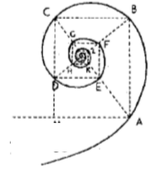

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Src Family Kinase Members Mediate Type I Interferon Production By Plasmacytoid Dendritic Cells

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Abstract

Plasmacytoid dendritic cells (pDCs) are the most powerful type I interferon (IFN-I) producing cells, playing important roles in defense against pathogens, tumor immune-surveillance and autoimmune diseases. Therefore, understanding pDC regulatory pathways to ultimately manipulate them holds great promise for immune-based therapeutics to attenuate several human illnesses. Here we investigated the role of Src Family Kinases (SFKs), a family of non-receptor tyrosine kinases, in pDC response to toll like receptor (TLR) stimulation. We observed for the first time that pDCs from either Fyn or Lyn deficient mice exhibited impaired IFN-I and pro-inflammatory cytokine production after TLR7 and TLR9 stimulation, whereas no difference was observed in conventional DCs. Consistently, treatment with either a pan-SFK inhibitor or a selective dual BCR-ABL/LYN inhibitor (i.e. Bafetinib) profoundly ablated TLR-induced IFN-I production in both a human pDC cell line (CAL-1) and in primary pDCs from human peripheral blood. Furthermore, genetic ablation of LYN by CRISPR/Cas9 in CAL-1 cells resulted in diminished TLR responses. Mechanistically, we showed that the inhibition of SFK activity by Bafetinib reduced the TLR-induced activation of IKKα/β, ERK1/2, P38 and IRF7, key elements downstream pDC TLR signaling, as well as those of MTOR and BCAP, an adaptor protein that bridges PI3K and TLR pathways. Taken together, our data indicate that SFKs, particularly LYN, promote pDC IFN-I and pro-inflammatory cytokine production and suggest that SFK inhibitory drugs, such as Bafetinib, could be considered for attenuating pDC response in autoimmune diseases.

Riassunto

Le cellule dendritiche plasmacitoidi (pDCs) sono le cellule che con più potenza producono gli interferoni di tipo I (IFN-I), e di conseguenza svolgono un importante ruolo nella difesa dai microorganismi patogeni, nell'immuno-sorveglianza dei tumori e nello sviluppo delle malattie autoimmuni. Una più profonda conoscenza dei percorsi regolatori dell'attivazione delle pDCs potrebbe portare allo sviluppo di nuove terapie per alleviare diverse malattie umane. In questo studio, abbiamo valutato il ruolo delle tirosin chinasi non recettoriali appartenenti alla famiglia Src (SFKs) nell'attivazione delle pDCs in seguito a stimolazione attraverso i recettori della famiglia toll-like (TLRs). pDCs ottenute da modelli murini geneticamente deficienti per Fyn o Lyn hanno mostrato una ridotta produzione dell'IFN-I e delle citochine pro-infiammatorie in seguito a stimolazione attraverso TLR7 e TLR9, mentre nessun difetto è stato riscontrato nelle cellule dendritiche convenzionali. In aggiunta, sia il trattamento con un inibitore generale delle SFKs che con un inibitore selettivo per BCR-ABL e LYN (Bafetinib) hanno profondamente ridotto la produzione di IFN-I da parte di una linea cellulare di pDCs (CAL-1) e di pDCs derivate dal sangue periferico umano. Ad ulteriore conferma, cellule CAL-1 in cui il gene di LYN era stato deleto tramite la tecnologia CRISPR/Cas9 hanno mostrato una riduzione nella produzione di IFN-I in seguito a stimolazione. Abbiamo inoltre dimostrato che bafetinib agisce riducendo l'attivazione di IKKα/β, ERK1/2, P38 e IRF7 indotta dalla stimolazione dei TLRs. Inoltre, anche MTOR e BCAP (una proteina adattatrice che collega i pathways di PI3K e dei TLRs) hanno mostrato una riduzione della loro attivazione. Pertanto, i nostri dati mostrano come le SFKs, e in particolare LYN, promuovano la produzione di IFN-I e citochine pro-infiammatorie da parte delle pDCs, e suggeriscono che inibitori delle chinasi della famiglia SRC, come bafetinib, possono essere considerati per l'attenuazione dell'attività delle pDCs nelle malattie autoimmuni.

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1. Introduction

In 1978, Trinchieri and colleagues identified for the first time a subset of non-T cell lymphocytes capable of mediating antiviral immune response and activate natural killer (NK)-cell-mediated cytotoxicity through the production, up to 1000 times higher than any other cell types, of interferon (IFN)-α [1]. Those cells were subsequently referred to as natural IFN-producing cells. However, for long a time they were not better characterized because of their rarity and only in 1999 it became clear that these cells were an effector cell type of the immune system [2,3], and that actually they were the same cell type described for the first time in the 1950s by Lennert and Remmele [4], and referred by pathologists as plasmacytoid T cells or plasmacytoid monocytes [5]. These cells were ultimately termed plasmacytoid dendritic cells (pDCs) due to their ability to mature, acquire dendritic cell morphology, present antigens, and stimulate T cells.

1.1 Plasmacytoid dendritic cells

1.1.1 Phenotype

Human pDCs lack the expression of the common lineage markers: CD3, CD19, CD14, CD16 and CD56. Moreover, they do not express CD11c, a common marker of the myeloid lineage [6]. Instead, they express CD32 (a low-affinity Fcγ receptor), CD123 (IL-3 receptor α subunit), CD4, high levels of human leukocyte antigen (HLA)-DR and several members of the leukocyte immunoglobulin-like receptors (LILR) family, such as LILRA4 (also known as immunoglobulin-like transcript 7, ILT7) and LILRB4 (or ILT3) [3,6,7]. The double positivity of CD123 and HLA-DR has been the historical panel of markers used to identify human pDCs. Recently, it has been shown that pDCs also selectively express C-type lectin domain family 4, member C (CLEC4C, also known as blood dendritic cell antigen 2, BDCA-2), a C-type lectin transmembrane glycoprotein able to internalize

antigens that will be presented to T cells, and Neuropilin 1 (NRP1, also known as BDCA-4), a neuronal receptor of the class 3 semaphorin subfamily [8]. Thus, just BDCA-2 or BDCA-4 alone can also be used to identify human pDCs in peripheral blood and bone marrow.

LILR family and BDCA-2 and -4 are proteins expressed only by human cells and are not expressed on murine pDCs. Consequently, other markers are necessary to identify them in mouse. Murine pDCs are defined as CD11c intermediate, CD11b$^-$, B220$^+$ and BST2$^+$ [6]. Other markers that can be used to identify them are sialic acid-binding immunoglobulin-like lectin H (Siglec-H), Ly6C and Ly49Q (also known as killer cell lectin-like receptor subfamily A member 17, KLRA17) [6,9]. Of note, although BST2 is fairly specific to pDCs and plasma cells in steady-state conditions, it is induced on many cell types following exposure to type I and type II IFNs [10]. Also Siglec-H expression is mainly confined to pDCs, but it is downregulated upon activation [11].

1.1.2 Development

pDCs and classical dendritic cells (cDCs) derive from a common DC progenitor (CDP), identified by lack of lineage markers (Lin$^-$), high expression of Fms-like tyrosine kinase 3 (FLT3) and macrophage colony-stimulating factor receptor (M-CSFR) and low levels of c-KIT. FLT3 ligand (FLT3L) is the most important cytokine involved in pDC and cDC development, controlling the expansion of the common progenitors and the peripheral DC homeostasis [12]. Indeed, Flt3l$^{-/-}$ mice showed a reduced number of pDCs in the lymphoid organs and bone marrow [13]. Whereas Flt3L induces proliferative expansion of CDP, little or no proliferation appears to occur during and after pDC commitment [12]. FLT3 signals through the activation of signal transducer and activator of transcription 3 (STAT3), and drives the expression of E2-2, a transcription factor member

of the E family and the master regulator of pDC development. Deletion of E2-2 in mature pDCs causes the loss of pDC-associated markers and generate a cDC-like cell type, with upregulated major histocompatibility complex (MHC)-II and increased ability to prime T cells [14]. By contrast, the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) activates STAT5 and through the overexpression of the inhibitor of DNA binding (ID) 2 inhibits the pDC developmental program activated by FLT3. ID2, and also ID3, are members of a family of transcription factors that counteract the activity of the E family members sequestering them through the formation of heterodimers [14].

E2-2 allows the transcription of several proteins involved in the development, homeostasis and functions of pDCs, including the transcription factors B cell lymphoma 11A (BCL-11A, necessary for the expression of IL-7 receptor and FLT3 in early hematopoietic progenitor cells), SPIB (important for the differentiation of hematopoietic stem cells into pDCs), IFN-regulatory factor 8 (IRF8, essential for pDC and CD8α$^+$ DC development since its deficiency results in the absence of pDCs and a reduction in the numbers of CD8α$^+$ DCs), Runt-related transcription factor 2 (RUNX2, necessary for the expression of several pDC-enriched genes, among which chemokine receptors CCR2 and CCR5) and class II transactivator (CIITA); the cell surface markers BDCA2, ILT7 and Siglec-H; and the nucleic acid sensors TLR7, TLR9 and the protein kinase C and casein kinase substrate in neurons protein (PACSIN1) [14,15].

1.1.3 Sensing of pathogens

The innate immune system responds to invading pathogens or danger signals through the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). These are detected by pattern recognition receptors (PRRs), such as the membrane

bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) or the cytoplasmic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [16].

pDCs are specialized in the recognition of viral pathogens, which sense mainly through TLR7 and TLR9, respectively a ssRNA and a dsDNA sensor [17] (Figure 1.1).

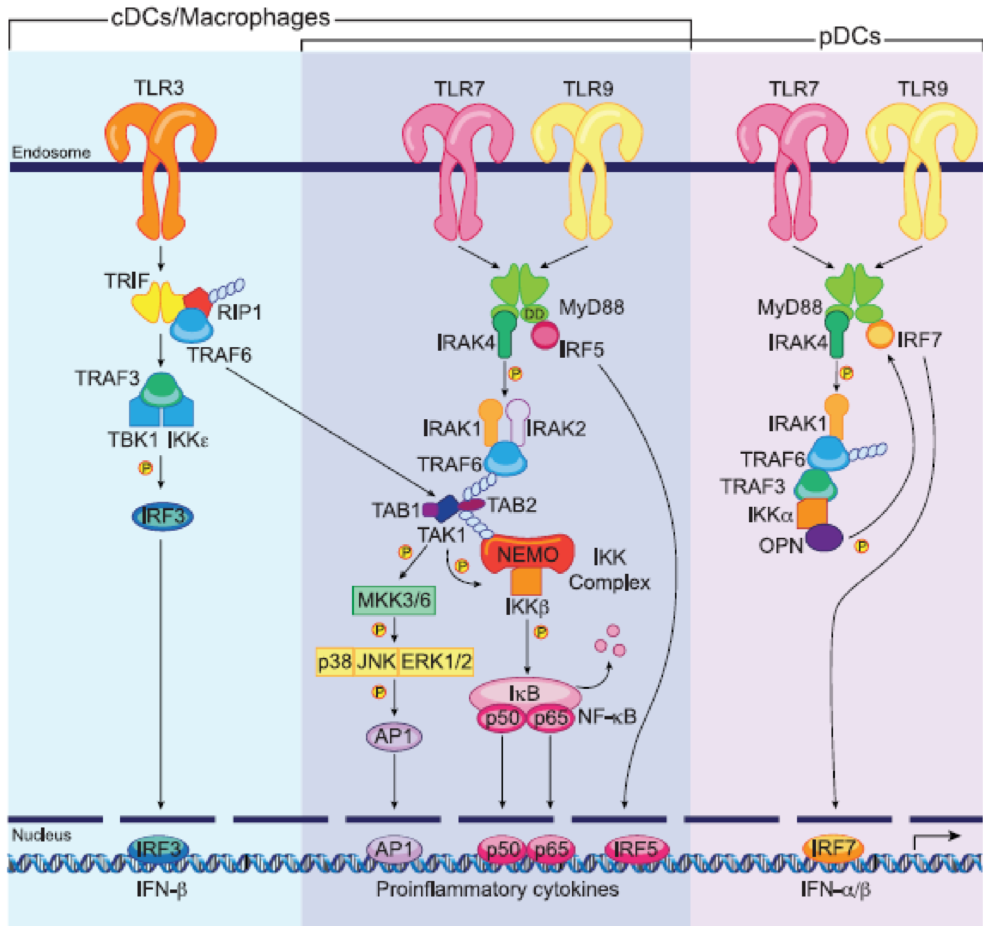

Figure 1.1: Intracellular TLR signaling.

Ligand binding to the leucine rich repeats positioned in the extracellular domain of the TLR is thought to induce a conformational change that allow the interaction between the TLR TIR intracellular domain and MYD88. This adaptor protein, through its N-terminal death domain,

interacts with IL-1R associated kinase 4 (IRAK4), which subsequently phosphorylate IRAK1 and IRAK2. The phosphorylation activates the kinases, which in turn phosphorylate and activate TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase. TRAF6 associates with UBC13 and UEV1A and polyubiquitin itself and NF-κB essential modulator (NEMO, also known as IKKγ). These two polyubiquitin chains are bound by TGF-β activated kinase 1 (TAK1)-binding protein 2 (TAB2), which acts as an adaptor protein and brings TAB1 and TAK1 near TRAF6 and IKKγ. In this way TAK1 can phosphorylate and activate IKKβ, which in turn phosphorylate IκB, leading it to degradation and release of NF-κB. TAK1 also activates MAPKK3 and MAPKK6, resulting in the activation of JNK, P38 and ERK1/2. IRAK1 can also interact with TRAF6, TRAF3, IKKα, osteopontin (OPN), leading to IRF7 phosphorylation and its translocation to the nucleus [18,19].
Figure from "Intracellular Toll-like receptors" by A. L. Blasius, 2010 [18].

TLR7 and TLR9 reside in the endoplasmic reticulum (ER), but they encounter their ligands only in the endosomal compartment. This trafficking is mediated by Unc-93 homolog B1 (UNC93B1), a residing ER protein which directly interacts with the transmembrane domain of intracellular TLRs and mediates their translocation from ER to endolysosomes [20]. Viruses and endogenous nucleic acids end up in the endosomal compartments through endocytic or phagocytic events. Hepatitis A virions enter pDCs bound to T cell immunoglobulin and mucin domain-containing 1 (TIM1) receptors [21], whereas hepatitis C virus through via exosomes released by infected cells [22]. When bound to antibodies, viruses and nucleic acids can enter pDCs through Fc receptors, such as FcγRIIA and FcγRIIB [23–25]. Also Siglec-H and BDCA-2 have been shown to play a role in the antigen uptake [11,26]. Instead, the internalization of large DNA-immunocomplex requires the convergence of the phagocytic and autophagic pathways, a process called microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP), which involved autophagy-related proteins, but not the conventional autophagic preinitiation complex [27]. Irrespective of the route of entrance, the encounter of TLRs with their ligands results in the activation of these

receptors, leading to type I IFN (IFN-I) production via the myeloid differentiation primary response protein 88 (MYD88)-IRF7 pathway and to pro-inflammatory cytokine production via MYD88-nuclear factor-κB (NF-κB) pathway (Figure 1.1) [17,18], but which pathway is activated depends on the endosomal maturation. CpG-A oligonucleotides (ODNs), a synthetic TLR9 agonist, aggregate and form multimeric particles in the early endosomes, which activate the MYD88-IRF7 pathway and consequently induce IFN-I production. Instead, CpG-B ODNs remain in a monomeric state and thereby are transferred quickly to late endosomes, where they trigger the MYD88-NFκB pathway and pro-inflammatory cytokine production [28] (Figure 1.2).

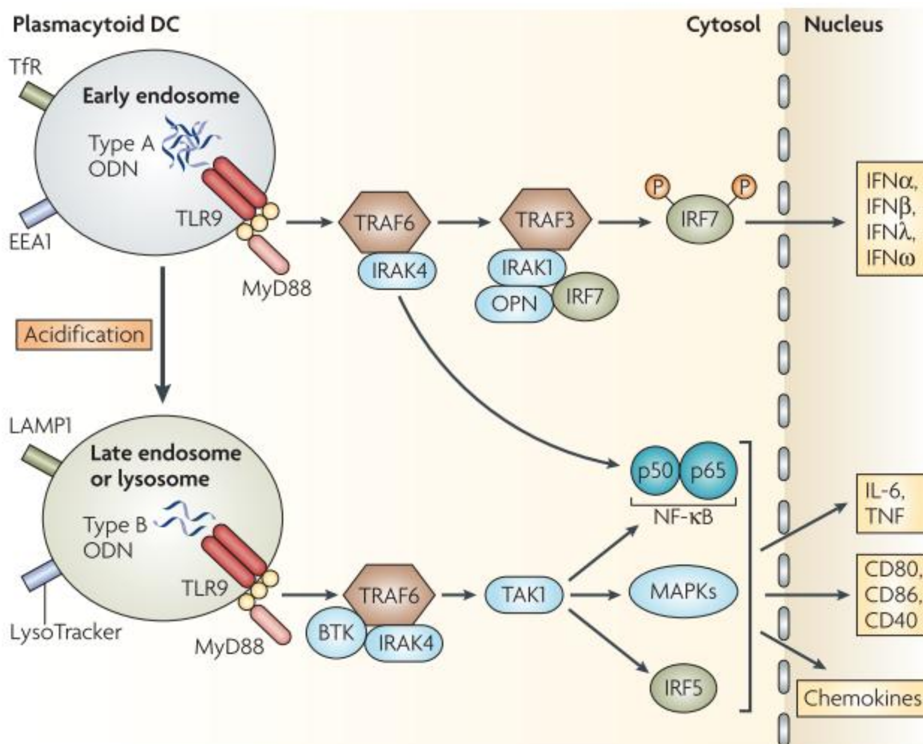

Figure 1.2: Signaling of CpG ODN classes in different endosomal compartments.

Figure from "Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases", by M. Gilliet, 2008 [17].

Although the TLR-mediated detection of invading pathogens elicits the strongest activation, pDCs can also sense replicating viruses in the cytoplasm through RIG-I, a member of the RLR family, and consequently to initialize a TLR-independent IFN-I production [29].

1.1.4 Regulation of TLR response: surface receptors

The regulation of the amplitude of the IFN-I production and, more generally, of the pDC activation state in response to TLR7 and 9 ligands is an important step, since dysregulation of this response can lead to either a defective immune response to pathogens and tumoral cells or autoimmunity [30–33]. Thereby, pDCs express on their surface several receptors capable to influence pDC activation [17]. These receptors usually either contain or couple with an adaptor protein that contain intracellular tyrosine-based activation motif (ITAM) or intracellular tyrosine-based inhibitory motif (ITIM), and the tyrosine in these sequences, once phosphorylated, acts as docking site for different proteins, leading to the activation of different pathways [17].
Human and mouse pDCs express DNAX activation protein 12 (DAP12) and the γ-chain of Fcε receptor (FcεRIγ), two ITAM-containing adapter proteins present at the cell membrane which associate with other surface receptors in order to transmit their signals. In human, DAP12 associates with NKp44 [34], an IL-3-induced receptor that after engagement inhibits pDC IFN-α production, whereas in mouse it couples with Siglec-H [35], again transmitting into the cell a signal that inhibits the TLR-mediated activation. Up to now, there are not any receptors known to associate with FcεRIγ in murine pDCs. However, it is different on the surface of human pDCs. BDCA-2 associates with FcεRIγ and they potently suppress the induction of IFN-I production after antigen capture, presumably by a mechanism dependent on calcium mobilization [8,36]. Also ILT7 decreases the TLR-

mediated pDC activation using the ITAM on FcεRIγ, again presumably through a similar mechanism [7,37]. Interestingly, the ligand of ILT7 is bone marrow stromal antigen 2 (BST2, also known as tetherin), a lipid raft associated protein with anti-viral property which is expressed mainly in response to IFN-I stimulation, suggesting its involvement in a negative feedback loop to tune the IFN-I production [37]. FcεRIγ can also associate with another member of the CLR family, the DC immunoreceptor (DCIR), leading to the inhibition of the TLR9-induced IFN-α production after ligand engagement [38]. The inhibition of pDC activation can be mediated also by immunoglobulin, which are detected by FcεRIα and FcγRIIB, suggesting an important mechanism by which the potentially deleterious effects of IFN-I are prevented during a secondary infection [23]. Finally, dual CD300a/c triggering by crosslinking antibody reduced TNF-α and increased IFN-α secretion by pDCs [39]. However, the signaling events responsible for such effect remain to be determined, given that CD300c contains 3 ITIMs whereas CD300a has a short intracellular domain with a charged amino acid in the transmembrane domain, which may allow the association with signaling adapters as DAP12 and FcεRIγ [39].

Conversely, not many ITIM-containing receptors have been identified on pDC surface, and just few of them have been characterized. Ly49Q, a C-type lectin-like membrane glycoprotein, is highly express on murine pDCs and its binding with H-2K^b, an MHC-I molecule, is essential for the IFN-α and IL-12p70 production after TLR stimulation [40,41], regulating the cytoskeletal architecture and the spatiotemporal trafficking of TLRs [9,42].

Although counterintuitive, all these striking evidences support a positive regulation of TLR signaling by ITIMs and a negative regulation by ITAMs, suggesting that pDCs evolved a peculiar way to exploit these pathways different from other immune subsets, maybe with the purpose to counteract an easy hijacking of the immune response by microbial and viral proteins.

The importance of tyrosine phosphorylation for the pDC activation has been

recently suggested again in a study by Bunin et al., where they showed that the absence of the receptor protein tyrosine phosphatase sigma (PTPRS) in human and of Ptprs and Ptprf, an homologous receptor phosphatase, in mice enhances IFN-I and TNF-α production [43].

1.1.5 Regulation of TLR response: intracellular molecules

In addition to the surface receptors, several intracellular molecules capable to influence the TLR7 and 9 signaling have been identified in pDCs. SCARB2, a scavenger receptor, and phopsholipid scramblase 1 (PLSCR1) are two proteins regulating TLR9 endosomal translocation and, consequently, they influence IRF7 nuclear translocation and IFN-I production [44,45]. Protein kinase C and casein kinase substrate in neurons protein 1 (PACSIN1) and dedicator of cytokinesis 2 (DOCK2) are endocytic adaptors which are necessary for the induction of IFN-I, but not of pro-inflammatory cytokines, after TLR9 stimulation, likely because of their role in the reorganization of the cell cytoskeleton [46,47]. Also Viperin, an IFN-I inducible antiviral protein, favors only IFN-I production, recruiting IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) near the TLRs and inducing IRF7 nuclear translocation [48]. Lastly, Bruton's tyrosine kinase (BTK) affects in a negative way TLR9, but not TLR7, signaling, although the mechanism is not clear [49].
A special mention in the regulation of the TLR-mediated pDC activation is due to the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB, also known as AKT)-mammalian target of rapamicin (MTOR) pathway. Inhibition of PI3K, MTOR or the downstream kinases p70 ribosomal s6 kinases p70S6K1 and p70S6K2 blocked the interaction of TLR9 with the adaptor MYD88 and the subsequent activation and nuclear translocation of IRF7, resulting in impaired IFN-I and TNF-α production [50–52]. The importance of the PI3K-AKT-MTOR pathway in the tuning of pDC activation is due to

the multitude of signals that can regulate it, thereby allowing to a series of factors not strictly related with the immune system, as the cell energy level, the hypoxia status, the cellular stress, the DNA damage, to influence the TLR-mediated activation (Figure 1.3).

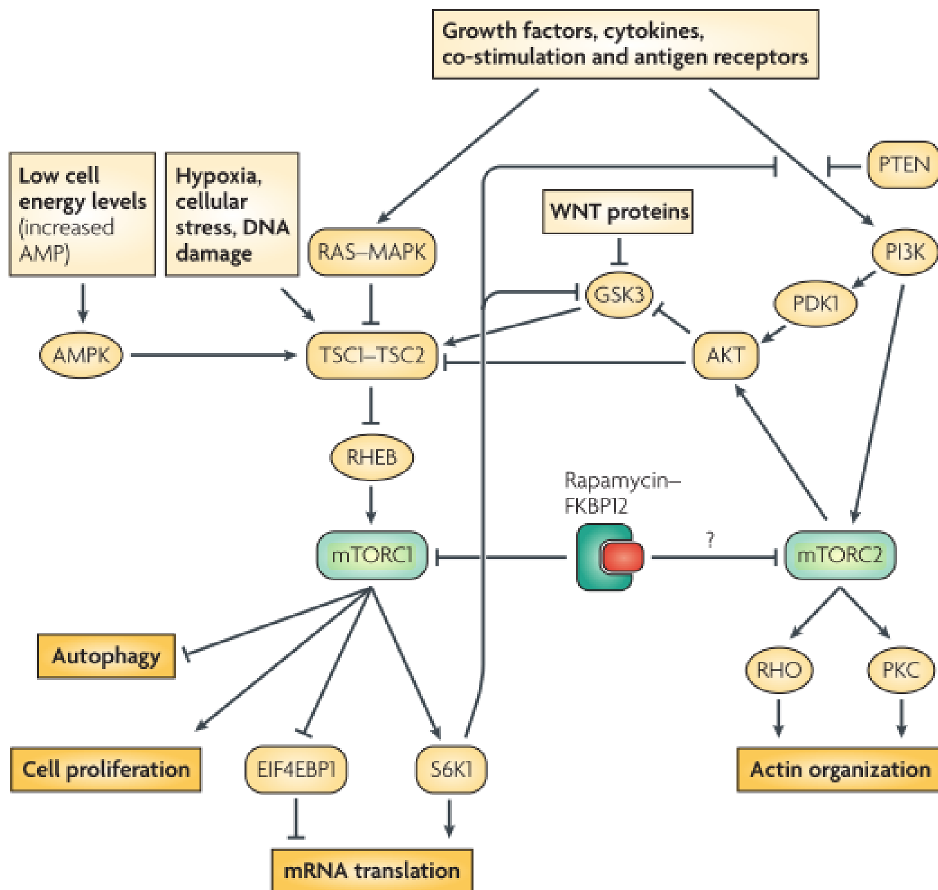

Figure 1.3: Regulators of PI3K-AKT-MTOR pathway.

Figure from "Immunoregulatory functions of MTOR inhibition", by A.W. Thomson 2009 [53].

Although it is not clear how TLR7 and TLR9 can lead to the activation of the PI3K pathway in pDCs, it has been recently suggested a role for the B-cell adaptor of PI3K (BCAP), since it is highly expressed in pDCs and in macrophages it has been shown to be able to interact with MYD88, through

a N-terminal TIR homology domain, and with PI3K, through a phosphorylated tyrosine which binds the SH2 domain of the regulatory subunit (p85) of PI3K [54,55].

1.1.6 pDCs and viral infections

As consequence of the PRRs primarily expressed, pDCs are mainly equipped to respond to viral pathogens.

During acute viral infection, pDC IFN-I production is primarily important during the early phase, whereas later on other cell types, such as macrophages and epithelial cells, become the most relevant source, mainly because they overcome pDCs in number [56]. In addition, also the route of infection is important, since the IFN-I production by pDCs is important during systemic infection, but only secondary during local viral infection [57]. Noteworthy, pDC responses to acute viral infections could be not always beneficial: excessive production of IFN-I during influenza virus infection can result in damage of bronchial epithelium as consequence of uncontrolled inflammation and cellular apoptosis mediated by TNF-related apoptosis-inducing ligand (TRAIL) [58].

The role of pDCs during chronic viral infection has been principally studied in the context of HIV. pDCs can sense HIV through TLR7, activating IFN-I production and trafficking to peripheral lymph nodes and gut mucosa. However, since HIV stimulated pDCs express low levels of maturation molecules, induce a weak T cell response and produce continuously IFN-I, it has been proposed that they may contribute to the chronicity of HIV infection [59]. Indeed, increased IFN-α expression in circulating peripheral blood mononuclear cells (PBMCs) correlates with HIV disease progression, and especially the IFN-α2b subtype is upregulated in HIV patients during the course of the disease [60]. Although this seems counterintuitive, since IFNs are the most important antiviral mediators and are capable to reduce

viral replication through the induction of a whole set of antiviral genes, it is not surprising. Indeed, it is precisely because of their importance as mediators of the antiviral response that current viruses needed to find a way to skew IFN-I activity in their favor to survive. For example, HIV takes advantage of the INF-α-mediated upregulation of TRAIL on uninfected $CD4^+$ T cells, which leads them to apoptosis and contributes to their wane and to the destruction of lymph node architecture, and of p53, which favors the apoptosis of virally infected cells [59].

During chronic HIV infection, circulating blood pDCs have diminished functionality in response to TLR7 and TLR9 stimulation [61,62]. This compromised functionality is thought to occur because of a greater refractoriness to stimulation, since they are exposed to HIV, bacterial DNA (as a consequence of the increased gut permeability) and inflammatory cytokines. Lastly, it is important to mention that HIV stimulated pDCs can also suppress the immune response through the non-canonical activation of NF-κB, which causes IDO expression and T reg cells induction [63].

The beneficial or detrimental effects of IFN-I in chronic viral infection depends also by the timing of action. It has been shown in SIV infection that exogenous administration of IFN-α2a early on increases the expression of antiviral genes and prevents systemic infection, whereas a continuous IFN-α2a treatment induces an IFN-I desensitization, leading to a decrease in the antiviral gene expression and to an increase in the viral load and in the loss of $CD4^+$ T cells [64]. The importance of the time when the IFN-I is produced has been also confirmed in a mouse model. pDC ablation, or just the loss of their ability to signal through TLR7, seems to have a negative effect on T cell priming and viral clearance [65,66]. Indeed, early administration of exogenous IFN-I prevents lymphocytic choriomeningitis virus (LCMV) infection. However late administration has no effects, and, actually, the blockade of IFN-I signaling throughout the infection improves T cell function and diminishes viral burden [67–69].

1.1.7 pDCs and autoimmunity

Considered the ability to produce high amount of IFN-I, pDCs have been suggested to play a role in the pathogenesis of autoimmune diseases that are characterized by IFN-I signature. Among these, the most important in term of prevalence is systemic lupus erythematosus (SLE), and always more compelling evidence are suggesting a role for pDCs in the onset of the disease.

SLE is a chronic disease of variable severity with a waxing and waning course, with significant morbidity that can be fatal in some patients [70]. The etiology of SLE includes genetic, epigenetic and environmental components, with female hormonal factors strongly influencing the pathogenesis. These factors lead to an irreversible break in immunological tolerance manifested by immune responses against endogenous nuclear antigens [71,72] (Figure 1.4).

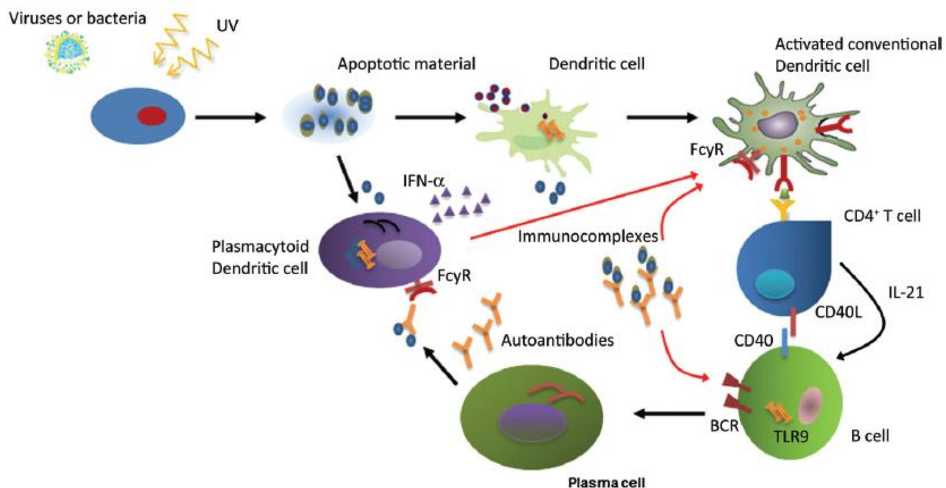

Figure 1.4: Pathogenesis of systemic lupus erythematosus.

Increased production of auto-antigens during apoptosis (UV-related and/or spontaneous) followed by a decreased disposal of these antigens by monocytes/macrophages and a deregulated internalization and presentation by antigen presenting cells, are important steps for the initiation of the autoimmune response. Apoptotic bodies, containing danger elements that can bind PRR as nucleosomes, are internalized by DCs and B cells through Fc

receptors and BCR and drive the production of IFN-I and auto-antibodies, respectively. Moreover, antimicrobial peptides released by damaged tissues such as LL37 may bind nucleic acids, inhibiting their degradation and facilitating their endocytosis by antigen presenting cells, as pDCs, thereby stimulating IFN-I production. Immune complexes and complement activation pathways mediate tissue injury, since the failure to clear immune complexes results in tissue deposition and consequent injury, mediated by the recruitment of inflammatory cells, reactive oxygen intermediates, production of inflammatory cytokines, and modulation of the coagulation cascade. Locally produced cytokines, such as IFN-α and tumor necrosis factor (TNF), contribute to affected tissue injury and inflammation.
Figure from "Systemic Lupus Erythematosus: Pathogenesis and Clinical Features.", by G. Bertsias 2012.

In patients with SLE, pDCs are continuously activated by circulating immune complexes composed by antibodies bound to self DNA and nucleoproteins, which are internalized by Fc receptors and translocated to endosomal compartment where they encounter TLRs [73]. It is important to note that in non-pathological conditions, antibodies bound to DNA and nucleoprotein alone are unable to protect extracellular DNA fragments from the activity of nucleases, as a consequence of the immune system efforts to impede the recognition of self DNA and RNA. However, in autoimmune disease as SLE several host factors, such as LL37 and high-mobility group box 1 (HMGB1), have been implicated in the nullification of these efforts and in the conversion of self DNA into triggers for pDC activation. LL37 is an antimicrobial peptide able to bind self-DNA fragments released by dying cells, forming large aggregates which block the degradation mediated by extracellular nucleases [74]. In addition, the LL37-self-DNA aggregate is retained longer in the early endosomes of pDCs, where TLRs trigger IRF7 pathway [74]. HMGB1 is a nuclear DNA binding protein that is released by dying cells. It binds only multimeric DNA aggregates, and subsequently HGMB1-DNA complexes bind the receptor for advanced glycation end-products (RAGE) on the pDC surface, entering the cells and potently enhancing IFN-I production [75]. The continuous synthesis of IFN-I induces

the activation and the maturation of DCs, which consequently stimulates and activates autoreactive T cells. In addition, IFN-I, combined with IL-6, stimulates the differentiation of autoreactive B cells into autoantibody-secreting plasma cells [72].
Studies in SLE murine models have showed that genetic deficiency or antibody blockade of the IFN-I receptor reduced serologic, cellular, and histologic disease manifestations and extended survival [76,77], confirming that IFN-I is essential to the disease pathogenesis. SLE murine models have also allowed to prove definitively the important role of pDCs in the onset of the disease. Mice lacking the IFN regulatory factor 8 (IRF8), and therefore lacking pDCs, or carrying a mutation affecting the peptide/histidine transporter solute carrier family 15, member 4 (SLC15A4), which turn off the TLR7/9-mediated pDC cytokine production, show almost complete absence of anti-nuclear, anti-chromatin, and anti-erythrocyte autoantibodies, along with reduced kidney disease [78]. However, it is fair to note that IRF8 is not essential only for pDC development and that SLC15A4 seems to be necessary also for TLR7-induced production of antibodies by B cells. Recently, three other studies with cleaner models have strongly proved the involvement of pDCs in the SLE onset. The first study exploited global or CD11c-specific E2-2 haplodeficiency, which impairs the development of pDCs but not B cells, in lupus prone mice and they observed an abolition of the key disease manifestations, including anti-DNA antibody production and glomerulonephritis [79]. The second and the third studies used lupus-prone mice in which pDCs can be selectively depleted in vivo through the diphteria toxin administration, and they showed that an early, transient ablation of pDCs, before disease initiation, resulted in reduced splenomegaly and lymphadenopathy, impaired expansion and activation of T and B cells, reduced antibodies against nuclear autoantigens and improved kidney pathology [80,81]. Taken together, these studies validate the role of pDCs in the onset of SLE, and they also suggest that

pDCs could be considered as disease target.

1.1.8 pDCs and tumors

pDCs have been characterized in a variety of human neoplasms, including carcinomas from different sites, melanoma, and hematopoietic malignancies [82]. Moreover, their presence has also been documented in tumor draining lymph nodes [83]. Although the clinical benefits of IFN-I administration to cancer patients have been shown [84], the presence of tumor-associated pDCs (TA-pDCs) is often associated with poor prognosis, since they tend to be tolerogenic rather than immunogenic. This polarization could depend to the alternative activation of TA-pDCs, which drives the expression of inducible T-cell co-stimulatory ligand (ICOSL) and the production of the immunosuppressive enzyme IDO. The expression of ICOSL on pDCs seems to correlate with breast cancer progression, as a consequence of the amplification of IL-10 production by T reg cells [85]. Also the IDO overexpression has been associated with worse clinical outcome in patients with breast carcinomas, and this because TA-pDCs in breast cancer are poor producers of IFN-I, favoring instead the expansion of T reg cells [86]. An additional tolerogenic mechanism proposed for pDCs is based on the secretion of granzyme B, which, in combination with IL-3 and IL-10, is capable to block T cell proliferation [87].

However, it is important to note that pDCs can still also promote immunogenic antitumor response, if appropriately triggered. Indeed, the injection of pDCs previously activated and loaded with tumor-associated antigens into metastatic melanoma patients leads to improvement in the $CD4^+$ and $CD8^+$ T cell response [88]. It has also been observed in an orthotopic murine mammary tumor model that intratumoral administration of TLR7 agonist results in a TA-pDCs activation with potent antitumoral effects [89]. Moreover, the administration of Imiquimod, a TLR7 agonist, to

a mouse model of melanoma in which the mice are injected with C57BL/6-derived B16 melanoma cell line, stimulates pDCs and mediate tumor regression through the expression of TRAIL and granzyme B and the activation of NK cells [90,91].
These data indicate that vaccination strategy using activated pDCs may be an attractive therapeutic strategy to overcome immune tolerance in certain types of cancer.

Up to now, many immune functions of pDCs have been described; however, there are still a lot of questions about their complex biology that need to be answered. With the recognition of pDC involvement in human pathologies, it became primarily important to increase our knowledge on pDCs biology, especially on how their immune activity is regulated, in order to find new therapeutic strategy for many of the disease scenarios discussed above.

1.2 Src Family Kinases

Src family kinases (SFKs) are non-receptor tyrosine kinases that are involved in several cellular process, as cytoskeletal assembly [92], cell-cell and cell-matrix interaction [93], DNA replication [94], cell survival and proliferation [95], cellular response to external stimuli [96].

This family is composed of nine members, but only eight of them are expressed in mammalian cells: Fyn, Src and Yes are expressed widely throughout the organism, whereas Blk, Fgr, Hck, Lck and Lyn are expressed only by the hematopoietic system [97].

All SFKs share a common structure [97,98]. At the N-terminal there are a glycine (position 2) and a cysteine (position 3) that function as acceptor sites for the addition of myristate and palmitate (palmitoylation does not occur on Blk and Src) [97,98]. These post-translational modifications promote association with the lipids in the membrane, especially at the level of the lipid rafts, and this association is essential to bring the kinases in contact with the receptors and the membrane proteins that are their substrates [97,98]. Then, there is an N-terminal unique domain, the less understood part of SFKs and the only part that highly differs among the family members, thereby it has been suggested to confer specificity. Src-homology 3 (SH3) domain and Src-homology 2 (SH2) domain follow the unique domain [97,98]. SH3 is a sequence of 40-70 aminoacids that recognizes proline-rich motif, and it can be found on a wide variety of proteins, where it favors the interaction among proteins. The solving of the SH3 domain by crystallography and nuclear magnetic resonance spectroscopy revealed a clasped hands interaction, whereby the proline-rich motif assumes a particular helical appearance and interdigitates with pockets and a salt bridge in the SH3 domain [99]. Instead, SH2 domain consists of about 100 aminoacids and recognizes with high affinity

particular sequences bearing a phosphorylated tyrosine [97,98]. Following, there is the catalytic domain, which moves a phosphate group from ATP to the substrate tyrosine [97,98]. Lastly, is the C-terminal regulatory domain. The SFK activity is regulated by two phosphorylations at two different sites [98,100,101]. With regard to Lyn, the phosphorylation at tyrosine 397, positioned into the activation loop of the kinase domain, has an activating effect, whereas the phosphorylation at tyrosine 508, in the C-terminal negative regulatory domain, causes a conformational change that closes the enzyme in an inactive form. The inhibitory phosphorylation is mediated by carboxy terminal Src-kinase (CSK), whereas the activating phosphorylation is, actually, an auto-phosphorylation mediated by the SFK itself. Instead, the inhibitory phosphorylation is removed by Protein tyrosine phosphatase, receptor type, C (PTPRC, also known as CD45), whereas the activating phosphorylation can be removed by Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) and PTPN6 [102] (Figure 1.5).

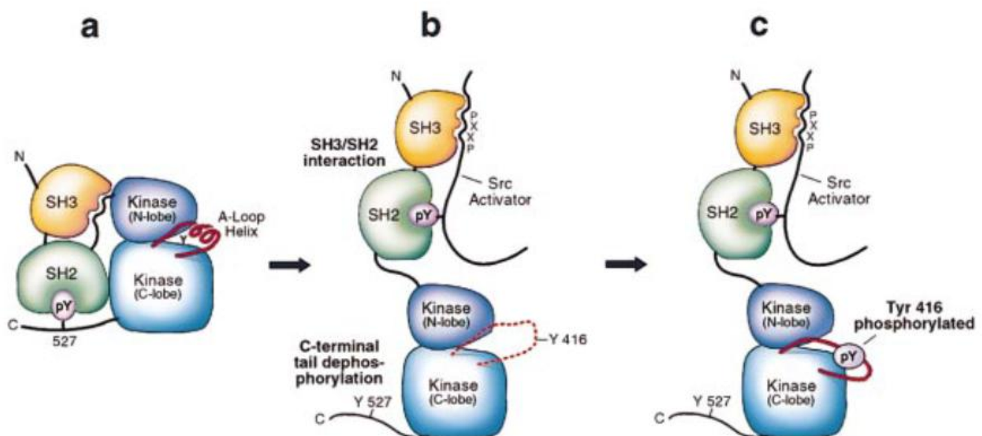

Figure 1.5: A model for SFKs activation

A: Intramolecular interactions involving SH2 and SH3 domains restrain SFKsin a closed conformation. In this state, an inhibitory conformation of the activation loop helps disrupt the kinase active site by interfering with substrate. In addition, the A loop helix protects Tyr-416 from phosphorylation. B: The dephosphorylation of C-terminal tyrosine or the competitive

binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, disrupting the A loop helix and exposing Tyr-416 to phosphorylation. C: The auto-phosphorylation at Tyr-416 causes a conformational changes that reorganizes the activation loop, relieving the steric barrier for substrate binding and reconstituting a fully active tyrosine kinase.
Figure from "Crystal Structures of c-Src Reveal Features of Its Autoinhibitory Mechanism", by W. Xu 1999 [101].

The complexity of the SFKs and their interactions with so many proteins related to signal transduction and cellular function place them into a very important position for the regulation of cell proliferation, differentiation, survival, metabolism, and other essential functions of the cells. One of the critical roles of SFKs is to regulate the inflammatory response.

1.2.1 SFKs and activating pathways

The engagement of the prototypical activating immunoreceptor leads to the SFK activation, with consequent phosphorylation of the ITAM. This motif, which the consensus sequence is YxxI/Lx$_{(6-12)}$YxxI/L, is present in the cytoplasmic tail of the activating immunoreceptor. Some immunoreceptors do not encode an ITAM, but instead they bear a positively charged transmembrane aminoacid, which mediates association with negatively charged transmembrane residues of ITAM-containing associate proteins, as the CD3γ/δ/ε chains for T cell receptors, the Igα/β chains for B cell receptors, the FcRγ chain for Fc receptors and DAP-12 for activating NK receptors [103]. The activation of SFKs is mediated by CD45, which de-phosphorylates the tyrosine in the C-terminal end [97,98]. Although the mechanism is not completely clear, the SFKs localization into the lipid rafts [104,105] and the fact that the disruption of the raft structures by pharmacological cholesterol depletion strongly reduces signaling [96,106], lets us suppose that this interaction takes place within the lipid rafts. In addition, the engagement of the immunoreceptor with its ligand causes a conformational change that favors the receptor complex dislocation into the

raft and, as a consequence of the kinase activation and the proximity with the immunoreceptor, the associate proteins undergo tyrosine phosphorylation into the ITAM sequences [97,98]. This leads to the recruitment of other kinases, usually spleen tyrosine kinase (Syk) or Zeta-chain-associated protein kinase 70 (Zap-70), which use the phosphorylated tyrosines in the ITAM sequences as docking sites for their SH2 domains [97,98]. The binding, and also another phosphorylation mediated by the SFKs [107,108], activates the kinases, which in turn phosphorylate various other scaffold proteins, such as linker of activated T cells (LAT), B cell linker (BLNK), lymphocyte cytosolic protein 2 (SLP-76) [97,98]. These proteins are the base from which the different pathways begin, leading to changes in cell morphology, transcriptional activity, proliferation, cytokine secretion and antibody secretion in lymphocytes or phagocytosis and degranulation in myeloid cells (Figure1.6).

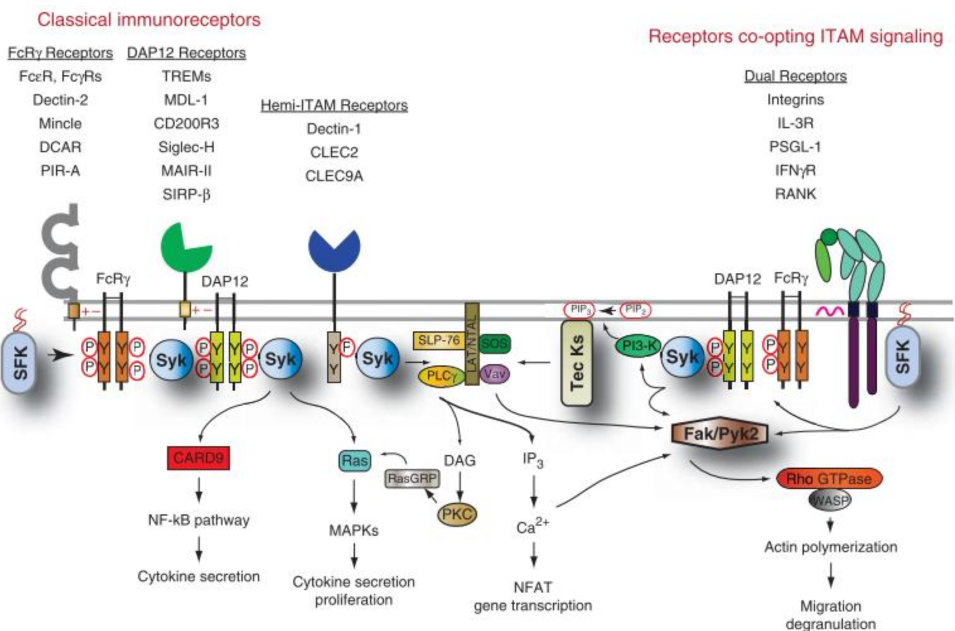

Figure 1.6: Activating signaling pathways utilizing ITAM-containing adapters. “Classical immunoreceptors” refers to those signaling molecules that are directly couples to ITAM adapters FcRγ or DAP12 through transmembrane charged residues. To the right are

shown examples of receptors that link to or co-opt the ITAM pathway, as leukocyte integrins. For all these receptors, it remain unclear how they are coupled to the ITAM adapters. Despite the difference in the coupling of these receptors to the adaptor proteins, the overall signaling events that follow receptor engagement by their respective ligands are similar, and as first step expects SFKs activation and phosphorylation of ITAM, leading to docking sites for Syk/Zap70 kinases.

Figure from "Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk", by C. A. Lowell 2011 [109].

1.2.2 SFKs and inhibitory pathways

Negative regulators in the form of receptors bearing one or more ITIM, whose consensus sequence is S/I/V/LxYxxI/V/L, are employed by the immune system [110]. Ligand engagement by these receptors results in ITIM phosphorylation by SFKs and recruitment of phosphotyrosine phosphatases (PTP) such as Protein Tyrosine Phosphatase, Non-Receptor Type 6 (PTPN6, also known as SHP-1) and PTPN11 (also known as SHP-2), or the inositol polyphosphate-5-phosphatase (INPP5, also known as SHIP-1). The PTP recruitment results in dephosphorylation of activation pathway effectors, as those described before [111,112], whereas SHIP-1 recruitment results in the prevention of calcium signaling and protein kinase C activation through the dephopshorylation of the phospholipase C substrate: phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [112].

The realization that SFKs play an important role in the activation of inhibitory signaling was reached during studies with $Lyn^{-/-}$ mice. These mice show a peculiar phenotype, with hyper-responsive B cells, increased Ig levels and autoimmunity, as a consequence of an exaggerated activating response due to a reduced phosphorylation of ITIM-containing receptors [113,114]. In addition, $Lyn^{-/-}$ mice lose also an important inhibitory loop used to control the SFK activity in the activating pathways: phosphorylation of phosphoprotein membrane anchor with glycosphingolipid microdomains 1 (Pag, also known as Cbp) by SFKs results in the recruitment of Csk to the

lipid rafts, which in turn phosphorylates and helps to inactivate the kinases [115]. In Lyn$^{-/-}$ mast cells, the decreased activity of Csk on Fyn results in the hyperactivation of the Gab2/PI3K pathway and subsequent increased degranulation [115].

Other than Lyn, also Fgr has been shown to be implicated in negative regulation, since Fgr$^{-/-}$ macrophages show an increased rate of uptake of IgG-coated red cells [116,117] (Figure 1.7).

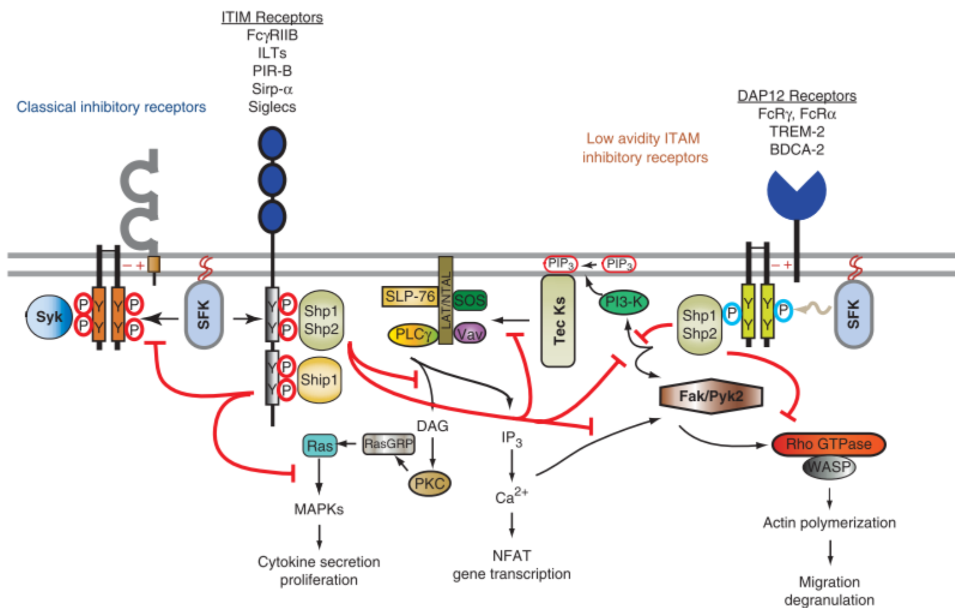

Figure 1.7: Inhibitory signaling pathways utilizing ITIM-containing adapters. To the left are classical inhibitory receptors that contain ITIM binding sequences within their cytoplasmic tails. Inhibitory receptors are often engaged simultaneously with activating receptors, though they recognize distinct sets of ligands. The ITIM domains on these proteins are then phosphorylated by SFKS, which results in the recruitment of tyrosine or lipid phosphatase. To the right are shown examples of inhibitory receptors that functions through “inhibitory” ITAM signaling. This pathway is believed to be engaged following low avidity interactions of these receptors with various ligands, which results in only weak phosphorylation of associated ITAM adapters. The partial phosphorylation of the ITAMs leads to SHP1/2 recruitment instead of Syk, hence engaging an inhibitory response analogous to an ITIM pathway.

Figure from “Src-family and Syk kinases in activating and inhibitory pathways in innate

immune cells: signaling cross talk", by C. A. Lowell 2011 [109].

1.2.3 SFKs and other pathways

The importance of SFKs is not limited to the immunoreceptors. There are several other pathways, within the immune system that exploit this kinase family.

SFKs have been implicated in the downstream signaling of chemokine G protein coupled receptors (GPCRs). In macrophages, both C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) activate MAPK and PI3K pathways through Lyn [118,119]. Also CCR2 and CXCR1 have been shown to require SFK activity for the full activation of their signaling [120,121]. In neutrophils, the combined absence of Hck and Fgr causes a functional defect following formyl peptide stimulation, which signals through GPCR coupled formyl peptide receptor [122].

Several GPI-linked proteins in innate cells, such as CD14 and plasminogen activator receptor (uPA-R), signal through tyrosine phosphorylation mediated by SFKs [123].

The study of knockout mice has underlined also a role for the SFKs in the immune cell integrin signaling. Hck- or Fgr-deficient neutrophils show normal response to the crosslinking of β_2 and β_3 integrins. However, double and triple knockout ($Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$) neutrophils have impaired migration and adhesion [124]. In addition, macrophages from these mice fail to rearrange their actin cytoskeleton when exposed to an extracellular matrix protein-coated surfaces, due to a reduced phosphorylation of the E3 Ubiquitin Protein Ligase c-Cbl and the subsequent reduced recruitment of PI3K p85 subunit to the plasma membrane [125].

Other than integrins, also selectins are dependent on the SFK activity. Recognition of E-selectin by leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) and CD44 leads to the tyrosine phosphorylation that are inhibited

by SFK specific inhibitors [126,127]. In this signaling response, Fgr seems to play the dominant role [127].

1.2.4 Activation and inhibition:- beyond the nomenclature

Unfortunately, the biology of the immunoreceptors is more complex than described. Indeed, we are now beginning to appreciate how ITAM can mediate inhibition and ITIM can mediate activation.

The first evidence about inhibitory ITAM came out from the study of the IgA signaling. For a long time it has been known that, in the absence of the antigen, monomeric IgA exert anti-inflammatory effects (downregulating IgG mediated phagocytosis, oxidative respiratory burst and cytokine release) whereas. multimeric IgA complexes, formed by several monomeric IgA aggregated with their antigen, exert a pro-inflammatory effect [128]. Although also other Ig isotypes can mediate inhibitory signals exploiting ITIM-bearing receptors to fulfill this purpose, no ITIM-bearing IgA receptors have been identified. Lately, this mechanism has been elucidated, and it has been shown that both the monomeric-inhibition and the multimeric-activation needs tyrosine phosphorylation of FcRγ, the ITAM-containing adaptor receptor of the IgA receptor FcαRI [129]. The trick is that monomeric-inhibition induces weakly the phosphorylation of the ITAM and allows the recruitment of both SHP-1 and Syk, whereas the multimeric-activation induces strongly the phosphorylation and results only in Syk recruitment [129]. This positive/negative regulation of the immune cells mediated by an high/low intensity stimulation of the Fc receptor associated with FcRγ has also been extended to IgE and FcεRI [130]. In pDCs, several surface receptors, as BDCA-2 and ILT7, exploit FcRγ to transmit inhibitory signals to the cell, as previously described [7,36].

The ITAM-containing adaptor protein DAP12 associates with several surface receptors to transduce their activating signals. However, DAP12

deficient macrophages produce higher amounts of pro-inflammatory cytokines in response to various TLR and FcRγ receptor stimulations, suggesting the existence of an inhibitory DAP12 signaling [131]. Noteworthy, this DAP12-dependent inhibition appears to be macrophage specific, since DAP12-deficient dendritic cells do not respond stronger to stimulation [131]. Also several pDC surface receptors, as Siglec-H and NKp44, exploit DAP12 to transmit inhibitory signal to the cell, as previously described [34,35].

Interestingly, an hidden ITIM is encoded in DAP12 ITAM, exactly on the first tyrosine of the consensus sequence (<u>s</u>p<u>Y</u>qe<u>L</u>qgqrsdvYsdL, uppercase the aminoacids for the consensus ITAM, underlined the aminoacids for the consensus ITIM). Whether this hidden ITIM can recruit inhibitory phosphatase is still unknown.

As ITAM does not transmit only activating signals, ITIM does not transmit only inhibitory signals.

The ITIM-containing protein TREM-like transcript-1 (TLT1) is present in the granules of resting platelets and its expression on the surface membrane increases after activation. Through its ITIM, TLT1 can recruits SHP-2 and enhance FcεRI-mediated calcium signaling [132].

On murine pDCs, the ITIM-containing receptor Ly49Q has been shown to be essential for the cell activation after TLR stimulation [40].

Therefore, several receptors on pDC surface exploit ITAM and ITIM sequences to signal and regulate pDC immune activity, suggesting a role for SFKs in the pDC biology.

2. Aim of the work

Plasmacytoid dendritic cells are the most powerful interferon type I (IFN-I) producers among the immune cell subsets. As a consequence of this peculiar ability, they play important role in the defense against pathogens, tumor immune-surveillance and autoimmune diseases. Since both a reduction and an increase in the IFN-I production by pDCs influence negatively the immune response and can lead to immune-pathologies, pDCs have evolved a complex system to modulate their activation status. Understanding this tuning and ultimately manipulate it holds great promises for immune-based therapeutics to attenuate several human illnesses. Up to now, it has been shown that several receptors on the surface of pDCs can modulate the cytokine production TLR-mediated, but the downstream pathway that they activate is still unclear. Although, it has been shown that their signaling often start from an immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibition motif (ITIM). Given that phosphorylation of ITAM and ITIM is often mediated by SFKs, a family of non-receptor tyrosine kinases, we decided to investigate the role of SFKs in pDCs biology. To evaluate the SFKs involvement in the pDC activation, we exploited mouse and human genetic models of SFK deficiency and we assessed the ability of pDCs to respond to TLR stimulation by different techniques. In addition, we confirmed our results using chemical inhibitors blocking the SFK activity. Lastly, we sought to identify the mechanisms by which SFKs affects TLR-mediated pDC activation.

3. Materials and Methods

3.1 Animals

Wild type (WT) C57Bl/6 and Fyn$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lyn$^{-/-}$ were a gift from Dr. Kawakami T. (La Jolla Institute for Allergy and Immunology, San Diego, CA). Mice were bred and maintained in a closed breeding facility, and mouse handling complied with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego. Six- to ten-week-old mice were used for the experiments.

3.2 Cell purification

Murine spleen were incubated with Collagenase D (1mg/mL, Roche, Indianapolis, IN) for 20 min at 37°C, passed through a 100 µm strainer in order to obtain a single cell suspension, and subsequently FACS purified using a BD ARIA (BD Biosciences, San Jose, CA) for B cells (CD19$^+$), T cells (Thy1.2$^+$), macrophages (Thy1.2$^-$CD19$^-$NK1.1$^-$CD11c$^-$CD11b$^+$), pDCs (Thy1.2$^-$CD19$^-$NK1.1$^-$CD11c$^+$CD11b$^-$B220$^+$PDCA$^+$), and cDCs (Thy1.2$^-$CD19$^-$NK1.1$^-$ and CD11c$^+$B220$^-$CD11b$^+$ or CD11c$^+$B220$^-$CD8$^+$).

Bone marrow (BM) cells were isolated from femurs and tibias and incubated in RPMI with 10% fetal-bovine serum (FBS) (Lonza containing 100 ng/mL of Flt3L (Amgen, Thousand Oaks, CA) for 8–10 days. At day 8, BM-derived DC were FACS purified for pDCs (CD11c$^{intermediate/dim}$CD11b$^-$B220$^+$PDCA$^+$), and CD11b$^+$ cDCs (CD11c$^+$B220$^-$CD11b$^+$CD8$^-$). Purity for all cell types was >95%.

3.3 Primary human cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from healthy volunteers using Ficoll-Paque PLUS (GE Healthcare). The UCSD Institutional Review Board approved study participants, protocols, and consent forms, and informed consent was

obtained from all participants.

3.4 Culture and cell line maintenance

CAL-1 cell line was cultured in RPMI 1640 (Lonza, Walkersville, MD, USA), supplemented with 2 mM l-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin (from Gibco, Grand Island, NY, USA), plus 10% heat-inactivated FBS (Lonza). LYN-deficient CAL-1 were generated by CRISPR/Cas9-mediated genome engineering following the protocol described by Ran et al. [133]. A target sequence in the fourth exon of human LYN (GTAGCCTTGTACCCCTATGATGG (PAM motif underlined) was chosen and appropriate oligonucleotides were cloned into the BbsI site of pSpCas9(BB)-2A-GFP plasmid obtained from the laboratory of Feng Zhang via Addgene (Cambridge, MA; plasmid ID: 48138). The plasmid was introduced into CAL-1 using the Amaxa human monocyte nucleofector kit (Amaxa), and one day later the GFP positive population was single cell sorted into 96-well plate, in order to obtain clonal populations. Cells were maintained in 175 cm2 flasks at a density of 1–2 x10^6 cells/mL in a total volume of 30 mL.

3.5 Nucleic acid isolation, reverse transcription and real-time quantitative PCR

Cell lines or primary purified cells were harvested in lysis buffer (Qiagen, Valencia, CA). RNA was purified using RNeasy extraction kits with a DNase (Qiagen) incubation step to digest any trace genomic DNA present. For RNA extraction from cell line lysates, 5 x 10^5 cells were extracted using RNeasy mini columns, and for primary cells, 5-10 x 10^4 cells were lysed and extracted using RNeasy micro columns. For standard qPCR analysis of relative mRNA expression levels, cDNA was synthesized using SuperScript III (Life Technologies, Carlsbad, CA) followed by incubation with RNase H

according to the manufacturer's protocol. All cDNA products were stored at -20°C. Quantification of cDNA was performed using SYBR Green PCR Kit or Real-Time PCR Detection System (Applied Biosystem, Carlsbad, CA). The set of primers used for this study is reported in Appendix A. The relative RNA levels were normalized against GAPDH RNA.

3.6 Cytokine measurements

For cytokine measurement in culture supernatants, 5 x 10^4 FACS-purified pDCs, 1 x 10^5 CAL-1 or 3 x 10^6 peripheral blood mononuclear cells were pre-treated for 1 hour with the SFKs inhibitor PP2 (Calbiochem), the inhibitor Bafetinib (Adooq) or the equivalent concentration of DMSO control, followed by 15 hours in media in presence or absence of 0.1 µM CpG-B ODN 1668, 100 µM loxoribine (murine cells) or 1 mg/mL R848 (human cells) (all from InvivoGen). Murine IFN-I bioactivity was measured with reference to a recombinant mouse IFN-β standard (Research Diagnostics, Concord, MA) using a L-929 cell line transfected with an interferon-sensitive luciferase. Human IFN-I bioactivity was measured with reference to a recombinant human IFN-β standard (InvivoGen) using HEK-Blue™ IFN-α/β cell line (InvivoGen). Murine and human TNF-α were measured in culture supernatants by ELISA (Mouse TNF alpha ELISA Ready-SET-Go!, eBioscience, and Human TNF-alpha ELISA MAX, Biolegend, respectively) as described in the manufacturer's protocol.

3.7 Flow cytometry

Prior to staining all cell preparations were blocked with either anti-mouse or anti-human CD16/CD32 (Fc block, BD PharMingen) in PBS containing 1% FBS for 10 min. For surface staining, cells were stained with saturating concentrations of the chosen antibodies in PBS containing 1% FBS for 30 min at 4°C. For intracellular cytokine staining, cells were stimulated at 37°C

for 6 hours and Brefeldin-A (10ng/mL) was added during the last four 4 hours. The staining was performed in the presence of 0.01% of saponin in order to permeabilize the cell membrane. For phosflow staining, the cells were fixed with BD Phosflow Lyse/Fix Buffer 5x for 10 minutes at 37°C, and then permeabilized with BD Phosflow Perm Buffer III for intracellular staining with Abs for phospho-proteins. Cells were acquired using the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed with the FlowJo software (Treestar, Inc., Ashland, OR). Surface marker and intracellular proteins were analyzed on viable cells by LIVE/DEAD® fixable aqua dead cell staining (Invitrogen). The set of antibodies used for this study is reported in Appendix B.

3.8 Cell lysates, immunoprecipitation and immunoblotting

CAL-1 cells were pre-treated with either bafetinib or DMSO control for 1 hours, stimulated with R848 (1 µg/mL) and subsequently lysed with either immunoprecipitation lysis buffer (Thermo Scientific) added with protease inhibitor cocktail set III (Calbiochem), phosphatase inhibitor cocktail set I and II (Calbiochem) or RIPA buffer (Thermo Scientific) added with protease inhibitor cocktail set III (Calbiochem). For immunoprecipitation, 1 mg of each lysate were incubated on a rotator for 1 hours at 4°C with the appropriate amount of the respective antibody. 30 µL of Protein G Sepharose Fast Flow (Sigma) were added, followed by 2 hours incubation on a rotator at 4°C, and were recovered with an equal amount of 4x laemmli sample buffer (Bio-Rad) containing 2-ME. Proteins were resolved by 8, 10, 12% SDS-PAGE, were electrophoretically transferred to PVDF membranes (EMD Millipore) and blocked with 3% BSA, 5% BSA or 5% non-fat dry milk (according to the primary antibody) in Tris-buffered saline containing 0.1% Tween-20; they were then probed with the desired primary antibody and an appropriate HRP-conjugated antibody and eventually

visualized by ECL Plus (Thermo Scientific). When necessary, membranes were stripped using Restore Plus Western Blot Stripping Solution (Thermo Scientific).

4. Results

4.14 SFKs are expressed in murine pDCs and in a human pDC cell line

SFKs play an important role in the regulation of the immune response and therefore they are widely expressed through the immune system populations. Every immune cell type shows a particular SFK pattern, which is used to generate a cell type-specific response to encountered stimuli. Up to now, SFK expression in pDCs has been assessed only for few members, such as Lyn and Fyn [36,134], therefore we decided to assess the mRNA expression levels of all the SFKs in murine and human pDCs and to compare them with other murine naïve immune subsets and human immune system-derived cell lines, in order to outline the pDC SFK pattern. Blk, Lck, Src and Yes were expressed at very low levels in both splenic and BM-derived murine pDCs, whereas Fgr, Fyn, Hck and Lyn were the members mainly expressed (Figure 4.1a). Intriguingly, murine pDC Fyn expression was several folds higher than any other cell types, suggesting that this kinase may be important for pDC function.

To assess the SFKs expression in human pDCs we exploited CAL-1 cells, a recently established [135], but already broadly used [15,43], human pDC cell line (Figure 4.1b). Similar to murine pDCs, CAL-1 cells showed a low expression of BLK and SRC and no detectable expression of LCK and YES. Furthemore, unlike murine pDCs, FGR and HCK were poorly expressed. Also FYN showed a low expression, although this was not surprising since it has already been reported that FYN is poorly expressed by human pDCs [36,134]. Finally, the mRNA levels of LYN were notable.

In conclusion, this data allowed us to outline the pDC SFK expression pattern and showed that Fyn and LYN are the SFK members mainly expressed by murine and human pDCs.

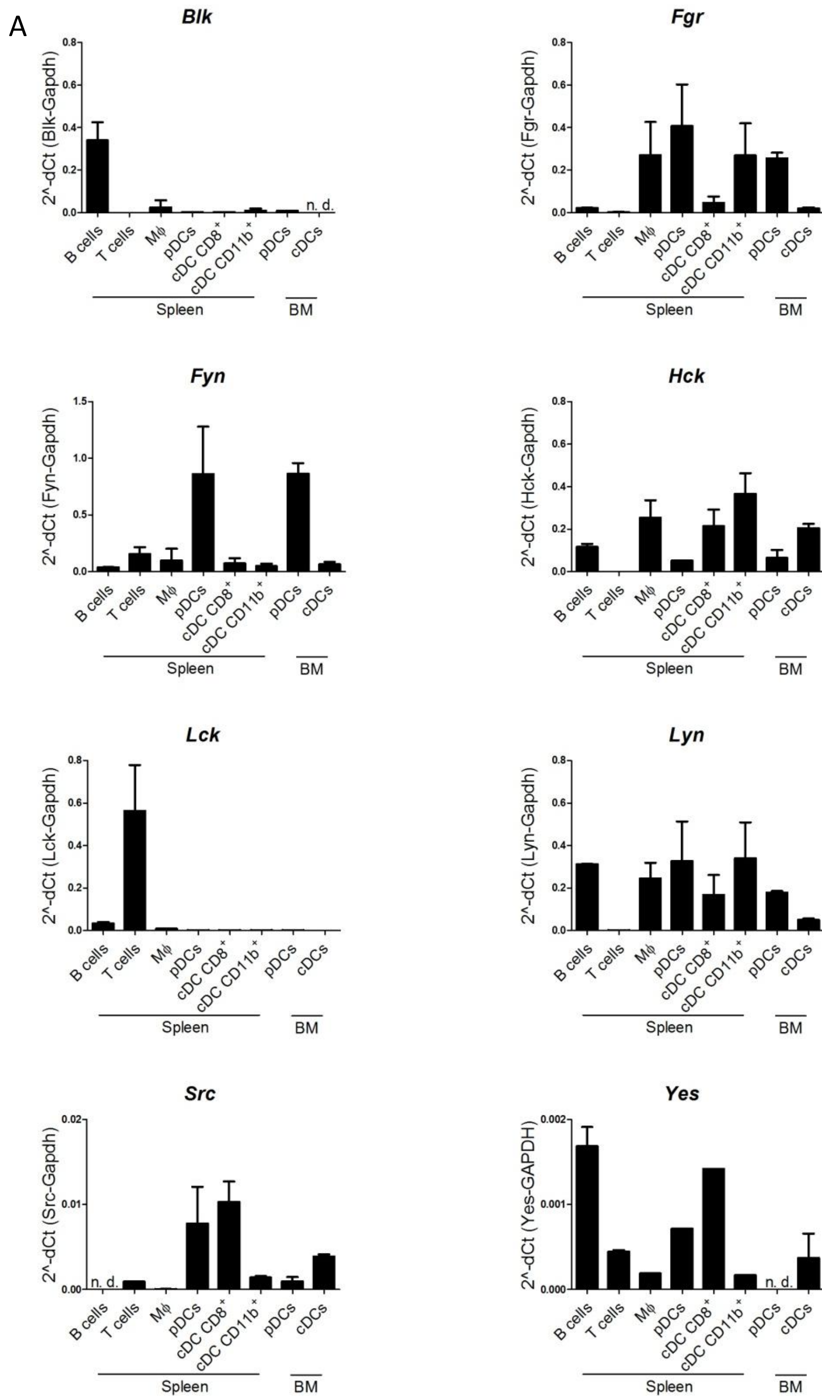

Figure 4.1: SFK expression in murine subsets and human cell lines.

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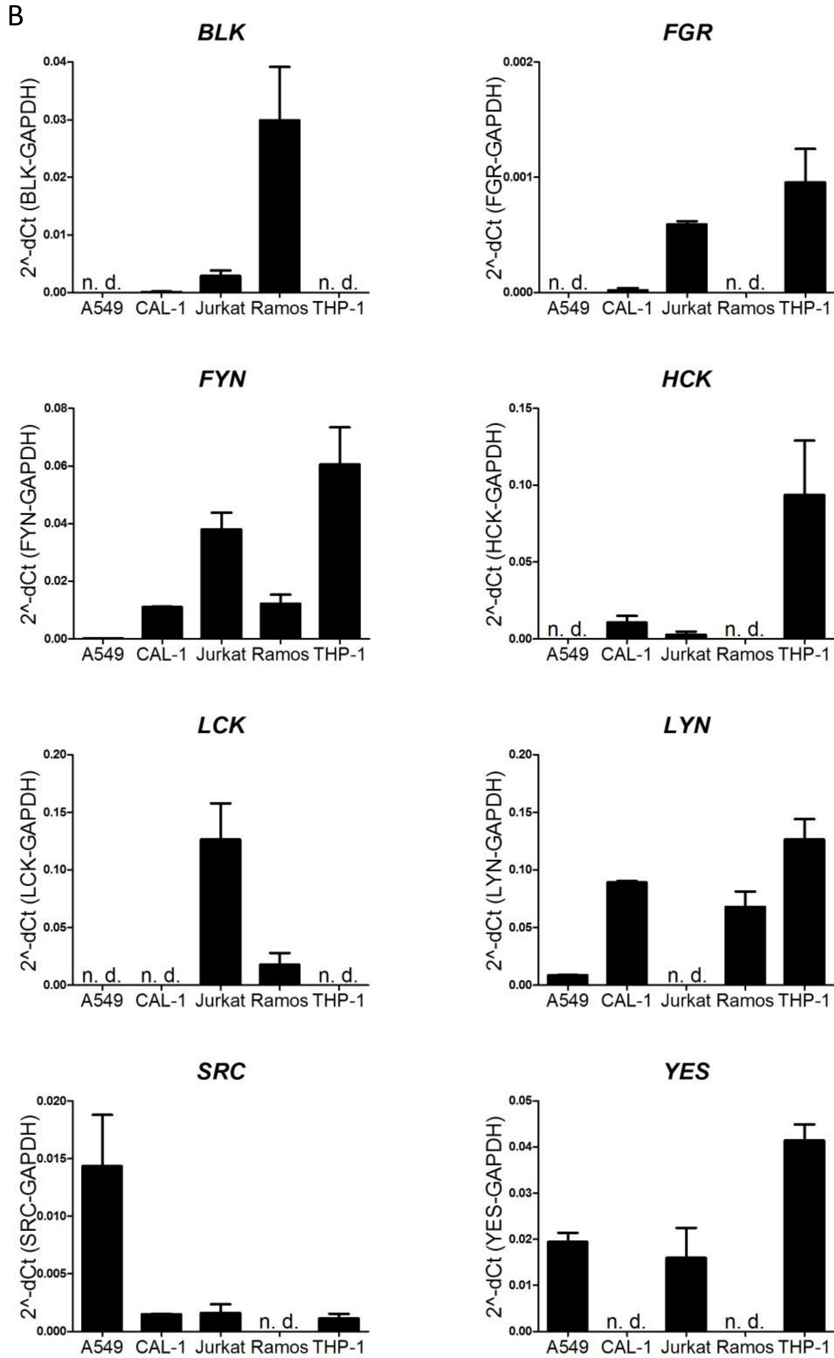

Figure 4.1: SFK expression in murine subsets and human cell lines.

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The expression of SFK mRNA was assessed by SYBR Green qPCR and it was normalized against the expression of GAPDH. **A:** Murine WT splenic mononuclear cells (SMCs) were FACS-purified for B cells (CD19+), T cells (Thy1.2+), macrophages (Thy1.2-CD19-NK1.1-CD11c-CD11b+), pDCs (Thy1.2-CD19-NK1.1-CD11c+CD11b-B220+PDCA+), and cDCs (Thy1.2-CD19-NK1.1- and CD11c+B220-CD11b+ or CD11c+B220-CD8+), followed by total mRNA extraction. Bone marrow from WT mice was cultured in Flt3L containing media for eight days and then FACS-purified for pDCs (CD11c+CD11b-B220+PDCA+) and cDCs (CD11c+CD11b+B220-), followed by total mRNA extraction. **B:** Cells from human cell lines were collected before reaching confluency and then total mRNA were extracted. A549: lung epithelial cell line; CAL-1: human pDC cell line; Jurkat: T cell line; Ramos: B cell line; THP-1: monocyte cell line.

4.2 SFKs are necessary to fully drive the cytokine production by murine pDCs

Confirming a significant expression of SFKs by pDCs, we subsequently evaluated whether they play a role in the pDC ability to sense and respond to invading pathogens. To answer this question, we treated BM-derived pDCs with PP2, a pan-SFK inhibitor, in order to inhibit simultaneously all the family members and to elude the grade of complexity due to the redundant activity among the SFKs, a common practice within this family. Intriguingly, the drug was able to decrease in a dose-dependent manner the IFN-I and TNFα production by BM-derived pDCs stimulated either with CpG-B, a TLR9 agonist, or Loxoribine, a TLR7 agonist, without affecting the viability of the cells (Figure 4.2a), proving that the activity of SFKs is necessary to drive the pDC response after TLR stimulation. However, considered the broad spectrum of action of PP2, this treatment did not allow us to attribute this defect in the cytokine production by pDCs to any single or group of SFKs. Since it has been proposed many times that pDCs exploit a BCR-like pathway [36,134,136], which involved Blk and Lyn as SFKs, and given that murine pDCs showed a very low Blk expression, we decided to investigate whether Lyn was the SFK necessary for the pDC

activation. As hypothesized, we observed a reduced production of IFN-I after CpG-B and Loxoribine stimulation and of TNFα after CpG-B stimulation by BM-derived pDCs from $Lyn^{-/-}$ mice compared to those from their WT littermate (Figure 4.2b). Conversely, no defect was observed in the cytokine production by cDCs, although a small increase in the TNFα production after TLR7 stimulation was observed (Figure 4.2c). To overrule the hypothesis that the pDC reduced cytokine production was caused by a developmental defect, we quantified the relative frequencies of pDCs and cDCs after 8 days of cultivation in presence of Flt3L, and we did not observed any difference in terms of relative frequencies (Figure 4.2d). However, the Lyn deficiency did not totally ablate the IFN-I production as the PP2 administration did, suggesting a redundancy with other SFKs. Although not involved in the BCR-like pathway, Fyn was the SFK most impressively expressed by murine pDCs, thereby we hypothesized its involvement in pDC activation. Consistently with our hypothesis, BM-derived pDCs from $Fyn^{-/-}$ mice stimulated with either Loxoribine or CpG-B showed generally at least a 50% reduction in the production of IFN-I and TNFα compared to WT BM-derived pDCs (Figure 4.2e). Additionally, this defect was restricted to the pDCs population, since cDCs did not show any impairment in the cytokine production (Figure 4.2f). Eventually, as for $Lyn^{-/-}$ pDCs, we did not observe any developmental defect (Figure 4.2g).

A

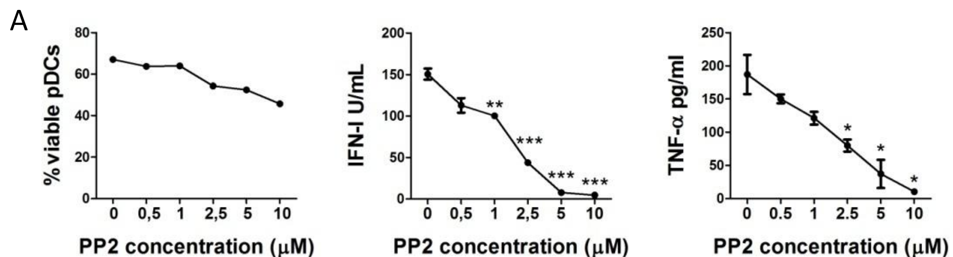

Figure 4.2: SFKs are necessary to fully drive the cytokine production by murine pDCs.

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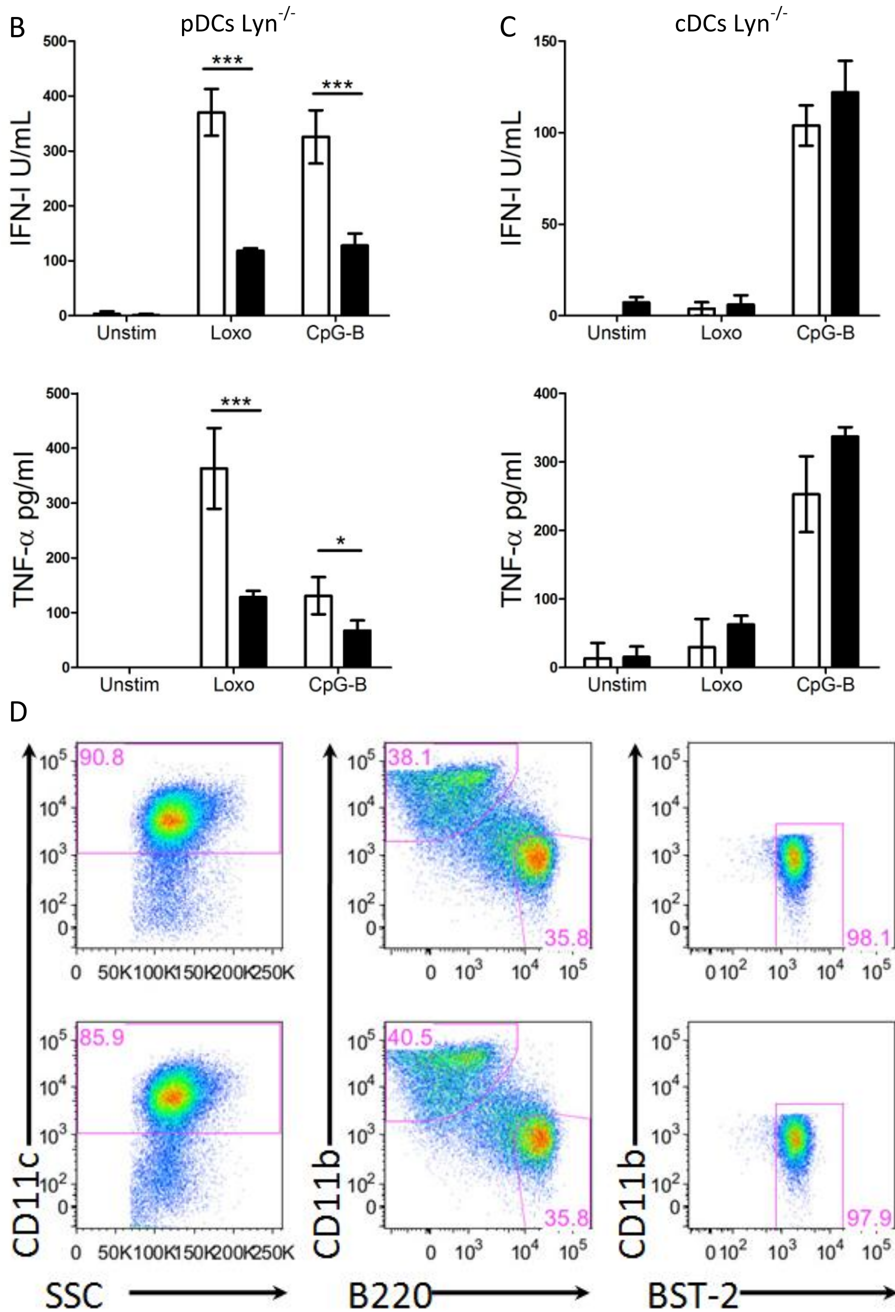

Figure 4.2: SFKs are necessary to fully drive the cytokine production by murine pDCs.

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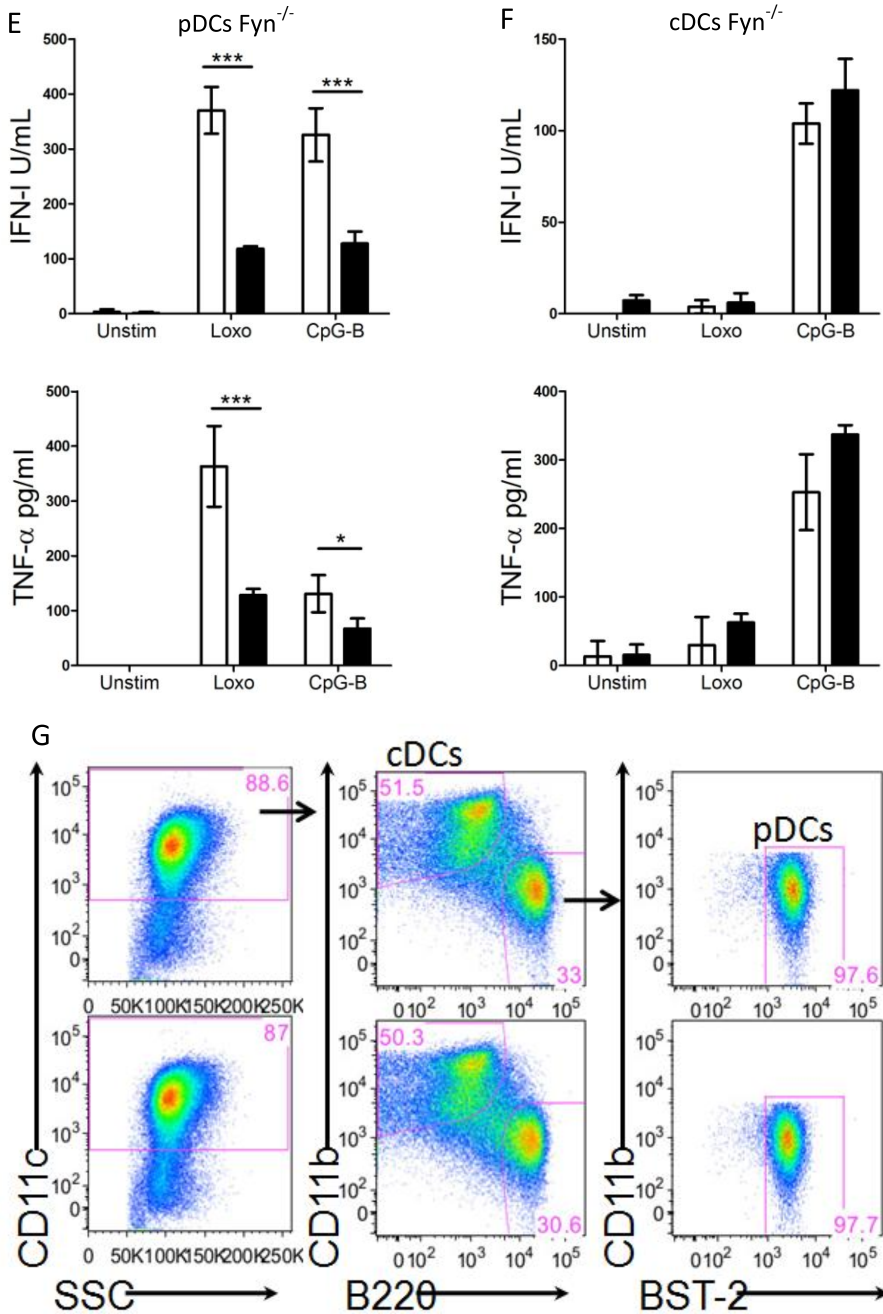

Figure 4.2: SFKs are necessary to fully drive the cytokine production by murine pDCs.

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A: Bone marrow-derived DCs were pre-treated for 1 hr with SFKs inhibitor PP2, or the equivalent concentration of DMSO control, followed by 15 hrs in media in presence or absence of 0.1 μM CpG-B ODN 1668. Supernatants were collected and IFN-I was measured by an interferon sensitive luciferase reporter bioassay, and TNF-α was measured by ELISA. Cell viability was assessed by live/death staining and analyzed by FACS. **(B-F)** Bone marrow from WT and $Lyn^{-/-}$ (**B-D**) or WT and $Fyn^{-/-}$ (**E-G**) mice was cultured in Flt3L containing media for eight days prior harvest and FACS-purification of pDCs and $CD11b^{+}$ cDCs. **B, E:** Sample FACS plot showing pre-FACS purification viability, distribution of DC subsets and sort gating strategy. **C, D, F, G:** FACS-purified pDCs (**C, F**) and cDCs (**D, G**) were cultured 15 hrs in media alone or stimulated with either 100 μM loxoribine (loxo) or 0.1 μM CpG-B ODN 1668. Supernatants were collected and IFN-I were measured by an interferon sensitive luciferase reporter bioassay, and TNF-α was measured by ELISA. White bar: WT mice; Black bar: KO mouse. Graphs show mean ± SD of n≥3/group and representative of 2-4 independent experiments. * p<0.05, ** p<0.01, ***p<0.001.

Taken together, our data showed that SFKs are necessary for cytokine production by murine pDCs after TLR stimulation, and that both Lyn and Fyn are involved, playing a partially redundant role.

4.3 SFKs positively regulate the activation of a human pDC cell line after TLR stimulation

To elucidate whether human pDCs require SFKs to promote the TLR-induced immune response, we evaluated IFN-I and TNFα production by the human pDC cell line stimulated with R848, a TLR7/8 agonist, after treatment with the pan-SFK inhibitor. Consistently with the murine data, the treatment caused a reduction in the TLR-induced CAL-1 cytokine production, in the absence of any effects on cell viability (Figure 4.3a).

With the purpose to narrow the SFK requirement to the kinases effectively involved, we restricted our attention to the SFK implicated in the BCR-like signaling and the ones we have already observed to be required for the murine pDC activation. Considering that BLK and FYN mRNAs seem expressed at negligible levels, we focused on LYN, and we established a

LYN$^{-/-}$ CAL-1 cell line (Figure 4.3b). We observed that the absence of LYN profoundly affects the IFN-β mRNA synthesis after TLR7 stimulation (Figure 4.3c). However, IFN-I was still detectable, although significantly reduced (Figure 4.3d), suggesting that also in CAL-1 there is a partial functional redundancy among SFKs.

Considering its peculiar ability to inhibit only some members of the SFKs, restriction hypothesized to be dictated by the requirement to have determined aminoacids in specific position of the ATP-binding site in the kinase domain, we decided to assess whether the selective dual ABL/LYN inhibitor bafetinib was capable of altering CAL-1 activation [137]. Interestingly, bafetinib was able to strongly inhibit in a dose-dependent manner the IFN-I production by CAL-1 after R848 stimulation without affecting cell viability (Figure 4.3e). In addition, a reduction in the TNFα production was also observed (Figure 4.3e).

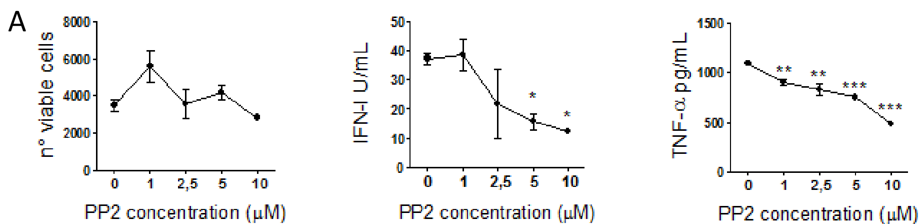

Figure 4.3: SFKs regulate positively the activation of a human pDC cell line after TLR stimulation.

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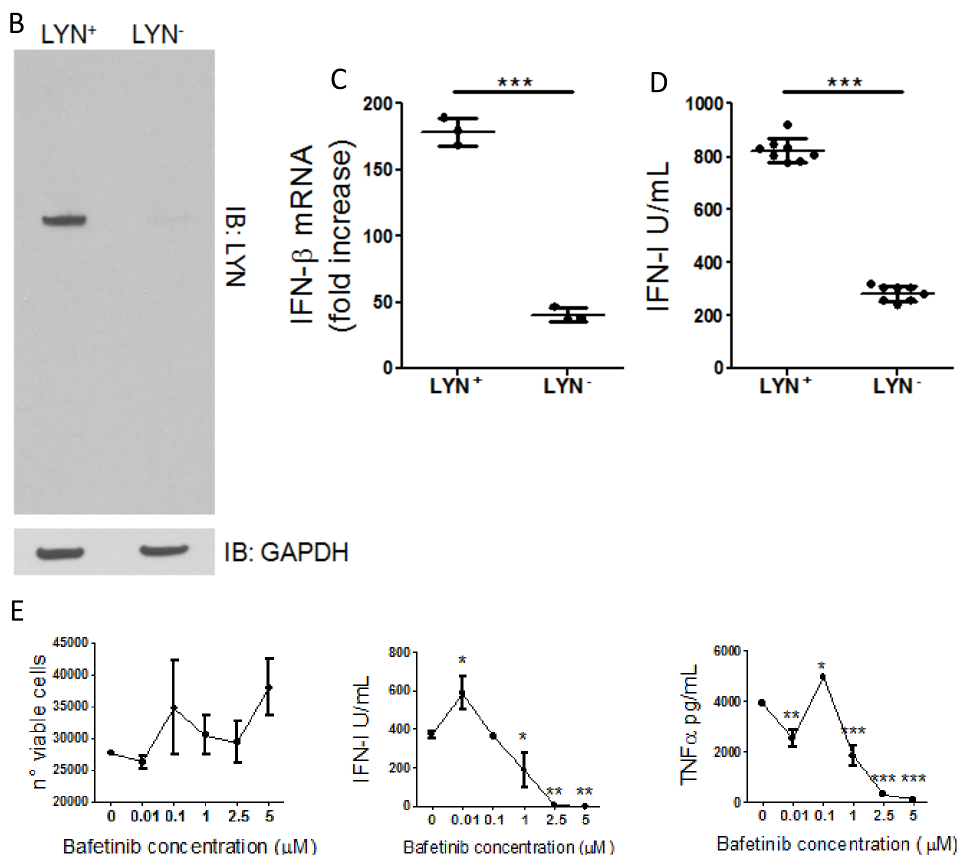


Figure 4.3: SFKs regulate positively the activation of a human pDC cell line after TLR stimulation.

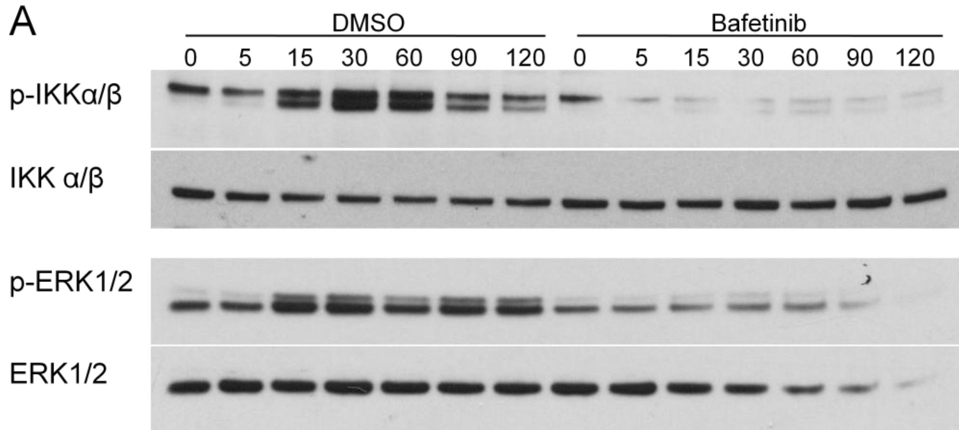
A, B: Human pDC cell line CAL-1 pre-treated for 1 hr with either the SFKs inhibitor PP2 (**A**) or the dual inhibitor ABL/LYN bafetinib (**B**), or the equivalent concentration of DMSO control followed by 15 hrs in media in presence or absence of R848 (1mg/mL). Supernatants were collected and IFN-I was measured by an interferon sensitive luciferase reporter bioassay, and TNF-α was measured by ELISA. Cell viability was assessed by live/death staining and analyzed by FACS. **C:** Whole-cell lysates were prepared and resolved proteins were probed with antibodies against LYN and GAPDH. **D, E:** Cells were cultivated in presence of absence of 1 mg/mL R848 for 1 hour (**D**) or 15 hours (**E**). **D:** total mRNAs were extracted and the expression of IFN-β mRNA was assessed by SYBR Green qPCR and it was normalized against the expression of GAPDH. The graph represents the IFN-β mRNA fold increase after 1 hour stimulation. **E:** IFN-I production was measured by an interferon sensitive luciferase reporter bioassay Graphs show mean ± SD of 3-5 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

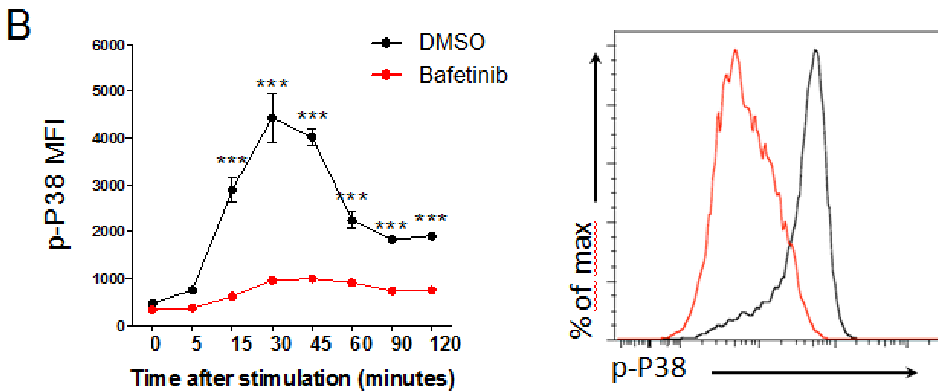
4.4 Bafetinib decreases the TLR pathway activation in a pDC cell line

TLR7 and 9 signal through similar pathways, which involve the phosphorylation or ubiquitination of kinases and adaptor proteins and end up with the activation of several transcription factors, including NF-κB, AP-1 and IRF7 [19]. To evaluate how the SFK activity influences this pathway, we stimulated bafetinib-treated CAL-1 cells with R848 and we analyzed the phosphorylation of different kinases downstream TLR7 by western blot and flow cytometry. We opted for bafetinib instead of PP2 since it proved to be more efficient to turn off the cytokine production. CAL-1 treated cells showed a slighter increase in the phosphorylation associated with the activation of IKKα/β, the kinase complex which leads NF-κB activation, mitogen-activated protein kinases ERK1/2 and P38, indicating a milder initiation of the TLR7 pathway (Figures 4.4a and 4.4b).

However, IFN-I can be produced by pDCs not only in response to TLR stimulation. Indeed, its production is also strongly driven by a positive feedback loop, in which the IFN-I produced early after TLR stimulation acts in an autocrine way binding the IFN-I receptors (IFNAR) present on the same cell and stimulating the transcription of new IFN-I and IFN-stimulated genes [28]. To assess an involvement of SFKs also in this pathway, we stimulated bafetinib-treated CAL-1 with different amounts of IFN-β. Interestingly, no difference in STAT1 phosphorylation, the main transcription factor downstream the IFNAR, were observed compared to the control-treated cells (Figures 4.4c), suggesting that SFKs are not involved in the IFNAR mediated IFN-I production.

Taken together, these data showed that SFKs are necessary to drive a complete TLR7 pathway activation.





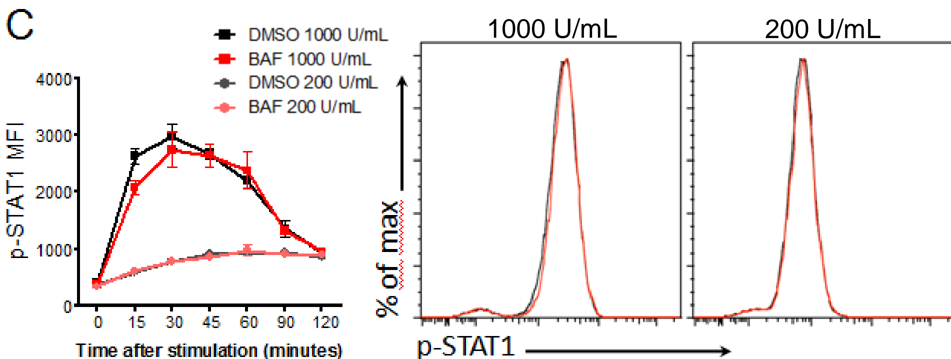


Figure 4.4: Bafetinib decreases the activation of several key signaling mediators downstream TLR stimulation.

A: CAL-1 cells were pre-treated for 1 hr with Bafetinib, or the equivalent concentration of DMSO control, and stimulated with R848 (1 mg/mL). Whole-cell lysates were prepared and

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resolved proteins were probed with antibodies against phopsho-IKK α/β (p-IKKα/β), IKKα/β, phopsho-ERK1/2 (p-ERK1/2), ERK1/2. **B:** cells were treated and stimulated as in **A**, directly fixed, subsequently methanol-permeabilized and stained with phospho-P38 (p-P38) antibody. The line graph shows the phosphorylation kinetic of P38 after stimulation. The histogram bar depicts a representative timepoint (15 minutes). Black line: DMSO control treated. Red line: Bafetinib treated. **C:** cells were treated as in **A** and stimulated with either 1000 or 200 U/mL of INF-I, directly fixed, subsequently methanol-permeabilized and stained with phospho-STAT1 (p-STAT1) antibody. The line graph shows the phosphorylation kinetic of STAT1 after stimulation. The histogram bars depict a representative timepoint (15 minutes). Graphs show mean ± SD of 2-3 independent experiments. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

4.5 Bafetinib affects PI3K-AKT-MTOR pathway and the adaptor protein BCAP

Recent discoveries have revealed the value of PI3K [50] and MTOR [51] for pDC IFN-I production, raising the importance of the PI3K-AKT-MTOR pathway within the pDC biology. Considering this, we decided to assess the MTOR activation kinetic after TLR7 stimulation in the presence of bafetinib. The treatment caused a reduction in the activating phosphorylation of MTOR, starting at 15 minutes and lasting for all the timepoints considered, suggesting that the SFK activity is necessary to lead to the MTOR activation (Figure 4.5a). However, the dynamics that bring SFKs to influence MTOR are not clear. Recently, two studies have identified B-cell adapter for phosphoinositide 3-kinase (also known as BCAP or PIK3AP1) as the protein connecting the PI3K-AKT-MTOR pathway to the TLR pathway [54,55]. This is an adaptor protein capable of interacting with MYD88, through a Toll-IL-1 receptor (TIR) domain, and with the regulatory subunit of PI3K, through a YxxM motif which acts as docking site for the PI3K Src-homology 2 (SH2) domain after phosphorylation. Which kinase phosphorylates BCAP is still unclear, and different studies have proposed a role for Lyn, Syk and Btk [55]. To assess whether SFKs are involved in this

phosphorylation in pDCs, we immunoprecipitated BCAP from either bafetinib- or control-treated CAL-1 which were kept unstimulated or were stimulated for 15 minutes with R848 and we performed western blotting to detect tyrosine phosphorylation. At the level of the BCAP molecular weight, the anti-phosphorylated tyrosine antibody detected a lower intensity when the cells were treated with the drug. Interestingly, this alteration was already present before the TLR stimulation (Figure 4.5b). These data suggest that SFKs are involved in BCAP phosphorylation and that upstream signals causing BCAP tyrosine phosphorylation are active in resting pDCs.

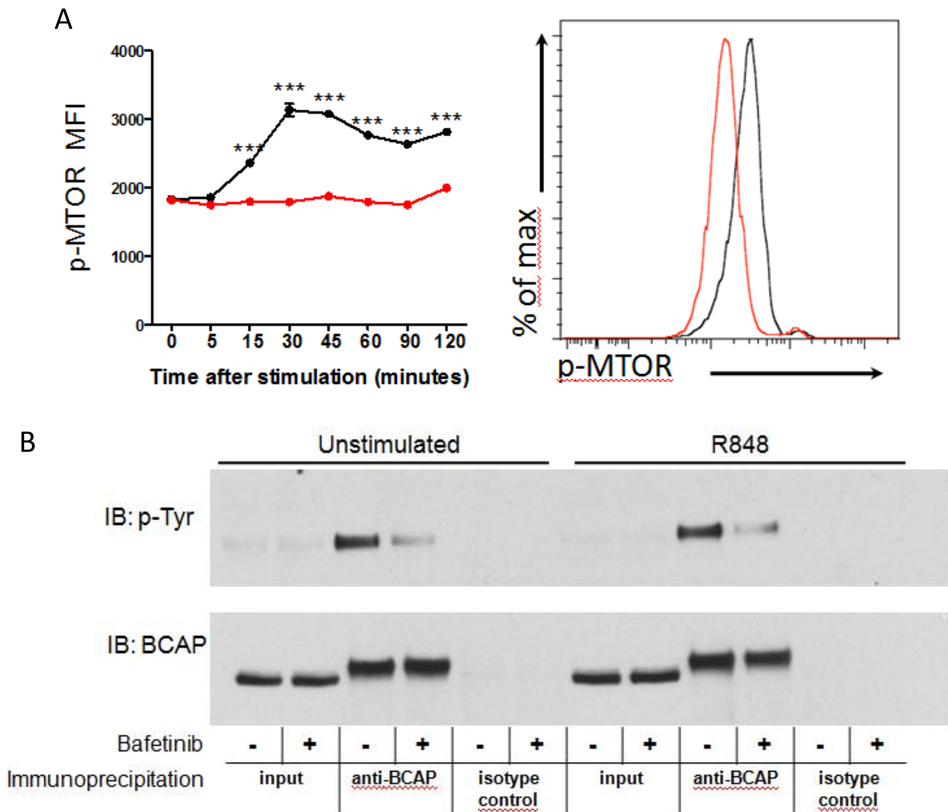

Figure 4.5: Bafetinib affects PI3K-AKT-MTOR pathway and the adaptor protein BCAP.

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A: CAL-1 cells were pre-treated for 1 hr with Bafetinib, or the equivalent concentration of DMSO control, and stimulated with R848 (1 mg/mL) for different time. Cells were directly fixed, subsequently methanol-permeabilized and stained for phospho-MTOR (p-MTOR). **B:** BCAP was immunoprecipitated from CAL-1 cells either unstimulated or stimulated for 15 minutes with R848 and probed with antibodies to phosphotyrosine or BCAP. p-Tyr: antibodies to phosphotyrosine. Graphs show mean ± SD of 2-4 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.6 SFKs are needed for complete IFN-I and TNF-α production after TLR stimulation by primary human PBMCs

To investigate the potential biological and clinical relevance of the observation noted above in the murine and in vitro model system, we determined whether the inhibition of SFK activity by PP2 or bafetinib can block the pDC activation after engagement of TLR by R848. Consistently, PP2-treated R848-stimulated peripheral blood mononuclear cells (PBMCs) from healthy donors showed a decreased production of IFN-I and TNF-α production despite any effect of the drug on the cell viability (Figure 4.6a). Intriguingly, bafetinib showed a deeper abolition of the cytokine production compared to PP2 (Figure 4.6b). To assess more precisely the effect of Bafetinib on pDCs in the PBMC mixed culture, we exploited flow cytometry to perform a per-cell analysis (Figure 4.6c). The bafetinib treatment caused a dose dependent decrease in the percentage of positive IFN-I and TNF-α pDCs, and, in addition to this, it caused also a decrease in the MFI of the positive populations, indicating that the response to TLR stimulation is smaller both in term of number and strength (Figure 4.6d). Moreover, we also confirmed that pDCs were the only source of IFN-I after 6 hours of R848 stimulation (data not shown), confirming that the decreased IFN-I production observed in the supernatant of the PBMC mixed culture was attributable to the bafetinib effect on pDCs.

These data suggested that SFK activity was necessary to drive a complete

cytokine production by pDCs derived from healthy donor after TLR7 stimulation.

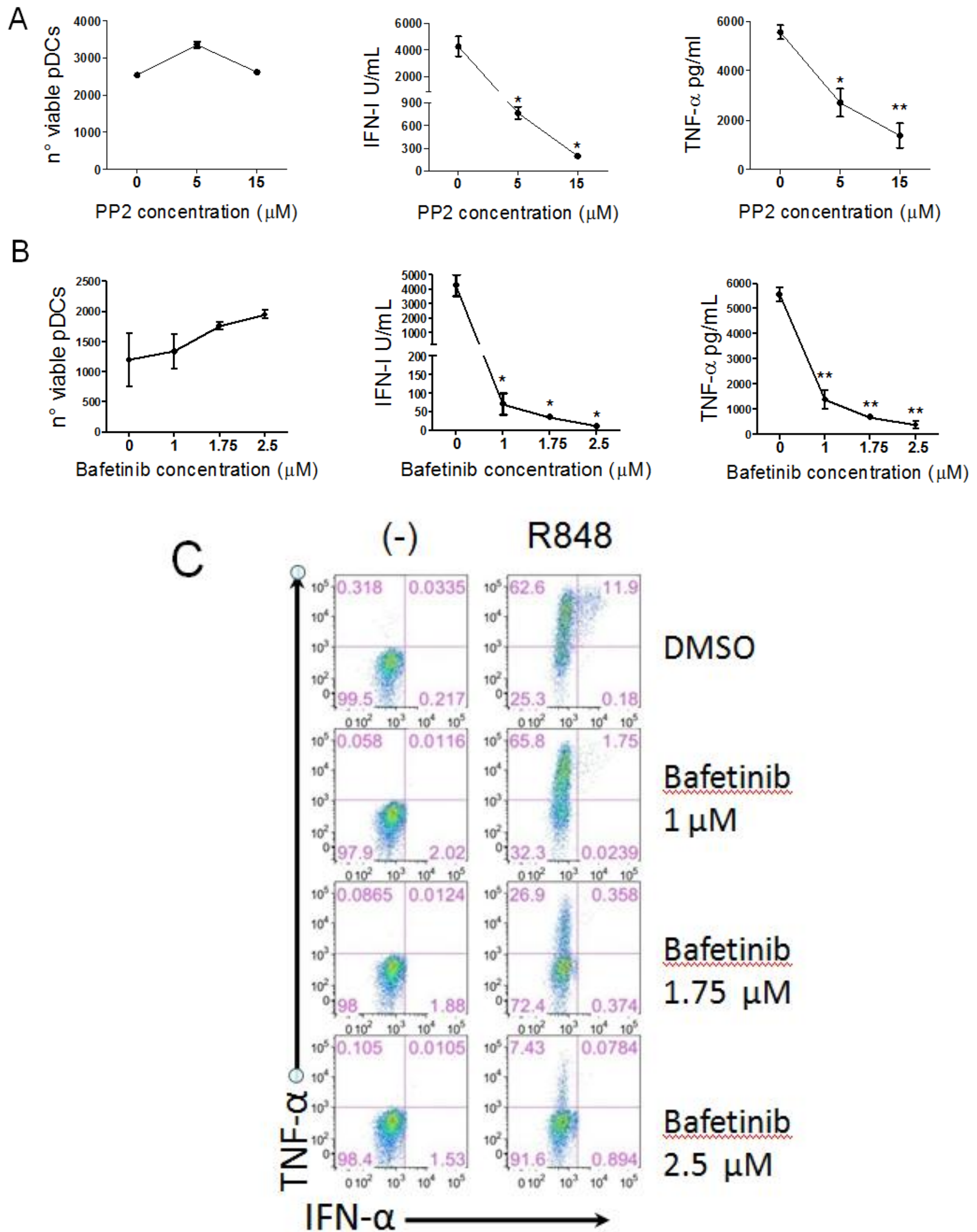

Figure 4.6: Bafetinib affects IFN-I and TNF-α production of primary human PBMCs.

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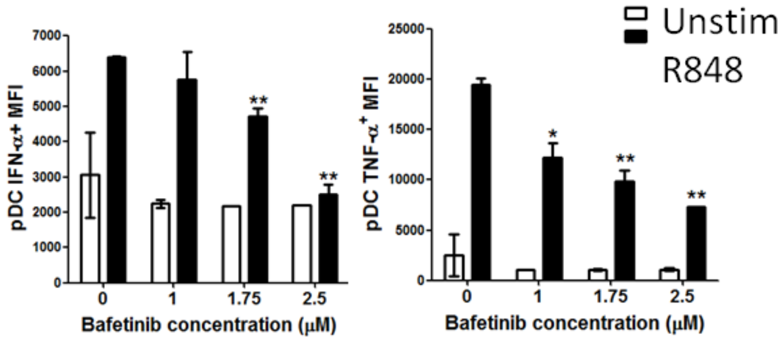

Figure 4.6: Bafetinib affects IFN-I and TNF-α production of primary human PBMCs.

Human peripheral blood mononuclear cells were pre-treated for 1 hr with either PP2 (**A**) or Bafetinib (**B**), or the equivalent concentration of DMSO control, followed by 15 hrs in media in presence or absence of R848 (1mg/mL). Supernatants were collected and IFN-I was measured by an interferon sensitive luciferase reporter bioassay, and TNF-α was measured by ELISA. Cell viability was assessed by live/death staining and analyzed by FACS. **C, D:** Human peripheral blood mononuclear cells were pre-treated for 1 hr with Bafetinib, or the equivalent concentration of DMSO control, followed by 6 hrs in media in presence or absence of either R848 (1 mg/mL). **C:** Sample FACS plot showing IFN-α and TNF-α expression in pDCS. **D:** IFN-α and TNF-α MFI of pDC IFN-α and TNF-α positive population. Graphs show mean ± SD of n≥2/group and representative of 2-4 independent experiments and n=4 human samples. * $p<0.05$, ** $p<0.01$, ***$p<0.001$

5. Discussion

pDCs are a subset of dendritic cells able to produce prodigious amounts of IFN-I after recognition of pathogen associated molecular patterns through TLR7 and TLR9, which lead to the activation of IRF-7, with subsequent production of IFN-I, and of NF-κB, with production of pro-inflammatory cytokines such as TNF-α. A fine tuning of this process is necessary, since a deregulated production of IFN-I can cause immunopathologies. Indeed systemic lupus erythematosus (SLE) has been associated with an IFN-I signature, and recently it has been demonstrated in murine models that pDC-produced IFN-I is one of the key elements driving SLE [79,80]. Instead, in the context of viral infection, the initial peak of IFN-I production seems to be required for a quick resolution of the infection, whereas the continuous low IFN-I production after the peak seems to favor the viral persistence [68,69]. Several receptors on the surface of pDCs modulate their cytokine production and, although the downstream pathway that they activate is still unclear, it has been shown that often it starts from an immunoreceptor tyrosine-based activation motif or immunoreceptor tyrosine-based inhibition motif. Given that phosphorylation of ITAM and ITIM motifs is often mediated by SFKs, a family of non-receptor tyrosine kinases, we decided to investigate the role of SFKs in pDCs biology.

At first, we confirmed at the mRNA level the SFK expression by murine pDCs and the human pDC cell line CAL-1, and we observed that the different members of the family are not all represented at the same degree. In mouse, the transcript level of Blk, Lck, Src and Yes were very low, suggesting that they are not primarily involved in the pDC biology. Instead, Fgr, Fyn, Hck and Lyn were the SFKs mainly expressed, with Fgr, Fyn and Lyn at a degree similar to or higher than any other immune cell types analyzed. Intriguingly, Fyn expression in murine pDCs was by several folds the highest among the immune subsets considered, suggesting an importance for this kinase in the murine pDC biology. In contrast, no SFKs were expressed at outstanding levels in CAL-1 cells compared to the other

human immune system-derived cell lines, and only LYN expression could be considered noteworthy. However, CAL-1 cells well resembled human blood-derived primary pDCs: indeed, previous studies showed that human pDCs express low BLK and FYN and high LYN both at the mRNA and protein level [36,134].
Interestingly, the SFKs play a role in the pDC biology, and they are necessary for the complete pDC activation after TLR stimulation. The administration of the pan-SFK inhibitor PP2 on murine pDCs showed that their activity is necessary to the full IFN-I and TNFα production after stimulation of TLR7 and TLR9. Taking advantage of murine genetic model of single SFK deletion, we tried to individuate the family members involved. At first, we identified the involvement of Lyn, thereby confirming the hypothesis that pDCs exploit a BCR-like signaling [36,134,136]. However, the inflammatory mediator production was not completely ablated, suggesting that other family members were playing a role. But since the other kinase usually associated with the BCR signaling is Blk and given that we detected a low Blk mRNA expression by murine pDCs, we instead decided to evaluate Fyn, which showed to be the SFK most impressively expressed by murine pDCs at the mRNA level. Consistently with our hypothesis, pDCs from $Fyn^{-/-}$ mice showed generally at least a 50% reduction in the production of IFN-I and TNFα compared to WT pDCs. Since the absence of neither Lyn nor Fyn was enough to totally ablate the pro-inflammatory cytokine production, we hypothesized a partial functional redundancy among the SFKs. Also human pDCs require SFK activity to fully drive the inflammatory mediator production after TLR stimulation. Indeed, the treatment of CAL-1 cells and human blood-derived primary pDCs with the pan-SFK inhibitor PP2 resulted in a lower IFN-I and TNFα secretion. Exploiting the CRISPR-Cas9 technology, we observed that the absence of LYN negatively impacted the human pDC response to TLR stimulation. Again, the deletion of LYN alone was not sufficient to totally

ablate the CAL-1 response to R848, suggesting that also in CAL-1 several SFKs exert a partially redundant role.
To further investigate the involvement of SFKs in the pDC biology, we decided to take advantage of the selective dual ABL/LYN inhibitor bafetinib [137]. A kinase inhibition profile, in which 79 tyrosine kinases were tested, showed that bafetinib, at the concentration of 0.1 μM, was able to inhibit almost totally the activity of ABL, ABL-related gene (ARG) and LYN [137]. In addition, it also inhibited more than 75% of the FYN activity and almost 50% of the BLK activity. Instead, it was not able to inhibit SRC and YES, the other SFKs tested, thereby showing different affinities for the different family members. We administered bafetinib to CAL-1 and human blood-derived primary pDCs and, intriguingly, we observed a strong reduction in IFN-I and TNFα production, even more profound than that observed with the PP2 treatment. This difference in the effects between these two compounds could be explained by the fact that bafetinib seems to be a better LYN inhibitor, and indeed its IC50 (the half maximal inhibitory concentration, or IC50, is a measure of how effective a drug is, and it indicates how much of a particular drug is needed to inhibit a given biological process, as an enzyme, by half) for LYN is 19 nM [137]. Although the PP2 IC50 for LYN has not been reported yet [138], its IC50 for LCK, the SFK bound with more affinity, is only 30-40 nM [139,140]. In addition, a recent study performed an ATP-site competition binding assay on several tyrosine kinases using 10 μM of PP2 and it showed that LYN was still retaining 10% of its activity [141]. Another explanation of the more profound effect seen with the administration of bafetinib could be connected with the ABL activity. However, pDCs derived from the peripheral blood of chronic myeloid leukemia patients treated with imatinib, a selective ABL inhibitor, showed IFN-α production after ex-vivo HSV stimulation higher than those of chronic myeloid leukemia patients refractory to bafetinib treatment and similar to those of healthy donors [142]. In addition, it has been reported

several times that also PP2 is able to inhibit ABL activity, and already at the concentration of 2 μM (as assessed in an in vitro kinase assay) [141,143]. These last observations lead us to speculate that the stronger inhibition seen with the administration of bafetinib is not related to ABL activity, although we know that further investigation are necessary to definitively rule out this scenario.

In conclusion, we exploited different inhibitors and several murine and human genetic models of SFK deletion to prove that SFKs activity is necessary to drive the full IFN-I and pro-inflammatory cytokine production after TLR stimulation by pDCs. However, how the SFK activity is necessary for the TLR-mediated pDC activation is not known.

To address this question, we performed several experiments to shed light on the mechanisms behind this phenotype. We decided to use bafetinib instead of PP2, since it has been shown to inhibit more profoundly the TLR-mediated activation. In addition, several studies pointed out that PP2 is not as selective as previously believed, and several off-target kinases (as ABL, CSK, CK1δ, P38) can be inhibited already at low drug concentration [97,140]. We observed that bafetinib administration reduced the TLR-mediated activating phosphorylation of P38, IKKα/β and ERK1/2, some of the main kinases downstream of TLR7 [19]. In contrast, the treatment did not cause any difference in STAT1 activating phosphorylation level after IFN-β stimulation, suggesting that SFKs affect pDC activation through the regulation of the TLR receptor pathway and not of the positive feedback loop driven by the autocrine action of the IFN-I produced early after stimulation [28]. In addition, we observed that TLR7-mediated stimulation of CAL-1 lead to increased activation of MTOR, and that bafetinib was able to inhibit this process, thereby furthermore influencing the pro-inflammatory cytokine production [51]. We hypothesized that the reduced MTOR activation could be the consequence of a defective recruitment of PI3K by TLR, since we observed a diminution of the tyrosine phosphorylation level

of BCAP, an adaptor protein that links TLRs to PI3K-AKT-MTOR pathway binding the PI3K SH2 domain with a phosphorylated tyrosine and bringing the phosphatidylinositol bisphosphate kinase near the activated TLRs and, subsequently, near its substrate [54,55]. Eventually, we observed that bafetinib was affecting BCAP tyrosine phosphorylation level also in the absence of TLR stimulation, leading us to speculate about the SFKs involvement in a tonic signal necessary to place the pDCs in a ready-to-respond state, a pre-condition necessary to respond to external stimulation. The need of a basal signaling tuning the cellular response to external stimulation has been already theorized and confirmed for many other immune cell types, and it always involved immunoreceptors and kinases belonging to either the Src-family or the Syk-family, as we hypothesized for pDCs [144–146].

Taken together, our data showed that SFKs are key elements involved in the tuning of the pDC activation after pathogen recognition by the intracellular TLRs, and considering that has been shown that pDCs play important roles in the defense against pathogens, tumor immune-surveillance and autoimmune disease, SFK activity manipulation holds great promise for immune-based therapeutics to attenuate several human illnesses.

6. Conclusion

Plasmacytoid dendritic cells are the most powerful interferon type I (IFN-I) producers among the immune cell subsets. In order to avoid a dysregulated production that could lead to immunopathologies, they exploit the activity of several surface receptors, which mainly signal through ITIM and ITAM, to modulate their activation status. Given that phosphorylation of ITAM and ITIM is often mediated by SFKs, a family of non-receptor tyrosine kinases, we decided to investigate the role of SFKs in pDCs biology.

At first, we showed that pDCs express a particular SFK expression pattern, different from the other immune subsets, and that Fyn and LYN were the SFK members mainly expressed by murine and human pDCs. Then, we showed that SFKs were required to induce pDC activation after TLR-stimulation, and that Fyn, in mouse, and Lyn, in both human and mouse, were involved. However, since the single deletion of these genes did not totally ablate the IFN-I and TNFα production, we hypothesized that several members of the family play a partial redundant activity. To test this hypothesis, we will assess the pDC activity in murine genetic models of double or triple gene deletion and we will perform a siRNA matrix on the human pDC cell line to silence more kinases at the same time.

Furthermore, we showed that the absence of the SFK activity diminished the activation of several kinases and transcription factors downstream the TLR pathway, such as IKKα/β, ERK1/2, P38 and IRF7, and that also MTOR activation was affected by SFK action, likely as consequence of the BCAP tyrosine phosphorylation, which is an adaptor protein that bridges TLR and PI3K-AKT-MTOR pathways. To understand which elements upstream SFKs regulate their activity, we have planned to immunoprecipitate several ITAM- and ITIM-bearing receptors in either presence or absence of SFK inhibitors and to evaluate their tyrosine phosphorylation status.

Lastly, we showed that inhibitors of SFK activity are able to affect pDC IFN-I and TNFα production during in vitro and ex vivo experiments. These intriguing results let us to suggest a possible clinical use of SFKs inhibitors

to attenuate pDC response in autoimmune diseases, although we are aware that further in vivo studies will be necessary.

7. References

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8. Appendixes

8.1 Appendix A

Primer used in the study:

	Primer Forward	Primer Reverse
Murine Blk	AAGCCACTGAAGCTGACTGAGA	TCACTGACCTGCCTCTTGCTG
Human BLK	CCTGCTCCACTGTAAGGGTG	TCCATTGGCCCTTGTCCTTC
Murine Fgr	AGATCCGAAAGCTGGACACG	AGAGTCTGGGGCTTAGTGGT
Human FGR	CCGGAAAAACTGGCTGCATT	GGTCTCGCTTTCCCGAATGA
Murine Fyn	AAGCACGGACGGAAGATGAC	ATGGAGTCAACTGGAGCCAC
Human FYN	ACAGCTCGGAAGGAGATTGG	CTGTCGCTCAGCATCTTTTCG
Murine Hck	GGCACGAATCATCGAGGACA	GTAAGGGATCCGGCCATAG
Human HCK	CCATCAAGTGGACAGCTCCT	AGCTCGGATCACTTCAGGGT
Murine Lck	TGGGACCTTCACCATCAAGTC	TGTCAGGTCTCACCATGCG
Human LCK	TCCTGCTGACGGAAATTGTCA	CACACTGCGCAGGTAGTCA
Murine Lyn	TGGCTAAGGGTAGTTTGCTGG	CGCAGATCACGGTGGATGTA
Human LYN	ACCAGGGAGGAGCCCATTTA	CTTCCGCTCGATGTATGCCA
Murine Src	GCCTCACTACCGTATGTCC	TTTTGATGGCAACCCTCGTG
Human SRC	GCTGTTCGGAGGCTTCAACT	CCAGTCTCCCTCTGTGTTGT
Murine Yes	TGAGGCTGCTCTGTATGGTC	GCATTCTGTATCCCCGCTCT
Human YES	GCTGCACTGTATGGTCGGTT	TCCTGTATCCTCGCTCCACT
Human IFN-β	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG
Human TNF-α	ACCCACACCATCAGCCGCAT	TGGCCAGAACCAAAGGCTCCCT

Human GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT
Murine Gapdh	TCCCACTCTTCCACCTTCGA	AGTTGGGATAGGGCCTCTCTT

8.2 Appendix B

Antibodies used in the study

For flow cytometry the following antibodies were used: anti-mouse Thy1.2 (clone 30-H12), anti-mouse CD11c (N418), anti-mouse B220 (RA3-6B2), anti-mouse CD8a (53-6.7), anti-human CD3 (UCHT1), anti-human CD14 (HCD14), anti-human CD16 (3G8), anti-human CD19 (HIB19), anti-human CD56 (MEME-188), anti-human CD11c (3.9), anti-human CD123 (6H6), anti-human CD304 (12C2), anti-human HLA-DR (L243), anti-human TNF-α (Mab11) from Biolegend; anti-mouse CD19 (eBio1D3), anti-mouse NK1.1 (PK136), anti-mouse CD11b (M1/70), anti-mouse CD317 (eBio927), anti-human p-MTOR (MRRBY) from eBioscience; anti-human p-STAT1 (612564) from BD Biosciences; anti-human p-IRF7 (REA310) and anti-human IFN-α (LT27:295) from Miltenyi Biotec; anti-human p-P38 (D3F9) from Cell Signaling Technology.
For immunoprecipitation and for immunoblotting the following antibodies were used: anti-human p-ERK1/2 (9101S) and anti-human p-IKKα/β (2694P) from Cell Signaling Technology; anti-human ERK1/2 (5AD13MA) from eBioscience; anti-human IKKα/β (sc-7607) from Santa Cruz Biotechnology; anti-human LYN (LYN-01) from Biolegend; anti-human PIK3AP1 (ab157151) from Abcam; anti-phospho tyrosine (4G10) from EMD Millipore.

9. Scientific productions and funds

Scientific productions related to this work

Conference attended as selected speaker:

- The American Association of Immunologists (AAI) - Toll-like Receptor Signaling, Signaling by Innate Receptors block symposium speaker, New Orleans, LA, 2015.

Conference attended as selected poster:

- The American Association of Immunologists (AAI), New Orleans, LO, 2015.
- La Jolla Immunology Conference, La Jolla, CA, 2015.

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