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Article Sub-Title		
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Journal Name	Journal of Clinical Immunology	
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Schedule
Received 11 Feb 2022
Revised
Accepted 12 Apr 2022

Abstract

Purpose:
STAT1 gain-of-function (GOF) and dominant-negative (DN) STAT3 syndromes share clinical manifestations including infectious and inflammatory manifestations. Targeted treatment with Janus-kinase (JAK) inhibitors shows promising results in treating STAT1 GOF-associated symptoms while management of DN STAT3 patients has been largely supportive. We here assessed the impact of ruxolitinib on the JAK-STAT1/3 pathway in DN STAT3 patients' cells.
Methods:
Using flow cytometry, immunoblot, qPCR, and ELISA techniques, we examined the levels of basal STAT1 and phosphorylated STAT1 (pSTAT1) of cells obtained from DN STAT3, STAT1 GOF patients, and healthy donors following stimulation with type I/II interferons (IFNs) or interleukin (IL)-6. We also describe the impact of ruxolitinib on cytokine-induced STAT1 signaling in these patients.
Results:
DN STAT3 and STAT1 GOF resulted in a similar phenotype characterized by increased STAT1 and pSTAT1 levels in response to IFNα (CD3+ cells) and IFNγ (CD14+ monocytes). STAT1-downstream gene expression and C-X-C motif chemokine 10 secretion were higher in most DN STAT3 patients upon stimulation compared to healthy controls. Ex vivo treatment with the JAK1/2-inhibitor ruxolitinib reduced cytokine responsiveness and normalized STAT1 phosphorylation in DN STAT3 and STAT1 GOF patient' cells. In addition, ex vivo treatment was effective in modulating STAT1 downstream signaling in DN STAT3 patients.
Conclusion:
In the absence of effective targeted treatment options for AD-HIES at present, modulation of the JAK/STAT1 pathway with JAK inhibitors may be further explored particularly in those AD-HIES patients with autoimmune and/or autoinflammatory manifestations.

Keywords (separated by '-') JAK-STAT pathway - DN STAT3 - STAT1 GOF - ruxolitinib

Footnote Information Pilar Blanco Lobo and Paloma Guisado-Hernández contributed equally.Peter Olbrich and Olaf Neth contributed equally.The online version contains supplementary material available at https://doi.org/10.1007/s10875-022-01273-x.

Journal of Clinical Immunology
https://doi.org/10.1007/s10875-022-01273-x

ORIGINAL ARTICLE

Ex vivo effect of JAK inhibition on JAK-STAT1 pathway hyperactivation in patients with dominant-negative STAT3 mutations

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Received: 11 February 2022 / Accepted: 12 April 2022

Abstract

Purpose STAT1 gain-of-function (GOF) and dominant-negative (DN) STAT3 syndromes share clinical manifestations including infectious and inflammatory manifestations. Targeted treatment with Janus-kinase (JAK) inhibitors shows promising results in treating STAT1 GOF-associated symptoms while management of DN STAT3 patients has been largely supportive. We here assessed the impact of ruxolitinib on the JAK-STAT1/3 pathway in DN STAT3 patients' cells.

Methods Using flow cytometry, immunoblot, qPCR, and ELISA techniques, we examined the levels of basal STAT1 and phosphorylated STAT1 (pSTAT1) of cells obtained from DN STAT3, STAT1 GOF patients, and healthy donors following stimulation with type I/II interferons (IFNs) or interleukin (IL)-6. We also describe the impact of ruxolitinib on cytokine-induced STAT1 signaling in these patients.

Results DN STAT3 and STAT1 GOF resulted in a similar phenotype characterized by increased STAT1 and pSTAT1 levels in response to IFNα ($CD3^+$ cells) and IFNγ ($CD14^+$ monocytes). STAT1-downstream gene expression and C-X-C motif chemokine 10 secretion were higher in most DN STAT3 patients upon stimulation compared to healthy controls. Ex vivo treatment with the JAK1/2-inhibitor ruxolitinib reduced cytokine responsiveness and normalized STAT1 phosphorylation in DN STAT3 and STAT1 GOF patient' cells. In addition, ex vivo treatment was effective in modulating STAT1 downstream signaling in DN STAT3 patients.

Conclusion In the absence of effective targeted treatment options for AD-HIES at present, modulation of the JAK/STAT1 pathway with JAK inhibitors may be further explored particularly in those AD-HIES patients with autoimmune and/or autoinflammatory manifestations.

Keywords JAK-STAT pathway · DN STAT3 · STAT1 GOF · ruxolitinib

Introduction

With the advances in high-throughput DNA sequencing, the number of patients identified with inborn defects in the Janus Kinase (JAK)-Signal Transducers and Activator of Transcription (STAT) pathway, or its regulatory components has markedly increased over the past few decades [1, 2]. Heterozygous autosomal dominant (AD) negative mutations in STAT3 have been recognized to cause hyper-IgE syndrome (AD-HIES), also known as Job syndrome [3–6]. Patients with dominant-negative (DN) mutations in STAT3 are susceptible to skin and pulmonary infections (frequently caused by *Staphylococcus aureus* or *Aspergillus fumigatus*) and chronic mucocutaneous candidiasis (CMC) [7, 8]. They also display elevated IgE serum levels (>2000 U/ml), reduced circulating Th17 and follicular T helper (Th) cells, decreased B and natural killer (NK) cell activation and function resulting in diminished vaccine responses [5, 9–12]. Features most likely not directly related to the immune system include skeletal

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and connective tissue abnormalities, vasculopathies, and malignancies [5, 11–13].

Diseases due to gain-of-function (GOF) mutations in STAT1 and DN mutations in STAT3 share a variety of clinical manifestations including infection susceptibility, predisposition to vascular complications as well as the immune phenotype [5, 14, 15]. Common mechanistic explanations for an impaired Th17 differentiation have been proposed including a decrease in STAT3-dependent transcription of retinoic acid receptor–related orphan receptor (ROR)-γt or the upregulation of programmed death-ligand 1 (PD-L1) on naïve T cells [16, 17]. Although in vivo PD-L1 inhibition was not protective in an oropharyngeal candidiasis mouse model, it was shown to be effective in restoring interleukin (IL)-17A (but not IL-17F) expression frequencies as well as PD-L1 and suppressor of cytokine signaling 3 (SOCS3) levels [17]. In addition, an increase in STAT1 phosphorylation (pSTAT1) after stimulation with IL-6, IL-21, IL-27, or interferon (IFN)-γ has been observed, both in patients with STAT1 GOF and DN STAT3 compared with healthy controls, suggesting the existence of a STAT3-dependent, STAT1 regulation mechanism [17].

In the setting of STAT1 GOF, targeted treatment with the JAK 1/2 inhibitor ruxolitinib has shown to control the paradigmatic hyperresponsiveness to type I and II IFNs and has resulted in improvement of clinical symptoms like CMC and autoimmune manifestations and in normalization of Th cell differentiation in some patients [18–23]. In contrast, current treatment for DN STAT3 patients is supportive and mostly limited to continuous antibiotic and antifungal prophylaxis, aggressive and early treatment of intercurrent infections and physical therapy [5, 24]. Hematopoietic stem cell transplantation has been performed in a limited number of patients with overall mixed results and non-hematological complications such as vasculopathy or bone-related complications remain likely unresolved [5, 24–26]. There is hence an urgent need for identifying novel therapeutic options for treating patients with DN STAT3 mutations.

Considering the similarities in clinical and immunological phenotypes found in STAT1 GOF and DN STAT3, we here sought to determine the response of DN STAT3 patient cells to type I and II IFNs, as well as IL-6 in the setting of JAK inhibition (Fig. 1). We observed an immunological phenotype characterized by high levels of total STAT1 and cytokine-induced pSTAT1 that result in upregulation of STAT1-downstream signaling. Correspondingly, the JAK1/2 inhibitor ruxolitinib reduces the cytokine hypersensitivity of immune cells in the setting of DN STAT3 indicating that this drug may be a potential directed treatment option for some of these patients.

Materials and methods

Study participants

Study subjects were diagnosed with Job Syndrome (AD-HIES, DN STAT3) using a diagnostic scoring system comprising immunological and non-immunological features [27] and identification of STAT3 mutations by Sanger sequencing. Patients with STAT1 GOF mutations (N658H, M202I, and P326S) were included as controls. The study protocol was approved by the Ethics Committee of the Hospitales Universitarios Virgen Macarena and Virgen del Rocío (0243-N-19). Specific informed consent forms were signed from all patients, family members, and healthy volunteers at each Spanish participating center (Seville, Malaga, Valencia, and Madrid). Experiments were always performed using a healthy control sample treated in the same way than samples from patients, including those that were sent from other hospitals.

Whole blood stimulation, cellular staining, and flow cytometry

Fresh heparinized whole blood samples from patients with DN STAT3 or STAT1 GOF mutations and healthy controls were transferred (100µL) to polystyrene round-bottom tubes (Falcon). Cells were then stimulated with IFNγ (400 UI/mL; Imukin, Horizon Pharma) or IL6 (100ng/m; PeproTech) for 15 min, or with IFNα (100ng/mL; PBL Assay Science) for 30 min at 37°C in the presence of different concentrations of ruxolitinib (0.1 µM, 0.5 µM, or 1 µM; Selleckchem), or vehicle (Dimethyl Sulfoxide; PanReac AppliChem). The cell suspensions were then incubated (15 min, room temperature) with 2 mL (1X) of lysis buffer (e-Bioscience, Invitrogen) and washed twice with RPMI 1640 (Biowest). For intracellular staining, an initial permeabilization step was performed. One-hour incubation with ice-cold methanol was followed by 2 washes with phosphate-buffered saline (PBS) and 2% fetal bovine serum (FBS; South America, Biowest) to remove any residual methanol. After three washing steps, cells were incubated with the following monoclonal antibodies for 1 h at 4°C: anti-human (h)CD14-FITC (clone M5E2, Becton Dickinson), anti-hCD3-APC-H7 (clone SK7, BD), anti-hCD4-BV711 (clone SK3, BioLegend), and anti-hCD8-PE-Cy7 (clone SK1, BioLegend), anti-hSTAT1 N-terminus-Alexa Fluor 647 (clone 1/STAT1, BD), anti-hSTAT1 (pTyr701)-PerCP-Cy5.5 (clone 4A, BD), anti-hSTAT3-PE (clone M59-50, BD), and anti-hSTAT3 (pTyr705)-BV421 (clone 13A3-1, BioLegend). Isotypes for mouse IgG1k1-Alexa Fluor 647 and IgG2a,k-PerCP-Cy5.5 (BD)

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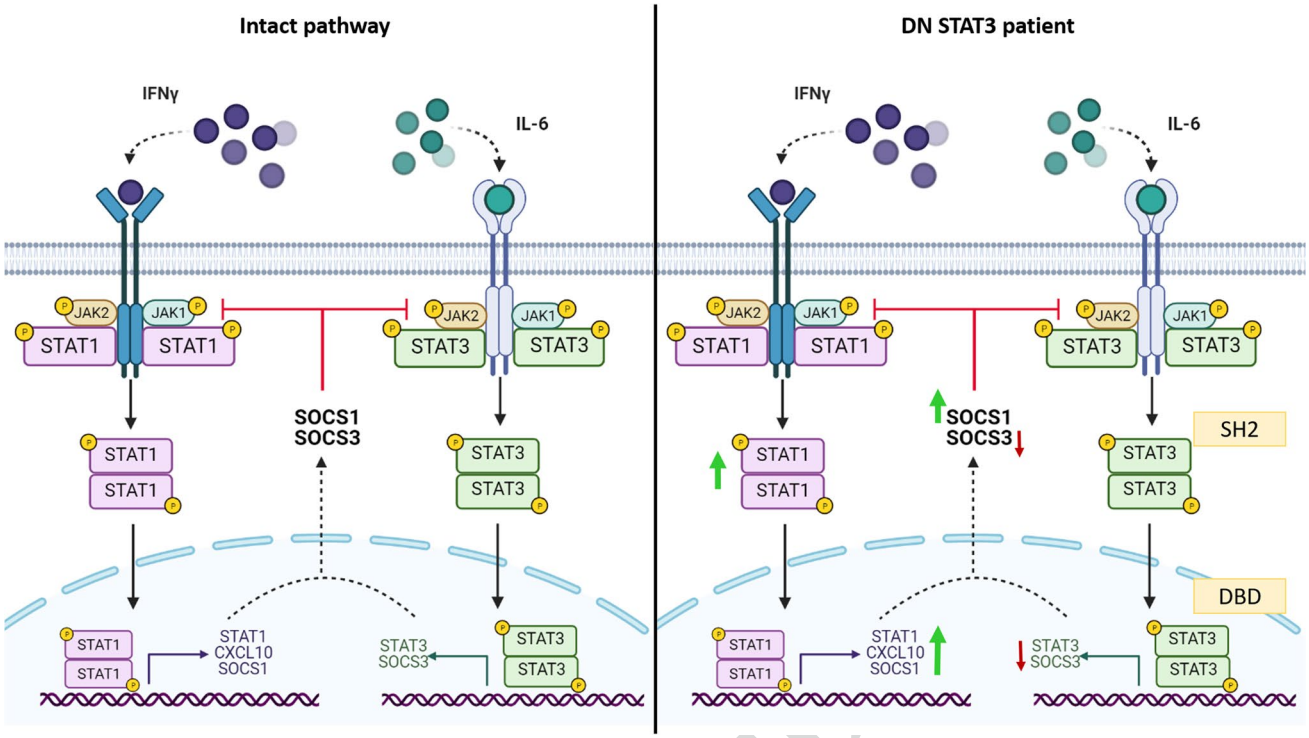

Fig. 1. Schematic representation of intact and altered JAK-STAT pathways potentially modulated by ruxolitinib. Healthy (left) and DN STAT3 (right) scenarios are represented. Binding of type I and II IFNs (only type II represented as IFN-γ) to its receptor leads to activation of JAK1/JAK2 and phosphorylation (p) of STAT1. Homodimers (pSTAT1/pSTAT1) translocate to the nucleus to activate interferon-stimulated genes (e.g. STAT1, CXCL10, or SOCS1). Binding of IL-6 to its receptor leads to activation of JAK1/JAK2 and phosphorylation (p) of STAT3. Homodimers (pSTAT3/pSTAT3) translocate to the nucleus to activate interferon-stimulated genes (e.g. STAT3 or SOCS3). DN mutations in the SH2 (impaired dimerization) and DNA binding domains of STAT3 result in a reduced expression of STAT3-dependent genes upon IL-6 stimulation (right). SOCS proteins are negative regulators of cytokine-induced signaling. Reduced levels of SOCS3 lead to higher activation of JAK1/2 and excessive accumulation of total STAT1 and phosphorylation. Higher activity in the JAK-STAT1 pathway after IFN stimulation allows for high level of STAT1-dependent genes (STAT1, CXCL10, and SOCS1). IFN: interferon; IL-6: interleukin 6; JAK: Janus Kinase; STAT: signal transducer and activator of transcription; suppressor of cytokine signaling (SOCS); C-X-C motif chemokine ligand 10 (CXCL10)

were used as controls. Stained cells were washed twice (PBS/2%FBS), re-suspended in paraformaldehyde 1% (PFA, Sigma), and stored in dark at 4°C until analysis. Data were collected using the BD LSRFortessa™ (Becton Dickinson) including the FACS DIVA (v8.0) software and analyzed with the FlowJo (v. 10.7.0, Treestar, Ashland, OR, USA) software package.

Immunoblotting assays

Peripheral blood mononuclear cells (PBMCs) from DN STAT3 and STAT1 GOF patients and healthy donors were isolated by density-gradient centrifugation using BD Vacutainer cell preparation tubes. PBMCs were then left unstimulated or stimulated with IFNα (100ng/mL; PBL Assay Science) with or without ruxolitinib (1μM) for 30 min at 37°C. Cells were lysed using RIPA buffer (NaCl 150mM, NP-40 Calbiochem 1%, DCO 0.5%, SDS 0.1%, Tris HCl 50mM pH 7.5) containing 1% proteinase inhibitors and phosphatase inhibitors (Sigma-Aldrich) on ice for 10 min. Lysates were then centrifuged at 15000 rpm for 15 min at 4 °C. Protein concentration was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher). Samples were diluted in Laemmli buffer (Sigma-Aldrich) and heated to 95 °C for 5 min. Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to PVDF membranes (Cytiva Amersham Hybond PVDF Membranes). Membranes were blocked in 200 mM Tris, 1500 mM NaCl (pH 7.6), 0.1% Tween 20, 5% serum bovine albumin and (T-TBS-albumin, AppliChem), for 30 min at room temperature. To detect STAT1 proteins, the membranes were incubated overnight at 4°C with antibodies directed against STAT1 (Cell Signaling 9172), pSTAT1 (Py701; Cell Signaling 9167) or β-actin (Cell Signaling 4967). Membranes were washed with T-TBS and incubated for 1 h at room temperature with polyclonal horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG (Cell Signaling 7074). Immunoreactivity was

Journal : Large 10875 | Article No : 1273 | Pages : 12 | MS Code : 1273 | Dispatch : 29-4-2022

assessed by chemiluminescence reaction using the enhanced chemiluminescence (ECL) blocking detection system (Bio-Rad). Densitometry was performed using the automated digitizing software (ImageJ, NIH, Bethesda, USA). All bands were normalized to relative protein levels using β-actin as housekeeping protein.

PBMCs stimulation for transcriptomic analysis and chemokine secretion assays

Fresh isolated PBMC were re-suspended in RPMI culture media (Biowest), supplemented with L-Glutamine (300 mg/L), penicillin (100 U/ml)/streptomycin (100 µg/ml; Gibco), and 10% FBS. PBMCs (2×10^6 cells/well, 12-well plate) were rested for 1 h and stimulated with IFNγ (400 UI/mL; Imukin, Horizon Pharma) for 4 h at 37°C, 5% CO2 in the presence of different concentrations of ruxolitinib (0.1 µM, 0.5 µM, or 1 µM; Selleckchem), or vehicle (Dimethyl Sulfoxide; PanReac AppliChem). One unstimulated sample was included for each patient and healthy control to assess the basal state.

Determination of mRNA levels by quantitative reverse transcription-polymerase chain reaction

Unstimulated and IFNγ-stimulated PBMCs were harvested and subjected to total RNA extraction using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. Complementary (c) DNA was generated from 100 ng of RNA using the reverse transcription kit (Applied Biosystems). Relative *STAT1*, *CXCL10*, *PD-L1*, *SOCS1*, and *SOCS3* mRNA levels were determined using the Gene Expression Assays (Hs01013996, Hs00171042, Hs00204257, Hs00705164, and Hs02330328, respectively; Thermo Fisher) and TaqMan Gene Expression Master Mix (Applied Biosystems) following manufacturer's instructions. β-actin (Hs01060665; Thermo Fisher) was used as endogenous control. PCR conditions consisted of polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Relative mRNA levels were analyzed using the comparative $2^{-\Delta\Delta ct}$ method.

Enzyme-linked immunosorbent assay

IFN-inducible CXCL10 protein levels were determined in PBMCs culture supernatant by enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher) following manufacturer's instructions. CXCL10 levels obtained from patients' cell supernatants were compared to the levels of the same-day healthy control.

Data analysis

Data from all experiments acquired on the same flow cytometer instrument, using the same settings, were analyzed in raw values of geometric mean of fluorescence (gMFI). Data were expressed either as direct gMFI of STAT1 and pSTAT1 levels or as percentage of the same-day healthy donor's level. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) data from patients were also normalized with the respective same-day healthy donor's level. Graphs were performed using the Prism software (version 8, GraphPad software). Statistical analysis was performed using the software RStudio Team (2021). Normality for quantitative variables was evaluated using Shapiro-Wilk. For inferential statistics, Wilcoxon and Kruskal-Wallis tests were used. *p* values lower than 0.05 were considered statistically significant.

Results

Increased STAT1 levels and cytokine-induced phosphorylation of STAT1 in patients with AD-HIES

We included 6 patients from 5 unrelated Spanish families with heterozygous DN STAT3 mutations located in the DNA binding and Src homology 2 (SH2) domains, all of them previously described to cause AD-HIES (Fig. S1) [3, 28–30]. Patients with STAT1 GOF mutations (N658H, M202I, and P326S) were included as controls. Because patients with STAT1 GOF and DN STAT3 mutations show a remarkable overlap in terms of clinical manifestations and cellular phenotypes [5, 14, 17, 31], we first sought to evaluate levels of total STAT1 in resting $CD3^+$ T cells and $CD14^+$ monocytes by flow cytometry (Fig. 2). DN STAT3 patients exhibited increased total STAT1 levels when compared to their corresponding same-day healthy controls, similar to those observed in STAT1 GOF patients. We then evaluated the pSTAT1 levels of DN STAT3 and STAT1 GOF patients' cells after stimulation with IFNα, IFNγ and IL-6, all of which being involved in the activation of the JAK-STAT signaling pathway (Fig. 3). When we evaluated the effect of individual cytokines on pSTAT1 levels in patient's cells compared to their basal state (Fig. 3A), we observed significantly augmented pSTAT1 levels when $CD3^+$ cells were stimulated with IFNα (p=0.013). Similarly, a pSTAT1 increase was observed after IFNγ (p=0.017) and IFNα (p=0.02) stimulation of $CD14^+$ monocytes whereas other cytokine-cell type combinations did not result in such an increase (Fig. 3A). In addition, pSTAT1 levels in $CD3^+$ cells from DN STAT3 patients were shown to be higher compared to their same-day healthy controls, after stimulation with IFNγ (p=0.036) or IFNα (p=0.036) and tended to be raised after IL-6 stimulation (p=0.059). In $CD14^+$ monocytes,

Journal : Large 10875 | Article No : 1273 | Pages : 12 | MS Code : 1273 | Dispatch : 29-4-2022

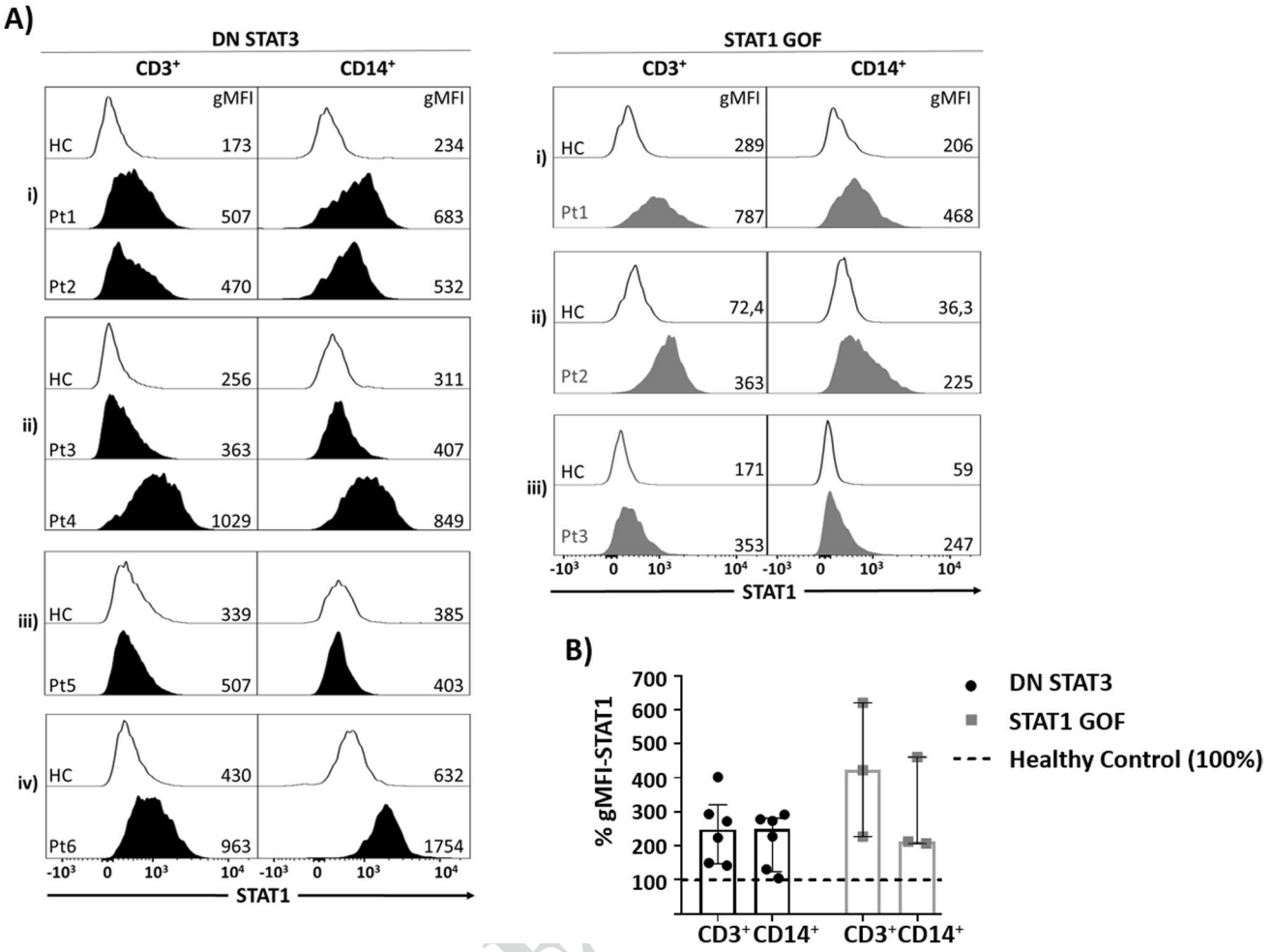

Fig. 2. STAT1 levels of patients with AD-HIES and STAT1 GOF. **A** Geometric mean fluorescence intensity (gMFI) of STAT1 in resting CD3$^+$ T cells and CD14$^+$ monocytes of healthy controls (white histograms), patients with DN STAT3 (black histograms), and STAT1 GOF (gray histograms). **B** Normalization of STAT1 levels at basal state in CD3$^+$ T cells and CD14$^+$ monocytes from DN STAT3 (black) and STAT1 GOF (gray) patients considering the gMFI value of the healthy control to be 100% (black dotted line)

pSTAT1 levels were significantly higher in patients than healthy controls after IL-6 stimulation (p=0.036) and tended to be higher after IFNγ (p=0.059) or IFNα (p=0.093) stimulation. Similar results were observed when testing pSTAT1levels in CD3$^+$ and CD14$^+$ obtained from three STAT1 GOF patients (Fig. 3B).

Ex vivo treatment with the JAK1/2 inhibitor ruxolitinib reduces the IFN-mediated STAT1 hyperphosphorylation in cells obtained from AD-HIES patients

Since ruxolitinib has shown to treat successfully clinical and immunological features of STAT1 GOF patients [18–23], we explored the potential utility of this small molecule inhibitor in the DN STAT3 setting. We tested the ex vivo effect of JAK inhibition (0.1 µM, 0.5 µM, or 1 µM ruxolitinib) on the cytokine-hyperresponsiveness found in DN STAT3 patients (Fig. 4A–B). By means of flow cytometry (see gating strategy in Supplementary Fig. S2A), we analyzed CD3$^+$ cells following stimulation with IFNα and CD14$^+$ monocytes with IFNγ as both stimuli allow for rapid, and reproducible STAT1 activation (Fig. 2) [32]. Overall, a dose-dependent ruxolitinib effect was observed and levels of pSTAT1 were generally reduced or normalized in those patients with high IFN-sensitivity by using concentrations between 0.5 and 1 µM, very similar to the pattern observed in a STAT1 GOF patient (Fig. 4C). Comparable findings were obtained when analyzing the effect of ruxolitinib on the IL-6-STAT1 axis (Fig. 4D) and after IFNα stimulation of CD4$^+$, CD8$^+$ T cells (Fig. S2) and CD14$^+$ monocytes obtained from DN STAT3 patients (Fig. S3). To confirm those findings, we measured total STAT1 and pSTAT1 levels in lysates of PBMC stimulated with IFNα in the presence of ruxolitinib by immunoblotting assays. We found increased constitutive STAT1 expression and higher phosphorylation of STAT1 in

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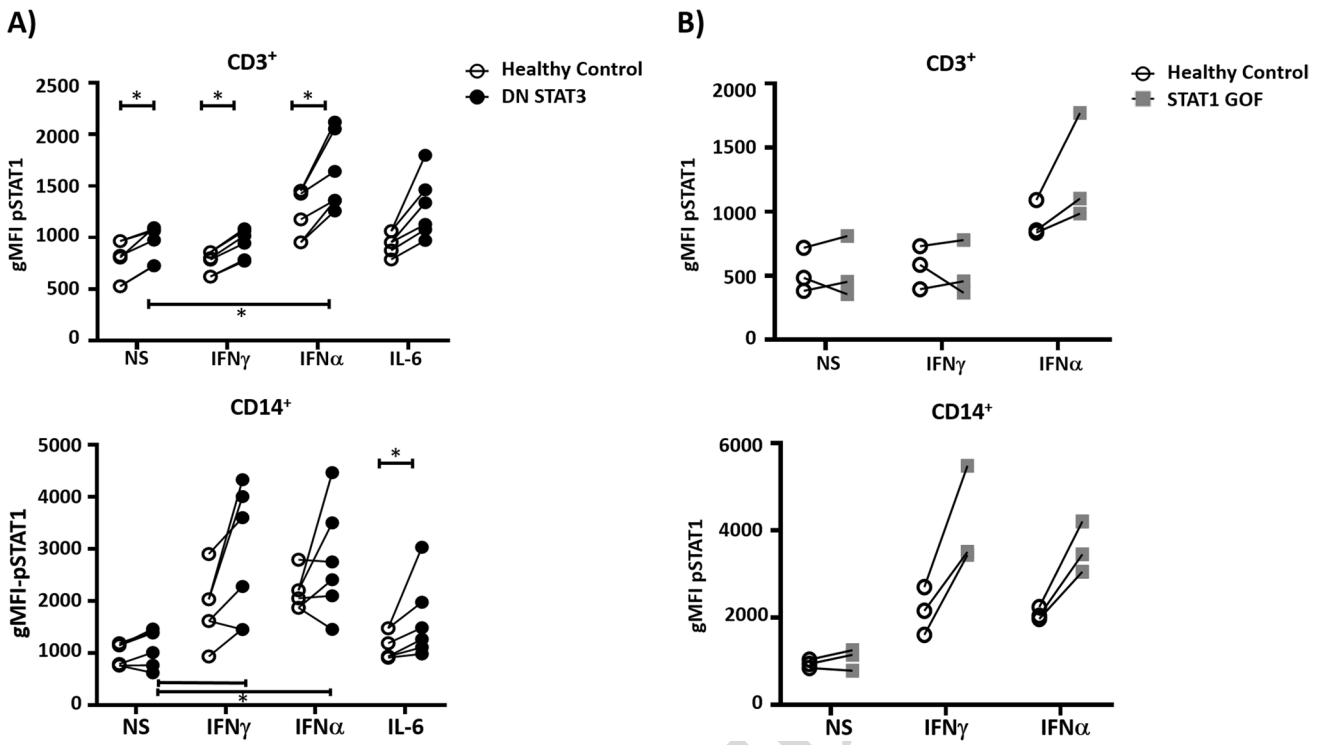

Fig. 3. Levels of phosphorylated STAT1 (pSTAT1) in patients with AD-HIES and STAT1 GOF. Geometric mean fluorescence intensity (gMFI) of pSTAT1 before (NS, unstimulated) or after IFNα IFNγ or IL-6 stimulation on $CD3^+$ and $CD14^+$ cells healthy controls (white circles), patients with DN STAT3 (black circles; A) and STAT1 GOF (gray squares; B). Black lines connect patient values with the same-day healthy control value

response to IFNα in DN STAT3 and STAT1 GOF patients than in healthy controls. In addition, IFNα-induced pSTAT1 levels were markedly reduced in the presence of 1 μM ruxolitinib (Figs. 5 and S2).

Ex vivo treatment with ruxolitinib normalizes the pSTAT1 downstream signaling in cells from AD-HIES patients

We next sought to explore whether the elevated total STAT1 and pSTAT1 levels found in DN STAT3 patients result in increased STAT1 downstream signaling. Furthermore, we aimed to determine the effect of ruxolitinib on different components of the JAK-STAT pathway. Following stimulation with IFNγ, PBMCs from all patients with DN STAT3 were found to have increased *STAT1* transcripts compared to healthy controls that resulted in overexpression of C-X-C motif chemokine ligand 10 (*CXCL10)* in 5 of them (Fig. 6). *PD-L1* and *SOCS1* tended to be higher in 4 out of 6 patients while *SOCS3* was overall reduced compared to healthy controls (Fig. 6). Adding ruxolitinib ex vivo also had a dose-dependent effect on STAT1-targeted genes, normalizing the expression of those highly abundant transcripts upon IFNγ stimulation (Fig. 6). Finally, secretion of CXCL10 was evaluated in supernatants of IFNγ-stimulated PBMCs from AD-HIES patients and controls in the presence of ruxolitinib. Four out of 6 patients displayed higher levels of CXCL10 compared to the respective healthy control that subsequently normalized upon ruxolitinib exposure (Fig. 7).

Inhibitory effect of ruxolitinib on the STAT3 axis

To evaluate the impact of ruxolitinib on STAT3 phosphorylation, we selected IL-6 and IFNα as the stimulating cytokines given their well-known activating effect on STAT3 signaling [33]. No marked differences were found in terms of total STAT3 expression and pSTAT3 levels (Figs. S4 and S5). AQ6 Although the addition of increasing concentrations of ruxolitinib (0.1 μM, 0.5 μM, or 1 μM) did not have a marked effect on STAT3 phosphorylation when determined by flow cytometry (Fig. S4), using the highest concentration of 1 μM ruxolitinib seemed to impact STAT3 phosphorylation when evaluated by western blot in both DN STAT3 and STAT1 GOF patients (Figs. 5 and S2).

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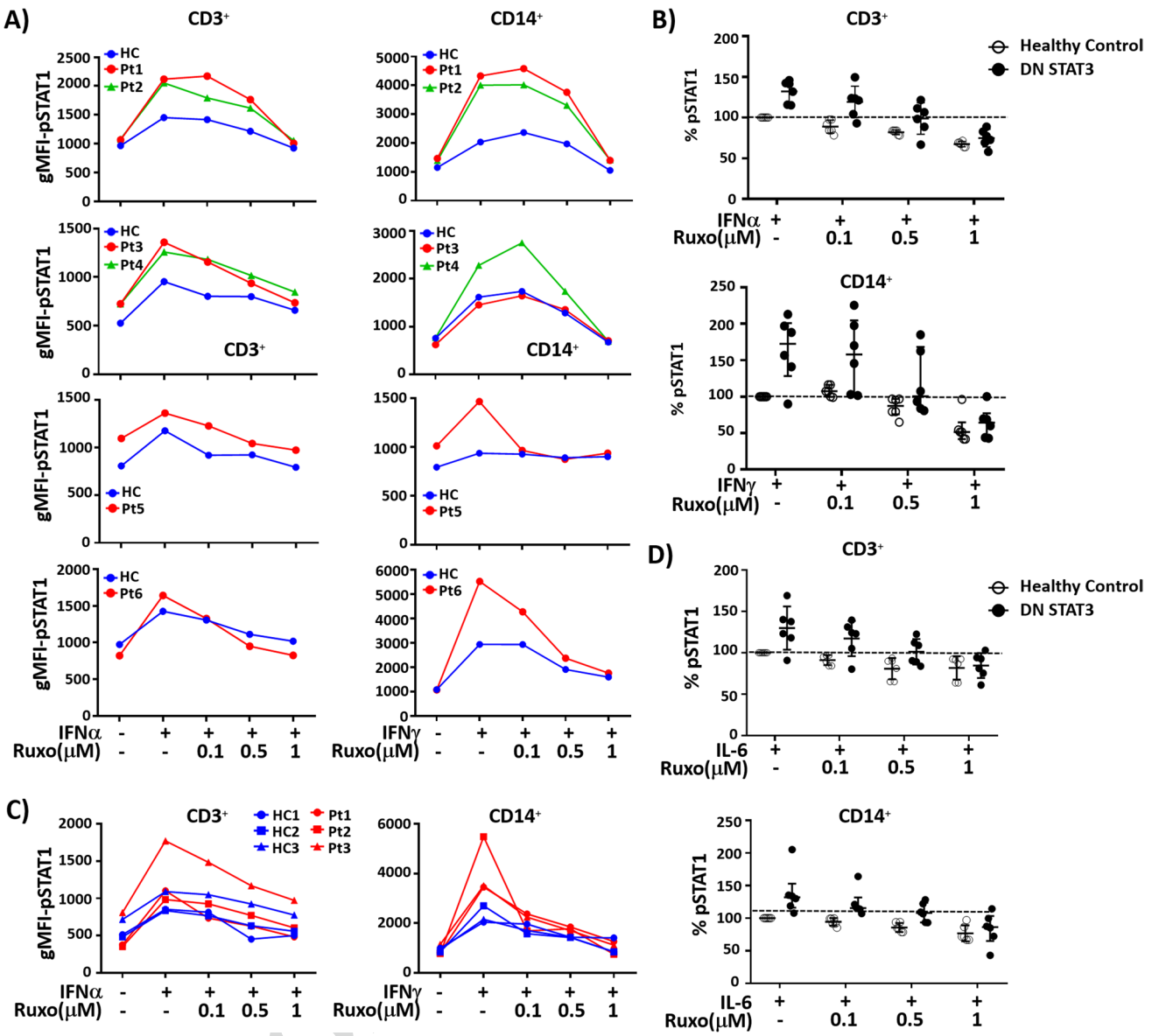

Fig. 4. Effect of ex vivo ruxolitinib treatment on pSTAT1 levels of AD-HIES and STAT1 GOF patient's cells. **A** Dose-related effect of ruxolitinib (Ruxo) on pSTAT1 levels (gMFI) of $CD3^+$ (left) and $CD14^+$ (right) cells of patients with DN STAT3 and healthy controls (HC) after stimulation with IFNα or IFNγ, respectively. Individual experiments are represented including the respective same-day healthy control. **B** Normalization of pSTAT1 (B) levels following stimulation with IFNα or IFNγ of DN STAT3 patients' cells considering the gMFI value of the healthy control to be 100%, in the absence of ruxolitinib (represented by a black dotted line). **C** Dose-related effect of ruxolitinib on pSTAT1 levels of $CD3^+$ (left) and $CD14^+$ (right) cells of patients with STAT1 GOF and healthy controls (HC) after stimulation with IFNα or IFNγ, respectively. **D** Normalization of pSTAT1 (B) levels following stimulation with IL-6 of DN STAT3 patients' cells, considering the gMFI value of the healthy control to be 100% in the absence of ruxolitinib (represented by a black dotted line)

Discussion

Patients with DN STAT3 and STAT1 GOF share several clinical and cellular phenotypes suggesting a common pathological mechanism [5, 14, 17, 31]. To date, only one study has evaluated the JAK-STAT1 signaling of DN STAT3 patients using peripheral blood mononuclear cells (PBMCs), reporting a shared cellular phenotype characterized by STAT1 hyperphosphorylation in response to cytokine stimulation in patients with either STAT1 GOF or DN STAT3 [17]. After confirming these results by immunoblotting, we sought to specifically investigate T cells and monocytes aiming to understand this initial observation in more detail. We observed that pSTAT1 levels in the investigated cell types

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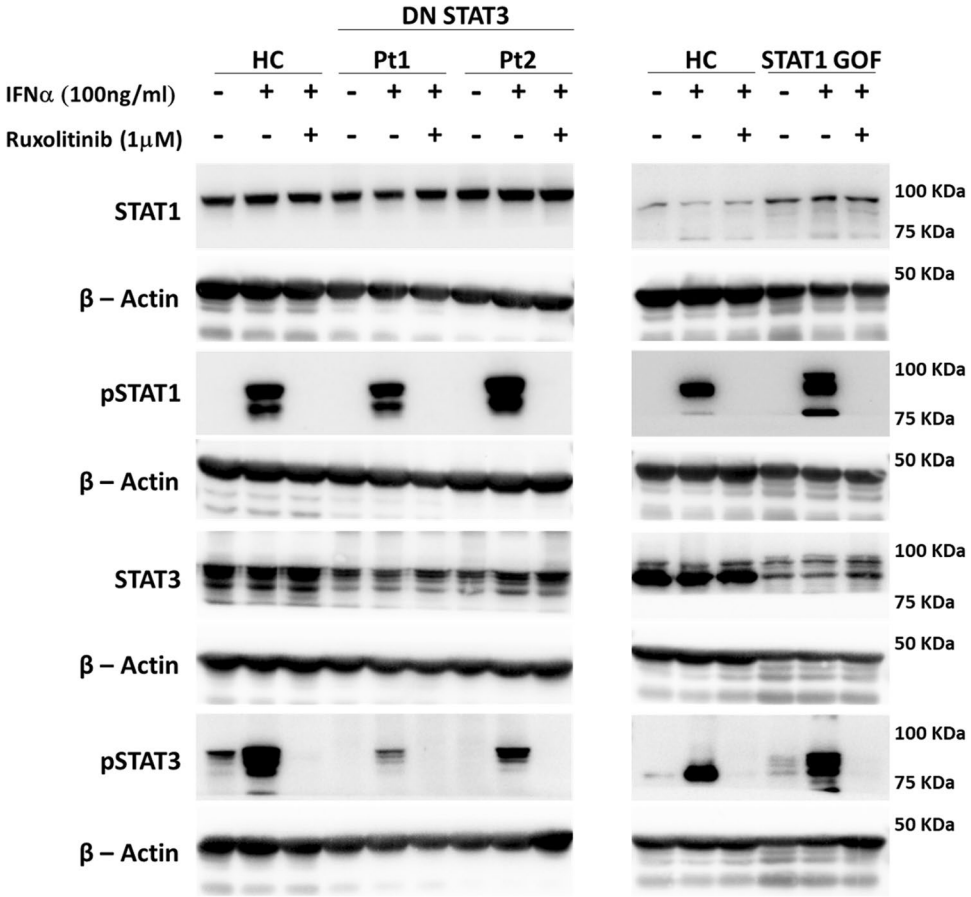

Fig. 5. Western blot analysis of STAT1 and STAT3 expression and phosphorylation. Immunoblot analysis of lysates of PBMCs from DN STAT3 (Pt1 and Pt2) and STAT1 GOF patients. PBMCs from two healthy volunteers were used as control (HC). Cells were left unstimulated or stimulated with IFNα (100ng/ml, 30 min) in the absence or presence of 1 μM ruxolitinib

differed according to the employed cytokines most likely due to cell type-specific physiological differences in density and distribution of cell surface receptors [32].

While Zhang et al. found only 1 out of 15 DN STAT3 patients displaying increased total STAT1 [17], our results show that patients with DN STAT3 not only have higher levels of pSTAT1 but increased STAT1 appears to be a common characteristic. Elevated total STAT1 in primary cells may explain the higher increment in pSTAT1 levels after cytokine stimulation (Figs. 2 and 3). This phenomenon has been previously proposed as a possible mechanistic cause of excessive pSTAT1 production after stimulation in STAT1 GOF patients [34]. We postulate that the increased activation of STAT1 found in DN STAT3 patients after cytokine stimulation might be related to the development of several clinical manifestations such as infectious susceptibility and the development of autoimmune and autoinflammatory manifestations. In fact, DN STAT3 patients also show clinical manifestations and immunological features commonly observed in patients with systemic lupus erythematosus such as increased IFN-stimulated gene expression and increased formation of neutrophil extracellular traps (NETs) and anti-NET autoantibodies when compared to healthy controls [35]. This observation, together with the reported clinical responses seen in patients with STAT1 GOF patients under JAK inhibitor therapy [18–23], encouraged us to explore the potential utility of the JAK1/2 inhibitor ruxolitinib in the setting of DN STAT3 (Figs. 4 and 5). We demonstrate the ability of ruxolitinib to effectively reduce the increased levels of pSTAT1 found in most of our patients, similarly to the well-described effect on the hyperactivation found in STAT1 GOF patients. We selected ruxolitinib concentrations based on previously published data [20, 36, 37]. In addition, studies in other settings such as myelofibrosis or in healthy volunteers have shown that a maximum plasma concentration of 0.5–1μM can be achieved using oral doses between 10 and 25mg/12h showing good safety and tolerability [38–40] indicating a potential clinical applicability of JAK inhibition as a suitable pharmacologic intervention for selected AD-HIES patients. One aspect to consider when using JAK inhibitors is the potential selectivity for certain JAKs or other kinases. Clinical responses under ruxolitinib therapy (improved CMC as well as autoimmune manifestations) have been reported even when reduced STAT3 phosphorylation was documented [18, 20]. We did not observe a marked pSTAT3 suppression using increasing ruxolitinib doses by flow cytometry (Fig. S4) but found an important reduction of pSTAT3 in the presence of high concentration of ruxolitinib

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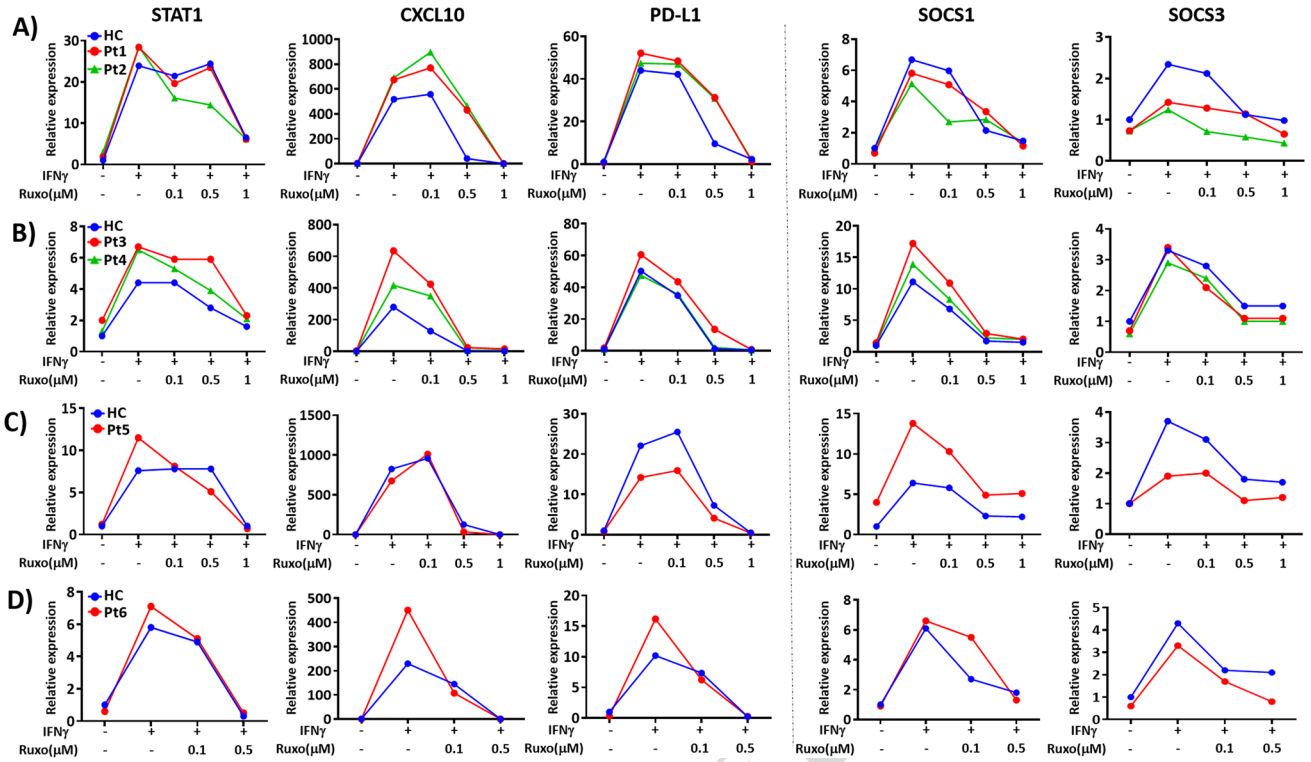
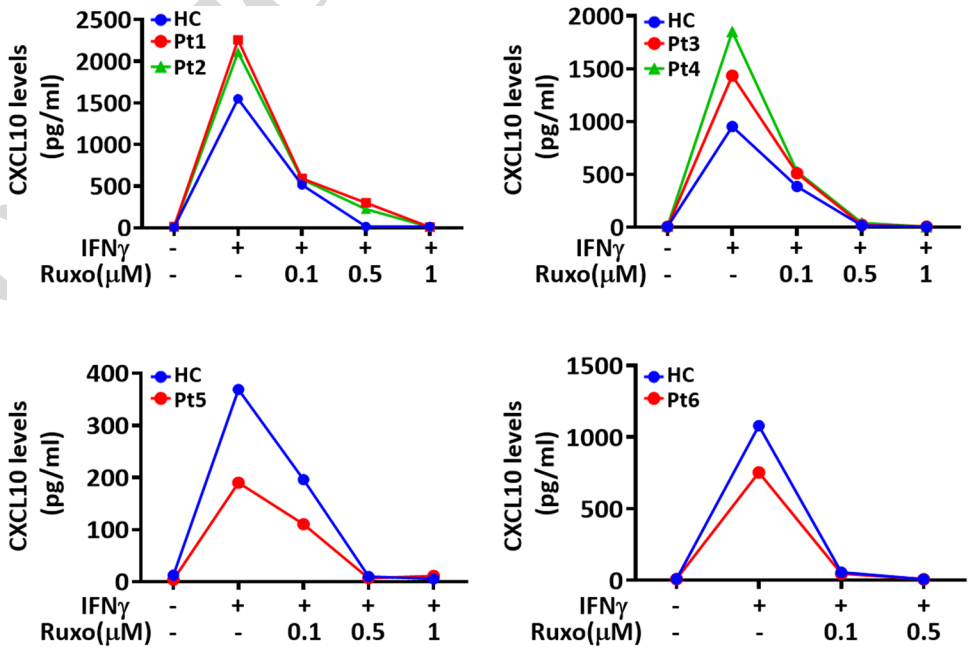


Fig. 6. Transcription levels of STAT1-dependent genes. Relative expression of *STAT1*, *CXCL10*, *PD-L1*, *SOCS1*, and *SOCS3* after 4h-stimulation of PBMCs with IFNγ in presence of different concentrations of ruxolitinib (0.1 μM, 0.5 μM, or 1 μM). Relative expression was calculated in triplicate after normalization to unstimulated *d* sample of healthy control using the comparative $2^{-\Delta\Delta ct}$ method

Fig. 7. CXCL10 secretion in response to IFNγ. Secretion of CXCL10 to supernatant of PBMCs stimulated with IFNγ for 4 h in presence of ruxolitinib (0.1 μM, 0.5 μM, or 1 μM) was evaluated by ELISA. Total levels (pg/ml) of CXCL10 from healthy controls and patients are represented. Samples were measured in triplicates; mean is represented



using western blot analysis (Fig. 5). Whether high ruxolitinib levels reduce STAT3 phosphorylation in certain cell types or if the observed differences are due to differences in the applied methodology as previously suggested in the setting of acute myelogenous leukemia [41] remains to be determined. Furthermore, the impact of potentially further reduced pSTAT3 levels in DN STAT3 patients is unknown and will need to be addressed in future preclinical studies.

We also found that DN STAT3 patients had higher levels of STAT1-related genes (*STAT1*, *CXCL10*, and *SOCS1*) transcripts as well as CXCL10 secretion compared to healthy controls (Figs. 6 and 7). In accordance with previous data Zhang et al. [17], most patients had increased *PD-L1* mRNA levels and this has been associated with impaired Th17 differentiation. These observations suggest an increase of STAT1 and STAT1 related molecules, not only at the protein level but also at the gene expression level in DN STAT3 patients. In line with the observations reported by Zhang et al. [17], *SOCS3* transcription was reduced in 5 out of the 6 patients (Fig. 6). Although the concrete mechanism remains to be elucidated, decreased SOCS3 expression has been described in the context of STAT1 hyperphosphorylation [17, 31, 42]. With respect to DN STAT3, the dominant-negative impact of STAT3 variants might result in reduced SOCS3 expression thereby reducing STAT1 inhibition. This would lead to increased pSTAT1 levels further enhancing STAT1 expression and signaling (Figs. 1 and 4). Our results show that overexpressed STAT1-dependent genes might be effectively modulated with ruxolitinib. However, in vivo studies are required to further evaluate the effect of this molecule on the regulation of STAT1 hyperactivation, since some authors have shown that IFN-related chemokine expression (e.g. CXCL10) is not always normalized under ruxolitinib therapy when using concentrations known to normalize pSTAT1 levels in the setting of STAT1 GOF [36, 43].

In our exploratory study, the relatively small sample size did not allow to test for *STAT3* mutation-specific effects on STAT1 or STAT3 expression. It therefore remains to be determined whether the type of *STAT3* mutations may differentially affect the cytokine induction of the JAK/STAT1 pathway and if there are domain-specific effects when adding ruxolitinib [44].

Our results confirm previous studies indicating STAT1-dependent hyperresponsiveness in AD-HIES patients. Furthermore, we here provide, for the first time, a detailed cell-specific analysis of the underlying JAK-STAT pathway alterations evaluating relevant immune cell populations and cytokine activation assays. Based on our experience and given the overlap in some of their clinical manifestations (e.g. CMC), clinicians should be aware that testing for STAT1 or pSTAT1 overexpression would not always distinguish between STAT1 GOF and DN STAT3. Considering our preliminary observations, and in the absence of effective directed treatment options for AD-HIES, modulation of the JAK/STAT1 pathway with ruxolitinib or other JAK inhibitors should be explored particularly in those AD-HIES patients with autoimmune or autoinflammatory manifestations. In addition, treatment of vasculopathies in AD-HIES remains a challenge and studies on primary prevention of vascular complications in these patients are limited [5]. In this regard, a recent study has identified JAK-STAT pathway-dependent alterations of the hematopoietic system on the onset and development of aortic aneurysms in patients [45]. Furthermore, Yokokawa et al. demonstrated the positive effects of ruxolitinib in preventing aneurysm formation in a murine model [45]. In STAT1 GOF patients, refractory CMC and a variety of autoimmune manifestations have clearly improved or resolved under ruxolitinib therapy in several patients [18–23]. Some patients with AD-HIES also suffer from these conditions and would be candidates to enroll in studies aiming to specifically test this hypothesis [35]. In addition, the reported risk of infectious complications (especially for fungal and herpesvirus infections) under JAK inhibition warrants close monitoring, which further highlights the complexity of the JAK-STAT pathway regulation and the need of controlled prospective multicenter clinical studies [36].

To date, there is no specific therapy for AD-HIES patients. We propose that those AD-HIES patients with autoimmune or autoinflammatory manifestations might potentially benefit from JAK inhibitor therapy although of course further preclinical work is needed to better understand the on- and off-target effects of JAK inhibitors in this specific population. However, once confirmed that this therapy indeed targets predominantly deleterious STAT1 hyperactivation, carefully performed controlled off-label studies may be indicated to assess the clinical value of this therapeutic intervention.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10875-022-01273-x.

Acknowledgements The authors would like to thank the patients and their families for participating in this study.

Author contribution PO and ON contributed to the conception of the work. PBL, PGH, and IV performed all sample processing and experiments. BdF organized sample shipping. CC, HR, BCG, AME, JML, POA, PO, and ON contributed to the diagnostic and inclusion of DN STAT3 patients. MJC contributed as technician of the cytometry core of the Institute of Biomedicine of Seville. JFNU contributed to the western blot performance analysis. All authors (PBL, PGH, IV, BdF, CC, HR, BCG, AME, JML, POA, MJC, JFNU, JDM, KM, OZ, AF, MSL, SMH, ON, and PO) contributed to the analysis or interpretation of the data, manuscript revision, read, and approved the submitted version. PBL wrote the first draft. PO and ON edited the manuscript.

Funding This work was supported by the Job Research Foundation (NY, United States); Consejería de Salud de la Junta de Andalucía (SA0051/2020 to O.N.); Agencia de Innovación y Desarrollo de Andalucía (PI-0184-2018 to P.O.), Instituto de Salud Carlos III, Madrid, Spain [Sara Borrell, CD20/00124 to P.B.L, Juan Rodés JR18/00042 to P.O, FIS PI19/01471].

Data availability The raw datasets generated and analyzed for this study and supporting the conclusions will be made available by the corresponding author without undue reservation and on reasonable request to any qualified researcher.

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Declarations

Ethics approval The study was approved by the Ethics Committee of the Hospitales Universitarios Virgen Macarena and Virgen del Rocío (0243-N-19).

Consent to participate All patients, family members, and healthy volunteers provided written and signed informed consent at each Spanish participating center (Seville, Malaga, Valencia, and Madrid). The authors affirm that human research participants or their legal guardians provided informed consent for participation and publication of their individual details.

Consent for publication All authors agreed with the submission and publication of this manuscript.

Conflict of interest The authors declare no competing interests.

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Journal:	**10875**
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