Universidade de Aveiro Departamento de Biologia
2015

SARAH RAQUEL MATOS ALMEIDA

S4D-SRCRB E SPα: PROPRIEDADES FUNCIONAIS E INTERAÇÃO COM RECETORES

UNCOVERING THE BINDING AND FUNCTIONAL PROPERTIES OF S4D-SRCRB AND SPα

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Liliana Sofia da Silva Martins de Oliveira, Investigadora Pós-doutoramento no Instituto de Investigação e Inovação em Saúde e pelo Doutor Mário Jorge Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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agradecimentos

À doutora Liliana Oliveira, por ter aceite o convite para orientadora desta dissertação de mestrado, por ter tornado este trabalho possível, acompanhando-me ao longo deste percurso, dando a orientação científica necessária, importante para o meu desenvolvimento tanto a nível pessoal como profissional.

Ao doutor Alexandre Carmo, por me ter recebido como aluna de mestrado no grupo de investigação Cell Activation and Gene Expression e pelas opiniões, sugestões e ensinamentos dados, essenciais para este trabalho e para a área de investigação em geral.

Ao doutor Mário Pereira, orientador desta dissertação, por toda a disponibilidade, atenção, orientação e conselhos prestados ao longo deste ano de trabalho.

A todos os elementos do grupo Cell Activation and Gene Expression, Rita Santos, Soraia Mendes e Patrícia Nogueira pela ajuda, partilha de opiniões e companhia fantástica.

Ao Joel Araújo pelo apoio, compreensão, entreajuda, motivação e otimismo essenciais ao longo deste percurso.

Aos meus pais por me apoiarem e ajudarem a concretizar os meus sonhos e conseguir ingressar no caminho que escolhi.

palavras-chave

Receptores *Scavenger* ricos em cisteína, S4D-SRCRB, Spα, Sistema imunitário, Recetores de reconhecimento de padrões, Inflamação

resumo

A superfamília de recetores *scavenger* ricos em cisteína é caracterizada por proteínas contendo domínios *scavenger* compostos por 100 aminoácidos com resíduos de cisteínas dispostos de uma forma bem conservada entre espécies. A superfamília encontra-se dividida em dois grupos, A e B, podendo as proteínas ser expressas em diversos tipos de células e serem secretadas ou estarem associadas à superfície celular mediando interações com outras proteínas e ligandos. Não há uma característica específica que unifique estas proteínas, exceto as propriedades estruturais dos domínios altamente conservados que apresentam. As proteínas pertencentes ao grupo SRCR B estão envolvidas em diversas funções como reconhecimento de patogénios, modulação da resposta imune, homeostasia do epitélio e desenvolvimento de tumores entre outros. Spα e S4D-SRCRB pertencem a este grupo. A proteína Spα tem sido associada a condições inflamatórias, como doenças metabólicas (aterosclerose e obesidade), cancro do pulmão e fígado, sendo também considerado um recetor de reconhecimento de patogénios para diversas bactérias e fungos. Apesar de estruturalmente semelhante ao Spα, pouco se sabe acerca do padrão de expressão da proteína S4D-SRCRB. As características de ambas proteínas Spα e S4D-SRCRB são importantes para a compreensão do tipo de papéis que estas proteínas têm na homeostasia do sistema imunitário. Enquanto que o papel de S4D-SRCRB permanece desconhecido, a importância de Spα como recetor de reconhecimento a patogénios e o seu envolvimento na inflamação foram já observados. No entanto, inúmeros mecanismos associados a estas proteínas permanecem desconhecidos.

O objetivo deste trabalho foi, por conseguinte, estudar o papel destas proteínas na inflamação. Para tal, diferentes técnicas foram utilizadas para a caracterização da expressão destas duas proteínas, a nível tecidular e celular num estado de repouso. Após isto, foi analisado também a variação da expressão destas proteínas após estímulos inflamatórios e anti-inflamatórios dados a células de origem epitelial ou hematopoiética.

Neste trabalho são reportadas novas localizações destas duas proteínas e demonstrado que a sua expressão parece ser diferente em resposta a condições de inflamação/anti-inflamação, sugerindo que possam ter um papel em diferentes localizações celulares e uma função na modulação da inflamação tanto na imunidade inata como adaptativa. Pela purificação da proteína recombinante Spα foi possível identificar células que expressam ligando(s) para esta proteína, um passo importante para entender a sua função.

keywords

Scavenger receptor cysteine-rich, S4D-SRCRB, Spα, Immunity system, Pattern Recognition receptor, Inflammation

abstract

The scavenger receptor cysteine-rich (SRCR) superfamily is characterized by proteins containing scavenger domains composed of about 100 amino acids with cysteine residues placed in a highly conserved manner among species. It is divided into two groups, A and B. The proteins of this superfamily can be expressed in diverse types of cells and be secreted or cell surface-bound, mediating protein-protein interactions and binding to (a) ligand(s). There is no special feature that unifies these proteins, except for the structural properties of the highly conserved domains. SRCR B proteins are involved in diverse functions such as pathogen recognition, modulation of immune response, epithelial homeostasis and tumor development among others. Spα and S4D-SRCRB belong to this group. Spα is being associated to inflammatory conditions, like metabolic disorders (atherosclerosis and obesity), liver and lung cancer, being a pattern recognition receptor for diverse bacteria and fungi. Although structurally similar to Spα, very little is known about the expression pattern and localization of S4D-SRCRB. The characterization of both Spα and S4D-SRCRB proteins is important to understand what kind of roles they have in the homeostasis of the immune system. While the role(s) of S4D-SRCRB remain largely unknown, the importance of Spα as a pattern recognition receptor (PRR) and its involvement in inflammation was already observed. Nonetheless, numerous mechanisms associated with these proteins remain unexplored.

The goal of this work was therefore to study the role of these proteins in inflammation. For that, different detection techniques were used for the characterization of the expression of the two proteins, at the tissue and cellular levels in a resting state. Then the variation of these proteins expression depending on inflammatory and anti-inflammatory stimuli given to epithelial or hematopoietic-origin cells was also analyzed.

In this work new localizations of these two proteins are reported and their expression was shown to be different in response to inflammatory/anti-inflammatory conditions, suggesting that they may have a role in different cellular locations and a function in the inflammatory modulation of both innate and adaptive immunity. Through the purification of the recombinant Spα, it was possible to identify cells that express ligand(s) for this protein, an important step to understand its function.

Alguns dos resultados deste trabalho estão incluídos no poster apresentado no 4º Congresso Europeu de Imunologia:

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A role for Spα in the control of infection and inflammation

4th European Congress of Immunology, Vienna, Austria. September 2015.

Spα is a soluble scavenger receptor cysteine-rich (SRCR) protein expressed by macrophages from different tissues. A role of Spα as a putative pathogen-recognition receptor has been previously suggested, but additional pleiotropic functions such as viability support in lymphocytes during thymic selection, lipid metabolism or anti-inflammatory effects in autoimmune pathologies have also been ascribed to Spα. We have expressed recombinant Spα and determined that it displays increased binding to pathogenic bacterial strains and probably plays a part in host defense mechanisms against microbial invasion. To elucidate the role of Spα in an inflammatory context, we activated a human monocytic cell line using different agonists and found that stimuli associated with the triggering and resolution of inflammation induced significant changes in Spα protein levels. Furthermore, we identified several human cell lines of multiple types and origins that produce Spα, revealing that the action of this protein may be broader than anticipated, but in agreement with the high abundance of Spα in human serum. Given that the cellular targets and receptors for Spα remain unknown, we used Spα tetramers containing fluorescent streptavidin to perform a screening of binding of the protein to different cell lines, and multiple cells from epithelial origin seemed indeed to express a putative receptor (s) for Spα. Further characterization showed that the interaction is Ca^{2+} dependent, possibly inducing conformational changes in the Spα SRCR domains to stabilize the protein. Our evidence thus points to the participation of Spα in pathogen neutralization and in the control of inflammation.

INDEX OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

LIST OF ABBREVIATIONS

AIM – apoptosis inhibitor expressed in macrophages

ALCAM – activated leukocyte cell adhesion molecule

APC – antigen presenting cell

ATCC – American Type Culture Collection

a.u. – arbitrary unit

BBB – blood-brain barrier

BCR – B cell receptor

BHI – brain heart infusion

BSA – bovine serum albumin

Ca^{2+} – calcium

$CaCl_2$ – calcium chloride

CD – cluster of differentiation

cDNA – complementary deoxyribonucleic acid

CFU – colony forming units

CO_2 – carbon dioxide

DAB – tetra-hydrochloride 3-3'-diaminobenzidin

DAMP – damage-associated molecular pattern

DC – dendritic cell

DEX – dexamethasone

DMBT1 – deleted in malignant brain tumor 1

DMEM – Dulbecco's modified eagle medium

DMSO – dimethyl sulfoxide

dNTP – deoxyribonucleoside triphosphates

DTT – dithiothreitol

ELISA – enzyme-linked immunosorbent assay

FACS – fluorescence activated cell sorting

FAS – fatty acid synthase

FBS – fetal bovine serum

GM-CSF – granulocyte macrophage-colony stimulating factor

Hb-Hp – hemoglobin-haptoglobin complex

HBS – HEPES buffered saline

hSpα – human secreted protein alpha

IFN-γ – interferon gamma

Ig – immunoglobulin

IL – interleukin

ITAM – immune-receptor tyrosine-based activation motif

Kd – dissociation constant

kDa – kilodalton

LB – Luria-Bertani

Lck – leukocyte-specific tyrosine kinase

LDL – low density lipoprotein

LPS – lipopolysaccharide

LRR – leucine rich repeat

LTA – lipoteichoic acid

MAP kinases – mitogen activated protein kinase

M-CSF – macrophage-colony stimulating factor

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

MOI – multiplicity of infection

Mϕs – macrophage

mRNA – messenger ribonucleic acid

MS – multiple sclerosis

mSpα – mouse secreted protein alpha

NF-kB – nuclear factor kappa-light chain enhancer of B cells

NK cell – natural killer cell

NLR – NOD like receptor

NO – nitric oxide

NOD – nucleotide-binding oligomerization domain like receptors

OD – optical density

Opti-MEM – Opti-minimum essential media

oxLDL – oxidized low density lipoprotein

PAMP – pathogen-associated molecular pattern

PBL – peripheral blood lymphocyte

PBMC – peripheral blood mononuclear cell

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PEI – polyethylenimine

PFA – paraformaldehyde

PHA – phytohemagglutinin

PMA – phorbol 12-myristate 13-acetate

pMHC – peptide-major histocompatibility complex

PRR – pattern recognition receptor

PST – proline-serine-threonine

RCA – regulators of complement activation

RIG – retinoic acid-inducible gene I

RLR – RIG-like receptor

ROS – reactive oxygen species

rpm – revolutions per minute

RPMI – Roswell park memorial institute

rSpα – recombinant secreted protein alpha

RT – room temperature

S4D-SRCRB – scavenger four domains-scavenger receptor cysteine rich group B

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SN-Spα – supernatant of secreted protein alpha

Spα – secreted protein alpha

SR – scavenger receptor

SRCR – scavenger receptor cysteine rich

SSc5D – soluble scavenger with 5 domains

TBS-T – Tris-Buffered Saline with Tween

TCR – T cell receptor

TGF – Transforming growth factor

TIR – Toll/IL-1 receptor

TLR – toll-like receptor

TNF – Tumor necrosis factor

WB – western blot

ZAP-70 – zeta-chain-associated protein kinase 70

I. Introduction

1. The immune system

The multicellular organisms have developed a defense system against pathogens that is able to distinguish between self and nonself molecules with the purpose of eliminating foreign threats. For that, the immune system comprises an array of cells and molecules that work together. The different cells that serve as effectors in the immune system originate in the bone marrow from pluripotent precursors: the hematopoietic stem cells. Classically, it is assumed that these cells can assemble two types of defense: innate and adaptive immunity [1, 2]. Nowadays, this separation is ambiguous or somewhat inaccurate because instead of working separately, the two defense systems cooperate to enhance the immune response. For example, cells belonging to the adaptive branch require signals from the innate immunity to be properly activated [3]. In order to simplify the explanation of the immune system, I will refer the two systems separately in the following sections.

1.1. Innate Immunity – the first line of defense

Innate immunity is an ancient first line of defense that provides a rapid and broad response to invading pathogens, independent from previous contact with foreign molecules, which also lacks immunologic memory. It is composed of physical (e.g., epithelial barriers) and chemical defenses (e.g., secreted mucus, soluble proteins, enzymes, membrane-bound pattern recognition receptors – PRR – that bind conserved pathogen-associated molecular patterns – PAMPs – expressed on the surface of pathogens). One fundamental aspect of innate immunity is that its quality and intensity does not change upon repeated interactions with pathogens [3, 4].

Granulocytes, mast cells, dendritic cells, natural killer cells and monocytes are the key players in establishing the innate immune response.

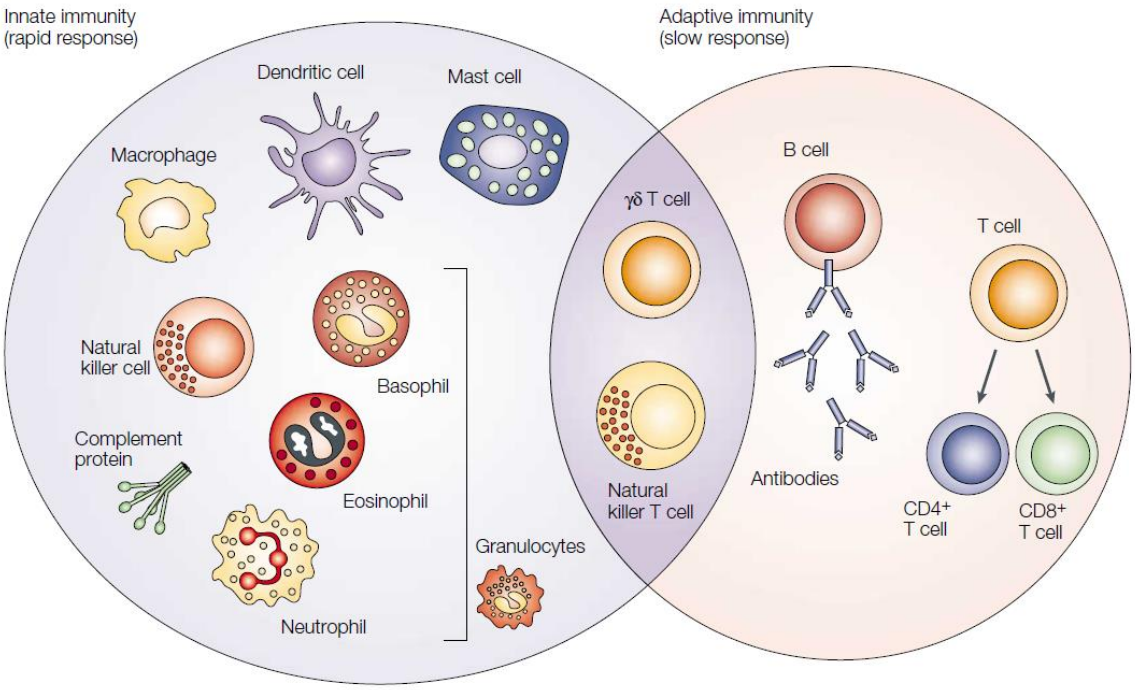


Figure 1: Cells from the immune system can be divided as belonging to innate or adaptive immunity. On the left are represented cells associated with the innate immune system: mast cells, dendritic cells, granulocytic cells, macrophages and natural killer cells. On the right are represented cells from the adaptive immune system, the T and B lymphocytes. In the middle of the scheme are γδ and natural killer T cells, cytotoxic lymphocytes found in the interface of these two defense systems. Adapted from Dranoff [1].

Granulocytes like neutrophils, eosinophils and basophils have a short life and are produced in a large scale. Neutrophils are the more abundant cell type and are responsible for the phagocytosis of microorganisms in an efficient way in early stages of inflammation. They produce reactive oxygen species – ROS – (cytotoxic for bacterial pathogens), neutrophil extracellular traps (capable of destroying extracellular bacteria) and can release granules in the extracellular environment to improve their response. Neutrophils can also produce enzymes for tissue repairing and remodeling after injury. They are cleared from the injured tissue by apoptosis. It is believed that eosinophils and basophils have a variety of enzymes and toxic proteins, acting against parasites that are hardly engulfed by neutrophils. Eosinophils can be found in large amounts in mucosal areas and are important in the triggering of allergic reactions and asthma. They are also very important in the combat of parasitic infection by antibody-dependent cell-mediated cytotoxicity. These cells can produce various cytokines and

pro-inflammatory mediators. Basophils can be found in smaller numbers representing only 1% of the peripheral blood leukocytes. They can be recruited to inflammatory sites, responding to immunoglobulin (Ig) E-antigen complexes and contribute to immediate hypersensitivity reactions. Mastocytes have a role in allergic and inflammatory responses, protecting internal surfaces of the organism against pathogens. Mast cells can be found in mucous membranes, under epithelial skin, blood vessels, having an important role in acute inflammation [5].

Monocytes may be found circulating in blood, bone marrow and spleen. They have an irregular shape, with a high cytoplasm-to-nucleus ratio. The circulating monocytes in the bloodstream migrate to tissues where they differentiate into macrophages – Mϕs. This differentiation is dependent on stimuli, which according to its nature can lead to a classical (M1 – a more pro-inflammatory phenotype) or an alternative activation (M2 – anti-inflammatory phenotype) [6-8]. The polarization into one of these two types of macrophages differs in the *in vivo* and *in vitro* contexts. M1 Mϕs are differentiated *in vitro* upon bacterial infection or presence of IFN-γ, TNF-α, GM-CSF. This type of Mϕs can produce toxic molecules (reactive oxygen species – ROS and nitric oxide – NO), inflammatory cytokines (TNF-α, interleukin (IL)-1, IL-6, IL-12, IL-23) and mediate resistance against intracellular parasites and tumors. M2 macrophages differentiate in response to IL-4, IL-10, IL-13, TGF-β, M-CSF and glucocorticoids. This phenotype is more frequently associated with allergies, parasite clearance, tissue remodeling, angiogenesis and tumor promotion. However, these two phenotypes are not static and M2 can change between the heal and growth promoting stage (its metabolism pass from arginine to ornithine and polyamines, promoting cell proliferation and repair) to M1, a more killing and inhibitory stage (where the metabolism pass to NO and citrulline, providing a microbicidal activity and cell proliferation inhibitory capacity) [9]. *In vivo* the two macrophage phenotypes are not well defined and often macrophages present a mixed phenotype, which reflects the complexity and the continuous changes in tissue microenvironment [9].

Natural killer cells mediate the defense against cells infected by viruses (causing cell lysis), bacteria, protozoa and tumor cells. They are characterized by a wide response inducing the recruitment of macrophages and neutrophils, activation of dendritic cells and B and T lymphocytes [5].

Dendritic cells (DCs) are located in peripheral tissues like the skin, intestine and liver, where they get into contact with pathogens, or capture substances through phagocytosis. DCs are regulated by a myriad of cytokines derived from macrophages or natural killer cells. Also,

they have a detrimental role in the activation of certain T lymphocytes like γδT and NK T cells through presentation of lipid antigens. Besides the interaction with T cells, DCs can also coordinate B lymphocyte responses. Both Mϕs and dendritic cells make the connection between the innate and adaptive immunity by presenting antigens to B and T cells in the lymph nodes, being considered antigen presenting cells – APC. In the other hand, cells from adaptive immunity enhance the activation of the innate immunity resulting in an increased efficacy in eliminating foreign molecules/particles [5, 10].

Apart from the cells that serve as effectors to the immune system, cytokines are also an important group of soluble proteins released by immune cells and constitute another layer of complexity in this system. These proteins mediate the interactions between cells and allow the communication among them. Cytokines act like a hormone having an autocrine, paracrine or endocrine action. Their action may be synergic or antagonist and trigger signaling cascades, where one cytokine can induce the production of others cytokines [11].

Cytokines can be divided into the following subgroups:

i) Pro-inflammatory cytokines – they are produced by activated macrophages, fibroblasts, endothelial cells, monocytes, dendritic cells and lymphocytes [12]. Their action involves up-regulation of inflammatory reactions. In an acute inflammation context, pro-inflammatory cytokines like IL-1 β and TNF-α play an important role, being considered potent inflammatory molecules that mediate this type of reaction induced by LPS (lipopolysaccharide), for example.

ii) Anti-inflammatory cytokines – immunoregulatory molecules that control pro-inflammatory cytokine production and secretion. IL-10 is produced by a variety of cells like macrophages, $CD4^+$ T cells, activated $CD8^+$ T cells and activated B cells, being considered an important anti-inflammatory stimulus. It is capable of inhibiting IFN-γ production by T cells, interrupt the synthesis of pro-inflammatory cytokines by monocytes and macrophages, among other functions [13].

iii) Chemokines – chemotactic cytokines that promotes activation and migration of leukocytes. Examples of this class of molecules are monocyte chemoattractant protein, IL-8 and lymphotactin [11].

1.2. Adaptive Immunity – The specialized line of defense

By contrast with innate immunity, the adaptive immune system represents a delayed but specific response, involving APCs that migrate to secondary lymphoid tissues where they will come into contact with B and T lymphocytes, activating these cells and originating a clonal expansion and memory. Naive lymphocytes are selected by its antigen specificity to differentiate into activated effector cells. The antigen specificity is provided by antigen specific receptors upon recombination of their genes, creating a myriad of cells whose receptors are capable of recognizing virtually any antigen [10].

The T cell receptor (TCR) is the antigen specific receptor expressed in T lymphocytes. The TCR only recognizes peptides, produced by proteolytic cleavage of antigens, when bound to the major histocompatibility complex (MHC) expressed in other cells. There are two types of MHC: class I and II. MHC class I presents molecules that are expressed in most cells of the organism and since they can collect peptides derived from cytosolic proteins, this allow the presentation of viral protein fragments at the surface. MHC class II molecules are only expressed by APCs and present peptides derived from endocytosed proteins that are subsequently degraded by acid-dependent proteases in endosomes.

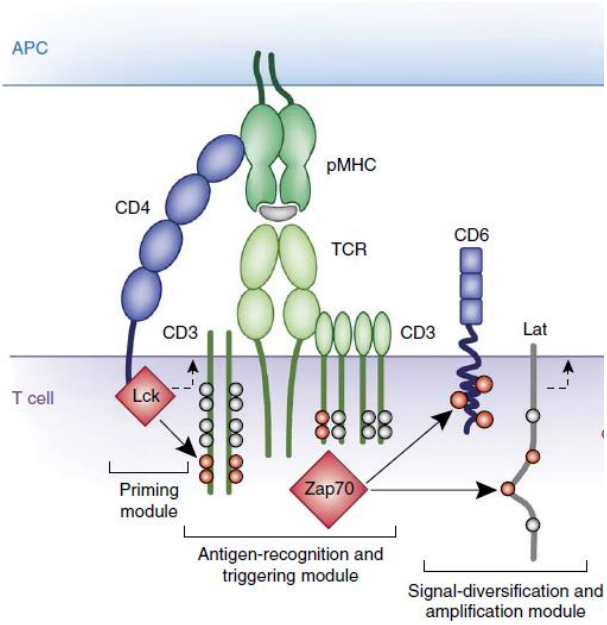


Figure 2: Schematic representation of the initial signaling events triggered by the interaction of the TCR with the peptide-MHC complex (pMHC). Several molecules are recruited inducing a signaling cascade, which ultimately leads to clonal proliferation and differentiation of lymphocytes. Adapted from Malissen *et al* [15].

T lymphocytes can be distinguished according to the expression of the co-receptors CD4 and CD8. $CD8^+$ T cells (also called cytotoxic T cells) are restricted to peptide presentation through MHC-I and are responsible for killing cells infected with intracellular pathogens. By contrast, $CD4^+$ T cells (T helper cells) recognize antigenic peptides bound to MHC-II and regulate cellular and humoral immune responses [14].

The TCR is associated with invariant accessory chains, the CD3 complex, as shown in Fig. 2 [15]. When the TCR contacts with MHC it may induce modifications in the CD3

complex, triggering phosphorylation and association with downstream proteins. CD3 chains have immune-receptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domain that associate with the Src-family tyrosine kinase Fyn. For the activation to occur, the co-receptors CD4 and CD8 bind to MHC molecules. These co-receptors have bound, in the cytoplasmic domain, another Src kinase leukocyte-specific tyrosine kinase – Lck – that phosphorylates the CD3 ITAMs. Another molecule named CD45 is important within this mechanism. CD45 contains a tyrosine phosphatase activity in the intracellular region that activates Lck and Fyn by removing the inhibitory phosphate groups from the kinases, thereby enabling their function in phosphorylating the CD3 ITAMs. These phosphorylations allow ZAP-70 (zeta-chain-associated protein kinase 70) docking, triggering a downstream cascade that will lead to the transcription of genes that control lymphocyte proliferation and differentiation [16].

However, for optimal T-cell stimulation, a second signal is required because when the TCR binds to an antigen-MHC complex, only a partial signal for cell activation is provided. To induce proliferation and other effector functions, a costimulatory molecule, like CD28, is required, that will bind to CD80 or CD86 expressed in the APC [3]. This kind of stimulation enhances TCR signaling in a quantitative way because CD28 binding will result in the recruitment of almost the same proteins involved in the signaling cascade recruited by the activated TCR complex as explained above [16].

B cells have immunoglobulin (Ig) as B cell receptors (BCR) that recognize antigens (native proteins and glycoproteins) and pathogens. The immunoglobulin is membrane bound, but upon activation, B cells start to secrete Igs (also known as antibodies). Igs are composed by two identical heavy chains and other two identical light chains, as represented in Fig. 3 [17]. There are five different types of heavy chains and each type originates one class of Ig: IgA, IgG, IgD, IgE and IgM. These chains have an amino-terminal portion that varies in the amino acid sequence from one antibody molecule to another, named variable region. Light and heavy chains loci are

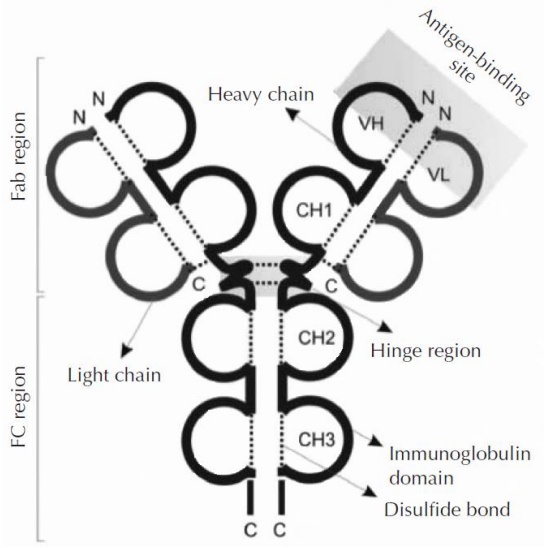


Figure 3: Schematic representation of an immunoglobulin. Adapted from Mesquita Junior *et al* [17].

composed by variable gene elements (V), diversity segments (D) for the heavy chain gene only, joining segments (J) and constant region (C) exons. The somatic combination of V-D-J creates the amino-terminal portion of each heavy chain. On the other hand, the combination of V-C genes creates the amino-terminal portion of the light chain. The variable region contains 3 subregions that are highly variable between different antibodies. When VDJ combine, a hypervariable region is formed which is important to the antigen-binding domain assembly.

B cells are activated after binding of the BCR to an antigen that can be whole microorganism, proteins or polysaccharides, like LPS. After the antigen recognition, the B lymphocyte will proliferate and differentiate into plasma cells ultimately leading to the secretion of Igs [3, 10].

1.3. Inflammation and receptors from innate immunity

In this non-specific response, both innate and adaptive immunity interact in order to fight pathogens and tissue malfunction by loss of homeostasis (injury, trauma or exposure to foreign particles), but with a particular difference: it can damage the self-tissue. Sometimes, this type of damage is deregulated leading to autoimmune diseases like multiple sclerosis, rheumatoid arthritis, type 2 diabetes, among others.

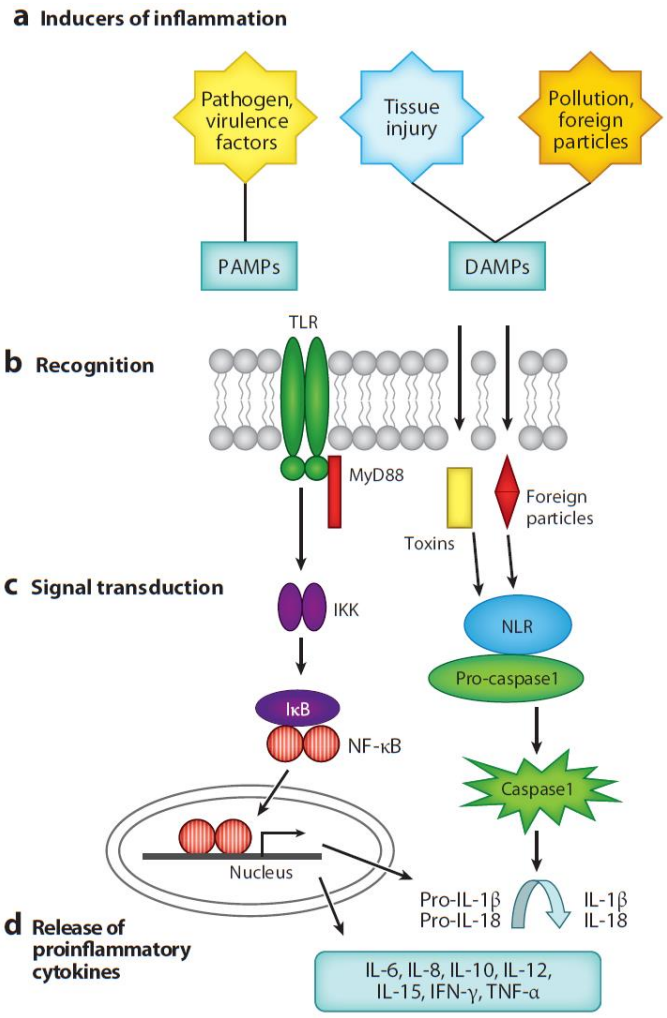


Figure 4: Illustration of the first part of the inflammatory cascade. Some inducers (PAMPs or DAMPs) are necessary to initiate this cascade. The recognition is made through TLR or NLR. The pathway via TLR induces the separation of IkB (an inhibitory protein) from NF-kB, leading to the production of interleukins. NLR activate caspase 1, which converts cytokines into an active form. Adapted from Ashley *et al* [18].

Once tissue dysfunction occurs, a highly conserved first response takes place, characterized by the recruitment of several repairing components, such as leukocytes, platelets or plasma proteins, increased fluid mediated by local vasodilation and an overall increase in blood flow [18].

The innate immune system relies on a limited number of invariable pathogen recognition receptors (PRR). These receptors recognize pattern-containing molecules expressed at the surface of the pathogen's membrane, like LPS (expressed in Gram-negative bacteria), LTA (expressed in Gram-positive bacteria) and others. Upon ligand binding with the PRR, a cascade of events is triggered, resulting in the production of mediators (pro-inflammatory, anti-inflammatory) that lead to eradication of the threat and the restauration of homeostasis. Thus, the inflammatory process begins with an inducer, which can be a PAMP or DAMP (damage-associated molecular pattern) and proceeds to their recognition by innate immunity through its receptors, as illustrated in Fig. 4 [19].

1.3.1. Toll-like receptors

Toll-like receptors (TLR) are a family of transmembrane glycoproteins characterized by an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail that contains a conserved region called Toll/IL-1 receptor (TIR) domain. They initiate the expression of diverse host defense genes leading to the transcription of inflammatory cytokines and antimicrobial peptides. Nowadays, 13 different TLRs have been described, according to their ligand specificities and localization [20]. Some are expressed in the plasma membrane and recognize bacterial components, like TLR4 that together with CD14 binds LPS, or TLR2 that by forming heterodimers with TLR1/6 recognizes a vast number of ligands (peptidoglycans, LPS, a certain component of the yeast cell wall, etc.) [21]. Other receptors are localized in the endolysosomal membrane (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13) where they can be activated by microbial nucleic acids. After the recognition made by these receptors, the pathway triggered leads to the activation of the transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) providing a rapid release of inflammatory mediators [22].

1.3.2. NOD-like receptors

The nucleotide-binding oligomerization domain-like receptors (NOD like receptors-NLR) constitute another family of PRRs. They are cytoplasmic receptors that can recognize PAMPs of internalized microbial components and DAMPs, frequently associated with cell stress, alerting the immune system to cell injury. The characteristic feature of NLRs is a central NOD (or NACHT) domain required for oligomerization, an N-terminal homotypic protein-protein interaction domain and a C-terminal series of LRRs involved in agonist sensing or ligand binding. Sensing of such ligands by NLRs leads to their oligomerization into large macromolecular scaffolds inducing i) the rapid activation of NF-kB or MAP kinases culminating in the production of inflammatory cytokines, or ii) the activation of inflammasomes (supramolecular complexes for activation of inflammatory caspases by cleavage). NOD 1 and NOD 2 are examples of NLRs, involved in the recognition of peptidoglycans, diverse microbial pathogens, pathogenic protozoal parasites and may direct autophagy to eliminate intracellular bacterial pathogens [23].

After the signal transduction initiated by the ligation of these PRRs to their ligands, there is an increase in the expression of pro-inflammatory cytokines like IL-1, IL-6, TNF-α, among others, as illustrated in Fig. 4. The cytokines, together with chemokines, allow the recruitment of effector cells like neutrophils and monocytes to the injured site.

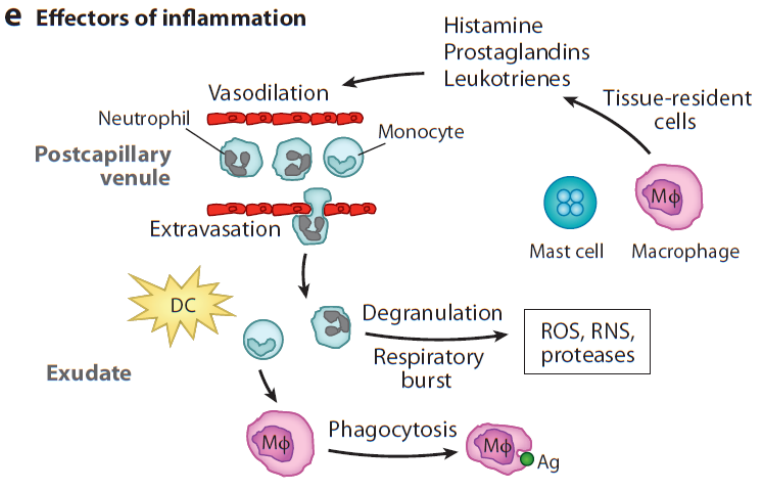


Figure 5: Effectors of inflammation. Neutrophils and monocytes migrate to damaged tissue, followed by protein rich fluid (exudate) that promotes swelling. Mast cells and tissue-resident macrophages promotes this migration through the release of histamine, prostaglandins and leukotrienes causing vasodilatation and an increase in vascular permeability. Adapted from Ashley *et al* [18].

At this point, the typical symptoms of local inflammation appear: heat, swelling, redness, pain and loss of function (Fig. 5).

If the response is successfully accomplished, the result is the elimination of the infectious agent. The resolution phase is characterized by the transition from pro-inflammatory prostaglandins to anti-inflammatory lipoxins that will inhibit the recruitment of

neutrophils and bring monocytes to the hound, for dead cells removal, and promotion of tissue remodeling [18].

1.3.3. RIG-I-like receptors

Retinoic acid-inducible gene I (RIG) like receptors (RLRs) are cytoplasmic RNA helicases expressed in almost all cells and that detect viral RNA in the cytoplasm of infected cells, resulting in inflammation. However, besides RNA, they can act against viral DNA and bacterial pathogens. The signaling pathway triggered leads to the transcription and induction of interferon-mediated antiviral responses, recruiting immune cells like macrophages and dendritic cells. Ultimately, the triggering of these receptors will originate apoptosis in infected cells, up-regulation of MHC class I and enhanced adaptive immune responses [24].

1.3.4. C-type lectins

C-type lectins are soluble or transmembrane receptors, capable of recognizing a wide range of molecules like carbohydrates, lipids and proteins. The carbohydrate recognition domain, with conserved motifs, enables carbohydrate specificity, allowing the recognition of viruses, bacteria or fungi. The pathway is complex and requires interaction with other PRRs to induce the phagocytosis of pathogens by dendritic cells and subsequent antigen presentation. Dectin-1 belongs to this family and is a type 2 transmembrane protein expressed by DC, monocytes, Mϕs and neutrophils. They act as antifungal defenses and may recognize *Listeria* and *Mycobacterium* [25].

1.3.5. Scavenger receptors

Scavenger receptors (SR) are a large family of structurally heterogeneous, transmembrane and cell-surface glycoproteins. They may contain in their structure collagen, C-type lectins, or scavenger receptor cysteine-rich (SRCR) domains, among others. SR can recognize different ligands from non-self to altered self-targets promoting the removal thereof. This is accomplished by endocytosis, phagocytosis, adhesion and signaling mechanisms. They were first described as receptors with the ability to uptake low density

lipoproteins (LDL) in macrophages, then several proteins have been classified as SR and new properties were discovered, for example as being involved in phagocytosis, antigen presentation, and clearance of apoptotic cells [26]. The nomenclature applied to scavengers receptors was type I and II macrophage scavenger receptors. As new receptors were discovered, the nomenclature changed to class A scavenger receptors I and II. Currently this large family is divided in ten classes (SR A-J) [27]. These receptors have an important role in innate immunity and the association of SR to pathogens and modified lipids implicates these proteins in inflammation and metabolic disorders.

As previously mentioned, the inflammatory process may lead to chronic inflammatory or autoimmune diseases when mediators that initiate and maintain inflammation and those promoting the resolution of inflammation are not well coordinated. An example of such imbalance is atherosclerosis, a disease where some SR play an important role, that results from the accumulation of lipids and fibrous elements in large arteries. Lipoproteins detained in the subendothelial space may go through modifications inducing an activation of the immune response. Monocytes are then recruited to the subendothelial space, differentiate into macrophages enabling the phagocytosis of the modified lipoproteins mediated by the SR CD36, together with Spα (further detailed in the section 3) promoting the transformation into foam cells. These cells secrete substances that are involved in plaque growth, leading to disease progression [28]. Multiple sclerosis (MS) is another inflammatory disease where a SR is involved. This chronic autoimmune disease results from a deregulation of inflammation in the myelinated axons of the central nervous system, ultimately leading to neurodegeneration. Recently it was found that a single nucleotide polymorphism in CD6, a member of the SR family, is associated with a lower risk for multiple sclerosis. CD6 has a ligand named CD166/ALCAM (leukocyte cell adhesion molecule) located in the epithelial layer of the blood-brain barrier (BBB) and when both molecules interact, allows the passage of $CD4^+$ T cell through the BBB. Therefore, a reduction in the ALCAM-CD6 interaction that can occur through increased skipping of the ALCAM-binding domain in CD6 may reduce the transmigration of T lymphocytes through the BBB protecting against MS [29].

Besides inflammatory diseases, SR may have a role in macrophage polarization too, as is the case of CD163, CD36 and SR-A1 that have their expression increased in M2 macrophages. SR-A1 and CD36 contribute to M2 macrophages clearance of apoptotic bodies and CD163 promotes an anti-inflammatory response [26].

2. Scavenger receptors cysteine-rich superfamily

The scavenger receptors cysteine-rich (SRCR) superfamily is characterized by proteins containing scavenger domains composed of about 100 amino acids having the cysteine residue positions highly conserved among species, already found in echinoderms, sponges, mammals, fish, avian and amphibian. The proteins of this family can be expressed in diverse types of cell and can be secreted or be cell surface bound mediating protein-protein interactions and ligand binding. SRCR proteins are involved in diverse functions such as pathogen recognition, modulation of immune responses, epithelial homeostasis and tumor development, among others. There are two groups in this superfamily, group A and group B. These two groups differ from one another in the number of exons that encode the scavenger domains and in the number of cysteines present in each domain. The members of SRCR A have their SRCR domains encoded by split exons and possess eight cysteine residues. On the other hand, SRCR B domains are encoded by a single exon and have six cysteine residues [30].

Different proteins from SRCR B have been described as having diverse functions; however there is no feature that unifies them, except for the structural properties of the highly conserved domains. Some of these proteins seem to be related to the innate and the adaptive immune systems. It was reported that members of this family are capable of detecting PAMPs, thus acting like PRRs [31]. For instance, deleted in malignant brain tumor 1 (DMBT1) is a glycoprotein that has 14 SRCR domains and plays a role in the interaction between tumor cells and the immune system. DMBT1 can be secreted or associated with the plasma membrane of macrophages, but can also be found in other types of cells, predominantly in epithelial-like tissue. It was reported that DMBT1 levels are higher on fetus than in the adult and the location of this protein varies according to the developmental stage. This protein also plays a role in virus and bacteria elimination, since it can bind, agglutinate and neutralize them, protecting the epithelial and mucosal tissues [32].

CD5 and CD6 are found at the surface of T lymphocytes and are important for the modulation of activation of these cells. Both molecules are membrane glycoproteins and have 3 extracellular SRCR domains, a transmembrane region and cytoplasmic tail for intracellular signal transduction. They have the ability to bind to conserved PAMPs from bacteria and fungi. CD6 can aggregate bacteria in a way to prevent its transversion through the epithelial barrier [4]. Transmembrane glycoprotein CD163 with 9 SRCR domains is exclusively expressed on monocytes and macrophages and act as an innate sensor against bacteria. Its

expression is upregulated by anti-inflammatory inducers (glucocorticoids, IL-10) and downregulated by pro-inflammatory agents (LPS, TNF-α). It also has a role in iron metabolism since this protein can bind to hemoglobin-haptoglobin (Hb-Hp) complexes leading to hemoglobin removal from plasma. CD163 has a soluble form circulating in the plasma resulting from the shedding of the ectodomain [33]. SSc5D is composed of five SRCR domains and is another member of this family for which very little is known. The SSc5D mRNA is abundant in monocytes, macrophages, T lineage cells and placenta, but not in B lymphocytes. These proteins and others not listed are found in human cells and are schematized in Fig. 6.

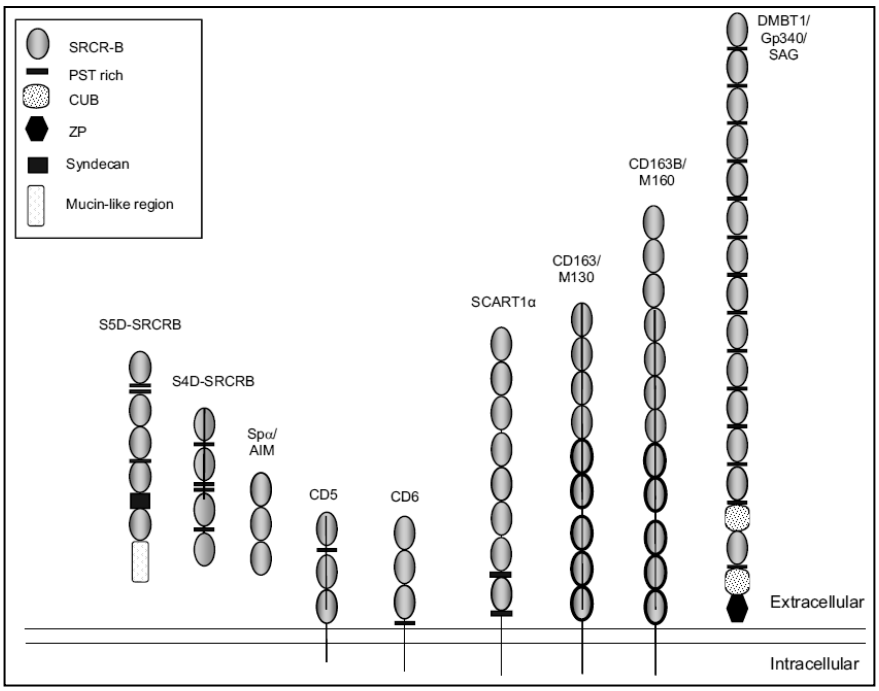


Figure 6: Representation of human SRCR B members. Their structure is composed of single or tandem repeats of SRCR domains and some are mosaic or multidomain proteins, providing variability.

The structures are:
CUB-C1r/C1s Uegf Bmp1 domain;
PST- proline-, serine-, and threonine-rich sequence;
ZP - zona pellucida domain.
Figure adapted from Martínez *et al* [4].

Both Spα (secreted protein alpha) and S4D-SRCRB (soluble protein with 4 domains SRCR) belong to SRCR B group [34] and are the subject of this work being more detailed in the next sections.

3. The Spα protein

Secreted protein alpha (also known as Api6 or CD5L) was first described in 1997 [35]. This human soluble glycoprotein (38 kDa) is not membrane bound and has a secretory signal sequence followed by three SRCR domains. The authors reported that Spα transcripts were restricted to lymphoid tissue (spleen, lymph nodes, thymus, bone marrow) but it could bind to resting myeloid cells and peripheral blood monocytes, suggesting that this protein may play a role in the regulation of the immune system. By contrast, the protein is not found in peripheral blood lymphocytes (PBL), appendix, prostate, testis, uterus, small intestine and colon [35]. Later it was found that it is an abundant circulating serum protein that is associated with other serum components like IgM [36].

Multiple roles for Spα were described and its role in inflammation is somehow confusing by the different reports showing that depending on the model studied, this protein can behave as a pro- or anti-inflammatory molecule (fig.7) [37].

Spα was described as a PRR because it could bind to LPS and LTA and also to a variety of Gram-positive and Gram-negative bacteria in a dose-dependent way [38].

It is a novel serum biomarker for liver fibrosis in hepatitis C [39] and for atopic dermatitis, since its presence in serum increases in these patients [40]. Furthermore, the increased expression of Spα may lead to lung inflammation and it can control epithelial cancerous transformation and myeloid cell proliferation by inhibiting apoptosis and activating oncogenic pathways in a lung adenocarcinoma context [41]. Recently it was demonstrated that Spα activates autophagy in Mϕs through the modulation of CD36 expression, thereby downregulating their inflammatory status by preventing the secretion of TNF and IL-1β (derived from the TLR pathway) and increasing the production of IL-10 [42].

AIM (apoptosis inhibitor expressed by macrophages) is the homologous protein of Spα in mice, sharing 70% similarity. This homologue is only expressed by tissue macrophages and appears to increase resistance to apoptosis suggesting an important role in inflammatory and infection sites [43, 44]. AIM was broadly studied in the last decade, being a model to the human Spα protein. In combination with TGF-β, this protein inhibits B lymphocyte proliferation induced by LPS stimulation [45].

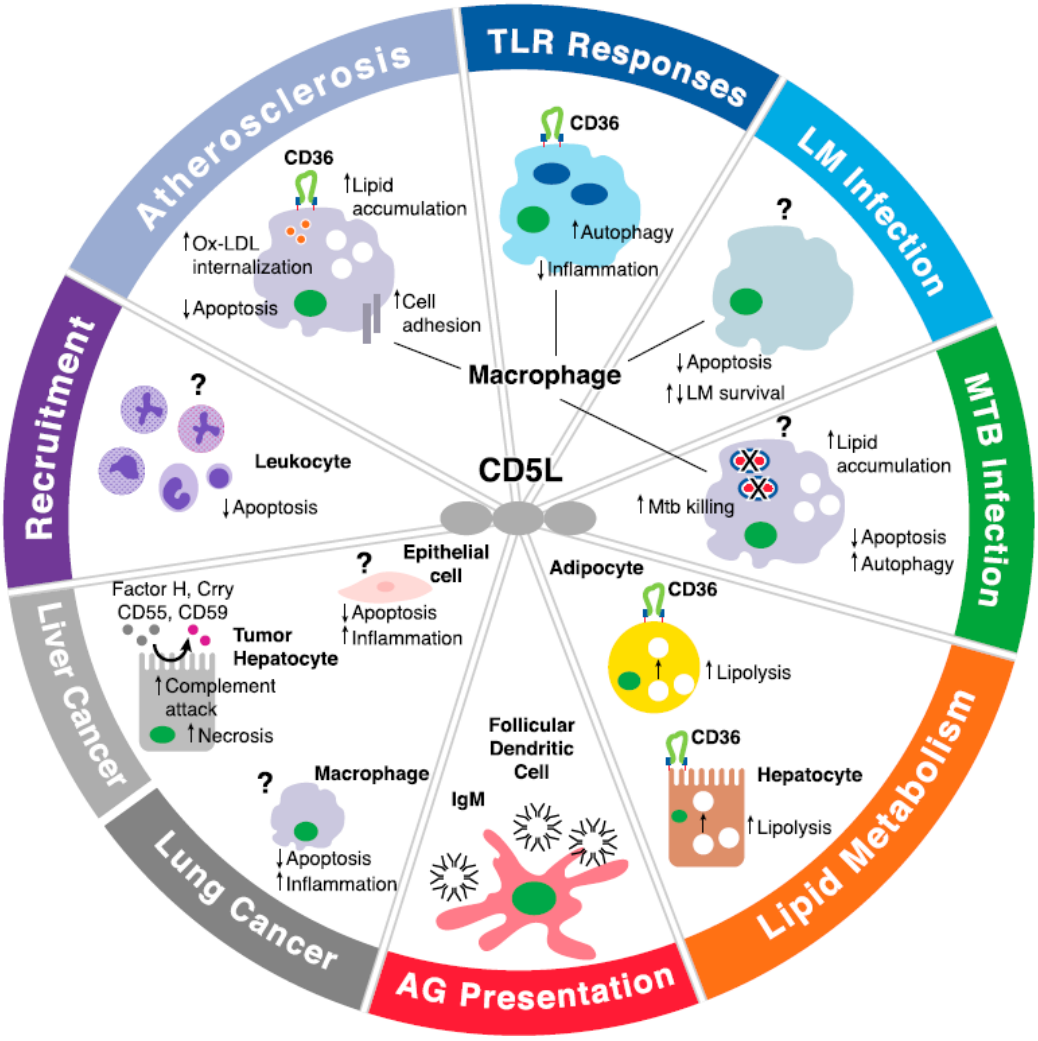


Figure 7: Different features of Spα in inflammation.
This protein may influence macrophage response in atherosclerosis, survival against bacteria and TLR response. Besides that, it promotes recruitment of leukocytes, Ag presentation and in adipocytes contributes for lipid uptake. In epithelial cells is associated to cancer in liver and lung. Adapted from Sanjurju *et al* [37].

AIM is being related to metabolic disorders such as atherosclerosis and obesity. In an atherosclerosis condition, monocytes migrate into the subendothelial space of blood vessel and differentiate into Mϕs. In hyperlipidemic conditions, Mϕs incorporate oxLDL via CD36. oxLDL causes cytotoxic effects that leads to apoptosis. AIM present in Mϕs promotes its survival against the apoptotic signals and Mϕs with oxLDL increase the expression of AIM. Ultimately, these effects cause the characteristic atherosclerotic lesions. Knockout mice for

AIM showed that these lesions are markedly decreased when compared with control $AIM^{+/+}$ mice, thereby demonstrating the role of AIM in this pathology [37, 46].

Another role for AIM in metabolic-associated disorders is given by a distinct report showing that soluble AIM is endocytosed into adipocytes via CD36 and within these cells, AIM associates with cytosolic fatty acid synthase (FAS), thereby decreasing FAS activity. This decreases lipid droplet size and prevents adipocyte maturation, suggesting that AIM specifically influences adipocyte status. Thus, this AIM function in adipocytes may be physiologically relevant to obesity progression [47].

The anti-apoptotic protein is also involved in the immune response against infection. Indeed, it was demonstrated that AIM contributes to autophagy mechanisms and participates in the vitamin D antimicrobial pathway in the response of macrophages to *Mycobacterium tuberculosis*. Furthermore, the expression of AIM in THP-1 cells renders them resistant to apoptosis induced by *M. tuberculosis* infection [48]. This protein is also a PRR for PAMPs from bacteria and fungi and besides mediating this recognition, AIM may reduce pro-inflammatory cytokines and chemokines secretion, benefiting the tissue protection against responses elicited by PAMPs [49].

4. The S4D-SRCRB protein

Although structurally similar to Spα, very little is known about the expression pattern and localization of S4D-SRCRB. This protein is composed of four SRCR domains with short proline-serine-threonine (PST) rich polypeptides between them. Its mRNA expression was analyzed in human tissues by Northern blotting and a wide distribution of S4D-SRCRB was observed in various tissues and cell lines of different origins: kidney, placenta, peripheral blood lymphocytes, small intestine, liver, spleen, colon, skeletal muscle, heart, brain, fetal liver and bone marrow. S4D-SRCRB mRNA has two major forms, one with 1.5 kb with a large distribution in different cell lines (HeLa, A549, HEp2, HEK 293T) and another with 2.8 kb with a more restricted distribution, being present only in HepG2 cell line. In order to correlate the S4D-SRCRB expression with the inflammatory context of cells, the expression of this protein was analyzed upon stimulation with the pro-inflammatory cytokines IL-1 and IL-6 or dexamethasone. However no change in the S4D-SRCRB mRNA levels was observed [50].

5. Objectives

The characterization of both Spα and S4D-SRCRB proteins is important to understand theirs contribution in immune responses. While the role(s) of S4D-SRCRB remain largely unknown, the importance of Spα as a PRR and its involvement in inflammation was already observed. Nonetheless, numerous mechanisms associated with these proteins remain unexplored.

With this work it is intended to unveil the functions of these proteins. In order to achieve this goal, the first step was to analyze the expression of these proteins in different human cell lines and tissues by mRNA, immunohistochemistry and western blotting. Cell lines and some primary cells were the first approach due to their availability and ease of use. To understand the dynamics of protein production and secretion depending on the inflammatory context, different stimuli and kinetics were employed to analyze changes in the tested protein levels.

The second part of the work is to identify possible ligands for Spα and S4D-SRCRB. For this purpose the recombinant proteins were produced and purified and binding assays to different human cell lines were then performed.

Following this path, hopefully the expectation is to find a clue about the function of these proteins and their role as pro- or anti-inflammatory molecules.

II. Material and Methods

1. Cell line culture

Epithelial cell lines used in this work were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) from Gibco, 1% penicillin and streptomycin (Gibco) corresponding to complete media, at 37 ºC and 5% CO_2. Hematopoietic-origin cell lines were grown in complete Roswell Park Memorial Institute (RPMI) media from Gibco also with the same components as described for DMEM. Each cell line was split when approaching approximately 90% confluence.

All cell lines were acquired from the American Type Culture Collection (ATCC).

2. SDS-PAGE and western blotting

Cell lysates were prepared by lysing 3 x 10^6 cells with lysis buffer (Triton X-100) for 45 minutes (min) on ice. Samples were centrifuged at 13,000 x g for 10 min at 4 ºC and the protein content from the supernatant was quantified by Bradford Assay (BioRad). 60 μg of each lysate was mixed to 6x Laemmli Sample buffer, denatured for 5 min at 95 ºC and loaded on a 10% SDS-polyacrylamide gel (SDS-PAGE) and separated for 45 min at 200 V. Samples were transferred to a nitrocellulose membrane (iBlot). The membrane was blocked with a solution of 5% non-fat milk in Tris-Buffered Saline and 1% Tween (TBS-T) for 1 hour at room temperature (RT), then incubated with the primary antibody in a solution of 3% non-fat milk in TBS-T overnight at 4 ºC. The membrane was washed 3x with TBS-T during 10 min and subjected to the HRP-conjugated secondary antibody in a solution of 3% non-fat milk in TBS-T during 1h at RT. ECL solution (GE Healthcare) was used to develop a chemiluminescent signal in an X-ray film (GE Healthcare) or using a digital acquisition devise (ChemiDoc, Bio-Rad). First and second antibodies used for protein detection were: mouse monoclonal anti-Spα IgG_{2a} (Santa Cruz Biotechnology), mouse monoclonal anti-HA (16B12, Abcam), mouse monoclonal anti human α-Tubulin (B-5-1-2, Sigma Aldrich) and goat anti mouse IgG-HRP (Sta Cruz Biotechnology).

3. Monocyte isolation and *in vitro* differentiation and activation of MΦs

Peripheral blood mononuclear cells (PBMCs) were obtained from human peripheral blood from healthy volunteers donors from Hospital de São João of Porto. The separation of

blood constituents was performed by density gradient centrifugation using Lympholyte®-H (Cedarlane). To eliminate erythrocytes, PBMCs were subjected to red blood cells lysis buffer and further washed with phosphate buffered saline (PBS, HyClone) to reduce platelet contamination. Cells were counted and seeded in a culture flask for monocytes to adhere for 40 min. The supernatant was removed and cells were tripsinized. A part of the monocytes were lysed as already described for western blotting (WB) and the other part was plated into a 6 well plate at a density of 0.5 x 10^6/mL in RPMI complete medium. To differentiate these cells into macrophages, GM-CSF (for a type 1 MΦs) was added to a final concentration of 20 ng/mL, or M-CSF (for a type 2 MΦs) at a final concentration of 50 ng/mL. Cells were incubated at 37 ºC, 5% CO_2 for 7 days (after 3-4 days of culture, new complete medium supplemented with GM-CSF or M-CSF was added to the culture). After 7 days, cells were used to extract total RNA.

4. Total RNA extraction and PCR

4.1. Total RNA extraction

Different cell lines and GM-CSF/M-CSF differentiated MΦs were incubated with TripleXtractor reagent (Grisp) at room temperature (RT) for 5 min to allow complete dissociation of nucleoprotein complexes. Isolation of total RNA was performed according to the manufacturer's instructions. Per 1 mL TripleXtractor was used 0.2 mL chloroform and the tubes were shacked vigorously for 15 seconds and incubated at RT for 3 min. After a centrifugation of 12,000 x g for 15 min the colorless part of the sample containing the RNA was transferred to a new RNase-free tube and 0.7 volumes of ethanol were added to a final concentration of 35%. Samples were transferred to a RNA mini spin column from the RNA Extraction kit (Grisp) and the manufacturer protocol was followed. Quantification was performed in a NanoDrop™ 1000 Spectrophotometer and 5 μg were collected to perform first-strand cDNA synthesis. RNA was stored at -80 ºC.

4.2. First-strand cDNA synthesis

Following manufacturer's procedures for first-strand cDNA synthesis, 5 μg of RNA were incubated with 1 μL of random primers, 1 μL of dNTPs (10 mM) and nuclease free

water to make up to 13 μL for 5 min at 65 ºC followed by incubation on ice for 1 min. A mixture of 4 μL 5x First-Strand buffer, 1 μL of DTT (0.1 M), 1 μL RNaseOUT™ Recombinant RNase Inhibitor and 1 μL of SuperScript™ III Reverse Transcription (200 units/μL) (Invitrogen) was added to the sample. Samples were incubated at 25 ºC for 5 min and then at 50 ºC for 60 min. Following reverse transcription, cDNA was stored at -20 ºC or used in a Polymerase Chain Reaction (PCR).

4.3. PCR amplification

For PCR amplification, a master mix containing 1.25 μL of forward primer and the same for reverse primer (table 1), 5 μL GoTaq® Flexi Buffer (Promega), 5 μL of $MgCl_2$, 1 μL DMSO, 1 μL of dNTPs, 0.15 μL GoTaq® DNA Polymerase (Promega) and 8.35 μL of nuclease free water for a final volume of 23 μL was prepared. To this mixture, 2 μL of cDNA was added. An optimized program for PCR was used starting with initial denaturation lasting 5 min at 95 ºC and followed by 35 cycles: 30 sec at 95 ºC (denaturation), 30 sec at 65 ºC (annealing) and 40 sec at 72 ºC (extension). The final extension took 5 min at 72 ºC. As control, a PCR reaction of the same samples with β-actin primers (0.5 μL) was also performed.

Samples obtained from PCR were analyzed by gel electrophoresis in a 1% agarose gel.

Table 1: Oligonucleotides and the respective sequences used in each PCR.

Oligonucleotide	Sequences
S4D-PCR-F	5' GGCGTCCACAATTGCTTTCA
S4D-R3	5' ACGGATCTGTCTGCCAAG
S4D-*MIu*I-F	5' TAGACGCGTATGCACAAGGAAGCAGAGA
S4D-c*Bam*HI-R	5'CTAGGATCCCGAGCGTAGTCTGGGACGTCGTATGGGTATGA AGGCTGGCACAGGACACT
M13-F	5' CCCAGTCACGACGTTGTAAAACG
M13-R	5' AGCGGATAACAATTTCACACAGGAA
SFFV-F	5' TGCTTCTCGCTTCTGTTCG
Citrine-R	5' GCTGAACTTGTGGCCGTTTA
actB-F	5' TGATGGTGGGCATGGGTCAGA
actB-R	5' AATGTCACGCACGATTTCCCG

5. Over expression of S4D-SRCRB protein in Caco-2 and Jurkat E6.1 cells

5.1. Cloning, transformation and plasmid preparation

Since S4D-SRCRB RNA is expressed in the HepG2 cell line, this was selected to perform a PCR reaction for cDNA amplification. The master mix contained 1.25 μL of forward primer and the same for reverse primer (table 1), 4 μL 5x Phusion Buffer (Thermo Scientific), 0.6 μL DMSO, 0.4 μL of 10 mM dNTPs, 0.2 μL Phusion DNA Polymerase (Thermo Scientific) and 16.3 μL of nuclease free water for a final volume of 24 μL was prepared. To this mixture, 1 μL of cDNA was added. An optimized program for PCR was used starting with initial denaturation lasting 30 sec at 98 ºC and followed by 35 cycles: 15 sec at 98 ºC (denaturation), 20 sec at 68 ºC (annealing) and 50 sec at 72 ºC (extension). The final extension took 10 min at 72 ºC.

The final product of PCR was analyzed in a 1% agarose gel and the band of interest was cut followed by DNA purification using a GRS PCR & Gel Band Purification Kit (Grisp). The purified gel band (5 μL) was incubated with 0.5 μL of TOPO® cloning vector and 0.5 μL of saline solution for 30 min RT. The mixture was transformed in TOP10 chemically competent *E. coli* by mixing 50 μL of cells with the sample. The preparation was incubated on ice for 30 min and heat shocked at 42 ºC for 60 sec and transferred to ice for 2 min. LB medium (500 μL) was added to the sample and incubated for 1 h at 37 ºC. Bacterial cells were then plated on LB agar containing 100 μg/μL of ampicillin and 50 μg/mL of XGal and grown at 37 ºC overnight.

5.2. Colony PCR

Using colony PCR analysis, 15 colonies were chosen for confirmation of *E. coli* transformation. A master mix mixture was made using 1 μL of each primer (M13R and M13F), 0.4 μL of dNTPs, 2 μL GoTaq® Flexi Buffer (Promega), 2 μL of $MgCl_2$, 0.4 μL DMSO, 0.06 μL GoTaq® DNA Polymerase (Promega) and 3.14 μL of nuclease free water for a final volume of 10 μL. An optimized program for PCR was used starting with initial denaturation lasting 5 min at 95 ºC and followed by 35 cycles: 30 sec at 95 ºC, 30 sec at 55 ºC

and 2 min at 72 ºC. The final extension took 5 min at 72 ºC. Final colony PCR products were analyzed in a 1% agarose gel.

Two positive colonies were inoculated in 5 mL of LB medium supplemented with 100 μg/mL of ampicillin and grown overnight at 37 ºC in an orbital incubator. Using GRS Plasmid Purification Kit from Grisp, mini-preps of these clones were made according to the manufacturer's procedure. Plasmid DNA was quantified using a NanoDrop™ 1000 Spectrophotometer and sent for sequencing.

After obtaining the results from sequencing, plasmid DNA from a positive colony was digested with *Mlu*I and *Bam*HI enzymes for 2 h at 37 ºC. The pHR-mCitrine vector was also digested with the same enzymes. A ligation protocol was performed for the S4D-SRCRB insert and pHR vector using T4 DNA Ligase (Fermentas) for 1 h at 22 ºC followed by transformation in TOP10 competent *E. coli* as described above. A new colony PCR was performed identically to the already described but with different primers: SFFV-F and mCitrine-R. Positive colonies were grown and plasmid was purified as describe above. The final sample was sent for sequencing.

5.3. Stable transfection

HEK 293T cells were counted and plated on 6 well plate at a concentration of 3 x 10^5 cell/mL in 2 mL of DMEM media and incubated overnight. Cells were transfected using 4.5 μL Lipofectamine® 2000 reagent (Invitrogen) and 100 μL of minimal essential medium (Opti-MEM, without supplements) with a mixture of 0.5 μg of three vectors needed for virus assembly (p8.91, pMD-G and pHR-citrine S4D-SRCRB). The mixture was incubated for 30 min at RT and added to HEK 293T cells. Cells were incubated for 48-72 h at 37 ºC to allow virus assembly and production into the supernatant. For stable over expression of S4D-SRCRB protein by lentiviral transduction, Caco-2 (epithelial like) and Jurkat E 6.1 (lymphoblast) cells were chosen. Caco-2 were counted (5 x 10^5 cells) and plated the day before transduction and Jurkat cells were plated in the same day of the transduction (1 x 10^6 cells). The supernatant from HEK 293T was filtered and added to Caco-2 and Jurkat cells followed by incubation at 37 ºC for 48 h.

5.4. FACS analysis for transduction efficiency monitoring

To access the lentiviral transduction efficiency, mCitrine fluorescence was used to measure the level of expression of S4D-SRCRB protein in the two cells by flow cytometry. 5 x 10^5 cells were washed with FACS buffer (PBS, 0.1% bovine serum albumin and 0.5% sodium azide) and filtered before flow cytometer analysis (FACSCalibur). Untransduced cells and cells transduced with pHR-mCitrine (empty) were used as controls. Cells were sorted (FACSAria) to obtain a homogenous population. Results were analyzed with FlowJo (version VX.0.7.).

6. Stimulation assays in THP-1 MΦs for Spα expression assessment

Monocytic THP-1 cells (1 x 10^6 cells/mL) were seeded in a 6-well plate with 2 mL of RPMI complete media and 10 ng/mL of PMA (phorbol 12-myristate 13-acetate), for differentiation of monocytes into macrophages, and incubated at 37 ºC and 5% CO_2 for 36 h, followed by media replacement to fresh media without PMA for 24 h. Cell were stimulated for 24 h with LPS (10 ng/mL and 1 μg/mL), IL-10 (10 ng/mL) and dexamethasone (DEX, 100 ng/mL) or left untreated. Cells were lysed and WB was performed to check for variation of Spα intracellular protein upon the different stimuli.

7. Activation of T cells

Jurkat E6.1 cells were subjected to different types of activation in a 6-well plate. Activation with αCD3/αCD28 required αCD3 antibody (OKT3 clone, ExBio) coating (5 μg/mL in 500 μL) and incubation overnight at 4 ºC the day before cell seeding. 1.5 x 10^6 cells were plated in 1.5 mL and αCD28 antibody (CD28.2, BioLegend®) was added in a final concentration of 2 μg/mL. Alternative stimulations were achieved using PMA+Ionomycin (10 ng/mL and 500 ng/mL, respectively) or PHA (phytohemagglutinin, 5 μg/mL), or left untreated. T cells were incubated for 6 h for Jurkat E6.1 S4D-SRCRB and 12/24/48 h for Spα analysis at 37 ºC with 5% CO_2.

8. Immunohistochemistry assay

The immunohistochemistry technique used was based on the basic mechanism of the binding between the antibody to an antigen by an indirect method. Histological tissue was deparaffinized and hydrated with a series of alcohols in decreasing concentration followed by antigenic recovery using sodium citrate buffer (10mM sodium citrate, 0.05% Tween 10, pH 6) for 30 min at 95-100 ºC. After cooling, the biologic material was submitted to a series of treatments according to the manufacturer's procedure (Thermo Scientific). First, inhibition of endogenous peroxidases was performed to avoid non-specific reaction from the chromogen agent (DAB – tetra-hydrochloride 3-3'-diaminobenzidin). A new incubation was done with Ultra V Block for 5 min at RT. For the assessment of S4D-SRCRB protein presence in tissues, slides were incubated with a monoclonal mouse anti-S4D-SRCRB antibody (Santa Cruz Biotechnology) (1:50 in PBS) for 1 h at RT. Sections were submitted to the Value Primary AB Enhancer then to Value HRP Polymer protected from light. For the development, a mixture of DAB Plus Chromogen to DAB Plus Substrate was applied to the slides for 5-15 min. After washing with water, counterstain with hematoxilin was performed. After washing, histologic sections were dehydrated with a series of alcohols in a crescent concentration and diaphanized with xilol. Slides were finally mounted using Vectashield (Vector). For each tissue, a negative control with an irrelevant antibody was also performed.

9. Immunofluorescence staining

Slides were placed in a 12-well plate for coating with fibronectin (Sigma-Aldrich) for 1 h followed by seeding of 1.5 x 10^5 cells/mL (Caco-2 and Jurkat E6.1 S4D-SRCRB). Cells were left to adhere overnight. For immunofluorescence staining, slides were fixed with PFA (4%) and blocked using PBS+0.5% BSA. Phalloidin-TexasRed (Molecular Probes, Life Tech) was used for F-actin staining and DAPI (Molecular Probes, Life Tech) for nucleus staining. Lamellae were mounted using Vectashield (Vector). Samples were observed in a fluorescence microscope (Axio imager Z1, Zeiss) for citrine visualization.

10. Infection of Caco-2 S4D-SRCRB cells

10.1. Bacterial preparation

Bacterial strains *E. coli* RS218 (a kind gift from Patrick Trieu-Cuot, Institut Pasteur, Paris) and *Listeria monocytogenes* EGD-e (kindly provided by Didier Cabanes, IBMC, Porto) were grown from −80 °C freezer stocks for each experiment and never passaged for longer than 48 h prior to infection experiments. *Escherichia coli* were grown on Luria-Bertani (LB)-agar plates while *Listeria monocytogenes* were grown in brain heart infusion (BHI)-agar plates and incubated at 37 °C. One colony of each bacterial strain was inoculated into liquid LB (BD, Fisher Scientific) for *E. coli* or BHI media (BD, Fisher Scientific) for *L. monocytogenes*. Cultures were grown on a shaking incubator at 37 °C overnight. The bacterial cultures were diluted into fresh bacterial medium and grown, as previously described, until the exponential phase (OD_{600} ≅ 0.3-0.4).

Bacteria were harvested by centrifugation at 4000 x g for 10 min at 4 ºC and washed three times in PBS. Samples were diluted in PBS to obtain an OD of 0.45 (corresponding to 2.5 x 10^8 colony forming units (CFU)/mL). Bacteria were then diluted in DMEM without FBS or antibiotics for the appropriate multiplicity of infection (MOI). Quantification of CFU was done by serial dilutions and plating bacteria in LB and BHI agar plates.

10.2. Infection assays

3 x 10^5 Caco-2 cells per well were seeded in a 12-well plate the day before infection. Cells were incubated with the bacterial suspensions for 1 h at 37 ºC. After this period, which was considered as 0 h, cells were extensively washed with PBS and treated cells were lysed as described above. The remaining cells were incubated with fresh DMEM without FBS containing 1% penicillin and streptomycin and 0.05% gentamycin (Gibco) for 2 h before lysis.

Cells were adhered to slides and prepared similarly for immunofluorescence staining but without performing cell lysis.

11. Protein production and purification

11.1. Histidine-Tagged recombinant protein purification

CHOK1 cells stably expressing recombinant Spα or S4D-SRCRB were cultured in complete DMEM supplemented with 2.5% FBS and 1% penicillin and streptomycin in 4-tray cell-factories for approximately 30 days at 37 ºC in 5% CO_2. Production of recombinant proteins was controlled once a week through WB of supernatant samples.

Supernatant was collected, 0.2 μm filtered and concentrated using a Vivaflow 200 with a 10 KDa cut-off (Sartorius) following the manufacturer instructions and dialyzed to binding buffer (20 mM sodium phosphate, 150 mM NaCl and 10 mM imidazole, pH 7.4). The concentrated supernatant was loaded into a HisTrap HP column (GE Healthcare) in a low-pressure liquid chromatography system. Washing was performed with buffers containing increasing concentrations of imidazole: 20 mM sodium phosphate, 150 mM NaCl and 10 /20/30/100/250/500 mM imidazole, pH 7.4. Using a BioRad BioLogic Fraction Collector, the components trapped in the column were separated and the proteins were purified. Fractions were analyzed with BioRad BioLogic LP data view software. Fractions of interest were selected for an SDS-PAGE and stained with Blue Safe (NzyTech). Selected samples were pooled, dialyzed against HEPES-buffered saline (HBS) (20 mM HEPES, 50 mM NaCl, pH 7.4) and stored at -80 ºC for posterior use.

11.2. Size exclusion chromatography purification

Recombinant Spα (rSpα) purified by affinity chromatography was subjected to a new type of purification for purity optimization using size exclusion chromatography in an AKTA Purifier 10 fast protein liquid chromatography high-pressure system (GE Healthcare). Samples were injected in a Superose 12 10/300 GL column (GE Healthcare) and flow of HBS was allowed to pass through the column for protein elution at pressure < 2.5 MPa using a fraction collector Frac 950 (GE Healthcare). Fractions were analyzed with the UNICORN software (GE Healthcare). Fractions of interest were selected for an SDS-PAGE and stained with Blue Safe (NzyTech). Selected samples were pooled, dialyzed against HEPES-buffered saline (HBS) (20 mM HEPES, 50 mM NaCl, pH 7.4) and stored at -80 ºC for posterior use.

11.3. Production of recombinant S4D-SRCRB protein by transient transfection

HEK 293T cells were seeded at 3.5 x 10^6 cells/mL in a petri dish with 8 mL of DMEM complete media. Transient transfection was made with PEI (polyethylenimine) using an optimized protocol. A mixture of PEI (1:10) and pEE14 S4D-SRCRB vector in opti-MEM was incubated at RT for 20 min and added to cells for 3 h at 37 ºC. Media was replaced with a fresh one and the culture maintained for approximately 2.5 weeks. After a week, protein expression of each petri dish was assessed by collecting a supernatant sample for WB.

All supernatants from the petri dishes were pooled and purified using a batch purification method based on nickel affinity following the manufacturer's instructions. Briefly, the supernatant was added to His-Selected® Nickel Affinity Gel (Sigma-Aldrich®) and gently mixed for 30 min in an orbital shaker (175 rpm) at 4 ºC. After centrifugation, the supernatant was removed and the gel washed 3 x with an appropriate buffer (50 mM sodium phosphate, pH 8, with 0.3 M sodium chloride). Using an elution buffer (50 mM sodium phosphate, pH 8, with 0.3 M sodium chloride and 250 mM imidazole) the purified protein was obtained. To confirm the purity and the protein yield, an SDS-PAGE was performed followed by Blue Safe coloration and WB.

12. Search for ligand binding to rSpα

12.1. Biotinylation of rSpα

An rSpα sample purified by size exclusion chromatography was subjected to buffer exchange to 10 mM Tris, pH 8, to a final concentration of 2 mg/mL. A Biotinylation Kit (Avidity) was employed for sample biotinylation (according to the manufacturer's procedures) and the mixture was incubated for 2 h at 30 ºC, then left overnight at 4 ºC in an orbital shaker. Biotinylation was confirmed using a pull-down assay with streptavidin beads (Sigma-Aldrich) and WB using streptavidin-HRP incubation. Protein sample was quantified by Bradford assay (Bio-Rad).

12.2. Tetramer preparation and binding assays

For tetramer assembly, a solution of molar excess of rSpα (200 μg/mL) was mixed with 50 μg/mL streptavidin-A647 (Molecular Probes) in HBS or streptavidin-A647 only, as negative control, and incubated in an orbital shaker for 1.5 h at 4 ºC. 2.5 x 10^5 cells were washed and incubated with 30 μL of tetramer mix or control for 45 min on ice. Cells were washed 3 x in FACS buffer and 4 μg/mL propidium iodide counterstaining was made for the exclusion of dead cells. For the assays in the presence of Ca^{2+}, 0.9 mM $CaCl_2$ was added to the HBS and to the FACS buffer during tetramer assembly and cell washes. Cells were then analyzed in a flow cytometer (FACS Calibur). Results were analyzed using FlowJo (version VX.0.7.).

III. Results and Discussion

1. Spα expression

Human Spα (hSpα) mRNA was found to be present in the bone marrow, spleen, lymph node, thymus and fetal liver but not in non-lymphoid tissues [35]. However, the majority of the characterization of this protein is being performed in the mouse context, where it was observed that the main cellular sources of mouse Spα (mSpα) are tissue macrophages [43]. However, it has been demonstrated that although similar, mSpα and hSpα have differences in their glycosylation patterns. Indeed, while hSpα has no N-glycosylation sites, mSpα has 3 sites and this difference may be relevant for the function of the protein since mutation in 2 N-glycosylation sites of mSpα affects its secretion while enhances its lypolytic activity in adipocytes [51].

In order to elucidate the expression pattern of hSpα at the protein level, a cell line screening using human cells from different origins was performed.

After analyzing the expression of hSpα, experiments with inflammatory and anti-inflammatory stimuli were made in a chosen cell line demonstrated to express this protein.

1.1. Cell line expression

hSpα is a very abundant protein in the serum (60 μg/mL) circulating in association with IgM [36, 52]. Through the use of an anti-human Spα monoclonal antibody, it is possible to easily detect this protein in human serum and plasma, in two different sample dilutions tested (1:10 and 1:50), as shown in Fig. 8. Analysis of the WB demonstrates the existence of two forms of hSpα having distinct molecular masses due to differences in sialic acid content, an observation previously reported by Sarrias *et al.* [36].

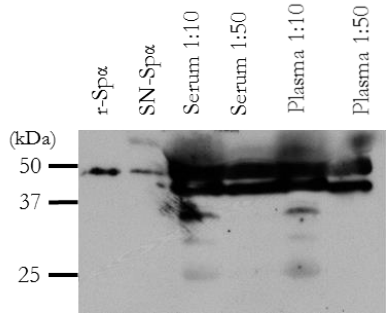


Figure 8: WB for the detection of Spα in human serum and plasma. Two different dilutions (1:10 and 1:50) of serum and plasma were subjected to WB. As control, the recombinant Spα protein (rSpα) and supernatant from cells over expressing it (SN-Spα) were used.

A WB of total lysates of different cell lines and primary cells was performed to assess potential tissues where hSpα could be expressed. All the cell lines used and their origin is detailed in Table 2. The results obtained indicated that Spα is expressed in epithelial cells (HeLa, HEK 293T, JEG-3, Caco-2, HCT 116, MDA-MB-231), lymphoblast (Jurkat E 6.1, K562), monocytic (THP-1) and in primary monocytes (Fig. 9). However, the protein levels were very low or undetected in HepG2 and TCCSUP cells.

Table 2: Origin of each cell line used in the WB analysis of Spα expression.
The characteristics of the cell lines like morphology and tissue origin are detailed.

Morphology	Cell line	Tissue origin
Epithelial	HeLa	Cervix
	HepG2	Liver
	HEK 293T	Human embryonic kidney
	Caco-2	Colon
	HCT116	
	JEG-3	Placenta
	TCCSUP	Unary bladder
	MDA-MB-231	Mammary gland
Lymphoblast	K562	Bone marrow
	Jurkat E 6.1	T lymphocyte
Monocytic	THP-1	Monocyte

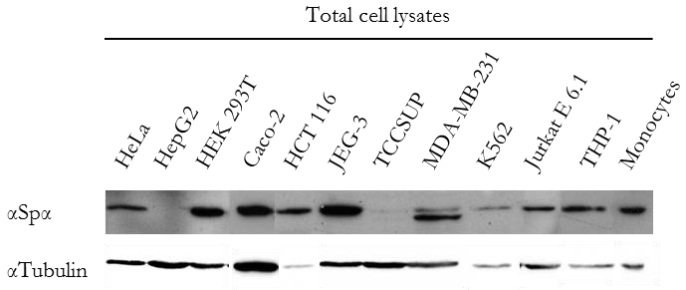


Figure 9: Spα expression in different total cell lysates. WB of lysates from each cell line detected with anti-Spα mouse monoclonal antibody. As loading control, detection of tubulin was also performed. The figure reflects one representative assay of multiple experiences made.

Although a previous report has showed that the mRNA for Spα was only present in lymphoid organs being undetectable in peripheral blood leukocytes and in several cell lines like HL60, Raji, Molt4, A549, SW480, GA361, K562 and HeLa [35], this may not be conclusive at the protein level. In fact, our observations show that the abundance of transcripts of several members of the SRCR superfamily does not relate with protein expression since the mRNA coding for these proteins is almost undetectable or is present at very low levels. This fact could explain the discrepancy between the reports that point to a unique expression of Spα in macrophages and our study showing that this protein is expressed in cells from different origins, particularly in epithelial cells. Our observations thus suggest that Spα may have a role in different cellular locations or even a different, previously unknown function according to the expression site.

1.2. Expression of Spα in response to inflammatory and anti-inflammatory stimuli

Since Spα expression is found in monocytes and these are key mediators of innate immune responses, the variation in the expression of this protein upon stimulation was performed in THP-1 monocytes. Although our initial screenings were performed using different stimuli (LPS, LTA, GM-CSF, M-CSF, IFN-γ, IL-4, IL-10, IL13, TGF-β, TNF-α and dexamethasone), here are presented the observations for three of them that gave more consistent results in the various experiments made.

THP-1 cells were firstly differentiated into Mϕs with phorbol myristate acetate (PMA, 10 ng/mL). This model is widely used since PMA can activate protein kinase C in monocytes, mimicking the physiological activator diacylglycerol resulting in a differentiated phenotype characterized by increased adherence, loss of proliferation, phagocytic activity, expression of CD11b and CD14, release of ROS and production of TNF-α upon LPS stimulation [53]. After differentiation, THP-1 cells were treated for 24 h with LPS (10 ng/mL), IL-10 (10 ng/mL), dexamethasone (DEX, 100 ng/mL) or were left untreated as control. After this period, total cell lysates were obtained for WB analysis of Spα expression (Fig. 10). The results obtained show that Spα expression is highly increased with DEX treatment while stimulation with LPS induced a more moderate effect. Treatment with IL-10, an anti-inflammatory cytokine, leads to a reduction of the intracellular amount of Spα. This reduction could be due to degradation of intracellular Spα or increased secretion. To elucidate this question, several attempts to detect Spα in the supernatant of treated cells were made. However, the protein

was never detected probably due to its low levels, a technical issue that despite our efforts, we could not solve yet.

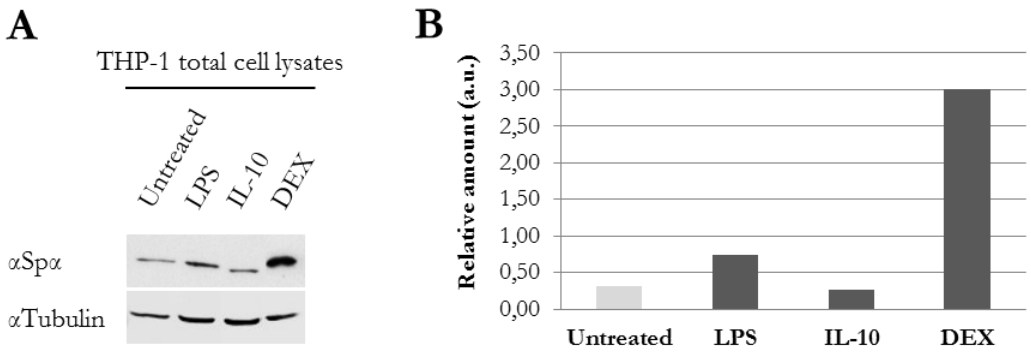


Figure 10: Spα expression in THP-1 total cell lysates upon stimulation. MΦs derived from THP-1 monocytes were treated with LPS (10 ng/mL), IL-10 (10 ng/mL), dexamethasone – DEX – (100 ng/mL) for 24 h or left untreated. A) WB of total cell lysates, representing Spα expression (top blot) and tubulin used as loading control (lower blot). B) Relative amount of Spα protein to tubulin upon different stimulation, calculated by densitometry analysis of band intensity. The figure reflects one representative assay of multiple experiences made.

We next tested whether a different concentration of LPS had any change in the dynamics of production and secretion of Spα. For that, differentiated THP-1 cells were treated for 24 h with LPS 10 ng/mL, 1 μg/mL, or left untreated as control. The results obtained showed that the stimulation with an increased LPS concentration led to a decrease of Spα intracellular amount comparing with the lower dose of 10 ng/mL (Fig. 11). This result can be explained by an increased secretion of Spα as a possible result of a more rapid production of this protein. In order to clarify this question, kinetic studies using different time points with different concentrations of LPS are being performed. A relationship between LPS and Spα is suggested by the fact that this protein is capable of binding the endotoxin as well as the component of Gram-positive bacteria, lipoteichoic acid (LTA) [38]. Furthermore, studies with mSpα show that its expression is up-regulated under inflammatory conditions of infectious origin, such as infection with *Listeria monocytogenes* [54], *Corynebacterium parvum* [55] or in an endotoxin-induced fulminant hepatitis [56]. Therefore, it is conceivable that once the presence

of LPS is detected by the cells, a positive feedback loop can induce the production and release of Spα as a defense mechanism designed to help the immune system to eliminate a bacterial threat.

The effect of LPS on the expression of SRCR-B proteins was also described for other members of this family. Studies performed with CD163 demonstrated that its expression is downregulated in monocytes when cells are subjected to low or high concentrations of LPS [57]. In contrast, the expression of secreted protein DMBT1 can be up-regulated by LPS *via* NF-kB. Once in the presence of DMBT1, the LPS interaction with cells inhibits TLR4-induced NF-kB gene activity, suggesting that this SRCR B protein acts as an anti-inflammatory molecule [58]. Therefore, both proteins modify their expression when in contact with LPS and they are clearly implicated in stablishing anti-inflammatory conditions. Our observation that the endogenous levels of Spα are altered in macrophages subjected to the pro-inflammatory stimuli LPS suggests that this molecule can have an anti-inflammatory role in the immune reaction against Gram-negative bacteria, probably involving TLR as described for DMBT1.

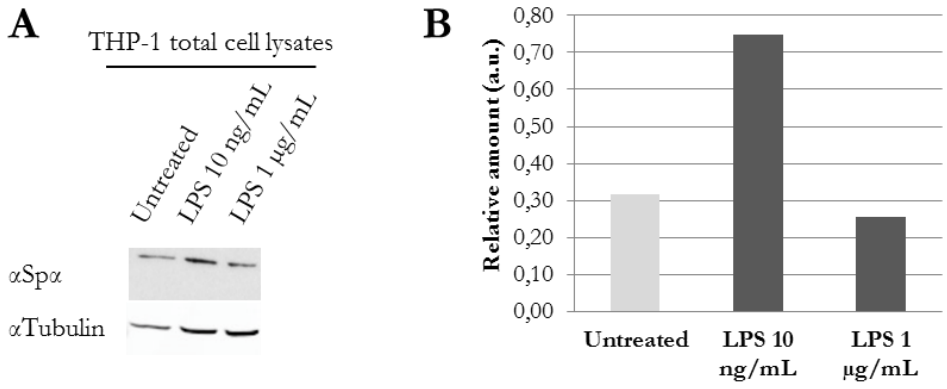


Figure 11: Comparative intracellular Spα amount in THP-1-derived MΦs upon treatment with LPS. MΦs were treated with two different concentrations of LPS (10 ng/mL and 1 µg/mL) for 24 h or left untreated. A) WB of total cell lysates, representing Spα expression (top blot) and tubulin used as loading control (lower blot). B) Relative amount of Spα protein to tubulin upon LPS treatment, calculated by densitometry analysis of band intensity. The figure reflects one representative assay of multiple experiences made.

The results observed in Fig. 10, suggesting a higher expression of Spα in the presence of DEX, led us to study this question in more detail by performing a kinetic study of Spα expression intracellularly in the presence of the glucocorticoid. After a 24 h treatment, the

intracellular amount of Spα increases significantly and is in accordance with the results depicted in Fig. 10. However, increased treatment times of 48 and 72 h with DEX led to a decrease in the intracellular amount of Spα over time, being lower than the untreated control cells at 72 h (Fig. 12). These results suggest that upon DEX treatment, an initial accumulation of Spα occurs as a result of increased protein expression but prolonged exposure times to the drug likely induce Spα secretion, although an intracellular degradation cannot be excluded. Dexamethasone is an anti-inflammatory and anti-proliferative glucocorticoid that can suppress the expression of pro-inflammatory mediators, but also induces the secretion of anti-inflammatory cytokines like IL-10 [59]. A similar overexpression induced by DEX treatment was previously described for CD163 in macrophages. The authors reported that the increase of CD163 expression helps in the internalization of toxic Hb-Hp complexes in a dose dependent manner, thus supporting an anti-inflammatory role for this SRCR B protein [60].

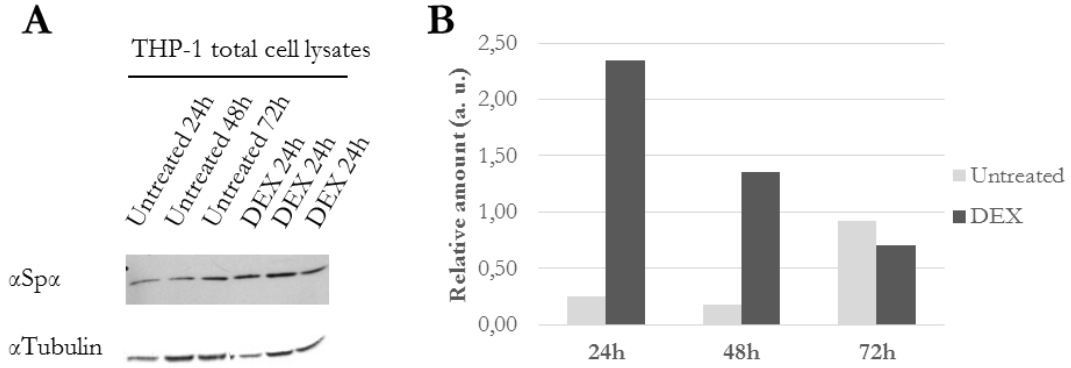


Figure 12: Kinetics of Spα expression in THP-1-derived MΦs upon treatment with dexamethasone. MΦs were treated with DEX for 24, 48, 72 h or left untreated. A) WB of total cell lysates, representing Spα expression (top blot) and tubulin used as loading control (lower blot). B) Relative amount of Spα protein to tubulin upon DEX treatment, calculated by densitometry analysis of band intensity. The figure reflects one representative assay of multiple experiences made.

Altogether the results obtained suggest that Spα may behave as an immune modulator of inflammation, whose expression is enhanced in an inflammatory context like the one resulting from an infection, but the protein can also have a synergistic role in a more anti-inflammatory environment and therefore its secretion is enhanced.

1.3. Activation of T cells and Spα expression

After exploring some dynamics of Spα expression in cells from innate immunity and since T cells also express Spα, we next investigated whether this protein also changes in cells from adaptive immunity upon activation. The strategy was then to activate Jurkat E 6.1 cells (a human T cell line), using three different activators and analyze Spα expression by WB.

T cell activation through αCD3/αCD28 treatment is the activation procedure that better mimics *in vitro* what happens *in vivo*. The antibodies will trigger an efficient activation due to the clustering of the TCR/CD3 complex and the co-stimulatory signal will be provided by the antibody against CD28 that acts as a secondary activation signal, required for full T cell activation [61]. Phytohemagglutinin (PHA) can activate T cells by binding to cell membrane glycoproteins, for example the TCR-CD3 complex, leading to the clonal proliferation of cells [62]. The last stimuli used was a combination of PMA, a protein kinase C activator, and ionomycin, a ionophore that opens Ca^{2+} channels present on the endoplasmic reticulum, an important event to the signaling pathway occurring upon activation of T cells. These two stimuli combined create a synergistic dynamic, increasing the signaling response in T cells [63].

The activation of T cells was confirmed by the analysis of CD69 expression by flow cytometry. CD69 is a human transmembrane C-type lectin protein that is a commonly used early activation marker of T cells [64].

The intracellular amounts of Spα in Jurkat T cells stimulated with αCD3/αCD28 antibodies decreased after 12 h of stimulation, comparing with the untreated control (Fig. 13). However, the intracellular levels of Spα were replenished with increased times of stimulation, having observed a higher expression at 24 and 48 h of treatment when comparing with the levels obtained after only 12 h of activation. The results suggest that the protein could have been rapidly secreted upon activation of cells followed by an increase in its production and consequent intracellular accumulation in later times reaching comparable levels to the untreated cells. A similar behavior in Spα intracellular levels was observed in T cells activated with PHA. A decrease in the levels of the protein was observed 12 h post-activation and these remained similarly low at least until 24 h after treatment. An increase in the Spα amount only occurred later and at 48 h protein levels were higher than in early times of treatment but remained lower than those observed for untreated cells. The activation of T cells with PMA and ionomycin resulted in kinetics of Spα expression similar to those observed with the αCD3/αCD28 treatment. Thus, a decrease in the intracellular levels of Spα happens after 12 h

but its expression increases in the next two time points tested (24 and 48 h), remaining higher than the intracellular amounts of untreated cells.

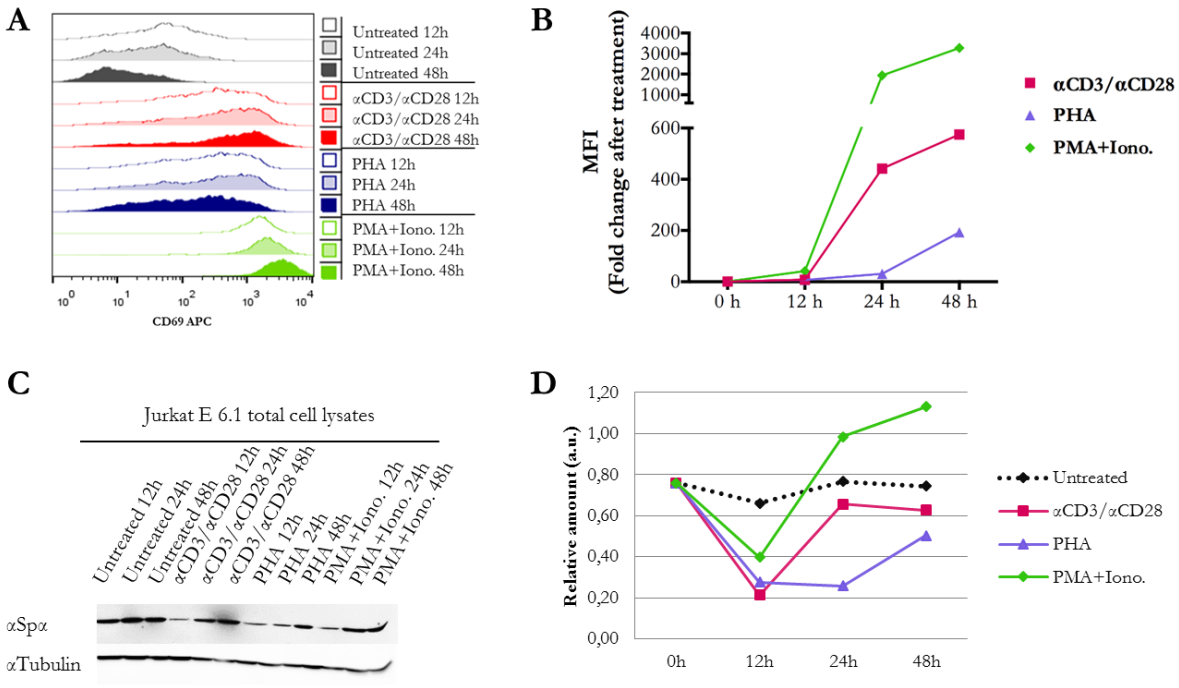


Figure 13: Kinetics of intracellular Spα levels in Jurkat T cells upon activation. Jurkat T cells were activated with anti-CD3 together with anti CD28, PHA, PMA and ionomycin for 12, 24 and 48 h or left untreated. A) Activation of T cells accessed by analysis of CD69 expression by flow cytometry. B) Mean fluorescence intensity (MFI) of CD69 staining of the cells over time for each treatment. The MFI of CD69 staining for each treatment was normalized to the isotype control and divided to the MFI of untreated cells, thus obtaining a fold change after treatment. C) WB of total lysates of cells to detect Spa expression (top blot) or tubulin as loading control (bottom blot). D) Relative amount of Spα protein to tubulin upon activation overtime, calculated by densitometry analysis of band intensity. The figure reflects one representative assay of multiple experiences made.

In the three types of stimulation, Spα seems to decrease after 12 h, a rapid response given by T cells after activation. With increased activation time, in all stimuli tested, the intracellular amount of protein increases, which raises the question whether a constant level of Spα in these cells is important for the homeostasis. Moreover, with the combination of PMA and ionomycin the protein increases to levels above the untreated. Since this type of activation

acts directly on the intracellular pathway triggered upon T cell activation, it could mean that there is also a rapidly activated pathway that leads to the increase in the levels of Spα in the cell, suggesting that this pathway and the one elicited through TCR activation could be related.

Other two members of SRCR B are expressed in T cells, CD5 and CD6 proteins, and Spα presents a SRCR domain organization similar to these two proteins [35]. It is well know that CD5 and CD6 proteins have an important role in T cell homeostasis. CD5 and CD6 can induce inhibitory signals in T cells, leading to a decrease in Ca^{2+} response and suppression of cytokines expression resulting in decreased T cell proliferation, a mechanism that involves a downstream pathway of the TCR/CD3 complex [65-67]. Due to the relevance of CD5 and CD6 in this type of cells and in the regulation of the immune response and taking into account the structural similarity with Spα, new studies with this protein could demonstrate an important role for Spα in adaptive immunity.

2. Searching for Spα ligands

Given the relative high abundance of Spα in circulation and its anticipated role as an immune modulator, the next objective was to identify possible ligands for Spα that would enable to better understand and explore the function of this protein.

2.1. Protein production and purification

Spα production and purification in recombinant forms is the first requirement to fulfill this task. The protein was produced from stably Spα expressing CHO-K1 cells, already available in the lab. These cells were constructed using the glutamine synthetase (GS)-based expression system for eukaryotic cell lines. The Spα cDNA was fused with an HA (N-terminal) and 6xHis (C-terminal) tags for detection and purification and a BirA recognition sequence (C-terminal) for biotinylation mediated by BirA (biotin ligase) enzyme (Fig. 14). Due to the presence of an engineered signal peptide (RaduSP) in the expression vector (that replaces the endogenous one present in Spα cDNA), the recombinant protein is secreted to the cell culture media facilitating its purification. Therefore, after recovery of this supernatant, two different purification techniques were performed: first by affinity chromatography

through the use of nickel media that binds with high affinity to the 6xHis tag present in Spα and secondly by size exclusion chromatography to increase the purity of the obtained protein.

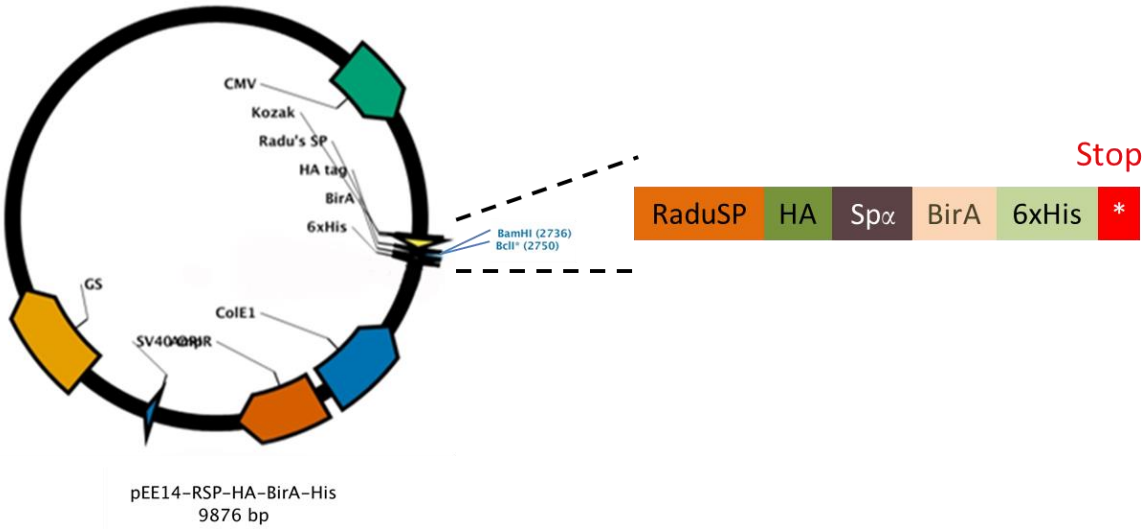


Figure 14: Schematic representation of the plasmid used to produce recombinant Spα in CHO-K1 cells. The Spα cDNA was fused with an engineered signal peptide (RaduSP) allowing the recombinant protein to be secreted to the cell culture media, an HA and 6xHis tags for detection and purification, respectively. The protein also has a BirA recognition sequence in the C-terminal for *in vitro* biotinylation.

Supernatant collected from transfected CHO-K1 cells consists in several components from the cell culture medium and proteins released from normal metabolism of these cells, besides the recombinant protein of interest. To obtain the recombinant Spα from this mixture, affinity chromatography is the first approach because it is based on the specific biological interaction between a ligand (nickel immobilized in the column) and the target protein, Spα, contained in the mobile phase (mixture). Upon extensive cycles of washing with increasing concentrations of an imidazole containing-buffer to eliminate contaminations that bind non-specifically to the column matrix, Spα was eluted with a 100 and 250 mM imidazole containing-buffers (Fig. 15). After purification, several fractions were analyzed on SDS-PAGE to check for the presence and purity of Spα. Since the recombinant Spα did not show the degree of purity required, fractions 40-60 were pooled and subjected to a different type of purification method.

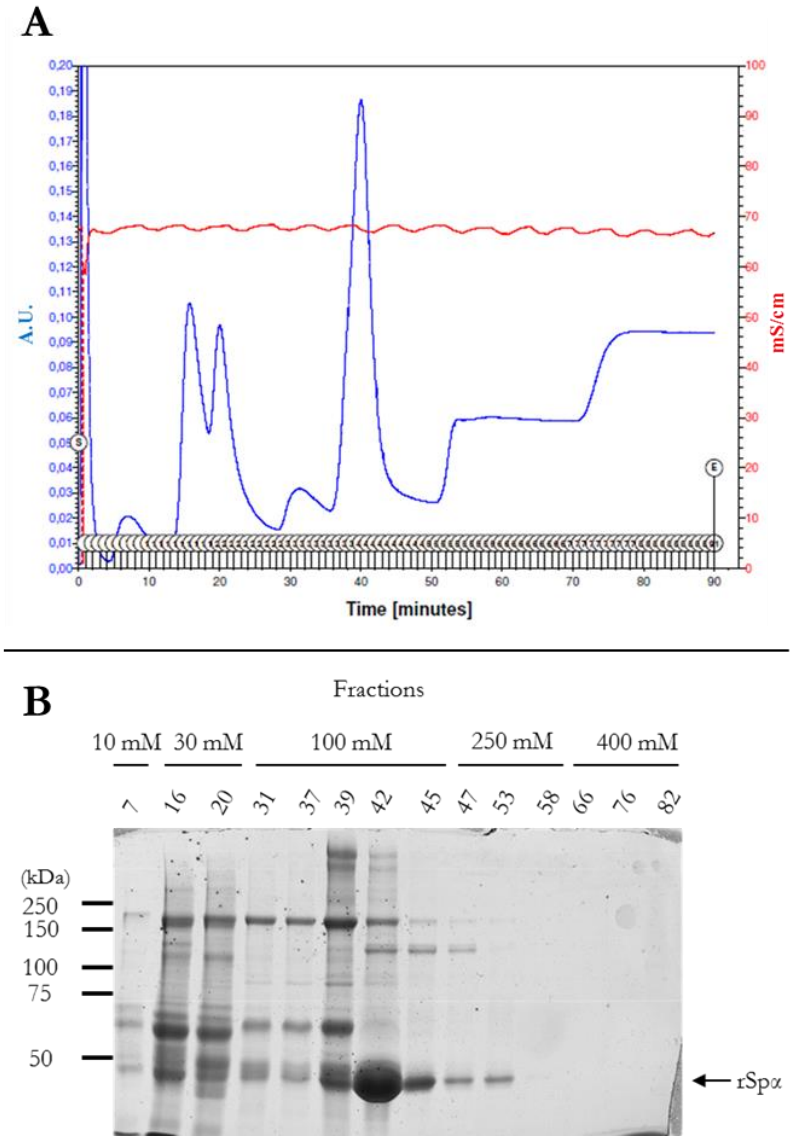


Figure 15: Affinity chromatography purification of Spα. A) Chromatogram demonstrating the different fractions collected during the washing and elution steps. Spα was eluted with a 100 and 250 mM imidazole containing-buffers. B) SDS-PAGE containing selected fractions after staining with BlueSafe to check the purity of selected fractions. Fractions 42-53 presented in this gel demonstrated the presence of Spα, still with some remaining impurities.

Size exclusion chromatography is a technique that allows the separation of molecules according to their size. The fractions selected to use in this purification have less different molecules than the original mixture, which facilitates the recovery of the recombinant protein with higher purity. Fig. 16 represents the results obtained after this technique. An SDS-PAGE analysis demonstrated that the recombinant protein present in fractions B6-B10 had the degree of purity required for the binding assays.

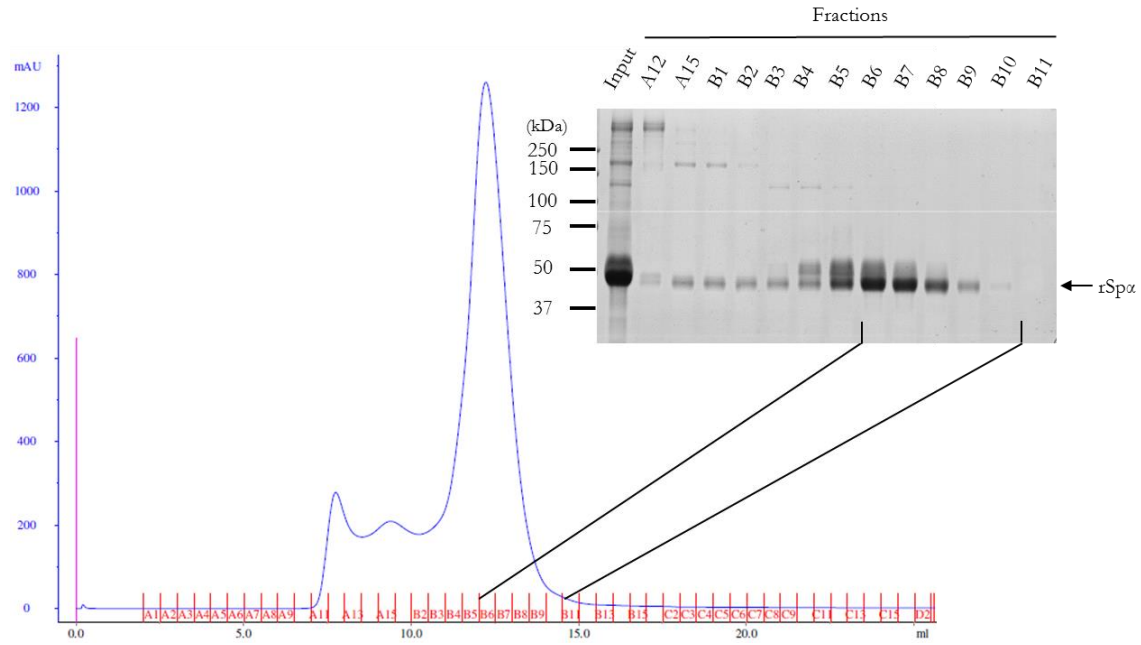


Figure 16: Size exclusion chromatography for rSpα purification. The figure represents the chromatogram of fractions obtained during the elution and the SDS-PAGE stained with Blue Safe used to check the purity of selected fractions. Fractions from B6 to B10 present the degree of purity required for the binding assays

2.2. Ligand binding assays

Due to the measured normally low dissociation constant (K_d) of the interaction between cell surface molecules, it is difficult to detect and identify the ligand for a given receptor of interest using affinity methods. In order to increase the sensitivity of binding detection of soluble Spα to different cell surfaces, we assembled this protein as tetramers containing a fluorescent label. To do this, we performed *in vitro* biotinylation through the use of the enzyme biotin ligase that specifically recognizes the BirA recognition sequence included in the C-terminal of recombinant Spα (Fig. 14). The resulting biotinylated Spα was mixed with AlexaFluor 647-coupled streptavidin, thereby resulting in a tetramer (Fig. 17).

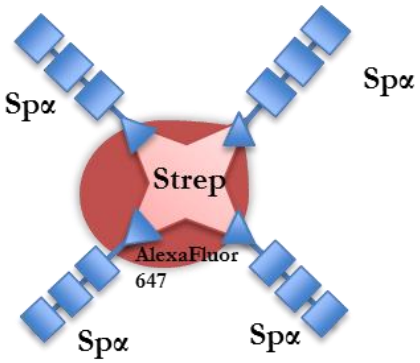


Figure 17: Illustration of the assembled Spα into tetramers. After the protein biotinylation step, samples were mixed with AlexaFluor 647-coupled streptavidin, containing 4 biotin-binding sites, to obtain fluorescent tetramers.

Several cell lines from different origins (epithelial, monocytic and lymphoid lineages) were tested for expression of ligands to Spα (Table 3). To do this, cells were incubated with fluorescent tetramers composed of Spα, human CD2 or empty, as a negative control, and subjected to flow cytometry analysis to assess the fluorescent intensity as a direct measure of binding. Human CD2 was used in these experiments as a control since the ligand for this molecule, CD58, is well known and characterized.

Table 3: Origins of each cell line used in the binding assay. The characteristics of the cell lines like morphology and tissue origin are detailed.

Morphology	Cell line	Tissue origin
Epithelial	HEK 293T	Human embryonic kidney
	HT-29 HCT 116 DLD1	Colon
	SNU638	Colon
	HCT116	Stomach
	HeLa	Cervix
	HepG2 Hep3b	Liver
	SKBR3 MDA-MB-231	Mammary gland
Lymphoblast	K562	Bone marrow
	Daudi EBV Raji	B lymphocyte
	Molt4 Jurkat E 6.1 PM1	T lymphocyte
	NK92	Natural killer cell
Monocytic	THP-1 U937	Monocyte

The results obtained show that Spα can bind to the epithelial cells HeLa, Hep3B and SKBR3 and moderately to HEK 293T, HCT116 and SNU638 (Fig. 18). In addition, binding was also observed in cells from hematopoietic origin like K562. Binding was not detected or was very low in other epithelial cells included in this screening like HT-29, DLD1, HepG2, MDA-MB-231 as well as in the cells from hematopoietic lineage Daudi, Molt4, PM1, NK92, EBV and U937. The binding of CD2 tetramers followed the expected pattern according to the expression of CD58 described. Given the broad expression of CD58, CD2 tetramers bound to the majority of the tested cell lines and fluorescence was not detected in some cells reported as not expressing or expressing very low levels of CD58, namely Daudi and Molt4.

It was previously reported that binding of Spα to Gram-positive and negative bacteria is only observed in the presence of Ca^{2+} [38]. We have therefore tested whether the presence of this divalent cation induces any change in the binding ability of Spα to the panel of cell lines included in the previous experiment. Using a buffer with $CaCl_2$ during the ligand binding assay, we observed that this ion can increase binding between Spα and all the cell lines tested, even in the ones that were negative for Spα binding in the absence of Ca^{2+} (Fig. 18).

Interestingly, the presence of calcium did not significantly change the binding intensity of the control CD2 tetramers to all the cells except for Hep3b, SKBR3, MDA-MB-231, DLD1 and SNU638 that had the higher MFI increase in the presence of calcium ions (Fig. 18).

These findings complete the preliminary information described for Spα that was reported as binding to K562, Raji, Hut78 and THP-1 [35].

Ca^{2+} is an important ion in the homeostasis of cells, regulating a myriad of cellular functions, affecting every aspect of the cell's life. In response to stimulus, Ca^{2+} can be released from internal storages or from the exterior of the cell. For instance, intracellular Ca^{2+} is released when needed through itself or by messengers. Extracellular Ca^{2+} enters into cells by plasma membrane channels in response to membrane depolarization, noxious stimuli, among others [68]. The binding of calcium to proteins can drive a modification in conformation and charge, important for signal transduction [69].

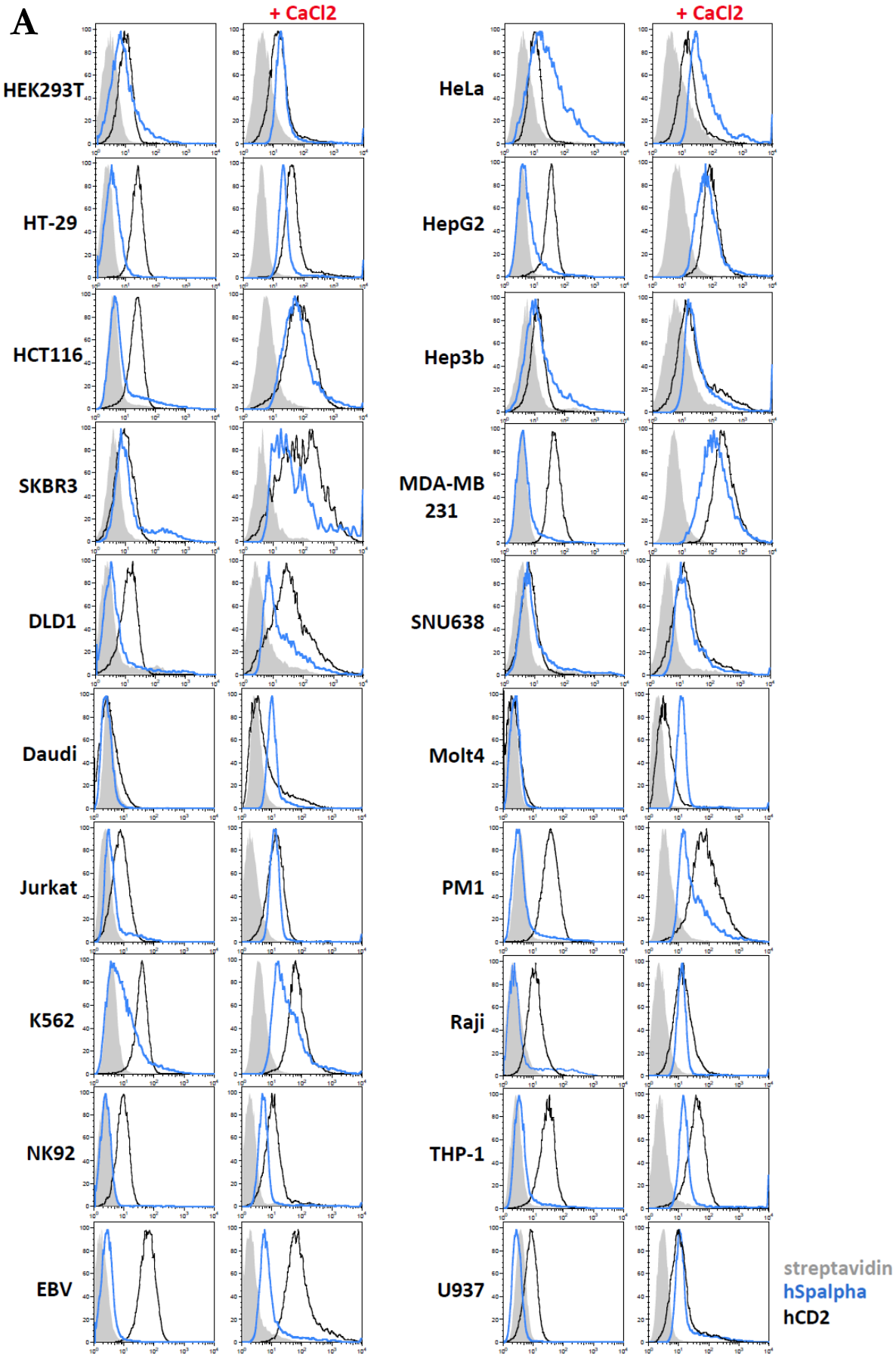
DMBT1 is a SRCR member known to be associated with innate immune response in the oral cavity. A study made with this protein revealed that in the presence of calcium, there are glycosylation (more predominantly O-glycosylation) and conformational changes in SRCR domains occurring in the DMBT1 protein [70]. Similarly, binding of calcium to some domains of CD163 induces a conformation change in this protein increasing the binding affinity to

haptoglobin-hemoglobin complexes [71]. By *in silico* analysis of the sequence of SRCR domains of CD163, the amino acids involved in this binding were identified and confirmed through different experiments. Sequence analogy also revealed that other members of the SRCR B family have potential calcium binding sites and Spα was no exception. Indeed, such binding sites are thought to be present in the SRCR domains 1 and 3 of Spα but experimental evidence is still missing [71]. Notwithstanding, these clues can explain our results showing that Spα binds more intensely in the presence of Ca^{2+}.

The results in Fig. 18, although suggesting the presence of one or multiple Spα ligand(s) in the tested cell lines, do not necessarily reveal the biochemical nature of this unknown molecule(s). Therefore, to confirm that this putative ligand(s) is of protein nature, K562 and HTC116 cells (positive for Spα binding) were subjected to a mild treatment with the protease trypsin prior to tetramer incubation. The results obtained show a decrease in the binding of Spα to both cell lines after treatment with trypsin confirming that the ligand(s) for Spα is a protein expressed on the cell surface (Fig. 19). There is still some binding of Spα tetramers to the cells because the trypsin treatment was moderate to prevent cell death but effective as can be observed through the reduction in CD2 tetramers binding to its ligand CD58.

Ligands for Spα were already reported in the literature. CD36 was the first molecule described as being a receptor for Spα endocytosis in macrophages [47]. CD36 recognizes multiple ligands allowing it to exert different functions. The ability of this protein to internalize modified lipoproteins like oxLDL and thus its association with the atherosclerosis is one of the most investigated functions. However, the nature of the Spα-CD36 binding and a possible modulation of the interaction of CD36 with other ligands remain largely unknown. In addition, an interaction of Spα with some regulators of complement activation (RCA) like CD55, Crry, CD59 and factor H was demonstrated by co-immunoprecipittation assays in transfected cells, even if further experimental evidences are needed to validate and characterize these interactions [72]. However, both CD36 and RCAs are not expressed in some of the cells positive for Spα binding in our screening, showing that other putative(s) ligand(s) exist adding another layer of complexity to the functions of this protein.

Even though this ligand(s) remains unknown, the next approach, already in course, is to identify this molecule(s) by a transcriptomic analysis of all the cell lines and confirm the top candidates through molecular and cellular techniques.


A
+ CaCl2
+ CaCl2
HEK293T
HT-29
HCT116
SKBR3
DLD1
Daudi
Jurkat
K562
NK92
EBV
HeLa
HepG2
Hep3b
MDA-MB 231
SNU638
Molt4
PM1
Raji
THP-1
U937
streptavidin
hSpalpha
hCD2

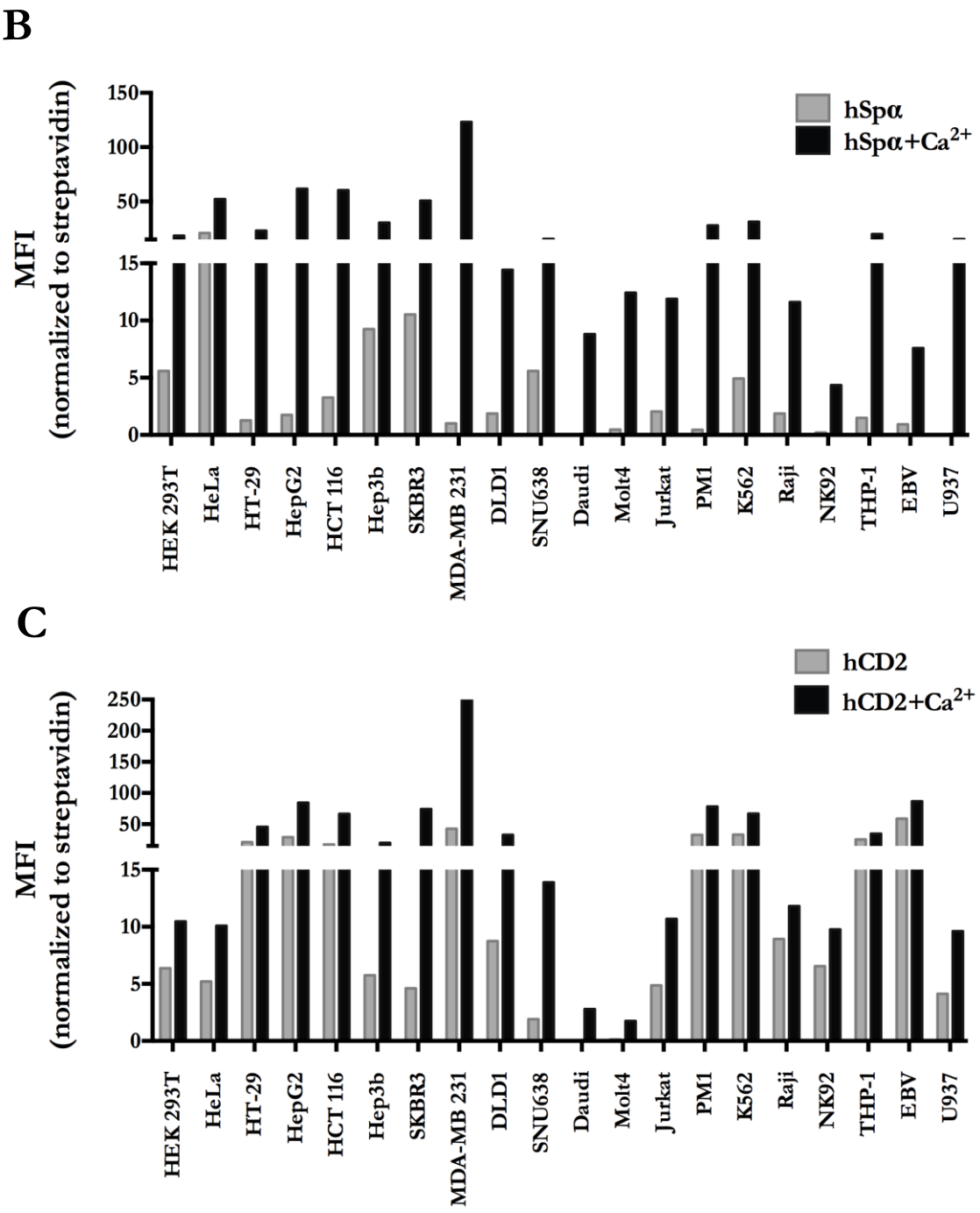


Figure 18: hSpα tetramers binding to the different cell lines tested. A) A panel of cell lines (from non-lymphoid, myeloblastic, monocytic and lymphoid lineages) were incubated with: i) streptavidin-Alexa647 (negative control); ii) tetramers composed of Spα and streptavidin-Alexa647 (hSpα); and iii) CD2 tetramers containing Alexa647-coupled streptavidin (hCD2) in the absence or presence of 0.9 mM $CaCl_2$ (+$CaCl_2$) and analyzed by flow cytometry. B) Mean fluorescence intensity (MFI) of hSpa tetramers binding to the cells in the presence or absence of Ca^{2+} normalized to the streptavidin empty tetramers. C) MFI of hCD2 tetramers binding to the cells in the presence or absence of Ca^{2+} normalized to the streptavidin empty tetramers.

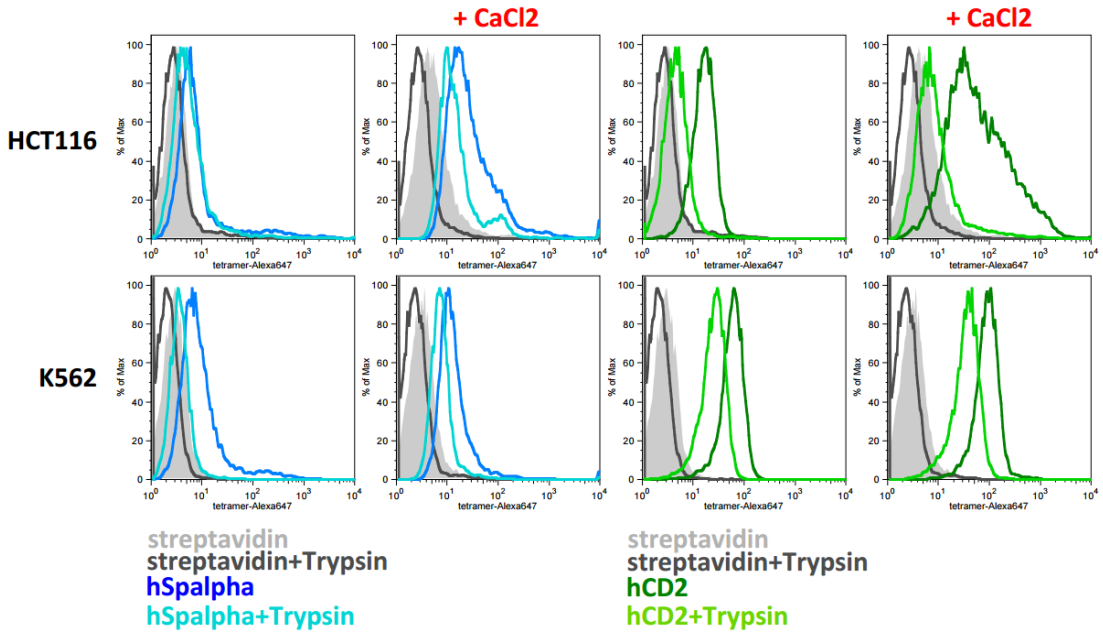


Figure 19: Binding of hSpα tetramers to cells after trypsin treatment. HCT116 and K562 cells were treated with 0.05 % trypsin for 20 min or left untreated. The cells were incubated with: i) streptavidin-Alexa647 (negative control); ii) tetramers composed of Spα and streptavidin-Alexa647 (hSpα); and iii) CD2 tetramers containing Alexa647-coupled streptavidin (hCD2) in the absence or presence of $CaCl_2$ (+ $CaCl_2$) and analyzed by flow cytometry.

3. Insights into S4D-SRCRB localization and function

3.1. S4D-SRCRB expression

Very little is known about S4D-SRCRB. We started with the same approach used for Spα, using WB and ELISA of different protein lysates to detect S4D-SRCRB expression. However, we were unsuccessful in detecting this protein in all our vast attempts, probably due to the quality of the commercially available antibodies or the low expression of this protein. To circumvent this problem, other techniques were used in order to find more about the location of expression of this protein and function(s).

The first approach was to analyze the expression of S4D-SRCRB, at the mRNA level, by PCR of cDNA of cells from different origins. S4D-SRCRB was detected in epithelial cells (HepG2, Caco-2, JEG-3, HEK 293T and HeLa) and in cells from hematopoietic origin (K562 and Jurkat E 6.1). The expression was not detected in Raji, TCCSUP or in primary monocytes differentiated into MΦs with GM-CSF or M-CSF as a way of inducing M1 and M2 macrophages, respectively (Fig. 20). These findings are in accordance to Padilla *et al.*, where by northern blotting analysis the expression of S4D-SRCRB was detected in K562, HeLa, HepG2 and HEK 293T [50]. In contrast with our results, these authors reported a lack of expression in Jurkat T cells that can be explained by a difference in the detection method.

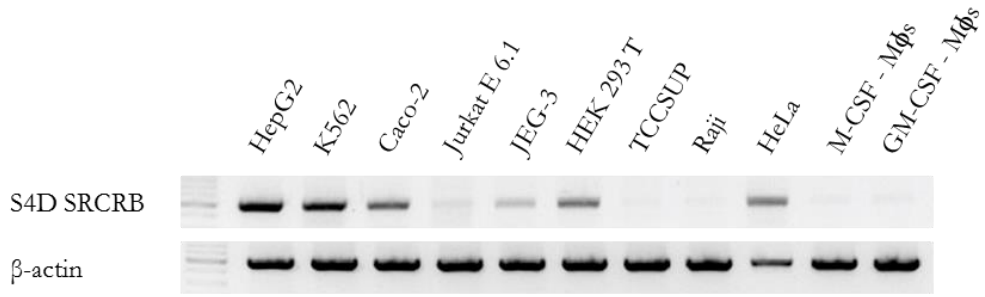


Figure 20: S4D-SRCRB mRNA expression in cells from different origins. Agarose gel from the result of a PCR used to detect S4D-SRCRB mRNA expression. As control, β-actin amplification was used.

Immunohistochemistry technique can be used to identify structures within tissues but also molecules within the cells. Following this line of thought, this approach was used to characterize S4D-SRCRB expression in some human tissues. The anti-S4D-SRCRB antibody that did not work in WB or ELISA, revealed to be suitable for immunohistochemistry analysis. This human protein was found in several different human tissues like lung, thyroid, skeletal muscle, stomach, liver, kidney and placenta (Figs. 21, 22 and 23) but not all types of cells within these tissues express the protein.

The lung histology (Fig. 21) revealed some tissue processing artifacts leading to some background after the antibody staining. However, there is a soft staining in pneumocytes from both types of epithelium (I and II). Type I pneumocytes form a thin barrier that enables gas exchange and at the same time prevents liquid passage. On the other hand, type II pneumocytes are responsible for the production of surfactant liquid, which prevents pulmonary alveolus from collapsing [73]. These two types of cells are in contact with substances from the exterior, which raises the question whether the presence of S4D-SRCRB protein is linked to the immune responses happening in the epithelial barriers of the lung. An alveolar macrophage present in the image corresponding to αS4D-SRCRB staining (indicated by a black arrow) does not seem to express this protein.

The thyroid is an endocrine gland important for the synthesis of hormones and present plenty of follicles in its composition. S4D-SRCRB is slightly expressed in the cytoplasm of follicular cells of this organ.

A skeletal muscle tissue sample was also analyzed for the expression of S4D-SRCRB. Surprisingly, S4D-SRCRB expression is clearly visible in the longitudinal section of skeletal muscle fibers presenting a "dotted" staining as illustrated in Fig. 21.

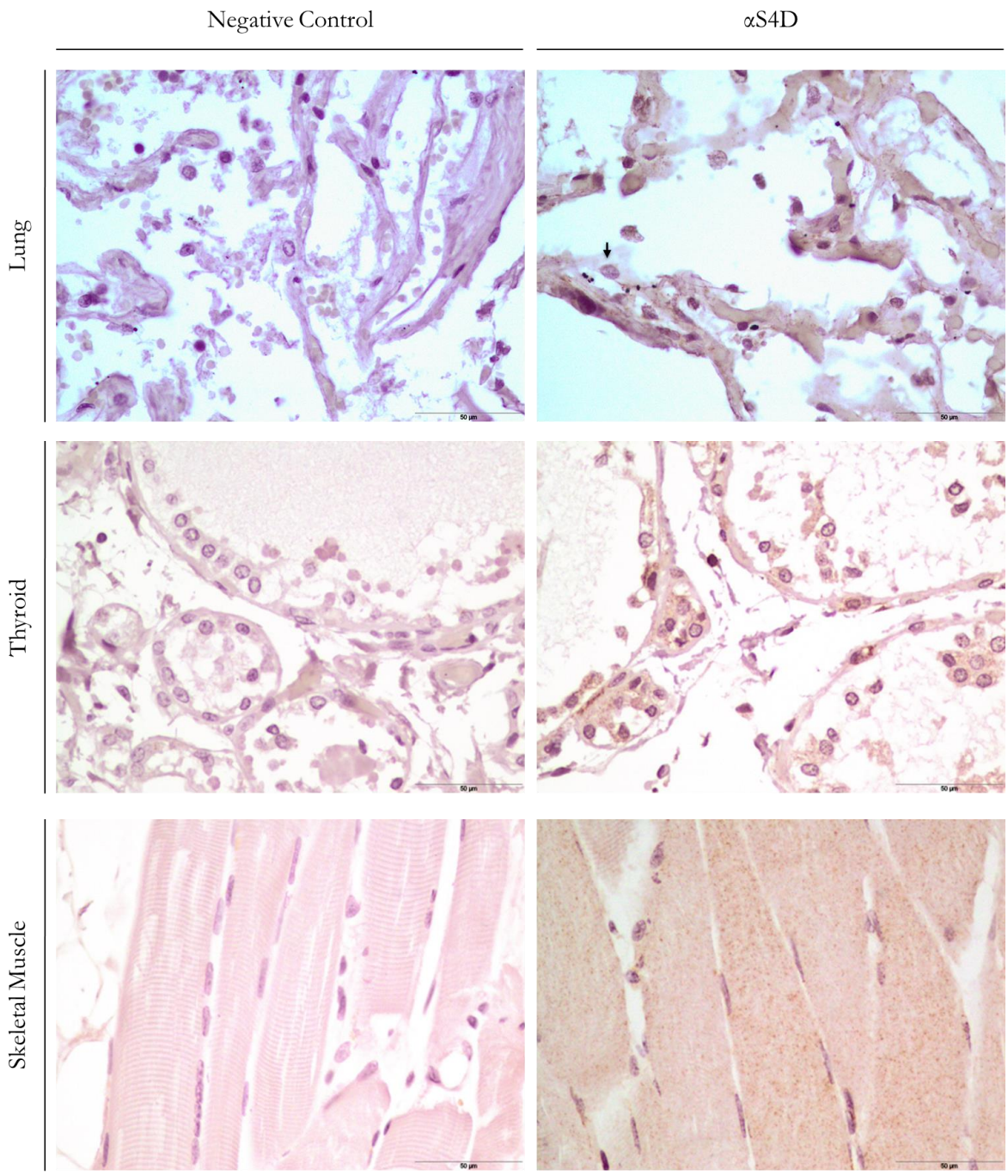


Figure 21: Photographs of histologic sections of lung, thyroid and skeletal muscle treated for S4D-SRCRB protein detection. On the left are represented tissues stained with a negative control antibody and on the right those incubated with an anti-S4D-SRCRB antibody. Scale bars, 50 μm.

The "dotted" staining pattern revealing the expression of this protein is more evident in the stomach tissue. A very strong staining is present at the base of the stomach gland from the body portion. The cells that stand out are peptic cells that are producers of pepsinogen and lipase, presenting a very characteristic morphology due to the active synthesis and export of proteins (Fig. 22) [73]. Expression of S4D-SRCRB is also observed in a few parietal cells (oxyntic), responsible for H^+ and Cl^- secretion.

The liver is the largest gland in the human body. This is an essential organ to neutralize and eliminate toxic substances. Analysis of the expression of S4D-SRCRB in this tissue reveals a strong staining in hepatocytes cytoplasm, but not in the interlobular connective tissue nor in central veins (Fig. 22). Hepatocytes have endocrine and exocrine functions and contribute to detoxification. In general, hepatocytes do not accumulate proteins in secretory granules intracellularly, they secrete proteins constantly into the blood stream [73]. This can explain why the "dotted" staining of S4D-SRCRB protein, observed in other tissues, is less visible in this organ.

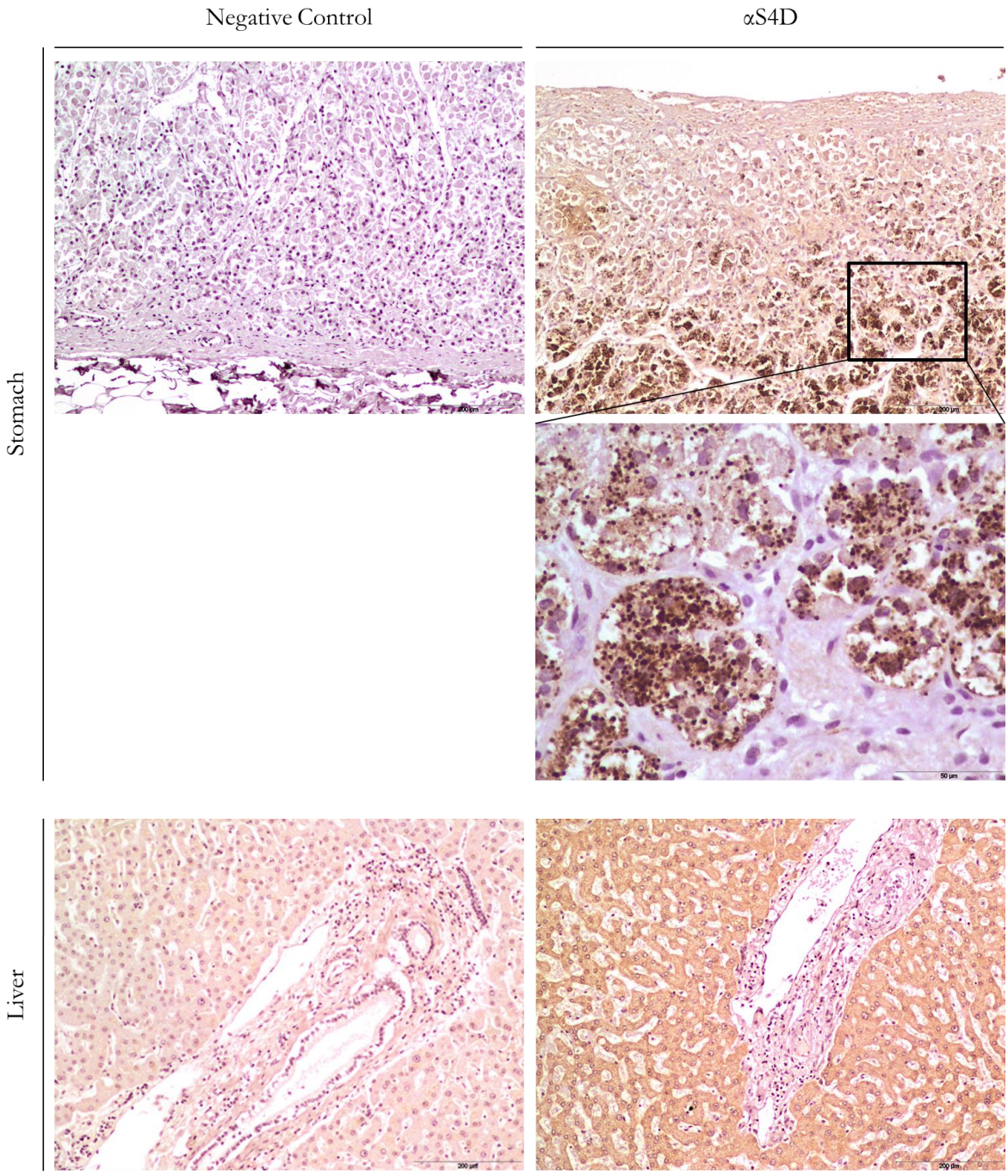


Figure 22: Photographs of histologic sections from stomach and liver treated for S4D-SRCRB protein detection. . On the left are represented tissues stained with a negative control antibody and on the right those incubated with an anti-S4D-SRCRB antibody. Scale bars, 200 μm and 50 μm for a close view of the stomach histology.

In the placenta, a strong staining at the cytoplasm of syncytiotrophoblast cells from the external cell layer of the chorionic villi reveals a high expression of S4D-SRCRB protein (green arrow in Fig. 23). Curiously, a macrophage from this tissue (Hofbauer cell) seems to express this protein as revealed by the "dotted" staining (black arrow) contrasting to the lack of expression of this protein in alveolar macrophages. A diffuse and less intense staining is observed throughout the intervillous space as shown in Fig. 23 (blue arrow).

The last histological tissue tested was the kidney. The histological section represented is from the cortical part, which is constituted by renal corpuscles (Bowman capsule and glomerulus), arterioles, proximal tubule, distal tubule, collector tubule and stellate veins. S4D-SRCRB has a high expression in the epithelium of tubules, mainly in proximal tubules, responsible for the beginning of absorption and excretion process, not being expressed in renal corpuscles.

In summary, these results indicate that S4D-SRCRB protein is mostly expressed in epithelial barriers demonstrated by PCR of different cell lines and by histological tissues analysis. These results are similar to those reported for human SSc5D and SCART1 proteins, members of SRCR B family. Both proteins are expressed in some epithelial tissues, such as kidney, placenta and stomach [74-76]. DMBT1 is also found in mucosal surfaces like lung, oral cavity, intestine, kidney and skin. This protein can be found in a secreted form, in the cytoplasm or on the cell surface. It is involved in the innate immune defense and it is considered a PRR [77, 78].

Besides being expressed mainly in epithelial barriers, the staining pattern of S4D-SRCRB protein has the characteristic of a dotted expression in certain tissues possibly indicating that the protein is stored in vesicles. In the future, tissue sections can be doubly stained with a lisossomal/endosomal marker and the anti-S4D-SRCRB antibody to analyze whether there is a co-localization indicative of in-vesicle storage of the protein. One interesting feature is its expression in the skeletal muscle, also with a "dotted" staining pattern, which transcends the view of its mucosal expression and is a characteristic never observed before for any of the other proteins from the SRCR B family.

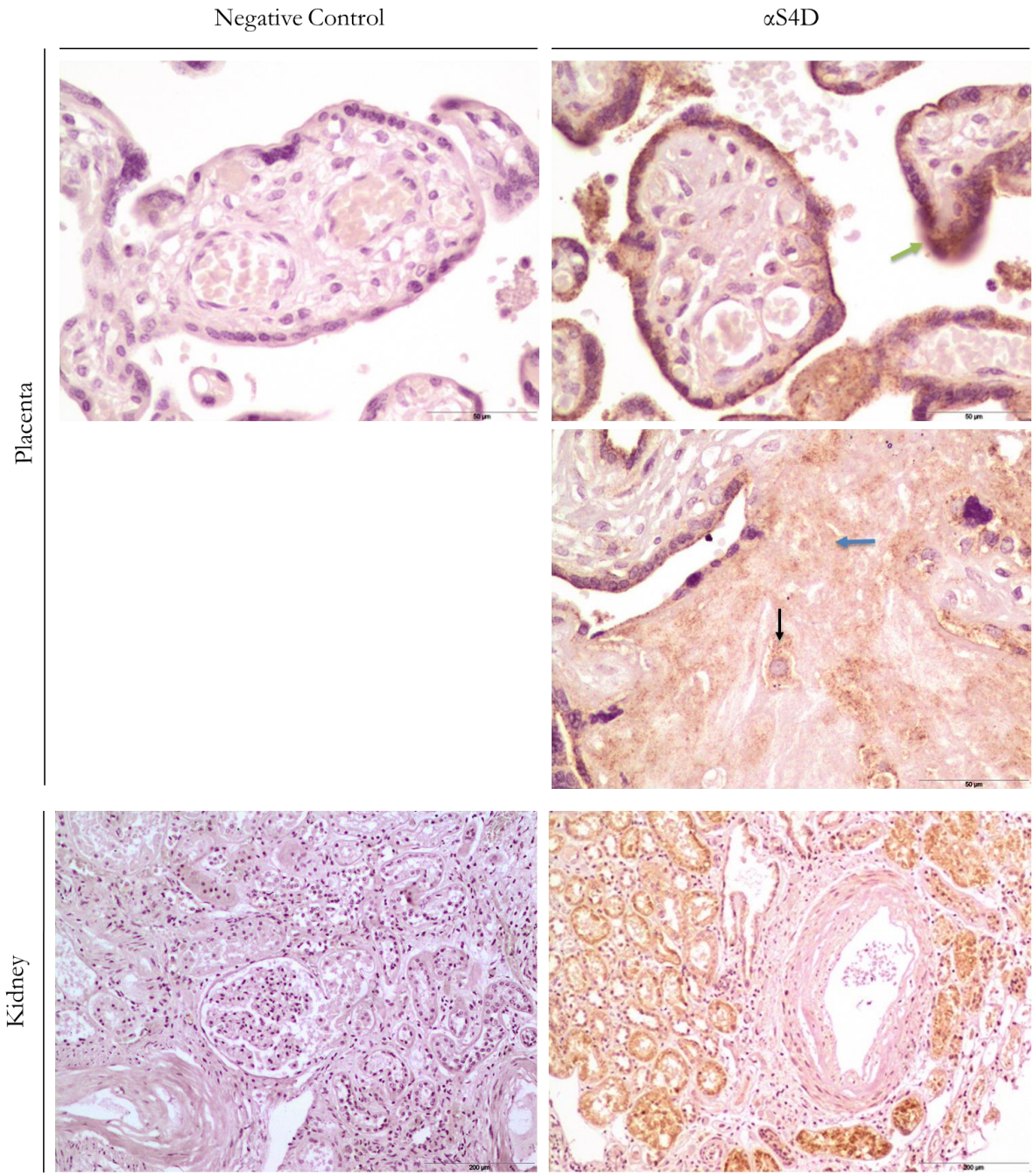


Figure 23: Photographs of histologic sections from placenta and kidney stained for S4D-SRCRB protein detection. On the left are represented tissues stained with a negative control antibody and on the right those incubated with a monoclonal mouse anti-S4D-SRCRB antibody. In the placenta, S4D-SRCRB has a strong staining in the cytoplasm of syncytiotrophoblast cells from the external cell layer of the chorionic villi (green arrow). The black arrow points to a macrophage from this tissue (Hofbauer cell) that seems to express this protein. The blue arrow points to the diffuse and less intense staining throughout the intervillous space. Scale bars, 50 μm in the placenta images and 200 μm for kidney images.

3.2. Over-expression of S4D-SRCRB in cell lines

Given the inability to use the commercially available antibodies in WB or ELISA based assays, we chose a strategy of S4D-SRCRB over-expression to try to uncover the functions of this protein.

S4D-SRCRB was thus expressed in two cell lines: the epithelial Caco-2 cells and Jurkat E6.1 T cells. These two cells were chosen given the preferential expression of the protein in epithelial sites but also to analyze its putative function in a completely different context, in one of the cells that constitute the adaptive immune response, and therefore infer whether this protein can have different functions depending on the cells that express it.

To overcome the antibody limitations, we cloned S4D-SRCRB in fusion with the HA tag and the fluorescent protein mCitrine in the C-terminal (Fig. 24) under the control of the spleen focus-forming virus (SFFV) promoter that allows the use of different experimental techniques.

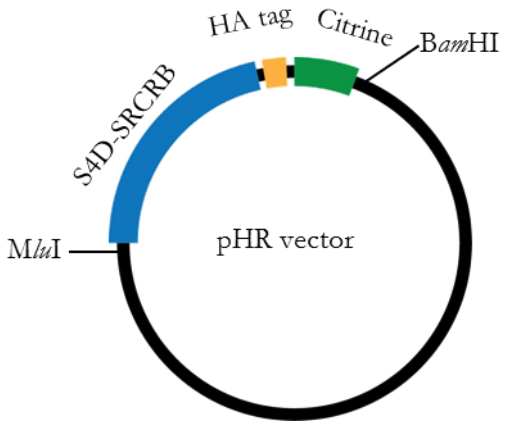


Figure 24: Schematic representation of the vector used to express the S4D-SRCRB protein. This protein was cloned in the pHR vector with a HA tag to facilitate WB detection and mCitrine for fluorescence assays, fused in the C-terminal of S4D-SRCRB.

The resulting pHR-S4D-SRCRB-mCitrine was introduced in Caco-2 and Jurkat E6.1 cells through lentiviral mediated transduction enabling its insertion in the genome and thus resulting in stably transfected cells expressing S4D-SRCRB constitutively (S4D-mCitrine). After transduction, the expression of the protein was analyzed by flow cytometry measuring mCitrine fluorescence (Fig. 25). The percentage of Caco-2 cells expressing S4D-mCitrine was 33.5% and 42% for Jurkat E 6.1. To increase the homogeneity of the population of cells, cell sorting based on mCitrine fluorescence was performed. The results obtained in Fig. 25 show that > 97% of the recovered cells have a high expression of S4D-mCitrine.

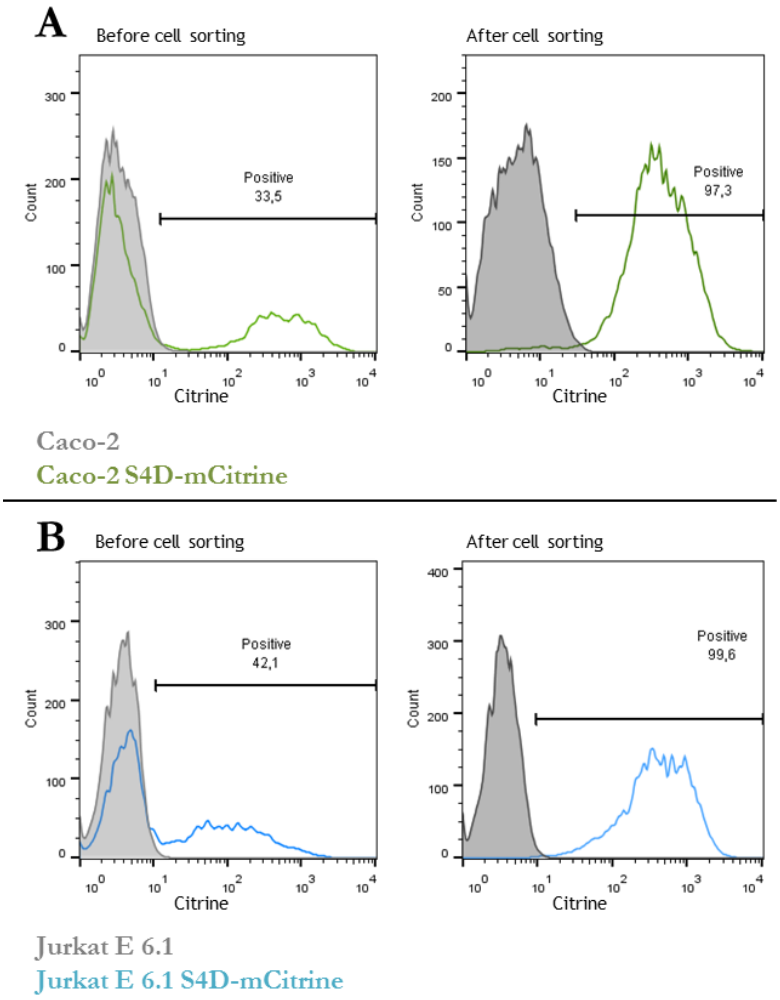


Figure 25: S4D-SRCRB expression in Caco-2 and Jurkat T cells after lentiviral transduction. A) Histograms of Caco-2 cells before and after cell sorting showing mCitrine fluorescence. Caco-2 untransduced cells were used as control. B) Histograms of Jurkat T cells before and after cell sorting showing mCitrine fluorescence. Jurkat E6.1 untransduced cells were used as control.

To analyze whether S4D-mCitrine is secreted, supernatant of these two cell lines was collected when the cells were at ≈ 90% of confluence and tested with an anti-HA antibody by WB (Fig. 26). The supernatant of Caco-2 cells revealed the presence of S4D, indicating that not only the protein is being correctly expressed but also effectively secreted. Surprisingly, S4D was not found in the supernatant of Jurkat E6.1 cells, which may indicate that although being correctly expressed in these cells as demonstrated by the flow cytometry analysis in Fig. 25, S4D-SRCRB secretion may be differently regulated in epithelial and lymphoid cells.

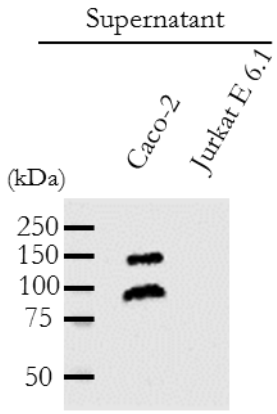


Figure 26: Detection of S4D-mCitrine in the supernatant of Caco-2 and Jurkat E6.1. S4D-mCitrine was detected with an anti-HA antibody.

We next assessed the pattern of expression and subcellular localization of S4D-mCitrine in Caco-2 cells and Jurkat E6.1 cells by immunofluorescence microscopy (Fig. 27). The expression of S4D-SRCRB protein in both cells is, as expected, cytoplasmic even if a "dotted" staining is observed more markedly in Jurkat E6.1 cells, which is consistent with a possible localization on endosomal vesicles. These findings can be related with the presence of S4D-mCitrine in the supernatant of Caco-2 cells and absence in Jurkat E6.1 cells: it is conceivable that a more active secretion would reduce intracellular localization in secretory vesicles in Caco-2 cells and, in contrast, a reduced secretion could result in a more pronounced accumulation of the protein intracellularly in Jurkat E6.1 cells. Of note, this punctate pattern of expression of S4D-SRCRB is consistent with the one observed in the immunohistochemistry staining of diverse tissues detailed in the section above.

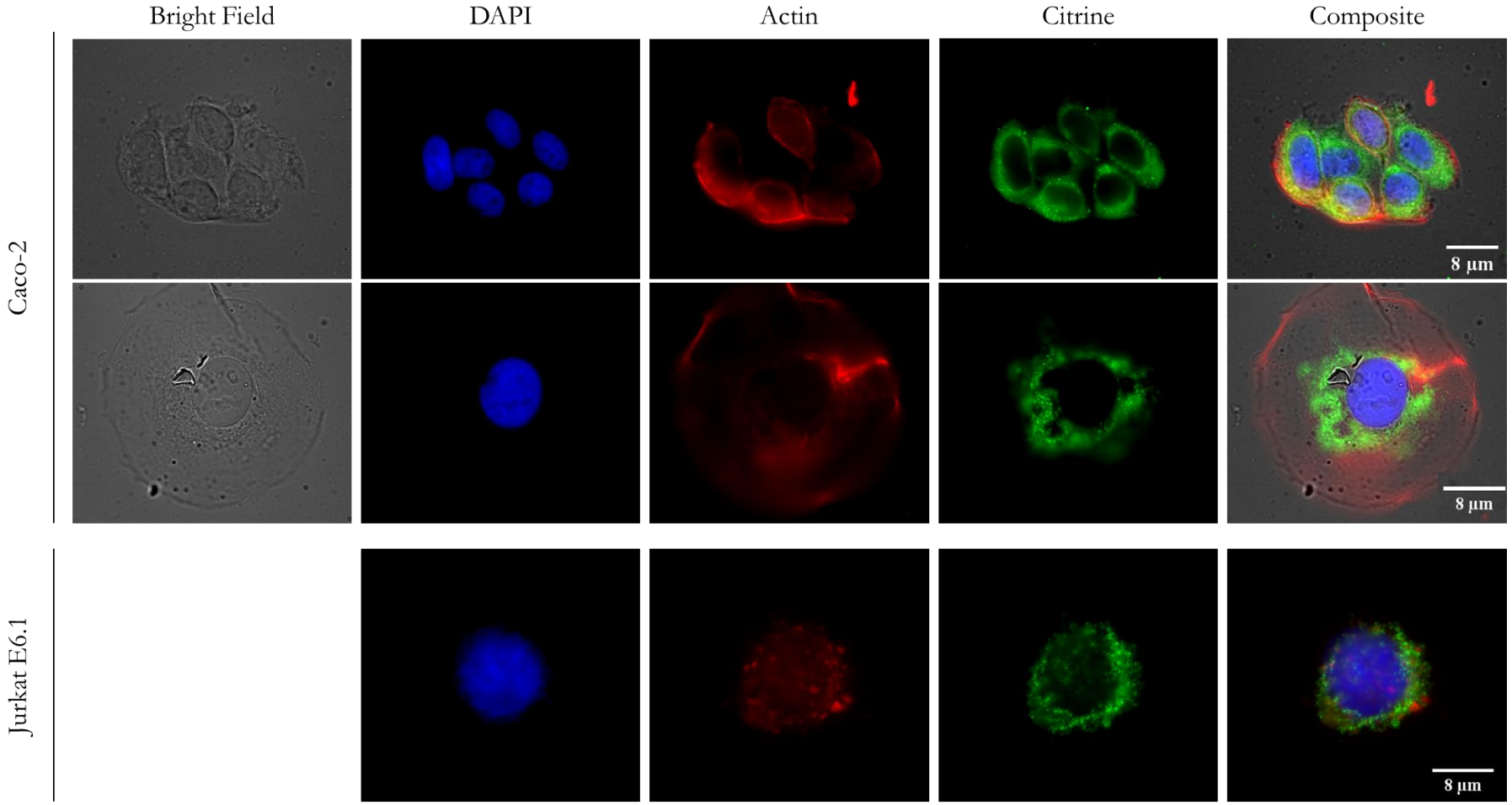


Figure 27: Immunofluorescence images of Caco-2 and Jurkat E6.1 cells expressing S4D-mCitrine. Cells were stained with DAPI and phalloidin-TexasRed as a probe to F-actin.

3.3. Expression and secretion of S4D-SRCR during infection

In a natural context, cells from the intestine are in contact with numerous microorganisms, acting as natural barriers preventing their access to the blood stream. Caco-2 are epithelial cells from the colon, commonly used as a model of the intestinal barrier [79]. Diverse SRCR B molecules like CD163, DMBT1, CD5, CD6 and Spα, were already classified as PRR because they can bind to Gram-positive and negative bacteria and/or fungi. Some of these members also have the property to aggregate bacteria, which may prevent them from crossing the epithelial barrier [4, 80, 81]. Given the important role of these other members of the SRCR family as PRR and the fact that S4D-SRCRB is naturally expressed in Caco-2 cells, we wanted to know whether this protein would have any role in an infection context. In these preliminary experiments, two types of bacteria were used: the extracellular *Escherichia coli* and intracellular *Listeria monocytogenes*.

Caco-2 cells expressing S4D-mCitrine were infected *in vitro* with *E. coli* and *L. monocytogenes* in a multiplicity of infection (MOI) of 50 for 1 h after which normal cell culture media containing antibiotics was added to the cells for extracellular elimination of bacteria, and this was considered time 0 of the experiment. Cells were then observed by fluorescence microscopy at time 0 and 2 h post-infection to compare the intracellular amount and localization of the protein within uninfected cells. Total lysates were simultaneously obtained for WB analysis.

Western blot of lysates demonstrated that 2 h after interrupting the contact between *E. coli* or *L. monocytogenes* with Caco-2 cells, the intracellular amount of S4D-SRCRB decreases (Fig. 28). *E. coli* strain RS 218 is an extracellular pathogenic Gram-negative bacterium that is used as a prototype of a microorganism causing neonatal meningitis. Upon contact with the intestinal barrier, these bacteria can cause its disruption, reach the blood stream and migrate to the brain [82]. *Listeria monocytogenes* is an intracellular Gram-positive bacterium capable of crossing the intestinal barrier. The important virulence factors internalin A and B expressed on the *L. monocytogenes* bacterial surface can bind to membrane receptors of human epithelial cells mediating its invasion followed by actin polymerization and membrane remodeling [83]. Our results suggest that S4D-SRCRB seems to be somehow involved in the cell response to this inflammatory stimulus, being likely secreted. Immunofluorescence analysis of Caco-2 cells indicate that upon infection with *E. coli* (0 h) there is an increase in the punctate-like

localization of S4D-SRCRB, when compared with the uninfected cells or to those infected with *L. monocytogenes*, consistent with a possible increased accumulation in secretory vesicles that will then lead to the secretion of this protein (Fig. 29). The difference between the possible localization of S4D-mCitrine in endosomal vesicles in Caco-2 cells upon infection with *E. coli* and not in those infected with *L. monocytogenes* could reflect different cellular mechanisms triggered as a result of an infection by an extracellular *vs.* an intracellular pathogen. In addition, a decrease in the intracellular amount of S4D-mCitrine can be observed in the cells 2 h post-infection either with *E. coli* or *L. monocytogenes*, in agreement with the results obtained with WB analysis (Fig. 28).

Caco-2 cells mimic an epithelial barrier and seem to secrete S4D-SRCRB. Our results indicate that the intracellular amount of this protein decreases intracellularly, probably due to secretion, being the first time that this protein could be implicated in this process. Considering that other members can interact with bacteria, this protein may have a similar role in infection.

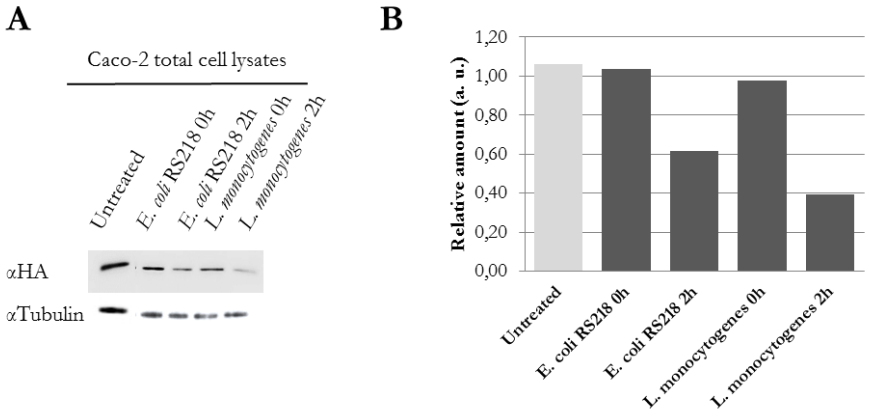


Figure 28: S4D-mCitrine intracellular levels upon Caco-2 infection. A) WB of total Caco-2 lysates from cells infected with *E. coli* or *L. monocytogenes* or untreated at 0 h and 2 h after infection to detect S4D-mCitrine levels using an anti-Ha antibody (top blot) or tubulin as loading control (bottom blot). B) Relative amount of S4D-mCitrine protein to tubulin upon activation, calculated by densitometry analysis of band intensity. The figure reflects one representative assay of multiple experiences made.

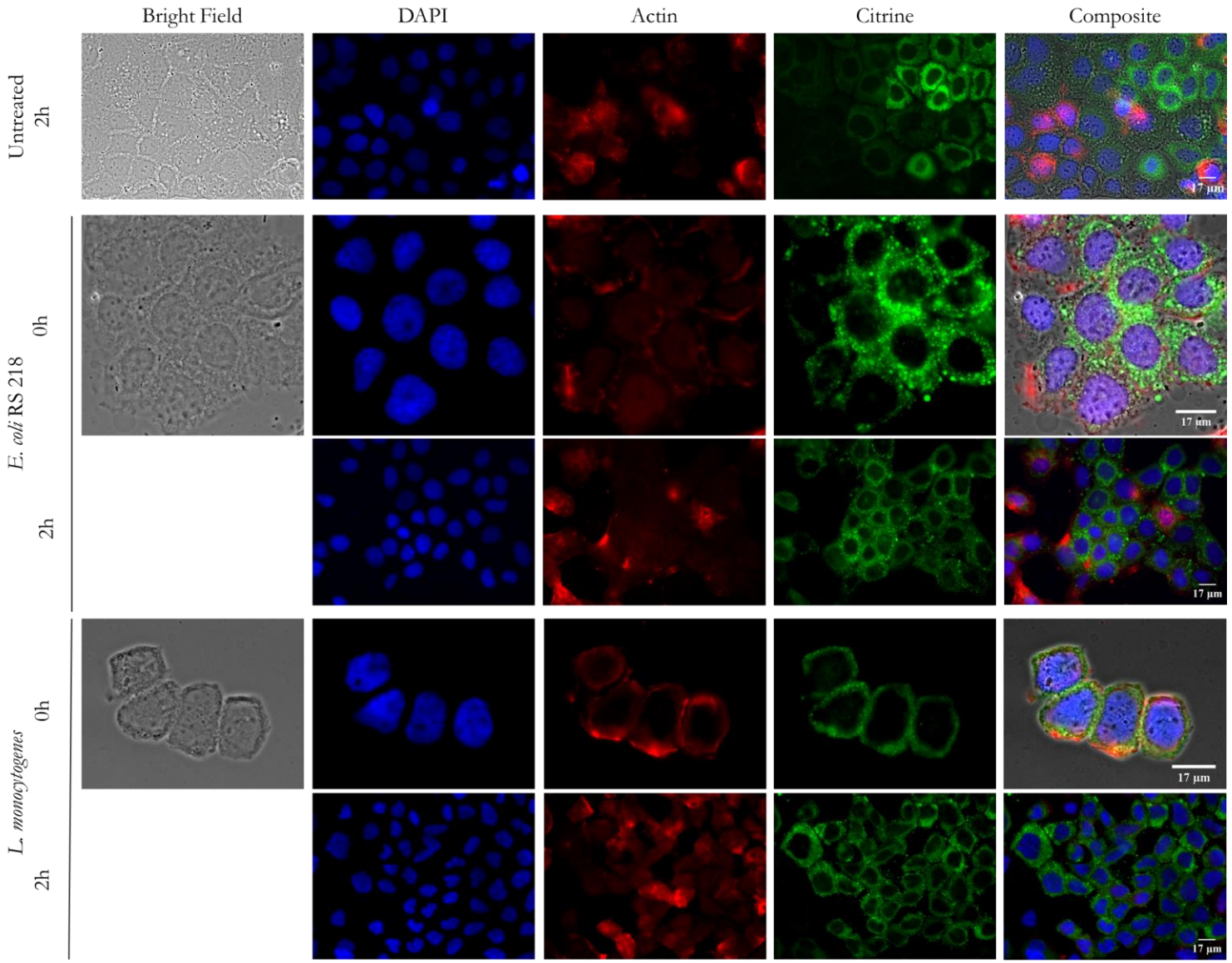


Figure 29: Immunofluorescence analysis of Caco-2 cells expressing S4D-mCitrine after infection. Cells were infected with *E. coli* or *L. monocytogenes* or left untreated, and were analyzed at 0 or 2 h after interrupting the contact between cells and bacteria. Cells were stained with DAPI and phalloidin-TexasRed as a probe to F-actin.

3.4. Activation of Jurkat T cells expressing S4D-mCitrine

S4D-SRCRB mRNA was found in Jurkat E 6.1. To assess the function of this protein in T cells, over-expression was induced, as mentioned above. Similarly to what was performed for understanding the behavior of Spα in T cells under different activations, Jurkat cells transduced with the S4D-SRCRB construct were activated with αCD3/αCD28, PHA, PMA and ionomycin, or left untreated for 6 h. The activation of T cells was confirmed by the analysis of CD69 expression by flow cytometry. As a control, the same experiment was performed with Jurkat cells containing the empty vector (pHR-mCitrine).

6 hours after activation with all treatments, a clear increase in the intracellular amount of S4D-SRCRB was induced, as demonstrated by WB using an anti-HA antibody (Fig. 30 A and B), where activation with PMA and ionomycin induced the higher increase in intracellular levels of S4D-mCitrine. Since the expression of the protein is under a foreign promoter and not the S4D-SRCRB endogenous promoter, this result can only be explained by a diminished secretion of this protein maybe due to intracellular interaction with some proteins, triggered by the activation of the T cells.

The effects of S4D-mCitrine expression in the activation of Jurkat T cells were also analyzed by the expression of the early activation marker CD69. All treatments induced the expression of this marker upon activation in the pHR-mCitrine control cells, confirming the efficacy of the stimuli used (Fig. 30 C and D). However, the activation of Jurkat T cells expressing S4D-mCitrine was impaired after treatment with all the activators, being more evident when PHA and PMA+ionomycin were used.

In basal conditions, Jurkat T cells did not seem to secrete S4D-SRCRB (Fig. 26), and upon T cell activation the protein amount in the cell is increased, which may suggest an intracellular role for this protein in the activation of the T cell. CD5 is capable of inhibiting the activation of the T cell through interaction of kinases involved in the signaling pathways downstream of TCR triggering [67]. In summary these observations raise the question about a possible role of S4D-SRCRB protein in the modulation of T cell activation.

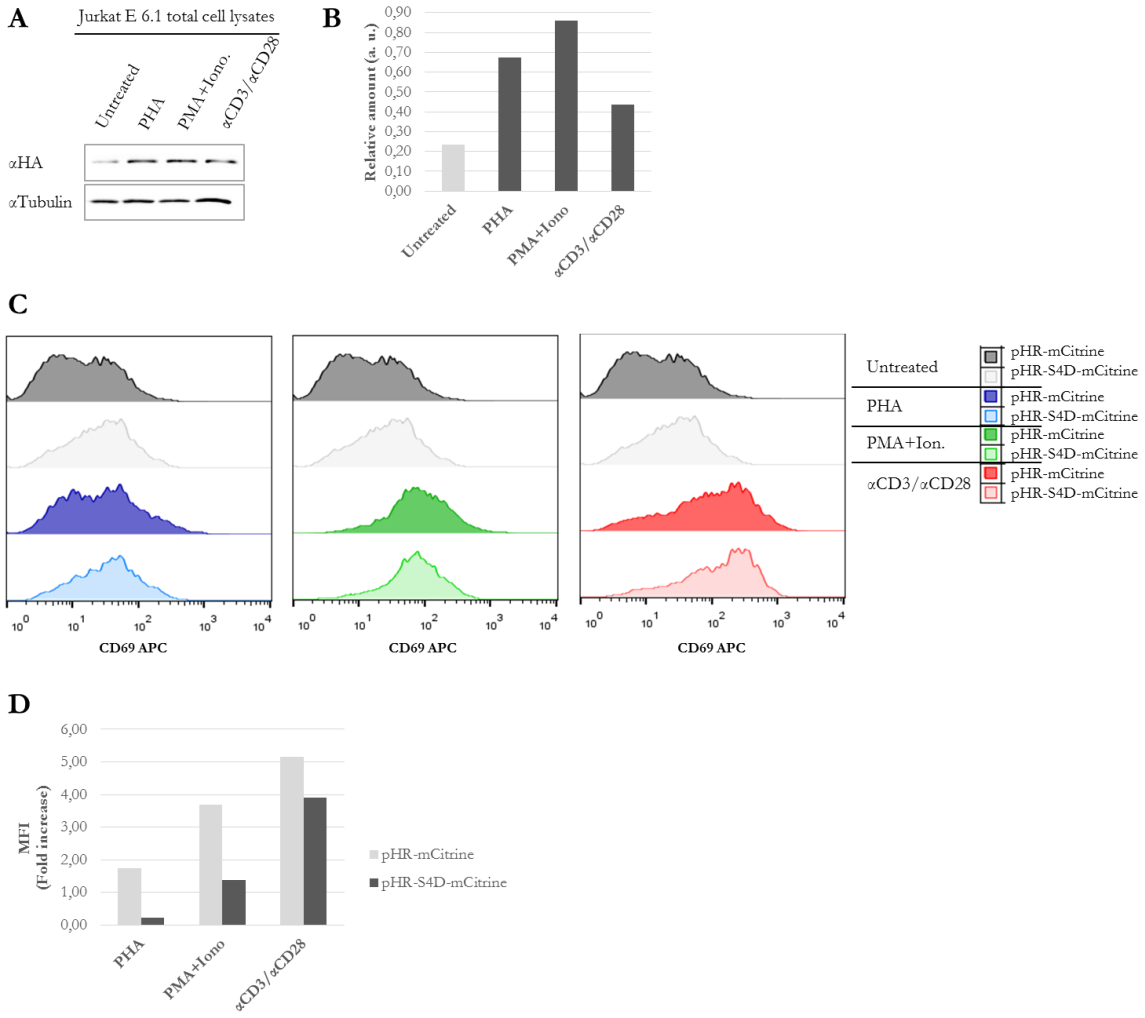


Figure 30: Activation of Jurkat T cells expression S4D-mCitrine. Jurkat T cells transduced with pHR-S4D-mCitrine or pHR-mCitrine as control were activated with anti-CD3 together with anti-CD28, PHA, PMA and ionomycin for 6 h or left untreated. A) WB of total lysates of Jurkat T cells expressing S4D-mCitrine detected with an anti-Ha antibody (top blot) or tubulin as loading control (bottom blot). B) Relative amount of S4D-mCitrine protein to tubulin upon activation, calculated by densitometry analysis of band intensity. C) Activation of Jurkat T cells accessed by analysis of CD69 expression by flow cytometry. D) MFI of CD69 staining of the cells for each treatment. The MFI of CD69 staining for each treatment was normalized to the isotype control and divided to the MFI of untreated cells, thus obtaining a fold change after treatment. The figure reflects one representative assay of multiple experiences made.

4. S4D-SRCRB production and purification for ligand binding assays

To perform the same binding experiments as those described for Spα, the same strategy was employed for S4D-SRCRB. CHOK1 stably expressing this protein with the same structure as recombinant Spα were already available (Fig. 31).

Supernatants from these cells were collected and purified similarly as described for Spα by affinity chromatography using nickel media. However, and despite several attempts to optimize this process, the final result of purification was still unsuccessful characterized by low protein yields and a low degree of purity of the protein (Fig. 32).

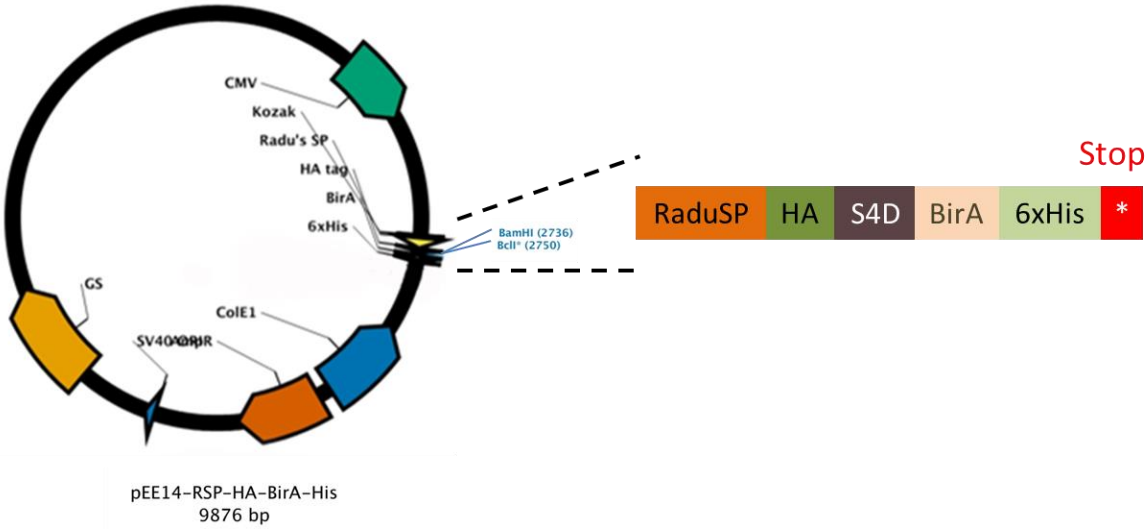


Figure 31: Schematic representation of the plasmid used to produce recombinant S4D-SRCRB in CHO-K1 cells. The S4D-SRCRB cDNA was fused with an engineered signal peptide (RaduSP) allowing the recombinant protein to be secreted to the cell culture media, an HA and 6xHis tags for detection and purification, respectively. The protein also has a BirA recognition sequence in the C-terminal for *in vitro* biotinylation.

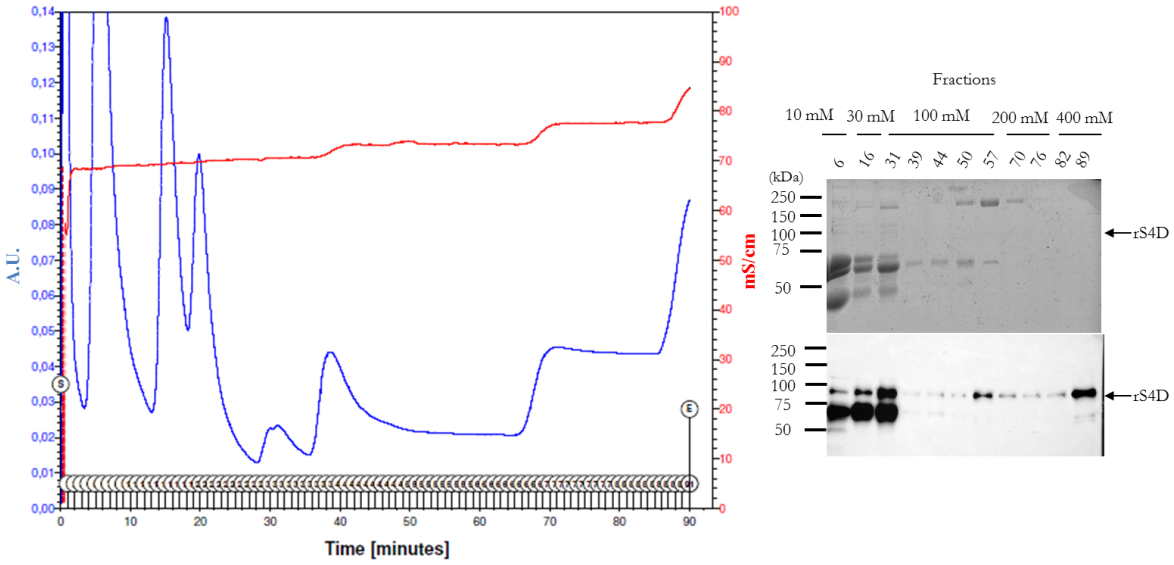


Figure 32: Affinity chromatography purification of S4D-SRCRB. On the left, a chromatogram demonstrating the different fractions collected during the washing and elution steps. S4D-SRCRB was eluted with a 100, 200 and 400 mM imidazole containing-buffer. On the top right panel, a SDS-PAGE containing selected fractions after staining with BlueSafe to check the purity of selected fractions. On the lower right is represented a WB to confirm the presence of this protein.

To overcome this problem, a new cell transfection approach was followed, using HEK 293T cells due to their ease of transfection to produce the protein in a transient manner. Inherent to this method, the production of the protein was made in a shorter time and a smaller amount of supernatant was recovered, which allows for affinity chromatography to be performed using beads in a batch method. To assess the purity of the eluted fractions, an SDS-PAGE was performed together with WB to check whether the protein of interest was present in the elutate (Fig. 33). Unfortunately, although rS4D-SRCRB protein was present, the sample presented a high degree of impurity. Other attempts to purify this protein were made, but none of them were successful and since the contaminants present in the mixture had a similar molecular weight of the S4D-SRCRB protein, it was not possible to use the size exclusion technic. The second objective of this work, the search for possible ligands of S4D-SRCRB protein was therefore not fulfilled.

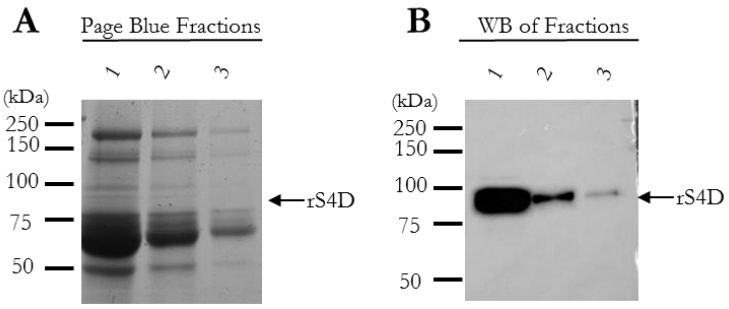


Figure 33: Purification of S4D-SRCRB recombinant protein using a transient transfection. A) SDS-PAGE of the 3 fractions obtained after purification stained with Blue Safe for sample purity assessment. B) WB of the 3 fractions using an anti-HA antibody to confirm the presence of the recombinant protein.

IV. CONCLUSION AND FUTURE PERSPECTIVES

Spα has an important role in the immune system. Results obtained in this work point to a role in innate and adaptive immunity, once we observed for the first time its expression in other cells of epithelial and hematopoietic origin, as well as in serum and plasma, showing that it is not restricted to macrophages as reported. Besides the sites where this protein is expressed, the variation in its expression upon inflammatory (LPS) and anti-inflammatory (DEX) stimuli indicates that this protein has a role in macrophage responses. Besides that, we also demonstrated for the first time that Spα may have a role in adaptive immunity, suggesting that there is a pathway capable of rapidly altering the levels of Spα in T cells by affecting its secretion and/or production upon TCR stimulation. Furthermore, we have here detailed the binding of Spα to a multitude of cell lines highlighting the presence of a putative ligand (s) of protein nature where calcium plays an important role in this interaction. Ongoing work will allow the identification of this ligand (s) using an approach based on the transcriptome of the tested cell lines.

S4D-SRCRB is closely linked to epithelial cells, which may indicate a role in innate immunity. The "dotted" pattern of staining suggests its storage in cytoplasmic vesicles. We have also demonstrated a variation in the intracellular levels of this protein upon infection suggesting a possible role in fighting pathogens at the level of the epithelial barriers. An intriguing finding was the association of S4D-SRCRB with T cell activation, thus unveiling a possible new role to this protein that seemed to be limited to the innate immune system.

This investigation contributed with valuable new information concerning the sites and levels of expression of these two proteins upon different stimuli. Despite this, this work is a first approach for understanding the behavior of the proteins, especially for S4D-SRCRB whose function and characterization remains poorly defined. However, this work requires new experiments to clarify the observations made, especially more efforts should be done to assess whether changes in the intracellular levels are due to increased protein expression or increased secretion. The next step in the study of these proteins would be to dissect the signaling pathways that alter the expression of Spα in macrophages and in T cells. For S4D-SRCRB, new more sensitive methods for detecting endogenous protein are needed. Also, it would be interesting to discover the purpose of S4D-SRCRB expression in tissues like the skeletal muscle that could reveal a structural function for this protein.

In conclusion, although preliminary in essence, this work provided important clues about the functions of these proteins that seem to be broader than initially expected.

V. REFERENCES

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