

alprazolam in a large group of patients with drug-resistant CU is necessary.

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## Signal transducer and activator of transcription 5 tyrosine phosphorylation for the diagnosis and monitoring of patients with severe combined immunodeficiency

To the Editor:

T<sup>+</sup>B<sup>+</sup> natural killer (NK)<sup>+</sup> severe combined immunodeficiency (SCID) is frequently caused by defects in the common  $\gamma$  chain ( $\gamma$ c) or the tyrosine kinase Janus kinase (JAK) 3. However, not all  $\gamma$ c or JAK3 SCIDs present with this typical phenotype because some might have low numbers of T cells, NK cells, or both. Other forms of SCID, such as IL-7 receptor alpha (IL-7R $\alpha$ ) SCID, might present in a similar manner.<sup>1</sup> A rapid screen to distinguish among these patients is essential to direct genetic analysis and initiate appropriate therapy.<sup>2</sup>

Signal transducer and activator of transcription (STAT) 5 is a member of the STAT family of transcription factors. STATs are downstream of a number of cytokine and growth factor receptors, including  $\gamma$ c receptors. In healthy individuals IL-2 binds a trimeric receptor consisting of the IL-2  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. This receptor complex then trimerizes, bringing together the tyrosine kinases JAK1 and JAK3. These cross-phosphorylate each other, resulting in their activation and enabling them to phosphorylate STAT5. Once phosphorylated, STAT5 dimerizes, translocates to

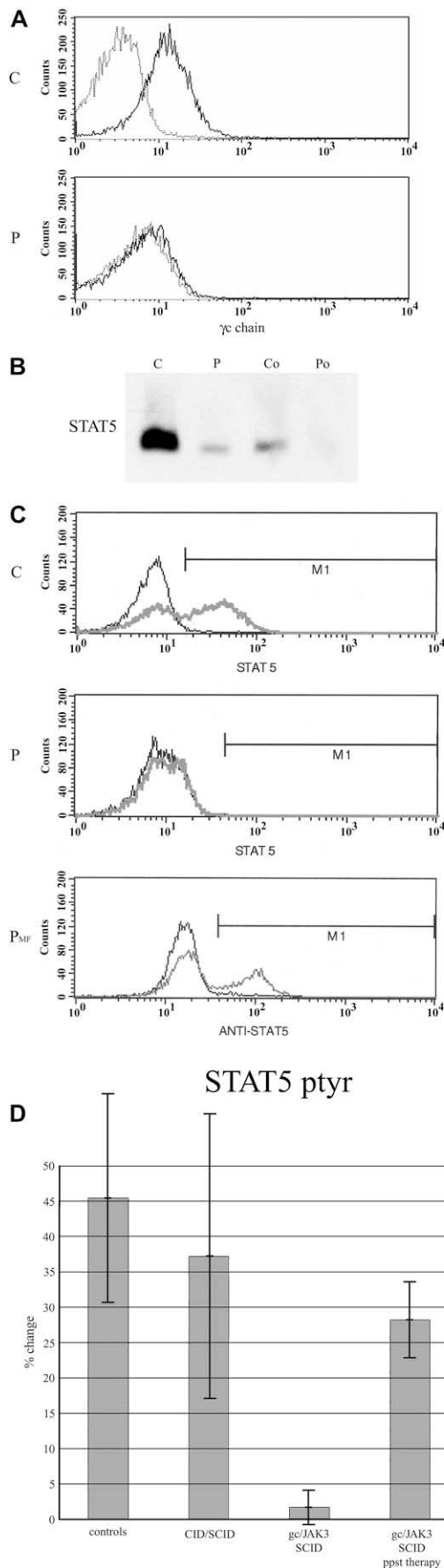
the nucleus where it binds a defined DNA sequence, and activates transcription.<sup>3</sup>

Detection of tyrosine-phosphorylated STAT5 after IL-2 stimulation is therefore an indication of a functional IL-2/JAK3 signal transduction pathway, and abnormalities in STAT5 tyrosine phosphorylation might identify patients with defects in this pathway. Historically, STAT5 activation has been assessed by using the electrophoretic mobility shift assay (EMSA). EMSA specifically detects STAT5 binding to its consensus DNA sequence<sup>3</sup>; however, EMSA is time-consuming and requires large volumes of blood.

A functional STAT5 tyrosine phosphorylation (ptyr) assay was established to detect abnormalities in signaling through the  $\gamma$ c/JAK3 pathway. Ethical approval and consent from parents/guardians was obtained for patients included in the study. Immunophenotyping by means of flow cytometry was performed with standard techniques to determine T, B, and NK cell numbers and  $\gamma$ c expression,<sup>2</sup> and phosphorylated STAT5 was detected by means of EMSA<sup>4</sup> and flow cytometry (FACS). To detect STAT5 ptyr by means of FACS, 100  $\mu$ L of blood was left unstimulated or stimulated with 10<sup>4</sup> units of IL-2, lysed, fixed, washed, and permeabilized before 5  $\mu$ L of antibodies (STAT5 ptyr, CD19 phycoerythrin, and CD4 peridinin-chlorophyll-protein complex; BD Biosciences, San Jose, Calif) was added. Ten thousand lymphocyte events were acquired and analyzed.

STAT5 ptyr by means of FACS was compared with results obtained from staining for  $\gamma$ c by means of flow cytometry, expression of JAK3 by means of immunoblotting, available genetic results, and detection of STAT5 by using EMSA. **Fig 1** is a representative experiment and shows  $\gamma$ c expression by FACS (**Fig 1, A**) in  $\gamma$ c-deficient SCID (patient 8 in **Table I**) in comparison with that seen in a healthy control subject and demonstrates the correlation between a STAT5 EMSA (**Fig 1, B**) and STAT5 ptyr (**Fig 1, C**) by means of flow cytometry. By using STAT5 ptyr analysis, there is a significant shift in fluorescence in the normal blood control compared with the patient sample (**Fig 1, C**). By means of EMSA analysis (**Fig 1, B**), a large band could be detected in the control subjects where phosphorylated STAT5 had bound to the oligonucleotide; the specificity of this band was demonstrated by adding unlabeled oligonucleotide, which competed most of the protein away from the labeled oligonucleotide. This band was faint or undetectable when unlabeled oligonucleotide was added in the patient sample. The faint remaining band is likely to be due to activation of STAT5 by  $\gamma$ c-independent signaling pathways, such as prolactin.

**Table I** summarizes the data from 32 patients with SCID. Twelve  $\gamma$ c and 4 JAK3 genetically defined SCIDs, including several patients who had atypical immunophenotypes (T<sup>low</sup>B<sup>+</sup>NK<sup>+</sup>), had absent or highly abnormal STAT5 tyrosine phosphorylation in their lymphocytes. In 2 patients who had high levels of maternal engraftment (patients 12 and 14), a negative STAT5 ptyr peak was present, as well as a smaller positive STAT5 ptyr peak presumed to be due to maternal cells (**Fig 1, C**). A further patient with a missense mutation allowing partial cytokine binding (patient 5) showed abnormal but not absent STAT5 ptyr. In patient 17 there was absent IL-2-induced STAT5 ptyr, but a confirmed  $\gamma$ c or JAK3 mutation has not been identified, although a number of sequence variants have been identified in his JAK3 gene. A second affected child carried these same variants, whereas an unaffected sibling did not. Forty control samples and 15 samples from other forms of SCID or undefined combined



immunodeficiency, including 2 IL-7R $\alpha$  SCIDs (patients 27 and 28) and 2 adenosine deaminase SCIDs (patients 18 and 19) had B-cell STAT5 tyrosine phosphorylation in response to IL-2. In 2 IL-7R $\alpha$  and 1 undefined combined immunodeficiencies there was decreased STAT5 ptyr, but the STAT5 ptyr levels were significantly higher than for  $\gamma$ c/JAK3 SCIDs. These data demonstrate the specificity of this FACS assay for detecting  $\gamma$ c/JAK3 SCID defects.

Definitive treatment for SCID is either by means of hematopoietic stem cell transplantation (HSCT) or gene therapy (GT). The success of these therapies is usually monitored by engraftment of donor cells; immunophenotyping for the presence of naive, memory, and effector T cells; and proliferation assays to assess cell function.<sup>5,6</sup> Five patients were analyzed before and after HSCT (patients 5 and 6) or GT (patients 7, 8, and 9) for IL-2-induced STAT5 tyrosine phosphorylation. As Table I shows, all pretreatment samples demonstrated absent/abnormal STAT5 ptyr, whereas all post-treatment samples showed restoration of IL-2-induced STAT5 tyrosine phosphorylation. This suggests that this assay should be evaluated further for monitoring immune reconstitution in this group of patients. Because the STAT5 assay requires fewer numbers of cells, it can be performed earlier after therapy than proliferation assays and has a shorter analytic time, and where necessary, individual T-cell populations can be examined.

Analysis of IL-2-induced STAT5 tyrosine phosphorylation is a rapid assay that determines the functionality of the IL-2 signaling pathway. If STAT5 tyrosine phosphorylation is normal, this demonstrates the integrity of the upstream signaling molecules ( $\gamma$ c and JAK3). If there is absent IL-2-induced STAT5 tyrosine phosphorylation, this implies a defect in this pathway, and further genetic investigations can focus on mutation detection in  $\gamma$ c or JAK3. Examination of the restoration of IL-2-induced STAT5 tyrosine phosphorylation might be a specific method to evaluate immune reconstitution in  $\gamma$ c and JAK3 SCIDs after HSCT or GT. This technique should prove useful for assessing the functionality of other STAT5 signaling pathways in the clinical setting.

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**FIG 1.** Tyrosine-phosphorylated STAT5 is detected in a control subject but not in a  $\gamma$ c-deficient patient. **A**,  $\gamma$ c staining. **B**, EMSA showing cells stimulated with IL-2. **C**, STAT5 ptyr staining on unstimulated (black) and IL-2-stimulated (gray) cells. **C**, Control; **P**, patient with  $\gamma$ c SCID (patient 1); **Co/Po**, control/patient plus specific oligonucleotide; **P<sub>MF</sub>**, patient with maternal fetal engraftment (patient 14). **D**, Graph showing the mean and SD percentage change in STAT5 ptyr in control subjects and subjects with combined immunodeficiencies/SCIDs,  $\gamma$ c/JAK3 SCIDs (samples with maternal-fetal engraftment not included), and  $\gamma$ c SCIDs after therapy.

TABLE I. Patient data

| Patient no.            | Diagnosis          | Sex    | abs     |         |          | STAT5    | Change | Genetic mutation                                                                                              |
|------------------------|--------------------|--------|---------|---------|----------|----------|--------|---------------------------------------------------------------------------------------------------------------|
|                        |                    |        | T cells | B cells | NK cells |          |        |                                                                                                               |
| 1                      | γc SCID            | Male   | 0.00    | 0.56    | 0.02     | Absent†  | 0.0%   | c.670C>T (p.Arg224Trp)                                                                                        |
| 2                      | γc SCID            | Male   | 0.02    | 1.65    | 0.09     | Absent   | 0.6%   | c.590G>A (Trp197X)                                                                                            |
| 3                      | γc SCID            | Male   | 0.31    | 3.08    | 0.00     | Absent   | 2.0%   | c.317T>C (Leu106Pro)                                                                                          |
| 4                      | γc SCID            | Male   | 0.07    | 0.61    | 0.00     | Absent   | 1.3%   | c.322T>C (Ser108Pro)                                                                                          |
| 5                      | γc SCID            | Male   | 0.92    | 1.06    | 0.02     | Abnormal | 5.3%   | c.272A>g(Tyr91Cys): a missense mutation with abnormal (not absent) α, β, and γ chains                         |
| 6                      | γc SCID            | Male   | 0.05    | 0.21    | 0.00     | Absent   | 3.3%   | c.296G>A (trp90X)                                                                                             |
| 7                      | γc SCID            | Male   | 0.00    | 1.16    | 0.00     | Absent   | 0.8%   | γc Gene deletion                                                                                              |
| 8                      | γc SCID            | Male   | 0.00    | 4.08    | 0.01     | Absent†  | 0.1%   | c.545G>A (Cys182Tyr)                                                                                          |
| 9                      | γc SCID            | Male   | 0.04    | 1.16    | 0.00     | Absent   | 3.4%   | c.3G>A (M1I)                                                                                                  |
| 10                     | γc SCID            | Male   | 4.53    | 1.39    | 0.01     | Absent   | 5.9%   | c.822-823delCAins/dup. 833-845 (Ile274MetfsX24)                                                               |
| 11                     | γc SCID            | Male   | 0.04    | 0.93    | 0.21     | Absent   | 2.6%   | γc Gene deletion                                                                                              |
| 12                     | γc SCID            | Male   | 0.19    | 0.01    | 0.00     | Absent*  | 25.2%  | c.676C>T (p.Arg226Cys)                                                                                        |
| 13                     | JAK3 SCID          | Male   | 0.00    | 1.02    | 0.46     | Absent   | 4.0%   | c.184+22 C>T/wt, c.184+40G>A/wt, c.184+42G>A/wt, c.1701+9 A>G/wt, c.1915-30C>T/wt, c.2978+32T>C/ c.2978+32T>C |
| 14                     | JAK3 SCID          | Male   | 1.82    | 1.38    | 0.04     | Absent*  | 32.8%  | JAK3 gene deletion                                                                                            |
| 15                     | JAK3 SCID          | Male   | 0.26    | 1.17    | 0.00     | Absent   | 0.2%   | c.421-10G>A, c.1701+9A>G, c.2680+3G>C                                                                         |
| 16                     | JAK3 SCID          | Male   | 0.02    | 1.19    | 0.00     | Absent   | 2.3%   | c.2350+1G>T/c.2350+1G>T, c.2350+6C>T/ c.2350+6C>T                                                             |
| 17                     | JAK3 SCID          | Male   | 0.00    | 0.14    | 0.01     | Absent   | -3.6%  | JAK3 sequence variants: IVS13-30c>t/wt, c.2625C>T (L875L)/wt, IVS 20+32T>C/wt (no mutation found in γc)       |
| Posttreatment analysis |                    |        |         |         |          |          |        |                                                                                                               |
| 5                      | γc SCID after HSCT | Male   | 1.26    | 0.65    | 1.44     | Normal   | 30.7%  |                                                                                                               |
| 6                      | γc SCID after HSCT | Male   | 1.01    | 0.32    | 0.09     | Normal   | 31.6%  |                                                                                                               |
| 7                      | γc SCID after GT   | Male   | 4.71    | 1.53    | 0.04     | Normal   | 33.5%  |                                                                                                               |
| 8                      | γc SCID after GT   | Male   | 3.12    | 1.98    | 0.02     | Normal   | 20.5%  |                                                                                                               |
| 9                      | γc SCID after GT   | Male   | 1.94    | 0.98    | 0.00     | Normal   | 25.3%  |                                                                                                               |
| Control samples        |                    |        |         |         |          |          |        |                                                                                                               |
| 18                     | ADA SCID           | Female | 0.12    | 0.2     | 0.05     | Normal   | 29.1%  |                                                                                                               |
| 19                     | ADA SCID           | Female | 0.14    | 0.32    | 0.36     | Normal   | 29.8%  |                                                                                                               |
| 20                     | Undefined CID      | Male   | 0.58    | 0.63    | 0.20     | Normal   | 11.7%  | No mutation found in γc (subsequently given a diagnosis of ALL)                                               |
| 21                     | Undefined CID      | Female | 2.78    | 0.23    | 0.10     | Normal   | 76.0%  | Polymorphisms in IL7RA                                                                                        |
| 22                     | Undefined CID      | Female | 0.23    | 0.00    | 0.06     | Normal   | 60.2%  | No RAG1 or RAG2 mutation                                                                                      |
| 23                     | Undefined CID      | Female | 1.12    | 0.24    | 0.04     | Normal   | 35.4%  | No RAG1 or RAG2 mutation                                                                                      |
| 24                     | Undefined CID      | Male   | 0.35    | 0.01    | 0.04     | Normal   | 48.2%  |                                                                                                               |
| 25                     | Undefined CID      | Male   | 2.27    | 0.16    | 0.33     | Normal   | 24.5%  | No mutation found in RAG1                                                                                     |
| 26                     | Undefined CID      | Male   | 0.19    | 0.05    | 0.05     | Normal   | 33.6%  |                                                                                                               |
| 27                     | IL-7Rα SCID        | Male   | 0.00    | 2.22    | 0.08     | Normal   | 8.2%   | c.221+2T>G, c.221+2T>G in IL7RA                                                                               |
| 28                     | IL-7Rα SCID        | Male   | NA      | NA      | NA       | Normal   | 11.7%  | Confirmed IL7RA mutation                                                                                      |
| 29                     | MHC II SCID        | Female | 2.04    | 1.35    | 0.02     | Normal   | 36.3%  |                                                                                                               |
| 30                     | RAG SCID           | Male   | 0.84    | 0.00    | 0.07     | Normal   | 77.7%  | c.1088G>A (p.Cys363Tyr)/C.1186C>T (p.Arg396Cys) in RAG1, no mutation found in RAG2                            |

(Continued)

TABLE I. (Continued)

| Patient no. | Diagnosis                   | Sex    | abs     |         |          | STAT5  | Change | Genetic mutation                                                                                                          |
|-------------|-----------------------------|--------|---------|---------|----------|--------|--------|---------------------------------------------------------------------------------------------------------------------------|
|             |                             |        | T cells | B cells | NK cells |        |        |                                                                                                                           |
| 31          | STAT5 SCID                  | Female | 1.64    | 1.09    | 0.18     | Normal | 19.2%  | Homozygous point mutation in exon 13 of <i>STAT5</i> that results in expressed protein that binds DNA in response to IL-2 |
| 32          | ZAP 70                      | Male   | 0.52    | 1.66    | 0.47     | Normal | 31.4%  |                                                                                                                           |
| Control     | Mean of 40 control subjects |        |         |         |          |        | 45.6%  |                                                                                                                           |

abs, Absolute counts in  $10^9$  cells/L; *HSCT*, hematopoietic stem cell transplantation; *GT*, gene therapy; *ADA*, adenosine deaminase; *CID*, combined immunodeficiency (abnormal T/B-cell numbers or function with abnormal humoral function); *ALL*, acute lymphoblastic leukemia; *RAG*, recombina-activating gene; *ZAP*, ZAP-70 kinase deficiency.

\*Small second peak detected by means of FACS because of confirmed maternal engraftment.

†Absent IL-2-induced STAT5, as measured by means of EMSA.

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