

Dual Role of the Tyrosine Kinase Syk in Regulation of Toll-Like Receptor Signaling in Plasmacytoid Dendritic Cells

Besma Aouar, Denise Kovarova, Sébastien Letard, Albert Font-Haro, Jonathan Florentin, Jan Weber, David Durantel, Laurence Chaperot, Joël Plumas, Katerina Trejbalova, et al.

► **To cite this version:**

Besma Aouar, Denise Kovarova, Sébastien Letard, Albert Font-Haro, Jonathan Florentin, et al.. Dual Role of the Tyrosine Kinase Syk in Regulation of Toll-Like Receptor Signaling in Plasmacytoid Dendritic Cells. PLoS ONE, Public Library of Science, 2016, 11 (6), pp.e0156063. 10.1371/journal.pone.0156063 . inserm-01357267

HAL Id: inserm-01357267

<https://www.hal.inserm.fr/inserm-01357267>

Submitted on 27 Sep 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



RESEARCH ARTICLE

Dual Role of the Tyrosine Kinase Syk in Regulation of Toll-Like Receptor Signaling in Plasmacytoid Dendritic Cells

Besma Aouar^{1,2,3,4}, Denisa Kovarova^{1,2,3,4,5,6}, Sebastien Letard^{1,2,3,4,7}, Albert Font-Haro^{5,6,8}, Jonathan Florentin^{1,2,3,4}, Jan Weber⁸, David Durantel⁹, Laurence Chaperot¹⁰, Joel Plumas¹⁰, Katerina Trejbalova⁵, Jiri Hejnar⁵, Jacques A. Nunès^{1,2,3,4}, Daniel Olive^{1,2,3,4}, Patrice Dubreuil^{1,2,3,4}, Ivan Hirsch^{1,2,3,4,5,6,8}*, Ruzena Stranska^{1,2,3,4}✉*

1 Centre de Recherche en Cancérologie de Marseille, Inserm U1068, Marseille, France, **2** CNRS, UMR7258, Marseille, France, **3** Institut Paoli-Calmettes, Marseille, France, **4** Aix-Marseille Université, UM105, Marseille, France, **5** Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic, **6** Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Prague, Czech Republic, **7** AB Science, Paris, France, **8** Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, **9** Centre de Recherche en Cancérologie de Lyon, Inserm U1052, CNRS UMR5286, Lyon, France, **10** UJF, INSERM U823, University Grenoble Alpes, EFS Rhone-Alpes, Grenoble, France

✉ These authors contributed equally to this work.

* ruzena.stranska@kuleuven.be (RS); ivan.hirsch@inserm.fr (IH)



OPEN ACCESS

Citation: Aouar B, Kovarova D, Letard S, Font-Haro A, Florentin J, Weber J, et al. (2016) Dual Role of the Tyrosine Kinase Syk in Regulation of Toll-Like Receptor Signaling in Plasmacytoid Dendritic Cells. PLoS ONE 11(6): e0156063. doi:10.1371/journal.pone.0156063

Editor: Roberto F. Speck, University Hospital Zurich, SWITZERLAND

Received: November 12, 2015

Accepted: May 9, 2016

Published: June 3, 2016

Copyright: © 2016 Aouar et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Grantova agentura Ceske republiky (Czech Science Foundation) 14-32547S (<http://gacr.cz/>) DK AFH KT JH IH. Fondation ARC pour la Recherche sur le Cancer, PJA 20131200304, (<http://www.fondation-arc.org/>) BA JAN DO IH RS. Fondation pour la Recherche Medicale, SPF20121226309, (<http://www.frn.org/>) RS. BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University“ (CZ.1.05/1.1.00/02.0109), from the European

Abstract

Crosslinking of regulatory immunoreceptors (RR), such as BDCA-2 (CD303) or ILT7 (CD85g), of plasmacytoid dendritic cells (pDCs) efficiently suppresses production of type-I interferon (IFN)- α/β and other cytokines in response to Toll-like receptor (TLR) 7/9 ligands. This cytokine-inhibitory pathway is mediated by spleen tyrosine kinase (Syk) associated with the ITAM-containing adapter of RR. Here we demonstrate by pharmacological targeting of Syk that in addition to the negative regulation of TLR7/9 signaling *via* RR, Syk also positively regulates the TLR7/9 pathway in human pDCs. Novel highly specific Syk inhibitor AB8779 suppressed IFN- α , TNF- α and IL-6 production induced by TLR7/9 agonists in primary pDCs and in the pDC cell line GEN2.2. Triggering of TLR9 or RR signaling induced a differential kinetics of phosphorylation at Y352 and Y525/526 of Syk and a differential sensitivity to AB8779. Consistent with the different roles of Syk in TLR7/9 and RR signaling, a concentration of AB8779 insufficient to block TLR7/9 signaling still released the block of IFN- α production triggered *via* the RR pathway, including that induced by hepatitis B and C viruses. Thus, pharmacological targeting of Syk partially restored the main pDC function—IFN- α production. Opposing roles of Syk in TLR7/9 and RR pathways may regulate the innate immune response to weaken inflammation reaction.

Regional Development Fund (<http://www.biocev.eu>) IH. Ligue contre le cancer (<http://www.ligue-cancer.net/>) BA. Algerian Ministry of Higher Education and Research, and Franco-Algerian Cooperation BA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. SL is an employee of AB Science. AB Science provided support in the form of salary for author SL, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of this author is articulated in the 'author contributions' section.

Competing Interests: SL is an employee of AB Science. PD is a consultant and a shareholder of AB Science. All other authors declared that no competing interests exist. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Introduction

Plasmacytoid dendritic cells (pDCs) are a highly specialized subset of dendritic cells that plays a central role at the interface of innate and adaptive immunity. They are important actors in antiviral and antitumor immunity but also potent inducers of autoimmune diseases [1–5]. They express endosomal Toll-like receptor (TLR) 7/9, recognizing ssRNA or CpG containing DNA. TLR signaling leads to secretion of proinflammatory cytokines and chemokines as interleukin (IL)-1, tumor necrosis factor (TNF)- α , IL-6, IL-8, and most importantly type I interferons (IFN)- α/β [6–8].

In addition to TLR7/9, pDC express multiple specific receptors that regulate pDC function and thus prevent aberrant immune responses. These include Fc (FcR) and C-type lectin (CLRs) receptors [9, 10], which signal through the B cell receptor (BCR)-like pathway involving Syk, Mek-Erk1/2, and BLNK [6, 10]. Signaling *via* pDC regulatory receptors (RR) attenuates TLR7/9-induced production of IFN and proinflammatory cytokines [6, 7, 10]. This physiological feedback mechanism of IFN control is hijacked in the pathogenesis of several chronic viral infections and cancers, leading to immune tolerance [7, 11–13]. We have previously shown that interaction of HCV envelope glycoprotein E2 with RR, BDCA-2 (CD303) and DCIR (CLECSF-6) activates B cell receptor (BCR)-like signaling that suppresses TLR7/9-mediated production of IFN- α [13]. We hypothesized that inhibition of BCR-like pathway could restore TLR7/9 signaling in pDCs exposed simultaneously to TLR7/9 and RR agonists [7].

Here, we demonstrate by pharmacological targeting of Syk that in addition to the negative regulation of TLR7/9 signaling *via* RR, Syk also positively regulates TLR7/9 pathway in human pDCs. While ample experimental evidence demonstrate negative effect of RR-mediated BCR-like activation of Syk on TLR7/9 signaling, the mechanism by which Syk acts as a positive regulator of TLR7/9 signaling in pDCs is much less clear. Novel highly specific Syk inhibitor AB8779 suppressed IFN- α and TNF- α production induced by TLR7/9 agonists in primary pDCs and in the pDC cell line GEN2.2 [14]. Triggering of TLR9 or RR signaling induced a differential kinetics of phosphorylation at Y352 and Y525/526 of Syk and a differential sensitivity to AB8779. Consistent with the different roles of Syk in TLR7/9 and RR signaling, a concentration of AB8779 insufficient to block TLR7/9 signaling still released the block of IFN- α production triggered *via* the RR pathway, including that induced by hepatitis B and C viruses. Opposing roles of Syk in TLR7/9 and RR pathways suggest that Syk may fine-tune the innate immune response to weaken inflammation reaction.

Materials and Methods

Ethics statement

Peripheral blood mononuclear cells (PBMCs) from healthy anonymous donors were obtained from the Etablissement Français du Sang (EFS). Blood samples were obtained after written consent following the approval of the EFS, Marseille, France and the Centre de Recherche en Cancérologie de Marseille (CRCM) in accordance to the convention signed the 20th May 2014. Human pDC line GEN2.2 was obtained from invaded peripheral blood of one patient as described previously [14].

Isolation and culture of primary pDCs and pDC line GEN2.2

pDCs from PBMCs of healthy donors were purified and cultured as described previously [15, 16]. Human pDC line GEN2.2 was grown in RPMI 1640 medium supplemented with L-glutamine, 10% FCS, 1% sodium pyruvate and 1% MEM nonessential amino acids, on a monolayer

of the murine stromal feeder cell line MS-5 as described previously [14]. For phosphoflow and western blot experiments, GEN2.2 cells were separated from MS-5 feeder cells and serum-starved overnight before stimulation.

Inhibitors, antibodies and reagents

Syk kinase inhibitor AB8779 was from AB Science (Paris, France). *In vitro*, AB8779 was shown to be as potent as Fostamatinib (R406) with $IC_{50} = 0.04 \mu\text{M}$ (S1 Fig and S1 Table). For *in vitro* pDC stimulation assays CpG-A (ODN 2216), CpG-B (ODN 2006), resiquimod (R848), PMA (all InvivoGen, San Diego, USA), BDCA-2 mAb (Miltenyi Biotech, Paris, France), ILT7 (CD85g) mAb and IgG1 isotype control antibody (eBioscience) were used.

In vitro pDC stimulation

To determine cytokine production, purified primary human pDCs (in the presence of IL-3) or GEN2.2 cells were kept at a concentration of 10^6 cells/mL aliquoted in 100- μL quantities in 96-well round-bottom culture plates, and stimulated with 4 $\mu\text{g}/\text{mL}$ CpG-A, 0.5 $\mu\text{g}/\text{mL}$ CpG-B, 0.5 μM R848, 25 ng/mL PMA, 10 $\mu\text{g}/\text{mL}$ of BDCA-2 or ILT7 antibody, or with HCV, or HBV overnight. In some experiments, BDCA-2 or ILT7-exposed cells were further crosslinked with goat-anti-mouse $F(ab')_2$ (15 $\mu\text{g}/\text{mL}$) (Jackson ImmunoResearch).

Production and purification of cell culture-derived HCVcc (JFH-1 3M) and HBV

JFH-1 3M HCVcc particles were prepared and purified as described previously [13, 15]. The HBV particles were concentrated from HBV stably transfected HepG2 cell line, clone 2.2.15 (HepG2.2.15) as described previously [17]. The 8% PEG8000 precipitated HBV supernatant purified by ultracentrifugation through 20, 30, 40, 50% sucrose was resuspended in RPMI 1640 medium to obtain a virus suspension containing 10^{12} HBV RNA copies/mL.

Determination of Syk phosphorylation by dynamic phosphoflow cytometry

Phosphoflow analysis of cells fixed, permeabilized, and incubated successively with phospho-Syk (Tyr525/526) (C87C1) (Cell Signaling Technology, Danvers, USA) rabbit mAb and anti-rabbit biotinylated antibodies was performed as previously described [13, 16, 18].

Determination of Syk phosphorylation by immunoblotting

Phosphorylation of Syk in the 25 μg cytoplasmic fraction in NP1 lysis buffer (Cell Signaling Technology) was analyzed by Western blotting using monoclonal phospho-Syk (Tyr525/526) (C87C1), or polyclonal phospho-Zap-70 (Tyr319)/Syk (Tyr352) Ab and Syk Ab, all from Cell Signaling Technology. In some experiments, the whole cell lysate was immunoprecipitated with phospho-Tyr mouse mAb (P-Tyr-100, Cell Signaling Technology) according to the manufacturer's instructions and the immunoprecipitate was analyzed by Western blotting using Syk Ab.

Determination of secreted IFN- α , TNF- α , and IL-6

The quantities of total IFN- α , TNF- α and IL-6 produced by pDCs or GEN2.2 were measured in cell-free supernatants using human ELISA kits (IFN- α and IL-6 from Mabtech, and TNF- α from BD Biosciences).

Statistical analysis

Quantitative variables are expressed as the means \pm SEM (standard error of the mean). To compare the levels of cytokine production by pDCs, Mann-Whitney two-tailed non-parametric test was used. Two-tailed unpaired Student's *t*-test was used to compare quantitative densitometric analysis of Western blots. Data were analyzed with GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). *p* value ≤ 0.05 was considered to be significant.

Results

Differential activation of Syk by TLR7/9 and RR agonists

To investigate the role of Syk in signaling triggered in primary pDCs by TLR7/9 and RR, we compared the kinetics of phosphorylation of Syk induced by agonists of both receptors. As in our previous studies, the insufficient quantities of primary pDCs available for biochemical analyses led us to employ dynamic phosphoflow cytometry [13, 16]. Our results show that triggering of TLR9 and RR signaling differentially induced kinetics of Syk phosphorylation (Fig 1A). TLR9 agonist CpG-A induced phosphorylation of Syk, which gradually increased for 30 min and then decreased to the initial MFI value. In contrast, triggering of RR by crosslinking of BDCA-2 induced rapid phosphorylation of Syk that peaked within 10 min of activation and then decreased during the next 50 min.

To facilitate biochemical analyses of cell signaling, which is still difficult to perform in rare and *in vitro* short living human primary pDCs, we performed our studies in human pDC line GEN2.2, which shares many features with human primary pDCs [14]. First, we compared the kinetics of the total phosphorylation of Syk by immunoprecipitation of the cell lysate with anti-Tyr Ab followed by blotting of immunoprecipitate with Syk mAb (Fig 1B). As in the primary pDCs, triggering of TLR9 and RR signaling in GEN2.2 cells induced differential kinetics of Syk phosphorylation. While after stimulation with CpG-A, total phosphorylation of Syk gradually increased up to 10 min, after stimulation with BDCA-2 mAb, the total phosphorylation of Syk peaked at 2 min.

When cytoplasmic fraction of GEN2.2 cells was probed for phosphorylation of the Syk at Y352 or Y525/526 (Fig 1C), the kinetics of phosphorylation was qualitatively similar to that in primary pDCs and to that of the total Syk phosphorylation in GEN2.2; it gradually increased after stimulation with CpG-A and peaked 2 min after stimulation with BDCA-2 (Fig 1D). While the phosphorylation at Y352, resembled that of the total Syk phosphorylation, the phosphorylation at Y525/526 showed much greater enhancement. In contrast to the total Syk phosphorylation, the phosphorylation of Syk at Y525/526 was more pronounced after stimulation with BDCA-2 than after stimulation with CpG-A, as in primary pDCs (Fig 1A). This result validates GEN2.2 cell line as an appropriate model for the study of cell signaling in pDCs.

The phosphorylation at Y525/526 in the kinase domain of Syk was sensitive to the Syk inhibitor AB8779 (Fig 1D and 1E), which is highly selective compared to Syk inhibitor fostatinib, R406 (S1 Fig and S1 Table). Densitometric analysis revealed that the inhibitory index defined by the ratio of $\text{pSyk}^{\text{Y525/526}}$ determined in the absence and the presence of AB8779 ($[\text{pSyk}^{\text{Y525/526}}]/[\text{pSyk}^{\text{Y525/526}}_{\text{AB8779}}]$) reached higher values in BDCA-2 (between 2.1 to 3.0) than in CpG-A-stimulated cells (between 1.5 to 2.1) (Fig 1E). This different effect of inhibitor on the phosphorylation induced by CpG-A and BDCA-2 was not observed for Y352. Taken together, triggering of TLR9 and RR induces differential activation of Syk with a differential sensitivity to AB8779.

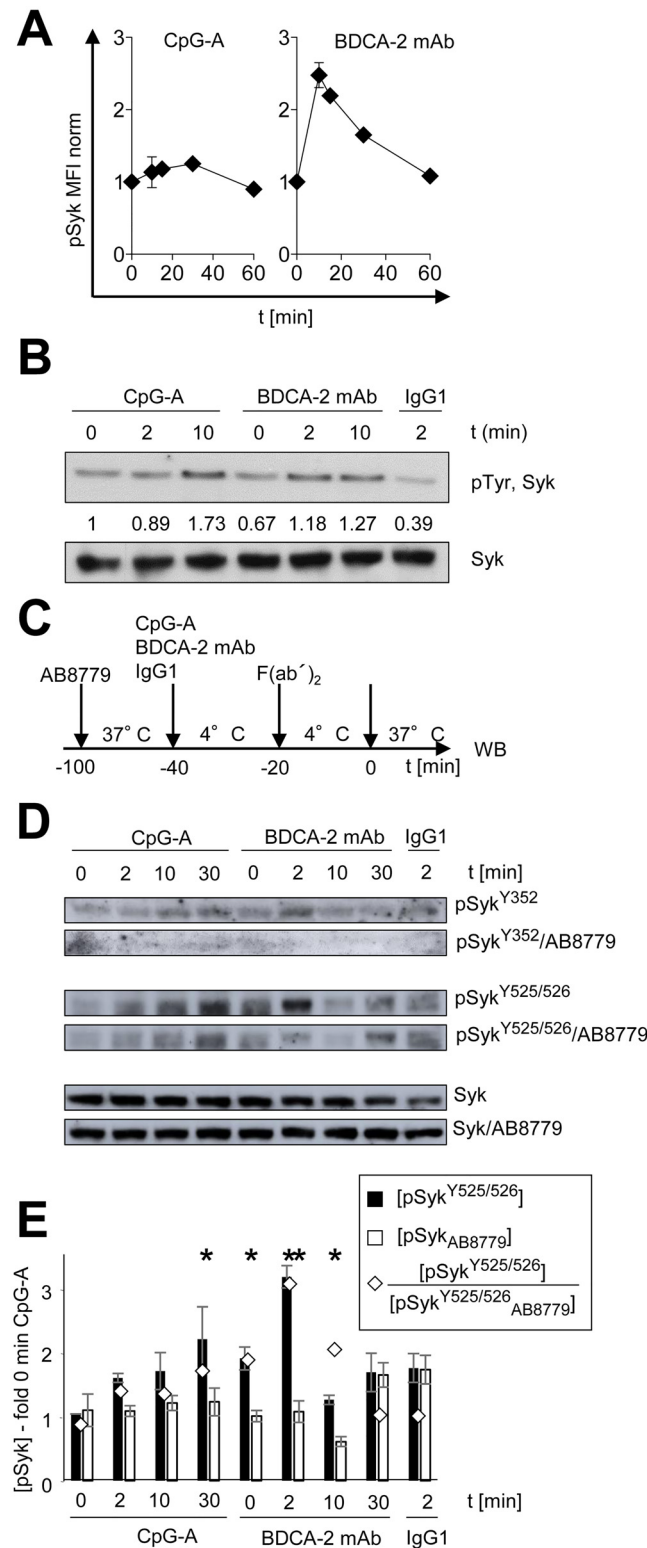


Fig 1. Phosphorylation of Syk in pDCs stimulated by CpG-A or crosslinked with BDCA-2 mAb. (A) Kinetics of phosphorylation of Syk (Y525/526) in the populations of magnetic bead-sorted pDCs exposed to CpG-A or crosslinked with BDCA-2 mAb was followed by flow cytometry (Phosphoflow). The data show means and SEM of three independent experiments with pDCs from different healthy donors. (B) Kinetics of the total Syk phosphorylation in GEN2.2 cells determined by immunoprecipitation of pTyr followed by

Western blotting with Syk Ab. Relative quantity of pSyk was determined by densitometry. Total Syk was used as a loading control. (C) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells and serum-starved overnight in RPMI were exposed or not to Syk inhibitor AB8779 for 1.5 h, and then to CpG-A at 4 μg/ml or to BDCA-2 mAb at 10 μg/ml for 20 min at 4°C. BDCA-2-treated cells were crosslinked with F(ab)₂ for 20 min at 4°C, and followed by analysis of phosphorylation of Syk by Western blotting. (D) Kinetics of phosphorylation of Syk Y352 (pSyk^{Y352}) and Syk Y525/526 (pSyk^{Y525/526}) in AB8779-treated or non-treated cells stimulated with CpG-A, BDCA-2 mAb or isotype control (IgG1) was followed by western blot. Total Syk was used as a loading control. Representative result of 3 independent experiments. (E) Quantitative densitometric analysis of phosphorylation of Syk Y525/526 (panel D) in the absence (full columns, [pSyk]) and presence (empty columns, [pSyk_{AB8779}]) of AB8779 normalized to the total Syk and expressed as fold increase compared to the control (CpG-A 0 min). ◇, inhibitory index defined by the ratio of pSyk/pSyk_{AB8779} densities. The data show means and SEM, N = 3. *, *p* ≤ 0.05; **, *p* < 0.01; two-tailed unpaired Student's *t*-test.

doi:10.1371/journal.pone.0156063.g001

Syk inhibitor blocks TLR7/9-mediated production of IFN-α and proinflammatory cytokines

We then investigated the effect of AB8779 on cytokine production in GEN2.2 cells stimulated with TLR9 agonists CpG-A and CpG-B, and with protein kinase (PKC) agonist PMA (Fig 2A). AB8779 inhibited IFN-α production with IC_{50,CpG-A} = 0.117 μM and IC_{50,CpG-B} = 0.215 μM, TNF-α production with IC_{50,CpG-A} = 0.006 μM and IC_{50,CpG-B} = 0.058 μM and IL-6 production

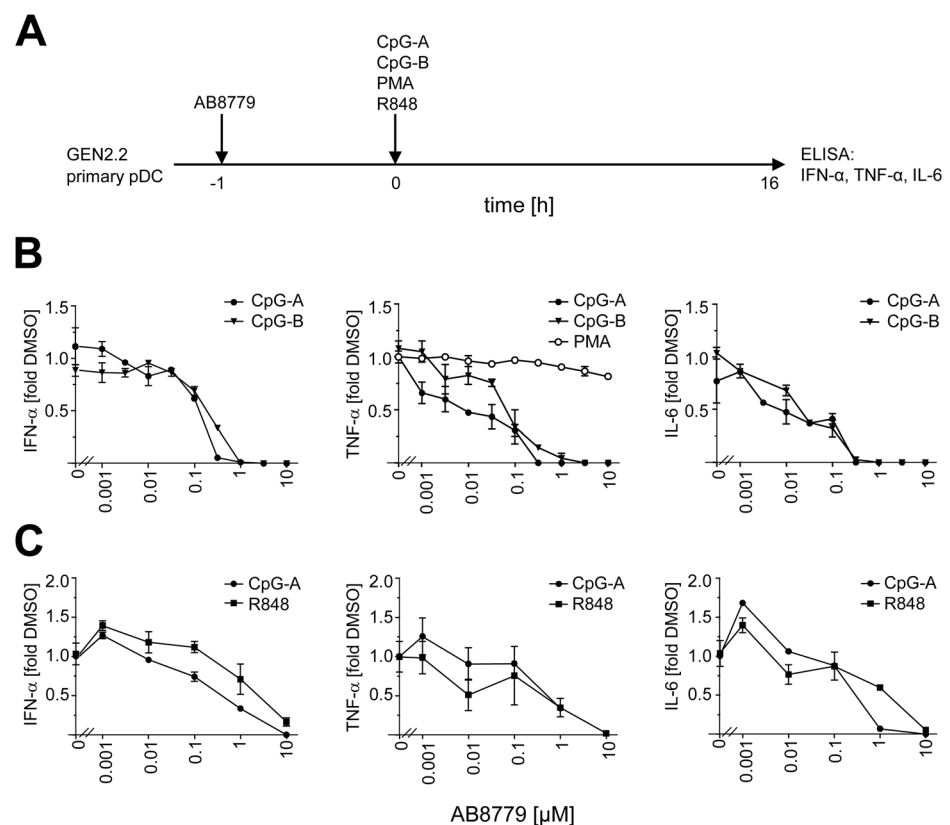


Fig 2. Effect of Syk inhibitor AB8779 on production of IFN-α TNF-α and IL-6 in pDCs. (A) Experimental outline. GEN2.2 cells (B), or primary pDC (C) were incubated with different concentrations of Syk inhibitor AB8779 for 1 hr before stimulation with CpG-A, CpG-B and PMA (N = 3) (B), or CpG-A and R848 (N = 2) (C). After 16 hr culture, IFN-α TNF-α and IL-6 production in GEN2.2 cells (B) or in primary pDCs (C) was determined in cell-free supernatants by ELISA and the results expressed as a multiple of control with the matching concentration of DMSO.

doi:10.1371/journal.pone.0156063.g002

with $IC_{50,CpG-A} = 0.023 \mu M$, and $IC_{50,CpG-B} = 0.021 \mu M$ (Fig 2B). In the same experiments, AB8779 only weakly inhibited production of TNF- α induced by PMA, which does not stimulate IFN- α and IL-6 production. In addition, we investigated effect of AB8779 on cytokine production in primary pDCs from healthy donors. Because synthetic agonists of TLR7 do not induce IFN- α secretion in GEN2.2 cells [14], we used primary pDCs, which permit to assess the effect of AB8779 on both TLR7 and TLR9-mediated cytokine production. As in GEN2.2 cells, AB8779 inhibited production of IFN- α , TNF- α , and IL-6 in primary pDCs stimulated with CpG-A; it inhibited also production of all three cytokines stimulated with synthetic TLR7 agonist R848 (Fig 1C). In sum, Syk inhibition specifically blocked TLR7/9-mediated production of IFN- α and proinflammatory cytokines.

Subliminal concentrations of Syk inhibitor enhance IFN- α production abrogated by crosslinking of RR by mAbs or by virus particles

Stronger inhibitory effect of AB8779 on Syk (Y525/526) phosphorylation induced by BDCA-2 than that induced by CpG-A (Fig 1D and 1E) suggested that subliminal concentrations of AB8779, which do not inhibit IFN- α production, could alleviate negative effect of BCR-like signaling on IFN- α production. To test this hypothesis, we exposed GEN2.2 cells pretreated with 0.01 μM AB8779 to ILT7 or BDCA-2 mAbs, or HBV or HCV particles, prior to stimulation with CpG-A (Fig 3A). As expected, in the absence of AB8779, IFN- α production was inhibited by RR crosslinking by mAbs or by HCV/HBV viral particles [12, 13, 15, 19–21] (Fig 3B). After standardization to the quantity of IFN- α produced in the absence of AB8779, pretreatment of GEN2.2 cells with 0.01 μM AB8779 significantly enhanced production of IFN- α (Fig 3C). IFN-

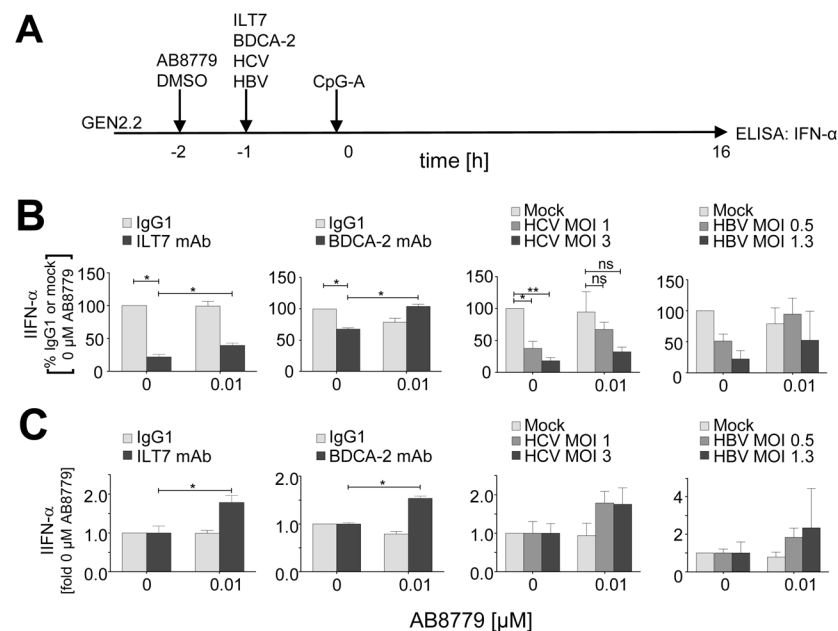


Fig 3. Subliminal concentrations of Syk inhibitor partially restore IFN- α production in GEN2.2 pDC cell line. (A) Experimental outline. After separation from MS-5 feeder cells, GEN2.2 cells were incubated with 0.01 μM AB8779 or with a matching concentration of DMSO for 1 hr before exposure ILT7 or BDCA-2 mAb or HCV or HBV particles and stimulation with CpG-A. (B) After 16 hr culture, IFN- α production was determined in GEN2.2 cell-free supernatants by ELISA, and the results were standardized to the quantity of IFN- α produced by GEN2.2 exposed to isotype control Ab or mock-infected culture in the absence of AB8779 (N = 3). (C) IFN- α production determined in GEN2.2 exposed to ILT7 or BDCA-2 mAb or HCV or HBV particles (shown in B) was normalized to IFN- α production in the absence of AB8779 *, $p \leq 0.05$; **, $p < 0.01$.

doi:10.1371/journal.pone.0156063.g003

α production increased in cells treated with ILT7 (1.8-fold, $p = 0.03$) and BDCA-2 (1.6-fold, $p = 0.04$), and it showed a tendency to enhanced production by cells treated with HCV particles (1.8-fold at both MOI = 1 and MOI = 3) and with HBV particles (1.8-fold at MOI = 0.5 and 2.3-fold at MOI = 1.3).

Discussion

Our results demonstrate that Syk is involved in pDCs in both RR and TLR7/9 signaling. While RR BCR-like pathway represents tolerogenic homeostatic functions of pDCs, TLR7/9 –a pattern recognition receptor pathway is responsible for pDC immunogenic activity. Thus, pharmacological targeting of Syk could be a useful tool to suppress overproduction of IFN-I in autoimmune diseases such as systemic lupus erythematosus and psoriasis, where pDCs play a critical role during initiation of the disease and are an attractive therapeutic target [4, 5].

The role of Syk in TLR signaling has been extensively studied in macrophages/monocytes in the context of the cell membrane localized TLR4 [22–24]. Syk activity is crucial for CD14-dependent endocytosis of TLR4 [22, 23]. Syk-deficient macrophages exhibited decreased TLR4-dependent activation of TBK1 signaling and production of type I IFNs, however, they showed an enhanced activation of TAK1 and increased production of proinflammatory cytokines, compared to that in wild-type macrophages. In contrast, the role of Syk in endosomal TLR7/9 signaling is much less understood. Several reports demonstrated that Syk is recruited to TLR9 upon CpG stimulation, and that this interaction can be blocked by Src kinases inhibitors [23–25]. Because membrane-associated Syk is recruited to ITAM-containing receptors, which is not the case of TLR9, it is likely that the TLR9–Syk association is indirect and that other proteins participate in the formation of a complex. This is compatible with different kinetics of Syk phosphorylation in GEN2.2 cells stimulated with TLR7/9 or RR agonists. Our results show that stimulation of TLR or BCR pathway in pDCs leads to phosphorylation of the activation loop tyrosines Y525/526 that is substantially greater than phosphorylation at Y352 (Fig 1D). A similar observation was made in B cells, where, phosphorylation at Y525/526 was shown to be required for sustained PLC- γ 2, Akt and ERK signaling, while phosphorylation at Y352 had a constitutive character [26].

Subliminal concentrations of AB8779, which only weakly inhibited production of IFN- α induced by CpG-A, significantly enhanced production of IFN- α blocked by triggering RR pathway. This suggests that concentration of Syk inhibitor that does not block TLR7/9 pathway ($\leq 0.01 \mu\text{M}$ AB8779) abrogates negative effect of RR BCR-like signaling on IFN-I production. This result is compatible with different sensitivity of Syk to AB8779 upon stimulation of GEN2.2 cells with TLR7/9 or RR agonists (Fig 1D and 1E); it suggests presence of different pools of Syk in pDCs, one controlling TLR7/9 and the other controlling RR pathway. Restoration of immunogenic activity by pharmacological targeting of Syk is of special interest in the case of pDCs exposed to HBV and HCV particles, where ligation of RR with viruses represents one of the immune escape mechanisms [7, 11–13, 15, 19, 20]. While in the era of the great success of direct acting antivirals against HIV and HCV, stimulation of IFN response might represent an adjuvant therapy, namely important in the case of the virus escape, induction of IFN-I in combination with existing antivirals may cure HBV infection [27].

Supporting Information

S1 Fig. Treepot™ interaction maps of AB8779 compared to R406 (Fostamatinib). *In vitro* kinase profiling by DiscoverX (Fremont, CA, USA). The result of a high-throughput system (KINOMEscan™) for screening of both compounds against large numbers of human kinases (442 kinases) developed by Ambit Biosciences are visualised using a TREEspot™ interaction

Maps. Kinases found to bind the compounds are marked with red circles, where larger circles indicate higher-affinity binding. The compounds were screened at the concentration of 1 μ M, and results for primary screen binding interactions are reported as percent control (% Ctrl), where lower numbers indicate stronger hits. DMSO is used as a negative control (100% Ctrl) while a high affinity compound is used as a positive control (0% Ctrl). % Ctrl is calculated as follow:

$$\frac{\text{test compound signal} - \text{positive control signal}}{\text{negative control signal} - \text{positive control signal}} \times 100$$

The S-score of AB8779 tested in this assay is shown in [S1 Table](#). These results clearly show that AB8779 is more specific than Fostamatinib (R406).

(PDF)

S1 Table. S-score table for AB8779 tested at 1 μ M. Selectivity (S)-Score is a quantitative measure of compound selectivity. It is calculated by dividing the number of kinases that compounds bind to by the total number of distinct kinases tested, excluding mutant variants. $S(35) = (\text{number of non-mutant kinases with \% Ctrl} < 35) / (\text{number of non-mutant kinases tested})$, $S(10) = (\text{number of non-mutant kinases with \% Ctrl} < 10) / (\text{number of non-mutant kinases tested})$, $S(1) = (\text{number of non-mutant kinases with \%Ctrl} < 1) / (\text{number of non-mutant kinases tested})$.

(PDF)

Acknowledgments

The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: IH RS BA DK PD. Performed the experiments: BA DK SL AFH JF RS. Analyzed the data: RS IH BA DK SL KT DO PD JAN. Contributed reagents/materials/analysis tools: DD LC JP JW JH PD. Wrote the paper: IH RS. Edited manuscript: JAN DO PD JW KT JH.

References

1. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med*. 2003; 198(3):513–20. PMID: [12900525](#).
2. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, et al. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med*. 2011; 208(10):1989–2003. Epub 2011/09/21. [jem.20101158](#) [pii] doi: [10.1084/jem.20101158](#) PMID: [21930769](#); PubMed Central PMCID: PMC3182061.
3. Sisirak V, Faget J, Gobert M, Goutagny N, Vey N, Treilleux I, et al. Impaired IFN-alpha Production by Plasmacytoid Dendritic Cells Favors Regulatory T-cell Expansion That May Contribute to Breast Cancer Progression. *Cancer Res*. 2012; 72(20):5188–97. Epub 2012/07/28. 0008-5472.CAN-11-3468 [pii] doi: [10.1158/0008-5472.CAN-11-3468](#) PMID: [22836755](#).
4. Rowland SL, Riggs JM, Gilfillan S, Bugatti M, Vermi W, Kolbeck R, et al. Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model. *The Journal of experimental medicine*. 2014; 211(10):1977–91. Epub 2014/09/03. doi: [10.1084/jem.20132620](#) PMID: [25180065](#).
5. Glitzner E, Korosec A, Brunner PM, Drobits B, Amberg N, Schonthaler HB, et al. Specific roles for dendritic cell subsets during initiation and progression of psoriasis. *EMBO molecular medicine*. 2014. Epub 2014/09/14. doi: [10.15252/emmm.201404114](#) PMID: [25216727](#).

6. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and auto-immune diseases. *Nat Rev Immunol*. 2008; 8(8):594–606. PMID: [18641647](#). doi: [10.1038/nri2358](#)
7. Hirsch I, Caux C, Hasan U, Bendriss-Vermare N, Olive D. Impaired Toll-like receptor 7 and 9 signaling: from chronic viral infections to cancer. *Trends Immunol*. 2010; 31(10):391–7. Epub 2010/09/14. S1471-4906(10)00108-0 [pii] doi: [10.1016/j.it.2010.07.004](#) PMID: [20832362](#).
8. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol*. 2015; 15(8):471–85. doi: [10.1038/nri3865](#) PMID: [26160613](#).
9. Cao W, Rosen DB, Ito T, Bover L, Bao M, Watanabe G, et al. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *J Exp Med*. 2006; 203(6):1399–405. PMID: [16735691](#).
10. Cao W, Zhang L, Rosen DB, Bover L, Watanabe G, Bao M, et al. BDCA2/Fc epsilon RI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells. *PLoS Biol*. 2007; 5(10):e248. PMID: [17850179](#).
11. Martinelli E, Cicala C, Van Ryk D, Goode DJ, Macleod K, Arthos J, et al. HIV-1 gp120 inhibits TLR9-mediated activation and IFN- α secretion in plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A*. 2007; 104(9):3396–401. PMID: [17360657](#).
12. Woltman AM, Op den Brouw ML, Biesta PJ, Shi CC, Janssen HL. Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function. *PLoS ONE*. 2011; 6(1):e15324. Epub 2011/01/20. doi: [10.1371/journal.pone.0015324](#) PMID: [21246041](#); PubMed Central PMCID: PMC3016396.
13. Florentin J, Aouar B, Dental C, Thumann C, Firaguay G, Gondois-Rey F, et al. HCV glycoprotein E2 is a novel BDCA-2 ligand and acts as an inhibitor of IFN production by plasmacytoid dendritic cells. *Blood*. 2012; 120(23):4544–51. Epub 2012/10/12. blood-2012-02-413286 [pii] doi: [10.1182/blood-2012-02-413286](#) PMID: [23053572](#).
14. Chaperot L, Blum A, Manches O, Lui G, Angel J, Molens JP, et al. Virus or TLR agonists induce TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. *J Immunol*. 2006; 176(1):248–55. PMID: [16365416](#).
15. Gondois-Rey F, Dental C, Halfon P, Baumert TF, Olive D, Hirsch I. Hepatitis C virus is a weak inducer of interferon alpha in plasmacytoid dendritic cells in comparison with influenza and human herpesvirus type-1. *PLoS ONE*. 2009; 4(2):e4319. PMID: [19183807](#). doi: [10.1371/journal.pone.0004319](#)
16. Dental C, Florentin J, Aouar B, Gondois-Rey F, Durantel D, Baumert TF, et al. Hepatitis C Virus Fails to Activate NF-kappaB Signaling in Plasmacytoid Dendritic Cells. *J Virol*. 2011; 86(2):1090–6. Epub 2011/11/18. JVI.05444-11 [pii] doi: [10.1128/JVI.05444-11](#) PMID: [22090103](#).
17. Luangsay S, Gruffaz M, Isorce N, Testoni B, Michelet M, Faure-Dupuy S, et al. Early Inhibition of Hepatocyte Innate Responses by Hepatitis B Virus. *J Hepatol*. 2015. doi: [10.1016/j.jhep.2015.07.014](#) PMID: [26216533](#).
18. Firaguay G, Nunes JA. Analysis of signaling events by dynamic phosphoflow cytometry. *Sci Signal*. 2009; 2(86):pl3. Epub 2009/09/03. scisignal.286pl3 [pii] doi: [10.1126/scisignal.286pl3](#) PMID: [19724061](#).
19. Shiina M, Reherrmann B. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. *Hepatology*. 2008; 47(2):385–95. PMID: [18064579](#).
20. Vincent IE, Zannetti C, Lucifora J, Norder H, Protzer U, Hainaut P, et al. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. *PLoS One*. 2011; 6(10):e26315. doi: [10.1371/journal.pone.0026315](#) PMID: [22046272](#); PubMed Central PMCID: PMC3201951.
21. Cao W, Bover L, Cho M, Wen X, Hanabuchi S, Bao M, et al. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *J Exp Med*. 2009; 206(7):1603–14. PMID: [19564354](#). doi: [10.1084/jem.20090547](#)
22. Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, et al. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell*. 2011; 147(4):868–80. Epub 2011/11/15. S0092-8674(11)01221-9 [pii] doi: [10.1016/j.cell.2011.09.051](#) PMID: [22078883](#); PubMed Central PMCID: PMC3217211.
23. Lin YC, Huang DY, Chu CL, Lin YL, Lin WW. The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Science signaling*. 2013; 6(289):ra71. Epub 2013/08/22. doi: [10.1126/scisignal.2003973](#) PMID: [23962979](#).
24. Sanjuan MA, Rao N, Lai KT, Gu Y, Sun S, Fuchs A, et al. CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. *The Journal of cell biology*. 2006; 172(7):1057–68. Epub 2006/03/29. doi: [10.1083/jcb.200508058](#) PMID: [16567503](#); PubMed Central PMCID: PMC2063763.
25. Rao S, Liu X, Freedman BD, Behrens EM. Spleen tyrosine kinase (Syk)-dependent calcium signals mediate efficient CpG-induced exocytosis of tumor necrosis factor alpha (TNFalpha) in innate immune

cells. *J Biol Chem*. 2013; 288(18):12448–58. Epub 2013/03/22. M113.454405 [pii] doi: [10.1074/jbc.M113.454405](https://doi.org/10.1074/jbc.M113.454405) PMID: [23515313](https://pubmed.ncbi.nlm.nih.gov/23515313/); PubMed Central PMCID: PMC3642293.

26. Carsetti L, Laurenti L, Gobessi S, Longo PG, Leone G, Efremov DG. Phosphorylation of the activation loop tyrosines is required for sustained Syk signaling and growth factor-independent B-cell proliferation. *Cell Signal*. 2009; 21(7):1187–94. doi: [10.1016/j.cellsig.2009.03.007](https://doi.org/10.1016/j.cellsig.2009.03.007) PMID: [19296913](https://pubmed.ncbi.nlm.nih.gov/19296913/).
27. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science*. 2014; 343(6176):1221–8. Epub 2014/02/22. doi: [10.1126/science.1243462](https://doi.org/10.1126/science.1243462) PMID: [24557838](https://pubmed.ncbi.nlm.nih.gov/24557838/).