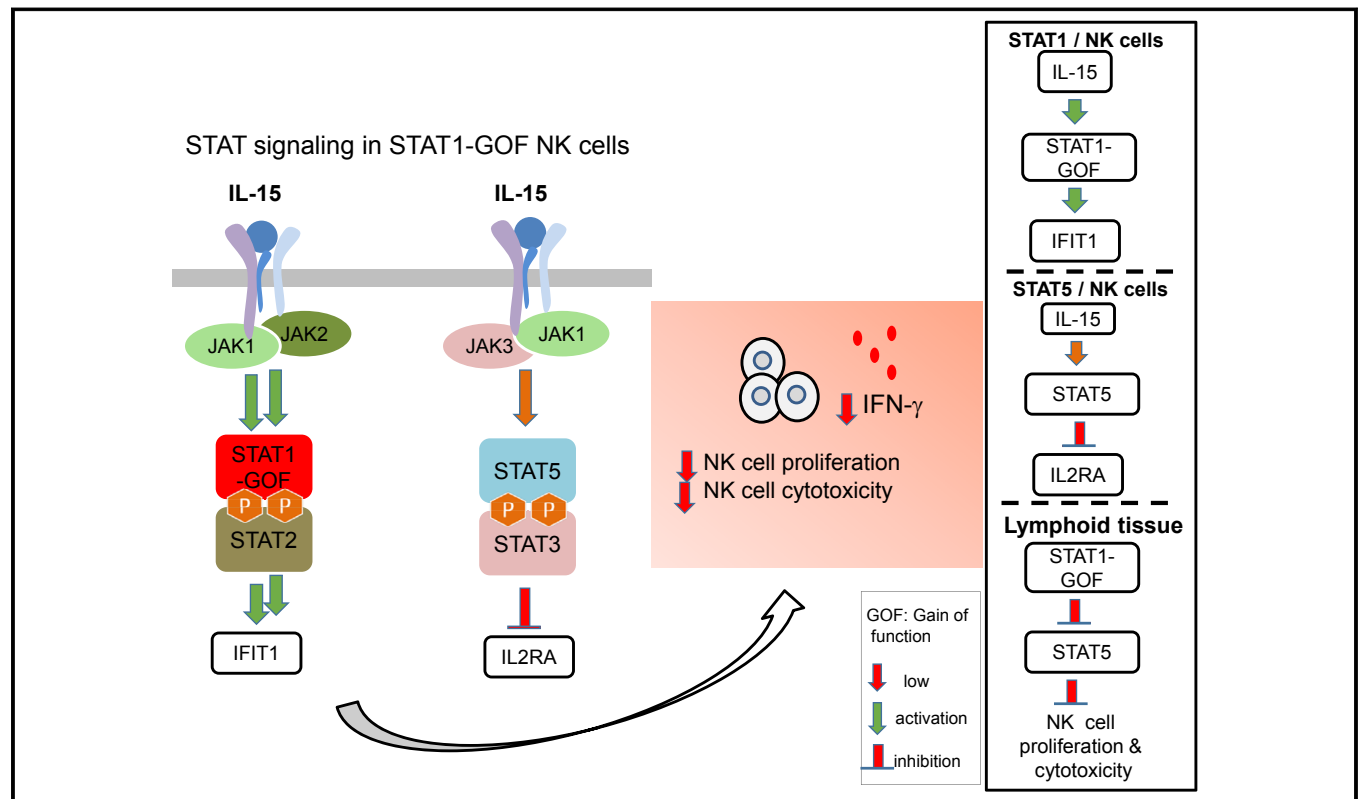


# Impaired natural killer cell functions in patients with signal transducer and activator of transcription 1 (*STAT1*) gain-of-function mutations

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## GRAPHICAL ABSTRACT



**Background:** Gain-of-function (GOF) mutations affecting the coiled-coil domain or the DNA-binding domain of signal transducer and activator of transcription 1 (*STAT1*) cause chronic mucocutaneous candidiasis disease. This condition is characterized by fungal and bacterial infections caused by impaired generation of  $T_H17$  cells; meanwhile, some patients with chronic mucocutaneous candidiasis disease might also have viral or intracellular pathogen infections.

**Objective:** We sought to investigate the effect of *STAT1* GOF mutations on the functioning of natural killer (NK) cells.  
**Methods:** Because *STAT1* is involved in the signaling response to several cytokines, we studied NK cell functional activities and *STAT1* signaling in 8 patients with *STAT1* GOF mutations.  
**Results:** Functional analysis of NK cells shows a significant impairment of cytolytic and degranulation activities in patients with *STAT1* GOF mutations. Moreover, NK cells from these

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patients display lower production of IFN- $\gamma$  in response to IL-15 and reduced proliferation after stimulation with IL-2 or IL-15, suggesting that STAT5 signaling is affected. In addition, signaling studies demonstrate that the increased phosphorylation of STAT1 in response to IFN- $\alpha$  is associated with detectable activation of STAT1 and increased STAT1 binding to the interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) promoter in response to IL-15, whereas STAT5 phosphorylation and DNA binding to IL-2 receptor  $\alpha$  (*IL2RA*) are reduced or not affected in response to the same cytokine.

**Conclusion:** These observations suggest that persistent activation of STAT1 might affect NK cell proliferation and functional activities. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

**Key words:** Natural killer cells, cytotoxic activity, IL-15, signal transducer and activator of transcription 1, signal transducer and activator of transcription 5, candidiasis

Chronic mucocutaneous candidiasis is a condition characterized by impairment of mucosal immunity against *Candida albicans*. It can be observed in patients with various inborn defects of the immune response, including disorders caused by mutations in autoimmune regulator (*AIRE*), signal transducer and activator of transcription 3 (*STAT3*), *IL12B*, *IL12RB1*, *IL17F*, *IL17RA*, or RAR-related orphan receptor C (*RORC*).<sup>1-3</sup> In 2011, 2 different groups reported that monoallelic mutations of *STAT1* account for a large proportion of patients with genetic predisposition to *C albicans* infections of the skin, nails, and mucous membranes.<sup>4,5</sup> These mutations, causing chronic mucocutaneous candidiasis disease (CMCD), were shown to be gain-of-function (GOF) mutations and to affect the coiled-coil or DNA-binding domain of *STAT1*. The discovery of these genetic defects and their pathogenesis have contributed to reveal the role of T<sub>H</sub>17 cells in the immune response against *Candida* species infections in patients with primary immunodeficiencies.<sup>4-10</sup>

STAT1 is a transcription factor that regulates many of the biological effects mediated by type I interferons and other cytokines in many cell types, including natural killer (NK) cells.<sup>11-13</sup>

NK lymphocytes constitute an important cell subset in the immune response against intracellular pathogens and virally infected cells.<sup>14</sup> Functioning of NK cells is controlled by an array of different activating and inhibitory receptors<sup>15</sup> that, on engagement by specific cell ligands, can either induce or suppress the process of killing. During early viral infection, STAT1 expression is dramatically induced and NK cell activity can be influenced by STAT1 phosphorylation. After type I interferon secretion, NK cells acquire potent effector functions and secrete immunomodulatory cytokines (ie, IL-15) that regulate NK cell expansion.<sup>13</sup>

Fungal infections constitute the most common manifestation of CMCD, but viral infections, including recurrent mucocutaneous infections caused by reactivation of varicella-zoster virus and herpes simplex viruses, debilitating *orf* infection, or severe invasive infection caused by chicken pox, cytomegalovirus, or EBV have also been reported.<sup>16-21</sup> The broad spectrum of infections observed in patients with CMCD suggests that susceptibility to pathogens is not entirely due to the lack of T<sub>H</sub>17 cells, but other cell types, particularly NK cells, might play a role in the pathogenesis of CMCD. To define the role of NK cells in the

#### Abbreviations used

ChIP:	Chromatin immunoprecipitation
CMCD:	Chronic mucocutaneous candidiasis disease
E/T:	Effector/target
FITC:	Fluorescein isothiocyanate
GOF:	Gain of function
IFIT1:	Interferon-induced protein with tetratricopeptide repeats 1
IL2RA:	IL-2 receptor $\alpha$
MFI:	Mean fluorescence intensity
NK:	Natural killer
PE:	Phycoerythrin
PRL:	Prolactin
SOCS:	Suppressor of cytokine signaling
STAT:	Signal transducer and activator of transcription

susceptibility of patients with CMCD to intracellular pathogens and viruses, we have investigated NK functional activities and STAT1 and STAT5 signaling in response to cytokines in these patients.

Here we report that NK cells from patients with *STAT1* GOF mutations display increased STAT1 phosphorylation in response to IL-2 and IL-15 but reduced STAT5 activation. This is associated with impaired NK cell proliferation in response to IL-2 or IL-15 and reduced IFN- $\gamma$  secretion in response to IL-15.

## METHODS

### Patients

We investigated 8 patients affected by CMCD from 7 families. Some of the patients had been previously screened for other mutations (ie, *AIRE* or *CARD9*) to rule out other causes of CMCD. In all patients described herein, we found heterozygous mutations in the coiled-coil or DNA-binding domains of *STAT1*: L283M in P1 and his mother P2, L351F in P3, L400V in P6, T837A in P8,<sup>21,22</sup> T385M in P4 and P5,<sup>23</sup> and A267V in P7.<sup>4</sup> Clinical data from patients were collected from medical records and are briefly summarized in Table I. Fungal and bacterial infections were observed in all patients with CMCD, whereas viral infections were observed in patients P3 and P8.<sup>21,22</sup>

More detailed information on the Methods used are reported in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## RESULTS

### STAT1 phosphorylation in NK cells of patients with *STAT1* GOF mutations

To investigate the mechanism of NK cells activation in response to cytokines, we explored STAT1 phosphorylation in response to IFN- $\alpha$  (40,000 U/mL), IL-2 (100 ng/mL), and IL-15 (50 ng/mL) by using flow cytometry in NK cells from both healthy control subjects and patients with *STAT1* GOF mutations. After stimulation, STAT1 phosphorylation was examined by staining with specific antibodies directed against phosphorylated tyrosine residues. Analysis of STAT1 signaling in freshly isolated NK cells (see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) showed higher STAT1 phosphorylation in response to IFN- $\alpha$  in cells from patients compared with those from control subjects, suggesting that *STAT1* GOF mutations result in abnormal functioning of STAT1 signaling in NK cells (Fig 1, A). This observation is in line with previous reports showing that *STAT1* GOF mutations are related to increased STAT1 phosphorylation.<sup>5,18,24,25</sup> Then we investigated STAT1

**TABLE I.** Genetics and main phenotypical features of 8 patients with *STAT1* GOF mutations

	P1	P2	P3	P4	P5	P6	P7	P8
Mutation (cDNA)	c.(847T>A)	c.(847T>A)	c.(1441G>T)	c.(1542C>T)	c.(1542C>T)	c.(1198C>G)	c.(801T>A)	c.G372C
Mutation (amino acids)	L283M	L283M	L351F	T385M	T385M	L400V	A267V	T387A
Affected domain	CCD	CCD	DBD	DBD	DBD	DBD	CCD	DBD
Sex	Male	Female	Male	Male	Male	Female	Female	Male
Age (y)	11	45	33	15	10	7	14	18
Clinical onset	1 year	1 year	6 months	5 years	2 months	2 years	6 months	7 years
Manifestations of CMC	Oral cavity, skin, nails	Oral cavity, nails	Oral cavity, esophagus	Oral cavity	Oral cavity, esophagus	Oral cavity, skin, nails	Oral cavity, genital mucosa	Oral cavity, nails, esophagus
Infections	URTI, pneumonia	URTI, pneumonia, skin abscess	URTI, pneumonia, otitis media, skin abscess, cryptococcal adenitis, leishmaniasis, skin HPV, recurrent mollusca	URTI, recurrent pneumonia	URTI, <i>Pneumocystis</i> pneumonia, otitis media, sepsis	URTI, pneumonia, otitis media	URTI, pneumonia	Skin abscesses, periodontitis, suppurative eyelid infection, mucocutaneous HSV infection, severe chicken pox
Inflammation	None	Reflux esophagitis, recurrent fever, chronic lung disease, bronchiectasis	Recurrent fever, chronic lung disease, bronchiectasis	Bronchiectasis, GERD anemia	GERD	Bronchiectasis, anemia	None	None
Endocrinopathy	Hypothyroidism	None	Hypothyroidism	None	None	None	None	None
Autoimmunity	None	None	Lupus-like skin lesion, ANA and dsDNA antibody positivity	ANA positivity, vitiligo	None	None	None	None
Lymphopenia	No	Yes	Yes	+/-	+/-	No	+/-	No

ANA, Antinuclear antibody; CCD, coiled-coil domain; CMC, chronic mucocutaneous candidiasis; DBD, DNA-binding domain; dsDNA, double-strand DNA; GERD, gastroesophageal reflux disease; HPV, human papilloma virus; HSV, Herpes simplex virus; URTI, upper respiratory tract infections.

phosphorylation in resting NK cells in response to IL-2 or to IL-15. We observed that phosphorylated STAT1 (p-STAT1) levels increased in response to both IL-2 and IL-15 in NK cells from patients with *STAT1* GOF mutations, whereas a remarkably lower phosphorylation in response to the same cytokines was detected in healthy control subjects (Fig 1, B-E;  $P < .05$ ). In addition, time-course analysis of STAT1 phosphorylation in response to IFN- $\alpha$  at 15, 30, 60, 90, 120, and 180 minutes in NK and T cells showed a slower kinetic in protein dephosphorylation in patients with *STAT1* GOF mutations compared with control subjects (see Fig E1, B-D). This observation is in accordance with previous studies showing a characteristic delay of STAT1 dephosphorylation.<sup>5,18,24,25</sup>

Moreover, we investigated STAT1 protein levels in primary resting NK and T cells from patients with *STAT1* GOF mutations. Surprisingly, flow cytometric analysis of STAT1 protein showed a 3-fold increase in STAT1 protein levels in resting NK and T cells from patients compared with protein levels detected in cells of healthy control subjects (Fig 1, F, and see Fig E1, E).

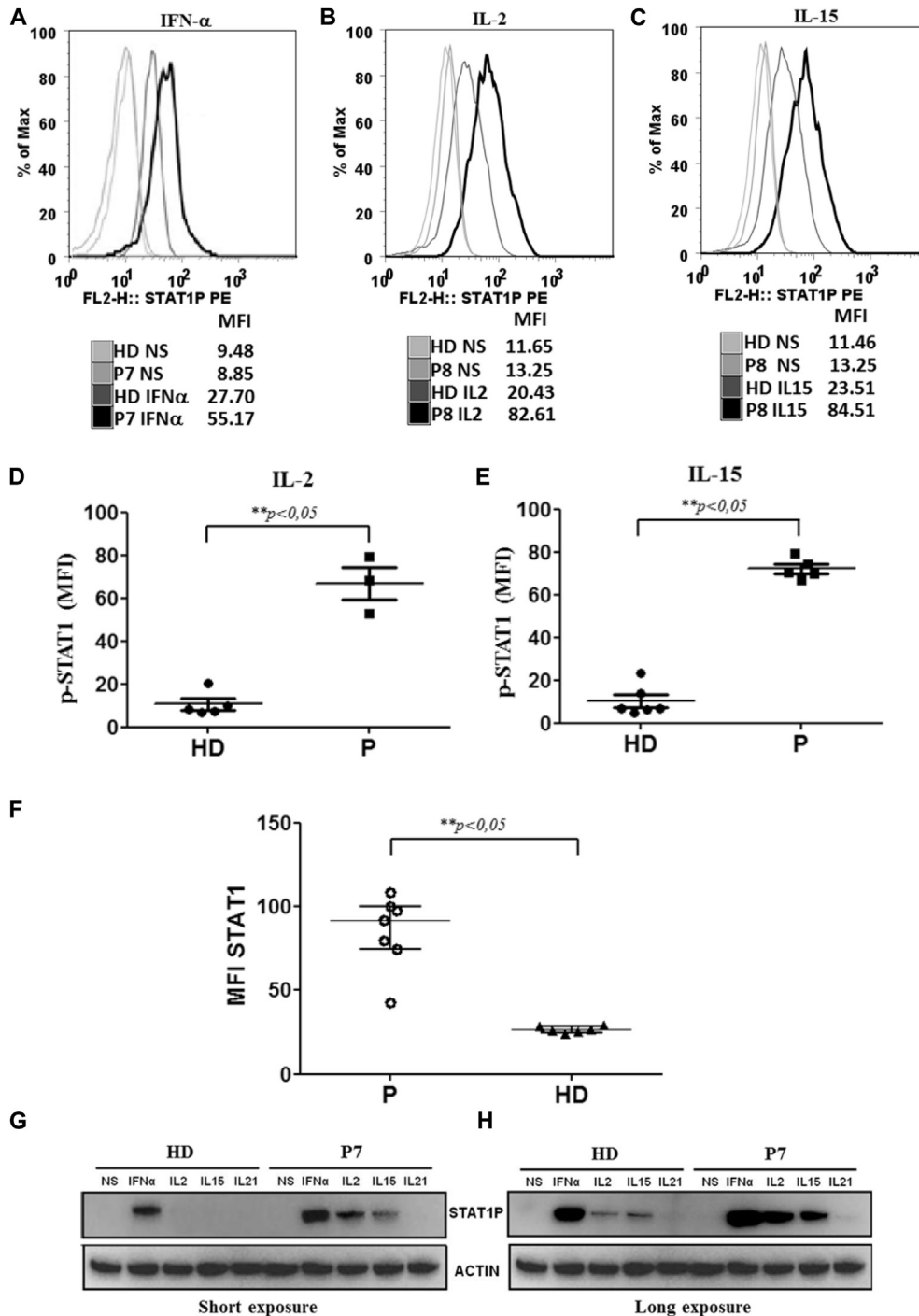
NK cells were isolated from PBMCs of patients with *STAT1* GOF mutations or from healthy control subjects by using magnetic beads and cultured with IL-2, as described in the Methods section, to perform Western blot experiments. However, we were unable to obtain long-term NK cultures from the majority of patients, with the exception of patient P7, because

of reduced proliferation of NK cells and prevalent contamination of T cells, suggesting an abnormal response of this cell type to IL-2. Western blot analysis of STAT1 in IL-2-activated NK cells from patient P7 showed that STAT1 protein levels were increased in the cells from this subject compared with cells from a healthy donor (see Fig E1, F).

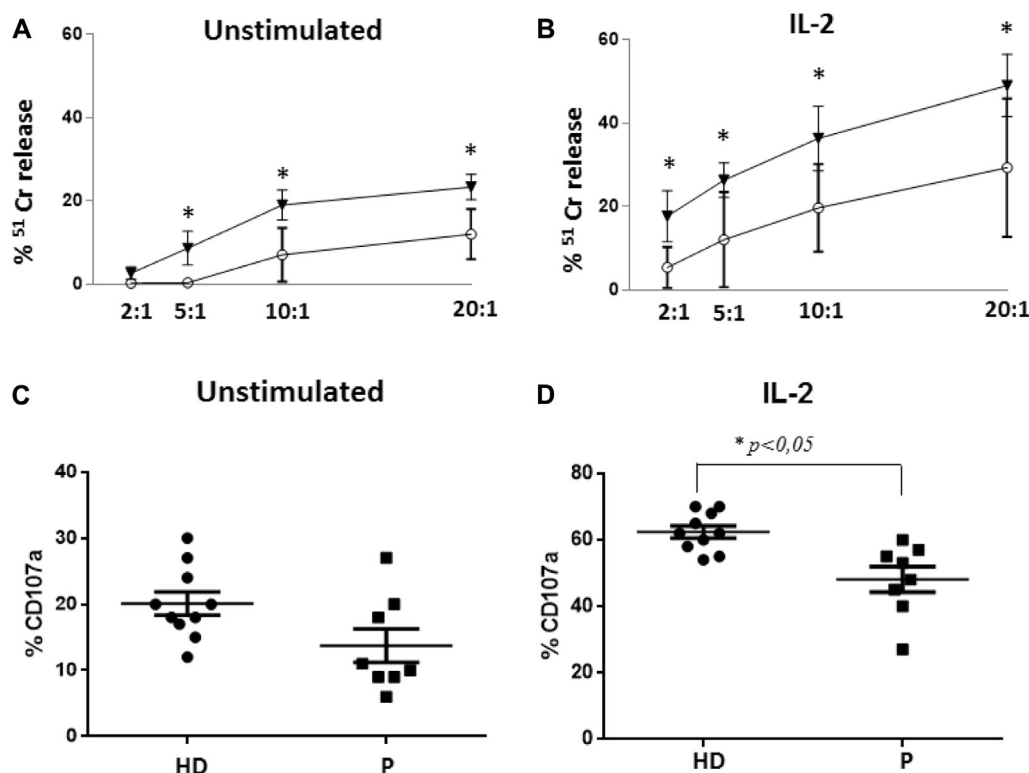
Furthermore, we performed Western blot analysis of STAT1 phosphorylation in response to IFN- $\alpha$ , IL-2, IL-15, or IL-21 in NK cell cultures obtained from patient P7 and a control subject. After withdrawal of IL-2 for 16 hours, IFN- $\alpha$  induced higher levels of p-STAT1 in cells from patient P7 compared with those in cells from a healthy control subject. In the same patient increased STAT1 phosphorylation in response to IL-2 and IL-15 was also observed, whereas a weaker response to the cytokine was detected in cells from the control subject only after prolonged exposure of the membrane (Fig 1, G and H). Analysis of STAT1 phosphorylation in response to IL-21 did not reveal STAT1 activation in either control cells or cells from the patient.

### NK functional activity and phenotype in patients with *STAT1* GOF mutations

To define the functional properties of NK cells derived from 8 patients with *STAT1* GOF mutations, we investigated NK cytolytic activity in freshly isolated PBMCs from patients and control



**FIG 1.** *STAT1* GOF mutations in patients with CMCD are associated with increased *STAT1* activation. **A-C**, NK cells derived from 5 patients with *STAT1* GOF mutations (P3, P5, P6, P7, and P8) and 6 healthy control subjects were stimulated with IFN- $\alpha$  (Fig 1, **A**), IL-2 (Fig 1, **B**), or IL-15 (Fig 1, **C**) or left unstimulated. Cells were stained with anti-pY701 *STAT1* and analyzed by using flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. The extent of p-*STAT1* intensity (MFI) of a representative patient is shown. **D** and **E**, *STAT1* activation in NK cells derived from patients (squares) and healthy donors (circles) after stimulation with IL-2 (Fig 1, **D**) or IL-15 (Fig 1, **E**). Mann-Whitney *U* test statistical analysis shows a significant difference in the extent of p-*STAT1* expression between patients with *STAT1* GOF mutations and control subjects ( $P < .05$ ). **F**, NK cells from 7 patients with CMCD (P1, P2, P3, P4, P5, P7, and P8) and healthy control subjects were stained with anti-*STAT1* and analyzed by means of flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. The extent of *STAT1* intensity is expressed as MFI. Mann-Whitney *U* test statistical analysis shows a significant difference in the extent of *STAT1* expression between patients with *STAT1* GOF mutations (open circles) and control subjects (triangles;  $P < .05$ ). **G** and **H**, IL-2-activated NK cells from a healthy control subject (HD) and patient P7 were stimulated with IFN- $\alpha$ , IL-2, IL-15, IL-21, or medium alone after IL-2 deprivation for 16 hours. *STAT1* phosphorylation was evaluated by means of Western blotting with an anti-Tyr-701 p-*STAT1* mAb. Actin expression was determined to measure protein loading. Long (Fig 1, **H**) or short (Fig 1, **G**) film exposures are shown.



**FIG 2.** Impaired NK cell cytotoxicity in patients with *STAT1* GOF mutations. **A** and **B**, Freshly isolated PBMCs derived from 3 control subjects (triangles) and 3 patients (circles) were incubated overnight, either without (Fig 2, **A**) or with (Fig 2, **B**) IL-2, and tested against the K562 target cell line at different E/T ratios. Data from the control group show means  $\pm$  SDs of 3 replicates. Asterisks indicate a significant difference between patients with *STAT1* GOF mutations and healthy subjects ( $P < .05$ ). **C** and **D**, Degranulation assay (as measured based on CD107a expression) of freshly isolated CD56<sup>+</sup>CD3<sup>-</sup> gated NK cells derived from 8 patients with *STAT1* GOF mutations (squares) and 10 healthy donors (HD; circles) after stimulation with the human K562 cell line. PBMCs were incubated overnight without (Fig 2, **C**) or with (Fig 2, **D**) IL-2. Mann-Whitney *U* test statistical analysis shows a significant difference in the extent of CD107a expression between patients with *STAT1* GOF mutations and control subjects in IL-2-stimulated samples ( $P < .05$ ). Results are presented as means  $\pm$  SDs.

subjects. NK cytotoxicity was evaluated in 3 patients and 3 healthy subjects by using a chromium release assay. In these experiments we observed a significant reduction in the extent of cytolytic activity of cells from patients compared with healthy control subjects (Fig 2, **A** and **B**). However, because variability in the number of NK cells can account for impairment of NK cell cytotoxicity, we investigated NK cell-surface degranulation by using flow cytometry after cell culture with the NK cell susceptible erythroleukemia K562 cell line. We observed that the degranulation activity of IL-2-activated NK cells was significantly lower in patients with *STAT1* GOF mutations compared with that of NK cells from healthy subjects ( $P < .05$ ; Fig 2, **C** and **D**). In contrast, intracytoplasmic perforin content was normal in the patients' NK cells (see Fig E2, **A**, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

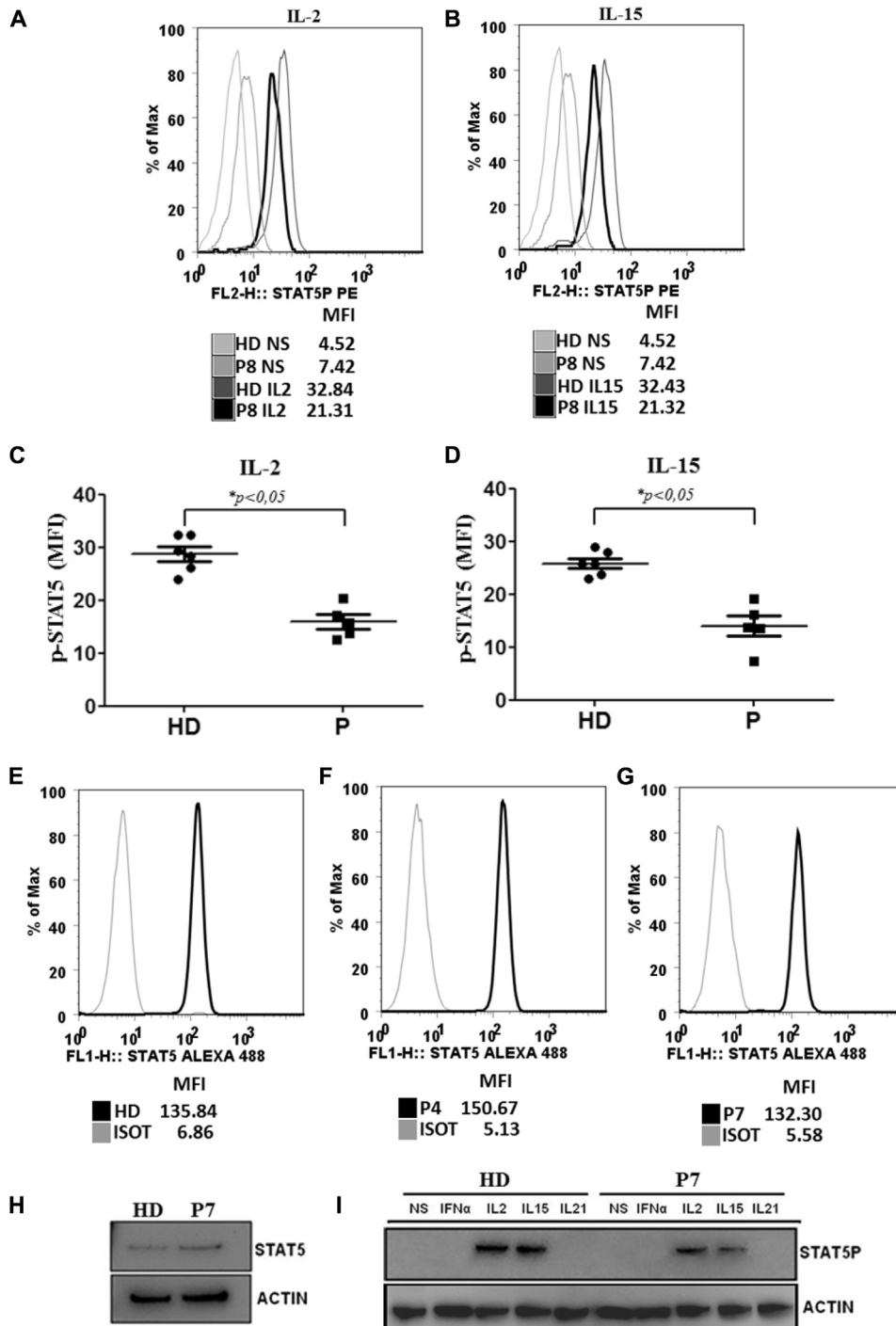
We analyzed NK cell subsets on the basis of CD56 and CD16 expression and the pattern of activating and inhibitory receptors in both *STAT1*-mutated patients and healthy donors. CD57 expression was decreased on CD56<sup>dull</sup> NK cells in 3 of 9 patients with *STAT1* GOF mutations, whereas killer cell immunoglobulin-like receptor (KIR) and NKG2A molecules were expressed at normal levels. Both CD56<sup>bright</sup> and CD56<sup>dull</sup> subsets of NK cells showed normal expression of NKG2D, the activating NK receptors NKp46 and

NKp30, and the coreceptors 2B4, NTB-A, and NKp80.<sup>26</sup> Likewise, the expression profile of the chemokine receptors CXCR1 and CCR7 of the 2 CD56 subsets were normal (see Fig E2, **A** and **B**).

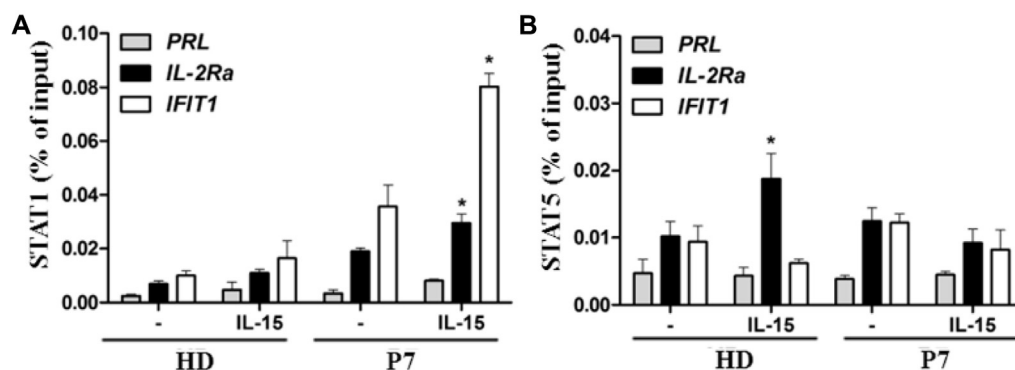
### STAT5 phosphorylation in NK cells of patients with *STAT1* GOF mutations

Because STAT5 mediates the activation of many functional activities of NK cells, we investigated STAT5 phosphorylation of IL-2-activated NK cells in response to IL-2 or IL-15 using flow cytometry. We observed reduced p-STAT5 levels in NK cells from patients with *STAT1* GOF mutations in response to both IL-2 and IL-15 compared with healthy control cells (Fig 3, **A-D**;  $P < .05$ ). STAT5 protein expression in NK cells, as measured by using flow cytometry, was similar in patients (Fig 3, **F** and **G**) and healthy control subjects (Fig 3, **E**). In addition, Western blot analysis of STAT5 expression on NK cell cultures derived from patient P7 and from a healthy donor showed similar levels of STAT5 expression in cells from a control subject and from a patient with a *STAT1* GOF mutation (Fig 3, **H**).

Then we investigated STAT5 phosphorylation by means of Western blotting in response to IFN- $\alpha$ , IL-2, IL-15, or IL-21 in NK cell cultures from patient P7 or a control subject. In NK cells



**FIG 3.** *STAT1* GOF mutations are associated with reduced STAT5 activation. **A** and **B**, NK cells from 5 patients with *STAT1* GOF mutations (P3, P5, P6, P7, and P8) and 6 healthy control subjects were stimulated with IL-2 (Fig 3, **A**) or IL-15 (Fig 3, **B**) or left unstimulated. Cells were stained with anti-pY694 STAT5 and analyzed by means of flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. A representative case is shown. The extent of p-STAT5 expression is calculated MFI. **C** and **D**, STAT5 activation in NK cells derived from patients with *STAT1* GOF mutations (squares) and healthy donors (circles) after stimulation with IL-2 (Fig 3, **C**) or IL-15 (Fig 3, **D**). Mann-Whitney *U* test statistical analysis shows a significant difference in the extent of p-STAT5 expression between patients with *STAT1* GOF mutations and control subjects ( $P < .05$ ). Data are presented as means  $\pm$  SDs. **E-G**, NK cells derived from patients P4 (Fig 3, **F**) and P7 (Fig 3, **G**) and a healthy control subject (Fig 3, **E**) were stained with anti-STAT5 and analyzed by means of flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. STAT5 levels are presented as MFI. **H** and **I**, Western blotting analysis of STAT5 expression was carried out on IL-2-activated NK cells derived from a healthy control subject (HD) and from patient P7 (Fig 3, **H**). After IL-2 deprivation for 16 hours, IL-2-activated NK cells derived from a healthy control subject (HD) and from patient P7 were stimulated with IFN- $\alpha$ , IL-2, IL-15, IL-21, or medium alone (Fig 3, **I**). STAT5 phosphorylation was investigated by means of Western blotting with an anti-Tyr-694 p-STAT5 mAb. Analysis of actin expression was determined to measure protein loading.



**FIG 4.** Abnormal STAT1 and STAT5 DNA-binding activity in IL-15-stimulated NK cells. IL-2-activated NK cells derived from a healthy control subject (HD) and patient P7 after IL-2 deprivation for 16 hours were stimulated with IL-15 (50 ng/mL) for 45 minutes or left unstimulated. ChIP assay was then performed with STAT1 (A) and STAT5 (B) antibodies; coimmunoprecipitated DNA samples were amplified by *IL2RA*, *IFIT1*, and *PRL* promoter-specific primers. Data are expressed as the percentage of total input. Bars represent SEs calculated from triplicate quantitative PCR reactions. A representative experiment of 2 performed is shown. Asterisks indicate a significant increase in STAT1 or STAT5 recruitment exerted by IL-15 stimulation: \* $P < .05$ , nonparametric Mann-Whitney *U* test. Statistical analysis was performed directly on cycle threshold values obtained from triplicate quantitative PCR reactions.

from the patient with a *STAT1* GOF mutation, we detected lower levels of p-STAT5 induction in response to IL-2 or IL-15, although no STAT5 phosphorylation was detected in response to IFN- $\alpha$  or IL-21 (Fig 3, I). In addition, image densitometry analysis showed that STAT5 phosphorylation was approximately 50% lower in NK cells from the patient compared with that in cells from control subjects.

Taken together, these results suggest that the increased STAT1 phosphorylation in response to IL-2 or to IL-15 might affect STAT5 activation in NK cells of patients with *STAT1* GOF mutations.

### Abnormal STAT1 and STAT5 DNA binding activity in NK cells of patients with *STAT1* GOF mutations

To evaluate the direct binding of STAT1 and STAT5 to specific genomic regions containing the canonical ISRE binding site or the STAT5 binding motif, such as the interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) and the IL-2 receptor  $\alpha$  (*IL2RA*) promoters,<sup>27,28</sup> we performed the chromatin immunoprecipitation (ChIP) assay in NK cells from patient 7 and from a control subject (Fig 4). We detected STAT1 binding to the *IFIT1* promoter in resting NK cells from the patient, probably because of the presence of constitutive activation of STAT1 in these cells (Fig 4, A). Moreover, IL-15 stimulation of NK cells from patients induced a stronger recruitment of STAT1 to the *IFIT1* and *IL2RA* promoters compared with NK cells from a healthy donor (Fig 4, A). Conversely, STAT5 binding to *IL2RA*, a gene strictly dependent on STAT5, was increased by IL-15 stimulation only in NK cells from the healthy donor but not in the patient's cells (Fig 4, B). This is in accordance with the observation that low levels of STAT5 phosphorylation are detected in IL-15-stimulated NK cells from patients (Fig 3).

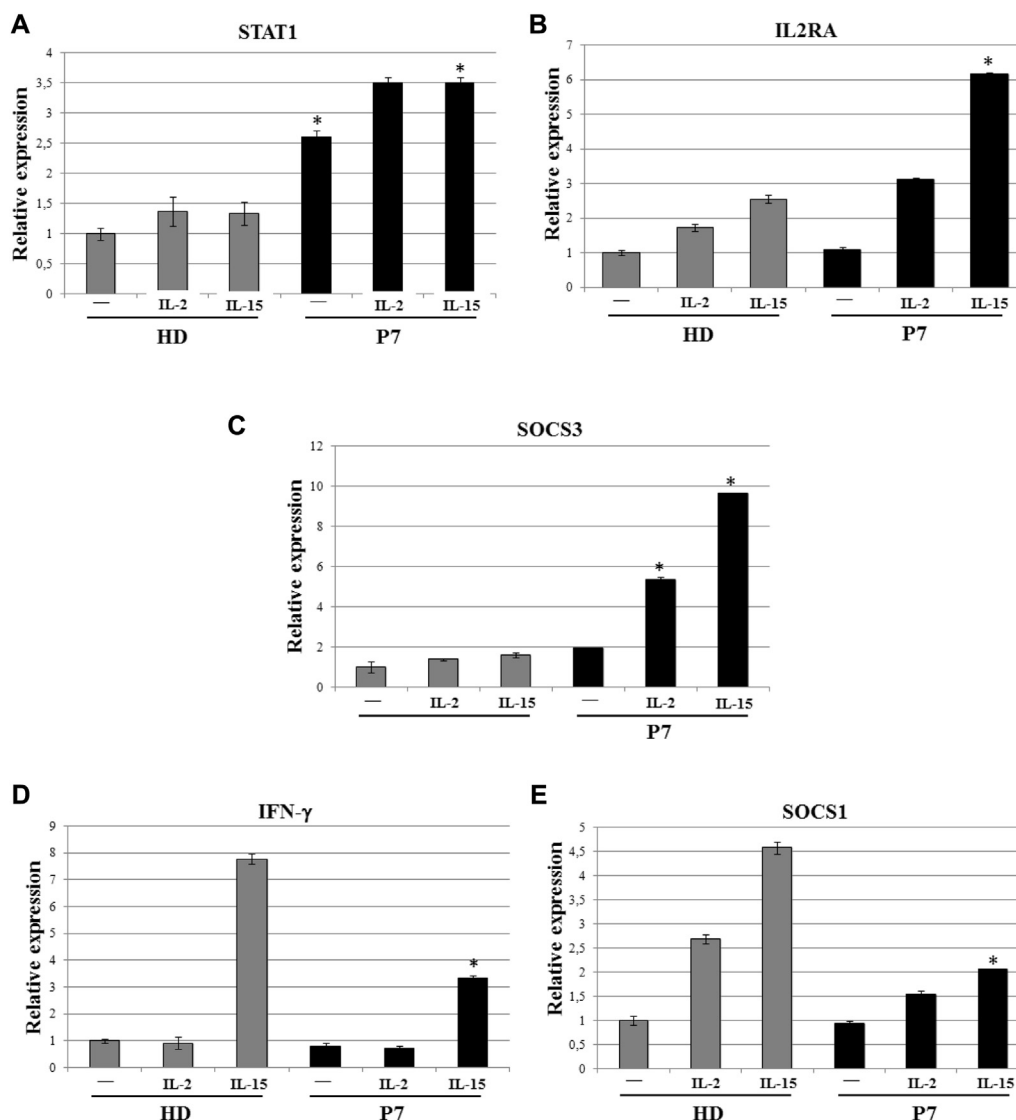
Evidence of increased STAT1 activation in response to IL-2 or IL-15 and decreased functionality of STAT5 suggest that abnormal STAT1 signaling might secondarily affect the STAT5-mediated response of NK cells to IL-15 and to IL-2 in patients with *STAT1* GOF mutations.

### Gene expression in NK cells of patients with *STAT1* GOF mutations

To address the effects of *STAT1* GOF mutations on gene expression, we performed real-time PCR experiments in NK cells activated with IL-2 for both patient P7 and a healthy control subject. Cells were cultured without IL-2 for 16 hours and then stimulated with IL-2 (100 ng/mL) or IL-15 (50 ng/mL) for 24 hours before analysis of *STAT1*, *IL2RA*, suppressor of cytokine signaling 3 (*SOCS3*), *IFNG*, and *SOCS1* mRNA expression (Fig 5). We observed that *STAT1* mRNA levels were already increased in unstimulated cells of the patient compared with those of the healthy control subject. This observation might account for the increased STAT1 protein levels in NK cells from the same patient. Moreover, stimulation of NK cells with IL-2 and IL-15 induced a further increase in mRNA expression in the patient compared with that seen in the control subject (Fig 5, A). Because patients' cells show a decrease in STAT5 phosphorylation after stimulation with IL-2 and IL-15, we evaluated *IL2RA* mRNA expression. After treatment with IL-2 or IL-15, we observed a greater increase in *IL2RA* mRNA expression in cells from the patient compared with cells from the control subject (Fig 5, B). Likewise, IL-2 and IL-15 stimulation of NK cells resulted in *SOCS3* mRNA upregulation in the patient but not in the control subject (Fig 5, C). Conversely, stimulation with IL-15 showed a marked increase in *IFNG* and *SOCS1* mRNA expression in normal control cells, whereas the increase in the patient's cells was significantly lower (Fig 5, D and E).

### NK cell proliferation and IFN- $\gamma$ production

Activated NK cells release cytokines that can modulate various effector functions of the immune system. To evaluate the biological response of freshly isolated NK cells of patients with *STAT1* GOF mutations to IL-15 and IL-12 stimulation, we analyzed IFN- $\gamma$  production after culture with IL-15 or with IL-12 and IL-18. We observed that IFN- $\gamma$  production in response to IL-15 was significantly reduced in NK cells from patients with *STAT1* GOF mutations compared with those from healthy control



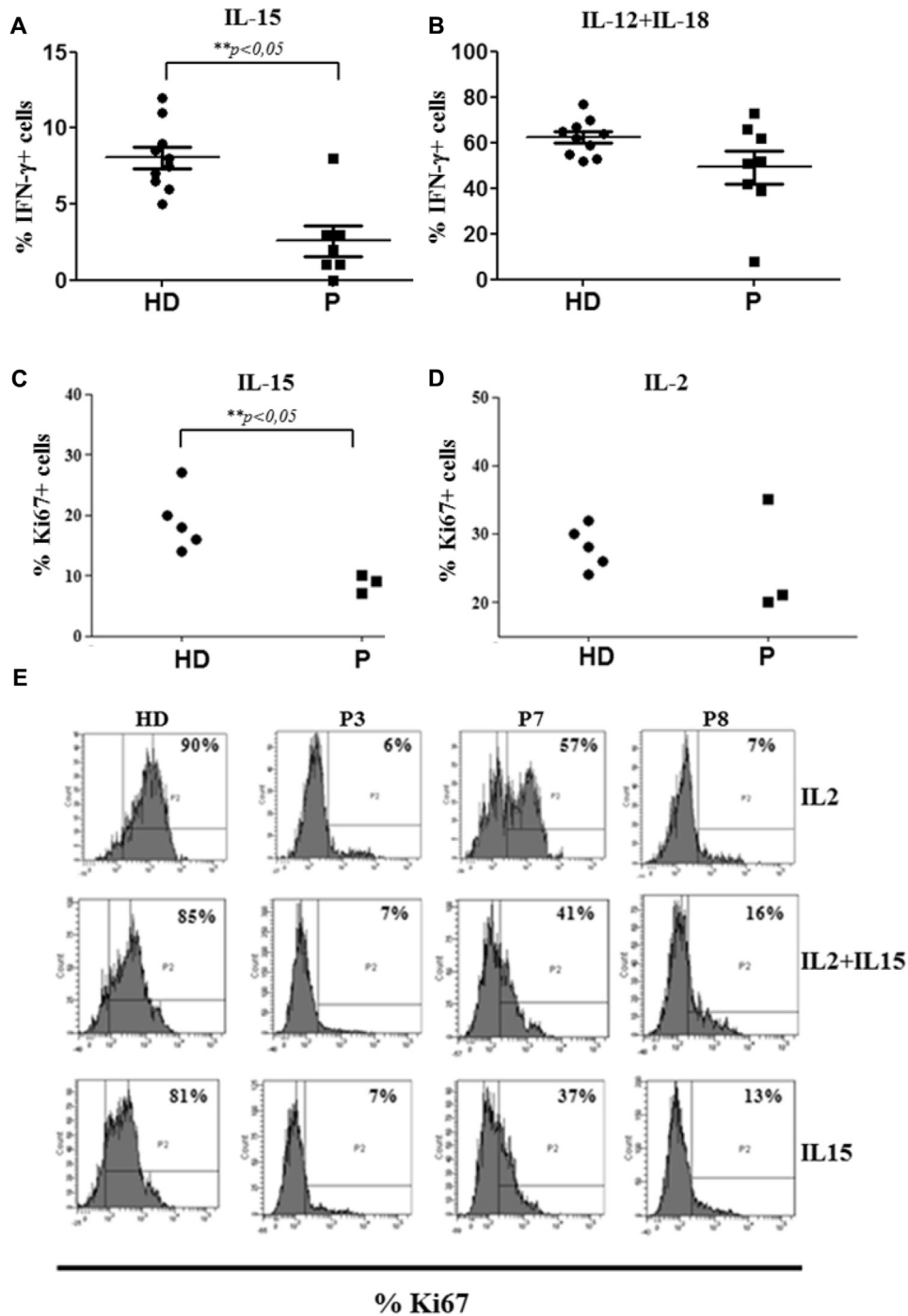
**FIG 5.** Abnormal gene response in NK cells of a patient with *STAT1* GOF mutations to IL-15. IL-2-activated NK cells from patient P7 and a healthy donor (HD) were stimulated after IL-2 deprivation for 16 hours with IL-2 (100 ng/mL) or IL-15 (50 ng/mL) or left unstimulated (-) for 24 hours. Relative expression of *STAT1* (A), *IL-2RA* (B), *SOCS3* (C), *IFNG* (D), and *SOCS1* (E) mRNAs was measured by using real-time PCR. Target gene expression was normalized with respect to expression of the housekeeping gene *GAPDH* and presented as the *n*-fold increase of mRNA expression over unstimulated cells from a healthy control subject. The experiment shown is representative of 3 independent experiments. Data are presented as means  $\pm$  SDs. Asterisks indicate a significant difference of mRNA levels between the patient and a control subject ( $P < .05$ ).

subjects ( $P < .05$ ; Fig 6, A). In contrast, IFN- $\gamma$  production after IL-12 and IL-18 stimulation was normal, suggesting a selective defect of the NK response to IL-15 stimulation (Fig 6, B).

As pointed out in the Results section, we were unable to maintain long-term cultures of IL-2-activated NK cells derived from patients with *STAT1* GOF mutations, suggesting a possible proliferation defect. Therefore we analyzed expression of the proliferation marker Ki67 after IL-15 or IL-2 stimulation of freshly isolated PBMCs for 72 hours. We observed that Ki67 induction in response to IL-15 by CD56<sup>+</sup>/CD3<sup>-</sup> cells was significantly lower ( $P < .05$ ) in cells from patients with *STAT1* GOF mutations compared with control NK cells (Fig 6, C and D).

Then we investigated the NK cell proliferative response to IL-2 or IL-15 in patients P3, P7, and P8 by means of analysis of the nuclear protein Ki67 expression after short-term cultures with IL-2 and withdrawal of IL-2 in the last 16 hours before stimulation. We observed that Ki67 expression was considerably decreased in NK cells derived from patients P3, P7, and P8 after activation for 48 hours with IL-2 or IL-15 compared with the response of NK cells derived from a representative healthy donor (Fig 6, E). However, broad variability was observed in the extent of Ki67 expression, suggesting interindividual variability in the proliferative response of NK cells. To evaluate a possible role of apoptosis in the regulation of NK cell homeostasis, we investigated spontaneous and cytokine-induced apoptosis in NK





**FIG 6.** Reduced IFN- $\gamma$  production and Ki67 expression by NK cells in patients with *STAT1* GOF mutations. **A** and **B**, IFN- $\gamma$  production was measured by means of intracellular staining from freshly isolated CD56<sup>+</sup>/CD3<sup>-</sup> NK cells derived from 8 patients with *STAT1* GOF mutations (squares) and 10 healthy donors (circles) after overnight stimulation with IL-15 (Fig 6, A) or IL-12 with IL-18 (Fig 6, B). The extent of production was measured as the percentage of IFN- $\gamma$ <sup>+</sup> cells. Mann-Whitney *U* test statistical analysis shows a significant difference in IFN- $\gamma$  production between patients and control subjects in IL-15-stimulated samples ( $P < .05$ ). Results are presented as means  $\pm$  SDs. **C** and **D**, Ki67 expression determined by means of intracellular staining was measured in CD56<sup>+</sup>/CD3<sup>-</sup> NK cells in PBMCs freshly isolated from 3 patients with *STAT1* GOF mutations (squares) and 5 healthy donors (circles) after stimulation for 72 hours with IL-15 (Fig 6, C) or IL-2 (Fig 6, D). The extent of Ki67 production was measured as a percentage of Ki67<sup>+</sup> cells. Mann-Whitney *U* test statistical analysis shows a significant difference in Ki67 expression between patients and control subjects in IL-15-stimulated samples ( $P < .05$ ). **E**, NK cells derived from 3 patients with *STAT1* GOF mutations (P3, P7, and P8) and 1 representative healthy donor were kept in culture with IL-2, maintained for 16 hours in complete medium without IL-2, and then stimulated with IL-2, IL-2, and IL-15 or IL-15 alone. After 48 hours, expression of the intracytoplasmic protein Ki67 was analyzed by using flow cytometry. The experiment we show herein is representative of 3 experiments that were performed.

cells of patients with *STAT1* GOF mutations. After PBMC culture for 48 hours with IL-15 or medium alone, analysis of Annexin V staining in NK cells showed comparable levels of apoptosis between patients and control subjects (see Fig E2, C). These results suggest that NK cells derived from patients with *STAT1* GOF mutations display an impaired response to cytokines that signal throughout STAT5, such as IL-2 and IL-15, resulting in abnormal proliferation and cytokine production.

## DISCUSSION

Several studies have shown that *STAT1* GOF mutations observed in patients with CMCD result in impaired generation of T<sub>H</sub>17 cells and increased risk of fungal and bacterial infections. In this study we report that patients with *STAT1* GOF mutations display abnormal NK cell function and proliferation.

In patients with primary immunodeficiencies, such as Wiskott-Aldrich syndrome, dedicator of cytokinesis 8 deficiency, or Hermansky-Pudlak type 2 syndrome, NK cells display abnormal functioning of the cytotoxicity machinery, whereas cellular response to IL-2 or IL-15 is usually normal.<sup>29-31</sup> Interestingly, in these conditions addition of IL-2 restores NK cytotoxicity, suggesting that the cell response to IL-2 was preserved in patients with these conditions.<sup>31,32</sup>

In contrast, NK cytolytic activity against the susceptible human erythroleukemia K562 cell line was reduced in NK cells activated with IL-2 in patients with *STAT1* GOF mutations. This is in accordance with a communication by Forbes et al,<sup>33</sup> who reported NK cytotoxicity impairment in patients with *STAT1* GOF mutations.

Moreover, the defect of NK cytotoxic activity in patients with *STAT1* GOF mutations was not related to abnormal expression, functioning, or both of NK receptors or to reduced perforin expression but to an impaired response of these cells to IL-2 or IL-15. Indeed, IFN- $\gamma$  production was reduced in NK cells from patients with *STAT1* GOF mutations after stimulation with IL-15 but was normal in cells activated with IL-12 and IL-18. This suggests that the NK cytotoxicity defect detected in patients with *STAT1* GOF mutations is probably related to abnormal response of these cells to the immunomodulatory cytokines IL-15 and IL-2.

NK cell functions are modulated *in vivo* by several cytokines, including IL-15 and IL-2. In particular, IL-15 is the most potent cytokine involved in the regulation of NK cell homeostasis, maturation, and activation.<sup>34</sup> IL-15 has a fundamental role in the development of NK cells and in their proliferation and/or survival in peripheral lymphoid cells. In fact, IL-15 promotes survival of mature NK cells through upregulation of antiapoptotic Bcl2 family members.<sup>35</sup> In addition, IL-15 controls both NK cell homeostatic proliferation and NK cell activation after bacterial, viral, or fungal infections and synergizes with IL-12 and IL-18 to induce IFN- $\gamma$  production by NK cells.<sup>36</sup>

IL-2 and IL-15 intracellular pathways share several signaling components, including the Janus kinase 1 and 3 molecules, IL-15 receptor  $\beta$ , the common  $\gamma$ c receptors, and the STAT5a and STAT5b transcription factors. Moreover, the importance of the STAT5 pathway for IL-15 biological activities on NK cells is also highlighted by many studies in mice and human subjects. In particular, there is evidence in STAT5b-deficient and, to lower extent, STAT5a-deficient animals that the number of NK cells is reduced<sup>37</sup> and that children carrying STAT5b mutations display severe immunologic dysfunctions characterized by moderate

lymphopenia and NK cytolytic activity defect.<sup>38,39</sup> Although STAT5 expression was normal in NK cells from patients with *STAT1* GOF mutations, the extent of STAT5 phosphorylation in response to IL-2 or IL-15 was significantly reduced, suggesting that abnormal functioning of the STAT1 pathway might affect the cellular response of NK cells to these cytokines. In fact, we have observed in NK cells from a patient with a *STAT1* GOF mutation that lower levels of STAT5 phosphorylation in response to IL-15 are associated with impaired upregulation of STAT5 binding to *IL2RA*, a gene strictly dependent on STAT5. Moreover, IL-15-dependent induction of the *IFNG* gene was abnormal in patients with *STAT1* GOF mutations, suggesting an interplay between STAT1 signaling and STAT5-mediated response of NK cells to IL-15. In particular, the increase of STAT1 binding to *IL2RA* promoter that is observed in NK cells after IL-15 activation might account for upregulation of *IL2RA* mRNA. An altered balance between STAT1 and STAT3 was observed in T cells of patients with *STAT1* GOF mutations stimulated with IL-6 and IL-27, suggesting that analogous mechanisms involving STAT1 and STAT5 might explain the observed *IL2RA* upregulation in IL-15-stimulated NK cells of patients with *STAT1* GOF mutation.<sup>40</sup>

In patients with CMCD, *STAT1* GOF mutations are associated with a characteristic delay of protein dephosphorylation, resulting in persistent activation of the signaling pathway.<sup>5,18,24,25</sup> In our study the time course analysis of STAT1 phosphorylation in response to IFN- $\alpha$  has shown a similar pattern in NK cells of patients with *STAT1* GOF mutations. However, we have also observed that both resting and IL-2-activated NK cells of these patients display increased STAT1 protein levels and higher expression of *STAT1* mRNA, suggesting that augmented STAT1 protein levels might concur with the increased response of NK cells to cytokines signaling throughout STAT1. Higher basal levels of STAT1 protein in NK cells of patients with *STAT1* GOF mutations prevent a correct analysis of p-STAT1 dephosphorylation kinetic after IL-15 or IL-2 stimulation. This experiment could not be performed in primary cells, thereby requiring a distinct experimental model that will be the object of a separate study.

Complete STAT1 deficiency is associated with impaired cytolytic activity of NK cells and reduced functional response to IL-15.<sup>41</sup> Lack of endogenous IL-15 results in reduced accumulation of NK cells in response to type I interferons, thus suggesting that the coordinated activities of IL-15 and interferons is required to induce NK proliferation during viral infections and that STAT1-mediated activation of NK cells has a priming effect on functional response to IL-15. Interestingly, in patients with *STAT1* GOF mutations, IL-15 or IL-2 stimulation of NK cells induces strong STAT1 phosphorylation, whereas a weak signaling response to IL-2 or IL-15 is detected in normal NK cells after prolonged exposure of the membrane. This suggests that GOF mutations of *STAT1* observed in these patients might confer an increased sensitivity of NK cells to STAT1-mediated signaling that will secondarily interfere with STAT5-dependent responses to IL-15 or IL-2. Likewise, in T cells of patients with *STAT1* GOF mutations, the increased and prolonged STAT1 phosphorylation is apparently affecting the STAT3-dependent responses to IL-23 or IL-6 required for induction of IL-17A and IL-17F. Although signaling mechanisms of these biological events remain largely speculative, it is likely that persistent STAT1 phosphorylation induced in response to various cytokines

in different cell types can interfere with important steps in the differentiation and functions of T and NK cells resulting in impaired generation of T<sub>H</sub>17 cells and reduced proliferation of NK cells.

Reduced proliferative response of NK cells associated with lower IFN- $\gamma$  production in response to IL-15 or IL-2 might result in an increased risk of invasive viral infection in patients with *STAT1* GOF mutations. This is in accordance with the observation that a significant proportion of patients with CMCD has been shown to be at risk of viral infections, suggesting that in patients with certain conditions, the abnormal function of NK cells might be related to specific clinical manifestations.

In addition, reduced IFN- $\gamma$  production by activated NK cells is observed in patients with *STAT1* GOF mutations, suggesting that response to intracellular pathogens might also be affected. Although STAT4, but not STAT1, is essential for IL-12-dependent NK cell IFN- $\gamma$  production, IL-15 might be important when IL-12 production is not sufficient to drive IFN- $\gamma$  secretion, as observed during intracellular parasite infections or during immunosuppressive treatment. This observation could explain why some patients with CMCD can have infections sustained by intracellular pathogens, such as *Cryptococcus*, *Leishmania*, or mycobacteria,<sup>42</sup> and might be helpful for the development of novel therapeutic strategies for these patients.

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**Clinical implications: GOF mutations of *STAT1* result in impaired proliferation, IFN- $\gamma$  production, and cytolytic activity of NK cells in patients with *STAT1* GOF mutations.**

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## METHODS

### mAbs

Characterization of NK cell-surface markers was performed by using the following mAbs generated in our laboratory (Department of Molecular and Translational Medicine, University of Brescia) or kindly provided by A. Moretta (Laboratory of Molecular Immunology, DIMES, University of Genoa): BAB281 (IgG<sub>1</sub>, anti-NKp46); AZ20 (IgG<sub>1</sub>, anti-NKp30); ON72 (IgG<sub>1</sub>, anti-NKG2D); SUS142 (IgG<sub>2b</sub>, anti-CD16); C227 (IgG<sub>1</sub>, anti-CD69); FS123 (IgG<sub>2a</sub>, anti-DNAM-1); 11PB6 (IgG<sub>1</sub>, anti-KIR2DL1/S1); GL183 (IgG<sub>1</sub>, anti-KIR2DL2/L3/S2); AZ158 (IgG<sub>2a</sub>, anti-KIR3DL1/S1/L2); Z199 (IgG<sub>2b</sub>, anti-CD94/NKG2A); and anti-XA147 (IgM, anti-CD57).

The following commercial antibodies were used in this study: anti-CXCR1 (IgG<sub>1</sub>; Santa Cruz Biotechnologies, Santa Cruz, Calif); anti-NKG2C (IgG<sub>2b</sub>; R&D Systems, Minneapolis, Minn); phycoerythrin (PE)-labeled anti-CD107a; PE-labeled anti-IFN- $\gamma$  (IgG<sub>1</sub>; BD Biosciences, Pharmingen, Calif); mixture of fluorescein isothiocyanate (FITC)-labeled CD3, PC5-labeled CD56, FITC-labeled CD14, and FITC-labeled CD20 (Beckman Coulter, Immunotech, Marseille, France); anti-hCCR7 (IgG<sub>2A</sub>; R&D Systems); PE-labeled anti-Perforin (Ancell, Bayport, Minn); anti-human Ki-67 antigen (IgG<sub>1</sub>; Dako, Copenhagen, Denmark).

### Cell preparations and flow cytometry

PBMCs derived from patients with *STAT1* GOF mutations and healthy donors, who were referred to the local hospital for minor trauma, were obtained from heparinized blood by means of density gradient centrifugation over Ficoll (Sigma, St Louis, Mo). PBMCs were resuspended in RPMI 1640 medium supplemented with 2 mmol/L glutamine, 50  $\mu$ g/mL penicillin, 50  $\mu$ g/mL streptomycin, and 10% heat-inactivated FCS (Sigma).

NK lymphocytes were isolated from PBMCs by using negative selection (NK cell isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured on irradiated feeder cells in the presence of 100 U/mL human IL-2 (Proleukin; Chiron, Emeryville, Calif) and 1.5 ng/mL PHA (GIBCO, Carlsbad, Calif) to obtain polyclonal NK cell lines.

For flow cytometric analysis, NK cells derived from peripheral blood were stained with the appropriate primary mAbs and then incubated with PE-conjugated isotype-specific goat anti-mouse secondary reagent (Southern Biotechnology, Birmingham, Ala). Cells were subsequently stained with a mixture of FITC-labeled CD3, PC5-labeled CD56, FITC-labeled CD14, and FITC-labeled CD20. NK cell analysis by means of flow cytometry was performed by gating on CD56<sup>+</sup>, CD3<sup>-</sup>, CD14<sup>-</sup>, and CD20<sup>-</sup> cells. Perforin expression analysis in NK cells was performed by using purified PE-labeled anti-perforin mAb after incubation with Cytotfix/Cytoperm (BD Biosciences).

Cell acquisition was performed on a FACSCanto flow cytometer, and data were analyzed by using the FACSDiva software (Becton Dickinson, Mountain View, Calif).

### Real-time PCR

Total RNA was extracted from IL-2-activated NK cells left unstimulated or stimulated with IL-2 (100 ng/mL) or IL-15 (100 ng/mL) for 24 hours by using the RNeasy Mini Kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. For RT-PCR experiments, 1  $\mu$ g of DNase-treated total RNA was used to synthesize the first strand of cDNA by using the ImProm-II Reverse Transcription System (Promega, Madison, Wis). For the RT-PCR analysis, Assays-on-Demand Products and TaqMan Master Mix from Applied Biosystems (Foster City, Calif) were used according to the instruction manual to analyze *STAT1*, *IFNG*, *IL2RA*, *SOCS1*, *SOCS3*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression. Target gene expression was normalized to expression of the housekeeping gene *GAPDH* and presented as *n*-fold increase over those in the healthy control subject by using  $2^{-\Delta\Delta C_T}$  evaluation.

### ChIP assays

ChIP experiments were performed, as previously described,<sup>E1</sup> with minor modifications. Briefly, after stimulation with the appropriate cytokine,

polyclonal NK cell lines were fixed by using 1% formaldehyde for 6 minutes, followed by incubation with 0.125 mol/L glycine for another 6 minutes to stop the cross-linking reaction. After 3 washes with ice-cold PBS, nuclear extracts were prepared from  $3.5 \times 10^6$  cells. Chromatin was then sheared by means of sonication and immunoprecipitated with 4  $\mu$ g of anti-STAT1 (sc-346) or 4  $\mu$ g of anti-STAT5 (sc-835; Santa Cruz Biotechnology). The precipitation signal was quantified also at the promoter of prolactin (*PRL*) to establish background levels of ChIP experiments. The coimmunoprecipitated material was subjected to quantitative PCR analysis by using the following promoter-specific primers (purchased from Life Technologies, Grand Island, NY): *IL2RA* forward, 5'-TTCCACAGTTCTGAGAAAGGTG-3'; *IL2RA* reverse, 5'-CATGGCAAGGGTTTATGAGG-3'; *IFIT1* forward, 5'-GGCAGCAATG GACTGATGTTTC-3'; *IFIT1* reverse, 5'-GGAACCGAAAGGGGAA AGTG-3'; *PRL* forward, 5'-AGGGAAACGAATGCTGATT-3'; and *PRL* reverse, 5'-GCAGGAAACACTTCACCA-3'. Data from quantitative PCR were expressed as the percentage over input DNA and are displayed as means  $\pm$  SEMs.

### Western blotting and flow cytometric analysis

After culture in IL-2 (1,200 U/mL)-containing medium, cells were starved to reduce basal phosphorylation for 16 hours at 37°C in RPMI containing 2% serum. For Western blot analysis, NK cells were left unstimulated or stimulated with IFN- $\alpha$  (10,000 U/mL for 30 minutes; PeproTech, Rocky Hill, NJ), IL-2 (100 ng/mL for 12 minutes, PeproTech), IL-15 (50 ng/mL for 12 minutes, PeproTech), and IL-21 (50 ng/mL for 15 minutes, PeproTech). After stimulation, cells were placed on ice and washed twice with cold PBS. Proteins were extracted with extraction buffer (RIPA Buffer; Millipore, Temecula, Calif) containing protease, phosphatase inhibitors (Thermo Scientific, Waltham, Mass), and EDTA (Thermo Scientific) and maintained on ice for 15 minutes with occasional mixing. Insoluble material was removed by means of centrifugation at 12,000g for 15 minutes at 4°C. Protein lysates (5  $\mu$ g per sample) were subjected to SDS-PAGE separation on 10% BIS-Tris gel (NuPage; Life Technologies), and proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare, Fairfield, Conn) and immunoblotted with primary antibody anti-pSTAT1-Tyr-701, anti-pSTAT5-Tyr-694 (both from Cell Signaling Technology, Danvers, Mass), anti-STAT5, anti-STAT1 (BD Transduction Laboratory), and anti- $\beta$ -actin or tubulin (Sigma-Aldrich, St Louis) as a loading control. Detection was carried out with horseradish peroxidase-conjugate by using the enhanced chemoluminescence system (Euroclone LITEAblot Extend), according to the manufacturer's instructions. All images were captured with the UVP Biospectrum AC Imaging System. Densitometric analysis was performed with Gel-Pro Analyzer Software, version 3.2. Actin was used as a loading control reference. STAT1 and actin expression in NK cells were calculated by using densitometric analysis; the STAT1/actin expression ratio was calculated in cells from a patient with *STAT1* GOF mutation and a control subject.

For flow cytometry, cells were left unstimulated or stimulated with IFN- $\alpha$  (40,000 U/mL for 30 minutes), IL-2 (100 ng/mL for 12 minutes), or IL-15 (50 ng/mL for 12 minutes) and stained simultaneously with an FITC-conjugated mouse anti-CD3 IgG mAb and an R-Phycoerythrin-5.1 (PerCP-Cy5.5)-conjugated mouse anti-CD56 IgG mAb. Cells were fixed and permeabilized according to the BD protocol (Protocol III) and stained with PE-conjugated mouse anti-pSTAT1-Tyr-701 and anti-pSTAT5-Tyr-694 IgG mAbs (BD Pharmingen) or isotype-matched mAb PE (BD Biosciences). Cells were analyzed by means of flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells with a FACSCalibur flow cytometer (BD Biosciences) and analyzed by using FlowJo version 7.5 Software (TreeStar, Ashland, Ore). The extent of STAT1 phosphorylation or STAT5 phosphorylation was calculated as the mean fluorescence intensity (MFI) after subtracting the MFI level of unstimulated cells stained with an isotype-matched mAb.

### Analysis of NK cell cytotoxicity, degranulation, and IFN- $\gamma$ production

For the degranulation assay against the erythroleukemia K562 human cell line, PBMCs derived from patients and healthy donors were incubated

overnight with or without 100 U/mL h-IL-2 (Proleukin; Chiron) at 37°C. Then samples were coincubated with target cells at an effector/target (E/T) ratio of 1:3 in a final volume of 200  $\mu$ L in round-bottom 96-well plates at 37°C and 5% CO<sub>2</sub> for 3 hours in culture medium containing CD107a-PE mAb. After 1 hour of coincubation, GolgiStop (BD Biosciences Pharmingen) was added at a 1:100 dilution. Surface staining was performed by incubating cells with CD3, CD14, CD20, and CD56 mAbs for 30 minutes at 4°C. Then cells were washed and analyzed by using flow cytometry (FACSCanto; Becton Dickinson). Analysis of NK cells was performed on CD56<sup>+</sup>CD3<sup>-</sup>CD14<sup>-</sup>CD20<sup>-</sup> gated cells.

NK cytotoxic activity was investigated in a 4-hour <sup>51</sup>Cr release assay, as previously described,<sup>E2</sup> by using  $5 \times 10^3$  cells/well at a final E/T ratio of 20:1 with unstimulated or IL-2-activated NK cells against K562 cells as a target.

PBMCs derived from patients and healthy donors were incubated overnight at 37°C with IL-15 (20 ng/mL; PeproTech), IL-12 (20 ng/mL; PeproTech), and/or IL-18 (100 ng/mL; PeproTech), as specified, to detect intracellular production of IFN- $\gamma$ . Then cells were washed, fixed, and permeabilized with Cytotfix/Cytoperm kit (BD Bioscience Pharmingen). IFN- $\gamma$  production was detected by using subsequent intracellular staining with anti-IFN- $\gamma$ -PE (BD Biosciences Pharmingen) on gating of CD56<sup>+</sup>CD3<sup>-</sup>CD14<sup>-</sup>CD20<sup>-</sup> cells. In the experiments of degranulation and IFN- $\gamma$  expression, the percentage of positive cells was calculated, subtracting the baseline CD107a or IFN- $\gamma$  expression in the absence of stimuli (target cells or cytokines).

### Analysis of cell proliferation and apoptosis

NK cell proliferation was investigated by intracellularly staining Ki67 protein. After stimulation for 48 hours with IL-2 or IL-15, primary NK cells were permeabilized with saponin (0.1% in PBS) at 4°C for 20 minutes. Then cells were incubated with anti-Ki-67 mAb (IgG<sub>1</sub>; mouse anti-human, 1:40 dilution; Dako) for 1 hour at 4°C. After washing, a secondary PE-conjugated isotype-specific goat anti-mouse (Southern Biotechnology)

was incubated for 30 minutes at 4°C before washing with PBS and flow cytometric analysis.

To analyze cell apoptosis, PBMCs derived peripheral blood of patients, and healthy donors were incubated with and/or without IL-15 (100 ng/mL) for 48 hours. Then PBMCs were resuspended in Apoptosis Binding Buffer (BD Biosciences) at  $1.5 \times 10^6$  cells/mL and incubated with 5  $\mu$ L of PE-conjugated Annexin V. Analysis of Annexin V binding was performed after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. Finally, cells were acquired by using the FACSCanto (BD Biosciences).

### Statistical analysis

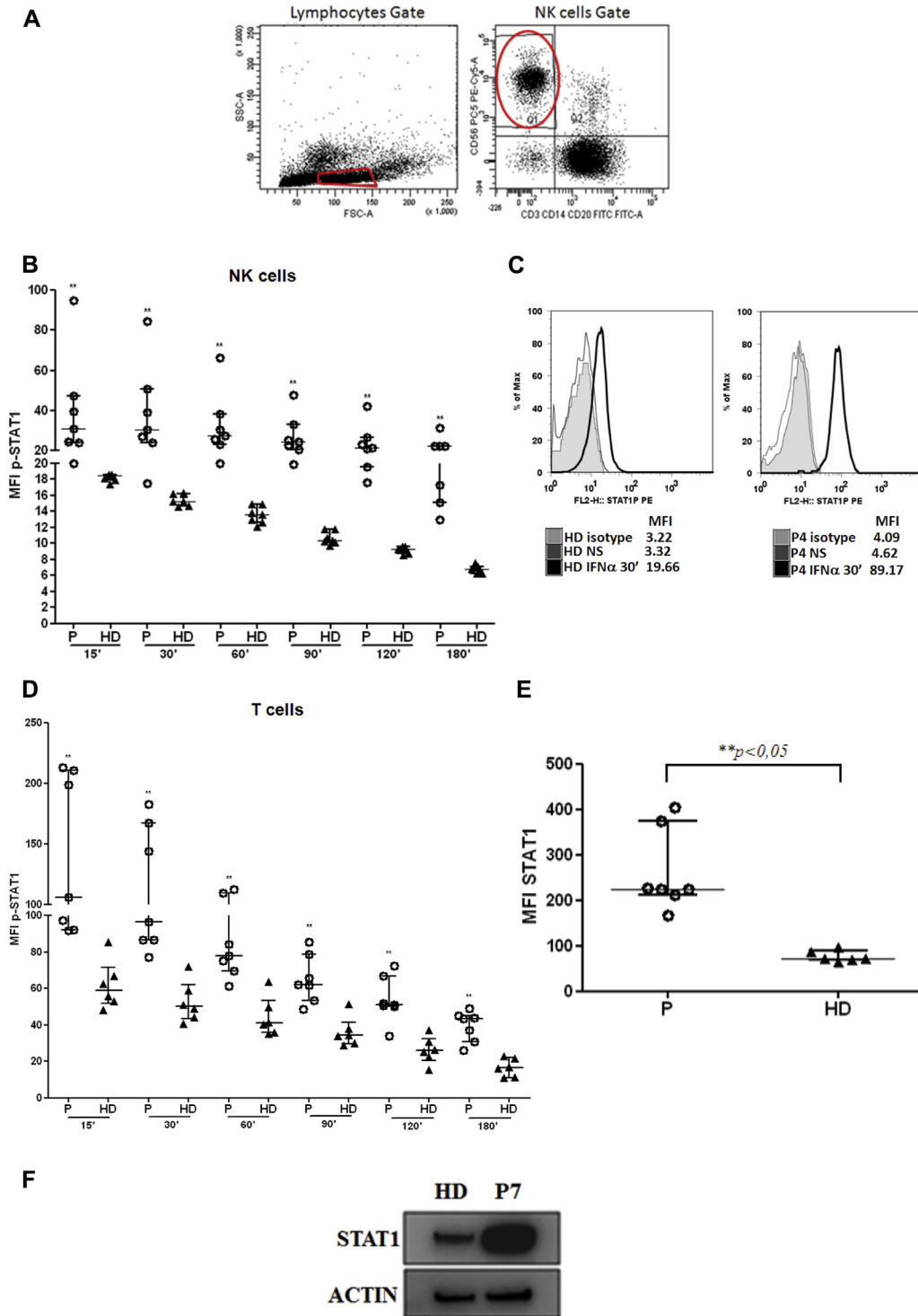
All results are shown as means  $\pm$  SDs. The nonparametric Mann-Whitney *U* test was used. *P* values of less than .05 were considered significant. Statistical analysis was carried out by using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, Calif).

### Study approval

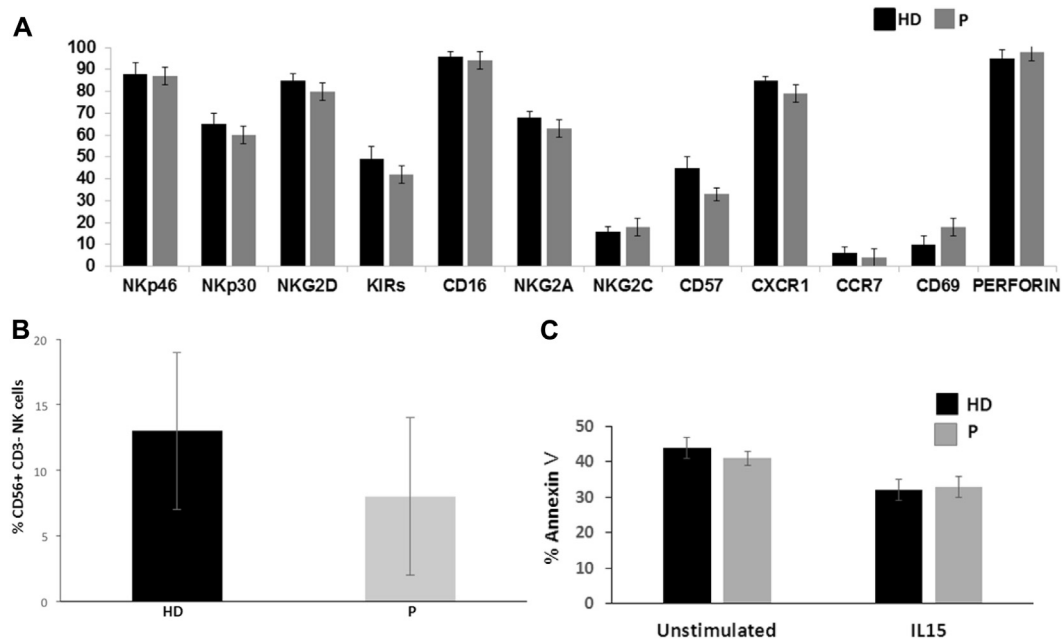
For PBMCs preparation, peripheral blood samples were collected from patients with *STAT1* GOF mutations and healthy donors and stored as established in the protocols of Spedali Civili (Brescia, Italy), according to the approval of the local ethics committee. Written informed consent was obtained for collection and storage of blood samples for genetic analysis and immunologic studies, for medical data recording from all patients or from their caregivers in the case of minors, and from healthy control subjects.

### REFERENCES

- E1. Tamassia N, Castellucci M, Rossato M, Gasperini S, Bosisio D, Giacomelli M, et al. Uncovering an IL-10-dependent NF-kappa B recruitment to the IL-1ra promoter that is impaired in STAT3 functionally defective patients. *FASEB J* 2010;24:1365-75.
- E2. Fontana S, Parolini S, Vermi W, Booth S, Gallo F, Donini M, et al. Innate immunity defects in Hermansky-Pudlak type 2 syndrome. *Blood* 2006;107:4857-64.



**FIG E1. A**, NK flow cytometric analysis. After lymphocyte gating on the basis of forward and side scatter (*left panel*), the NK cell gate was placed on the CD56<sup>+</sup>CD3<sup>-</sup>CD14<sup>-</sup>CD20<sup>-</sup> subpopulation (*right panel*). **B**, STAT1 phosphorylation kinetics in NK cells from patients with *STAT1* GOF mutations. STAT1 phosphorylation levels were assessed at different time points (15, 30, 60, 90, 120, and 180 minutes) in NK cells from 7 patients (P1, P2, P3, P4, P5, P7, and P8; *open circles*) and 6 healthy control subjects (*triangles*) after IFN- $\alpha$  stimulation (40,000 U/mL) for 30 minutes. Cells were stained with anti-pY701 STAT1 and analyzed by using flow cytometry after gating CD56<sup>+</sup>CD3<sup>-</sup> cells. *Asterisks* indicate a significant difference in p-STAT1 levels between patients and healthy subjects ( $P < .05$ ). Data are presented as means  $\pm$  interquartile ranges. **C**, The extent of p-STAT1 intensity (MFI) of a representative patient and representative healthy control subject is shown in response to IFN- $\alpha$  at 30 minutes. **D**, STAT1 phosphorylation kinetic in T cells from patients with *STAT1* GOF mutations. STAT1 phosphorylation levels were assessed at different time points (15, 30, 60, 90, 120, and 180 minutes) in T cells from 7 patients (P1, P2,



**FIG E2.** **A**, Comparison of NK cell marker expression in patients with *STAT1* GOF mutations and 10 healthy control subjects. Extent of expression is presented as the percentage of positive cells  $\pm$  SD. SDs are shown for values derived from healthy donors and patients with *STAT1* GOF mutations. Perforin expression was evaluated by using intracellular staining. **B**, Expression of CD56 by NK cells from patients with *STAT1* GOF mutations (*gray columns*) and healthy donors (*HD*; *black columns*). **C**, Analysis of NK cell apoptosis evaluated as percentage of Annexin V<sup>+</sup> cells after culture with IL-15 or medium alone for 48 hours from patients with *STAT1* GOF mutations (*gray columns*) or healthy donors (*HD*; *black columns*).

P3, P4, P5, P7, and P8; *open circles*) and 6 healthy control subjects (*triangles*) after IFN- $\alpha$  stimulation (40,000 U/mL) for 30 minutes. Cells were stained with anti-pY701 STAT1 and analyzed by means of flow cytometry after gating CD3<sup>+</sup> cells. *Asterisks* indicate a significant difference in p-STAT1 levels between patients with *STAT1* GOF mutations and healthy subjects ( $P < .05$ ). Data are presented as means  $\pm$  interquartile ranges. **E**, T cells from 7 patients with *STAT1* GOF mutations (P1, P2, P3, P4, P5, P7, and P8) and healthy control subjects were stained with anti-STAT1 and analyzed by means of flow cytometry after gating CD3<sup>+</sup> cells. The extent of STAT1 intensity is expressed as MFI. Mann-Whitney *U* test statistical analysis shows a significant difference in the extent of STAT1 expression between patients with *STAT1* GOF mutations (*open circles*) and control subjects (*triangles*;  $P < .05$ ). Data are presented as means  $\pm$  interquartile ranges. **F**, Western blot analysis of STAT1 expression was carried out on IL-2-activated NK cells derived from a healthy control subject (*HD*) and patient P7 after IL-2 deprivation for 16 hours.