

ORIGINAL ARTICLE

Validation of flow cytometric phospho-STAT5 as a diagnostic tool for juvenile myelomonocytic leukemia

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To diagnose juvenile myelomonocytic leukemia (JMML) is sometimes challenging, because around 10% of patients lack molecular abnormalities affecting Ras-MAPK (mitogen-activated protein kinase) pathway and other diseases such as cytomegalovirus infection can mimic clinical signs of JMML. In order to validate a phospho-specific flow cytometry assay assessing phospho-signal transducer and activator of transcription factor 5 (p-STAT5) as a new diagnostic tool for JMML, we examined 22 samples from children with JMML and 47 controls. CD33 + /CD34 + cells from 22 patients with JMML showed hyperphosphorylation of STAT5 induced by sub-saturating doses of granulocyte-macrophage colony-stimulating factor (GM-CSF). Using a training set of samples (11 JMML and 23 controls), we identified a threshold for p-STAT5-positive after stimulation with 0.1 ng/ml GM-CSF (17.17%) that discriminates JMML from controls. This threshold was validated in an independent series (11 JMML, 24 controls and 7 cases with diseases other than JMML) where we demonstrated that patients with JMML could be distinguished from other subjects with a sensitivity of 91% (confidence interval (CI) 59–100%) and a specificity of 87% (CI 70–96%). Positive and negative predictive values were 71% (CI 42–92%) and 96% (CI 82–100%), respectively. In conclusion, flow cytometric p-STAT5 profiling is a reliable diagnostic tool for identifying patients with JMML and can contribute to consistency of current diagnostic criteria.

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INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is an aggressive myeloproliferative neoplasm of childhood characterized by uncontrolled proliferation of monocytic and granulocytic cells.^{1–2} Hematopoietic precursor cells of JMML often show *in vitro* hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).³ GM-CSF binds to the alpha and beta subunits of its cell surface receptor, triggering two distinct signaling pathways: the Ras-MAPK (mitogen-activated protein kinase) pathway and the JAK-STAT (Janus-activated kinase–signal transducer and activator of transcription factor) pathway. In patients with JMML, Ras-MAPK signaling is constitutively activated by a spectrum of usually mutually exclusive mutations affecting genes, such as *NRAS*, *KRAS*,^{4,5} *NF1*,⁶ *PTPN11*⁷ and *CBL*.^{8,9}

Current diagnostic criteria for JMML are based on clinical features and laboratory findings.¹⁰ However, it is sometimes challenging to confirm the diagnosis in children who lack any of the known mutations, because clinical and laboratory findings of JMML can be mimicked by other diseases, such as viral infections.¹¹ In fact, GM-CSF hypersensitivity has been reported to be also induced by human herpes virus-6¹² and cytomegalovirus (CMV)¹³ infections. Moreover, GM-CSF hypersensitivity assay requires monocyte depletion and takes up to several weeks of culture.³

Recent advances in flow cytometry have allowed simultaneous analysis of cell phenotype and aberrant cell signaling.¹⁴

Taking advantage of phospho-specific flow cytometry, we and the others have demonstrated that JMML cells exhibit an aberrant response of phospho-STAT5 (p-STAT5) to sub-saturating doses of GM-CSF.^{15,16} Based on these results, p-STAT5 profiling is expected to contribute to a rapid and accurate diagnosis of JMML. In this study, we analyzed 83 specimens with the aim of validating the ability of a phospho-flow assay specific for p-STAT5 in the perspective of utilizing it in the diagnostic work-up of JMML.

MATERIALS AND METHODS

Clinical samples

Clinical samples were obtained from 22 patients at the time of diagnosis of JMML and from 47 children without hematological malignancies as controls. Three patients with JMML (JMML Nos. 1, 2, 3) have been reported previously.¹⁶ JMML samples were either bone marrow (BM; $n = 17$; 10 fresh and 7 frozen) or peripheral blood (PB) cells ($n = 5$; 4 fresh and 1 frozen). When paired BM and PB samples taken at the same time were available (6 patients, Supplementary Table S1), we considered only the value obtained from BM for the analysis. Fresh samples were processed within 48 h from collection, while frozen samples were processed and cryopreserved within 24 h from collection. The diagnosis of JMML was based on the criteria proposed by the JMML Working Group during the second International JMML Symposium.¹⁰ Spontaneous growth of colony-forming units-granulocyte/macrophage (CFU-GM) in the absence of exogenous growth factors was also assessed according to published methods.^{17,18} Clinical and

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laboratory characteristics of JMML patients are shown in Table 1. All control samples were BM cells (26 fresh and 21 frozen) collected from the following subjects: healthy sibling donors of patients given BM transplantation, non-leukemic children presenting with suspected symptoms of leukemia, such as bleeding diathesis or arthralgia (Supplementary Table S1). We also studied 14 BM samples (11 fresh and 3 frozen) with suspected diagnosis of JMML. These subjects were eventually diagnosed with diseases other than JMML, such as CMV infection, secondary chronic myelomonocytic leukemia and osteopetrosis (Supplementary Table S1).

Table 1. Clinical and laboratory characteristics of JMML patients (n = 22)	
Age at diagnosis (months), median (range)	13 (2–109)
<i>Gender</i>	
Male	16
Female	6
WBC ($10^9/l$), median (range)	31.3 (5.4–226.6)
Monocytes ($10^9/l$), median (range)	4.38 (1.15–53.2)
HbF (%), median (range)	16.5 (0.6–69)
<i>Karyotype</i>	
Normal	13
– 7	6
Both – 7 and + 8	1
Not determined	2
<i>Mutations</i>	
NRAS	5
KRAS	2
PTPN11	10
NF-1 phenotype	2
No mutations	3
<i>Spontaneous growth assay</i>	
Positive	18/22
ND	4/22

Abbreviations: HbF, fetal hemoglobin; JMML, juvenile myelomonocytic leukemia; ND, not determined; NF-1, neurofibromatosis type 1; WBC, white blood cells.

All samples included in this study were used after obtaining written informed consent from parents or legal guardians of each patient in accordance with the Declaration of Helsinki. This study was approved by the local institutional review board.

Cell preparation

Samples were prepared using a density-gradient separation of BM or PB cells (Ficoll-Paque; GE Healthcare, Milan, Italy). Frozen samples were cryopreserved in 90% fetal bovine serum (Lonza Walkersville Inc., Walkersville, MD, USA) and 10% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA) in liquid nitrogen. They were thawed quickly at 37 °C with RPMI 1640 medium (EuroClone spa, Milan, Italy) containing 50% fetal bovine serum and then washed twice. Cell viability was determined in both fresh and thawed samples by trypan blue (Sigma-Aldrich) dye exclusion assay and/or with aqua fluorescent reactive dye (LIVE/DEAD) Fixable Dead Cell Stain Kit (Molecular Probes, Eugene, OR, USA).

Cytokine stimulation and phospho-specific flow cytometry

Freshly isolated or thawed mononuclear cells were starved in serum-free medium (X-VIVO; Lonza, Walkersville, MD, USA) at a concentration of 1–2 million per ml and rested at 37 °C for 1 h, and then stained with anti-CD14 APC-H7 (clone M ϕ P9, BD Biosciences, San Jose, CA, USA) in order to avoid non-specific staining after fixation and permeabilization. Cells were stimulated with escalating doses (from 0.01 to 10 ng/ml) of GM-CSF (ImmunoTools, Friesoythe, Germany) for 15 min at 37 °C to allow signal transduction. Stimulations were performed in duplicate. Cells were then fixed with 1.5% paraformaldehyde (Sigma-Aldrich) and permeabilized with 90% ice-cold methanol (Carlo Erba Reagents, Val de Reuil, France). Samples were incubated with anti-phospho-STAT5 (p-STAT5) Alexa 488 (Y694, clone 47, BD Biosciences) or isotype IgG₁k Alexa 488 (clone MOPC-21, BD Biosciences), anti-CD33 PE (clone P67.6, BD Biosciences), anti-CD34 APC (clone 8G12, BD Biosciences), anti-CD45 PerCP (clone 2D1, BD Biosciences) and anti-CD38 PE-Cy7 (clone HB7, BD Biosciences) antibodies. All the surface antibodies were previously tested for their resistance to the fixation and permeabilization treatment. Samples were acquired on a FACSAria flow cytometer (BD Biosciences) equipped with 488-, 633-, and 405-nm lasers. Data were collected (at least 200 000 events) and analyzed using FACSDiva software (BD Biosciences). An example of flow cytometric gating strategy adopted in this study is depicted in Figure 1.

Statistical analysis

Response to stimulation at each GM-CSF dose was calculated as proportion (%) of p-STAT5 expressing cells within the CD33⁺/CD34⁺ subset and

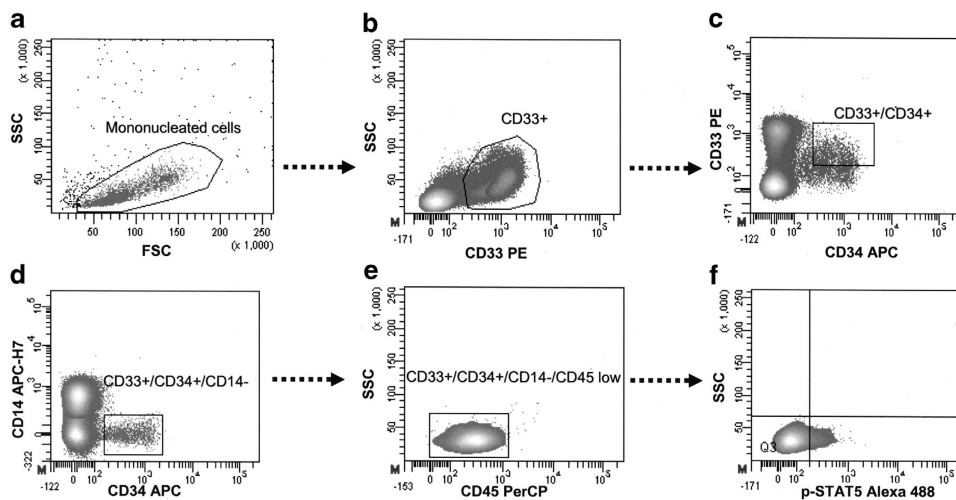


Figure 1. Flow cytometric gating strategy adopted to identify CD33⁺/CD34⁺ precursor cells. A representative JMML patient is shown. Mononuclear cells were initially gated to exclude debris and residual granulocytes by physical parameters (a); all myeloid cells were selected by their reactivity to anti-CD33 antibody (b); myeloid precursors were then identified as CD33⁺/CD34⁺ double positive cells (c). CD33⁺/CD34⁺ cells were further checked for their negativity to anti-CD14 antibody (d) and low expression of CD45 (e), as features of myeloid precursor cells. p-STAT5 response was then measured on these selected cells by dual SSC/STAT5 cytogram (f). In panel (e), only CD33⁺/CD34⁺/CD14⁻ gated cells are shown. In panel (f), only CD33⁺/CD34⁺/CD14⁻/CD45^{low} gated cells are shown.

then quantified by scaling the maximum percentage of p-STAT5+ cells at 100 and the unstimulated p-STAT5+ cells to 0.

The statistical analyses were performed using the R software. The comparisons between the two independent groups were performed using the Wilcoxon's test. Multiplicity corrections were adopted to control false positives using Holm's method. The adjusted *P*-values were deemed to be significant if below the alpha level 0.05.

In order to develop an algorithm for discriminating JMML patients using p-STAT5 values, we used two independent series of patients (training and validation sets; Supplementary Table S1). JMML and non-JMML samples were randomly assigned to the training and validation sets in a balanced way (50% of JMML samples to the training set and the other 50% of JMML samples to the validation set; the same splitting was applied to the non-JMML samples). Using the training set, JMML and non-JMML samples were compared at each dose of GM-CSF in order to identify the dose with the lowest significant adjusted *P*-value. At such dose, a threshold was determined as the mean between the lowest p-STAT5 value of the JMML samples and the highest of the non-JMML samples. In the validation set, we declared the subjects with p-STAT5 value over this threshold as 'JMML' and the subjects with p-STAT5 values below this threshold as 'non-JMML'. The performance of this algorithm was evaluated for sensitivity, specificity, positive and negative predictive values. Confidence intervals (CIs) for these quantities were determined according to the method described by Simel *et al.*¹⁹

RESULTS

Training set analysis

To assess the best threshold for distinguishing JMML from control specimens based on GM-CSF-induced p-STAT5, we analyzed a training set consisting of 11 JMML (8 BM; 3 PB) and 23 controls samples (all BM). Age means (in months) were 28 (range 2–109) and 65 (range 5–187), respectively ($P > 0.05$). As shown in Figure 2, we identified 0.1 ng/ml as the best dosage of GM-CSF to distinguish JMML from control samples ($P < 0.01$) by comparing STAT5-phosphorylated cells at each dose of stimulation. Flow cytometric analysis of p-STAT5 response in two representative cases (1 JMML and 1 control) is shown in Figure 3. In this condition, the best threshold of p-STAT5-positive cells to discriminate between JMML patients and controls was calculated to be 17.17% (the arithmetic mean of the lowest p-STAT5 value obtained for the JMML samples and the highest p-STAT5 value obtained for controls).

Validation set analysis

To validate the p-STAT5 threshold found in the training set, we tested an independent series comprising 11 JMML (9 BM; 2 PB),

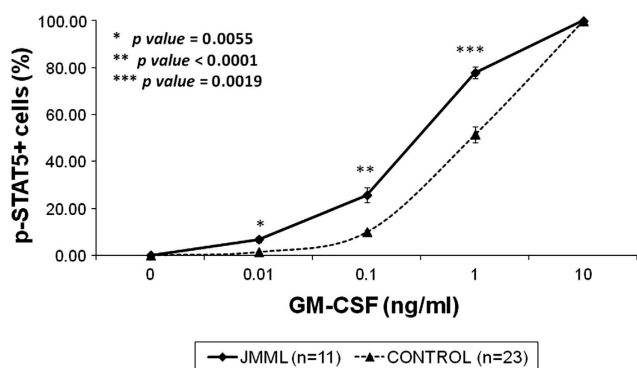


Figure 2. Training set samples assessed the best threshold. The p-STAT5 responses were measured at each GM-CSF concentration in the training set of samples (11 JMMLs and 23 controls). The best dose to distinguish JMML from non-JMML samples was identified at 0.1 ng/ml of GM-CSF ($P < 0.0001$). p-STAT5-positive cells (%) were quantified by scaling the maximum % of p-STAT5+ cells at 100 and the unstimulated p-STAT5+ cells to 0.

24 controls (all BM) and 7 subjects with diseases other than JMML (6 BM; 1 PB). Age means were 20 (range 2–76), 79 (range 8–188), and 26 (range 3–119) months, respectively ($P < 0.05$ for JMML vs controls and $P > 0.05$ for JMML vs diseases other than JMML).

From this series, we found that JMML could be distinguished from patients with other diseases and from controls with a sensitivity of 91% (CI 59–100%) and a specificity of 87% (CI 70–96%). Positive and negative predictive values were 71% (CI 42–92%) and 96% (CI 82–100%), respectively (Figure 4).

Robustness of p-STAT5 profiling according to the different type of materials

In order to assess whether the performances of our p-STAT5-based algorithm could be influenced by the different type of materials (BM or PB), we dissected the samples (both validation and training set) accordingly. As shown in Figure 5, using PB instead of BM did not influence test sensitivity and specificity. In fact, the percentage of p-STAT5-positive cells remained above the diagnostic threshold of 17.17% in all JMML PB samples tested ($n = 5$). Next, we assessed whether GM-CSF-induced p-STAT5 response was affected by using freshly isolated or thawed mononuclear cells, because frozen cells will be used for flow cytometric testing when fresh cells are not available. We analyzed separately JMML fresh samples ($n = 13$) and JMML thawed samples ($n = 9$), and we did not observe any significant difference in p-STAT5 response: mean 29.30% (range 4.83–60.39) vs 29.04% (range 17.20–42.64), respectively ($P = 0.968$).

p-STAT5 profiling in CD33+/CD34+ and CD33+/CD34- cells

We evaluated p-STAT5 responses in the subset of CD33+/CD34+ cells. Although this subset represents a rare cell population, its percentage among total mononuclear cells was always $\geq 0.3\%$, thus allowing flow cytometric analysis in all studied samples. The median percentage of CD33+/CD34+ cells in samples from JMML ($n = 22$), healthy controls ($n = 47$) and subjects with other diseases ($n = 14$) were 2.55% (range 0.50–21.10%), 1.1% (range 0.30–3.70%) and 1.0% (range 0.36–9.20%), respectively. The median number of events (that is, CD33+/CD34+ cells) acquired in the flow cytometer from these three subgroups were 5313 (range 389–20172), 2098 (range 523–10056) and 3075 (range 1358–5819), respectively.

In agreement with our previous results,¹⁶ low-dose GM-CSF-induced hyperphosphorylation of STAT5 in CD33+/CD34+ precursor cells was confirmed in the overall JMML series ($n = 22$) analyzed in this study as compared with controls ($n = 47$) (Supplementary Figure S1A). We also analyzed p-STAT5 response in CD33+/CD34-/CD14+/CD38low population that represents the more mature monocytic cells (Supplementary Figure S2). The median percentage of this subset in samples from JMML ($n = 18$) and controls ($n = 34$) were 5.15% (range 1.00–19.30%) and 2.7% (range 1.00–12.70%), respectively. Although Kotecha *et al.*¹⁵ reported that a higher p-STAT5 response was observed in CD33+/CD34-/CD14+/CD38low cells from JMML patients as compared with controls, we did not find significant differences, because responses to low doses of GM-CSF in mature monocytic cells from normal controls were as vigorous as those from JMML subjects (Supplementary Figure S1B).

Comparison of cytometric STAT5 hyperphosphorylation with spontaneous growth assay

Spontaneous growth of CFU-GM in the absence of exogenous growth factors was compared with GM-CSF-induced p-STAT5 results in 18 out of 22 JMML patients. In 16 out of 18 samples (88.8%), we obtained a concordant result (that is, positive spontaneous growth and p-STAT5 $\geq 17.17\%$). In two samples (JMML Nos. 5 and 18), p-STAT5 was under the threshold, whereas spontaneous CFU-GM growth was present. However, of these two

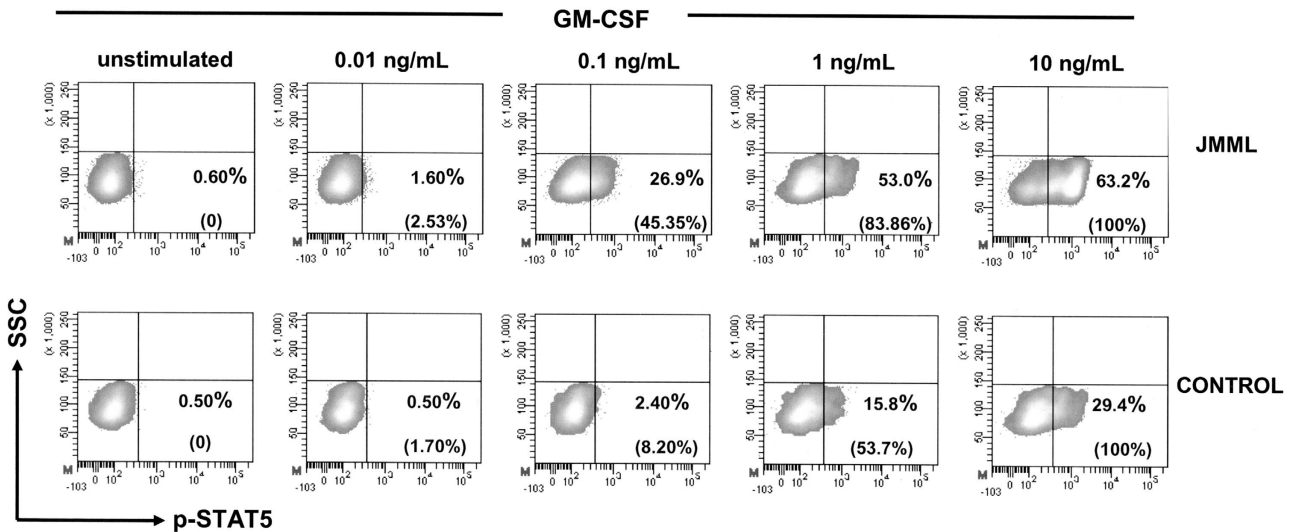


Figure 3. Representative flow cytometric contour plots of p-STAT5 response in CD33⁺/CD34⁺ cells. Dual SSC/STAT5 cytograms from a JMML (upper panels) and a control (lower panels) are shown. Contour plots are referred to CD33⁺/CD34⁺ cells identified by gating strategy described in Figure 1. For each dose of GM-CSF, the raw percentage of responding p-STAT5-positive cells is shown. Response to stimulation at each GM-CSF dose was then quantified by scaling the maximum percentage of p-STAT5⁺ cells at 100 and the unstimulated p-STAT5⁺ cells to 0. According to this criteria, calculated p-STAT5 responses are indicated in parenthesis for each stimulation dose.

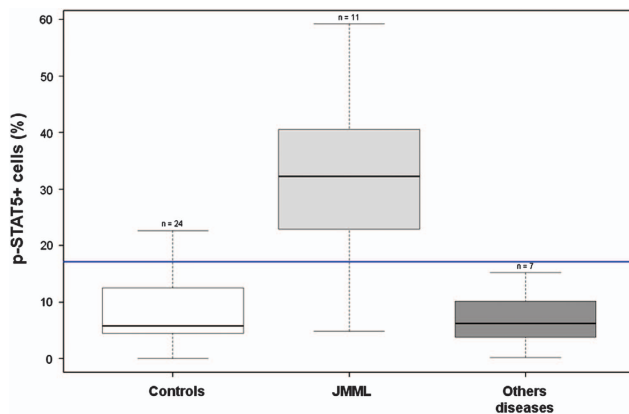


Figure 4. Comparison of p-STAT5-positive cells (%) induced by 0.1 ng/ml of GM-CSF in the validation series comprising 11 JMML samples (central box plot), 24 controls (left box plot) and 7 samples from patients with other diseases mimicking JMML at presentation (right box plot). The discriminating threshold (17.17% as assessed in the training set) is indicated. The bold line inside each box plot indicates the median level, while the upper and lower lines indicate the maximum and minimum observed values, respectively. There are no outliers.

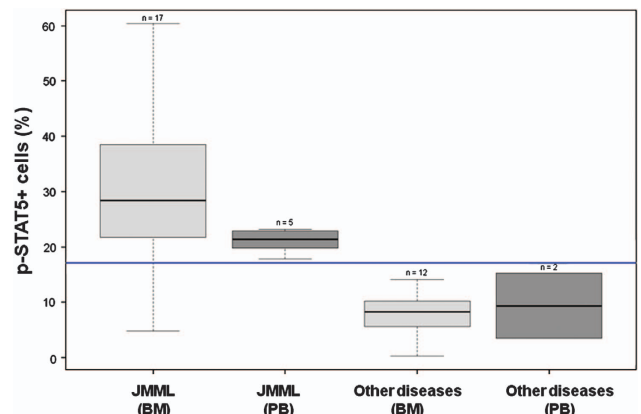


Figure 5. Comparison of p-STAT5-positive cells (%) induced by 0.1 ng/ml of GM-CSF according to the different cell source. In all, 17 JMML BM samples (left box plot), 5 JMML PB samples (middle left box plot), 12 BM samples and 2 PB samples (middle right and right box plot, respectively) from patients with other diseases mimicking JMML are shown. The discriminating threshold (17.17% as assessed in the training set) is indicated. The bold line inside each box plot indicates the median level, while the upper and lower lines indicate the maximum and minimum observed values, respectively. There are no outliers.

discordant cases, one (JMML No. 5) had CFU-GM spontaneous growth only in cells from PB but not in BM. Of the 14 samples from patients with a final diagnosis of diseases other than JMML, 8 samples were tested for spontaneous CFU-GM growth and all these samples showed concordant negative results.

DISCUSSION

Alterations in cell signaling can disrupt maturation, proliferation and survival of cells and thus have important roles in the pathogenesis of various cancers.^{20,21} Multi-parameter flow cytometry is a powerful tool for assaying intracellular levels of phosphoproteins and can be used to simultaneously determine immunophenotype and signaling activity in individual cells.¹⁴

Using this technique, we and the others have previously demonstrated in preliminary studies that JMML cells show aberrant responses of p-STAT5 to low doses of GM-CSF.^{15,16} In the present study, we tested the reliability and utility of p-STAT5 profiling as a new and rapid diagnostic tool for JMML. Using a simple algorithm, we identified a threshold p-STAT5 value after low-dosage stimuli with GM-CSF for discriminating JMML from control specimens. We then validated this threshold in an independent series. Importantly, however, each laboratory should determine and validate its own threshold value according to an appropriated validation procedure.

One of the laboratory hallmarks of JMML is the hypersensitivity of myeloid progenitor cells to GM-CSF in colony-forming assay.³

However, as this assay could test positive in certain viral infections, it is considered a sensitive but non-specific assay.¹¹ Moreover, differentiation between CMV-related disease and JMML in infants excreting CMV is sometimes difficult,^{22–24} because clinical and laboratory findings of CMV infection can overlap with those of JMML.¹¹

It should be noted that the algorithm developed here could also discriminate diseases mimicking JMML, such as CMV infection and other diseases. Moritake *et al.*¹³ also reported a patient with CMV infection resembling JMML, who showed GM-CSF hypersensitivity by *in vitro* colony assay but not by phospho-specific flow cytometry. Conversely, Nishio *et al.*²⁵ argued that p-STAT5 evaluated by phosphoflow might not be suitable to distinguish JMML from a CMV infection. We presume that these discrepancies might result mainly from differences in the examined cell population. In our study, we analyzed CD33+/CD34+ myeloid precursors by an accurate immunological gating strategy, by contrast Nishio *et al.*²⁵ analyzed all nucleated cells based on a broad SSC (side scatter)-based physical gating, thus including monocytes and residual granulocytes.

As JMML is a disease of early childhood, patients with this diagnosis could not be completely age-matched with the subjects of control. However, the differences between the two groups were either limited (validation set) or not significant (training set). Yet, patients with diseases mimicking JMML, but discriminated by p-STAT5 assay, had a mean age closely matching that of JMML patients.

Kotecha *et al.*¹⁵ studied p-STAT5 response in CD33+/CD34-/CD14+/CD38low cells. Our results show that this population (monocytic cells) responded vigorously to low doses of GM-CSF in specimens from both JMML subjects and normal controls (Supplementary Figure S1B), thus discrimination between JMML and controls at low doses of GM-CSF is not as clear as seen in CD33+/CD34+ precursor cells. It has been demonstrated that JMML CD34+/CD38- cells have the capacity to initiate and promote the disease development in a murine model,²⁶ and CD34+ cells have been thought to be involved in the pathogenesis of JMML.^{26–28} Along this line, it seems reasonable to consider that the subset of CD33+/CD34+ cells represents the most accurate one to assess hyper-phosphorylation of STAT5. However, we cannot exclude that mature monocytic progeny of CD33+/CD34+ cells can also maintain p-STAT5 hyper-responsiveness as shown by Kotecha *et al.*¹⁵ by using a different approach.

In acute myeloid leukemia, specific patterns in phosphoprotein responses have been shown to correlate with genetic and clinical phenotype.²⁹ Our analyses of JMML patients, however, did not show any differences in p-STAT5 values according to mutational status. Kotecha *et al.*¹⁵ reported that two patients with clinically aggressive JMML harboring the *KRAS*^{G12D} mutation did not exhibit p-STAT5 hyper-responsiveness and inferred that *KRAS*^{G12D} mutation might lead to differential activation of p-STAT5 as compared with other mutations than those deregulating Ras signaling.¹⁵ We examined five patients with *NRAS* mutations (G12V; *n* = 2, G13R; *n* = 1) and two with *KRAS* mutations (G13D and G12C). We did not find differences in p-STAT5 responses between samples with *NRAS* mutations and those with *KRAS* mutations. In addition, these samples with *NRAS* and *KRAS* mutations did not behave *in vitro* differently from samples harboring other genetic alterations. Although further data will be required to elucidate the correlation between genotypes and phosphoprotein profiles, it seems that different RAS pathway-associated mutations do not impact on JAK-STAT signaling.

Some technical considerations should be taken into account for the correct interpretation of the p-STAT5 data in JMML patients. First, although they fulfilled the proper diagnostic threshold, PB samples responded less strongly than BM. It is well known that PB and BM from patients with JMML behave similarly.³⁰ Although we

could not find a reasonable explanation for the observed difference between PB and BM specimens, we recommend that BM be the cell source preferentially used for investigating p-STAT5 profiling whenever possible. Second, although our cytometric procedure performs robustly, accurate internal methodological standardization is strongly recommended in order to avoid misclassification especially of borderline cases. Finally, the time elapsing from collection to either processing (≤ 48 hours) or freezing (≤ 24 hours) is crucial for proper assessment of p-STAT5 responses (data not shown).

In conclusion, JMML patients show p-STAT5 hyper-responsiveness to low doses of GM-CSF. p-STAT5 response to 0.1 ng/ml of GM-CSF assayed in CD33+/CD34+ cells by phospho-specific flow cytometry can be useful for the diagnosis of children with JMML. We developed and validated an original cytometric procedure based on an accurate gating strategy as well as a simplified calculation algorithm. Upon its further validation in a larger cohort of patients, this assay could represent an adjunctive tool improving sensitivity and specificity of the current diagnostic criteria for JMML, especially for those patients still lacking known genetic alterations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DH, CB and GG performed the research, analyzed the data and wrote the paper. MG, FL, AB and GtK analyzed the data and wrote the paper. SB performed the research and analyzed the data. DL, CM, UR, AB, GB and FL collected samples and clinical data. CB, GB, AB, GtK and GG designed the research study. All authors participated in editing the manuscript and approved the final version.

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