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## Multisystem autoimmune disease caused by increased STAT3 phosphorylation, and dysregulated gene expression

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**Brief Report**

**Immunobiology and Immunotherapy**

**Multisystem autoimmune disease caused by increased STAT3 phosphorylation, and dysregulated gene expression.**

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**Running title:** Multisystem autoimmunity due to STAT3 mutation

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**Key point**

Multisystem autoimmunity caused by a novel heterozygous mutation of STAT3 results in STAT3 phosphorylation, and gene activation in resting cells.

**Case report**

Signal Transducer and Activator of Transcription (STAT) 3 is a member of the STAT family, and plays a major role in various immunological mechanisms<sup>1</sup>. Mutations in STAT3 are associated with a broad spectrum of manifestations including immunodeficiency, autoimmunity, and malignancy<sup>2</sup>. In particular, heterozygous germline Loss-Of-Function (LOF) mutations cause Hyper-IgE Syndrome (HIES)<sup>3-5</sup>, while heterozygous germline Gain-Of-Function (GOF) mutations have recently been associated to multi-organ autoimmune manifestations (i.e. type 1 diabetes, enteropathy, cytopenia, interstitial lung disease, hypothyroidism), lymphoproliferation, short stature, and recurrent infections (OMIM #615952)<sup>6-8</sup>.

We report a 7-year-old boy who presented with early-onset severe enteropathy, and diffuse eczematous dermatitis since birth. During the first weeks of life Hirschsprung disease was also suspected and surgically treated. Gastrointestinal and cutaneous manifestations were first ascribed to food allergy with a quite good response to amino acid-based formula. In the following months, the patient developed failure to thrive, and respiratory tract infections. At two years, the patient presented with progressive interstitial lung disease, characterized by lymphocytic interstitial infiltration leading to pulmonary hypertension, tricuspid insufficiency, and right ventricular heart failure with hepatomegaly. Because of the increased risk of infections, he received IV immunoglobulin infusions (400 mg/kg), prophylaxis with cotrimoxazole and

fluconazole. Methylprednisolone at 0.3 mg/kg/day was also given to treat autoimmune manifestations.

At later times, growth hormone (GH) deficiency, hypothyroidism and type 1 diabetes were diagnosed.

Informed consent was obtained from parents of the patient. Ethical approval was obtained by the Institutional Review Board of Spedali Civili of Brescia. Genomic DNA was extracted from peripheral whole blood, Whole Exome Sequencing (WES) was performed while the identified mutation was validated by Sanger sequencing<sup>5</sup>. STAT3 phosphorylation was analyzed by flow cytometry on whole blood after stimulation with interferon (IFN)- $\alpha$  (40,000 U/ml) or interleukin (IL)-27 (100ng/mL) for 30' according to BD protocol: cells were acquired using FACSCalibur (BD Bioscience) and analyzed by FlowJo version 7.5 Software (TreeStar). Epstein-Barr virus (EBV)-immortalized B cells Western Blot assay was performed after overnight starvation as previously described<sup>5</sup>.

Reverse transcription quantitative real-time PCR (RT-qPCR) was performed with specific probes (Applied Biosystems) on CFX96 RT System (BIO-RAD). EBV cells were stimulated with 200ng/mL of IL-6 or IL-10 for 1 hour with/without overnight starvation. Results are shown as mean normalized expression (MNE) units after GAPDH normalization.

Chromatin immunoprecipitation (ChIP) assay was performed on  $5 \times 10^6$  EBV-transformed cell lines stimulated with IL-10 or IL-6 for 45' and executed as described<sup>9</sup>. To establish the background levels of ChIP experiments, STAT3 was quantified also at the promoter of prolactin (PRL) since it is completely silent in leukocytes. Additional details can be provided upon request.

Data Sharing Statement: For original data, please contact [raffaele.badolato@unibs.it](mailto:raffaele.badolato@unibs.it)

Because of the association of severe early-onset enteropathy and autoimmune manifestations, Immunodeficiency Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome or other IPEX-like inherited disorders were suspected. But the search for mutations in *FOXP3*, *CD25* and *STAT5b* resulted negative<sup>10,11</sup>. Subsequently, WES was performed and a *de novo* heterozygous missense *STAT3* mutation was identified that clustered in the DNA-binding domain and caused methionine-to-arginine substitution (c.1986T>G) at position 329 (p.M329R). Lymphocyte subsets analysis revealed normal levels of regulatory (CD4+ CD25<sup>hi</sup> FOXP3+) and effector (CD45RA-CCR7-) CD3+ T lymphocytes.

*STAT3* activation in response to cytokines such as IL-6, IL-10, IL-21, IL-23 and IFN- $\alpha$  is characterized by phosphorylation of tyrosine 705 (pY705) by associated Janus activating kinases (JAKs) and subsequent *STAT*-*STAT* dimerization and nucleus translocation<sup>12</sup>. We comparatively evaluated by ELISA assay plasmatic cytokines concentration in the patient and in age-matched healthy donors. Detectable levels of IL-6 (72.04 pg/ml), IL-10 (32.5 pg/ml) and IL-17(55.82 pg/ml) were observed in the patient, but not in the healthy donors (data not shown). The flow cytometry analysis of CD3<sup>+</sup> lymphocytes showed detectable levels of *STAT3* phosphorylation (p*STAT3*) in the patient, but not in healthy donors. Increased p*STAT3* levels were observed both in unstimulated conditions and after IFN- $\alpha$  or IL-27 stimulation (**Figure 1a**). Next, we analyzed *STAT3* phosphorylation in unstimulated cells or after 15, 30, 60, 90 and 120 minutes after stimulation of EBV-transformed cell lines with IFN- $\alpha$  (20000U/mL). The patient showed a delayed dephosphorylation of *STAT3*, especially 90' after stimulation (**Figure 1b**).

Finally, we investigated the levels of p*STAT3* by Western Blot assay in unstimulated cells, and following IL-6 or IL-10 stimulation in EBV-transformed cell lines. Analysis of cells derived from the patient with GOF mutation showed detectable levels of p*STAT3* also in unstimulated conditions, which increased after IL-6 (200ng/mL) or IL-10 (200ng/mL) stimulation. This was associated with increased levels of *STAT3* protein as compared to the healthy subjects (**Figure 1c**).

In order to evaluate STAT3 binding capacity to specific DNA sequences involved in the regulation of inflammation and immune response (e.g. SOCS3 and SNBO2), we performed a chromatin immunoprecipitation (ChIP) assay in EBV-transformed cells from the patient with STAT3 GOF mutation and from a healthy subject. We observed that unstimulated cells from the patient showed enhanced STAT3 binding to SOCS3 and SBNO2 promoters. But, after stimulation with IL-6 or IL-10, STAT3 binding was weakly increased as compared to cells from healthy controls (**Figure 1d**).

Moreover, we evaluated the expression of STAT3, IL-10, BCL-3, IL2RA and SOCS3 mRNAs by -RT-qPCR. Analysis of mRNAs in unstimulated cells from the patient showed high level expression of STAT3, IL-10, BCL-3, IL2RA, and SOCS3 at baseline, and further increase of SOCS3 and IL10 mRNAs after stimulation with IL-6 or IL-10. While, cell starvation did not influence the extent of gene expression in the cells of the patient (**Figure 2a-e**).

Patients with GOF-STAT3 mutation share similar phenotypic manifestations (i.e. eczema, autoimmunity) with STAT5b deficient patients<sup>13</sup>, suggesting that STATs proteins phosphorylation and expression can be negatively regulated by STAT3-mediated induction of SOCS3<sup>14</sup>. Therefore, we investigated cytokine-induced phosphorylation of both STAT1 and STAT2, and the expression of the total amount of STAT1 protein in the EBV-transformed cell line from the patient with STAT3 GOF mutation and from a healthy subject.

After preparation of cytoplasmic extracts, we observed that STAT2 phosphorylation levels were lower in STAT3-mutated cells after IFN $\alpha$  stimulation, as compared to healthy control. In contrast, the level of phosphorylated and total STAT1 was similar in both control and patient EBV-transformed cell lines. (**Figure 2f**). These results suggest that the STAT3 GOF mutation p.M329R affect not only STAT3, but also STAT2 mediated-response.

Immunological studies in this patient revealed normal T cell subsets, but severe depletion of both plasmacytoid (BDCA2<sup>+</sup>/CD123<sup>+</sup>/CD4<sup>+</sup>) and myeloid dendritic (CD1c<sup>+</sup>/CD4<sup>+</sup>/CD19<sup>-</sup>/CD14<sup>-</sup>) cells: pDC 0.06%, mDC 0.05% of total PBMC as compared to healthy subjects (pDC n.v. 0.16-0.7%, mDC n.v. 0.18-0.92%). This is in contrast with two other cases that showed increased T effector cell counts and a reduction of T regulatory cells<sup>1,15</sup>, but these differences might be related to inter-individual variations. Moreover, high plasmatic concentrations of IL-6, IL-10 and IL-17 in this patient were also observed, suggesting that these cytokines might be involved in the mechanism of the autoimmune diseases. All these atypical immunological features are probably related to the novel STAT3 mutation which was associated with increased phosphorylation of STAT3. While most of patients with STAT3 GOF mutations display normal STAT3 phosphorylation, but abnormal gene regulation<sup>7</sup>.

Current therapeutic of multiple organ autoimmune diseases in patients with STAT3 GOF mutations include immunosuppressive drugs, and hematopoietic stem cell transplantation in selected patients<sup>16</sup>. Because tyrosine kinases inhibitors, such as ruxolitinib, have been extensively used in conditions characterized by abnormal STAT activation, their use could be favorably extended to immunological diseases associated with increased STAT3 phosphorylation such as observed in this case<sup>17</sup>.

### **Authorship Contributions**

FT and MP carried out the culture of the EBV cell lines and performed the phosphorylation assays; FT wrote the paper. NT and EG executed the Real-Time, WB and ChIP assay. DM performed immunophenotypic analysis. LD and FC contributed to the clinical characterization of the patient.

AG performed ELISA assays. MG contributed in flow cytometry data acquisition. PL, RBC and EG performed patient clinical follow up. MC supervised signaling experiments.

RB supervised the work and designed the experiments.

**Conflict of Interest Disclosures**

No conflict of interest to disclose



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## Figure Legends

### **Figure 1. STAT3 phosphorylation and chromatin immunoprecipitation assay in the STAT3 GOF patient .**

(A) STAT3 phosphorylation in T cells (CD3<sup>+</sup> cells) in response to IFN $\alpha$  and IL-27 stimulation or medium alone. Cells from whole blood samples were lysed, fixed and permeabilized, then stained with anti-p-STAT3 and anti-CD3 antibodies. Extent of p-STAT3 is shown on y- axis as mean fluorescent intensity. (B) Time course of STAT3 phosphorylation of EBV-transformed B cell lines obtained from a control subject or from the STAT3 GOF patient. Cells were kept in culture with serum free medium over-night and stimulated with IFN $\alpha$  for 15, 30, 60, 90 or 120 minutes. Then, cells were permeabilized and stained with anti-p-STAT3. Extent of p-STAT3 is shown on y-axis as mean fluorescent intensity. (C) Western Blot analysis of STAT3 tyrosine phosphorylation in EBV-transformed B cell lines from a control subject or from the STAT3 GOF patient. Cells were kept in culture with serum free medium overnight and then either untreated or stimulated with IL-6 (200 ng/mL) and IL-10 (200 ng/mL) for 30 minutes.(D) Evaluation, by ChIP analysis, of STAT3 recruitment to the SOCS3, SBNO2, IL-10 and PRL promoters of EBV-transformed B cells cultured in the presence or absence of IL-6 (200ng/mL) and IL-10 (200ng/mL) for up to 1 h. Co-immunoprecipitated DNA was expressed as percent of the total input. Error bars represent standard errors calculated from triplicate qPCR reactions.

### **Figure 2 Analysis STAT3, IL10, BCL3, IL2RA, and SOCS3 mRNAs expression in EBV-transformed B cell lines**

A-E) EBV-transformed B cell lines from 2 healthy donors or the STAT3 GOF patient were kept overnight in serum-free medium and then either untreated or stimulated for 1 hour with IL-6 (200ng/mL) and IL-10 (200ng/mL) to investigate STAT3, IL10, BCL3, IL2RA, and SOCS3 mRNAs by RT-qPCR. F) Western Blot analysis of STAT1 and STAT2 levels in cytoplasmic extracts from EBV-transformed B cells from a control subject or the STAT3 GOF patient. Cells were cultured overnight without serum and then stimulated with IFN $\alpha$  (1000 U/ml) for 30 min. Levels of p-STAT1, p-STAT2, total STAT1 and STAT2 in the cytoplasmic fractions were evaluated. Samples were also immunoblotted for tubulin, a cytoplasmic marker.



