

Letter to the Editor

Flow cytometric measurement of STAT1 and STAT3 phosphorylation in CD4⁺ and CD8⁺ T cells—clinical applications in primary immunodeficiency diagnostics

To the Editor:

It is well known that cytokines achieve their biologic function by triggering Janus kinases, which are a family of proteins that regulate signal transducer and activator of transcription (STAT) proteins pathways, in order to control cellular activation processes.¹ Loss- or gain-of-function (GOF) gene mutations in *STAT1* and *STAT3* were described in primary immunodeficiencies (PIDs).² Patients with autosomal-dominant (AD) loss-of-function *STAT1* mutation are predisposed to mycobacterial infections, whereas the autosomal-recessive (AR) form is accompanied by mycobacterial and viral infections.^{2,3} In contrast, GOF *STAT1* mutations may cause chronic mucocutaneous candidiasis (CMC).^{4,5} Dominant-negative mutations in *STAT3* were found in the AD form of hyper-IgE syndrome (HIES), also called Job syndrome.⁶ *STAT1* and *STAT3* proteins have been studied by different methods, that is, genome sequencing and Western blot (WB), which is limited due to

its time-consuming nature and the requirement of extensive cell numbers.⁷ Therefore, we established a rapid flow cytometric whole blood assay to evaluate *STAT1* and *STAT3* protein phosphorylation and to provide reference values based on 21 healthy adult controls (for characteristics, see Table E1 in this article's Online Repository at www.jacionline.org). In addition, we evaluated the test in 4 patients with PID selected by their clinical representation: recurrent opportunistic infections, candidiasis, or an HIES phenotype. As a result, we conclude that our approach provides a diagnostics benefit by avoiding labor and time consumption needed for WB or other classical protein analyses, thereby achieving earlier diagnoses, potentially leading to improved treatment options and patient outcome. Written informed consent was obtained from all included individuals.

To evaluate phosphorylation of *STAT1* (pSTAT1) expression, peripheral EDTA blood was treated with IFN- α (PBL science, Piscataway, NJ, 40,000 U/mL), whereas IL-6 (BD Biosciences, Heidelberg, Germany, 100 ng/mL) was applied to stimulate the *STAT3* signal cascade. Live and dead cells were discriminated on the basis of BD Horizon Fixable viability stain 450 (FVS450).

The phosphorylation status of *STAT1* and *STAT3* in CD4⁺ and CD8⁺ T cells was analyzed both by flow cytometry (FCM) and by WB (see Table E2 and Fig E1 in this article's Online Repository at www.jacionline.org). All measurements were adapted according

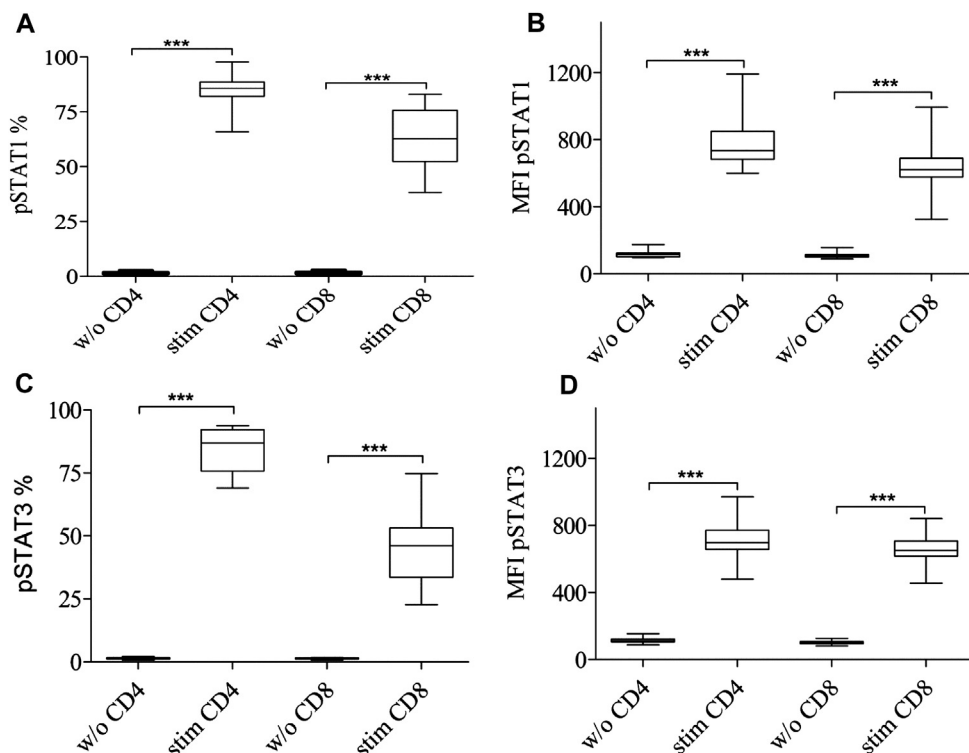


FIG 1. Summary of pSTAT1 and pSTAT3 expression analysis by flow cytometry (n = 21). **A**, Percentage of cells positive for pSTAT1 expression. **B**, MFI of cells positive for pSTAT1 expression. **C**, Percentage of cells positive for pSTAT3 expression. **D**, MFI of cells positive for pSTAT3 expression. The bold line inside each box plot shows the median level, and upper and lower lines indicate the maximum and minimum values, respectively. (***) *P* value = .001. *stim*, Stimulated; *w/o*, without stimulation.

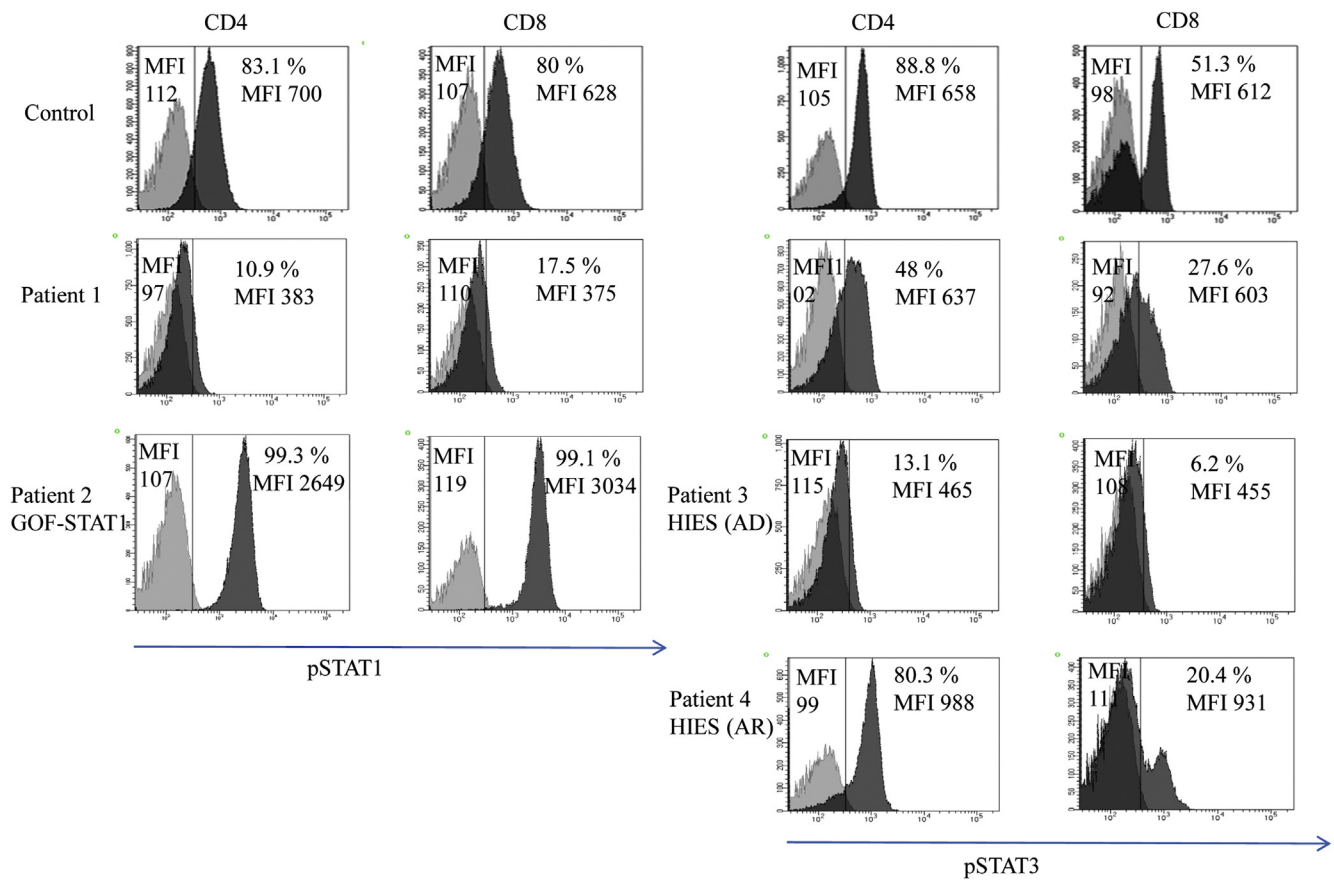


FIG 2. Detection of pSTAT1 and pSTAT3 by flow cytometry in 4 patients with PID in comparison with healthy controls. Human peripheral blood was either left unstimulated (gray histogram) or stimulated for 15 minutes as indicated (black histogram). Percentages and MFI of cells positive for pSTAT1 and pSTAT3 are detailed within the histogram boxes.

to DIN EN ISO 15189 requirements. The validation of our data set included the definition of intraassay and interassay precision values.⁸

Response to stimulation (phosphorylation) was calculated as percentage (%) and increase in mean fluorescence intensity (MFI) of pSTAT1 and pSTAT3-expressing cells within CD4⁺ or CD8⁺ subsets. The adjusted *P* values were considered significant based on Wilcoxon test when less than .05 (Graph Pad Prism, San Diego, Calif). The summary of FCM analysis showed that IFN- α and IL-6 induce a significant increase in pSTAT1 and pSTAT3, respectively, magnitude (vs unstimulated cells), as depicted in Fig 1 (A-D; see Tables E3 and E4 in this article's Online Repository at www.jacionline.org). However, pSTAT3 responsiveness of CD8⁺ cells was moderate and produced a bimodal peak (see Fig E2, A, in this article's Online Repository at www.jacionline.org). More specifically, because of this bimodal peak within CD8⁺ subsets, we investigated which phenotype-naïve or memory cells were involved in phosphorylation of STAT3. Thus, these cells were stained for CD45RA expression to discriminate between naïve (CD45RA⁺) and memory T cells (CD45RA⁻). Following stimulation with IL-6, the expression of pSTAT3 in naïve CD8⁺ subsets was more pronounced compared with memory subsets, in contrast to a very weak phosphorylation of STAT3 in unstimulated controls (see Fig E3 in this article's Online Repository at www.jacionline.org). Oberg et al⁹

described that IL-6 induces intracellular signals by ligand-binding to IL-6R- α chain (CD126) and gp130 (CD130), while only about 50% of human CD8⁺ lymphocytes express the surface markers CD126 and CD130, in contrast to about 80% on CD4⁺ T cells. Moreover, a study by Betz and Muller¹⁰ described that peripheral T cells with a cell surface marker profile of memory cells exhibited a gp130⁻ and IL-6R- α ⁻ immune phenotype due to downregulation upon activation. This suggests that naïve subsets are responsible for most pSTAT3 signals in CD8⁺ cells (see Fig E3 in this article's Online Repository at www.jacionline.org).

To examine whether our results were specific, the samples were also analyzed by WB in parallel. As displayed in Fig E4, B, in this article's Online Repository at www.jacionline.org, IFN- α -stimulated cells generated specific 92-kDa protein bands (the upper line) representing pSTAT1, in contrast to untreated cells, which depicted very weak or absent bands. However, stimulated CD4⁺ cells exhibited a strong increase in phosphorylation as indicated by specific band intensities, according to FCM analysis. In contrast, the CD8⁺ subsets presented a slight increase in phosphorylation as indicated by the band intensities, compared with FCM (see Fig E4, A).

For pSTAT3 (see Fig E2, B), the bands in the upper line illustrated a strong expansion of pSTAT3 within CD4⁺ and a moderate pSTAT3 increase in CD8⁺ T cells corresponding to a 92-kDa

fragment. β -Actin blotting was performed as loading control. The band intensities correlated with a strong increase in $CD4^+$ and a moderate increase in $CD8^+$ T cells, respectively, detected by FCM analyses (see Fig E2, A).

Subsequently, we investigated 4 patients with PID with defects in the STAT1 or STAT3 signaling pathway.

Patient 1 (recurrent opportunistic infections, candidiasis) exhibited almost completely deficient STAT1 phosphorylation in both $CD4^+$ and $CD8^+$, in contrast to normal pSTAT3 expression (Fig 2). This result resembles AR STAT1 deficiency, which is characterized by the occurrence of recurrent infections with opportunistic bacteria.³

Patient 2 (patient with CMC), who was diagnosed with a GOF-STAT1 mutation, exhibited a strong increase in MFI of pSTAT1 in response to IFN- α stimulation (Fig 2). In healthy controls, additional treatment with staurosporine (tyrosine kinase inhibitor, Sigma-Aldrich, Carlsbad, Calif) inhibited a pSTAT1 overexpression, in contrast to patient 2 with GOF-STAT1 mutation. The MFI of pSTAT1 remained at a high level (see Fig E5 in this article's Online Repository at www.jacionline.org).

As illustrated in Fig 2, we were also able to distinguish between different HIES traits: in patient 3 (AD with STAT3 mutation) pSTAT3 was defective, in contrast to patient 4 (AR without STAT3 mutation) with normal pSTAT3 expression. Similar results were also described by Yong et al.⁶

In conclusion, disorders in the Janus kinases/STAT signal pathway in $CD4^+$ and $CD8^+$ T cells may result in insufficient response to pathogens. Therefore, FCM-based pSTAT1 and pSTAT3 profiling is an effective tool for clinical laboratory diagnostics to (1) understand the susceptibility to recurrent opportunistic infections, (2) identify patients with functional $CD4^+$ and $CD8^+$ T-cell defects, and (3) identify and characterize well-known PIDs such as CMC, AD-HIES, or AR-HIES.

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Michael Bitar, MSc^{a*}
Andreas Boldt, PhD^{a*}

Stefanie Binder, PhD^a
Michael Borte, MD^b
Karim Kentouche, MD^c
Stephan Borte, MD, PhD^{b,d}
Ulrich Sack, MD^a

From ^athe Medical Faculty, Institute of Clinical Immunology, University of Leipzig and ^bImmuno Deficiency Center Leipzig (IDCL) at Hospital St Georg gGmbH Leipzig, Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Leipzig, Germany; ^cthe Department of Pediatrics, Jena University Hospital, Jena, Germany; and ^dthe Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden. E-mail: Michael.Bitar@medizin.uni-leipzig.de.

*These authors contributed equally to this work.

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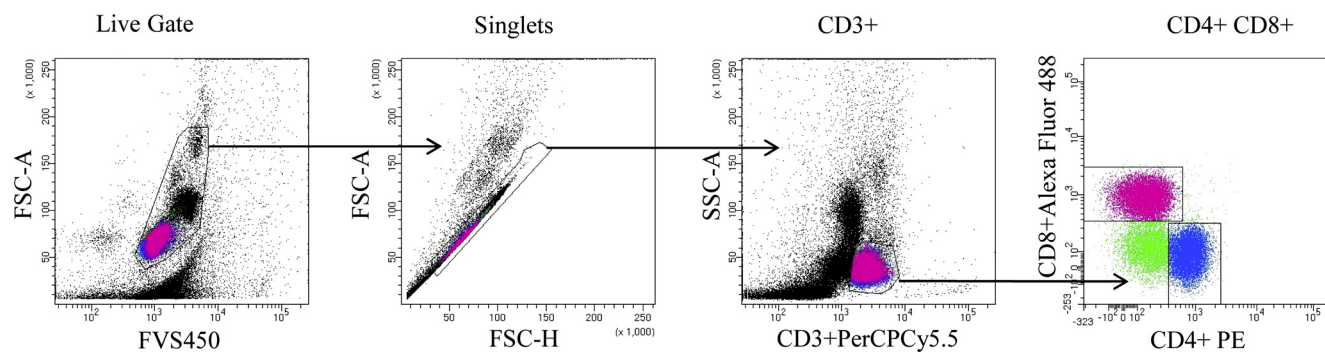


FIG E1. Flow cytometric gating strategy to exclude dead cells and doublets. PBMCs were stimulated with IFN- α for STAT1 phosphorylation, with IL-6 for STAT3 phosphorylation, or left unstimulated. Following staining with FVS450 according to manufacturer's specifications (BD Biosciences), live and dead cells can be discriminated by measurement of the fluorescence intensity. Dead cells have 10- to 20-fold higher fluorescence intensity than do live/intact cells (live gate—first left panel). Doublets were excluded on the basis of FSC area (FSC-A) versus FSC height (FSC-H) (singlets gate—second left picture). Additional staining for CD3⁺, CD4⁺, and CD8⁺ cells helps to identify the T-cell subsets (third left picture and right picture). *FSC*, Forward scatter.

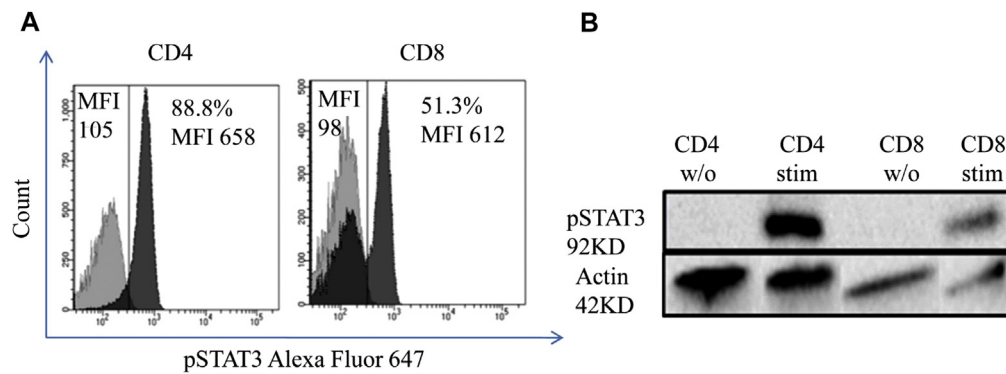


FIG E2. Detection of pSTAT3 in CD4⁺, CD8⁺ subsets by FCM and WB. **A**, Phosphorylation analysis of STAT3 by FCM; human peripheral blood was either left without stimulation (gray histogram) or stimulated with IL-6 (black histogram). STAT3 phosphorylation is presented as both percentage and the MFI of cells positive for pSTAT3. **B**, WB analysis, pSTAT3 bands (upper line at 92 KDa), β -actin blotting (bottom). *stim*, Stimulated; *w/o*, without stimulation.

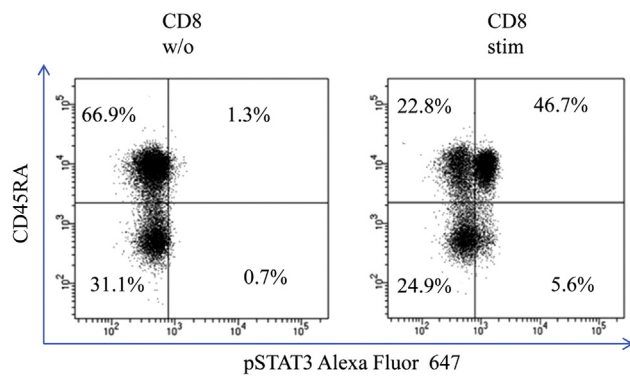


FIG E3. Evaluation of pSTAT3 in CD8⁺ CD45RA⁺ as naive cells and CD8⁺ CD45RA⁻ as memory cells. After IL-6 stimulation, an efficient expression of pSTAT3 within CD8⁺ CD45RA⁺ naive cells was increased distinctly rather than memory cells CD45RA⁻. Percentages of naive and memory cells that were involved in phosphorylation of STAT3 are given within the histogram boxes. *stim*, Stimulated; *w/o*, without stimulation.

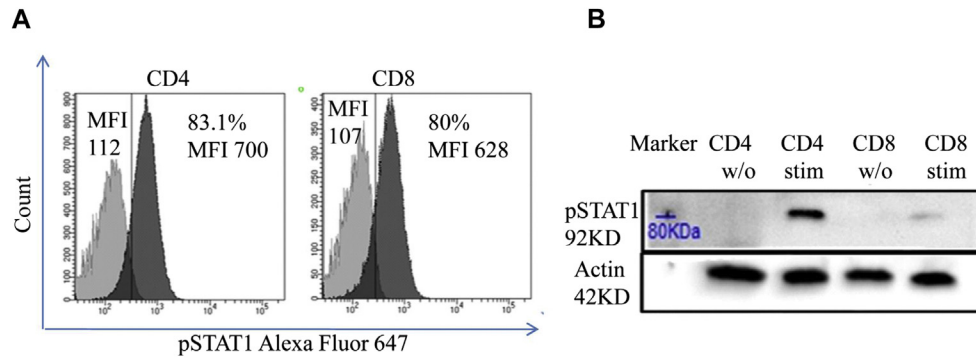


FIG E4. Detection of pSTAT1 in CD4⁺, CD8⁺ subsets by FCM and WB. **A**, Phosphorylation analysis of STAT1 by FCM; human peripheral blood was either left without stimulation (gray histogram) or stimulated with IFN- α (black histogram). STAT1 phosphorylation is presented as both percentage and the MFI of cells positive for pSTAT1. **B**, WB analysis, pSTAT1 bands (upper line at 92 KDa), β -actin blotting (bottom). *stim*, Stimulated; *w/o*, without stimulation.

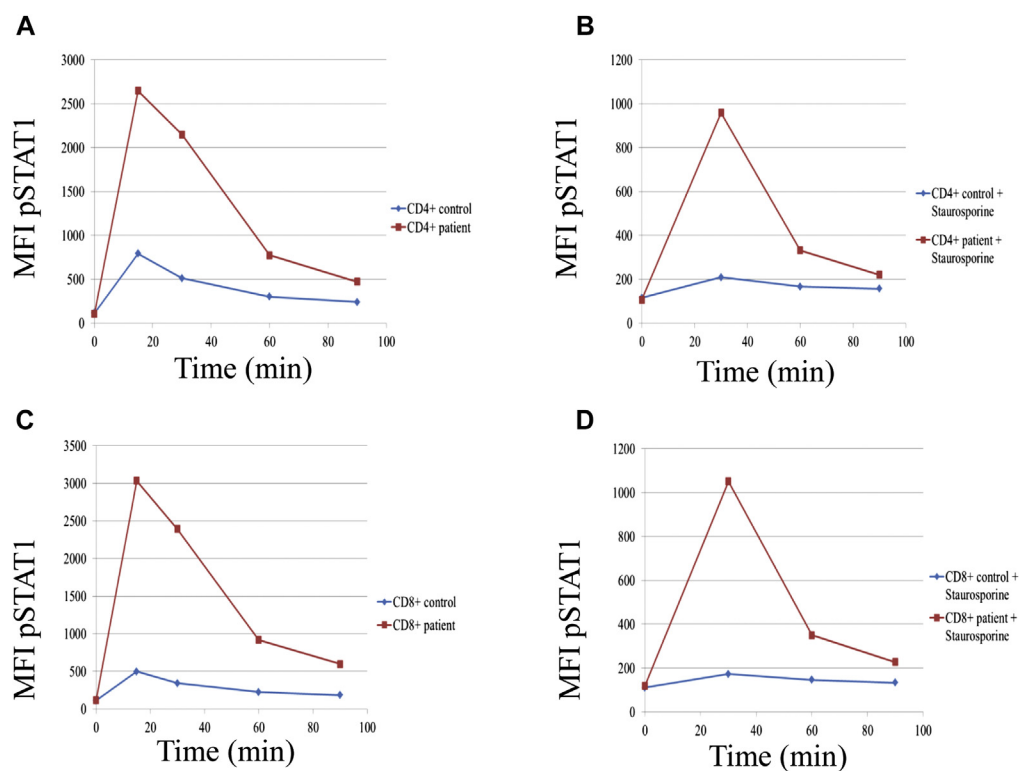


FIG E5. Time-dependent STAT1 phosphorylation and its inhibition in a patient with GOF-STAT1 mutation compared with healthy control. PBMCs were isolated and stimulated with IFN- α for 15, 30, 60, and 90 minutes. After reaching the maximum of pSTAT1-MFI at 15 minutes, the phosphorylation decreased to a normal level in healthy control and in the patient, in both CD4 $^{+}$ (A) and CD8 $^{+}$ (C) T cells. Interestingly, the maximum pSTAT1-MFI was multiple higher in the GOF-STAT1 patient compared with healthy control (Fig E5, A and C). Furthermore, PBMCs were stimulated with IFN- α and after 15 minutes treated with staurosporine (B and D). Staurosporine treatment inhibited strongly the STAT1 phosphorylation in healthy control in contrast to the patient with GOF-STAT1 mutation (Fig E5, B and D).

TABLE E1. Clinical characteristics of 21 adult healthy controls

Characteristic	Controls (n = 21)
Age (y), mean $\pm$ SD	35.1 $\pm$ 9.8
Sex	Male: 8/Female: 13
Immune diseases/medical treatment	No

TABLE E2. The antibodies used for staining and measurement of the cells by FCM

Antibody	Clone	Volume (μL)
BD phosflow PerCP-Cy 5.5 mouse anti-human CD3	UCHT1	2.5
BD phosflow Alexafluor 488 mouse anti-human CD8	RPA-T8	2.5
BD phosflow PE mouse anti-human CD4	RPA-T4	5
Alexa Fluor 647 mouse anti-human STAT1 (PY701)	Clone 4a	10
Alexa Fluor 647 mouse anti-human STAT3 (PY705)	4/P-STAT3	10
BD Horizon V450 mouse anti-human CD45RA	HI100	2.5

TABLE E3. Expression of pSTAT1 (% of positive cells and MFI) in adult healthy control whole blood samples

Control (n = 21)	CD4 ⁺ pSTAT1 w/o	CD4 ⁺ pSTAT1 IFN- α -stimulated	CD8 ⁺ pSTAT1 w/o	CD8 ⁺ pSTAT1 IFN- α -stimulated
Percentage*	1.4 $\pm$ 0.36	84.65 $\pm$ 8.34	1.47 $\pm$ 0.45	62.3 $\pm$ 13.8
MFI†	115 $\pm$ 17	778 $\pm$ 154	109 $\pm$ 14	641 $\pm$ 131

w/o, Without stimulation.

*Percentage of cells positive for pSTAT1; the value was calculated as mean $\pm$ SD.†MFI for entire CD4⁺, CD8⁺ populations, calculated as mean $\pm$ SD.

TABLE E4. Expression of pSTAT3 (% of positive cells and MFI) in adult healthy control whole blood samples

Control group (n = 11)	CD4 ⁺ pSTAT3 w/o	CD4 ⁺ pSTAT3 IL-6-stimulated	CD8 ⁺ pSTAT3 w/o	CD8 ⁺ pSTAT3 IL-6-stimulated
Percentage*	1.42 ± 0.38	84.3 ± 8.43	1.32 ± 0.27	44.9 ± 14
MFI†	112 ± 15	707 ± 104	101 ± 10	660 ± 92

w/o, Without stimulation.

*Percentage of cells positive for pSTAT3; the value calculated as mean ± SD.

†MFI for entire CD4⁺, CD8⁺ populations, calculated as mean ± SD.