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SLAM and CD31: Signaling molecules involved in cytokine secretion during the development of innate and adaptive immune responses

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Abstract

Immune cells are modulated through the crosslinking of receptors named ''immunoreceptors''. Ligation of immunoreceptors by their ligands induces a tyrosine-phosphorylation signal that is essential for cell activation or inhibition. Physiologically, immunoreceptor triggering is not enough for cell activation, and stimulation of co-receptors is necessary for antigen-evoked cytokine production. Thus, signal transduction pathways mediated by proteins that regulate cytokine secretion are critical to achieve an effective immune response of the host, where the balance between positive and negative signaling allows effective immune responses, preventing tolerance and autoimmunity. This review deals with recent studies based on the role of the receptor signaling lymphocytic activation molecule (SLAM), a signaling protein that modulates cytokine secretion by immune cells, and the transmembrane glycoprotein CD31, which plays multiple roles in cellular signaling events by modulating the balance between inhibitory and stimulatory signals to immune cells. Recent studies have shed light on the ability of these molecules to transmit different signals that regulate the ability of innate and adaptive immune cells to synthesize stimulatory and inhibitory cytokines.

Keywords: Cytokines; Signaling proteins; CD31; SLAM; Immune response

1. Introduction

During the past years, novel findings increased our understanding of the mechanisms by which cells integrate and modulate their responses to the complex array of environmental signals to which they are exposed. These studies allowed advances in the characterization of costimulatory molecules and inhibitory receptors which are responsible of delivering intracellular signals and modify cellular functions including activation, differentiation and cytokine secretion. In this regard, several advances have contributed to the understanding of the mechanisms by which cells physically organize signaling molecules into multimolecular complexes (through the action of adapter/scaffold proteins and lipid rafts), thus allowing an efficient cross-talk between a wide variety of signals.

Immune cells are modulated following the triggering of receptors termed ''immunoreceptors'' [1,2]. These receptors include antigen receptors on B (BCR) and T cells (TCR), Fc receptors on mast cells and macrophages, stimulatory natural-killer (NK) cell receptors and dendritic cell (DC) receptors. Ligation of some inmunoreceptors by their specific ligands induces a tyrosine-phosphorylation signal that is essential for cell activation [2]. Although immunoreceptors have no intrinsic protein tyrosine kinase (PTK) activity, they associate with subunits bearing immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular domain. ITAMs initiate cellular activation by modulating three classes of PTKs: the src, ZAP70/SYK and BTK families.

Physiologically, immunoreceptor triggering is typically not sufficient for cell activation. In fact, additional positive and negative signals are required to modulate effector cell

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functions. This is particularly true for T lymphocytes, given that these cells must be exposed to at least two different stimuli to trigger an efficient immune response. The first signal is generated by the interaction between the TCR and MHC plus antigenic peptide complex on antigen presenting cells (APC), and ensures the specificity of the activation signal transduced through the TCR. In addition to the interaction between the TCR and MHC–peptide complex, a second signal is crucial to potently stimulate T cells. This second signal is delivered by costimulatory molecules expressed on APCs that interact with their cognate receptors on T cells. Costimulatory receptors expressed on the surface of T cells include CD28 and inducible costimulator (ICOS), as well as the signaling lymphocytic activation molecule (SLAM), and their corresponding receptors on APC [3], which transduce signals necessary to fully activate T cells. Moreover, multiple cell surface receptors contribute to the active down-regulation or inhibition of T-cell responses, such as CTLA-4 and PD-1 [4] or the transmembrane glycoprotein CD31, with a similar counterregulatory role in T-lymphocyte activation [5]. The balance between positive and negative signals allows the immune system to provide control mechanisms that lead to effective immune responses, preventing tolerance and autoimmunity, but promoting clonal expansion of antigen-specific T cells and differentiation into effector or memory cells.

In this scenario, DCs, the most potent APCs of the immune system, regulate both adaptive and innate immune responses. Much of DCs effects are executed at the interface of DCs and lymphocytes, following the integration of multiple activating or inhibitory signals from pattern recognition receptors, cytokine receptors, and co-receptors molecules involved in DC–T-cell communication. Thus, depending on the nature of the pathogenic insult, tissue factors and co-receptors, DCs should orchestrate an effector response that should be appropriate to eliminate intracellular or extracellular pathogens, or to suppress self-reactive responses. Activation of immature DCs through signaling via Toll-like receptors (TLRs), pro-inflammatory cytokines or interaction with co-receptor molecules expressed on activated T cells induces maturation of DCs, modulating their ability to compromise or polarize T-cell responses [6–8].

In this review, we focus on recent advances on the immunobiology of SLAM and CD31, two signaling molecules which have been shown to regulate the amount and pattern of cytokines produced by immune cells during innate and adaptive immune responses.

2. The role of SLAM as a signaling molecule in the immune system

2.1. The SLAM family of receptors

The SLAM family comprises a group of receptors which have been shown to positively or negatively modulate the fate of immune cells [9,10]. This modulatory effect seems to be due to the capacity of these receptors to interact with SLAM-associated protein (SAP)-related molecules, a group of src homology 2 (SH2) domain adaptors that will be discussed later in this review. The SLAM family includes SLAM (CD150), 2B4, CD84, NK-, T- and B-cell antigen (NTBA; also known as SLAMF6 or Ly108 in mice), Ly9 (CD229), and CD2-like receptor activating cytotoxic cells (CRACC or CD319). These receptors are expressed on the surface of a diversity of immune cell types. They show immunoglobulin-like domains in their extracelullar region, a single transmembrane segment and a cytoplamic domain bearing tyrosine-based motifs. Almost all SLAM family receptors are involved in homotypic self-associations through the extracellular domain (except for 2B4 which specifically recognizes CD48).

We will focus here on recent advances concerning the role of SLAM in innate and adaptive immune responses. SLAM is a 70 kDa transmembrane type I glycoprotein of the CD2 subfamily which functions through bi-directional signaling after SLAM–SLAM associations. This receptor is also the lymphoid-specific receptor for measles virus and other morbolliviruses [11]. Splice variants of SLAM-related receptors exist and most of them differ in the cytoplasmic regions, although the biological significance of these variants still remains unknown [12].

2.2. SLAM during innate immune responses

2.2.1. SLAM expression and regulation

SLAM is a costimulatory molecule that mediates CD28-independent proliferation and IFN-γ production of T cells and was originally identified as a lymphocyte activation molecule. However, it has become increasingly clear that SLAM is also expressed on mature DCs and activated monocytes. Importantly, SLAM is different from other monocyte activation markers since its expression on monocytes is readily induced by bacteria-derived ligands of TLRs, but not by single stimulation with inflammatory cytokines [9]. In this regard, it has been suggested that the presence of monocytes-expressing SLAM may represent a sign of innate immune cell activation following infection [13], and that these monocytes may costimulate SLAM-expressing T and B cells.

On DCs, SLAM is expressed upon DCs maturation and is up-regulated by IL-1β [14]. Moreover, cell-surface expression of SLAM on DCs was observed by stimulation with TLR agonists, pro-inflammatory cytokines or CD40 ligation [14,15]. Thus, it has been proposed that SLAM expression on mature DC might play a role in facilitating the ability of DCs to initiate inflammatory immune responses by increasing local cytokine concentrations that may impact the nature and magnitude of the adaptive T-cell response [15]. In contrast, other studies showed up-regulation of SLAM following simultaneous treatment of

DCs with lipopolysaccharide (LPS) plus IL-10, suggesting the possibility that SLAM may be a negative regulator, with a complex role in regulating immune functions [16]. Whatever the case may be, the regulation of SLAM expression either on DCs or other cells interacting with DCs may have strong impact on DCs functions. In addition, SLAM levels on APCs would be sensitively modulated by inflammatory mediators or other tissue-specific factors [17].

2.2.2. SLAM signaling

SLAM can also regulate APC functions. In fact, it has been demonstrate that SLAM engagement in CD40L-activated DCs, augments IL-12 and IL-8 production, but not IL-10 secretion suggesting a pro-inflammatory effect of SLAM activation [15]. Furthermore, SLAM ligation in mouse peritoneal macrophages triggers the production of IL-6 and IL-12 [18], whereas in SLAM-deficient mice, IL-12 production by LPS-stimulated macrophages is markedly reduced and IL-6 is increased [18]. However, a recent study that avoided the use of anti-SLAM monoclonal antibodies, showed that SLAM–SLAM interactions inhibit CD40-induced signal transduction in DCs. This effect was not seen in earlier studies using specific neutralizing antibodies [17]. The results demonstrated that CD40L-induced IL-12, TNF-α and IL-6 production by DCs was potently inhibited by SLAM engagement. Consequently, DCs that matured in the presence of sustained SLAM–SLAM interactions were less potent inducers of differentiation of naïve T cells into IFN-γ-producing Th1 effector cells. In contrast, when the effect of SLAM engagement on LPS-induced activation via TLR signals was studied, LPS-mediated IL-12 expression was not inhibited in the presence of SLAM–SLAM association. These results suggest that SLAM ligation in DCs could induce a negative feed-back loop on CD40L-induced inflammatory signals, while such a mechanism would not operate to down-regulate inflammatory responses induced by bacteria [17]. Thus, signaling via SLAM–SLAM interactions was postulated to regulate DCs functions in a complex manner that might be different from the previously described effect of SLAM monoclonal antibodies [14,15]. In fact, SLAM ligation using these antibodies on antigen-stimulated DCs from tuberculosis patients increased the synthesis of pro-inflammatory cytokines (Garcia et al., personal communication). Thus, it has been hypothesized that SLAM up-regulation and pro-inflammatory cytokine production by DCs after *M. tuberculosis*-stimulation may allow their interaction with antigen-specific T cells. Then, SLAM–SLAM interactions would increase IFN-γ secretion in the local microenvironment, creating a positive feedback loop (Garcia et al., personal communication). Taken together, these data imply that SLAM can regulate the type of cytokines released by APCs [12], and that SLAM might possibly contribute to the capacity of DCs to stimulate T lymphocytes depending on their maturation and differentiation stage.

2.3. SLAM during adaptive immune responses

2.3.1. Regulation of SLAM expression

SLAM is expressed constitutively on immature thymocytes, peripheral blood $CD45RO^+$ T cells, T-cell clones, and on a proportion of B cells [9,19]. SLAM is rapidly induced on naive T cells after activation and up-regulated in all T-cell subsets following activation [19]. Accordingly, T-cell surface expression of SLAM in tuberculosis patients is directly correlated with responsiveness of these patients to *M. tuberculosis* antigens [20].

Furthermore, the investigation of the role of SLAM in leprosy demonstrated that two factors might be involved in regulating SLAM expression: TCR activation and IFN-γ production. Tuberculoid leprosy patients, whose T cells proliferate and rapidly produce IFN-γ in response to *M. leprae*, up-regulate SLAM in response to the pathogen. In contrast, lepromatous patients whose T cells show only weak proliferative and Th1 responses to *M. leprae*, do not up-regulate SLAM under the same conditions. However, co-culture of PBMC with IFN-γ and *M. leprae* did up-regulate SLAM expression in lepromatous patients up to the level of tuberculoid patients, suggesting that IFN-γ production is critical for induction of SLAM expression [21]. Lesions from tuberculoid patients contained antigen-responsive, IFN-γ-producing T cells resulting in SLAM expression, whereas lepromatous lesions contained *M. leprae*-unresponsive T cells, lacked IFN-γ production but expressed Th2 cytokines, including IL-10 [22], a cytokine that has been shown to inhibit SLAM expression on activated T cells [23]. The relative amounts of SLAM expression may vary during lymphocyte activation [24]. For example, pro-inflammatory Th1 cytokines increased SLAM levels in patients with mycobacterial diseases [20,21], whereas Th2 cytokines decreased SLAM expression [23]. Hence, expression of SLAM can be tightly modulated by the cytokine microenvironment during T-cell activation and differentiation.

2.3.2. The SLAM-SAP signaling pathway

Although T-helper cells go through a differentiation process that ''programs'' their cytokine production upon TCR stimulation, additional factors can influence the level and pattern of cytokines produced by activated T cells. One of those factors is SLAM. In fact, ligation of SLAM redirects Th2 responses to a Th1 or Th0 phenotype [25], given that SLAM–SLAM interactions heighten proliferation and support IFN-γ secretion [9,26]. These data suggest that SLAM mediates context-dependent functions in lymphocytes. Surprisingly, in null-mutant mice, SLAM deficiency had little effect on IFN-γ secretion by activated T cells, although it severely compromised IL-4 and IL-13 production [18,27]. However, it was hypothesized that in SLAM-deficient mice, SLAM might be primarily involved in promoting Th2-cytokine secretion, or, as an alternative, that other SLAM family receptors could compensate for SLAM deficiency to promote the secretion of Th1 but not Th2

cytokines [12]. Nevertheless, it should be kept in mind that the results observed in mice might significantly differ from the results obtained in humans.

SLAM displays the ability to interact with SAP, a molecule composed of a SH2 domain and a short C-terminal tail [28]. SAP is expressed on T cells, NK cells, NKT cells, eosinophils, platelets and some B cells [28–30]. SAP is mutated in the X-linked lymphoproliferative (XLP) syndrome, a human immunodeficiency characterized by a dysregulated immune response to Epstein–Barr Virus (EBV) infection. As a result of the alterations in SAP in XLP patients, the polypeptide is absent, unstable or functionally inert. Studies in SAP-deficient mice had shed light on the probable immunological basis of XLP and led to understanding of the role of SAP in normal immunity. These experiments revealed increased IFN-γ secretion and deficient IgE production, either at baseline or after infection with various agents [31,32], suggesting that the lack of SAP expression results in skewing of the immune response toward a Th1 phenotype. Naïve $CD4^+$ T cells from SAP-deficient mice have markedly reduced production of Th2 cytokines in response to peptide–MHC or TCR-complex-specific antibodies [27,31]. These mice also display severely reduced IgE and IgG, decreased germinal-center formation and pronounced loss of long-term antibody-secreting plasma cells and memory B cells [27,29,31,33,34]. On the other hand, activated T cells from mice over-expressing wild type SAP displayed an increase in IL-4 production as well as a decrease in IFN-γ secretion [35]. In addition, $CD4^+$ T cells from XLP patients showed a decrease in IL-10 production and a significant reduction in ICOS expression [12]. Therefore, the abnormalities in cytokine secretion that occur both in SAP-deficient humans and mice, might result from defects in the propagation of SLAM-induced signals [32].

The presence of SAP enables ligand-stimulated SLAM to mediate tyrosine phosphorylation signals. This relates to the ability of SAP to recruit FYNT, a SRC-related PKT. This function is mediated by a direct interaction between the SH2 domain of SAP and the SH3 domain of FYNT. The existence of this binding surface, together with the phosphotyrosine-binding fold of the SAP SH2 domain, allows a single SAP molecule to bind simultaneously to SLAM and FYNT [36,37]. It has been reported that both the relative amounts of SAP and SLAM expression may vary during lymphocyte activation and in some autoimmune disorders [24], and that the ratio of SLAM:SAP abundance could have a role in the context-dependent functions of SLAM [32]. In fact, the differential expression of SLAM and SAP was proposed to be related to the activation state of immune cells [38]. In mice, SAP expression is rapidly down-regulated *in vitro* following activation, whereas SLAM is up-regulated early during T-cell activation. SLAM–SLAM interactions might arise from T cells interacting with other activated T cells, or with DCs [15]. The synergism of SLAM and TCR signaling overcoming the regulatory effect of SAP on IFN-γ production is also suggested by the studies of Howie and colleagues [39]. The authors reported that T cells from $SAP^{-/-}$ mice produced significantly higher levels of IFN-γ than wild-type littermates upon anti-CD3-stimulation, but following stimulation with a combination of anti-CD3 and anti-SLAM antibodies both wild-type and $SAP^{-/-}$ T cells produced significantly higher amounts of IFN-γ [39]. In this regard, a model has been proposed in mice and subsequently demonstrated in patients in which SAP recruits FYNT to SLAM and regulates IFN-γ [36,37]; this effect prevents binding of SHP-2 to SLAM and subsequent signaling for IFN-γ secretion [37] (Fig. 1).

Finally, experiments with $CD4^+$ T cells lacking SLAM, SAP or FYNT, or expressing SAP that is defective in FYNT-binding have provided clear evidence that Th2 cytokine production relies heavily on the SLAM-SAP-FYNT pathway [12].

2.3.3. SLAM in human disease

It has been demonstrated that the activation of SLAM on T cells not only promotes Th1 responses in human disease, but also has the capacity to shift existing Th2 responses towards a Th1-dominant phenotype in HIV-I infected patients [40] and allergic individuals [41]. Further evidence implicating SLAM in directing Th1 cytokine responses in human disease emerged from the detailed study of the XLP syndrome [28], which results from a deficiency in SAP and promotes dysregulation of SLAM signaling.

By studying the SLAM–SAP interactions during human tuberculosis, an inverse relationship between SAP protein expression and IFN-γ production by antigen-stimulated T cells was demonstrated in patients with active disease and also in XLP patients [20]. These data confirm studies in SAP-deficient mice showing that the absence of SAP caused an excessive IFN-γ secretion by T cells [31] (Fig. 1). In contrast to SAP, it has been demonstrated that T-cell expression of SLAM is directly correlated with the responsiveness of T cells to *M. tuberculosis* antigen, suggesting that expression of SAP interferes with Th1 responses, while SLAM expression contributes to Th1 cytokine responses in tuberculosis [20]. These findings, together with the studies in mice, strengthen the hypothesis that SAP attenuates Th1 responses [26]. In this regard, it has been proposed that the regulation of IFN-γ production by signaling molecules in tuberculosis is primarily dependent on T-cell recognition of mycobacterial antigens. T cells responding to *M. tuberculosis* antigen rapidly up-regulate SLAM and these two signals act together to promote IFN-γ production. At the same time, SAP is transiently down-regulated in response to T-cell activation. This cascade of signaling is stalled in antigen unresponsive donors, because lack of T-cell responsiveness prevents up-regulation of SLAM and the existing SAP prevents IFN-γ production. If SLAM ligation is restored (e.g. using an anti-SLAM monoclonal antibody), IFN-γ levels are significantly increased [20] (Fig. 2).

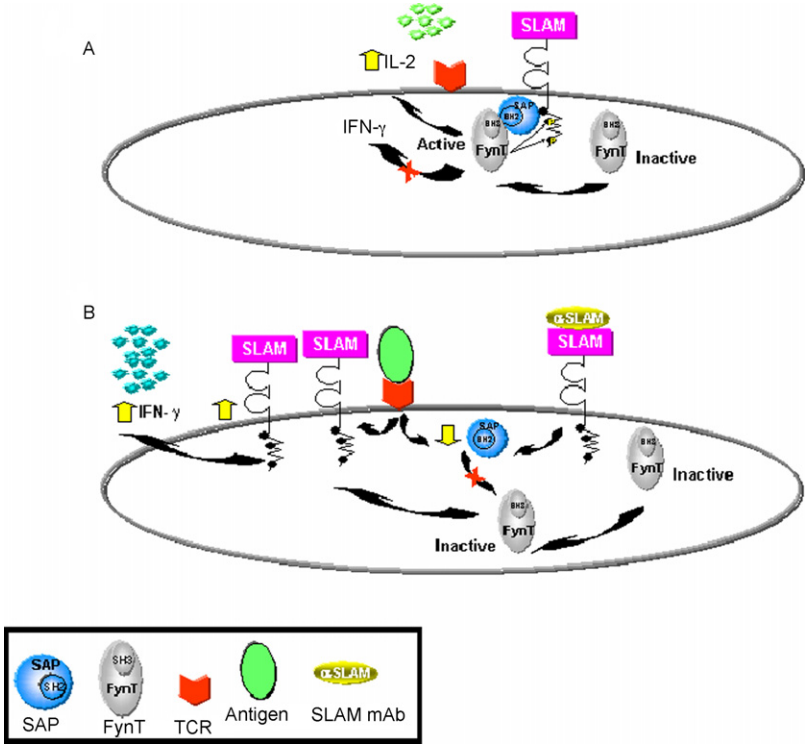

Fig. 1. SAP mediates recruitment of FynT to SLAM in mycobacterial infection. (A) In mice, FynT combines with SAP to form a complex with SLAM that inhibits IFN-γ production [36]. We demonstrated that FynT participates in the SLAM-SAP pathway during human *M. leprae* infection [37]. (B) T-cell activation induced during TCR signaling and SLAM costimulation in antigen-stimulated cells down-regulate SAP expression, preventing the SAP-mediated recruitment of FynT to SLAM and thus allowing IFN-γ production.

It has been also shown that SAP expression on *M. leprae*-stimulated cells from leprosy patients is inversely correlated with IFN-γ production, but SLAM ligation or exposure of cells from lepromatous patients to a proinflammatory microenvironment down-regulated SAP expression [37]. Moreover, SLAM activation induced a sequence of signaling events, including activation of the NF-κB complex, phosphorylation of Stat1, and induction of T-bet expression,

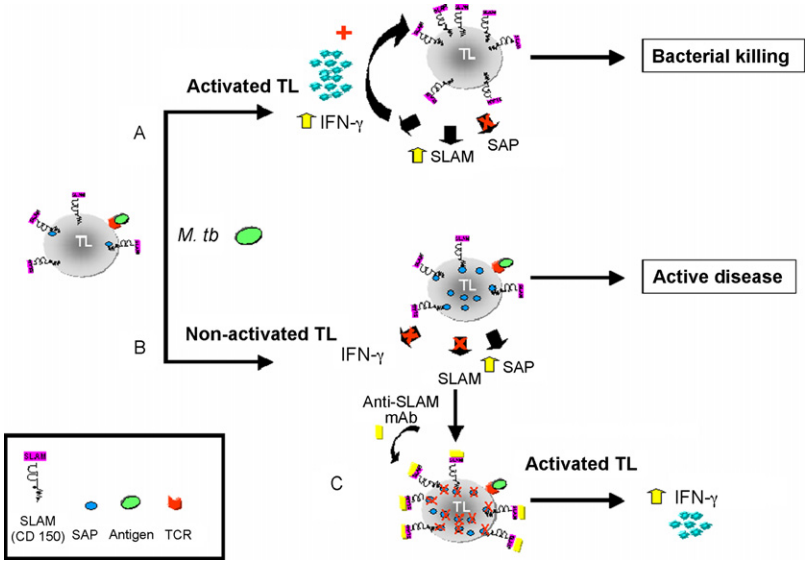

Fig. 2. Regulation of IFN-γ production by the SLAM-SAP pathway in tuberculosis. We propose a model in which the regulation of IFN-γ is primarily dependent on T-cell recognition of antigen. (A) T cells responding to *M. tuberculosis* antigen rapidly up-regulate SLAM and these two signals combine to promote secretion of IFN-γ. At the same time, SAP is transiently down regulated in response to T-cell activation. (B) This cascade of signaling events is stalled in antigen unresponsive donors, because the lack of T-cell responsiveness prevents up-regulation of SLAM and the existing SAP prevents IFN-γ production. (C) If SLAM ligation is restored by anti-SLAM monoclonal antibodies, then IFN-γ levels are significantly increased.

resulting in the promotion of IFN-γ synthesis; this pathway remains quiescent in response to antigen stimulation in lepromatous patients. Taken together, these findings reveal a cascade of molecular events triggered that cooperate to induce IFN-γ production during signaling through SLAM in leprosy [37].

Thus, SLAM might be a potential target for therapeutic modulation of T-cell cytokine responses in diseases characterized by dysfunctional Th2 responses. Caution must be used however, in designing strategies to manipulate SLAM in order to favor Th1 responses since inappropriate expression of SLAM might result in overproduction of IFN-γ and autoimmune pathology. For example, when T cells recognize self-antigen in disorders such as rheumatoid arthritis and multiple sclerosis, these cells experience a significant up-regulation of SLAM expression and IFN-γ production [25,42]. Thus, through the induction of IFN-γ, SLAM can either enhance anti-microbial immunity or promote autoimmunity, depending on the physiopathological context of the immune response.

3. The role of CD31 as a signaling molecule in the immune system

3.1. CD31 (PECAM-1): a versatile receptor

CD31 (PECAM-1) is a glycoprotein of approximately 130 kDa. This member of the inmmunoglobulin-superfamily contains six immunoglobulin-like domains, a short transmembrane domain of 19-residues and a 118-amino-acid cytoplasmic tail with alternative splicing of exons 10–16 and potential sites for phosphorylation and post-translational modifications [43]. The large cytoplasmic tail of CD31 has numerous potential phosphorylation sites which upon phosphorylation can interact with phosphatases and kinases, implying a role for CD31 in signal transduction [44]. Variations in molecular weight between different cell types have been found and are thought to be due to glycosylation and splice variants.

CD31 can engage in both homophilic and heterophilic interactions with other cell surface molecules [43]. Active binding sites for homophilic interactions have been proposed to reside within the domains 2–3 and 5–6. The heterophilic interactions have been shown to be mediated by the second immunoglobulin-like domain. The heterophilic ligands proposed include a molecule expressed by parasitized red blood cells [45], the ADP-ribosyl cyclase CD38 [46], and a CD31 ligand on T cells [47].

CD31 concentrates at the junctions of endothelial cells in all vessel types and it is also expressed on bone marrow precursor cells, platelets, monocytes, polymorphonuclear leukocytes, certain subsets of lymphocytes and in some tumor cell lines [48–50]. CD31 expression on endothelial cells is constitutive but there are few factors described that can modulate expression, such as thrombospondin-1, irradiation and hypoxia [43]. Cytokines however can modulate CD31 expression. For example, TNF-α and IFN-γ induce a redistribution of this molecule away from intercellular junctions [51]. However, the combination of both cytokines caused the disappearance of CD31 from the membrane [52]. This effect was related to the internalization and degradation of pre-existent CD31 and inhibition of its synthesis [52]. Thus, mechanisms of endocytosis and recycling can modulate the expression levels of CD31 [53,54].

It is known that the route to endosomes is specified by sorting motifs in the cytoplasmic tails of proteins [55]. Traffic of internalized membrane proteins is determined by a variety of tyrosine and leucine-based sorting motifs. Four types of endocytic sorting signals have been identified: (i) YXXZ/NXXY, where Z indicates one of the hydrophobic amino aids: L, I, V, C or A; (ii) dileucine LL-containing signals; (iii) a phosphorylated serine-rich domain at the COOH-terminus of many G-protein coupled receptor (GPCRs); (iv) a motif involving ligand-induced phosphorylation of serine residues and the ubiquitination machinery [55,56]. Several of these endocytic-sorting motifs can be found in the cytoplasmic tail of CD31, supporting the concept of an endocytosis and recycling mechanism for CD31.

CD31 mediates a range of different functions, including recruitment of leukocytes to inflammatory sites [57], vasculogenesis [58], angiogenesis [59], and regulation of monocyte, polymorphonuclear neutrophil, and T-cell activation [44,60]. In addition, this molecule can regulate the maintenance of adherent junction integrity and permeability, the organization of the cytoskeleton and can also have transcriptional activities and participate in the signaling of different STATs [61]. Therefore, in contrast to previous assumptions, CD31 function is not restricted to its adhesive properties. In fact, it is intimately involved in mediating different signal transduction pathways; this property is mediated by a number of interactions with adaptor molecules, mainly through phosphorylation of specific tyrosine residues located in an ITAM in the CD31 cytoplasmic tail. Moreover, CD31 has also been shown to be associated with other adaptor molecules in a tyrosine-phosphorylation-independent manner [61]. CD31 tyrosine phosphorylation and its ability to associate with the SH2 domain led to the assumption that this receptor could interact with SH2-containing adaptor molecules. Indeed, when tyrosine and threonine residues in the cytoplasmic tail of CD31 are phosphorylated, this effect leads to the recruitment of cytoplasmic signaling and adaptor molecules, including the phosphatases SHP-1 and SHP-2 as well α- and β-catenin. Furthermore, CD31 has also been assigned to the immunoreceptor tyrosine-based inhibitory motif (ITIM) superfamily, since it is characterized by the presence of intracytoplasmic ITIM (I/VxxYxxL/V/Ix > 20I/VxxYxL/V/I) that recruits and activates protein-tyrosine phosphatases [62] and modulates ITAM-dependent signaling cascades

[60]. Interestingly, those ITIM motifs allocate the endocytic-sorting motifs described above.

The ITIM domain is able upon the recruitment of SH2-containing adaptor molecules, to affect a wide range of cellular events. Specifically, ITIM-containing proteins have been implicated in the inhibition of cytokine-mediated signaling, proliferation and cellular activation [61], and attenuation of T-cell receptor-mediated signal transduction [63]. The relevance of CD31 and its inhibitory motifs became clear from co-ligation experiments, since the engagement of CD31 on some tumor cells inhibited cell proliferation [50]. A modulatory effect of CD31 antibody engagement on CD31 has been also described for lymphocytes [64], monocytes, neutrophils [65], NK cells [66] and endothelial cells [67] which leads to protection from starvation-induced apoptosis [68,69]. However, it has been shown that stable transfection of a truncated CD31 gene construct in colon carcinoma cells, results in decreased cell proliferation by increasing programmed cell death [70].

On the other hand, other studies showed that CD31 engagement resulted in proliferation, chemokine and cytokine secretion and up-regulation of CD25 on T cells, a hallmark characteristic of co-stimulatory ITAM-containing receptors [44]. Thus, it appears that the ITAM/ITIM tyrosine core residues 663 and 686 of CD31 may transmit either inhibitory or stimulatory signals, depending on the cell type and the different biological settings [61].

3.2. CD31 as a signaling molecule in the innate immune response

Studies on CD31 as a modulator of cell adhesion were extended to demonstrate that CD31 engagement results in the generation of other activation signals [44]. In neutrophils, ligation of CD31 using plate-coated CD31 monoclonal antibody to domain 1 (PECAM-1.3) and domain 2 (hec7) resulted in activation of these cells, whereas a monoclonal antibody directed against other domains were ineffective. Moreover, when a monoclonal antibody to domains 1 and 2 were used in solution, they were completely ineffective, indicating that cross-linking of CD31 would be necessary for neutrophil activation. Furthermore, soluble CD31 was unable to activate neutrophils either in soluble or plate-bound form [44]. In the same studies, it was shown that monoclonal antibody directed to domains 1 and 2 stimulated TNF-α production by monocytes. As observed with neutrophils, anti-CD31 Fab fragments were ineffective when used in solution and either plate-bound or soluble CD31 were unable to stimulate TNF-α from monocytes [44]. Collectively, these data demonstrate that only engagement of domains 1 and 2 of CD31 may result in successful signaling in leukocytes [44].

The infiltration of polymorphonuclear leucocytes into tissues is a prominent feature of inflammation and the mechanisms of leukocyte recruitment rely on chemotactic factors and adhesion molecules expressed on endothelial cells. By investigating the regulation of the chemokine CXCL8 in DCs by engagement of cell surface adhesion molecules, it has been demonstrated that CD31 participates in the adhesion of immature DCs to the endothelium [71]. Moreover, engagement of domains 1–3 of CD31 decreases the production of CXCL8 by immature/precursor but not mature DCs, which display lower CD31 levels than immature DC [71] and a high level of CXCL8. Furthermore, culture supernatants from CD31-co-ligated immature/precursor DCs (with lower CXCL8 levels) showed a reduced ability to induce leukocyte migration. These data suggest that CXCL8 production by immature/ precursor DCs might be regulated by signaling through CD31 during their migration through the vascular endothelium [71]. On the contrary, activation and maturation signals on DCs will increased the production of CXCL8 by these cells, which is not modulated by CD31 signaling. Thus, mature DCs not only play a key role in the induction of a specific immune response, but may also modulate the innate immune response by facilitating the recruitment of polymorphonuclear leukocytes.

By serving as a scaffolding molecule, CD31 also mediates tyrosine phosphorylation of two members of the Stat family, Stat3 and Stat5 [72]. During acute infection, binding of pathogen-associated molecular patterns (PAMPs) such as LPS and lipoteichoic acids to the TLR family of pattern recognition receptors initiates the acute phase response. Although this innate immune response is necessary for host survival during severe infection, impaired regulation of the acute phase response can lead to septic shock [73], like activation of TLR on macrophages that leads to local release of pro-inflammatory cytokines. Recently, it was demonstrated that CD31-deficient mice were markedly more sensitive to LPS-induced shock as compared to wild-type animals. Moreover, in response to LPS, these mice demonstrated reduced survival, increased vascular permeability and apoptosis in solid organs, elevated serum levels of TNF-α, IFN-γ, MCP-1 MCP-5 and IL-6 and decreased levels of phosphorylated Stat3, indicating a novel role for CD31 in the maintenance of endothelial integrity, prevention of apoptosis and Stat3-mediated acute phase responses that promote survival during septic shock [61].

3.3. CD31 as a signaling molecule during adaptive immune responses

Upon interaction with the specific antigen in the context of major histocompatibility complex (MHC), T cells receive signals through both activating and inhibitory receptors. The relative strength of the signal delivered by these receptors determines whether the degree of stimulation reaches a critical threshold required for commitment to activation. Active mechanisms that prevent or terminate T-lymphocyte responses include different signaling pathways that counteract the initial phase of T-cell activation (e.g. CTLA-4, PD-L1), and regulatory feedback systems whose primary

function is to control the late stages of T-cell proliferation and differentiation [74].

In this regard, a previous study reported that CD31 engagement on T lymphocytes results in costimulation of T-cell proliferation when sub-optimal doses of CD31 monoclonal antibody were used for the assays. Remarkably, this costimulatory effect was accompanied by secretion of IL-2, IFN-γ, TNF-α, TNF-β and several chemokines. However, of the four monoclonal antibodies that were used in this study, only the antibodies directed to domain 1 (PECAM-1.3) and 2 (hec7) was able to activate human T lymphocytes [44]. Later it were suggested that the ITAM/ITIM tyrosine core residues 663 and 686 of CD31 were responsible of generating either inhibitory or stimulatory signals [61].

CD31 signaling in T cells was proposed to be a candidate negative regulatory pathway (similarly to CTLA-4) that increases the threshold for T-cell activation, and prevents stimulation by low-strength TCR signals. In this regard, CD31 signaling might have preferential effects on different T-cell subsets, becoming operative after certain types of antigen exposure, and contributing to the development of different effector functions [75]. The pattern of expression of CD31 on T lymphocytes also suggests a potential inhibitory function for this receptor: the majority of $CD4^+$ and half of $CD8^+$ T cells lose CD31 expression as they make the transition from naïve to memory cells. Accordingly, it has been demonstrated that CD31 cytoplasmic domain becomes tyrosine phosphorylated in response to cross-linking of the TCR or CD31, with subsequent recruitment of the inhibitory phosphatase SHP-2. When CD31 and SHP-2 are brought in close proximity with the TCR, it promotes the attenuation of TCR-mediated release of calcium from intracellular stores [63].

CD31 down-regulates its expression after T-cell activation and maturation into Th1 effector cells [76]. Accordingly, by studying CD31 levels on the surface of T lymphocytes from tuberculosis patients, we found that *M. tuberculosis* significantly decreased CD31 expression upon antigen activation in patients with high cell-mediated immunity (CMI) against the pathogen. In contrast, the antigen promotes an increase in CD31 levels in individuals displaying weak CMI to the bacteria. Moreover, costimulation of *M. tuberculosis*-stimulated cells from tuberculosis patients with anti-CD31 monoclonal antibody significantly inhibits IFN-γ production from T lymphocytes, indicating that CD31 participates in the regulation of IFN-γ secretion against *M. tuberculosis* (Chuluyan and García, personal communication).

4. A potential relationship between the SLAM/SAP pathway and CD31

It is known that CD31 interferes with TCR-mediated signal transduction [63] and SAP inhibits IFN-γ secretion during mycobacterial infection [20,37]. Moreover, it has been suggested that CD31 and SAP might be able to bind to each other [77]. Therefore, the role of CD31 on lymphocyte activation, in the context of its possible interaction with SAP, was investigated to elucidate the pathways that lead to cytokine production during *M. tuberculosis* infection. After

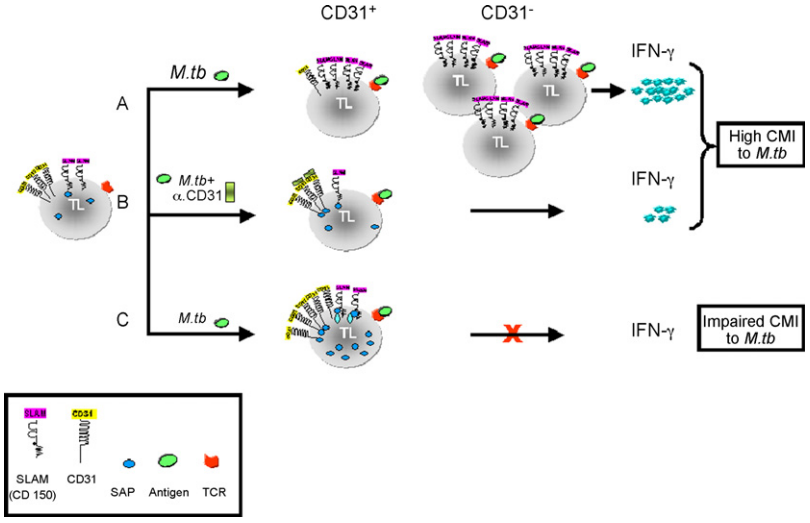

Fig. 3. A model for IFN-γ production in patients with tuberculosis through the signaling of SLAM and CD31. (A) *M. tuberculosis* (*M. tb*) down-regulates SAP in responder tuberculosis patients and decreases CD31 levels, augmenting SLAM expression on the surface of T cells. In turn, $SLAM^+$ $CD31^-$ T cells interact through SLAM producing high levels of IFN-γ against the pathogen. (B) However, if anti-CD31 mAb or recombinant CD31 are added to cell cultures together with *M. tuberculosis*, the antigen is not able to decrease CD31 expression and CD31 engagement decreases the levels of $SLAM^+$ T cells. Thus, some SAP might remain bound to CD31 or/and might recruit FynT and bind to the remaining SLAM, resulting in few $CD31^-$ $SLAM^+$ T cells producing IFN-γ. (C) On the other hand, in unresponsive tuberculosis patients (low CMI to the antigen), *M. tuberculosis* induces a weak signal through the TCR, which can not increase SLAM levels, but is able to up-regulate SAP and CD31 expression, and thus no $CD31^-$ T cells are present. Therefore, the high levels of SAP present are associated with CD31 or have recruited FynT to associate to SLAM, leading to IFN-γ inhibition, since there are no $CD31^-$ $SLAM^+$ T cells capable of producing this cytokine.

a short period of antigen-stimulation, a marked association between CD31 and SAP was found in tuberculosis patients. However, longer antigen stimulation decreases CD31 and SAP in patients with high CMI against the pathogen (responder individuals) but IFN-γ and SLAM were increased. In contrast, unresponsive patients with weak CMI to *M. tuberculosis*, displayed augmented SAP and CD31. Interestingly, these molecules were found to be associated and no significant increase in SLAM expression or IFN-γ secretion was observed after 5 days of antigen-stimulation (Fig. 3).

Lymphocyte subpopulations display differential basal levels of SAP expression in responder patients. Only $CD31^-$ lymphocytes (the majority $SLAM^+$ T cells) produced IFN-γ in response to *M. tuberculosis*. Accordingly, it has been demonstrated that the majority of T helper activity for B-cell dependent IgG synthesis and memory function to recall antigens such as tetanus toxoid, was provided by $CD31^-$ $CD4^+$ T cells [78]. In line with this evidence, CD31-deficient mice showed elevated IFN-γ plasma levels in response to systemic LPS-stimulation [79].

Co-ligation of CD31 in *M. tuberculosis*-stimulated lymphocytes from responder individuals did not decrease IFN-γ, but simultaneous signaling through CD31 and the TCR was able to reduce the levels of SLAM expression and IFN-γ production (Fig. 3). These results show that the regulation of the expression of CD31 and SAP during antigen stimulation is inversely associated with IFN-γ production in a time-dependent manner, indicating that CD31 and SAP may participate in the regulatory pathway that leads to IFN-γ production in activated T lymphocytes. Moreover, these data suggest that $CD31^-$ $SLAM^+$ lymphocytes produce the protective Th1 phenotype against *M. tuberculosis*, and that a cross-talk between CD31 and SAP might regulate IFN-γ production in response to microbial antigen stimulation (Chuluyan and García, personal communication). Supporting these data, it has been shown that T-cell activation is controlled by several inhibitory receptors, including CTLA-4 and PD-L1 [63]. Moreover, CD31 has been shown to attenuate cellular activation stimulated by ITAM-containing stimulatory receptors [63]. Furthermore, TNF-α-stimulation has been shown to regulate CD31 expression on human dendritic cells [71], thus decreasing the levels of inhibitory molecules on the surface of antigen-presenting cells, an event that might enhance T-cell activation during antigen presentation.

5. Conclusion

As a better understanding of the dynamic interactions between positive and negative regulatory molecules (SLAM, SAP and CD31) will be reached, a clear appreciation of these signaling pathways during cytokine production in the immune response of the host will develop. This fast increasing information might lead to consider these proteins as potential focal targets for novel therapeutic approaches aimed at modulating cytokine responses in human disease.

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