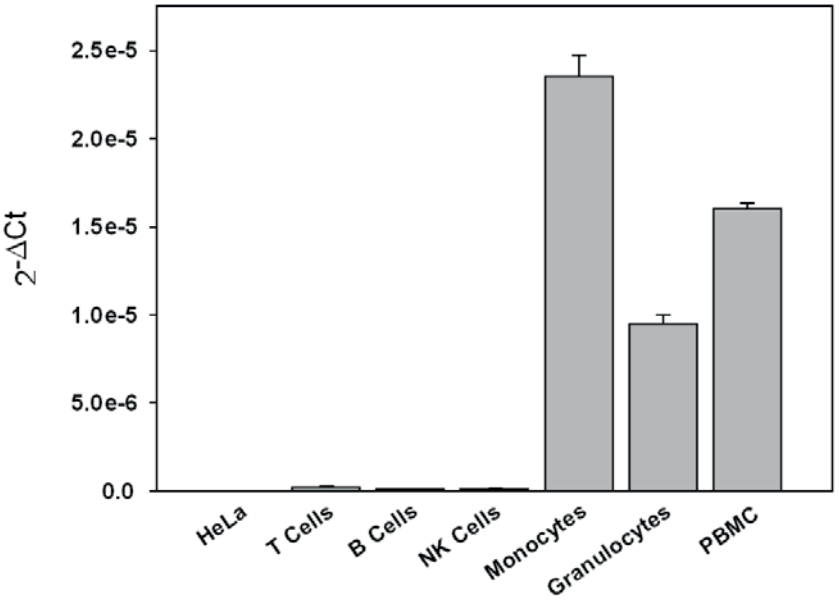


Figure 18: CD300d mRNA was detected by RT-PCR in cells of myeloid lineage. TaqMan analysis of CD300d expression in human purified leukocyte populations.

Similarly to CD300e, CD300d transcript was absent in cell lines from myeloid origin (THP-1, U937, HL-60 and MonoMac6) in basal conditions (data not shown). Based on these data, CD300d expression seems to be restricted to cells of the myeloid lineage, as previously reported for CD300b, CD300e and CD300f [215 201 217].

To further study the expression distribution of the receptor, fresh monocytes from healthy donors were obtained and differentiate *in vitro* into different subtypes of activated macrophages (M1, M2a, and M2c). mRNA was obtained from all the cells types to perform RT-PCR assays. The amplification data showed a significant decrease of CD300d expression in all types of *in vitro* derived macrophages when compared with freshly isolated monocytes. In fact, CD300d transcript was undetectable in IL-13 driven type M2a macrophages (**Figure 19**).

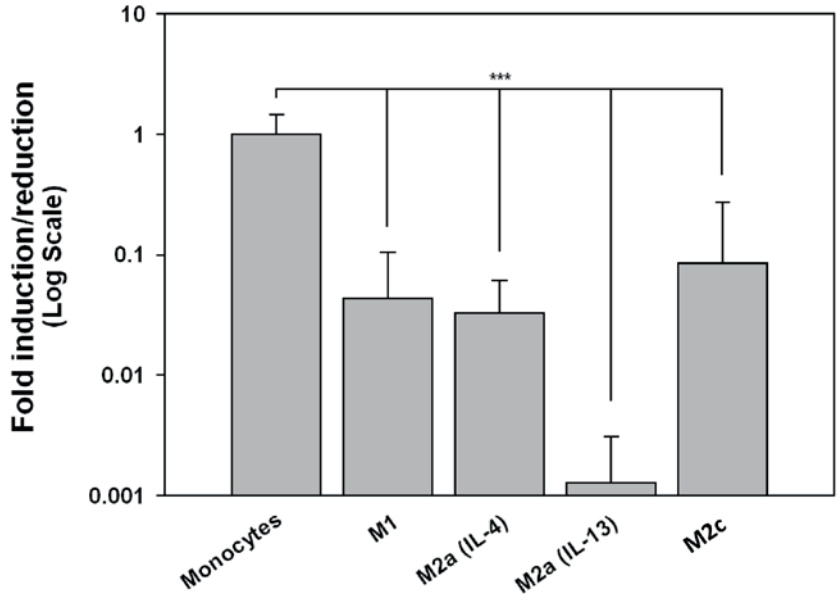


Figure 19: CD300d mRNA decreased in differentiated macrophages. TaqMan analysis of CD300d expression in different macrophage populations differentiated *in vitro* from fresh monocytes from healthy donors (n=7), (***=p≤0.005).

Biochemical characterization of CD300d

As commercial antibodies against CD300d were not available, the open reading frame (ORF) of CD300d (discarding the signal peptide) was cloned into the pDisplay expression vector to tag the protein with the hemagglutinin (HA) epitope at the N-terminal extreme of the protein (HA-CD300d). The pDisplay vector enables the expression of a protein under the control of a CMV promoter and allows the cell membrane localization through the Ig-κ signal peptide. Thus, by using commercial antibodies against the HA epitope the tagged protein could be specifically detected.

Subsequently, COS-7 cells were transiently transfected with the HA-CD300d encoding construct. Forty-eight hours post-transfection, cells were lysed and subjected to anti-HA immunoprecipitation (IP) and SDS-PAGE analysis. Empty vector was used as negative control and a HA-CD300c encoding construct as positive control, due to the high homology with CD300d in terms of predicted molecular weight and structure.

Despite CD300d had a predicted molecular weight of 21.5 kDa, the molecule migrated as a discrete pattern of two bands around 30 and 34 KDa, which looked like one sharp band depending on the experimental conditions. On the other hand, the electrophoretic pattern of CD300d differed considerably from that of CD300c, which showed a more complex pattern of bands, with a retarded electrophoretic mobility (**Figure 20**). These data suggested that both molecules were submitted to different posttranslational modifications.

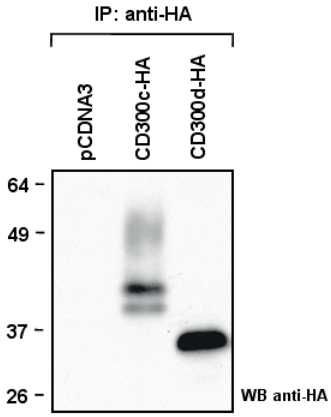


Figure 20: CD300d electrophoretic pattern differed from the one of CD300c. COS-7 cells were transiently transfected with HA-tagged CD300d and CD300c. Cells lysates were immunoprecipitated with anti-HA mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to a PVDF filter and probed with anti-HA (12CA5).

CD300d is N-glycosylated

The presence of two N-X-S/T consensus motifs for N-linked oligosaccharides within the Ig domain of CD300d (**Table 19**) prompted the investigation of putative N-glycosylations in CD300d, by using transfected cells. The lysate of HA-CD300d transfected cells were treated with the glycoaminidase PNGase F. As expected the treatment produced a significant change in the electrophoretic pattern of the receptor, indicating that in normal conditions HA-CD300d was posttranslationally modified by N-glycosylations (**Figure 21**).

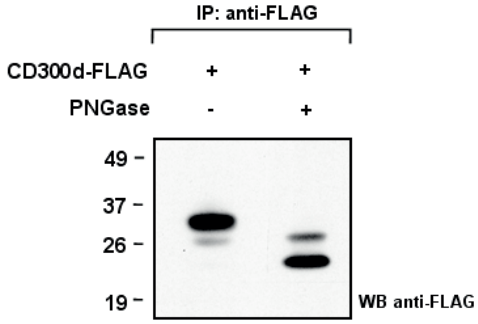


Figure 21: CD300d was posttranslationally N-glycosylated. COS-7 cells were transiently transfected with FLAG-tagged CD300d. Cells lysates were immunoprecipitated with anti-FLAG, subjected to PNGase F (NEB) treatment in non-denaturing conditions, transferred to a PVDF filter and probed with anti-FLAG.

Subcellular distribution of CD300d

HA-CD300d was expected to be expressed on the cell surface of transfected COS-7 as it occurred with the rest of HA-CD300 encoding constructs used in the laboratory [215 201 218]. It is noteworthy that CD300d was expressed efficiently in transfected cells, as comparable amounts of CD300c and CD300d could be detected when the expression was monitored by WB (**Figure 20**). However, FACS analysis showed undetectable levels of CD300d on the cellular surface of transfected COS-7 (4% of positive cells), compared to the positive control CD300c (23% of positive cells) (**Figure 22**).

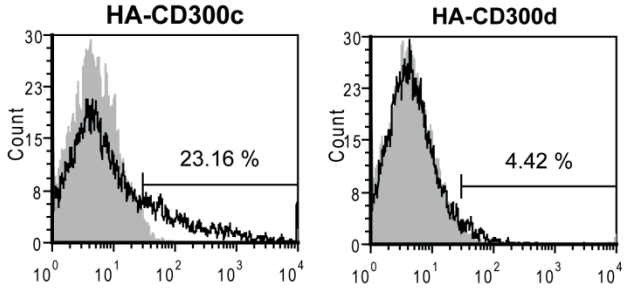


Figure 22: Surface expression of HA-CD300d compared with HA-CD300c. COS-7 cells were transiently transfected with HA-tagged CD300d and CD300c. Surface expression was monitored by flow cytometry using anti-HA (12CA5) mAb (white histogram) and an isotypic mAb as negative control (grey histogram).

These data suggested that HA-CD300d was expressed efficiently but its traffic towards the cell membrane was blocked. Considering that CD300d N-deglycosylation resulted in a molecular electrophoretic pattern close to the polypeptide backbone (**Figure 21**), it was feasible that the receptor was blocked in the endoplasmic reticulum (ER). Accordingly O-glycans could not be transferred to the immature protein in the Golgi apparatus, explaining the different mobility of CD300d in SDS-PAGE gels when compared with CD300c (**Figure 20**).

Intracellular retention of CD300d in the ER

To test the ER-blockade hypothesis, the CD300d receptor was fused to GFP to allow the protein visualization by means of fluorescence microscopy.

The confocal microscope analysis of the transiently transfected COS-7 cells demonstrated that CD300d-GFP was markedly accumulated in a perinuclear region, colocalizing with the GRP-78 staining (ER marker). Conversely, CD300c-GFP showed a more disseminated expression pattern and significant cell membrane distribution (**Figure 23**). The fact that CD300d was retained intracellularly when the expression of all CD300 molecules were driven by the same promoter and an exogenous signal peptide, suggested the existence of a retention motif in the sequence of CD300d.

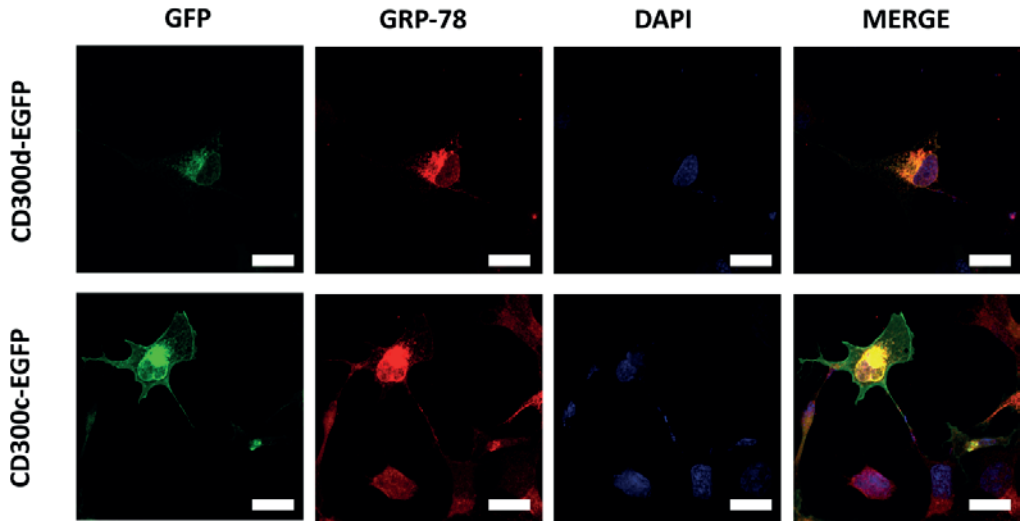


Figure 23: Intracellular retention of CD300d in the ER. COS-7 cells were transfected with CD300d-EGFP or CD300c-EGFP and analyzed by confocal microscope. Cell nuclei were stained with DAPI and endoplasmic reticulum (ER) with anti-Grp-78 antibody. Scale bar: 20 µm.

Intracellular retention motifs within CD300d sequence

The comparison of the cytoplasmic tail sequences of CD300d and CD300c showed that CD300d tail was shorter (7 aa) than CD300c (19 aa). 6 out of 7 aa present in CD300d cytoplasmic tail were identically found in CD300c. However, position 6+ showed a difference between both receptors, being a serine (S) in CD300c and an arginine (R) in CD300d (**Figure 24**). Interestingly, the arginine (R193 of CD300d) in combination with the surrounding residues, constitutes a putative ER retention motif (–RRS-) which is absent in CD300c. In order to determine whether this residue constituted a real ER retention motif, a HA-CD300d substitution mutant was carried out. HA-CD300d R137S was unable to reach the cell surface upon cell transfection (**Figure 25**, first column), indicating that other domains in CD300d had to be involved in the intracellular retention.

A further difference between the CD300d and CD300c receptors relied in the trans-membrane region, where the CD300d exhibited the –FLF- amino acid sequence, the CD300c displayed –LLV- (**Figure 24**). These variations could have implications in the tertiary structure and function of the protein. Leucine (L) and valine (V) are small hydrophobic amino acids, whilst phenylalanine (F) is a much bigger hydrophobic amino acid, as it carries a benzyl side chain, with aromatic properties. It was hypothesis that the change of both residues (F168 and F170) to (L) and (V) respectively could have an important effect on the CD300d folding, by promoting the receptor to reach the cell surface. However, when the double CD300d mutant F168L plus F170V was tested in transfected COS-7, membrane expression of the receptor was not detected. These data indicated that, although the F169

and F170 might be important for the tertiary structure of the receptor, they did not have any effect on the retention phenotype of CD300d (**Figure 25**, first column).

Since these punctual mutants gave no significant information about the retention phenotype of the CD300f receptor, a new strategy was designed by performing mutations affecting the whole domains of the receptors.

First, it was evaluated the possibility that instead of the ER retention motif in CD300d there were a ER retrieval motif in the cytoplasmic tail of CD300c. With this purpose the deletion mutant of CD300c lacking the cytoplasmic tail (CD300c ΔCyto) was used to evaluate the capability of the receptor to access to the cell surface, compared to the CD300c WT. No difference between the expression levels of both constructs was observed (**Figure 25**, second column).

Afterwards, a chimera containing the immunoglobulin domain and transmembrane region of CD300c plus the cytoplasmic tail of CD300d was generated (chimera c-d). Chimera c-d showed a normal surface expression compared to CD300c (**Figure 25**, second column), demonstrating that the cytoplasmic tail of the CD300d, on the whole, was not responsible for the retention of the receptor in the ER.

Finally, in order to analyze if the extracellular region of CD300d was involved in the intracellular entrapment of the receptor, a chimerical protein, containing the complete CD300d extracellular sequence and the transmembrane and cytoplasmic tail of CD300f receptor (Chimera d-f), was generated. The chimera was detected on the surface of COS-7 transfected cells similarly to CD300f WT, indicating that CD300d immunoglobulin domain and/or stem region were not involved in the retention phenotype (**Figure 25**, third column).

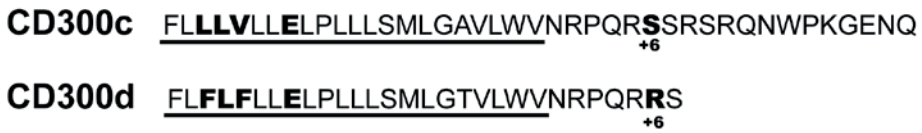


Figure 24: Transmembrane and cytoplasmic tail alignment of CD300c and CD300d. The transmembrane region is underlined and the residues considered for mutation are shown in bold type.

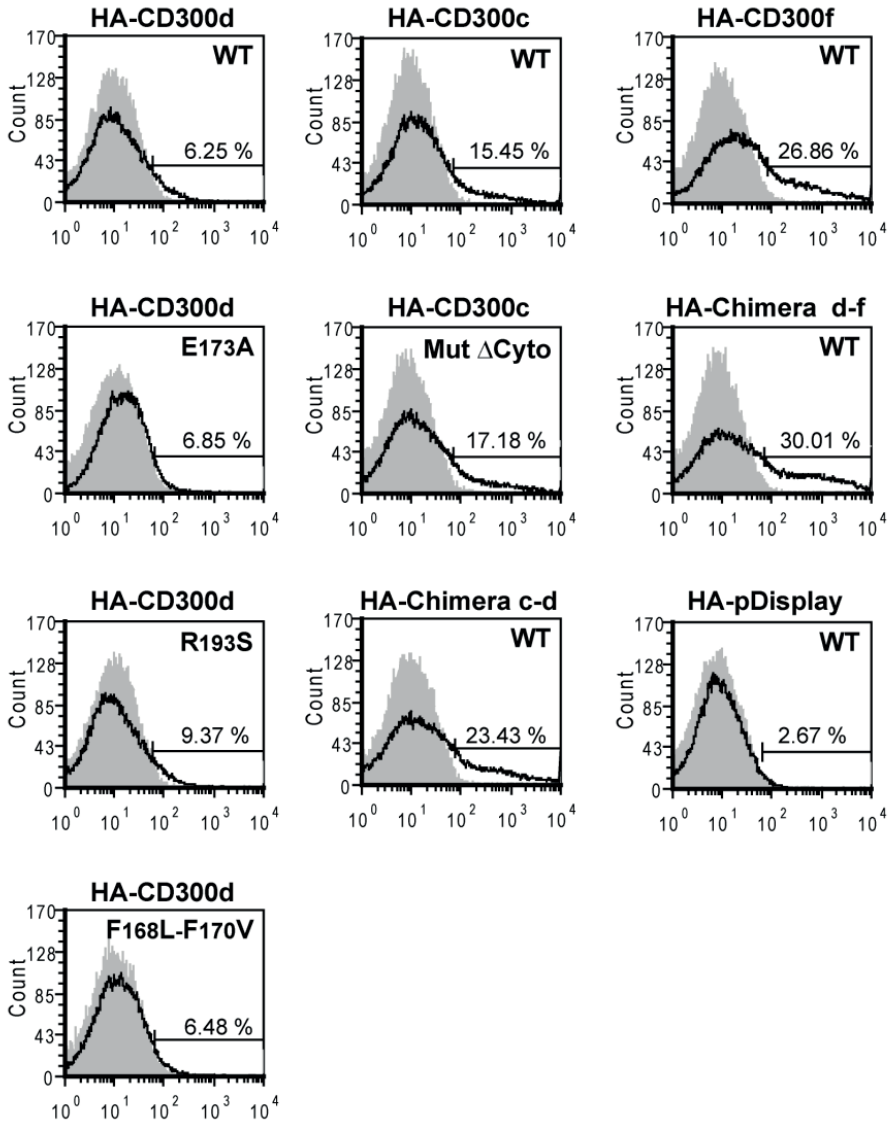


Figure 25: Cytometry analysis of different mutants of CD300d, CD300c and CD300f. COS-7 cells were transiently transfected with HA-tagged CD300c, CD300d and CD300f WT or mutants constructs. Forty-eight hours post-transfection cells were subjected to flow cytometry to assess the cell surface expression of the desired molecules. CD300 receptors were stained using anti-HA (12CA5) (white histograms). An isotype mAb was used as negative control (grey histograms).

These data strongly suggested the existence of diverse retention-retrieval motifs present in more than one domain of CD300d that might be responsible for the intracellular retention observed for the protein.

FcRγITAM-bearing adapter recruitment by CD300d

Some members of the CD300 family, like CD300c or CD300b, have the capacity to deliver activating signals or increase their surface expression through the recruitment of the ITAM-bearing adapter molecules FcRγ or DAP12 [176] respectively. It is noteworthy that the binding between CD300c and FcRγ is not based in the classical positive-negative charge complementation at the transmembrane region found in most of the activating immunoreceptors. In fact, CD300c transmembrane glutamic acid, which is shown to be essential for the functionality of the receptor, is not necessary for FcRγ recruitment.

Due to the structural similarity between CD300c and CD300d, it was checked by cytometry analysis whether co-transfection of CD300d with myeloid transmembrane adapter proteins could avoid ER retention. The result indicated that at least the FcRγ adapter was favoring the surface expression of the receptor (**Figure 26**, left panel, second column). It is worth mentioning that CD300c localized on the cell surface in the absence of FcRγ, however co-transfection with the adapter polypeptide enhanced its surface expression too, indicating the importance that FcRγ could have in the signaling within the CD300 family receptors (**Figure 26**, left panel, first column). Other myeloid transmembrane adapter molecules such as DAP12 or DAP10 had slight or no effect on both receptors. Importantly the cell surface expression of the adapter molecules remained constant in all the cases (**Figure 26**, right panel).

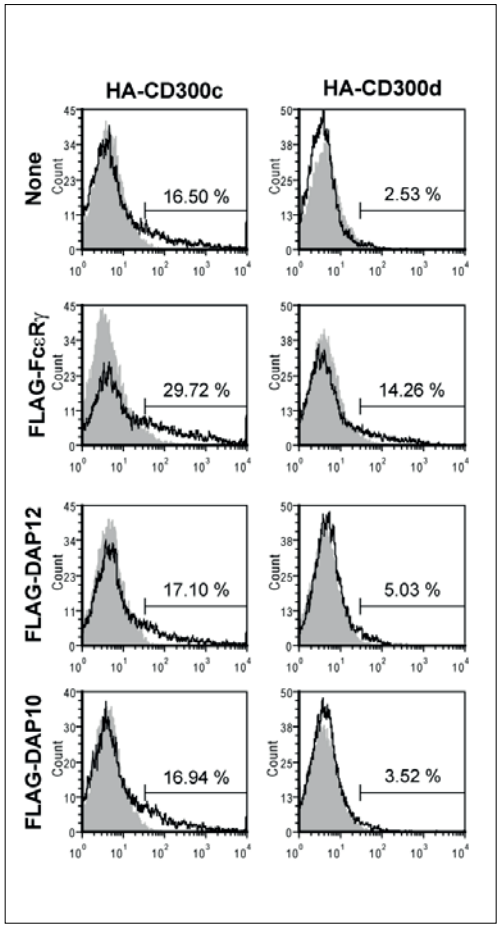
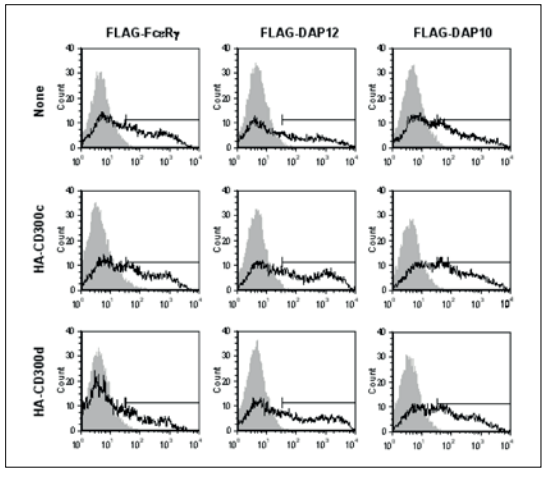


Figure 26: FcRγ promoted CD300d cell surface expression. COS-7 cells were transiently co-transfected with HA-tagged CD300c or CD300d together with FLAG-tagged transmembrane adapter molecules. Forty-eight hours post-transfection cells were subjected to flow cytometry to assess the cell surface expression of the desired molecules. Left panel: CD300 receptors were stained using anti-HA (12CA5) (white histograms), isotypic mAb was used as negative control (grey histograms). Right panel: Adapter polypeptides were stained using anti-FLAG (M2) (white histograms), isotypic mAb was used as negative control (grey histograms).



In order to explore whether the membrane localization of CD300d promoted by FcRγ was due to a direct interaction between both molecules, an IP assay was carried out, using the lysates of COS-7 co-transfected with HA-CD300d or HA-CD300c together with FLAG-FcRγ or FLAG-DAP12. Whereas the assay confirmed the direct interaction of CD300d with FcRγ, surprisingly it was observed that CD300d was able to co-precipitate DAP12 also (**Figure 27**). Indeed it was demonstrated that FcRγ or DAP12 were recruited by CD300d as efficiently as by CD300c (**Figure 27**).

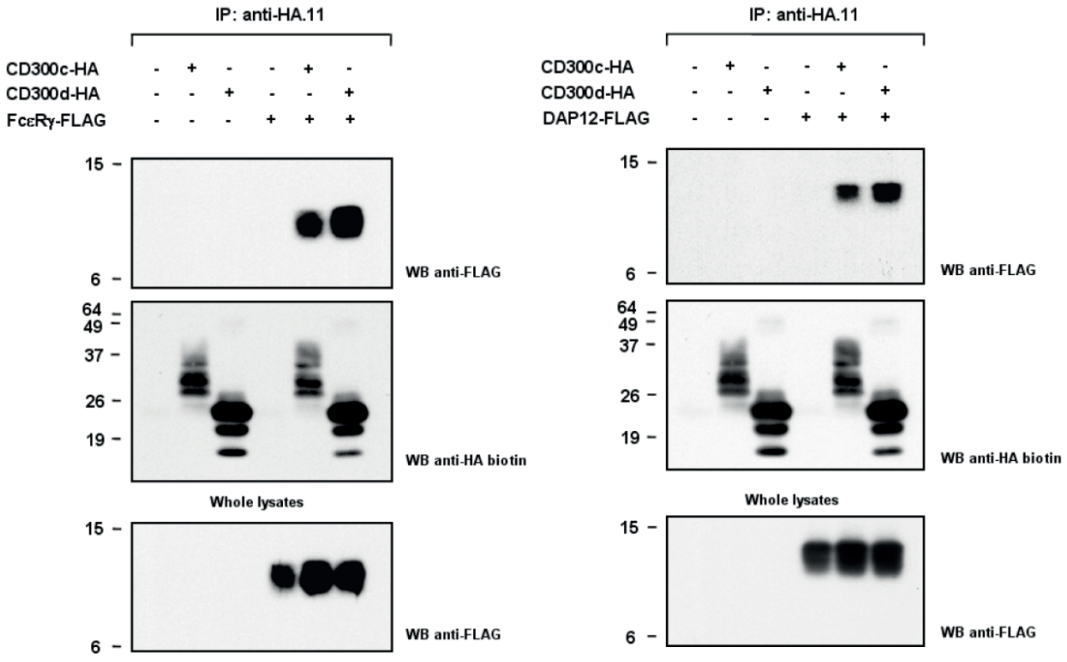


Figure 27: Direct interaction between CD300d and FcRγ. COS-7 cells were transiently co-transfected with HA-tagged CD300c or CD300d and/or FLAG-tagged transmembrane adapter molecules FcRγ or DAP12. Forty-eight hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 15% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Mapping of the interaction between CD300d and FcRγ

The interaction between the CD300d receptor and the adapter molecule FcRγ demonstrated to have a biological relevance on the subcellular localization of CD300d. To further characterize that interaction, it was investigated whether the negatively charged residues on the trans-membrane region of both molecules were involved. For that purpose, the mutants CD300d E174A and FcRγ D29A were obtained by substituting the trans-membrane negative charged residues, glutamic acid (E) and aspartic acid (D) respectively, with the non-polar amino acid alanine (A). An IP assay was performed to study the interaction capability of the mutants. The results showed that the interaction between CD300d and FcRγ was not interfered by the mutation E174A affecting the receptor. Nevertheless, the FcRγ D29A mutant showed a significantly reduced interaction with CD300d, indicating that the aspartic residue on the transmembrane domain of FcRγ is essential for a complete interaction (**Figure 28**). However, the interaction was not totally abrogated, indicating that some other residues might be involved.

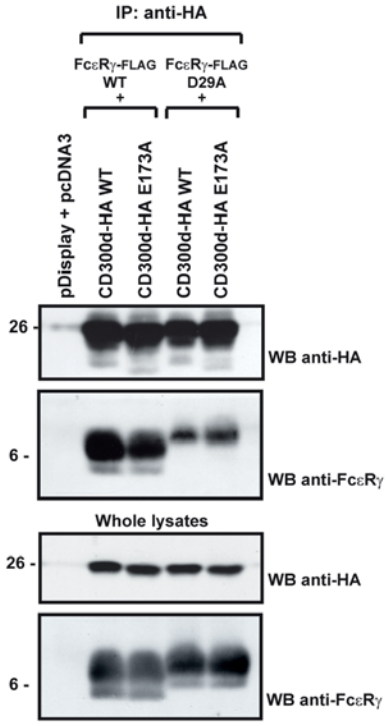


Figure 28: Mapping between CD300d and FcRγ interaction. COS-7 cells were transiently co-transfected with HA-tagged CD300d WT or CD300d E173A together with FLAG-tagged transmembrane adapter molecules FcRγ WT or FcRγ D29A. Seventy-two hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 15% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

CD300d interaction with the other members of the CD300 family receptors

CD300 receptors are able to interact with each other, even with themselves, forming both homo and heterodimers. These complexes are formed intracellularly, and the combination of CD300 receptors in a complex differentially modulates their signaling outcome [176]. As far as the interactoma between CD300 receptors was determined for all members but CD300d, which was not cloned at that moment, the capability of CD300d to interact with other members of the family was investigated, using the COS-7 cells overexpression system. Surprisingly, CD300d was capable of interacting with all the members of the family apart from CD300c (**Figure 29**, left panel). In addition, CD300d was able to form homocomplexes (**Figure 29**, right panel). Indicating that surface expression of CD300d might depend on the interaction with other CD300 receptors.

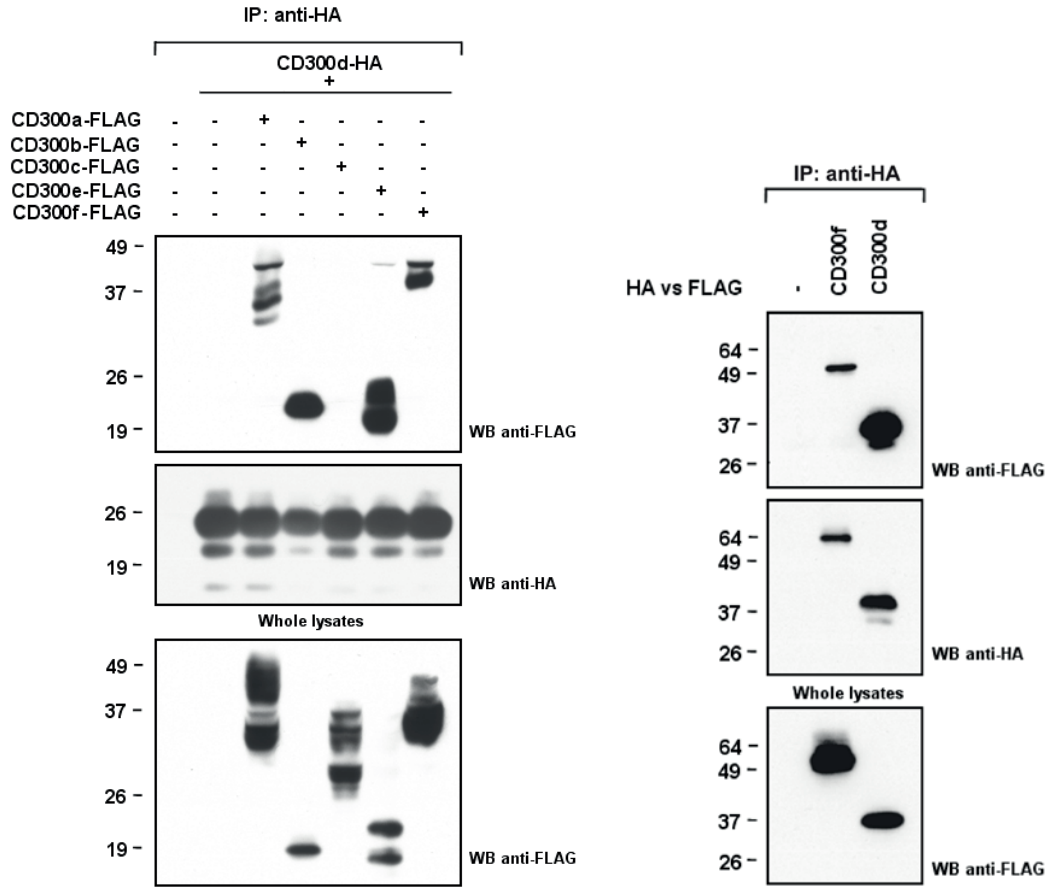


Figure 29: CD300d interacted with other CD300 family receptors and with itself. COS-7 cells were transiently co-transfected with HA-tagged CD300d together with FLAG-tagged CD300a, CD300b, CD300c, CD300d, CD300e and CD300f. Seventy-two hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Stable transfected CD300d is not present in the surface of RBL-2H3 cell line

In order to force the surface expression of CD300d two strategies were tested based in the following arguments. First, it was reasoned that transfection of HA-CD300d in a cell line expressing the adapter molecule FcRγ would lessen the retention phenotype of CD300d. Second, it was provable that co-transfection of CD300d with another CD300 receptor able to reach the cell membrane, would end with the surface expression of CD300d, by forming a complex with the other CD300 receptor. Thus, the basophilic cell line RBL-2H3 was chosen for the experiments, because are easily transfected and endogenously express the FcRγ adapter molecule (**Figure 30**). Subsequently, CD300d was stable transfected into RBL-2H3 cells by electroporation and it expression was proved by western-blotting. However, flow cytometry analysis failed to detect the receptor on the surface of the cells. These data suggest that the presence of endogenous FcRγ was not enough to avoid the ER retention of CD300d.

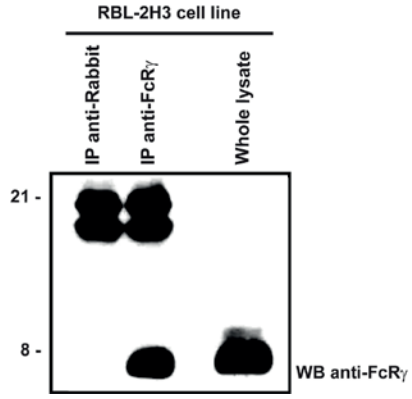


Figure 30: Endogenous expression of FcRγ in RBL-2H3 cell line. Cell lysate was immunoprecipitated with anti-FcRγ or isotopic control anti-Rabbit immunoglobulin mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as control.

Next, it was examined whether the interaction of CD300d with other members of the CD300 family receptors could stimulate its surface expression using the same cell system. RBL-2H3 cells stably expressing Myc-CD300f (test) or Myc-CD300c (used as negative control of interaction, as previously demonstrated) were transfected with HA-CD300d. The expected result was that complex formation between CD300f and CD300d would force the surface expression of the CD300d receptor, while cells carrying CD300c would not show any CD300d on the membrane. The presence of CD300d was confirmed by WB analysis of the whole cell lysates (**Figure 31**, upper panel). Surprisingly, cytometry analysis revealed no detectable CD300d on the cell membrane of any transfected RBL-2H3 cells (**Figure 31**, bottom panel, upper line). However, it should be noticed that the CD300f receptor was expressed at higher levels when transfected alone that when co-transfected in combination with CD300d. On the contrary, the expression of CD300c was not significantly altered in the presence or absence of the CD300d (**Figure 31**, bottom panel, bottom line). These results highlight a new and unexpected concept related to the function of the CD300d receptor. The retention phenotype of CD300d into the endoplasmic reticulum might be related to its biological function; the receptor might be negatively regulating the surface expression of the rest members of the CD300 family receptors.

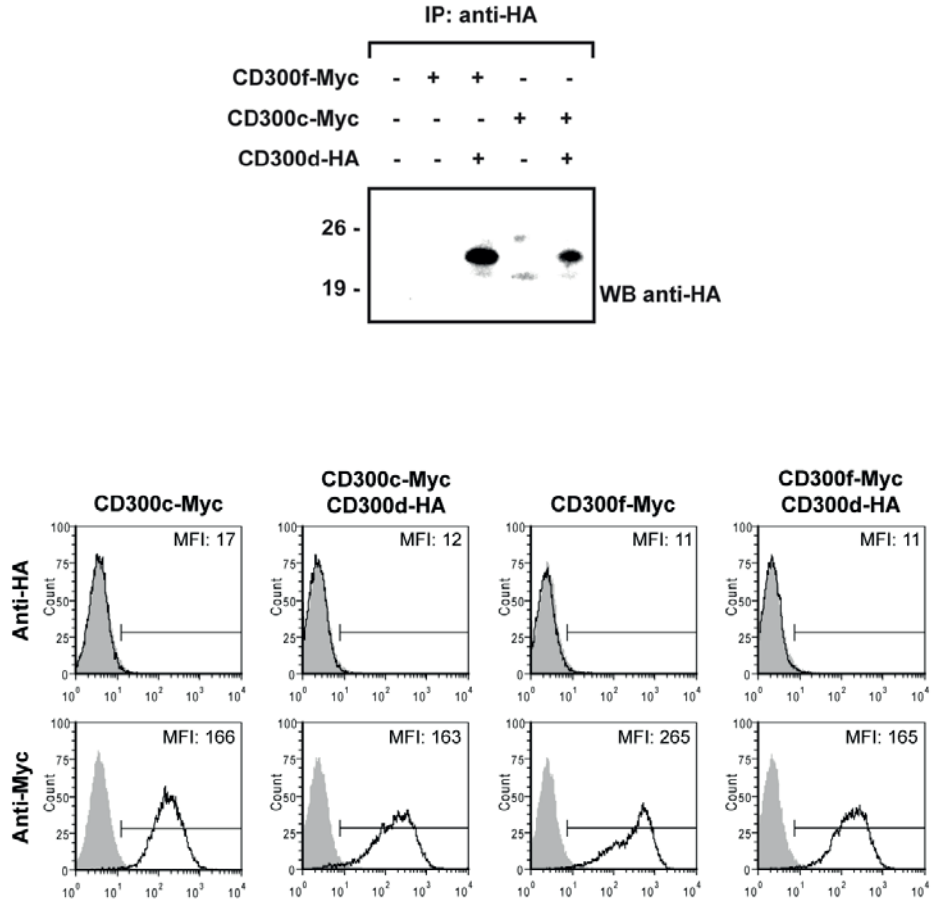


Figure 31: CD300d altered CD300f surface expression. RBL-2H3 cells stably expressing Myc-CD300f or Myc-CD300c on the cell membrane were transfected with HA-CD300d. Upper panel: cells lysates (CHAPS 1%) were immunoprecipitated with anti-HA (11) mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to PVDF filters and probed with the indicated antibody. Bottom panel: surface expression of CD300 molecules was monitored by flow cytometry using anti-HA (12CA5) or anti-Myc (9E7) (white histogram) or an isotype mAb as negative control (grey histograms). (MFI = median fluorescence intensity).

Downregulation of CD300f surface expression by CD300d in transiently transfected COS-7

To statistically verify the downregulating effect of CD300d in the surface expression of CD300f, COS-7 cells were transiently transfected with Myc-CD300f (test) or Myc-CD300c (used as negative control of interaction) in combination with HA-CD300d.

When assessed by WB of the whole cell lysates, equivalent amounts of CD300d were detected in any transfected cell (**Figure 32**, upper panel). Co-transfection of HA-CD300d significantly decreased the presence of Myc-CD300f on the cell surface. As expected, HA-CD300d had no effect on Myc-CD300c surface expression (**Figure 32**, bottom panel). The strongly significant statistical analysis reinforced the concept that the function of CD300d was to negatively regulate the cell surface expression of the rest of CD300 receptors.

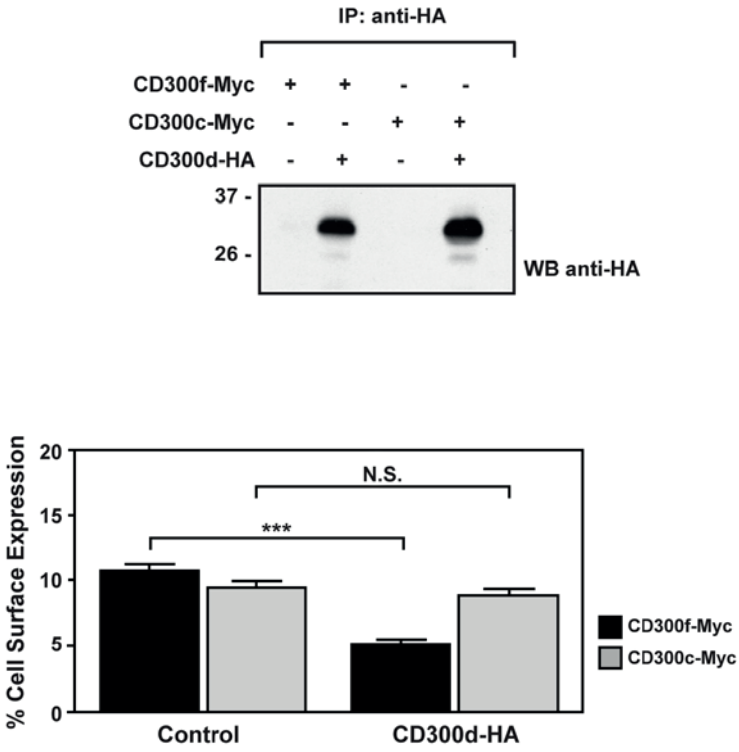


Figure 32: CD300d significantly downregulated CD300f surface expression in COS-7. Cells were transiently transfected with Myc-CD300f or Myc-CD300c, alone or in combination with HA-CD300d. Upper panel: cell lysates (CHAPS 1%) were immunoprecipitated with anti-HA (11) mAb and analyzed by 12% SDS-PAGE under reducing conditions. Proteins were transferred to PVDF filters and probed with the indicated antibody. Bottom panel: surface expression of CD300 molecules was monitored by flow cytometry using anti-Myc (9E7) or an isotype mAb as negative control. Each assay was set up in triplicate. Data are presented as mean ± SEM of 3 independent experiments. Significance was determined by Student's t-test. (***= $p \leq 0.0005$, N.S= non-significant).

CHARACTERIZATION OF CD300f

Identification of new signaling molecules of the CD300f pathway

Three-hybrid screening between CD300f and a library of PMA activated monocytes

With the aim of exploring the activating/inhibitory duality of the CD300f receptor [218] new molecules involved in the signaling pathway of the receptor needed to be identified. A Gal4 three-hybrid assay was performed on the CG1945 yeast strain between the cytoplasmic domain of CD300f (CD300f-Cyto) and a library of PMA activated monocytes, as detailed in methods. The assay gave two hundred and eighty yeast clones, from which one hundred and thirty were positive for both reporter genes (histidine and β-galactosidase). Subsequently, the cDNA of the positive clones was obtained to perform a Blast analysis, in order to determine the identity of each clone. One of them encoded for a polypeptide of 261 nucleotides (87 amino acids) identified as the FcRγ subunit.

X-gal semiquantitative liquid assay in yeast

The specificity of the interaction between CD300f and FcRγ needed to be verified. With that aim, the β-galactosidase reporter gen was used to perform a semi-quantitative X-gal substrate liquid assay in co-transformed yeast using different combinations of constructs (**Figure 33**). A plasmid containing the signaling molecule SHP-1 was used as positive control for the interaction with CD300f, as SHP-1 has been described previously to interact with the receptor mediating the inhibitory pathway [217]. Furthermore, phosphorylation of tyrosine residues within the cytoplasmic tail of the CD300f receptor is required for the interaction with SHP-1 or PI3 Kinase [218]. Thus, to explore the involvement of phosphorylation within CD300f in the interaction with FcRγ, constructions codifying for the kinase Fyn, constitutively active or mutated, were used. The results demonstrated strong interaction between the cytoplasmic tail of CD300f and FcRγ (**Figure 33**, column 4), comparable to the interaction between CD300f and SHP-1 (**Figure 33**, column 3). Unexpectedly, the phosphorylation of the receptor interfered in the interaction with FcRγ (**Figure 33**, column 5), suggesting that the CD300f interaction with FcRγ could be independent on CD300f cytoplasmic tyrosine phosphorylation. To further characterize the interaction between CD300f and FcRγ, it was decided to analyze it in a cell system different from the yeast model.

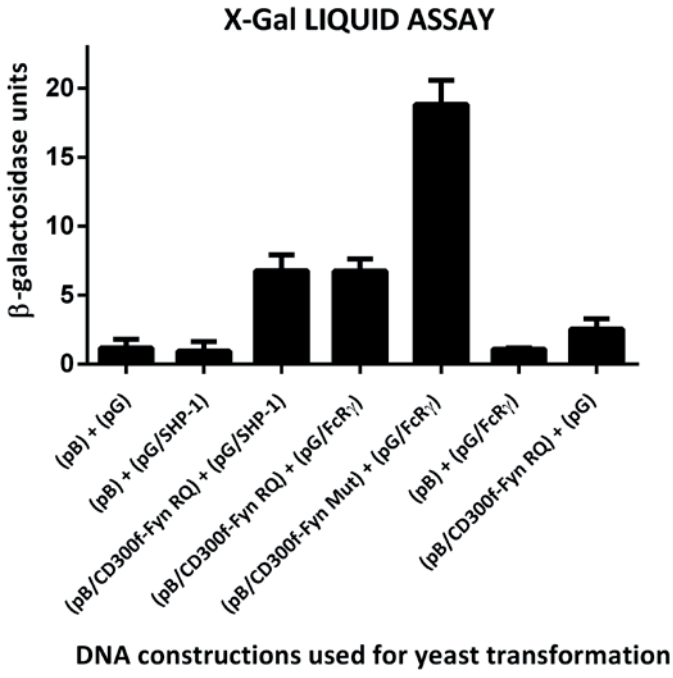


Figure 33: Interaction between CD300f and FcRγ was independent of receptor phosphorylation. CG1945 yeast strain was co-transformed with different combinations of the following constructs: pGAD containing SHP1 or FcRγ; pBridge containing CD300f-Cyto plus Fyn RQ or Fyn Mut. Empty pBridge (pB) or pGAD (pG) were used as negative controls; while (pGAD/SHP-1) construction was used as a positive control to interact with ((CD300f-Ig + Fyn/pBridge). The β-galactosidase reporter activity of the yeast cells were tested using X-gal liquid substrate. For each transformation, at least three independent colonies were tested in the assay. The results are representative of three independent experiments.

Characterization of the interaction between adapter molecule FcRγ and CD300f

Interaction test between FcRγand CD300f in transfected mammal cells

In order to test whether the interaction between FcRγ and CD300f was maintained in the COS-7 cells overexpression system, the cells were co-transfected with FcRγ and CD300f tagged with FLAG and HA epitopes respectively. HA-CD300a was used as negative control, as it was reported to be an inhibitory receptor [223], hence it was not expected to interact with mediators of activating pathways. The results demonstrated interaction between the adapter molecule and the CD300f receptor, confirming in a mammalian model the previously result obtained with the yeast system (**Figure 34**).

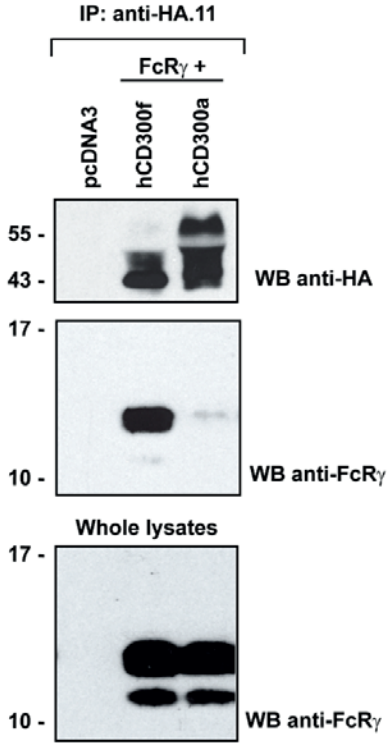


Figure 34: Interaction between CD300f and FcRγ was maintained in COS-7 cells. Cells were transiently co-transfected with HA-tagged CD300f or CD300a together with FLAG-tagged FcRγ. Seventy-two hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Effect of phosphorylation in the interaction between FcRγand CD300f

To investigate the effect of tyrosine phosphorylation on the interaction between FcRγ and CD300f, plasmids codifying for constitutively activated Fyn or Syk kinases were used together with the receptor and the adapter molecule in COS-7 overexpression system, to perform co-IP assays. The results showed that the amount of immunoprecipitated FcRγ was independent on Fyn or Syk kinases activity, indicating that the phosphorylation of the receptor did not affect the interaction with the adapter molecule (**Figure 35**).

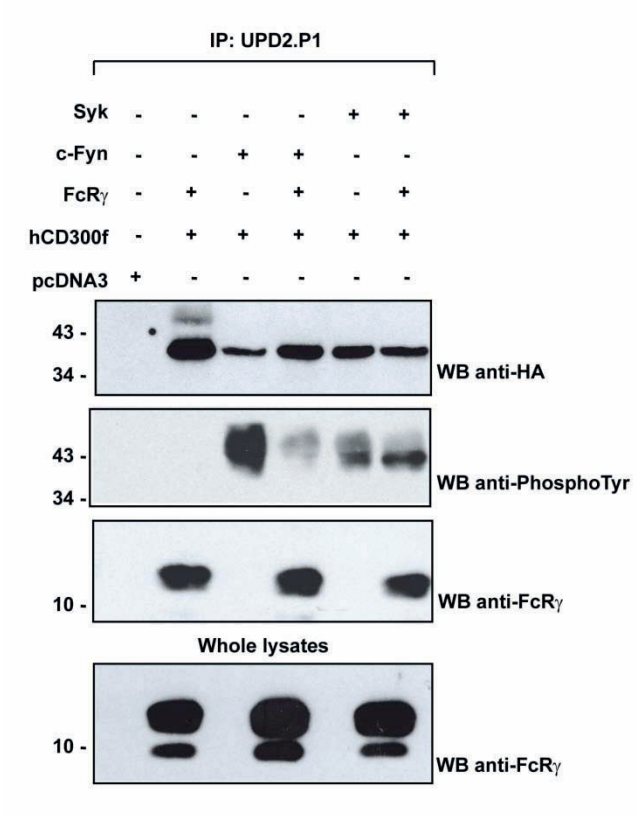


Figure 35: Phosphorylation of CD300f did not affect the interaction with FcRγ. COS-7 cells were transiently co-transfected with HA-tagged CD300f or CD300a together with FLAG-tagged FcRγ in the presence or absence of constitutively active forms of Fyn and Syk kinases. Seventy-two hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Identification of the interaction between the CD300f receptor and the adapter molecule DAP12

It is noteworthy that , apart from FcRγ, other adapter molecules like DAP12 and DAP10 are expressed in cells from the myeloid linage [232] [143]. Thus, to investigate the specificity of the interaction between the CD300f receptor and the FcRγ adapter, COS-7 cells were transfected with CD300f in combination with DAP12 or DAP10. FcRγ was used as a positive control. Surprisingly, the results demonstrated that DAP12 but not DAP10 was able to interact with CD300f (**Figure 36**). Interestingly, DAP12 was previously demonstrated to be crucial in mediating the positive signaling of different CD300 family members like CD300b [201] and CD300e [215]. Consequently, further experiments were designed to compare the strength of the interaction between the receptor and the adapters DAP12 and FcRγ, in order to determine whether one of the interactions would be favored endogenously.

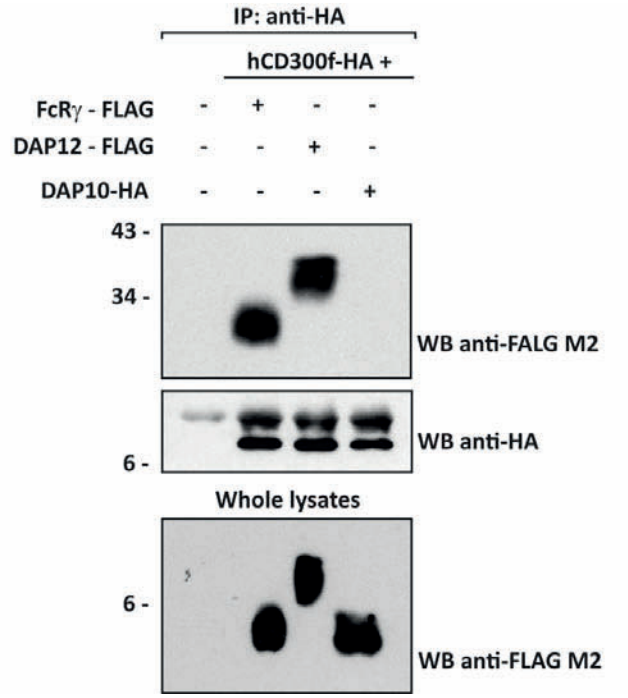


Figure 36: CD300f was able to interact with FcRγ as well as with DAP12. COS-7 cells were transiently co-transfected with HA-tagged CD300f together with FLAG-tagged FcRγ, DAP12 or DAP10. Seventy-two hours post-transfection cells lysates were immunoprecipitated with anti-HA (11) mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Effect of detergent and IP antibody in the interaction between CD300f and the adapter molecules

Detergents are commonly added to lysis buffers, to enable the disruption of the cell membrane. However, detergents also could break weak interactions between proteins, thus, the strength of the detergent might change the result of an IP assay.

DAP12 and FcRγ are very similar molecules in term of structure and both showed interaction with the CD300f receptor in the COS-7 cell overexpression system. However, a putative endogenous interaction has never been characterized. In an effort to discriminate which adapter interacted in a more specific way with the receptor, two different detergents were tested in the lysis process: 1% CHAPS *vs.* 1% Triton 100X. The results of the IP showed that FcRγ was expressed at higher levels than DAP12, even though the amount of plasmidic DNA used for transfection was the same (**Figure 37**). The data also indicated that the interaction among the adapters and the CD300f receptor was independent on the detergent used in the cell lysis.

Additionally, different antibodies against the same protein could bind to different epitopes, introducing variability in the results of an IP assay. For that reason, two different antibodies were used for the IP of the receptor: anti-HA *vs.* UPD2. The data confirmed that anti-HA Ab was more efficient than UPD2 to immunoprecipitate CD300f and co-precipitate both adapters (**Figure 37**). Interestingly, the CD300f immunoprecipitated bands were slightly different when using anti-HA or UPD2. Specifically, additional bands were visible in the lane corresponding to the anti-HA immunoprecipitation. Taken together, the results obtained showed that at least in the COS-7 cells transfection model, CD300f interaction was equally stable with FcRγ and DAP12.

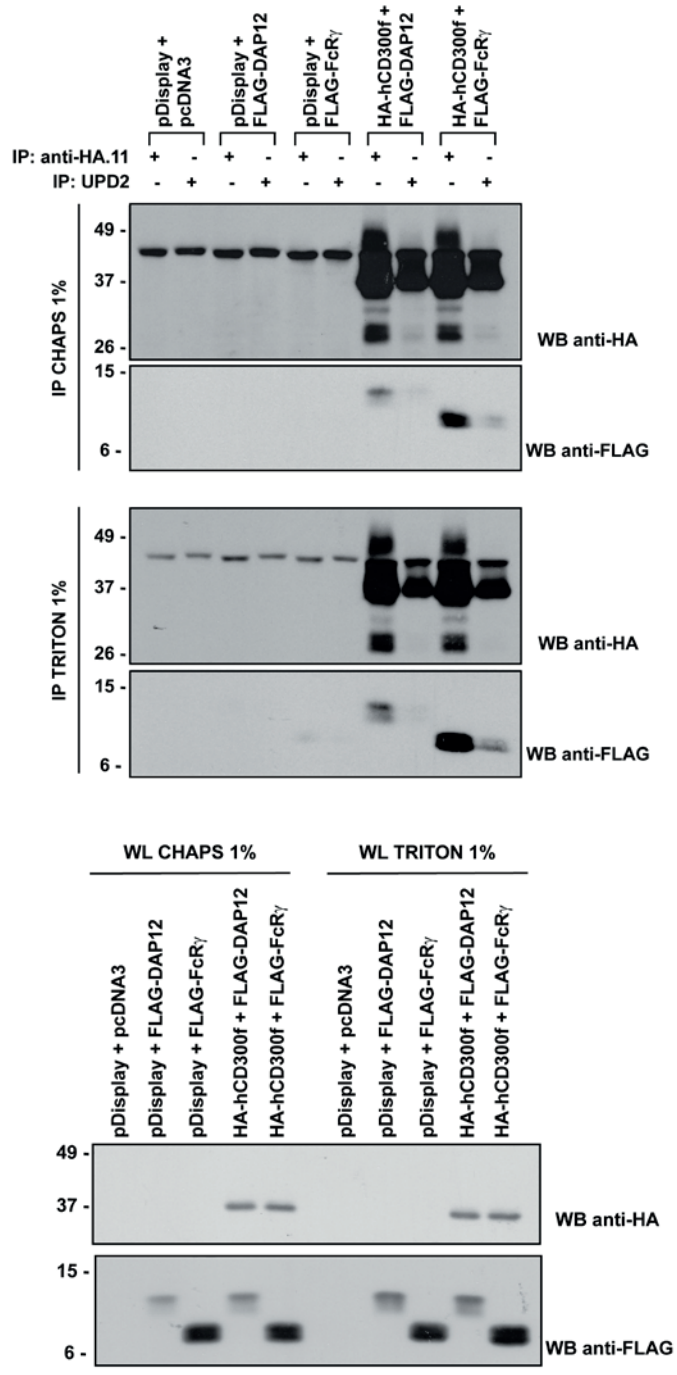


Figure 37: Effect of detergent and IP Ab in the interaction between CD300f and the adapter molecules FcRγ or DAP12. COS-7 cells were transiently co-transfected with HA-tagged CD300f together with FLAG-tagged FcRγ or DAP12. Seventy-two hours post-transfection cells lysates were obtained using 1% CHAPS or 1% TRITON-100X lysis buffer and then immunoprecipitated with anti-HA (11) or UPD2 mAbs. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Mapping the interaction among CD300f and the adapter molecules

The cytoplasmic domain of the CD300f was shown to be sufficient for the interaction with the adapter molecule FcRγ in the three-hybrid screening (**Figure 33**). Therefore, to determine the minimum domain required for the interaction of CD300f with adapter molecules, a CD300f devoid of the cytoplasmic tail (CD300f ΔCyto) was generated and used in COS-7 cells. Nevertheless, the mutant revealed no significant differences when compared to the WT receptor in the interaction with FcRγ (**Figure 39**, left panel) or Dap12 (**Figure 39**, right panel). Therefore, the existence of at least two independent interacting regions between the receptor and the adapter molecules was speculated: one interaction point might located in the cytoplasmic tail, responsible of the interaction detected in the yeast assay; another interaction point might reside in the transmembrane or extracellular domains, explaining the co-IP results with the CD300fΔCyto mutant in COS-7 cells overexpression system.

Involvement of CD300f transmembrane region on the interaction with FcRγand DAP12

To explore the existence of an interaction point, among CD300f and FcRγ or DAP12, out of the immunoglobulin domain or the cytoplasmic tail of the receptor, the Chimera a-f was generated and cloned into the pDisplay vector. The Chimera a-f contained the immunoglobulin domain of CD300a and the transmembrane and cytoplasmic tail of CD300f. The correct folding and cell surface localization of the Chimera a-f was confirmed by flow cytometry analysis, in terms of normal surface expression compared to CD300f or CD300a (**Figure 38**). Afterward, by deleting the tail of Chimera a-f, the mutant Chimera a-f ΔCyto was obtained. An IP assay was performed in COS-7 cells to check the ability of the Chimera a-f WT or ΔCyto mutant to recruit FcRγ or DAP12. The CD300a receptor was used as a negative control of the interaction and CD300f as a positive control. The results showed that Chimera a-f WT as well as the ΔCyto mutant maintained the capability to co-immunoprecipitate FcRγ and DAP12 (**Figure 39**, panel left and right respectively). This result pointed out that the CD300f immunoglobulin domain was not involved in the interaction with the adapter molecules, whereas the CD300f transmembrane region was sufficient for the binding.

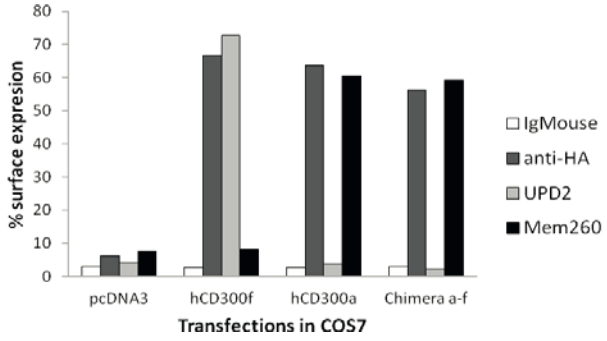


Figure 38: Chimera a-f showed correct folding and cell surface localization. COS-7 cells were transiently co-transfected with HA-tagged CD300f, CD300a or Chimera a-f. Forty-eight hours post-transfection cells were subjected to flow cytometry to assess the cell surface expression of the molecules. Cells were stained using anti-HA mAb (dark grey bar), UPD2 mAb (light grey bar), Mem 260 mAb (black bar) and isotypic mAb was used as negative control (white bar).

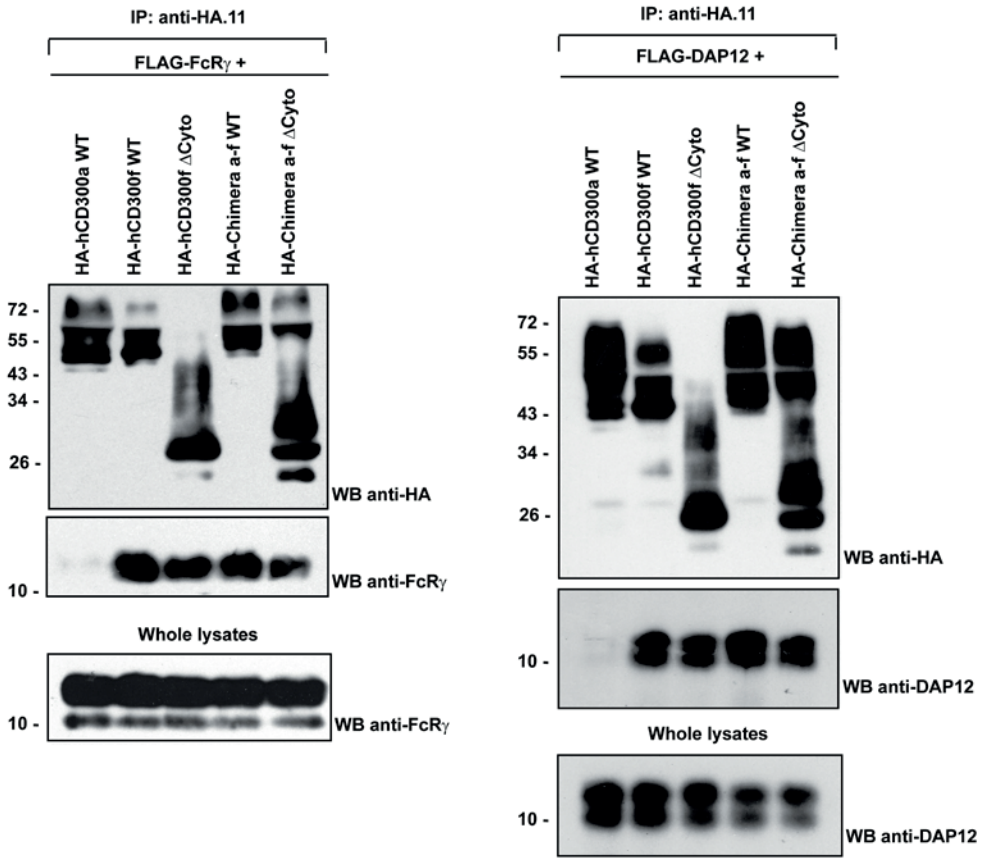


Figure 39: CD300fΔCyto mutant was able to interact with FcRγ and DAP12. COS-7 cells were transiently co-transfected with HA-tagged CD300f, CD300a, CD300fΔCyto, Chimera a-f or Chimera a-f ΔCyto together with FLAG-tagged FcRγ (left panel) or DAP12 (right panel). Seventy-two hours post-transfection cells lysates were obtained using 1% CHAPS lysis buffer and then immunoprecipitated with anti-HA (11) Ab. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Importance of the aspartic transmembrane residue within FcRγ for the interaction with CD300f

Classically the interaction between a receptors and the adapter molecules takes place throw attraction between opposite charge residues located in the transmembrane region of both molecules [120]. CD300f displays no positive charged residues in the transmembrane domain; however the amino acids on that region had a hydrophobic character that might generate a suitable environment for the negative charge residues (D29) of the FcRγ (**Figure 40**).

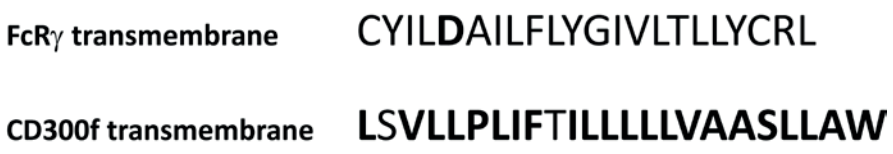


Figure 40: Amino acid sequence of the transmembrane region of FcRγ and CD300f. The bigger size and bold letters represent the negative charge residue D29 of FcRγ (top line) and the hydrophobic amino acids of the CD300f receptor (bottom line).

To explore the involvement of the D29 residue of FcRγ in the interaction with CD300f, the mutant FcRγ D29A was generated and cloned into the pFLAG-CMV2. The correct surface expression of the mutant FcRγ D29A was confirmed compared to the WT in COS-7 cells by cytometry (**Figure 41**). Interestingly, the result of the co-IP assay demonstrated that the D29A mutation in the transmembrane domain of FcRγ totally abrogate the interaction between the adapter molecule and the CD300f receptor (**Figure 42**), indicating that a main point of interaction between FcRγ and CD300f involved specific residues located within the transmembrane domains of the receptor and the adapters.

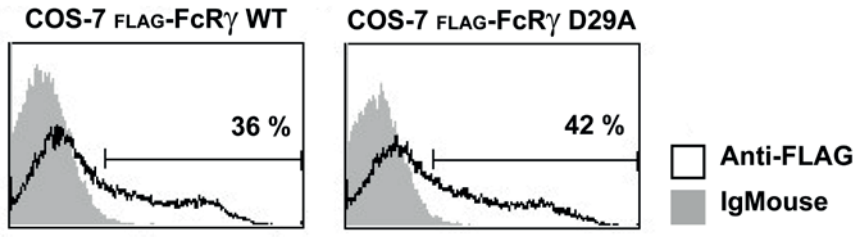


Figure 41: Surface expression of D29A FcRγ mutant. COS-7 cells were transiently co-transfected with FLAG-tagged FcRγ WT or FcRγ D29A mutant. Forty-eight hours post-transfection cells were subjected to flow cytometry to assess the cell surface expression of the molecules. Cells were stained using anti-FLAG (M2) (white histogram) and isotypic mAb was used as negative control (grey histogram).

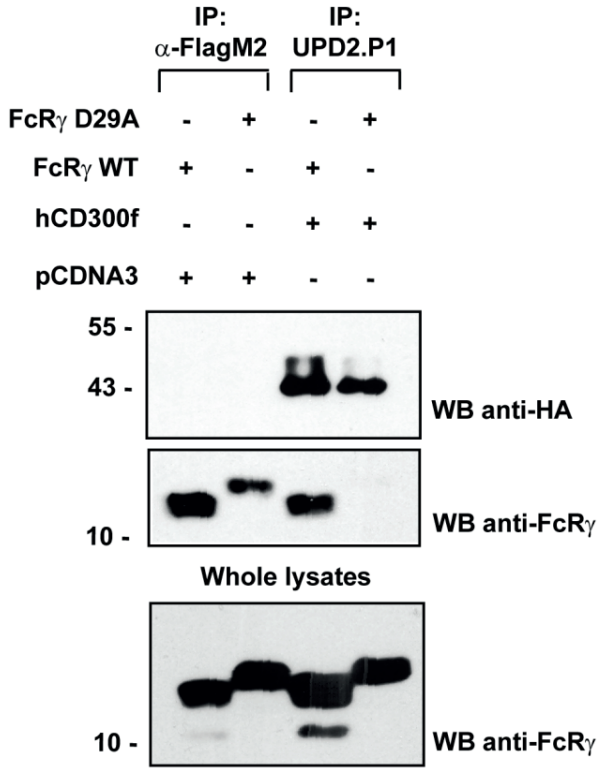


Figure 42: D29A mutation in FcRγ disrupted the interaction with CD300f. COS-7 cells were transiently transfected with, FLAG-tagged FcRγ WT or FcRγ D29A mutant alone or in combination with HA-tagged CD300f. Seventy-two hours post-transfection cells lysates were obtained using 1% CHAPS lysis buffer and then immunoprecipitated with anti Flag (M2) mAb or UPD2 mAb. Proteins were analyzed in 12% SDS-PAGE under reducing conditions, transferred to PVDF and probed with the indicated antibodies. Whole cell lysates (2%) were included as controls.

Pro-inflammatory activity of endogenous CD300f

As previously publish, the CD300f receptor transfected into RBL-2H3 cell line exhibited inhibitory effect on IgE stimulation [217]. However, the engagement of the CD300f mutant devoid of tyrosine residues within its cytoplasmic tail, generate an activating response by itself in the same cell model [218]. For that reason, the possible dual function of the endogenous CD300f receptor in activating/inhibiting the inflammatory response was investigated.

UPD1 and UPD2 antibodies are specific for CD300f receptor

In order to stimulate the endogenous CD300f receptor to induce a response, specific antibodies against CD300f were required. UPD1 and UPD2 monoclonal antibodies were previously isolated in our laboratory and both showed reactivity against CD300f and did not cross-react with the CD300e [217]. However, new CD300 receptors have been described since the isolation of UPD1 and UPD2: CD300a, CD300b, CD300c, and CD300d. Therefore the Ab specificity needed to be rechecked to exclude possible cross-reactions. To prove UPD1 and UPD2 reactivity, both mAb were used to stain RBL-2H3 cells stably transfected with CD300a, CD300b, CD300c, CD300e, and CD300f. In the case of CD300d, the specificity of the antibodies was analyzed by transfecting the Chimera d-f (harboring the Ig domain of CD300d and the transmembrane and cytoplasmic domains of CD300f) in COS-7 cells. The results obtained from the flow cytometry analysis demonstrated that UPD1 and UPD2 specifically recognize CD300f (**Figure 43**), therefore the monoclonal Abs were suitable to address the role of the endogenous CD300f receptor on myeloid cells.

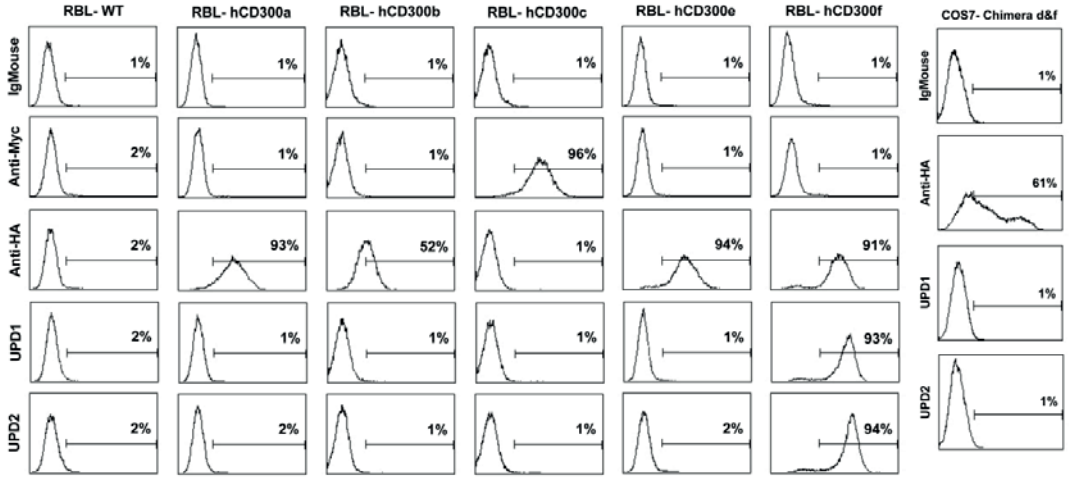


Figure 43: UPD1 and UPD2 mAbs were specific for CD300f. RBL-2H3 cells WT or stably expressing HA-tagged CD300a, CD300b, CD300e, CD300f or Myc-tagged CD300c (six first columns from the left) were used; in addition COS-7 cells were transiently transfected with HA-tagged Chimera CD300 d-f (last column). Cells were stained using anti-Myc (9E7), anti-HA (12CA5) or the isotypic control mAb to assess the cell surface expression of the molecules. In parallel the staining with UPD1 mAb and UPD2 mAb determines the specificity of the mAbs against CD300f.

Endogenous CD300f promotes TNFα release in primary monocytes

It was previously described that primary monocytes and macrophages express the CD300f receptor [217]. Thus, in order to determine the activity of endogenous CD300f, primary monocytes, from a fresh Buffy Coat, were isolated and stimulated using UPD1 mAb. After 48 hours of treatment the supernatant from the cells was recovered and used for an ELISA assay, to detect the presence of the TNFα pro-inflammatory cytokine. Production of TNFα was interpreted as the evidence of the activating inflammatory pathway generated after the receptor engagement. The results showed a significant production of TNFα, in primary monocytes stimulated with UPD1 compared to the isotopic control, supporting the pro-inflammatory function of CD300f (**Figure 44**, first two columns). Furthermore, to analyze the possibility that CD300f could mediate both activating and inhibitory signals depending on the cell activating status, CD300f was engaged in monocytes pre-elicited with lipopolysaccharide (LPS) obtained from Gram-negative bacteria. The results indicated that LPS-induced TNFα production was not diminished in the cells in which CD300f was cross-linked (**Figure 44**, last two columns), confirming that CD300f acts exclusively as activating receptor in human primary monocytes, independently on the pre-activating status of the cells.

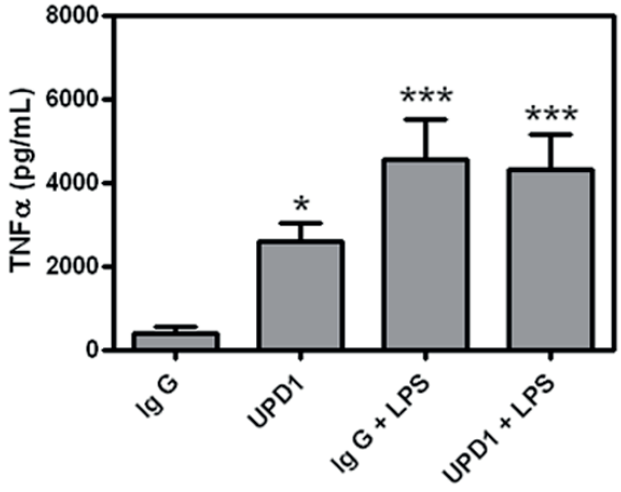


Figure 44: Endogenous CD300f induced the production of TNFα in primary monocytes. Primary monocytes were cultured on plates coated with UPD1 mAb or an isotypic control antibody (IgG). When needed LPS (10 ng/ml) was added 30 minutes after cell plating. After 48 hours amount of TNFα released in the supernatant was determined by ELISA. Data are presented as mean + SEM of at least 3 independent experiments. Significance was determined by one-way ANOVA, *P< 0.05, ***P < 0.001 compared with the control.

Endogenous CD300f expression in myeloid cell lines

To further study the activity of the endogenous CD300f receptor using a cell line model, the myeloid cell lines MM6, HL-60, THP-1 or U937 were tested for the expression levels of the endogenous receptor in the membrane. The results of cytometry assays revealed that the U937 cell line expressed the highest levels of CD300f in the surface (**Figure 45**). Furthermore U937 was described to express endogenously FcRγ [233] and DAP12 [234]. Consequently, U937 cell line was chose as the model to work with in future experiments to evaluate the function of CD300f on myeloid cells.

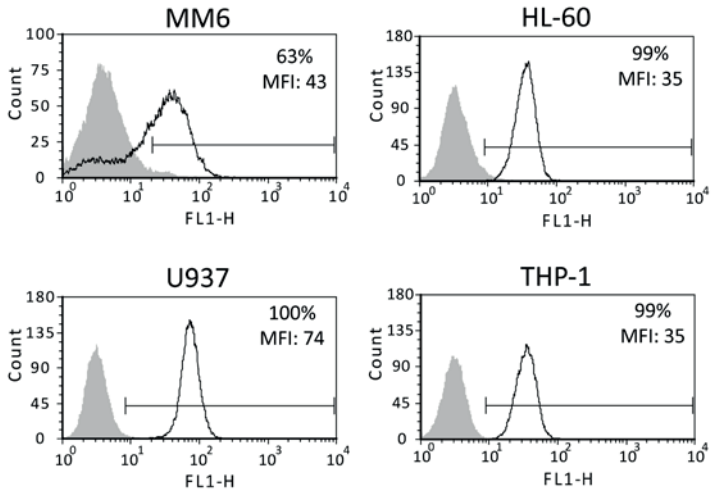


Figure 45: Myeloid cell lines MM6, HL-60, U937 and THP-1 expressed endogenous CD300f in the cell surface. Cells were subjected to flow cytometry to assess the cell surface expression of the endogenous CD300f receptor using UPD2 mAb staining (white histogram). Isotypic mAb was used as negative control (grey histogram).

Induction of pro-inflammatory cytokines production mediated by CD300f in U937 cell line

U937 cells in basal state were unable to elicit activating response, thus U937 cells were induced to a pre-activated status by phorbol 12-myristyl 13-acetat (PMA) treatment for 24 hours, as previously reported [235]. Subsequently, engagement of endogenous CD300f was performed using UPD1 mAb. An isotopic antibody was used for the negative control in every experiment. Afterwards, the mRNA from stimulated cells was obtained to perform a RT-PCR assay. Amplification data showed a significant increase of a set of pro-inflammatory cytokines, including TNFα, IL-1β and IL-6 and of the COX-2 enzyme, whose function is closely related to inflammation. In agreement, it could not be detected any change in the transcription rate of the anti-inflammatory cytokine IL-10 (**Figure 46**). Furthermore, supernatant from CD300f engaged U937 cells were harvested to perform an ELISA assay, in

order to quantify TNFα and IL-1 at protein levels. As expected, the results confirmed that CD300f engagement on U937 induced a pro-inflammatory response (**Figure 47**).

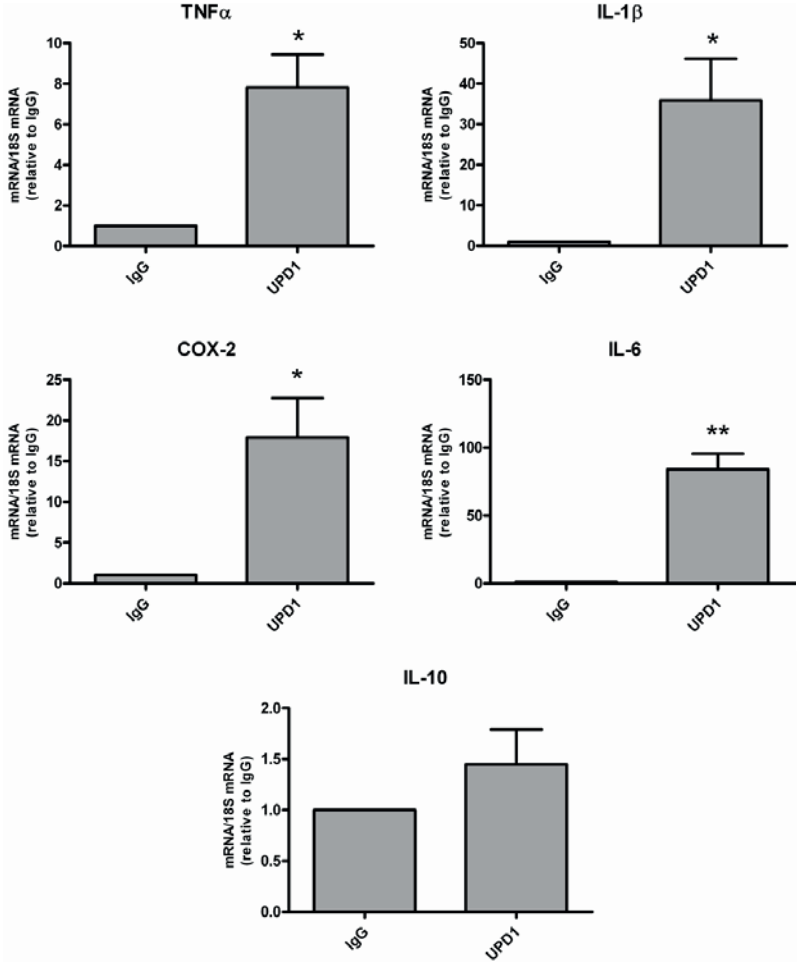


Figure 46: Endogenous CD300f engagement induced production of pro-inflammatory cytokines mRNA in U937 cells. Cells were differentiated for 24h with PMA and cultured on plates coated with UPD1 mAb or isotypic control antibody (IgG). After 6 hours of incubation, total cellular RNA was prepared and gene transcription of inflammatory mediators was analyzed by real-time RT-PCR. Data are presented as mean + SEM of at least 3 independent experiments. Significance was determined by Student's t-test, $P < 0.05$, ** $P < 0.01$ compared with the control.

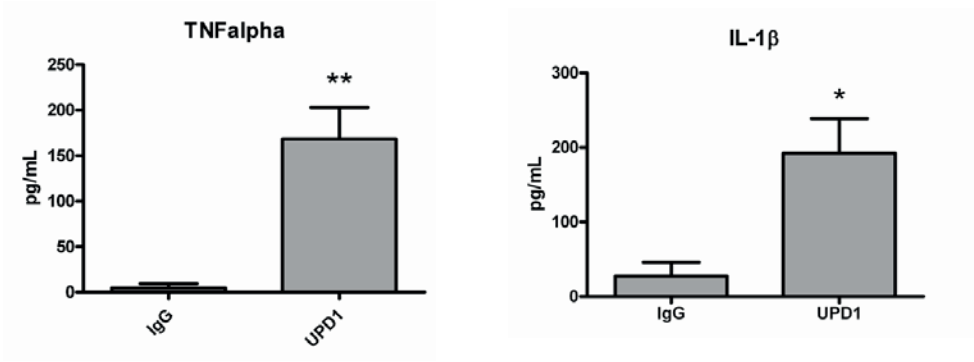


Figure 47: Endogenous CD300f engagement induced production of the pro-inflammatory cytokines TNFα and IL-1β in U937 cells. Cells were differentiated for 24h with PMA and cultured on plates coated with UPD1 mAb or isotypic control antibody (IgG). Twenty-four hours post-stimulation supernatants were harvested and protein levels of TNFα and IL-1β were determined by ELISA. Data are presented as mean ± SEM of at least 3 independent experiments. Significance was determined by Student's t-test *P < 0.05, ** P < 0.01 compared with the control.

Molecules involved in the pro-inflammatory pathway of CD300f

Involvement of FcRγand DAP12 in the pro-inflammatory pathway of CD300f

In order to know whether FcRγ was mediating the activating signals after CD300f engagement, U937 cells were transduced with a lentiviral vector coding for a shRNA against FcRγ or scramble, the levels of specific inhibition were tested by WB (**Figure 48**, left panel). It is of mention that CD300f cell surface expression was not affected by FcRγ silencing (data not shown). Changes in IL-1β, IL-6, TNFα and COS-2 mRNA levels and TNFα protein levels were quantified by real-time PCR and ELISA respectively, upon CD300f engagement in PMA-differentiated U937 cells, transduced with scrambled or specific shRNAs. Surprisingly, the decrease in FcRγ protein levels did not modify the pro-inflammatory signature triggered by CD300f (**Figure 49**) and (**Figure 50**, left graphic).

Next, it was analyzed the role of DAP12 in the CD300f activating functions using a similar approach (**Figure 48**, right panel). As observed for FcRγ, DAP12 knock-down did not affect CD300f-induced TNFα production in PMA-differentiated U937 cells (**Figure 50**, right graphic).

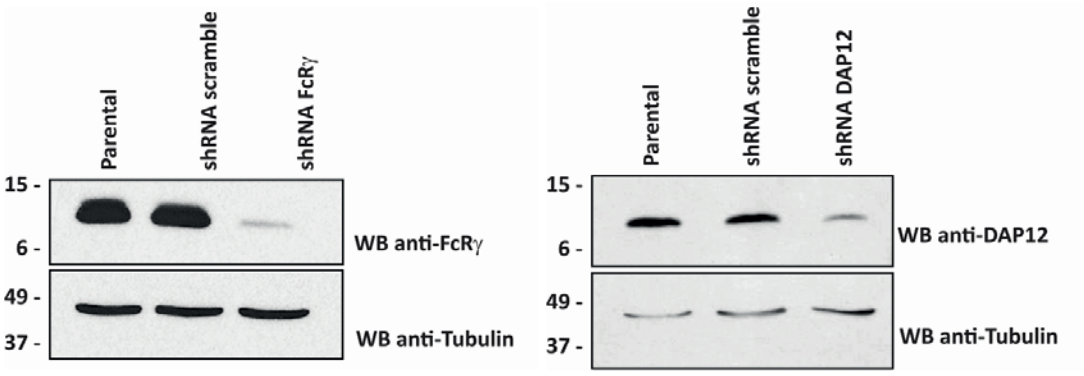


Figure 48: shRNA against FcRγ or DAP12 worked specifically in U937. Cells were transduced with lentiviral vectors encoding for shRNA against FcRγ (left panel), DAP12 (right panel) or scramble as negative control. Protein inhibition was assessed by Western blot.

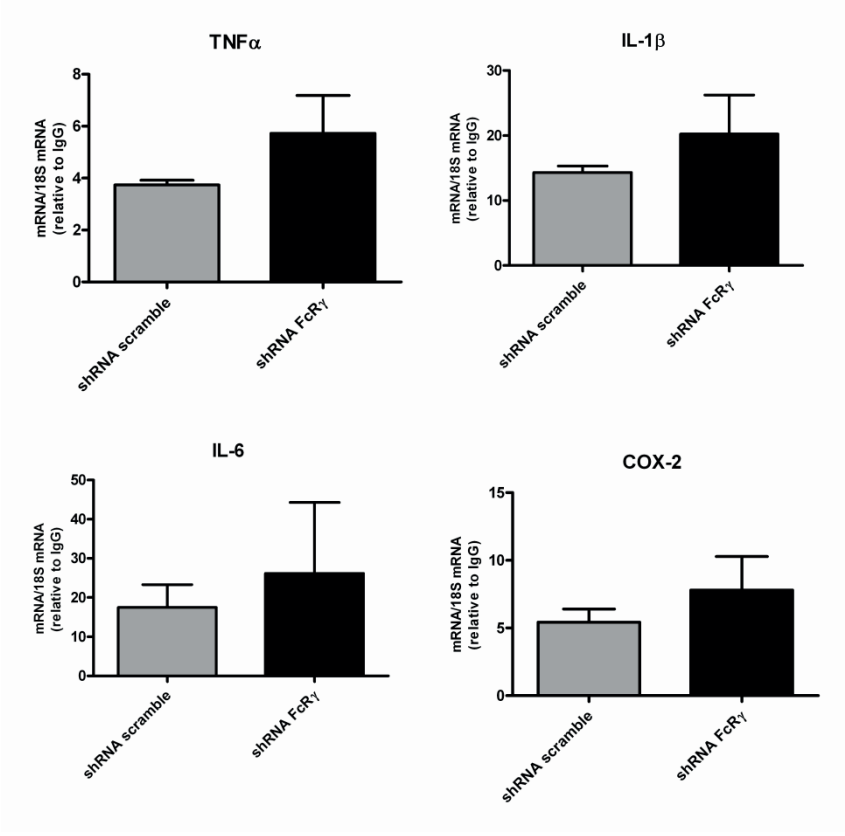


Figure 49: Treatment with shRNA against FcRγ did not affect CD300f ability to generate pro-inflammatory phenotype at mRNA level. U937 cells were transduced with lentiviral vectors encoding for shRNA against FcRγ or scramble. Cells were differentiated with PMA for 24 hours and engaged with plastic-bound UPD1 mAb or purified mouse IgG. Six hours post-stimulation, total RNA was prepared and gene transcription of TNFα, IL-1β, IL-6 and COX-2 inflammatory mediators was analyzed by real-time PCR. Data are presented as mean ± SEM of 3 independent experiments. Significance was determined by Student's t-test.

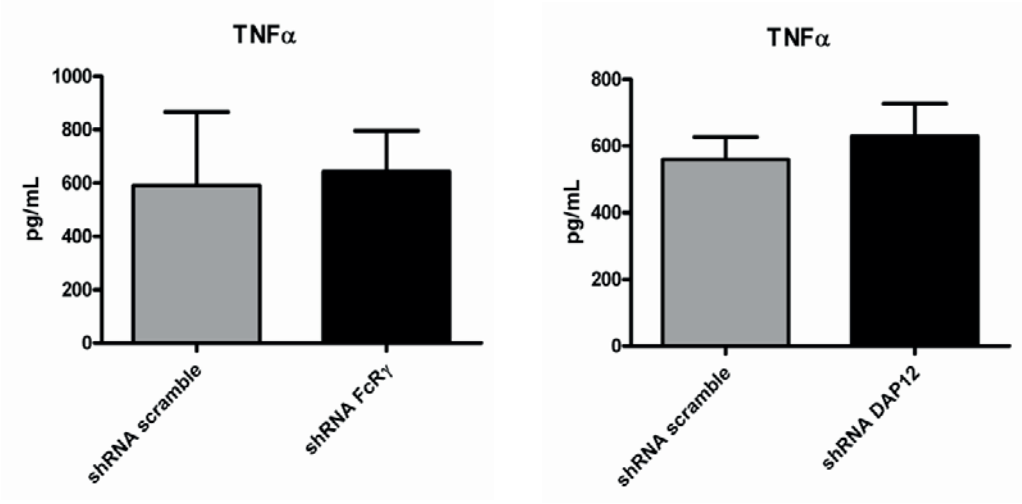


Figure 50: Treatment with shRNA against FcRγ or DAP12 did not affect CD300f ability to generate pro-inflammatory phenotype at protein level. U937 cells were transduced with lentiviral vectors encoding for shRNA against FcRγ, DAP12 or scramble. Cells were differentiated with PMA for 24 hours and engaged with plastic-bound UPD1 mAb or purified mouse IgG. Protein levels of TNFα in the supernatant were quantified by ELISA 24 hours after cell plating. Data are presented as mean ± SEM of 3 independent experiments. Significance was determined by Student's t-test.

Effect of PMA pre-treatment on U937

In order to understand why downregulation of the adapters did not have any effect in CD300f activating signaling, the effect of PMA-treatment on the expression of the adapter molecules was analyzed. To address that question, parental cells or cells infected with specific shRNA against FcRγ/DAP12 were treated with PMA and compared with not treated cells. Unexpectedly, PMA-treatment dramatically enhanced the expression levels of both ITAM-bearing adapters in U937 cells, compared with non-treated cells, interfering dramatically on the shRNA-mediated silencing, as observed by WB (**Figure 51**). Consequently, although the hypothesis was that the receptor was signaling through the studied adapter molecules, the shRNA technology was not suitable to determine the involvement of the adapters on the CD300f receptor signaling in this cell model.

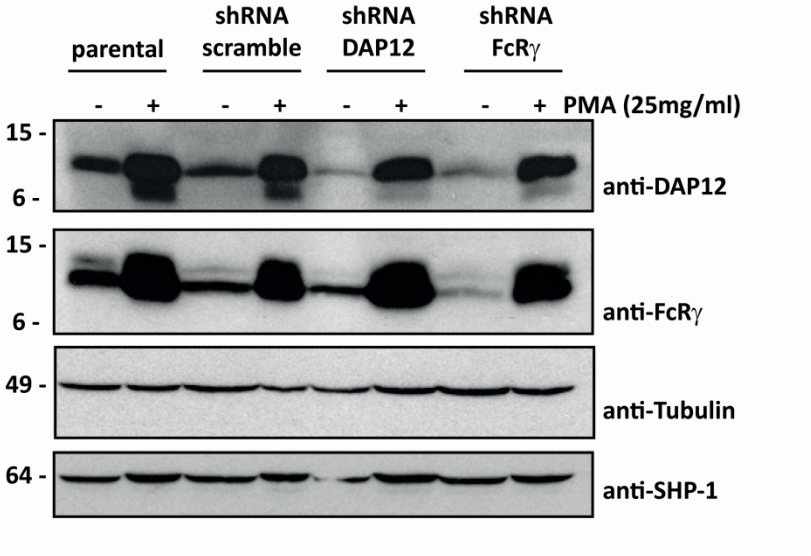


Figure 51: Effect of PMA on FcRγ and DAP12 in U937 cells. Cells were transduced with lentiviral vectors encoding for shRNA against FcRγ, DAP12 or scramble. Cells were differentiated with PMA for 24 hours or not. Parental U937 were added as control. Protein levels of FcRγ and DAP12 were assessed by WB of the whole cell lysates. The levels of Tubulin was used as loading controls for the WB and the level of SHP-1 was analyzed to demonstrate the specificity of the PMA effect on the adapter molecules.

Involvement of the Syk in cell activation after CD300f crosslinking in U937 cells

Data pointed out the possibility that both DAP12 and/or FcRγ could mediate the activating signals delivered by CD300f in U937 cells. To confirm the hypothesis, it was decided to block the signaling capability of both ITAM-bearing adapters simultaneously by means of chemical inhibitors. Syk kinase is known as a key mediator of FcRγ and DAP12 signaling in myeloid cells [120]. In order to inhibit the kinase, PMA-differentiated U937 cells, were treated with the Syk inhibitor R406/RT788 [236]. The ELISA analysis of the cell supernatants indicated that the treatment with R406 abrogated TNFα production induced by CD300f engagement, demonstrating the contribution of Syk in the pro-inflammatory pathway of the receptor (**Figure 52**).

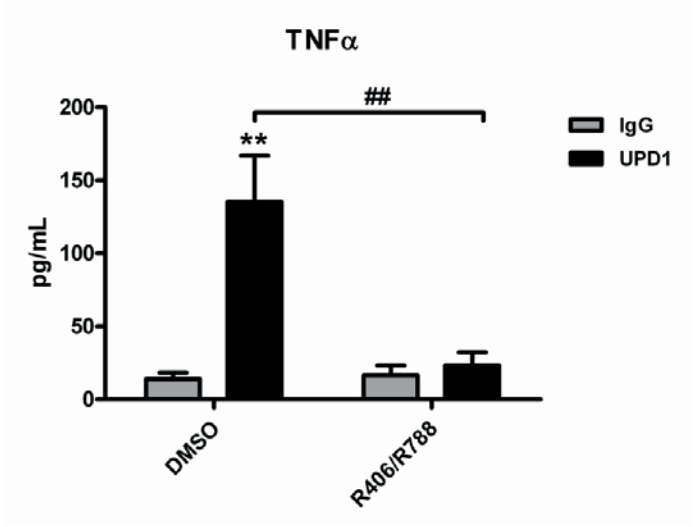


Figure 52: R406 inhibitor effect on CD300f activity in U937 cells. PMA-differentiated U937 cells were treated with Syk inhibitor R406 (10 μM) or vehicle before CD300f engagement. Supernatants were harvested 24 hours afterwards and TNFα protein levels were determined by ELISA. Data are presented as mean ± SEM of at least 3 independent experiments. Significance was determined by Two-way ANOVAs with Bonferroni post-test. ## $P < 0.01$ compared with the vehicle and **$P < 0.001$ compared with the IgG.

PI3, PKC and JNK kinases were involved in CD300f signaling

In order to further determine the signaling pathways implicated in the CD300f-elicited activating signals, PMA-differentiated U937 cells were treated with the selective inhibitors SP600125, Wortmannine and Bisindolylmaleimide I (BisI), that impair c-Jun N-terminal kinase MAP kinase (JNK), PI3 kinase (PI3K) and protein kinase C (PKC) activity, respectively. Whereas PI3K inhibitor did not affect significantly TNFα secretion, JNK inhibition produced a decrease of about 70% in TNFα levels and PKC inhibition totally abrogated the CD300f-induced production of this cytokine (**Figure 53**).

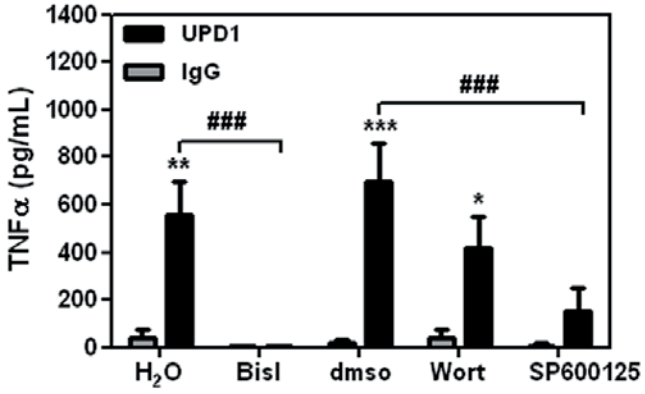


Figure 53: Effect of the inhibition of the kinases PI3K, PKC or JNK on CD300f activity in U937 cells. PMA-differentiated U937 cells were treated with PKC inhibitors Bisindolylmaleimide (BisI) (10 μM), PI3K inhibitor Wortmannine (Wort) (100 nM), JNK kinase inhibitor SP600125 (10 μM), or vehicles before CD300f engagement. Supernatants were harvested 24 hours afterwards and TNFα protein levels were determined by ELISA. Data are presented as mean ± SEM of at least 3 independent experiments. Significance was determined by Two-way ANOVAs with Bonferroni post-test. ###P<0.001 compared with the vehicle and *P<0.1, **P<0.01, ***P < 0.001 compared with the IgG.

To further investigate the involvement of PKC kinases on CD300f signaling, a dose dependent treatment was performed in the U937 cells, demonstrating that as little as 0,1μM of the drug BisI was sufficient to reach a significant inhibition in the CD300f pro-inflammatory pathway (**Figure 54**).

BisI is a reversible PKC inhibitor that shows selectivity for classical (α and β1) and novel (γ, δ and ε) PKC isoforms. In order to define which of the PKC isoforms were involved in CD300f signaling, specific PKC inhibitors Gö6976 and Gö6983 were used. The first chemical inhibits conventional PKC isoforms α, β1 and atypical μ [237] [238]; while Gö6983 blocks classical PKC isoforms α, β1 and γ, the novel isoform δ and the atypical isoform ζ [238]. The results demonstrate that only Gö6983 inhibited CD300f-induced TNFα secretion in PMA-treated U937 cells (**Figure 55**). The fact that PKCγ expression is restricted to central nervous system

[239] and PKCζ is not a target for BisI [240] strongly suggested that the novel PKCδ isozyme was involved in CD300f activating signaling.

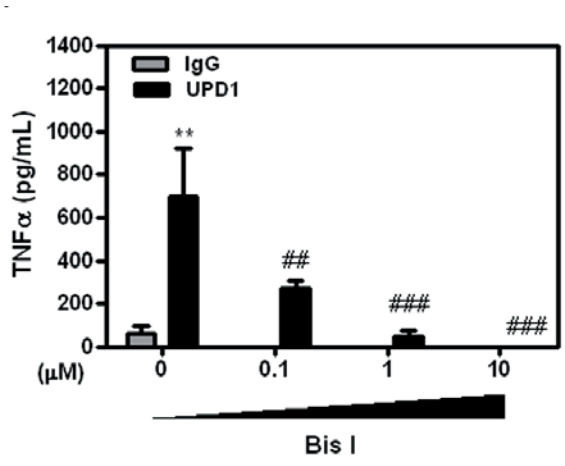


Figure 54: BisI inhibited CD300f activity in U937 cells. PMA-differentiated U937 cells were treated with PKC inhibitors Bisindolylmaleimide (BisI) at different concentrations before CD300f engagement. Supernatants were harvested 24 hours afterwards and TNFα protein levels were determined by ELISA. Data are presented as mean ± SEM of at least 3 independent experiments. Significance was determined by Two-way ANOVAs with Bonferroni post-test. ##P<0.01, ###P<0.001 compared with the vehicle and **P<0.01 compared with the IgG.

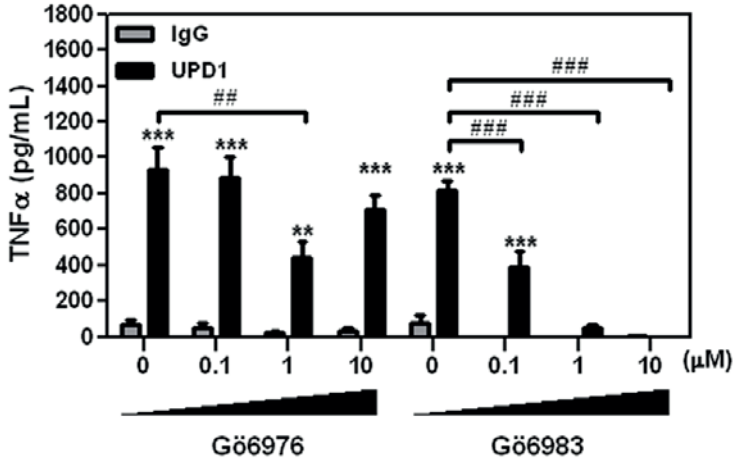


Figure 55: Specific isozyme PKC inhibitor Gö6983 affects CD300f activity in U937 cells. PMA-differentiated U937 cells were treated with specific isozymes PKC inhibitors Gö6976 or Gö6983 at different concentrations before CD300f engagement. Supernatants were harvested 24 hours afterwards and TNFα protein levels were determined by ELISA. Data are presented as mean ± SEM of at least 3 independent experiments. Significance was determined by Two-way ANOVAs with Bonferroni post-test. ##P<0.01, ###P<0.001 compared with the vehicle and **P<0.01, ***P<0.001 compared with the IgG.

CD300f activity in THP-1 cell line

The endogenous CD300f receptor was demonstrated to elicit pro-inflammatory activity on primary monocytes and U937 cell line. Nevertheless, previous publications using the myeloid cell line THP-1 demonstrated that engagement of CD300f, with soluble Abs, blocks cell activation produced by LPS-stimulation [220], indicating an anti-inflammatory activity of the receptor in that context. However, the apparent contradiction between U937 and THP-1 cellular models might support the dual property of the CD300f receptor in the inflammatory pathway. To further explore that possibility, it was tested whether the experimental conditions in which the receptor delivered activating signals in the cell line U937 reproduced the inhibitory effects previously observed in THP-1 cells.

It is noteworthy that two main differences exist between the U937 and the THP-1 cellular models: the use of soluble or plastic-attach Ab and the requirement of PMA pre-treatment on U937 cells to enable the secretion of cytokines, which is not required for THP-1 cells. To test the importance of those methodological differences in the final activity of the CD300f receptor, multiple experimental conditions were tested in THP-1 cell line. In every case, the amount of TNFα produced after CD300f crosslinking was quantified, by ELISA assay of the cell supernatants, and used as indicator of the pro- or anti-inflammatory capability of the receptor.

Preliminary results demonstrated that the engagement of CD300f with plastic attach Abs (UPD1 and UPD2), in THP-1 devoid of PMA pre-treatment, did not induce the release of TNFα (**Figure 56**). Furthermore, receptor cross-linking inhibits LPS-stimulated TNFα release (**Figure 56**), accordingly to previously published results [220], which related the CD300f receptor with anti-inflammatory activity.

Nevertheless, the effect of PMA pre-treatment on the THP-1 cells remained unknown. Interestingly, data demonstrated that under PMA experimental conditions the engagement of CD300f promoted TNFα release in THP-1 cells; and CD300f cross-linking did not block LPS- stimulated TNFα release (**Figure 56**), reproducing the pro-inflammatory activity of CD300f in the U937 model.

When taken together, the results obtained in THP-1 cells demonstrated that PMA pre-treatment was responsible of the switch of the CD300f activity, from anti-inflammatory to pro-inflammatory. Noteworthy, in U937 cell model it was observed that PMA up-regulated the expression of the FcRγ and DAP12 adapter molecules, thus the effect of PMA in the expression pattern of intracellular mediators involved in the CD300f signaling was studied in the THP-1 cell model. As expected, WB analysis revealed that the addition of PMA promoted the up-regulation of FcRγ and DAP12 in THP-1 cells, while the amount of SHP-1 mediator remained constant (**Figure 57**). Concluding, it might be established a direct dependence between the CD300f pro-inflammatory pathway and the availability of the adapter molecules FcRγ and DAP12 in the cell.

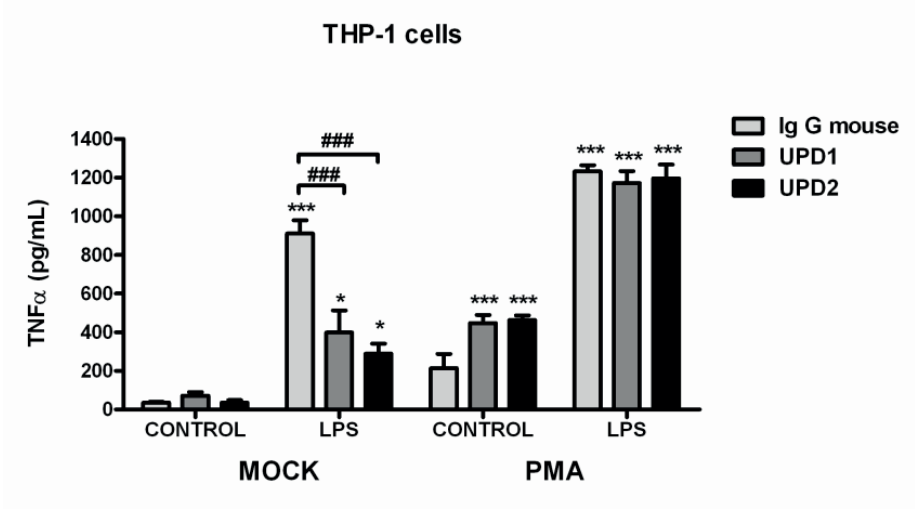


Figure 56: CD300f behaved differently in PMA-differentiated and non-differentiated THP-1 cell line. Non-treated (mock) and PMA-treated THP-1 cells were cultured on plates coated with UPD1, UPD2 Ab or purified mouse IgG in the presence or absence of LPS (10 ng/mL). TNFα protein levels in supernatants were determined by ELISA 24 hours after plating. Data are presented as mean ± SEM of 3 independent experiments, and data were analyzed by Two-way ANOVAs with Bonferroni post-test. *P < 0.05 and ***P < 0.001 compared with IgG, and ### P < 0.001, compared with IgG + LPS.

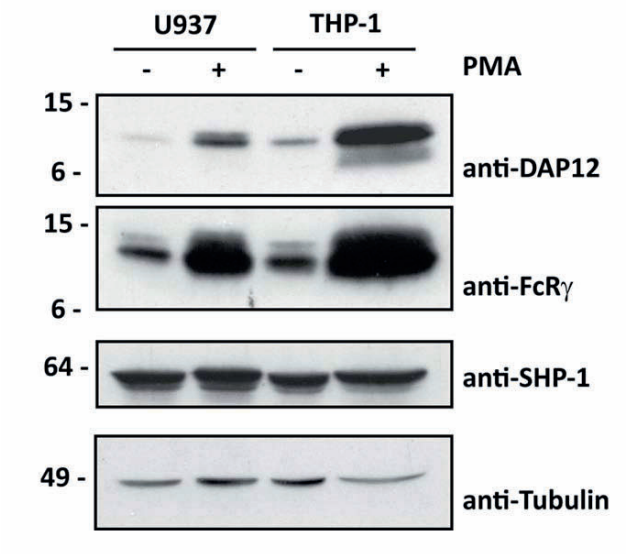


Figure 57: Effect of PMA on FcRγ and DAP12 in U937 cells compared to THP-1 cells. U937 or THP-1 cells were differentiated with PMA for 24 hours or not. Protein levels of FcRγ, DAP12 and SHP-1 were assessed by WB of whole cell lysates. The levels of Tubulin were used as loading control.

DETECTION OF SOLUBLE VARIANT FORMS OF CD300f

Molecular evidences about the existence of soluble variant of CD300f

Involvement of Splicing and Proteolysis in the generation of sCD300f

It has been shown that the knockout mice for CD300f (CLM1) exhibit an increased neuropathology in response to the induction of Experimental Autoimmune Encephalomyelitis (EAE). Accordingly, a worsened phenotype was also noted when these authors administrated a soluble CLM1-IgG fusion protein to wild type animals [229]. These data strongly suggest that the existence of natural soluble forms of CD300f could play a role in some autoimmune diseases affecting the CNS or any other inflammation process with pathological consequences.

There are two possible ways in which CD300f soluble proteins could be generated. First, secretion of soluble CD300f could be regulated through gene transcription. At least four different splicing variants of CD300f have been described. Two of them encode for molecules devoid of the transmembrane domain [217]. These proteins, in the case of being produced would not get stuck to the cell membrane and could be secreted to the extracellular compartment. A second process known as ectodomain shedding might be involved in the generation of soluble forms of the receptor, promoting the proteolysis of the extracellular domain of the receptor once CD300f is anchored to the cell membrane.

Development of an ELISA assay to detect sCD300f

Beside the clinical qualification of a biomarker, the analytical assay validation is a key element on biomarker research. Biological molecules are generally measured by immunoassays. Thus it was interesting to develop and validate an ELISA to determine the existence of sCD300f in human plasma or cerebrospinal fluid. With that aim, it was essential to generate molecular tools to approach a specific assay.

Production of CD300f-IgG2a fusion protein

A synthetic protein containing the extracellular domain of CD300f was required, to be used as a positive control of the assay. The recombinant protein CD300f-IgG2a, including the immunoglobulin domain of CD300f fused to the heavy chain of a mouse IgG2a immunoglobulin was generated, produced at high amounts in the supernatant of stable transfected CHO-K1 cell line and purified afterwards as described previously in the methods of the thesis.

Combination of Abs to detect the sCD300f

Antibodies for plate-coating and detection steeps of the ELISA were essential to approach the assay. UPD1 and UPD2 mAb had proved previously their ability to recognize the extracellular domain of the CD300f receptor specifically, so they were excellent candidates to be used in the assay. Nevertheless, it was important to verify whether each mAb joined different epitopes of the receptor avoiding steric interferences when bound to the same receptor molecule. Furthermore, the antibody required for the detection steep of the assay needed to be labeled in order to get a detectable read-out after the interaction. Thus, both UPD1 and UPD2 antibodies were biotinylated as described in the methods of the thesis.

Settings adjustment for the sCD300f ELISA

It was checked out if the combination of the antibodies worked to detect the soluble receptor and whether it was better to use UPD1 as capturing Ab and biotinylated-UPD2 as detecting Ab, or the opposite settings. Thus, a sandwich ELISA assay was carried out to test both conditions, using saturated concentration of CD300f-IgG2a as sample of the assay and purified mouse immunoglobulin (Ig Mouse) as negative control. The result showed that UPD1 and UPD2 bound to different epitopes of the extracellular domain of the CD300f receptor, being suitable to be used together in the ELISA assay. Furthermore, it was determined that to use UPD1 as capturing Ab and biotinylated-UPD2 as detecting Ab was the best combination to detect the putative sCD300f (**Figure 58**). In addition, other parameters of the assay were established: the use of UPD1 Ab at 10µg/ml in coating buffer (O/N at 4ºC); the use of 10% FBS in PBS 1X for the blocking steep (1h at RT), the use of biotinylated-UPD2 at 1µg/ml in PBS 1X (1h at RT), and the use of streptavidin-HRP at 1:5000 in PBS 1X (1h at RT).

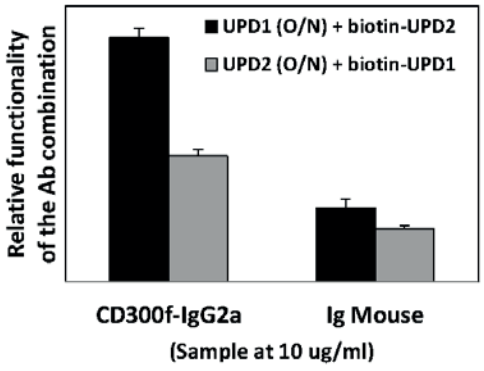


Figure 58: Settings analysis of the ELISA. UPD1 (black bars) or UPD2 (grey bars) mAb were coated on the ELISA plate (at 10µg/ml in coating buffer, O/N at 4ºC). After blocking (10% FBS in PBS 1X, 2h RT), saturating concentration of CD300f-IgG2a fusion protein or Ig Mouse negative control was added (at 10 µg/ml in PBS 1X, 1h at RT) . After washing the samples, biotinylated-UPD2 or biotinylated-UPD1 was added respectively (at 1µg/ml, 1h at RT), subsequently streptavidin-HRP was incubated (1:5000 in PBS 1X, 1h). Finally the Substrate Solution was added to detect enzymatic activity. Data are presented as mean + SD (n=1, duplicate).

Limit of detection of the sCD300f ELISA

A range of concentrations of the recombinant protein was assessed and compared with saturated concentration of the negative control in order to establish the limit of detection of the ELISA assay. The results indicated that the limit of detection for the assay was around 100 ng/ml of the CD300f-IgG2a protein (**Figure 59**), which simulated the soluble CD300f, thus it was speculated that similar concentrations of the soluble receptor would be detected with that ELISA.

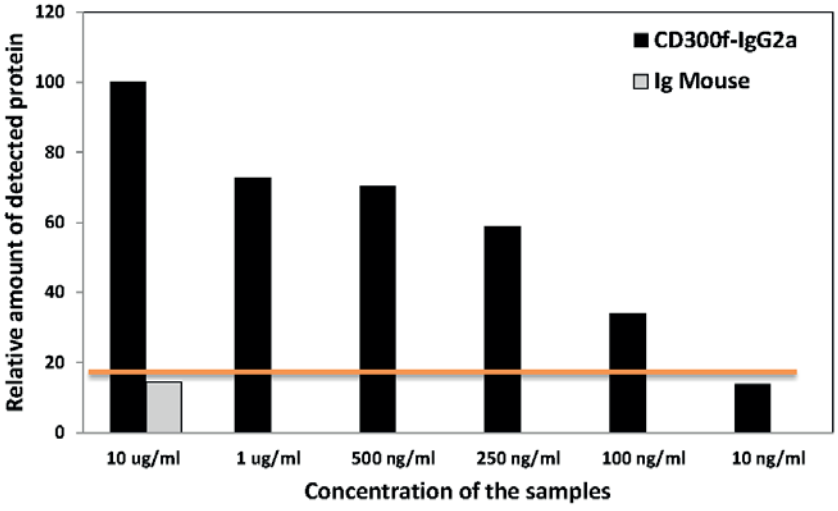


Figure 59: Limit of detection of the ELISA assay. A range of concentrations of the CD300f-IgG2a recombinant protein, from 10 µg/ml to 10 ng/ml, was assessed on the ELISA assay. Ig Mouse at highest concentration was used as negative control (n=1).

Detection of sCD300f in human fluids

Detection of sCD300f in serum samples from Antiphospholipid syndrome subjects

After the optimization of the ELISA assay, three random blood samples, concretely the serum fraction from the blood, were analyzed to detect the soluble CD300f receptor. PBS 1X was used as negative control of the assay. From the results, it was observed that the sample number 3 gave higher signal compared to the others, indicating that sCD300f might be detected in human blood (**Figure 60**). Subsequently, the origin of the samples 3 was investigated revealing that the donor suffered from a rare autoimmune disorder called Antiphospholipid syndrome (SAP), also known as the Hughes syndrome, which causes frequently clotting in arteries and veins due the presence of auto-antibodies affecting coagulation processes [241]. The presence of antibodies against apolipoprotein H or cardiolipin are the best markers known to diagnose SAP, however the clinical assays to discriminate these biomarkers are not consistent when used to analyze whole human serums [242]. Thus, to identify new biomarkers for the SAP syndrome might contribute to

improve its diagnosis. With that aim, more samples from SAP subjects were analyzed by sCD300f ELISA. The results demonstrated that SAP subjects showed significant higher amounts of sCD300f in blood serum, compared with the control samples (**Figure 61**). Interestingly, one of the controls exhibited high amount of sCD300f as well, indicating that some other pathological situations apart from the autoimmune SAP syndrome might induce an increase in the production of the soluble variants of the receptor. Nevertheless, to confirm sCD300f as a biomarker for SAP syndrome a large number of samples must be analyzed in the future.

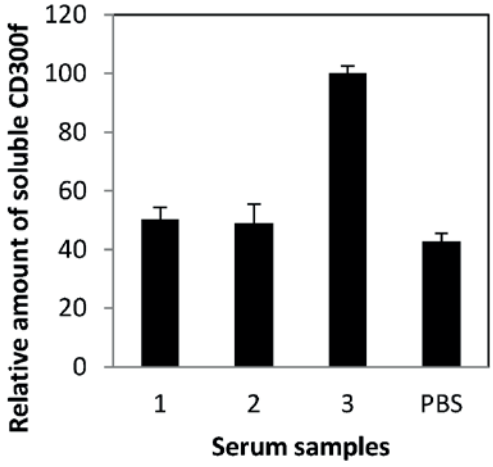


Figure 60: Analysis of sCD300f in human serum samples. Serum samples obtained from random subjects were diluted 1:2 in PBS 1X and sCD300f was quantified by ELISA. PBS 1X was used as negative control for the assay. sCD300f values are given as mean + SD (n=1, triplicate).

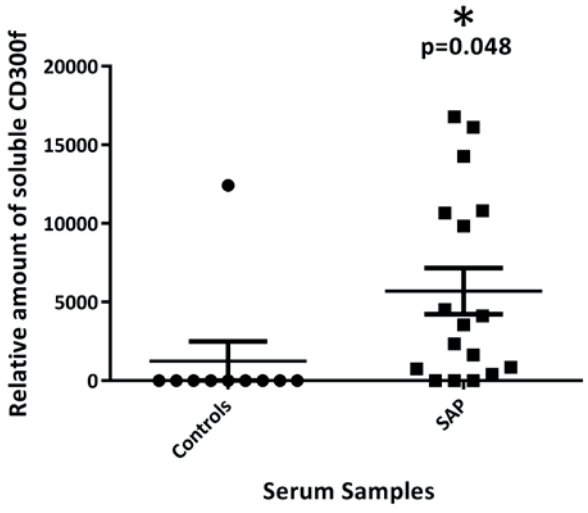


Figure 61: Elevated levels of sCD300f were found in the serum of SAP subjects. Serum samples obtained from control donors or SAP subjects were diluted 1:2 in PBS 1X and sCD300f was quantified by ELISA. sCD300f values are presented as mean ±SD. Significance was determined by Student's-test *p<0.05 (n=1, triplicate).

Detection of sCD300f in serum samples from Multiple Sclerosis subjects

With the aim of investigating the present of sCD300f in subjects with other inflammation diseases apart from SAP, it was hypothesize the existence of the soluble receptor in the blood from Multiple Sclerosis (MS) subjects [243]. The hypothesis was supported by evidences based on the neuroprotective role of the CLM1 receptor (the human CD300f ortholog in mice) in the EAE mice model [229], used to reproduce the Multiple Sclerosis disease in humans. Contrary to the premises, the ELISA results indicated no significant differences between the amount of sCD300f from controls and MS subjects (**Figure 62**). Nevertheless, one MS sample exhibited significant soluble receptor amount compared to the rest of the serums, indicating that the existence of the sCD300f might depend on unknown factors, present in the majority of SAP subjects and absent in MS subjects.

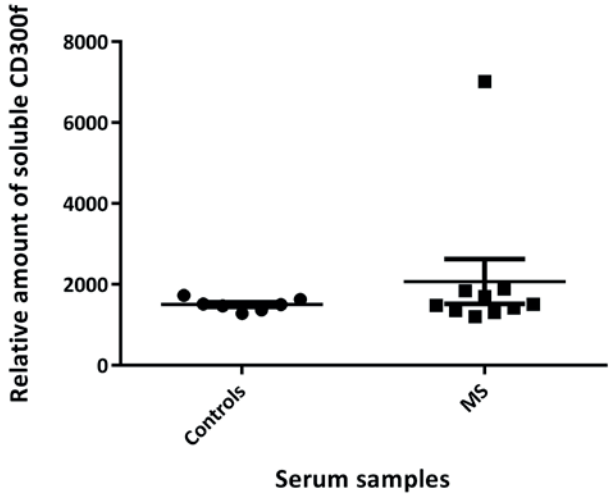


Figure 62: No significant sCD300f was detected in the serum of MS subjects. Serum samples obtained from control donors or MS subjects were diluted 1:2 in PBS 1X and sCD300f was quantified by ELISA. sCD300f values are presented as mean ±SD. Significance was determined by Student's-test (n=1, triplicate).

Detection of sCD300f in serum samples from other autoimmune diseases

In parallel serum samples from other autoimmune diseases were considered for analysis. The results from the ELISA assays demonstrated that sCD300f was abundant in the blood from Celiac disease patients. Furthermore, some subjects suffering from Rheumatoid Arthritis and Systemic Lupus Erythematosus diseases had important concentration of the sCD300f in the blood (**Figure 63**). Contrary serum samples from Diabetes Mellitus, Autoimmune Thyroid disease, Graves disease or Systemic Vasculitis PR3 patients showed no significant levels of sCD300f (**Figure 63**). Therefore, to understand the biological significance behind the fluctuating sCD300f levels in biological fluids is indispensable to determine the common factors between Antiphospholipid Syndrome, Celiac disease, Rheumatoid Arthritis and Lupus autoimmune diseases. Further studies are required to determine the use of sCD300f molecule as a potential biomarker for concrete autoimmune diseases.

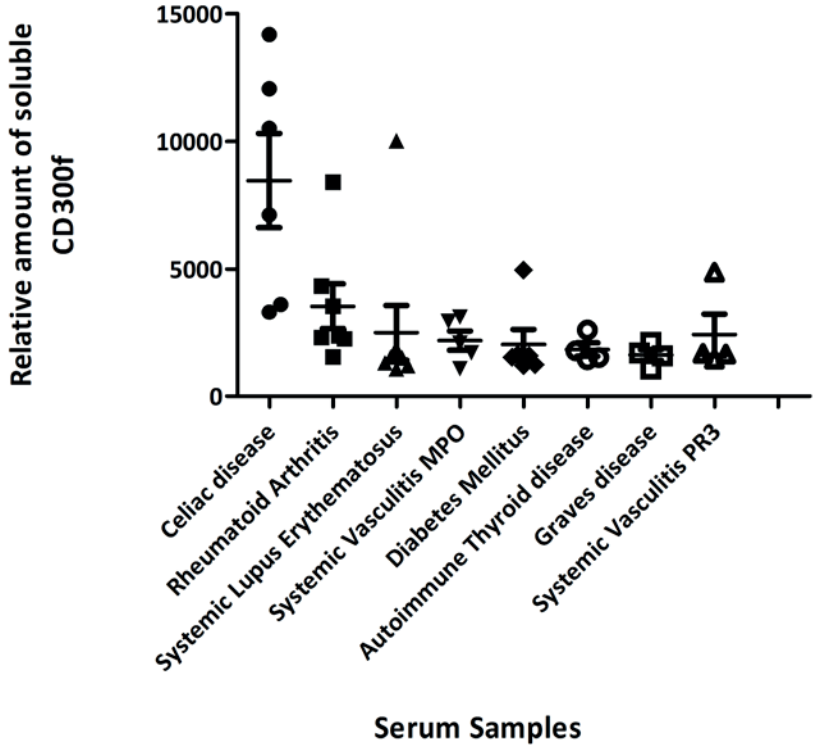


Figure 63: Significant sCD300f was detected in the serum of some autoimmune diseases subjects. Serum samples from Celiac disease, Rheumatoid Arthritis, Systemic Lupus Erythematosus, Systemic Vasculitis MPO, Diabetes Mellitus, Autoimmune Thyroid disease Graves disease, and Systemic Vasculitis PR3 subjects were diluted 1:2 in PBS 1X and sCD300f was quantified by ELISA. sCD300f values are presented as mean. (n=1, triplicate).

Discussion

The main goal of this thesis was to investigate the function of the endogenous CD300f. Initially, CD300f was described as an inhibitory receptor, although some data generated in our lab strongly suggested the possibility that the receptor could trigger activating signals in specific situations. Due to the fact that, all previous data from the lab was based in the work with transfected CD300f in different cell lines, it was decided to analyze the function of CD300f in cells that express the receptor endogenously. For that purpose, it was planned to use two monoclonal antibodies (UPD1 and UPD2), that recognize the extracellular domain of CD300f. Both antibodies were raised in the past, to help in the characterization of CD300f and where selected in our lab, based in their ability to recognize CD300f and do not cross-react with the CD300 molecules known at that moment (CD300a, CD300b, CD300c and CD300e). However, at the time that we started to work in this thesis, our lab had recently cloned a cDNA encoding for the CD300d molecule, so it was essential to check if UPD1 and/or UPD2 cross-reacted with CD300d. Nevertheless, CD300d was unable to reach the cell surface in transfected cells, so it was not possible to check if the antibodies recognize it. That result prompted us to further investigate and characterize the CD300d receptor. Finally, the cloning and characterization of CD300d has contributed to complete the description of the human CD300 locus, containing six members (from CD300a to CD300f), clustered in a 450 Kb region of the human chromosome 17.

- The most interesting finding regarding the CD300d receptor was its inability to reach the cell membrane, when transfected in multiple cell types. CD300d was retained intracellularly and more specifically within the endoplasmic reticulum (ER) (**Figure 23**). Different hypothesis have been considered to explain that phenotype.

Proteins need to acquire a transport-competent state before they can leave the ER. N-glycosylation is one of the modifications required for the correct folding of secretory proteins and CD300d was modified by N-glycans as shown in (**Figure 21**). However, not all the N-glycosylated proteins are translocated to the Golgi apparatus, as demonstrated for the VIP-L protein [244], indicating that specific signals exist that distinguish proteins to be maintained in the ER organelle.

Type I transmembrane ER retained proteins display a consensus di-lysine (-KKXX) motif in the carboxy-terminal extreme, which appears to be conserved across eukaryotes, although some substitutions of lysine by arginine are permitted. Anyway, the di-lysine motif was not observed in the CD300d sequence. On the contrary, the di-arginine (-XXRR) ER retention motif was present in the carboxy-terminal tail of the CD300d. That motif was initially described for the type II transmembrane proteins [245], but later on other studies demonstrated the presence of the motif in some type I transmembrane proteins, like VIP-L [244] or TMX4 [246]. The fact that the -RR- motif was not present in the CD300c receptor, which has no problems to reach the cell surface, made this motif suitable for study in the ER retention phenotype of CD300d (**Figure 24**). However, disruption of di-arginine motif by mutagenesis was not conducive to CD300d surface expression (**Figure 25**).

The carboxy-terminal –KDEL sequence is another consensus ER retention motif, not only described for soluble proteins found in the ER lumen, but also proposed to be involved in the ER retention in mammalian type II membrane proteins [245], however, it was not found in the CD300d tail. Finally, a comparative analysis between the cytoplasmic domain of CD300d and CD300c, demonstrated that apart from the di-arginine motif, the remaining residues were coincident (**Figure 24**). Thus, the CD300d cytoplasmic tail was ruled out to regulate the trafficking of the receptor by harboring ER retention motifs.

In this context, it is important to note that protein export from ER is a selective process driven by coat protein complex II (COPII), which forms transport vesicles from the ER and collects the appropriate cargo proteins into these vesicles. The COPII coat consists of three modules (Sar1, Sec23–Sec24 complex and Sec13–Sec31 complex) that are sequentially recruited to the ER membrane surface [247]. Importantly, ER-export cargo proteins possess surface residues or short and linear sequence motifs called ER export motifs, usually in the C-terminal region of the protein, which are able to bind Sar1–Sec23–Sec24 pre-budding complexes directly or indirectly. These pre-cargo complexes are then gathered by the Sec13–Sec31 complex into nascent vesicles, to extract the specific cargo from the ER [248 249]. The conserved di-acidic (DXE) motif is found as an export motif in secreted type I transmembrane proteins. Further types of transport signals have been identified in membrane cargos that exit the ER, these generally consist of a pair of huge hydrophobic residues and have been described as di-aromatic or di-hydrophobic motifs [250]. Nevertheless, CD300 receptors do not seem to bear ER export motifs within intracellular domains because the CD300c and CD300f cytoplasmic deletion mutants maintained a cell surface phenotype (**Figure 25**).

There are other mechanisms that could explain the ER retention phenomenon observed in CD300d, of special interest is the case of the high affinity receptor for IgE (FcεRI). In humans, this receptor is found in two alternative forms. The trimeric form is composed by the alpha IgE-binding subunit (FcεRα) and a disulfide-linked homodimer of γ-chains (FcεRγ/FcRγ) [251]; whereas the tetrameric form contains an additional tetraspanning β-chain (FcεRβ) [252]. Similar to CD300d, FcεRα is blocked in the ER and, contains an acidic residue in its transmembrane domain and exhibits a short cytoplasmic tail. Therefore, the study of FcεRα ER retention mechanisms could contribute to understand the CD300d phenotype.

Cotranslational assembly of FcεRI subunits (α, γ and β) was described as the first quality control mechanism for the generation of functional FcεRI receptor at the cell surface [253]. In agreement, FcεRα display two di-lysine ER retention motifs in its cytoplasmic tail, which become masked by the association with FcRγ, allowing its targeting to the cell membrane [254]. Nevertheless, FcεRα is accumulated in intracellular compartments in eosinophils [255] and megakaryocytes [256], despite the presence of FcRγ. Altogether, indicated the existence of additional mechanisms regulating the surface expression of the FcεRα chain. Among them, it is publish that glucose trimming within FcεRα immunoglobulin domain is required to overcome the ER quality control [257]; it is described that FcεRα signaling peptide impairs its own surface expression [258]; and it is demonstrated the importance of the D194 residue,

within the transmembrane domains of FcεRα, in determining IgE receptor subunit interactions, cell surface localization and initiation of downstream signaling events [259] [260].

Analyzing the involvement of similar regulating mechanism on CD300d, it was evident that post-translational modifications in the extracellular domain of the receptor were not responsible for CD300d retention, because substitution of Ig domain and stem regions of CD300f by CD300d resulted in a chimerical protein able to reach the cell membrane (**Figure 25**). The signal peptide might also be ruled out, because the entrapment occurred equivalently using three different constructs in which the CD300d receptor was driven by different signal peptides (Igκ-chain leader sequence in pDisplay, CD8α leader sequence in pCDNA3-FLAG, and its own signal peptide in pEGFP-N3). Interestingly, experiments with chimerical proteins pointed out to the transmembrane domain of CD300d as the ER retention unit. Although, the substitutions of the two phenylalanine residues (F168 and F170) and the glutamic acid (E173) present in this region were not able to induce the trafficking toward the cell surface (**Figure 25**). Taken together, these data suggest a complex scenario where the combination of different retention-retrieval motifs in different domains of CD300d might be responsible for the intracellular location of this protein.

Furthermore, it has been suggested that transmembrane adapter proteins might act not only as signaling modules but also as chaperones for certain immunoreceptors. Early events in the folding of the receptors are probably rate-limiting, thus receptor folding intermediates are retained in the ER until they can adopt the correct conformation and the fully glycosylated pattern. The formation of stable receptor-adapter modules is thought to assist this process and prevent intracellular degradation. This is the case for NKG2C [122], Ly-49 [121] and PDC-TREM [129], whose expression in the membrane is dependent on DAP12. In agreement, the presence of FcRγ was able to overcome CD300d ER retention in transfected COS-7 cells (**Figure 26**). FcRγ could mask CD300d putative retention motifs in a similar way to what has been described for FcεRα, even though, no receptor was detected in the surface of RBL-2H3, which express endogenous FcRγ [176]. Additionally, it is of note that all CD300-activating members (CD300b, CD300c, and CD300e) could be expressed on the surface of transfected COS-7 cells independently of the presence of the transmembrane adapter polypeptides to which they associate with, although the presence of those ITAM-bearing adapters could enhance the levels of expression on the cell surface. Curiously, whereas co-transfection of CD300d and CD300c with FcRγ enhanced the surface expression of both receptors, co-transfection with DAP12 did not produce the same effect. By contrast, it is described that the presence of DAP12 augments the presence of CD300b on the surface of co-transfected COS-7 cells. These data strongly suggest that the interaction between ITAM-bearing adapters and receptors is tricky. As commented in the introduction, immunoreceptors recruiting ITAM-bearing adapters could be classified according to the structural elements involved in the establishment of the interaction. The classical immunoreceptors bind to these adapters through a mechanism of positive-negative charge complementation at the transmembrane level, like the KIR [261] [262], ILT [263], and TREM [127] families of receptors. The non-classical immunoreceptors bind to the signaling adapters

independently of the presence of charged residues within the transmembrane domain of the receptors, although the presence of a negative charge in the adapters seems to be important for the interaction in some cases. This second group of receptors comprise integrins [264], growth factor receptors [265], and MHC proteins [122]; and could include those receptors bearing a negative charge within their transmembrane domain, such as CD300c, CD300d, and the alpha IgE-binding subunit (FcεRα). Thus, within the CD300 family, some members bind the adapter polypeptides both in a charge-dependent (CD300b [201] and CD300e [215]) and charge-independent (CD300c [176] and CD300d) manner.

As a final point, the lack of specific antibodies against CD300d makes difficult to define the location of this receptor in monocytes and granulocytes, which express the receptor at the mRNA level. Molecular experiments showed that CD300d was able to recruit the ITAM-bearing adapter FcRγ, suggesting that if this receptor is able to reach the cell surface, it could trigger activating signals after engagement with a specific ligand, but clearly the cellular compartmentalization would determine the CD300d function. Furthermore, recently it has been shown that CD300 molecules can interact with each other through their Ig domains. Thus, the combination of CD300 receptors in the cell surface of the cells modulates differentially the signaling out coming after specific receptor stimulation [176], suggesting a new mechanism by which CD300 complexes could finely regulate the activation of myeloid cells upon interaction with their natural ligands. In this context, the CD300d ER confinement could control the extracellular export of some CD300 receptors and, as a consequence, organize the composition of CD300 surface complexes. Indeed, ER export has been shown to be a rate-limiting step for the cell surface transport of the receptors [253] [266]. This hypothesis was validated both in COS-7 and RBL-2H3 cells, where CD300d reduced CD300f cell surface levels (**Figure 31**) (**Figure 32**). Accordingly, as a consequence of the lack of interaction between CD300c and CD300d, membrane content of CD300c was not modified by the presence of CD300d. In addition, there exists the possibility that, in primary cells, the CD300d surface expression requires the formation of intracellular complexes with other CD300 molecules, such as CD300a, CD300b, or CD300e. Even though, the presence of CD300f did not induce the surface expression of CD300d in co-transfected COS-7 cells.

- The possibility that CD300f could deliver both activating and inhibitory signals was proposed by our lab, based in the capability of the receptor to recruit both activating and inhibitory mediators such as SHP-1 [217], p85, and Grb2 [218]. Initially, it was demonstrated that the co-engagement of CD300f with specific antibodies in transfected RBL-2H3 cells impaired the secretion of β-hexosaminidase mediated by the FcεRI receptor. Interestingly, triggering of a CD300f mutant, devoid of the two binding site for the tyrosine phosphatase SHP-1, induced cell degranulation in the same cell line [218], reveling the potential of CD300f as activating receptor.

Some studies supported the inhibitory role of the endogenous CD300f receptor. For example, CD300f blocked ERK- and PI3K- mediated BAFF signaling pathway [219] or impaired MyD88- and TRIF- mediated TLR signaling pathway [220], when it was stimulated in THP-1 and U937 cells. On the contrary, activating capability of the endogenous CD300f was sustained by studies demonstrating the cytotoxic and apoptotic processes mediated by antibodies against the receptor. This experiments were done with blast from acute myeloid leukemia origin, pointing out the immunotherapy potential of those antibodies as anticancer agents [225].

All these data showed a potential dual activating/inhibitory functionality of the CD300f molecule and raised a huge number of questions, about the mechanisms that could regulate the final outcome of CD300f triggering in a specific situation. For instance, the fact that mutations, affecting the CD300f residues involved in the binding with SHP-1, were enough to produce a switch from an activating to an inhibitory behavior, suggested that, cellular events modifying the availability of specific signaling molecules and the accessibility of the interacting domains in CD300f, could be responsible for the final outcome of the receptor.

Nevertheless, no one of these preceding works demonstrated the duality of the endogenous CD300f receptor in the same cell system, either analyzed in detail the molecular components implicated in that activating facet of the receptor. An approximation was achieved in a study of the CLM1 receptor, the mouse ortholog of CD300f. Importantly, the authors demonstrated that in bone marrow-derived mast cells (BMMCs), the engagement of the endogenous CLM-1 in the presence of LPS enhance the IL-6 cytokine production, in a process dependent on FcRγ adapter molecule. At the same time that, the triggering of endogenous receptor delivered inhibitory function on FcεRI-mediated cytokine production in the same BMMCs [226].

The work presented in this thesis demonstrates that in THP-1 cell line a pre-treatment with PMA (phorbol 12-myristate 13-acetate) is enough to produce a dramatic change in the function of endogenous CD300f, from a receptor that blocks LPS-mediated TNFα production in the absence of PMA, to a receptor that promotes the secretion of the same pro-inflammatory cytokine after the PMA pre-treatment (**Figure 56**).

U937 and THP-1 cell lines have been used extensively as a model to study the behavior and differentiation of monocytes for more than 30 years. Whereas U937 cell line was isolated from the histiocytic lymphoma of 37 years old male patient [267], THP-1 cells derived from the peripheral blood of 1 year old human male with acute monocytic leukemia [268]. Both cells lines can be induced to mature by phorbol esters, like PMA, from a promonocytic into a monocytic stage of development. This process is accompanied by the acquisition of a number of morphological and functional attributes normally associated with mature monocytes/macrophages [269] [29] [270].

The culturing of U937 and THP-1 cells confirmed that treatment with PMA produces similar morphological changes in both cell lines. Thus, whereas not differentiated cells grew in

suspension, PMA-treated cells attached to the plastic substrate with a macrophage-like shape. However, a big difference regarding how they respond to LPS in terms of cytokine secretion was observed between both cells lines. Non PMA-differentiated U937 were unable to secrete TNFα in response to LPS, whereas differentiated cells promote cytokines release upon LPS treatment (**Figure 46**) (**Figure 47**). By contrast, LPS-stimulated THP-1 cells were able to secrete TNFα without requiring a PMA differentiation process (**Figure 56**). Altogether, indicated divergence in the basal state of differentiation between U937 and THP-1 cell lines and also highlight the importance of little variations in the activating status of a cell, affecting the reaction capabilities after the contact with a given stimulus.

Related to the previous observation, triggering of CD300f induced pro-inflammatory cytokine secretion in primary monocytes (**Figure 44**). These cells were isolated from PBMCs by plastic attachment and presented a macrophage-like morphology at the moment of the assay. In fact, it has been accepted for a long time that monocyte attachment in plastic surfaces for itself results in their pre-activation [271]. All these data suggest that the differentiation/activation processes induce cell changes that could modify CD300f function.

Focusing in the duality of the CD300f receptor activity, observed in the THP-1 cell due to the PMA pre-treatment, it was hypothesize if the change of function could be related to other phenomenon independent of CD300f itself. As mentioned before, immunoglobulin domains of some members of the CD300 family are extremely similar. Consequently, it could be feasible that antibodies used to engage the CD300f (UPD1 and UPD2) were interacting with other members of the family, which could be up-regulated as a consequence of the differentiation process induced by PMA. However, that hypothesis was rule out after demonstrating the specificity of the UPD1 and UPD2 for the CD300f receptor (**Figure 43**).

Later on, another possibility was explored linked to the PMA effect on the availability of signaling molecules related to CD300f. The result showed a dramatic increase in the expression of ITAM-bearing adapters FcRγ and DAP12, after THP-1 differentiation by PMA treatment (**Figure 57**), suggesting that both molecules could be responsible for the switch of CD300f function. Furthermore, data showed that inhibition of Syk kinase totally abrogated CD300f-induced TNFα production (**Figure 52**), whereas PI3 kinase inhibition did not significantly diminished CD300f signaling (**Figure 53**). Taking into account the signaling cascade downstream the adapter molecules, it is well known that Syk kinase plays a crucial role in all the signaling pathways involving ITAM or ITAM-like signaling adapters, including classical immunoreceptors (FcRs, Dectin-2, TREMs)[272] [273] [274], non-immunoreceptors (integrins, selectins, IL-3 receptor) [264] [57] [131] and C-type lectin receptors with hemi-ITAM sequences within their cytoplasmic tail (Dectin-1, CLEC-2) [275] [82]. Therefore, the idea that positive signaling of CD300f is mediated through DAP12 or FcRγ was reinforced. Moreover, not only Syk but the involvement of PKCδ in the signaling of CD300f in PMA-differentiated cells (**Figure 55**) pointed out to the same direction, as described for C-type lectin receptors [276]. Although, there were some open questions referred to the involvement of DAP12 and FcRγ in the signaling of CD300f.

First, ITAM's bearing adapters (DAP12, FcRγ and CD3ζ) were initially described as signaling partners of activating classical immunoreceptors, that bind to these adapters through a mechanism of positive-negative charge complementation at the transmembrane level. Interestingly, CD300f lacks any positive charged residue in its transmembrane, has structural characteristics of an inhibitory receptor and elicits some inhibitory functions, however was found to associate with FcRγ and DAP12 (**Figure 36**). In accordance with that finding, a group of non-classical immunoreceptors (integrins, growth factor receptors and MHC proteins) has been defined recently, due to their capability to bind to adapter signaling mediators independently of the presence of positively charged residues within their transmembrane domain. Even the mechanism of interaction is not fully understood, it has been demonstrated that negatively charged residue within the transmembrane domain of the adapters are indispensible for maintaining the association with these receptors, in agreement D29 FcRγ transmembrane residue was found crucial for the binding to CD300f (**Figure 42**). As well, using different mutants of the CD300f receptor and chimeric molecules with other CD300 molecules, the transmembrane domain of the CD300f was demonstrated to be sufficient to enable interaction with FcRγ (**Figure 39**). However, considering that the intracellular tail of the CD300f receptor was the only domain of the receptor used as bait in the three-hybrid screening; a secondary point of contact need to be located in the cytoplasmic tail of the receptor, indicating that the mechanism of interaction between FcRγ and CD300f is more complex than expected. Thus, other molecular experiments would be required to elucidate how the association between both molecules is achieved.

Second, the fact that FcRγ was cloned in a three-hybrid screening in the presence of Fyn suggested that tyrosine phosphorylation of CD300f and/or FcRγ would be required for the interaction. However, data clearly demonstrated that tyrosine phosphorylation of CD300f was not required for FcRγ recruitment (**Figure 33**); although it is possible that CD300f phosphorylation would be necessary for the activating signaling triggered by the endogenous receptor *in vivo*. It is of note that the pattern of CD300f tyrosine phosphorylation by Syk or Fyn was different (**Figure 35**). In fact, it is conceivable that binding of Grb2 and P85 subunit to the cytoplasmic tail of CD300f could be part of a signaling pathway initiated by action of Syk trough the adapters DAP12 and/or FcRγ, whereas specific phosphorylation of the ITIMs by other kinases could initiate the inhibitory responses mediated by CD300f.

Third, it was necessary to explain why the knocking-down of DAP12 or FcRγ had no effect in the CD300f cell signaling. A possible explanation was related with the idea that both adapters could perform complementary functions, thus the lack of one would be carried out by the other. However, it was indispensible to analyze the consequence of PMA treatment on knocked-down cells, as it was demonstrated in WT cells that the treatment strongly increased the expression of both adapters. As predicted, the low levels of adapters, achieved by shRNA technology, were dramatically enhanced after PMA differentiation (**Figure 51**), terminating the knocked-down effect and clarifying the lack of CD300f signaling impairment in these cells.

The last open question was referred to the demonstration of endogenous association between CD300f and DAP12/FcRγ. Whereas the binding was easily detected in transfected COS-7 cells, the association of CD300f with FcRγ/DAP12 in THP-1 or U937 monocytic cell lines was not observed. It is of note that a variety of experimental strategies was tried to overcome the problem, including the use of PMA treated or not treated cells, the crosslinking of surface CD300f before the cell lysis or changing the composition of the lysis buffer by using different detergents. However, in any case the co-precipitation between endogenous CD300f and the adapters was not detected. Accordingly, similar results had been described before for the murine ortholog of the receptor by other authors; it was published that transfected Flag-tagged CD300f recruited FcRγ in murine bone marrow-derived mast cells (BBMCs), whereas no data showing co-precipitation with endogenous CD300f was shown in the same study [226]. Afterwards another hypothesis was considered, related with the antibodies used to immunoprecipitate CD300f, because in transfected cells it was used an anti-tag antibody (anti-HA), while UPD1 or UPD2 mAbs were use to pull-down the endogenous receptor. To investigate whether the lack of co-immunoprecipitation in non-transfected cells was due to the used Abs, an experiment was performed in transfected cells using different combinations of antibodies and detergents. The data showed that anti-HA Ab was more efficient than UPD2 to pull-down CD300f and consequently co-precipitated better both adapters. Interestingly, the CD300f pulled-down bands were different depending on the used Ab, thus some additional bands were visible in the lanes corresponding to the anti-HA immunoprecipitation, indicating that each antibody had affinity for different posttranslational conformations of the receptor (**Figure 37**). It might be proposed that certain modifications of the receptor would be necessary to permit interaction with the adapter molecules. In addition, it might be that the interaction between endogenous CD300f and DAP12/FcRγ just occurs when the receptor is contained in complexes (homocomplexes or heterocomplexes with other CD300 receptors). Nevertheless, the formation of these complexes could mask the CD300f epitopes recognized by UPD1 and UPD2 antibodies, impairing the immunoprecipitation of the endogenous receptor. The design of new antibodies able to recognize endogenous CD300f complexes are required to answers these questions in the future.

- The existence of soluble CD300f proteins were hypothesized based in the presence of mRNA splicing variants of human, mouse and rat CD300f, encoding for putative proteins lacking their transmembrane domain, whereas maintaining the signal peptide and extracellular Ig domain. Moreover, due to the described neuroprotective role of CLM1 receptor (mouse ortholog for CD300f) in a mice model of Multiple Sclerosis (MS) termed EAE [229], it was hypothesized the involvement of soluble CD300f in the human disease. That publication also showed that the CLM1 knockout mice exhibited an increased neuropathology in response to the induction of EAE. Accordingly, a worsened phenotype was noted when a soluble CLM1-IgG fusion protein was administered to wild type animals. With the objective of obtaining a tool to analyze the presence of soluble CD300f in biologic

fluids, it was developed and ELISA technique using the UPD1 and UPD2 antibodies. Furthermore, the optimization of the ELISA protocol using the CD300f-IgG2a recombinant protein ensured the absence of soluble CD300f in serum samples from healthy donors, with a limit of detection at 100 ng/ml.

Contrarily to the hypothesis, just one of the seventeen MS serum samples analyzed by ELISA exhibited high amount of the sCD300f receptor (**Figure 62**), thus the results were not conclusive. MS is a chronic autoimmune demyelinating disease of the central nervous system (CNS) that mainly affects young adults and may lead to a significant disability over time [277]. The inflammatory cells in MS have been well described and include CD4+ and CD8+ T lymphocytes, microglia and macrophages [278]. Importantly, humoral immunity has also been described as a main component in the pathophysiology of MS [279]. It is noteworthy that the majority of the MS samples analyzed were from patients of relapsing-remitting multiple sclerosis (RRMS) subtype, apart from two cases of primary-progressive multiple sclerosis (PPMS). Immunological studies conducted on blood and cerebrospinal fluid samples showed differences between PPMS cases and other MS phenotypes, compatible with a different stage activation of the immune system [280]. Thus, it might be feasible that the importance of sCD300f would be related to PPMS subtype and not to the RRMS. Consequently, to discard the implication of sCD300f in MS disease a range of samples from distinct MS subtype would need to be analyzed in the future. In parallel, it would be essential to analyze the sCD300f level in the cerebrospinal fluid from MS subjects.

On the other hand, patients with Antiphospholipid Syndrome (SAP) showed higher levels of the soluble CD300f in relation with control subjects (**Figure 61**). The term SAP was coined in the early 1980s to describe a unique form of autoantibody induced thrombophilia, whose hallmarks are recurrent thrombosis and pregnancy complications affecting young adults [281]. The mechanisms by which antiphospholipid antibodies (aPL) cause the symptoms are poorly understood, but probably include inhibition of natural anticoagulants, activation of platelets and endothelial cells, blocking of the fibrinolytic system, and triggering of the complement cascade by monocytes [282] [242]. Furthermore, it is well known that aPL from subjects with SAP preferentially target negatively charged phospholipids such as cardiolipin, or their complex with plasma proteins such as β2-glycoprotein-I (β2GP-I) [242]. Additionally, antibodies against phosphatidylethanolamine (PE) in SAP sera have been detected [282]. PE is the main lipid component of the microbial membrane and is largely found in mitochondria. PE together with phosphatidylserine (PS) are located in the inner leaflet of cell membranes from living cells, but are exposed in the outer leaflet when cells become apoptotic [195] [196], being one of the mechanisms leading to phagocytic recognition of dead cells. Furthermore, PS is exposed by damaged endothelial cells and activated platelets during blood coagulation, in this situation membranes containing PS provide the catalytic surface that serves as a point of assembly for prothrombinease complex [283]. On the other hand it has been shown that human monocytes and platelets, when activated, generated four analogous PE lipids [284]. Thus, it could be proposed that uncontrolled activation of immune response, could mediate an increase in the production of phospholipids mediated by

monocytes and platelets, consequently a deregulation in the coagulation cascade leading to thrombophilic events could occur. Interestingly, CLM-1, the murine ortholog of CD300f, was demonstrated to promote phagocytosis of apoptotic cells in a process involving the recognition of PS [230]. The interaction of human CD300f with PS or PE has not been checked thus far, but it would be reasonable. Accordingly, soluble forms of the receptor might act as antibodies against these phospholipids giving rise to the development of SAP. Further molecular and *in vivo* experiment would be required to demonstrate this hypothesis.

Furthermore, patients with other autoimmune disease knew as Celiac disease (CD), exhibited significant amount of the soluble receptor in comparison with healthy donors or in comparison with other autoimmune diseases like Diabetes Mellitus, Autoimmune Thyroid disease, Graves's disease or Systemic Vasculitis (**Figure 63**). Celiac disease is a heritable chronic inflammatory condition of the small intestine, derived from a complex interplay between genetic and environmental factors [285]. Both innate and adaptive immune responses are involved in the course of the disease producing permanent intolerance to gluten (prolamin/gliadin) [286]. Interestingly, some cases indicated association between SAP and CD diseases; the connection has been observed in CD patients which show SAP symptoms as portal vein thrombosis [287], or nodular regenerative hyperplasia of the liver due to the presence of anti-cardiolipin antibodies [288]; other patients diagnosed with SAP show CS serological hallmarks, as the presence of anti-endomysial antibodies distinctive from CS patients in individuals with cutaneous necrosis typical from SAP [289].

Finally, even the main samples from patients diagnosed with Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) were negative for sCD300f, punctual individual present detectable amount of the soluble receptor (**Figure 63**). SLE is a chronic, autoimmune, connective tissue disorder affecting multiple organ systems, often with a relapsing-remitting clinical course, which presents abnormalities of the immune system at multiple levels. Pathogenic factors include influences from the environment, genetics, and epigenetic modifications including DNA hypomethylation [290]. Auto-antibodies implicated in the disease include antibodies against double-stranded DNA [291] or against extractable nuclear antigens as Ro, La or Sm. Additionally, antibodies against nucleosome, NMDA receptor, phospholipid and α-actinin are reported to play a role in the SLE disorder [292]. The autoantibodies are also drivers of the RA disease, which is a chronic debilitating autoimmune illness that results in systemic inflammation, synovitis and structural destruction of the joints [293]. A hallmark of RA pathogenesis is an imbalance between the bone-forming and bone-resorbing osteoclast driven by inflammatory processes, resulting in elevated bone resorption. Over time, irreversible bone and cartilage destruction occurs, leading to accrual of disability [294].

It is of note that clinical association between SAP, RA, CD and SLE disease has been reported in different studies [295] [296] [297]. Moreover, some of the diseases or syndromes have unique and overlapping manifestations, like the enhanced risk of atherosclerotic cardiovascular diseases in RA and SLE[298]; while others are proposed to be different clinical presentations of a single disease, as in the case of APS and SLE [299]. The molecular

mechanisms linking all these chronic syndromes and diseases are not really understood. However, the involvement of similar auto-antibodies affecting diverse biological functions is the main hypothesis. For example it has been demonstrated that auto-antibodies against nucleosome or chromatin characteristic from SLE are found in primary SAP patients, some of which subsequently develop the SLE disease [300]. Consequently, the identification of new serological hallmarks, able to discriminate between some of these autoimmune diseases, would facilitate a quick diagnosis and treatment; in this context, it would be interesting to determine the potential of sCD300f as a biomarker for autoimmune diseases and the effect of its accumulation.

An open question is related with the origin of the soluble forms of the receptor. As commented before, two putative sCD300f variants could be originated by alternative splicing of the CD300f gene. Nevertheless, the sequences of these variants were not found in any expressed sequence tag (EST) database, indicating that are not commonly translated in normal conditions. However, it could be that the expression of these splicing forms was restricted to anomalous situations as autoimmune diseases.

In addition, another cause is proposed for the origin of the soluble CD300f receptor, related with the existence of shedding processes. Proteolytic release of membrane proteins from the cell surface, also termed ectodomain shedding is an evolutionarily conserved post-translational modification, by which transmembrane molecules are converted into soluble forms. This process has been observed for many membrane proteins, including cytokines, growth factors, adhesion molecules and their receptors. Ectodomain shedding is a critical regulatory step affecting physiological and pathological processes, with two direct consequences: the adaptation of the cell phenotype by reducing the amount of surface-expressed membrane proteins and the release of soluble mediators capable of acting on other cells [301]. Interestingly, diverse soluble molecules with distinct functionality can be achieved from the same transmembrane protein. It is the case for the soluble variants generated from the low affinity IgE receptor (CD23), which either stimulate or inhibit the synthesis of IgE. Furthermore, the soluble molecules can be endocytosed, permitting them to modify intracellular signaling pathways by interacting with proteins form the cytosol or the nucleus, as is the case of Galectin-3 [302].

If shedding processes are responsible for the presence of sCD300f, it would be important to concrete the cell type from which it is generated. Taking into account that sCD300f is detected in serum sample from human origin, it is reasonable that monocytes or granulocytes would be the main source of sCD300f, as they express the CD300f receptor in the cell surface at high levels. However, also platelets could be responsible for the presence of sCD300f. This hypothesis is based in two premises: first, sCD300f is up-regulated in diseases involving a deregulation of the coagulation process, as SAP or SLE; second, ectodomain shedding is a major mechanism to modulate platelet receptor signaling and platelet reactivity [303]. Nevertheless, it has never been studied or published the expression of CD300f in platelets.

Apart from determining the cell origin of the sCD300f, it would be interesting to decode the molecular mechanism behind the proposed shedding process. In general terms, the cleavage of an extracellular domain from a transmembrane protein can be carried out by distinct proteinases, acting exclusively in concrete residues like serine, cysteine, aspartate; or proteinases dependent on the presence of metal ions, as MMPs (matrix metalloproteinase) and ADAM (A desintegrin and metalloproteinase). Furthermore, it is known that the shedding of a protein can be constitutive and/or inductive. For example, PMA and the Ca^{2+} ionophore Ionomycin are considered two common chemical stimulators of receptors shedding [301]. Additionally, it is known that PMA-induced shedding is a critical hallmark of ADAM-mediated shedding, which in turn depends on protein kinase C (PKC) activity [304]. In this context, ADAM17 and ADAM10 activities are known to modulate the inflammatory response, being the major sheddases for diverse cytokines as, TNFα, IL-1, IL-6 and IL-15 and also for the cytokine receptors TNFRI, TNFRII, IL-6R and IL15R [305].

Bearing in mind the involvement of PMA treatment in the activating function of transmembrane CD300f in THP-1 cells and its dependence in PKCδ activity, it is hypothesized that the ADAM metalloproteases could mediate the putative shedding process of the CD300f receptor in a pro-inflammatory environment. In agreement with this theory, an increase in the expression of ADAM17 is described in various autoimmune diseases such as Rheumatoid Arthritis [306], Sjögren's syndrome[307] or Multiple Sclerosis [308] among others, what in turn could explain the increase of sCD300f observed in some of these diseases. Altogether indicates that the CD300f soluble receptor would be a consequence, and not the direct cause of these diseases; even so, molecular studies are needed to confirm this hypothesis.

Conclusions

- The CD300d gene spans a 12.5 kb region on chromosome 17 (position 17q25.1), has an open reading frame of 585 bp and encodes for a protein of 194 amino acids.
- The CD300d gene codifies for a type I transmembrane protein; composed by an immunoglobulin-like extracellular domain stabilized by two disulphide bonds, a transmembrane domain containing a negative charged residue and a short cytoplasmic tail without known signaling motifs.
- CD300d has a predicted molecular mass of 21.5kDa, however presents two distinct mature forms of 30 and 34 KDa in transfected COS-7 cells. Both conformations are posttranslationally modified by N-glycosylations in the Ig domain.
- The CD300d receptor is expressed by primary monocytes and granulocytes. Conversely, T, B or NK lymphocytes do not express the receptor.
- Unlike previous described CD300 receptor, which exhibit surface expression, CD300d is retained in the endoplasmic reticulum of transfected cells. Even so, the interaction with the FcRγ adapter permits a slight surface recovery of the receptor in COS-7 cells but not in RBL-2H3 cells.
- In addition, CD300d is able to interact *in vitro* with the rest of known CD300 family receptors, with the exception of CD300c. Therefore, CD300d could play a role in the formation of CD300 complexes on the cell surface and consequently could modulate the state of activation of myeloid cells.
- The CD300d and CD300f immunoglobulin domains present a 71% of identity. Nevertheless, UPD1 and UPD2 mAb recognize exclusively the CD300f receptor.
- The stimulation of the endogenous CD300f receptor induces the production of TNFα pro-inflammatory cytokine in primary monocytes, giving support to the dual activity of the receptor, initially described as inhibitor.
- Crosslinking of endogenous CD300f, in PMA differentiated U937 or THP-1 monocytic cells, induce the production of TNFα and IL-1β pro-inflammatory cytokines.
- The activating functions of the CD300f could be mediated through its association with FcRγ and/or DAP12 adapter molecules, proved *in vitro*. The binding involves the transmembrane charged residue of the adapters and the transmembrane domain of the receptor.
- PMA pre-treatment upregulates the expression of endogenous FcRγ and DAP12 adapter molecules in U937 and THP-1 cells. In addition Syk kinase, JNK kinase and PKCδ are involved in the activating signaling mediated by CD300f. Altogether, supports the functional association of FcRγ and/or DAP12 with the CD300f receptor.
- Surprisingly, the engagement of CD300f inhibits LPS-stimulated TNFα release in THP-1 cells devoid of PMA differentiation. Indicating that the CD300f function switch, from an

activating receptor to an inhibitor, is controlled by the state of differentiation of the cells.

- Soluble forms of the CD300f receptor (sCD300f) exist and are detectable in human serum by an ELISA assay.
- The concentration of sCD300f is variable depending on the origin of the sample. Interestingly, high amounts of sCD300f are present in the majority of patients with Antiphospholipid syndrome (SAP) and Celiac disease (CD). In some subjects with other autoimmune diseases the level of sCD300f in blood is high, even so is not the case for the great majority of the analyzed samples.
- sCD300f has potential as biomarker for autoimmune disease.

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Thesis summary in Catalan

INTRODUCCIÓ

Receptors de membrana en leucòcits

Els leucòcits presenten receptors en la seva membrana superficial que els permeten rebre informació de l'exterior i transmetre-la a l'interior a través de complexes vies de senyalització, amb la finalitat de dirigir l'activitat cel·lular. Segons la informació rebuda, es mantindrà un estat d'homeòstasi o s'iniciarà una resposta immunològica. Majoritàriament, aquests receptors formen part del conjunt de molècules anomenades receptors de reconeixement de patrons o PRR (de l'anglès: *pattern recognition receptors*). Tot i que la majora de PRR s'expressen a la superfície cel·lular, alguns altres s'expressen en vesícules intracel·lulars com endosomes, lisosomes i el reticle endoplasmàtic [309].

Els dominis extracel·lulars dels receptors són responsables de la interacció amb els lligands. Per altra banda, les estructures o motius presents en els dominis transmembrana i citoplasmàtic dels receptors permeten classificar-los entre receptors activadors o inhibidors.

Els motius ITIM (de l'anglès: *immunoreceptor tyrosine based inhibitory motifs*) i ITAMs (de l'anglès: *immunoreceptor tyrosine-based activating motifs*) són seqüències d'aminoàcids concretes que es troben en les cues citoplasmàtiques dels receptors o les molècules adaptadores que s'hi associen. Aquests motius són responsables de propagar les vies senyalitzadores que s'inicien una vegada el receptor interacciona amb un dels seus lligands.

Receptors activadors

Clàssicament els receptors activadors es caracteritzen per tenir una cua citoplasmàtica curta i presentar un aminoàcid carregat positivament en el seu domini transmembrana, que permet la seva associació amb molècules adaptadores (Ex: la molècula adaptadora DAP12 (de l'anglès: *DNAX-activating protein 12*), la FcRγ o la CD3ζ) [55].

Generalment els receptors activadors no posseeixen motius ITAM, per contra aquestes són presents en les molècules adaptadores associades. La seqüència ITAM canònica és YxxL/Ix6-8YxxL/I (on x representa qualsevol aminoàcid). Normalment l'estimulació d'un receptor activador afavoreix l'activitat de quinases associades, com pot ser el cas de la família de quinases Src (SFKs), les quals una vegada activades fosforilen les dues tirosines contingudes en el motiu ITAM de la molècula adaptadora. Aquesta fosforilació converteix aquests residus tirosina en llocs diana per la interacció amb dominis (SH2), continguts en quinases de segón ordre, com les de la família Syk, que un cop activades fosforil·lerán altres substrats [57].

La fosforilació dels motius ITAM és necessària i suficient per la inducció de la cascada de senyalització intracel·lular i conseqüentment activa proteïnes efectores ben conegudes, com

la fosfolipasa Cγ, la proteïna quinasa C, la quinasa fosfatidilinositol-3 (PI3K), les proteïnes Ras, les proteïnes quinases MAPK (de l'anglès: *mitogen-activated protein kinase*), el factor nuclear NF-κB o el factor nuclear per l'activació de cèl·lules T (NFAT) entre d'altres [55]. Els efectors finals de la via regulen la mobilització de calci, l'activació transcripcional, la producció de citocines, la migració, la proliferació i/o diferenciació de la cèl·lula.

Receptors inhibidors

Clàssicament els receptors inhibidors es caracteritzen per tenir una cua citoplasmàtica llarga, la qual conté diversos motius ITIM [144]. La seqüència ITIM canònica és Ile/Val/Leu/Ser-X-Tyr-X-X-Leu/ Val (on X és qualsevol aminoàcid).

La interacció del receptor inhibidor amb un dels seus lligands resulta en la fosforilació de les tirosines contingudes en el motiu ITIM de la seva cua citoplasmàtica, normalment a través d'una quinasa de la família Src. Les tirosines fosforilades es converteixen en llocs diana per a dominis SH2 continguts en fosfatases citoplasmàtiques [145].

Majoritàriament existeixen dues classes de molècules inhibitòries efectores amb motius SH2: la fosfatasa de tirosines SHP-1 i la fosfatasa d'inositol SHIP [146] [147]. Una vegada aquestes fosfatases s'activen tenen la capacitat de defosforilar mediadors clau de vies activadores com són les molècules Syk, LAT, BLNK/SLP-76, Vav o PI3K, interferint conseqüentment l'activació cel·lular.

S'ha descrit que els motius ITIM són capaços d'interaccionar amb una altre fosfatasa coneguda com SHP-2, tot i així el rol d'aquesta molècula en funcions inhibidores és menys conegut i fins i tot es diu que pot participar en vies activadores [148].

Família de receptors CD300

La família de molècules CD300 esta formada per diferents proteïnes reguladors, normalment presents a la membrana cel·lular de cèl·lules mieloides humanes. Alguns membres de la família actuen com a inhibidors de la resposta immune i altres com a activadors, així doncs la coordinació en l'activitat de totes les molècules CD300 com a conjunt permet modular la resposta immunitària [167] .

Els receptors CD300 formen part de la superfamília d'immunoglobulines (IgSF) [168]. Cada proteïna CD300 s'anomena alfabèticament: CD300A, -B(LB), -C, -D(LD), -E i -F(LF); en referencia a l'ordre en el que els seus gens es troben situats en el cromosoma humà 17 [171]. En el ratolí existeix un clúster de gens ortòlegs a la família humana CD300, en aquest cas les molècules s'anomenen CLM (de l'anglès: *CMRF-like molecules*). El clúster CLM es localitza concretament a la regió cromosòmica 11D del genoma del ratolí i s'hi han identificat 9 receptors, del CLM-1 al CLM-9 [172].

Els ortòlegs entre les molècules humanes i de ratolí es varen establir seguint criteris d'organització genètica i anàlisis filogenètics [167], així doncs no tenen que tenir necessàriament funcions ortòlogues. A més, dins les molècules de ratolí, cada membre de la família pot patir petites modificacions en la seva seqüència, depenent de la soca de ratolí de la qual hagi estat clonat. Així doncs, les molècules aïllades a partir de la soca C57BL/6 s'anomenen CLMs, mentre que les provinents de la soca CBA/J s'anomenen LMIRs (de l'anglès: *Leukocyte Mono-Ig-like Receptors*).

Característiques generals dels receptors CD300

Totes les molècules CD300 són glicoproteïnes transmembrana tipus I, les quals estan formades per un domini extracel·lular simple tipus immunoglobulina (*IgV-like)*, seguit d'una regió proximal de membrana rica en prolines, serines i treonines. La regió pròximal permet la unió del domini immunoglobulina amb la regió transmembrana del receptor, després de la qual s'hi troba la cua citoplasmàtica.

El CD300b, el CD300c i el CD300e tenen la típica estructura de receptors activadors, amb cues citoplasmàtiques curtes sense motius de senyalització coneguts. La seva regió transmembrana conté una aminoàcid carregat, el qual permet l'associació amb altres molècules transmembrana, com poden ser les molècules adaptadores.

Per contra, el CD300a i el CD300f tenen la típica estructura de receptors inhibidors. Així doncs, en les seves cues citoplasmàtiques contenen motius ITIMs, els quals inicien la cascada senyalitzadora a través d'interaccions directes amb molècules senyalitzadors com fosfatases, quinases i altres pèptids.

Formació de homo- i heterocomplexes entre els membres de la família CD300

Inicialment es va accetar que les molècules CD300 concordaven amb el model clàssic de immunoreceptors activadors i inhibidors. Aquesta idea es suportava en el fet que, comparant els dominis extracel·lulars dels diferents membres es podien establir parelles que podrien compartir el lligand, tenint estructures funcionals oposades d'activador i inhibidor. Per exemple, la seqüència extracel·lular del receptor inhibidor CD300a presenta una identitat molt alta amb la mateixa seqüència del receptor activador CD300c, així doncs el CD300a i el CD300c es consideraven parella funcional [175]. El mateix es podia observar en el cas de l'inhibidor CD300f i el receptor activador CD300b. Tot i això, actualment s'ha evidenciat que la família de receptors CD300 té un comportament més complex de l'esperat, degut a la seva habilitat per interaccionar lateralment els uns amb els altres a través dels seus dominis immunoglobulina. Es sap que la formació d'aquests complexes és independent de ponts disulfur i que pot tenir lloc intracel·lularment, fet pel qual els receptors serien exportats a la superfície en forma de complexes preformats. També existeixen evidències de la importància de certs ions metàl·lics en la formació d'aquests complexes.

Cal remarcar la importància de la formació d'aquests complexes en la funció final de cadascun del receptors CD300 que en formen part, permeten relacions de sinèrgia o processos d'antagonisme. Per exemple s'ha observat que la estimulació del CD300c o el CD300b, en cèl·lules transfectades per separat amb cadascún dels receptors, porta a nivells similars d'activació cel·lular. Per contra, estimulant específicament el CD300c en cèl·lules transfectades amb els dos receptors alhora s'observa un nivell d'activació doble, demostran la suma de funcions entre els dos receptors [176]. Així doncs, s'evidencia que la formació de complexes entre els membres de la família CD300 pot ser un mecanisme de regulació funcional global per aquests receptors.

Característiques molecular i funcionals d'alguns membres CD300 i els seus ortòlegs murins

CD300d

La seqüència del receptor humà CD300d, també anomenat IREM4, va ser identificada el 2006 en el nostre laboratori. Tot i així, degut a les seves propietats estructurals la seva funció ha estat desconeguda fins avui, sent un dels objectius d'aquesta tesis.

CLM-4

El receptor CLM-4, també anomenat MAIR-II o LMIR2, és considerat l'ortòleg murí del CD300d [167], tot i que algunes publicacions el consideren també ortòleg del CD300c [198]. Aquesta confusió és deguda a les similituds estructurals entre els dos receptors humans. L'homologia entre el domini immunoglobulina del CLM-4 i els mateixos dominis en el CD300c o CD300d són molt alts, tot i així si s'analitza l'estructura total dels receptors es poden observar diferències remarcables: mentre que el CLM-4 presenta un residu carregat positivament a la regió transmembrana, els receptors humans hi mostren un residu carregat negativament, fet que pot tenir gran rellevància a nivell funcional.

Referent a l'expressió i funció del CLM-4, aquest receptor es troba en un subtipus de cèl·lula B i en macròfags peritoneals i de la melsa [198]. En aquestes darreres cèl·lules, el CLM-4 interacciona exclusivament amb l'adaptador DAP12, iniciant una cascada d'activació cel·lular que permet la secreció de TNFα. Per altra banda, en macròfags peritoneals el receptor interacciona tant amb el DAP12 com amb la molècula adaptadora Fc$R\gamma$, estan ambdós mediadors involucrats en la secreció de citocines induïda pel CLM-4. Cal remarcar que en els macròfags peritoneals l'expressió superficial del receptor pot ser potenciada per LPS en un procés que requereix la participació del la molècula Fc$R\gamma$. Per altra banda i de forma complementaria s'ha demostrat que el residu lisina de la transmembrana del receptor juga un paper clau en la interacció amb les dues molècules adaptadores [213].

En contra de la funció activadora descrita per el CLM-4 en macròfags, s'ha descrit que en cèl·lules B el receptor CLM-4 pot inhibir la proliferació cel·lular induïda per els receptors BCR i TRL-9, a través d'un mecanisme que involucre el reclutament de la fosfatasa SHP-1 a través del DAP12[214].

CD300f

El receptor CD300f també es pot anomenar IREM1. Es un receptor que presenta quatre variants de splicing alternatiu, dos de les quals codifiquen per formes completes del receptor que es diferencien en el seu inici de transcripció, tot i que tansols un dels dos sería funcional. Les altres dues variants codifiquen per formes truncades del receptor, a les quals els mancaria la regió transmembrana, possibilitant l'aparició de formes solubles del receptor.

La forma completa del CD300f es caracteritza per un pèptid senyal de 18 aminoàcids, una regió extracel·lular de 141 aminoàcids que conté un domini immunoglobulina variable, una regió transmembrana de 22 aminoàcids i una cua citoplasmàtica de 113 aminoàcids. El pes molecular de l'esquelet polipeptídic del receptor es de 32 kDa, mentre que el pes del receptor madur apareix com a dues bandes diferenciades de 53 kDa i 59 kDa, degut a diferents nivells de N- i O-glicosilasions.

El CD300f s'expressa en monòcits de sang perifèrica i granulòcits, així mateix com en diferents línies monocítiques. És d'interès el fet que la seva expressió es veu disminuïda en cèl·lules dendrítiques derivades de monòcits diferenciats *in vitro* [217].

La cua citoplasmàtica del receptor conté cinc tirosines, dues de les quals (la Y205 i la Y249) formen part de motius ITIM i una tercera (la Y284) forma part d'un motiu ITSM, el qual té caracterísitques funcionals similars al motiu ITIM, tot i mostrar diferències en quant a la seqüència aminoacídica. La fosfatasa SHP-1 és reclutada per el CD300f, sent la Y205 el principal lloc d'anclatge per aquesta interacció. De totes maneres s'ha demostrat que les altres tirosines Y249 i Y284 del CD300f també són importants per la seva funció inhibidora.

Sorprenentment, per a un receptor descrit inicialment com a inhibidor, les altres dues tirosines de la cua citoplasmàtica (Y236 i Y263) formen part de motius d'unió per a la molècula senyalitzadora PI3K, concretament per a la subunitat reguladora P85 de la quinasa. A més la Y263 també forma part d'una seqüència consens per al reclutament de la molècula adaptadora Grb2. Així doncs, es va proposar la possible participació de les molècules PI3K i el Grb2 en una possible funció activadora del receptor, posant de manifest el possible rol dual del receptor CD300f [218].

Existeixen un gran nombre de publicacions que defensen la funció inhibidora del receptor. Inicialment es va provar la seva capacitat per inhibir l'alliberació d'hexosaminidasa mediada per el receptor FcεRI en cèl·lules de la línia RBL-2H3, transfectades amb el receptor CD300f [217]. Posteriorment es va descriure la seva capacitat per inhibir la via activadora mediada per el factor BAFF en cèl·lules B [219] i es va demostrar la seva capacitat per bloquejar la senyalització de receptors TLR mediada per MyD88 i TRIF [220] [223]. Més recentment, en un model de dany cerebral agut en rates, s'ha demostrat que un increment en l'expressió del receptor té un efecte neuroprotector [224].

Per contra, la funció activadora del receptor també ha estat observat en altres estudis. Així mateix, s'ha demostrat que l'estimulació del receptor en cèl·lules mieloides, provinents de

leucèmies, provoca la mort cel·lular i un retard en el creixement dels tumors *xenograft*, en un procés depenent de mort cel·lular per citotoxicitat cel·lular, citotoxicitat depenent de complement o per mort directe per apoptosis [225].

CLM-1

El receptor CLM-1, també anomenat MAIR-V o LMIR3, es considera l'ortòleg murí del CD300f humà. Curiosament, el domini immunoglobulina del receptor CLM-1 presenta una identitat del 91% amb el mateix domini del receptor CLM-5 [173], considerat l'ortòleg murí del receptor CD300c.

Pel que fa a les similituds entre el CD300f i el CLM-1, s'observa una diferència important en la regió citoplasmàtica. El CLM-1 conté tan sols quatre tirosines, enlloc de les cinc presents en el CD300f, tot i així el CLM-1 també mostra dos motius ITIM i un tercer motiu (YxxM) descrit com a consens per a la interacció amb molècules de vies activadores com la PI3K; la cuarta tirosina de la cua del CLM-1 es troba en un motiu ITSM.

El pes molecular de la proteïna CLM-1 madura és de 60 KDa. El receptor s'expressa en cèl·lules dendrítiques primàries, macròfags, granulòcits, mastòcits i en un petit subtipus de limfòcits.

Pel que fa a les funcions inhibidores del receptor, s'ha publicat la seva capacitat per bloquejar la diferenciació d'osteoclasts [172]. Així mateix la producció de citocines (IL-6 i TNFα) induïda per l'estimulació del receptor FcεRI en cèl·lules mononuclears de medula òsea es veu inhibida per l'activitat del CLM-1 transfectat en aquestes cèl·lules. Per contra, en les mateixes cèl·lues, l'estimulació d'un mutant del CLM-1, en el que els residus tirosina han estat substituïts per fenilalanines, indueix per si mateix la producció de IL-6 en un procés depenent de la molècula adaptadora FcRγ; posant de manifest la possible dualitat del receptor. Altres assajos però varen demostrar la importància d'aquests residus tirosina en la producció de IL-6 mediada per CLM-1 en presència de la molècula coestimuladora LPS. Per contra, la presència d'altres molècules agonistes de receptor TLR, diferents a LPS, donaven lloc a funcions inhibidores del receptor CLM-1 [226].

Altres estudis suporten la funció activadora del receptor CLM-1. Per exemple, s'ha demostrat que l'estimulació del receptor en macròfags peritoneals indueix mort cel·lular en un procés similar a la mort per apoptosis induïda per raig UV [228]. També s'ha demostrat que en algunes línies cel·lulars l'estimulació del receptor promou la fagocitosis de cèl·lules apoptòtiques, les quals presenten alts nivells de fosfatidilserina (PS) a la superfície cel·lular [230].

Per contra, assajos *in vivo* usant un model murí, de l'enfermetat humana d'esclerosis múltiple, anomenat encefalomielitis experimental autoimmune (EAE), suporten que el receptor CLM-1 té una funció inhibidora i neuroprotectora en processos autoimmunes desmielinitzants. S'ha demostrat que en ratolins *knock out* per el gen CLM-1 s'observa un increment en la severitat de la malaltia, els seus leucòcits produeixen nivells de òxid nítric i citocines pro-inflamatòries més elevats i s'observa una major desmielinització [229].

MATERIALS I MÈTODES

Una gran varietat de tècniques i metodologies han estat usades al llarg d'aquesta tesis: cultiu *in vitro* de diferents línies cel·lulars o cèl·lules primàries, clonatge de molècules per PCR, tècniques de mutagènesis per PCR, assaig triple híbrid en llevats, PCR quantitativa en temps real, transfecció transitòria de la línia cel·lular COS-7, transfecció estable de la línia cel·lular RBL-2H3, transducció de la línia cel·lular U937 amb *shRNA* contra molècules concretes, utilització d'inhibidors químics contra molècules senyalitzadores en la línia cel·lular U937, generació de bacteris competents, transformació de bacteris competents mitjançant xoc tèrmic i recuperació del DNA plasmídic, immunoprecipitació de proteïnes, separació de proteïnes per *SDS-PAGE*, detecció de proteïnes per *western blot*, anàlisis de molècules de la superfície cel·lular per citometria de flux, tinció per immunofluorescència, assajos ELISA per detecció de citocines pro-inflamatòries o formes solubles de receptors i biotinilació d'anticossos, entre d'altres.

OBJECTIUS

- Caracterizar el nou receptor CD300d a nivell molecular i funcional.
- Verificar la possible dualitat funcional (inhibidora/activadora) del receptor CD300f endogen. A més de concretar els mediadors moleculars involucrats en aquesta possible via activadora del receptor.
- Desenvolupar un assaig capaç de detectar possibles formes solubles del receptor CD300f en fluids humans, per tal de poder provar la seva existència i estudiar la seva relació amb malalties d'origen autoimmune.

RESULTATS I DISCUSSIÓ

L'objectiu principal d'aquesta tesis era investigar la funció del receptor CD300f endogen. Inicialment, el CD300f es va descriure com a receptor inhibidor, tot i que alguns resultants del nostre laboratori suggerien la possibilitat que el receptor podia desencadenar respostes cel·lulars activadores en algunes circumstàncies. Degut al fet que tots els resultats obtinguts fins al moment en el laboratori s'havien basat en models cel·lulars en els que el CD300f s'introduïa per transfecció, es va decidir analitzar la funció del CD300f quan es trobava de

forma endògena. Amb aquest propòsit, es va decidir usar dos anticossos monoclonals (UPD1 i UPD2), que tenien la capacitat de reconèixer el domini extracel·lular del CD300f. Ambdós anticossos es varen obtenir en el nostre laboratori anteriorment, amb l'objectiu de permetre caracteritzar el receptor CD300f; varen ser seleccionats segons la seva capacitat per reconèixer el CD300f i no cross-reaccionar amb la resta de molècules CD300 conegudes fins al moment (CD300a, CD300b, CD300c i CD300e). Tot i així, poc temps abans de començar a treballar en aquesta tesis, en el laboratori s'havia clonat un nou membre de la família CD300, anomenat CD300d. Així doncs era necessari comprovar si els anticossos UPD1 o UPD2 cross-reaccionaven amb aquest nou membre de la família. Malauradament, el receptor CD300d era incapaç d'expressar-se a la membrana de cèl·lules transfectades, així doncs no era possible verificar si els anticossos el reconeixien. Aquest resultat ens va portar a investigar en més profunditat el CD300d. Finalment, el clonatge i caracterització del receptor CD300d ha contribuït a la descripció del locus humà CD300, que conté sis membres (del CD300a al CD300f) i ocupa una regió de 450 Kb en el cromosoma 17.

- El descobriment més important relacionat amb el CD300d fa referència al fenomen de retenció intracel·lular de la molècula, concretament en el reticle endoplasmàtic, impedint l'accés del receptor a la membrana extracel·lular. Per explicar aquest fenomen es varen considerar diferents teories.

L'existència de motius de retenció de reticle endoplasmàtic en la seqüència del CD300d podria ser una de les explicacions. Però mitjançant experiments de mutagènesis, es va demostrar que cap dels possibles motius de retenció identificats en la seqüència del receptor eren responsables d'aquest fenomen.

També es va considerar l'opció oposada, en la que s'hipotetitzava la presència de motius d'exportació en la resta de membres de la família CD300, els quals estarien absents en el CD300d. Però aquesta opció es va desestimar després de l'estudi del comportament de molècules quimera ,entre el CD300d i la resta de receptors CD300, i l'estudi de molècules mutants de diferents membres de la família CD300. En qualsevol cas les molècules aconseguien ser exportades a la membrana extracel·lular en tots els casos sense problema.

Una tercera opció per explorar era la implicació de certes modificacions post-traduccionals en el procés de retenció. Així doncs mitjançant experiments amb molècules quimeres es va demostrar que modificacions post-traduccionals afectant el domini immunoglobulina del receptor CD300d no eren responsables del fenomen de retenció. Així mateix també es va raonar que el pèptid senyal de la molècula no podia ser el responsable.

Es va concloure finalment, que la combinació dels diferents motius estudiats podria ser el responsable de la localització intracel·lular de la proteïna CD300d, així com també la presència d'altres motius de retenció intracel·lulars desconeguts.

Posteriorment es va estudiar l'efecte positiu que podia tenir la coexpressió del receptor CD300d amb diferents molècules adaptadores, facilitant la seva expressió superficial. D'aquesta manera es va demostrar que la co-transfecció del CD300d amb la molècula

adaptadora FcRγ permetia una petita expressió superficial del receptor, mentre que aquest efecte no s'aconseguia amb la molècula adaptadora DAP12. En un primer moment explicar la interacció entre el receptor CD300d i la molècula adaptadora FcRγ no va resultar fàcil ja que no concordava amb el model clàssic d'interacció entre un receptor i una molècula adaptadora. Anteriorment s'havia assumit que aquest tipus d'interacció tenia lloc a través de residus amb càrregues oposades a la regió transmembrana, una càrrega positiva del receptor i la negativa de l'adaptador. Però el CD300d no presentava un residu positiu a la transmembrana sinó negatiu. Tot i així, més recentment s'havia descrit un grup de immunoreceptors no clàssics, anomenats *non-immunoreceptors*, que interaccionaven amb les molècules adaptadores de forma alternativa. En concordança amb el fenòmen observat per el CD300d, en alguns casos, els *non-immunoreceptors* enlloc de presentar residus carregats positivament a la regió transmembrana, presenten residus carregats negativament, donat credibilitat a la interacció del receptor CD300d amb la FcRγ.

Amb la intenció d'anar més enllà en la caracterització del receptor CD300d, es volia definir en quins tipus cel·lulars s'expressava de forma endògena. Mitjançant assajos de PCR en temps reals es va determinar la seva presència en monòcits i granulòcits, però aquests resultats no es van poder confirmar a nivell proteic mitjançant citometria de flux degut a la manca d'anticossos específics contra el receptor. A més, cal afegir que si el receptor endogen es troba retingut en compartiments intracel·lulars de la mateixa manera que passa en cèl·lules transfectades, podria ser molt complicada la seva detecció mitjançant tècniques de citometria, tot i que es podrien fer aproximacions mitjançant immunocitoquímica.

Pel que fa a la funció del receptor, es va hipotetizar que si en algun moment el receptor era capaç d'accedir a la membrana extracel·lular podria tenir funcions activadores degut a la seva estructura molecular i la seva capacitat d'interaccionar amb la molècula adaptadora FcRγ. Tot i així calia tenir molt en compte que aquesta situació potser no es donava mai i que per tant la funció del CD300d podia ser una altra de molt diferent. En aquesta mateixa direcció s'havia demostrat feia poc la capacitat dels diferents membres de la família CD300 per formar homo- i heterocomplexes entre ells i es va hipotetitzar que la interacció del receptor CD300d amb altres membres de la família, podia facilitar l'expressió superficial del CD300d. En aquest context, es va demostrar la capacitat del CD300d per formar complexes amb qualsevol membre de la família CD300, amb accepció del CD300c, tot i així en cap cas es va aconseguir observar expressió superficial del CD300d. Aquesta troballa va posar de manifest una possible funció reguladora del CD300d en la formació del complexes i més important en el control sobre la expressió a nivell extracel·lular de complexes CD300 preformats.

- Pel que fa al receptor CD300f, la troballa més important va ser poder demostrar la hipòtesis d'una funció dual del receptor segons l'estat de preactivació de la cèl·lula.

La funció dual del CD300f va ser proposada per el nostra laboratori anteriorment, degut a la capacitat del receptor per reclutar tant molècules mediadores de vies activadores, com la

subunitat p85 de la quinasa PI3 o l'adaptador Grb2, com per reclutar molècules mediadores de vies inhibidores com la fosfatasa SHP-1. Així mateix, tot i que diferents publicacions aportaven informació sobre la via inhibidora o activadora del receptor, cap explicava la dualitat del receptor CD300f endogen un un únic model cel·lular ni analitzava en detall els components moleculars implicats en la faceta activadora del receptor.

El treball presentat en aquesta tesis demostra que, en la línia monocítica THP-1, el pretractament amb PMA és suficient per produir un canvi dramàtic en la funció del receptor CD300f endogen, des de un receptor amb capacitat de bloquejar la producció de TNFα induida per LPS en absència de PMA, fins a un receptor que promou la secreció de la mateixa citocina inflamatòria en cèl·lules pretractades amb PMA. Aquest mateix efecte no es va observar en la línia monocítica U937. Aquesta diferència va permetre profunditzar en la comprenció de la dualitat del receptor CD300f.

Les dues línies cel·lulars U937 i THP-1 han estat usades extensivament com a model per l'estudi del comportament i diferenciació de monòcits durant més de 30 anys. La línia U937 va ser aïllada d'un limfoma histiocític d'un pacient de 37 anys, mentre que la línia THP-1 es va aïllar de sang perifèrica d'un individu d'un any d'edat que patia leucèmia aguda. En tots dos casos, es pot induir la maduració de les cèl·lules usant ésters de forbol, com el PMA, des d'un estat pro-monocitic a un estat monocític. Aquest procés de desenvolupament està acompanyat per l'adquisició de diferents canvis morfològics i funcionals atribuïts normalment a cèl·lules madures com els macròfags.

El cultiu *in vitro* de les cèl·lules U937 i THP-1 va confirmar que el tractament amb PMA produïa canvis morfològics similar en les dues línies cel·lulars. Així doncs, mentre les cèl·lules no diferenciades creixien en suspensió, les cèl·lules tractades amb PMA s'adherien al substrat plàstic de manera similar a com ho fan els macròfags.

Tot i les similituds entre les dues línies cel·lulars monocítiques, també es varen observar certes diferències entre elles, sobretot pel que fa a la capacitat de secretar citocines en resposta a l'estimulació amb LPS. Es va veure que cèl·lules U937 no tractades amb PMA no eren capaces de secretar TNFα en resposta a l'estímul amb LPS, mentre que les cèl·lules una vegada diferenciades si que podien secretar la citocina en resposta al mateix estímul. Per contra, les cèl·lules THP-1 podien secretar la citocina TNFα en resposta a LPS en absència d'un procés previ de diferenciació per PMA. Conjuntament, aquests resultats indicaven la divergència que hi havia, entre les línies cel·lulars U937 i THP-1, en termes de estat de diferenciació basal; alhora que posaven de manifest la importància de peites variacions en l'estat d'activació d'una cèl·lula, en quan a la seva capacitat de resposta davant d'un estimul concret.

Relacionat amb les observacions anteriors, l'estimulació del receptor CD300f induïa la secreció de TNFα en monòcits primaris, aïllats a partir de PBMCs usant la seva capacitat per adherir-se al plàstic. Cal remarcar que actualment s'ha acceptat que la interacció dels monòcits als substrats plàstics té efectes en el seu estat basal d'activació, convertint-los en

monòcits preactivats. Això suporta la hipòtesis que els processos de diferenciació o activació que pateix una cèl·lula mieloide podrien modificar la funció del receptor CD300f.

Centrant-nos en la dualitat del CD300f endogen, en la línia THP-1 degut a la presència o absència d'un procés de diferenciació mediat per PMA, es va proposar que el canvi de funció del CD300f podria estar relacionat amb altres fenòmens independents al receptor en si mateix. En concordança amb aquesta idea, es va demostrar que el tractament amb PMA potenciava fortament l'expressió de les molècules adaptadores FcRγ i DAP12 endògenes, indicant que aquestes molècules podien ser responsables de la funció activadora del receptor. En concordança amb aquesta troballa, l'ús d'inhibidors químics contra molècules habitualment presents en les vies de senyalització iniciades per la FcRγ i el DAP12, com ara la quinasa Syk o la PKCδ, tenien efectes inhibitoris en la producció de TNFα mediada pel receptor CD300f.

A favor d'aquesta possible via d'activació del CD300f mediada per les molècules adaptadores FcRγ i DAP12, es va demostrar la seva associació a través d'assajos moleculars per immunoprecipitació del receptor i co-immunoprecipitació dels dos adaptadors, en cèl·lules transfectades. Tot i així, hi havia certes qüestions pendents de justificar o raonar per poder acceptar aquest resultat com a vàlid.

La primera qüestió a resoldre, feia referència al fet que el mecanisme d'interacció entre les molècules adaptadores FcRγ i DAP12 amb el receptor CD300f no seguia el model clàssic d'interacció; ja que el receptor no presenta cap residu transmembrana carregat positivament capaç d'interaccionar amb el residu transmembrana negatiu de les molècules adaptadores. Així doncs, igual que succeïa amb el receptor CD300d, el CD300f formaría part d'aquest nou grup de receptors anomenants *non-immunoreceptors* que interaccionen amb les molècules adaptadores de formes alternatives a la clàssica. En concordància amb aquest raonament, a través de diferents aproximacions moleculars es va desmostrar la importància de la regió transmembrana del CD300f i del residu carregat negativament de la FcRγ per mantenir la interacció entre les dues molècules. A més sembla ser que la fosforilació del CD300f o de la FcRγ són innecessaris per tal que es doni la interacció *in vitro*, però no es pot descartar que fos necessari per a la funció activadora *in vivo* del receptor.

Una altra qüestió important a clarificar, és el fet que al inhibir l'expressió de les molècules adaptadores DAP12 o FcRγ, mitjançant *shRNA,* no es va observar cap efecte sobre la capacitat del CD300f per senyalitzar com a activador. Inicialment, una possible explicació per aquest fet seria que els dos adaptadors tinguessin funcions complementaries, i per això la falta d'un podia ser suplert per l'altre. Apart d'això, posteriorment es va demostrar que tot i aconseguir cèl·lules amb molt baixos nivells de DAP12 o FcRγ, una vegada aquestes cèl·lules es tractaven amb PMA l'efecte del shRNA queda totalment anul·lat i s'observava una recuperació dels nivells d'expressió dels dos adaptadors molt superior als nivells basals. Fet que explicava clarament perquè no es veia un efecte inhibitori sobre la capacitat activadora del receptor tot i tractar-lo amb shRNA contra les molècules adaptadores.

L'últim punt important per resoldre era demostrar la interacció del receptor CD300f endogen amb les molècules adaptadores endògenes. Mentre que la interacció era fàcilment detectable en cèl·lules COS-7 transfectades, en les línies monocítiques THP-1 i U937 no es va poder observar la interacció de les molècules endògenes. Una de les possibles explicacions recau en el fet que els receptors endògens poden estar formant complexes entre ells, fent que els anticossos que són capaços de reconèixer el monòmer CD300f no puguin detectar el receptor quan aquest està inclòs en un complex, així doncs al fer la immunoprecipitació amb aquests anticossos no s'aconseguiria immunoprecipitar el receptor endogen i per tant no s'observa la co-immunoprecipitació de les molècules adaptadores.

- Referent a les formes soluble del receptor CD300f, el desenvolupament i optimització d'un assaig ELISA va permetre la detecció d'aquestes molècules en el sèrum humà amb un limit de detecció mínim de 100 ng/ml.

Sorprenentment, pacients amb el síndrome antifosfolipídic (SAP), mostraven alts nivells de la forma soluble en relació amb els individus control. El terme SAP va ser usat per primera vegada a principis de 1980 per descriure una forma única d'autoanticòssos que induïen trombofília, amb trombosis recurrent i complicacions en l'embaràs, sobretot afectant a adults joves. El mecanisme a través del qual els anticossos antifosfolipídics causen el síndrome és bastant desconegut, però segurament implica la inhibició del procés normal de coagulació, l'activació de plaquetes i cèl·lules endotelials, bloqueig del sistema del fibrinogen, i l'estimulació de la via de senyalització del complement per part dels monòcits. A més, es sap que els anticossos antifosfolipídics d'individus amb el síndrome SAP tenen afinitat per fosfolípids carregats negativament com ara la cardiolipina, o el seu complex amb la proteïna plasmàtica β2-glicoproteïna-I. Apart els individus amb SAP també presenten autoanticóssos contra la fosfatililetanolamina (PE). La PE és un dels components majoritaris de la membrana de molts microbis i també es molt abundant en la membrana dels mitocondris. La PE conjuntament amb la fosfatidilserina (PS) es localitzen en la part interior de la membrana citoplasmàtica de les cèl·lules eucariotes vives, però aquests fosfolípids passen a la part externa de la membrana quan les cèl·lules pateixen processos d'apoptosis, sent un dels mecanismes que permeten a les cèl·lules fagocítiques identificar les cèl·lules mortes. A més, la PS s'exposa a la superfície de cèl·lules endotelials danyades i això provoca l'activació de les plaquetes durant la coagulació sanguínia, en aquest context les membranes que exposen PS es converteixen en superfícies catalítiques que permeten l'anclatge del complex de la protrombinasa. Per altra banda s'ha demostrat que tan els monòcits com les plaquetes, quan s'activen generen quatre lípids anàlegs de la PE, així es podria proposar la hipòtesis que l'activació incontrolada de la resposta immune podria provocar un increment en la producció de fosfolípids a través dels monòcits i les plaquestes, fet que conseqüentment desregularia la cascada de coagulació donant lloc a fenòmens de trombosis. En aquest context, el receptor CLM-1 va demostrar la seva capacitat per promoure la fagocitosis de cèl·lules apoptòtiques en un procés que involucrava el reconeixement de la PS. La interacció entre el receptor CD300f amb la PS o la

PE no ha estat estudiada en profunditat, tot i així seria possible que formes solubles del receptor poguessin actuar com anticossos contra aquests fosfolípids, donant lloc al desenvolupament del síndrome SAP. Per tal de demostrar aquesta hipòtesis caldria fer més assajos moleculars i experiments *in vivo*.

Per altra banda, degut al paper neuroprotector del receptor CLM-1 en ratolins en els que s'havia induït un model murí de esclerosis múltiple (MS), es va hipotetitzar la participació de formes solubles del CD300f en la malaltia humana de MS. La MS és una malaltia desmielinitzant autoimmune crònica, que afecta al sistema nerviós central i es dóna sobretot en adults joves, provocant discapacitat significativa a mesura que passa el temps. Es creu que les cèl·lules inflamatòries que juguen un paper important en aquesta malaltia són sobretot els limfòcits T CD4+ i CD8+, la micròglia i els macròfags. A més la immunitat humoral també ha estat descrita com a un component important de la patologia. Tot i així, del total de mostres de sèrum de pacients amb MS analitzades, només una mostrava nivells alts del sCD300f. Cal destacar que la majoria de mostres MS analitzades eren de pacients del subtipus remitent recurrent (RRMS), apart de dos tipus de casos del subtipus primari progressiu (PPMS). Estudis immunològics, fets en sang i fluid cerebroespinal han demostrat diferències entre els casos PPMS i altres fenotips de la malaltia MS, compatibles amb diferents estats d'activació del sistema immune. Així doncs podria ser que la importància de formes solubles del CD300f estiguessin relacionades amb el subtipus PPMS i no amb el RRMS. Conseqüentment, per descartar la implicació de sCD300f en la malaltia MS seria necessari analitzar un rang de mostres de diferents subtipus de MS. En paral·lel, seria essencial analitzar els nivells de sCD300f en el fluid cerebroespinal d'individus MS.

Pacients amb una altra malaltia autoimmune, anomenada malatia celíaca (CD), mostraven quantitats significantives del receptor soluble en comparació amb donants sans o amb comparació amb mostres d'altres pacients amb diabetis o la malaltia autoimmune de les tiroides, la malaltia de Graves o vasculitis sistèmica. La CD és una malaltia hereditària crònica que afecta a l'intestí prim i que deriva d'una complexe combinació entre factors genètics i ambientals, on tant la resposta inflamatòria innata com la adaptativa estan involucrades en el desenvolupament de la malaltia, produint una intolerància permanent al gluten. És interessant destacar que en alguns casos clínics s'ha observat associació entre individus amb el síndrome SAP i la malaltia CD. Així doncs s'ha vist que alguns malalts amb CD tenen síntomes típics del síndrome SAP, com pot ser trombosis en la vena portal o hiperplàsia regenerativa nodular del fetge, degut a la presència d'anticossos contra la cardiolipina. D'altra banda, alguns pacients diagnosticats amb SAP posseeixen marcadors serològics de la malaltia CD, com la presència d'anticossos contra endomesi, característics d'individus amb CD, en pacients amb necrosis cutània típica d'individus amb SAP.

Finalment, tot i que la majoria de mostres de pacients diagnosticats amb artritis reumatoide (RA) i lupus sistèmic eritematós (SLE) varen resultar negatives per el sCD300f, individus puntuals presentaven quantitats significatives de la forma soluble del receptor.

La SLE és una malaltia autoimmune crònica en la qual es dóna un desordre en el teixit conectiu que acaba afectant a un gran nombre d'òrgans i normalment presenta un curs clínic remitent recurrent. El SLE presenta moltes anormalitats a diferents nivell del sistema immunitari. Els factors patològics presents en el SLE inclouen factors ambientals, genètics i modificacions epigenètiques, incloent la hipometilació del DNA. S'han descrit molts autoanticossos relacionats amb la malaltia, com ara anticossos contra la cadena doble de DNA, contra els antígens nuclears Ro, La o Sm, així com anticossos contra el nucleosoma, contra el receptor NMDA, contra fosfolípids i contra la α-actina, entre d'altres.

Els autoanticossos també juguen un paper molt important en el desenvolupament de la malaltia RA, que és una autoimmunitat crònica debilitant que dóna lloc a una inflamació sistèmica i una destrucció estructural de les articulacions. Una característica típica de la RA és un desequilibri en el sistema immune que porta a una descordinació entre els osteoclast responsables de formar l'os i els responsables de reabsorbir-lo, provocant una reabsorció desmesurada de l'os. A mesura que la malaltia avança, el desequilibri comporta una destrucció irreversible de l'os i el cartílag donant lloc a les discapacitats motores. En aquest punt, cal destacar que s'han observat associacions clíniques entre les malalties de RA, SLE, CD i SAP. Així doncs, algunes de les malalties tenen manifestacions molt similars, com ara un elevat risc d'arteriosclerosis cardiovascular en el cas de les RA i SLE; mentre que altres malalties s'ha proposat que són diferents manifestacions clíniques d'una única malaltia, com en el cas del SAP i el SLE.

Els mecanismes moleculars que uneixen els diferents síndromes i malalties dels que hem parlat no estan massa clars. Tot i així, la hipòtesis principal apunta que autoanticossos similars són els responsables de diferents efectes perjudicials sobre la biologia dels individus. Per exemple, s'ha demostrar que els autoanticossos contra el nucleosoma o la cromatina, característics d'individus amb SLE, també es poden trobar en individus amb SAP, els quals finalment poden acabar desenvolupant ambdues malalties. Conseqüentment, la identificació de nous marcadors serològics capaços de discriminar entre algunes d'aquestes malalties autoimmunes facilitaria un diagnostic ràpid i un millor tractament; en aquest context seria interessant determinar el potencial de les formes solubles del sCD300f com a biomarcador per a malalties autoimmunes.

Una qüestió pendent de comentar és la referent a l'origen de les formes solubles del receptor CD300f. Ja ha estat comentat amb anterioritat la possibilitat de que, dues formes d'splicing alternatiu del receptor CD300f podrien donar lloc a formes solubles. Malauradament, les seqüències per aquestes variants no es van trobar en cap base de dades de EST (de l'anglès: *expressed sequences tag*). Tot i així, podria ser que l'expressió d'aquestes variants d'splicing es donés exclusivament en situacions concretes, com és el cas de processos de inflamació crònics.

A més, una altra hipòtesis apunta a l'existència d'un fenomen de proteòlisi (o conegut com al terme anglès: *ectodomain shedding*) per explicar l'existència de les formes solubles del CD300f. Aquest procés consisteix en la proteòlisis de molècules expressades a la membrana

cel·lular, que permeten l'alliberació dels dominis extracel·lulars d'aquestes molècules. Les conseqüències directes d'aquesta modificació posttraduccional són dues: l'adaptació del fenotip cel·lular, reduint la quantitat de proteïnes expressades a la membrana exterior; i l'alliberació de molècules mediadores solubles capaces d'actuar en altres tipus cel·lulars com a lligands.

Apart de determinar si l'origen molecular de les formes solubles del CD300f, també seria important definir les cèl·lules capaces de segregar aquestes formes solubles. Així mateix, es proposa que els monòcits i els granulòcits podrien ser la font principal d'aquestes formes solubles, ja que ambdós tipus cel·lulars expressen el receptor CD300f a la seva membrana cel·lular en grans quantitats. Tot i així, també es podrien considerar les plaquetes com a possible font de les formes solubles, ja que s'observa un augment en la quantitat de sCD300f en malalties en les quals es dona una des-regulació dels processos de coagulació, com ara el SAP o el SLE; a més, la proteòlisis de molècules de membrana és un mecanisme que es dona habitualment en la membrana de plaquetes, per tal de regulat la funció dels receptors presents en aquest tipus cel·lular. De totes maneres, mai s'ha publicat l'expressió del CD300f en plaquetes.

CONCLUSIONS

- El gen del receptor CD300d ocupa 12,5 kb en la posició 17p25.1 del cromosoma 17 humà. El gen del CD300d te un marc obert de lectura de 585 pb i codifica per una proteïna de 194 aminoàcids.
- El CD300d és una proteïna transmembrana tipus I, composada per un domini extracel·lular immunoglobulina estabilitzat per dos ponts disulfur, un domini transmembrana que conté un residu carregat negativament i una cua citoplasmàtica curta sense motius de senyalització coneguts.
- El CD300d té un pes molecular predit de 21.5 kDa, tot i així presenta dos formes madures de 30 i 34 kDa, quan s'aïlla de cèl·lules COS-7 transfectades. Les dues conformacions són modificades post-traduccionalment per N-glicosilasions en el domini immunoglobulina.
- El receptor CD300d s'expressa en monòcits primaris i granulòcits. Contràriament, els limfòcits T, B o NK no expressen el receptor.
- A diferència de la resta de receptors de la família CD300 descrits prèviament, els quals s'expressen a la superfície de la membrana cel·lular, el CD300d és retingut intracel·lularment en el reticle endoplasmàtic de cèl·lules transfectades. La co-expressió del CD300d amb la molècula adaptadora FcRγ afavoreix l'expressió del receptor a nivell superficial en cèl·lules COS-7 transfectades, però no en cèl·lules RBL-2H3 transfectades.

- El CD300d és capaç d'interaccionar *in vitro* amb la resta de receptors CD300 coneguts, amb l'excepció del CD300c. Així, es proposa que el receptor CD300d podria jugar un paper important regulant la formació dels complexes CD300 i la seva exportació a la membrana extracel·lular.

- Els dominis immunoglobulina dels receptors CD300d i CD300f presenten una identitat del 71%. Tot i així, els anticossos monoclonals UPD1 i UPD2 reconeixen exclusivament el receptor CD300f.

- L'estimulació del receptor endogen CD300f indueix la producció de la citocina pro-inflamatòria TNFα en monòcits primaris, donant suport així a la dualitat en l'activitat del receptor, inicialment descrit com a inhibidor.

- L'estimulació del receptor CD300f endogen, en cèl·lules de les línies monocítiques U937 o THP-1 pretractades amb PMA, indueix la producció de les citocines pro-inflamatòries TNFα i IL-1β.

- La funció activadora del CD300f podria estar mediada per les molècules adaptadores FcRγ i DAP12, ja que s'ha provat *in vitro* la interacció entre el receptor i aquests adaptadors. La interacció involucra les regions transmembranes tant del receptor com dels adaptadors, sent de vital importància el residu transmembrana carregat negativament dels adaptadors.

- El pretractament amb PMA incrementa l'expressió de les molècules endògenes FcRγ i DAP12, en les línies cel·lulars U937 i THP-1. A més s'ha demostrat la participació de la quinasa Syk, la JNK i la PKCδ en la via senyalitzadora mediada per el CD300f. Fets que conjuntament donen suport a l'associació funcional entre el receptor CD300f i les molècules adaptadors FcRγ i DAP12.

- Per contra s'ha vist que l'estimulació del CD300f inhibeix l'alliberació de TNFα induïda per LPS en cèl·lules THP-1 en les quals no s'ha realitzat un pretractament amb PMA. Aquest fet, indica que el canvi de funció del CD300f, d'inhibidor a activador, és conseqüència de l'estat de diferenciació de les cèl·lules.

- Per altra banda, s'ha demostrat l'existència de formes solubles del receptor CD300f (sCD300f) en mostres de sèrum humà, mitjançant el desenvolupament d'un assaig ELISA.

- La concentració de les formes solubles del receptor CD300f varia depenent de l'origen de la mostra analitzada. Cal destacar, que s'han observat alts nivells de sCD300f en la majora de pacients del síndrome antifosfolipídic (SAP) i malalties celíaques (CD). En alguns individus amb altres malalties autoimmunes els nivells de sCD300f en sang també són alts, tot i així no és el cas de la majoria de les mostres analitzades.

- Les formes solubles del CD300f tenen un gran potencial com a biomarcadors en malalties autoimmunes.

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