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Single-Cell Profiling Identifies Aberrant STAT5 Activation in Myeloid Malignancies with Specific Clinical and Biologic Correlates

Nikesh Kotecha,[1,2] Nikki J. Flores,[5] Jonathan M. Irish,[1,3] Erin F. Simonds,[1] Debbie S. Sakai,[5] Sophie Archambeault,[5] Ernesto Diaz-Flores,[5] Marc Coram,[4] Kevin M. Shannon,[5,6] Garry P. Nolan,[1,7,*] and Mignon L. Loh[5,6,7,*]

[1]Department of Microbiology and Immunology
[2]Biomedical Informatics Program
[3]Department of Medicine
[4]Division of Biostatistics
Stanford University School of Medicine, Stanford, CA 94305, USA
[5]Department of Pediatrics
[6]UCSF Comprehensive Cancer Center
University of California, San Francisco, San Francisco, CA 94143, USA
[7]These authors contributed equally to this work
*Correspondence: gnolan@stanford.edu (G.P.N.), lohm@peds.ucsf.edu (M.L.L.)
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SUMMARY

Progress in understanding the molecular pathogenesis of human myeloproliferative disorders (MPDs) has led to guidelines incorporating genetic assays with histopathology during diagnosis. Advances in flow cytometry have made it possible to simultaneously measure cell type and signaling abnormalities arising as a consequence of genetic pathologies. Using flow cytometry, we observed a specific evoked STAT5 signaling signature in a subset of samples from patients suspected of having juvenile myelomonocytic leukemia (JMML), an aggressive MPD with a challenging clinical presentation during active disease. This signature was a specific feature involving JAK-STAT signaling, suggesting a critical role of this pathway in the biological mechanism of this disorder and indicating potential targets for future therapies.

INTRODUCTION

Myeloproliferative disorders (MPDs) are clonal malignances characterized by overproduction of immature and mature myeloid cells showing organ infiltration. In particular, juvenile myelomonocytic leukemia (JMML) and chronic myelomonocytic leukemia (CMML) are characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that variably retain the capacity to differentiate (Arico et al., 1997; Onida et al., 2002). Children suspected of having JMML often present with failure to thrive, fever, infection, splenomegaly, and a high white blood cell count with monocytosis. Current diagnostic criteria are imprecise and consist of major and minor requirements that are in large part based on excluding other conditions (Niemeyer et al., 1997). The major requirements include an absolute monocytosis > 1,000/μl, fewer than 20% bone marrow blasts, and absence of the t(9;22) or *BCR-ABL* fusion gene. Patients must also meet two of the minor criteria, including an elevated fetal hemoglobin level for age, circulating myeloid precursors, a total white blood cell count > 10,000/μl, and in vitro hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).

Extensive molecular data implicate genetic lesions that deregulate Ras signaling as key initiating events in JMML, with studies showing that 60% of patients harbor an oncogenic mutation in *PTPN11*, *NRAS*, or *KRAS* while another 15% have clinical

neurofibromatosis type 1 and/or demonstrate loss of the wild-type *NF1* allele in their diseased bone marrow (Emanuel, 2004; Flotho et al., 2007). Patients with the myeloproliferative subtype of CMML exhibit *NRAS*, *KRAS*, and *JAK2* mutations (Levine et al., 2005; Onida et al., 2002). A cellular characteristic of both JMML and CMML is the formation of abnormal numbers of granulocyte-macrophage colony-forming units (CFU-GM) in methylcellulose cultures containing subsaturating concentrations of GM-CSF (Cambier et al., 1997; Emanuel et al., 1991), which has led to suggestions that alterations downstream of the activated GM-CSF receptor collaborate to drive inappropriate cell growth and survival. One important primary signaling event following binding of GM-CSF to the GM-CSF receptor is activation of the JAK-STAT pathway (Paukku and Silvennoinen, 2004). JAK2 transphosphorylates the β common chain of the GM-CSF receptor, which creates docking sites for adaptors and signal relay molecules, resulting in activation of Ras and downstream Ras effectors including ERK and S6 ribosomal protein (see Figure S1 available online) (Irish et al., 2004; Kunz and Ibrahim, 2003; McCubrey et al., 2000; Rane and Reddy, 2002; Shuai and Liu, 2003).

CMML is an adult MPD that is clinically similar to JMML and shares certain genetic features such the frequent presence of *RAS* mutations (Onida et al., 2002). *JAK2* mutations are rare in JMML and only slightly more common in CMML patients (Levine et al., 2005; Steensma et al., 2005; Zecca et al., 2007), whereas *PTPN11* mutations are almost nonexistent in CMML (Loh et al., 2005). Both JMML and CMML can progress to M4 or M5 acute myeloid leukemia (AML), which comprise the myelomonocytic (M4) and monocytic (M5) subtypes (Arico et al., 1997). Furthermore, somatic *NRAS*, *KRAS*, and *PTPN11* mutations occur frequently in the M4 and M5 subtypes of AML (Bacher et al., 2006; Loh et al., 2004).

Currently, it takes up to 3–4 weeks to confirm a suspected diagnosis of JMML with a CFU-GM assay. As early allogeneic hematopoietic stem cell transplant (HSCT) is the only potentially curative therapy for JMML (Locatelli et al., 2005), it is important to quickly and accurately diagnose these patients in order to deliver appropriate therapy in a timely fashion. In addition, monitoring disease burden during treatment is challenging in patients with JMML due to imprecise clinical definitions of response. Current allele-specific PCR methodologies to detect minimal residual disease are only applicable to approximately 60% of patients (Archambeault et al., 2008). Importantly, because JMML and CMML exhibit considerable cellular heterogeneity, it has been difficult to elucidate the biologic features of cells that contribute to the cancer phenotype in vivo and of precursor populations that might carry genetic lesions predisposing cells to an oncogenic fate.

Assays for identifying therapeutic agents and assessing efficacy in these patients based on the biochemical consequences of lesions in the GM-CSF and Ras signaling networks are few. Recent advances in flow cytometry, however, have made it possible to simultaneously measure cell type and aberrant cell signals (Irish et al., 2006) arising as a consequence of these lesions. We used this approach to profile signaling at the single-cell level (Irish et al., 2004; Van Meter et al., 2007), including molecules downstream of the GM-CSF receptor and molecules closely associated with Ras signaling, for the presence of primary JMML

cells with altered signaling behavior that correlate with disease physiology. Our cohort of 52 samples included patients diagnosed with JMML, healthy individuals, infants with other MPDs, and children initially suspected of having JMML who were subsequently diagnosed with other disorders.

RESULTS

A Flow Cytometry-Based Signaling Assay Can Be Used to Measure GM-CSF Hypersensitivity

We used phosphospecific flow cytometry (Irish et al., 2004, 2006) after exposure to increasing concentrations of GM-CSF to interrogate evoked signaling responses in JMML cells. In a first test of GM-CSF-induced phosphorylation of STAT5, we observed the dose-dependent appearance of a population of cells in a JMML bone marrow sample compared to normal healthy bone marrow (Figure 1A). This leukemia was also assessed via the traditional methylcellulose assay and exhibited hypersensitive colony formation (defined as clusters of >50 cells) at increasing concentrations of GM-CSF (Figures 1C and 1D), as described previously (Emanuel et al., 1991).

We then investigated 11 additional JMML samples at diagnosis and compared these leukemias to normal samples (n = 8), other childhood MPDs (these cases included 8 patients with Noonan syndrome/MPD [NS/MPD] or Down's syndrome with transient myeloproliferative disorders [DS/TMD]), and 4 children with an initial clinical suspicion of JMML who were subsequently found to have another diagnosis. We observed an induced phosphorylated STAT5 (p-STAT5) population in the majority of JMML samples that were exposed to low levels of GM-CSF, but not in the other samples interrogated. The data were quantified as a relative percentage of p-STAT5-responsive cells as outlined in the Experimental Procedures (Figure 1B).

p-STAT5 Response to Low Doses of GM-CSF Indicates JMML Status

The combination of CD38 and p-STAT5 best stratified the GM-CSF-hyperresponsive population, which was measurable in both peripheral blood and bone marrow samples. Representative samples from the patient cohort are shown in Figure 2A. The signature was present in both fresh and previously frozen primary samples (details for each sample are provided in Table S1).

p-STAT5 Signaling Cells in JMML Samples Are of Myeloid Origin and Require JAK2 Activity

Immunophenotyping revealed that the p-STAT5-responsive cells were of myeloid origin (CD33+CD14+), CD34−, and CD38lo (Figure S2). The involvement of JAK-specific activation of the p-STAT5 response in these cells was first confirmed by exposing primary samples to a chemical JAK2 inhibitor for 30 min before GM-CSF stimulation at 10 ng/ml for 15 min (Figure S3). Similarly, exposing JMML cells to a 5 μM concentration of the oral JAK2 inhibitor XL019 (Exelixis) inhibited STAT5 and ERK phosphorylation in response to a saturating concentration of GM-CSF, whereas the MEK inhibitor CI-1040 (Pfizer) failed to alter the p-STAT5 response despite inhibiting p-ERK (Figure 2B).
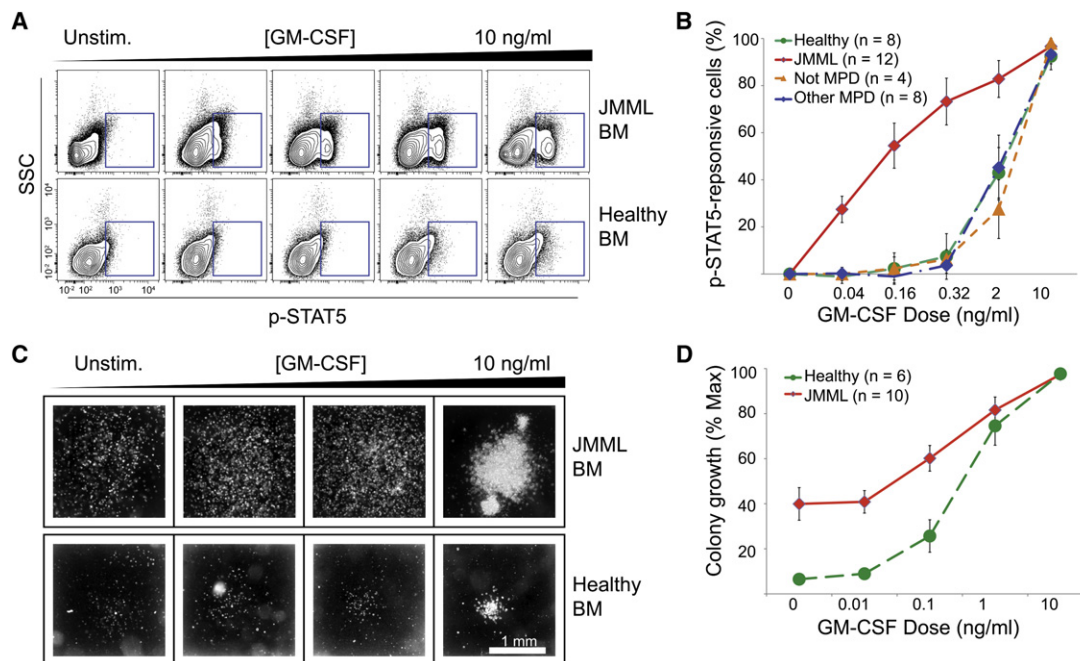
Figure 1. Hyperphosphorylation of STAT5 Is Seen in Primary JMML Cells at Low Concentrations of GM-CSF

The diagnosis of juvenile myelomonocytic leukemia (JMML) is confirmed through an in vitro granulocyte-macrophage colony-forming unit (CFU-GM) assay showing colony formation at low concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF). Colonies form over 2 weeks at various doses of GM-CSF.

(A) Phosphospecific flow cytometry-based assay demonstrating that GM-CSF hypersensitivity can be measured via phosphorylated STAT5 (p-STAT5). Primary cells from JMML and normal bone marrow (BM) were stimulated at varying concentrations of GM-CSF. The JMML samples show a subset of cells responding via p-STAT5 at low concentrations of GM-CSF.

(B) Increase in p-STAT5 response to GM-CSF concentration was quantified as a function of maximal response. A higher percentage of p-STAT5+ cells were present at 0.16 and 0.32 ng/ml GM-CSF concentrations in JMML samples (n = 12) than in healthy samples (n = 8), samples with Noonan syndrome or Down's syndrome-related transient myeloproliferative disorders ("Other MPD," n = 8), or non-MPD samples that were initially suspected to be JMML (n = 4).

(C) Colonies formed in a JMML sample and a normal sample at increasing doses of GM-CSF. Samples shown are the same ones measured using phosphospecific flow cytometry in (A).

(D) Colony formation from JMML patients (n = 10) was quantified as a function of maximum colony growth, plotted over varying concentrations of GM-CSF and compared against growth from healthy samples (n = 6).

Error bars in (B) and (D) represent standard error of the mean (SEM).

p-STAT5 Response Can Be Summarized using the 95th Percentile

To visualize the data set from all patients studied, we calculated ratios of the 95th percentile of p-STAT5 activity to the unstimulated sample and displayed the results in heat map format (see Experimental Procedures). Eleven of twelve JMML samples showed highly sensitive activation of p-STAT5 (at 0.32 ng/ml GM-CSF) as compared to lack of activation in the majority of the control specimens. Diagnostic samples from infants with other MPDs, including those with NS/MPD and DS/TMD, did not demonstrate a p-STAT5 population hyperresponsive to GM-CSF (Figure 3A). In addition, samples obtained from children suspected of having JMML but subsequently found to have other disorders did not demonstrate the induced p-STAT5 population. The absence of the p-STAT5 signature in conditions that are phenotypically similar to JMML highlights the importance of the signaling disruptions that we observe specifically in JMML.

Overall, 11 of 12 JMML diagnostic samples showed a GM-CSF-hypersensitive population, 7 of 8 healthy samples did not show a GM-CSF-hypersensitive population, 8 of 8 NS/MPD or DS/TMD samples did not show a GM-CSF-hypersensitive population, and 4 samples from patients suspected of having JMML but later found not to have a MPD did not show a GM-CSF-hypersensitive population (Figure 3A). Based on this analysis, the p-STAT5 diagnostic assay has a sensitivity of 91% and a specificity of 95%. An unsupervised grouping of these patients was calculated by normalizing the response of p-STAT5 (as measured by the 95th percentile) to GM-CSF concentrations within a patient (Figure 3D). Using this metric, samples with a response > 40 showed a JMML-like evoked signaling pattern. Further information about these samples is given in Table S1. Elevated levels of p-ERK and basal activation of p-S6 were detected in some JMML samples, but these alterations were a less consistent feature of JMML than hypersensitive signaling at the level of p-STAT5 (Figures S4–S7).

p-STAT5 Response to Low Doses of GM-CSF Is Detectable in CMML and M4/M5 AML

We also analyzed evoked p-STAT5 responses in CMML and AML and found that 5 of 5 samples from adults with CMML and 4 of 8 samples from patients with AML showed a hyperresponsive population (Figure 3C; Figures S8 and S9). In AML,
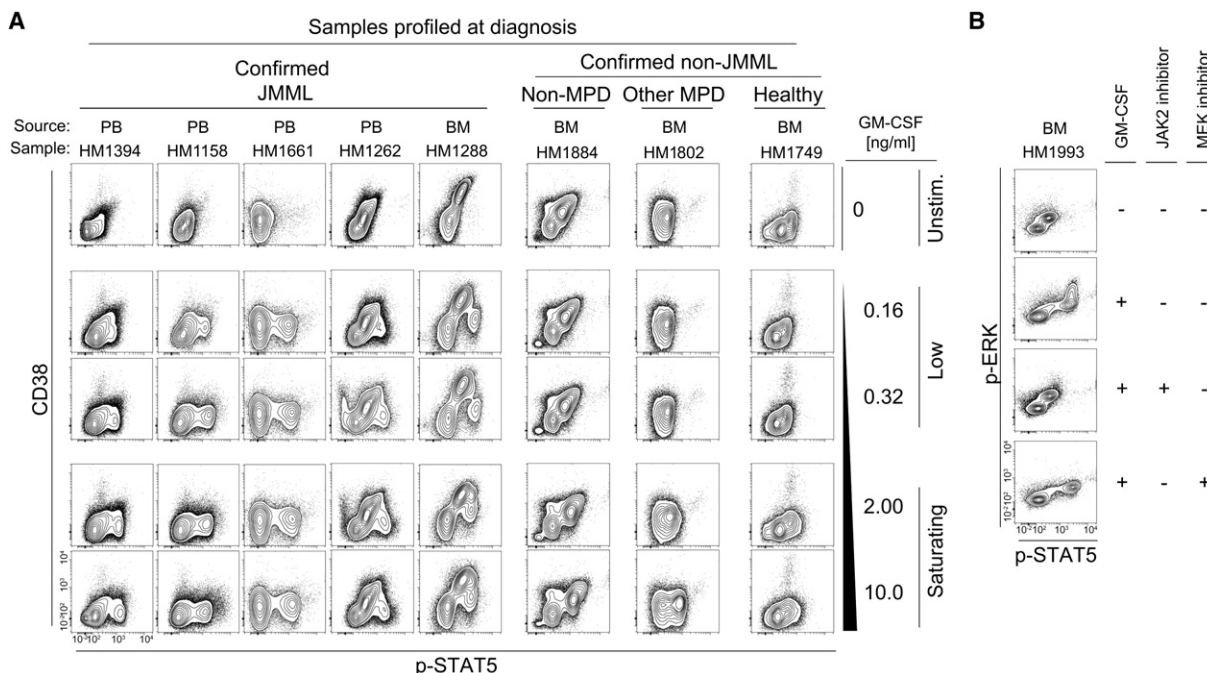
Figure 2. The p-STAT5-Responsive Population Can Be Used to Distinguish JMML from Other Myeloproliferative Disorders of Childhood at Diagnosis
(A) CD38 and p-STAT5 stratify the responsive population present in JMML peripheral blood (PB) and bone marrow (BM) samples at diagnosis.
(B) Exposure to an oral JAK2 inhibitor, XL019, abrogated both the p-STAT5 and p-ERK response while exposure to a MEK inhibitor, CI-1040, inhibited only the p-ERK response.

the presence of an aberrant p-STAT5 response to subtherapeutic concentrations of GM-CSF correlated with the French-American-British M4/M5 morphologic subtype (n = 4) and was not detected in non-M4/M5 AML samples (n = 4). The eight AML samples were assessed for lesions in *PTPN11*, *NRAS*, *KRAS*, and *FLT3*. The genotype data were less predictive of the signaling phenotype than the cell type, as two patients with non-M4/M5 AML who were found to harbor an *FLT3* internal tandem duplication (ITD) and one patient with an *NRAS* mutation did not exhibit the p-STAT5 signature. Further information is given in Tables S1 and S2.

Signaling Response Can Indicate Disease Status
To determine whether the signaling signature was maintained or altered over time, we followed two JMML patients through a series of samples obtained at various time points during therapy. In particular, patient HM1158 achieved clinical remission, underwent matched unrelated HSCT, and remains in remission. The p-STAT5-hyperresponsive population has been absent in this patient's remission samples (Figure 4). In contrast, patient HM1394 responded poorly to chemotherapy, never achieved complete clinical remission, and relapsed shortly after undergoing a first matched related HSCT. Following a second HSCT, this patient progressed to M4 AML. Over time, subsequent samples demonstrated an increasing p-STAT5 response at low concentrations of GM-CSF (Figure 4). To further demonstrate that the p-STAT5 population was of malignant origin, genomic DNA was isolated from the responsive population after sorting on

CD38 and p-STAT5 at 0.32 ng/ml of GM-CSF in a patient harboring a somatic $NRAS^{G12D}$ mutation. These cells contained the *NRAS* mutation, whereas DNA extracted from the patient's T cells did not (data not shown).

DISCUSSION

Based on the presence of Ras pathway mutations in JMML (reviewed in Emanuel, 2004; Flotho et al., 2007), the characteristic GM-CSF hypersensitivity of myeloid progenitors isolated from JMML patients in methylcellulose culture, and compelling data from *Kras*, *Nf1*, and *Ptpn11* mutant mice linking genetic lesions found in JMML to MPD and GM-CSF hypersensitivity (Araki et al., 2004; Braun et al., 2004; Chan et al., 2004; Le et al., 2004; Mohi et al., 2005), we used phosphospecific flow cytometry to assay p-ERK and p-S6 levels in patient samples. The finding that a small proportion of CD33⁺CD14⁺CD38^lo cells exhibited hyperphosphorylation of p-STAT5 in response to subsaturating concentrations of GM-CSF was unexpected. Whereas studies of JMML patient samples and mice lacking either *Gmcsf* or the β common chain of the murine GM-CSF receptor (Birnbaum et al., 2000; Kim et al., 2007) provide strong evidence that an aberrant response to GM-CSF is integral to the pathogenesis of JMML, the role of STAT5 has not been explored.

Our data raise the intriguing possibility that Ras-GTP is upstream of JAK2-STAT5 activation in the aberrant response of JMML cells to GM-CSF. Ras pathway mutations might potentiate JAK-STAT signaling by stabilizing or directly activating the
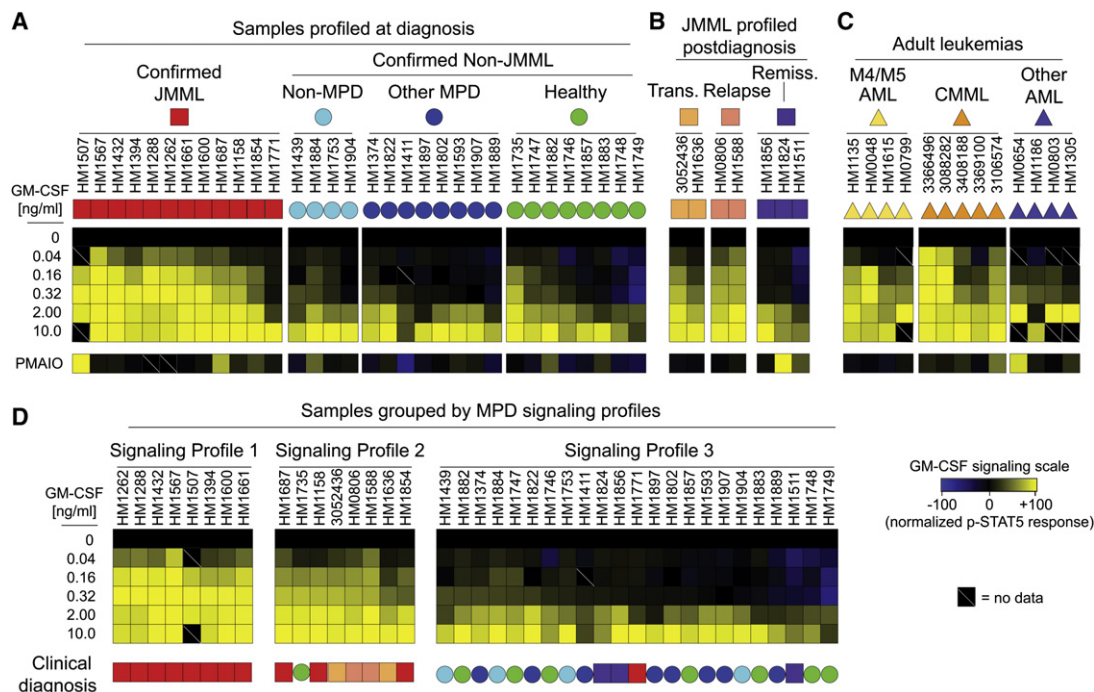
Figure 3. The p-STAT5-Responsive Population Is Indicative of Active JMML

(A) Clinically similar myeloproliferative disorders (MPDs) such as Noonan syndrome/MPD and Down's syndrome/TMD are distinguishable from JMML via phosphoflow cytometry. Eleven of twelve JMML patient samples at diagnosis showed a GM-CSF-hypersensitive population. Seven of eight normal bone marrow samples did not have a GM-CSF-hypersensitive population.

(B) Postdiagnosis samples show a p-STAT5 response in patients who have relapsed or transformed into M4/M5 acute myeloid leukemia (AML). Samples in remission do not show the p-STAT5 response.

(C) The signature was also present in chronic myelomonocytic leukemia (CMML, a similar disease to JMML that is diagnosed in adults) and M4/M5 AML (myeloid leukemia with myelomonocytic and monocytic differentiation).

(D) Samples sorted by normalized p-STAT5 response at 0.32 ng/ml. Samples > 40 showed JMML activity (signaling profiles 1 and 2). Eight of twelve JMML samples at diagnosis had a normalized p-STAT5 response > 95 (signaling profile 1).

Trans., transformation to AML; Remiss., remission; PMAIO, PMA/ionomyocin.

GM-CSF receptor or its associated signaling molecules. The SHP-2 phosphatase, which is deregulated by JMML-associated mutations, is recruited to phosphorylated tyrosine residues on the activated β subunit of the GM-CSF receptor and is essential for efficient STAT5 activation in myeloid cells that are stimulated with interleukin-3 (Yu et al., 2003). Ras localizes to activated receptor complexes, and elevated levels of Ras-GTP might in turn increase the degree and/or duration of JAK2 kinase activity.

We considered the possibility that hyperactivation of Ras/Raf/MEK/ERK signaling in JMML cells might overwhelm a negative regulatory molecule that normally suppresses GM-CSF-induced JAK-STAT signaling. If so, inhibitors of this effector pathway would be expected to interfere with the hypersensitive response of STAT5 to GM-CSF. However, exposing primary JMML cells to CI-1040 failed to alter the p-STAT5 response despite inhibiting p-ERK. As a number of reports have shown that STAT5 is essential for establishing murine MPDs, including *Bcr-Abl*-positive chronic myelogenous leukemia (CML) and CMML (Baker et al., 2007; Cain et al., 2007; Ilaria and Van Etten, 1996; Paukku and Silvennoinen, 2004; Van Etten, 2004), understanding the biochemical mechanism underlying elevated p-STAT5 levels in myeloid malignancies with mutations in Ras signaling genes has therapeutic implications.

We also observed discrepancies between p-STAT5 activation in response to low concentrations of GM-CSF and a hypersensitive pattern of CFU-GM colony growth in three types of patients. This is perhaps not unexpected, as the CFU-GM assay interrogates a population of cultured myeloid progenitors that form colonies after 2 weeks, whereas phosphospecific flow cytometry measures a specific biochemical response of a more mature monocytoid cell population to a burst of GM-CSF. It is also important to recall that a hypersensitive pattern of CFU-GM colony growth in methylcellulose is neither necessary nor sufficient to establish a diagnosis of JMML. With these caveats in mind, the rare patients who showed differences between the two assays raise interesting questions that have implications for diagnosis and disease management.

Infants with NS/MPD are an interesting group. Whereas myeloid progenitors from these patients display hypersensitivity to GM-CSF in CFU-GM assays, we did not detect the p-STAT5 signature in our phosphoflow assay. Importantly, it is now recognized that, like the transient MPD seen in neonates with DS, the MPD that occurs in infants with NS usually resolves without treatment (Bader-Meunier et al., 1997; Kratz et al., 2005). Current management of these patients involves watchful waiting. Germline *PTPN11* mutations encode weaker gain-of-function alleles
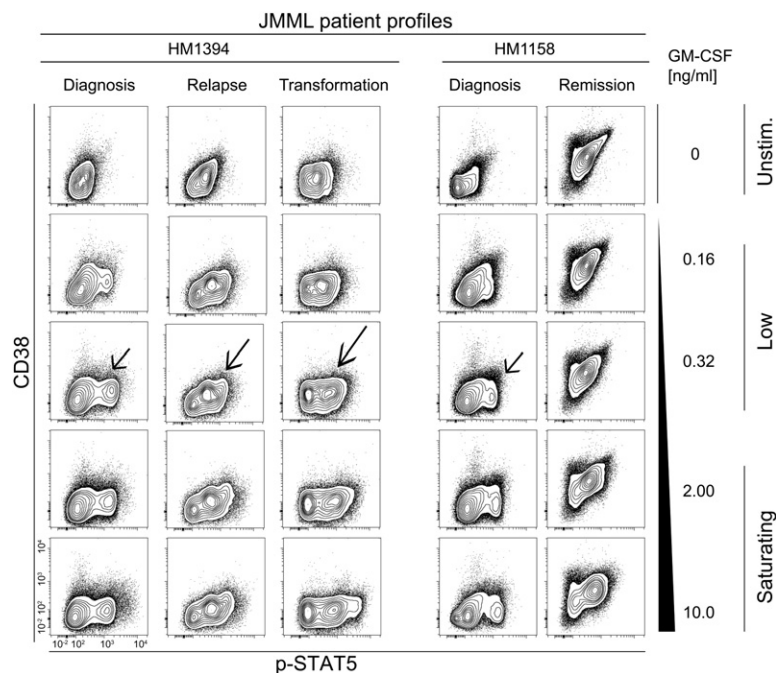
Figure 4. The p-STAT5-Responsive Population Disappears in Remission and Reappears during Relapse
Cells from JMML patients were profiled at diagnosis and again following a change in disease status. Both patients exhibited the characteristic JMML signature of p-STAT5 hypersensitivity to low GM-CSF doses (0.16 and 0.32 ng/ml) at diagnosis. Patient HM1394 responded poorly to upfront therapy, relapsed, and eventually transformed to M4 AML. As the disease worsened, a greater percentage of cells were hyperresponsive. In contrast, patient HM1158 achieved clinical remission and no longer displayed the JMML signature.

than somatic leukemia-associated mutations by a number of criteria, including progenitor colony growth (Araki et al., 2004; Mohi et al., 2005; Schubbert et al., 2006). Based on these data, it is likely that phosphoflow cytometry provides a more specific readout of the rewired signaling networks found in JMML, an aggressive clonal myeloid malignancy, versus the transient MPD that occurs in patients with NS. An interesting question for future studies is whether phosphoflow cytometry can prospectively identify those rare infants with NS/MPD who will manifest an aggressive clinical course.

We also encountered a child without NS (patient HM1753) who met diagnostic criteria for JMML early in life but improved without treatment. When we evaluated this patient at 2 years of age, he demonstrated modest GM-CSF hypersensitivity but did not exhibit the phosphoflow signature. Molecular analysis revealed an $NRAS^{G13D}$ mutation. Interestingly, other cases of JMML associated with somatic $NRAS$ mutations have been reported to spontaneously clinically regress over time (Flotho et al., 2008; Matsuda et al., 2007) but retain their RAS lesions. The molecular basis for this is unclear but could involve the initiating $NRAS$ mutation occurring in a cell lacking unlimited self-renewal potential (i.e., a non-stem cell). Alternatively, since Ras proteins with amino acid substitutions at codon 13 exhibit higher intrinsic GTPase activity than codon 12 mutant proteins (Ahmadian et al., 1999), it is possible that some mutant RAS alleles are insufficiently activated to cause aggressive JMML. This possibility is consistent with an infant with NS and a moderately activating germline $KRAS^{T58I}$ mutation who showed spontaneous regression of a JMML-like MPD during the first year of life (Schubbert et al., 2006) and with the NS/MPD paradigm discussed above. Importantly, our analysis of the patient with an $NRAS^{G13D}$ mutation further suggests that a normal phosphoflow signature may identify children with $NRAS$ and $PTPN11$ mutations who will have a benign clinical course and can be observed closely

without aggressive treatment. Further studies of additional patients will be necessary to confirm this hypothesis.

The only case in our group with aggressive JMML who did not exhibit the characteristic GM-CSF/p-STAT5 phosphoflow signature harbored a $KRAS^{G12D}$ mutation. In recent studies of additional cases, we have confirmed that the vast majority of children meeting the clinical criteria for JMML exhibit aberrant p-STAT5 activation in response to GM-CSF, with the exception of a second patient with a $KRAS^{G12D}$ mutation who did not show this signaling abnormality. Although these data need to be prospectively validated in a larger cohort study, they suggest that the $KRAS^{G12D}$ mutation, which is phenotypically aggressive in murine models (Braun et al., 2004; Chan et al., 2004), is less dependent upon JAK2-STAT5 signaling than other Ras pathway mutations. Interestingly, phosphosignaling analysis of c-kit+/lineage^{lo/−} cells from $Kras^{G12D}$ mice with MPD did not reveal abnormal STAT5 activation by GM-CSF (data not shown; Van Meter et al., 2007). Our data therefore raise the intriguing possibility that myeloid malignancies with mutations in $KRAS$ show differential activation of p-STAT5 (and perhaps other signaling molecules) compared to cells that express oncogenic $NRAS$ or $PTPN11$.

A recent publication of World Health Organization 2008 classification guidelines (Tefferi et al., 2007; Tefferi and Vardiman, 2008) suggests diagnostic approaches to combine molecular pathogenesis along with histology for both classic and atypical MPDs. Discoveries of somatic mutations in Ras signaling molecules have improved diagnostic capabilities for JMML but are still not universally applicable. In addition, following patients on therapy remains challenging in the absence of tractable markers. Our data imply that advances in proteomic and single-cell flow cytometry technologies will add to the genetic mapping of these disorders, allow tracking of rare cell populations that would be difficult to observe in bulk assay approaches (RNA expression or mass spectrometry), and allow us to measure specific activity at the protein level. We have demonstrated the diagnostic value of such information in JMML and CMML and anticipate that further phosphoflow cytometry-based assays will allow for direct measurements of the key signaling events required for disease maintenance. Finally, the results suggest that JMML, CMML, and M4/M5 AML are related entities in which hyperactive Ras and aberrant JAK2-STAT5 signaling are early or initiating events (Braun et al., 2004). As such, M4/M5 AML might be distinct from

other subtypes of AML, in which aberrant transcription factor fusions such as *PML-RARA* and *AML1-ETO* likely represent primary leukemogenic events (Gilliland and Griffin, 2002). This has important therapeutic implications, as M4/M5 AML might be highly dependent on Ras and JAK2-STAT5 signaling and therefore sensitive to inhibitors of these pathways. Revealing cell subpopulations associated with disease opens additional avenues for measuring minimal residual disease, assessing the biochemical effects of targeted therapies at the single-cell level, and understanding drug action and mechanisms in diseases of heterogeneous origins and manifestations in diverse patient populations.

EXPERIMENTAL PROCEDURES

Sample Collection

Fresh bone marrow or peripheral blood samples were obtained from children suspected of having JMML or another MPD related to a congenital syndrome, including NS or DS. In addition, the following hematopoietic tissues were analyzed in order to demonstrate the specificity of the JMML phosphoprotein signature: (1) archived frozen bone marrow products from healthy sibling donors for hematopoietic stem cell transplants, (2) normal bone marrow taken from children suspected of having a metastatic solid tumor, (3) diagnostic bone marrow or pheresis samples from children or adults with AML, and (4) archived bone marrow or peripheral blood samples from adult patients with CMML. All samples were obtained with informed consent. This study was approved by the UCSF Committee on Human Research. Samples were collected in sodium heparin, and mononuclear cells were isolated according to standard methods.

Frozen samples were cryopreserved in 90% FBS/10% DMSO. Table S1 indicates fresh or frozen status of samples used for phosphoflow analysis; however, all colony assays were performed only using fresh material. Table S2 lists cytogenetic information about the AML samples.

Genotyping

All patients referred for workup of a JMML diagnosis were genotyped for *PTPN11* exons 3 and 13, *NRAS* exons 1 and 2, and *KRAS* exons 1 and 2 according to previously published methodologies (Kalra et al., 1994; Loh et al., 2004; Meshinchi et al., 2003). Genomic DNA was prepared using Puregene reagents (QIAGEN). Data were also collected for family history of and World Health Organization criteria for neurofibromatosis type 1. The eight patients with AML were additionally genotyped for *FLT3* ITD and *FLT3* D835 according to previously published methodologies (Meshinchi et al., 2003; Zwaan et al., 2003).

CFU-GM Assay

Mononuclear cells were isolated from fresh bone marrow samples and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) + 2% FBS. Cells were suspended at a concentration of 200,000 cells/ml. 154 μl of the cell suspension was added to a tube with the following: 1.2 ml MethoCult H4230 methylcellulose (Stem Cell Technologies, Cat. 04230), 15 μl of 100× penicillin/streptomycin, 30.8 μl of human GM-CSF titration (PeproTech, Cat. 300-03), diluted in water and IMDM to complete the tube volume to 1.54 ml. The solution was vortexed for 15 s and rested for 15 min. 1.1 ml was plated into a 35 × 10 mm Petri dish (BD Falcon, Cat. 351008) placed into a 150 × 15 mm Petri dish (BD Falcon, Cat. 351058) with another dish containing sterile water, and placed into an incubator at 37°C, 5% CO_2. After 14 days, plates were removed from the incubator, and colonies (clusters of 50 cells or more) were counted under a microscope at 40× magnification. Data are presented in Table S1. Hypersensitive growth (+2) corresponds to ~50% of maximal growth, whereas hypersensitive growth (+3) corresponds to maximal growth.

Cytokine Stimulation and Intracellular Phosphoprotein Analysis Using Flow Cytometry

Reagents used for flow cytometry included 16% paraformaldehyde (EM grade, Electron Microscopy Sciences, Cat. 15710), methanol (Electron Microscopy Sciences, Cat. 18510), StemSpan H3000 (Stem Cell Technologies, Cat. 09800), and FACS rehydration/staining buffer (HBSS plus 4% FBS). Human GM-CSF (Peprotech, Cat. 300-03), PMA (Sigma Technologies, Cat. P8139), and ionomycin (Sigma Technologies, Cat. I0634) were used to stimulate cells at the concentrations indicated in the text. A chemical JAK2 inhibitor (Calbiochem, Cat. 420099), the oral JAK2 inhibitor XL019 (Exelixis), and the oral MEK inhibitor CI-1040 (Pfizer) were used as described. Antibodies used for phosphoprotein detection were p-STAT5 Alexa 647 (BD Biosciences, Cat. 612599, 10 μl per sample), CD34-PerCPcy5.5 (BD Biosciences, Cat. 347213, 7 μl per sample), CD38-PECy7 (BD Biosciences, Cat. 335790, 5 μl per sample), CD11b-Pacific blue (BD Biosciences, Cat. 558123, 8 μl per sample), CD33-PE (BD Biosciences, Cat. 347787, 5 μl per sample), CD3-Pacific blue (BD Biosciences, Cat. 558117, 5 μl per sample), and CD14-APC Cy7 (BD Biosciences, Cat. 557831, 5 μl per sample). Primary antibody against p-ERK (p-p44/42 MAPK) was from Cell Signaling (Cat. 9101, used at 1:100). FITC-labeled anti-rabbit IgG secondary antibody was from Jackson ImmunoResearch (Cat. 711-096-152, used at 1:400). Primary antibody against p-S6 was from Cell Signaling (Cat. 4856, used at 1:50). FITC-labeled anti-rabbit IgG secondary antibody was from Jackson ImmunoResearch (Cat. 711-096-152, used at 1:400).

Freshly isolated or defrosted mononuclear cells were suspended in prewarmed StemSpan H3000 at a concentration of 1–2 million cells/ml and rested at 37°C for 1 hr. The monocytic cell line U937, which harbors a *PTPN11* exon 3 mutation (178G→C, G60R), was used in each assay as a positive control.

Cells were transferred as 1 ml aliquots into flow cytometry test tubes (BD Biosciences, Falcon 2052) and stimulated with various concentrations of human GM-CSF (PeproTech, Cat. 300-03) for 15 min. Cells were fixed by adding 100 μl of 16% paraformaldehyde (PFA, Electron Microscopy Sciences) at room temperature for 10 min. Cells were washed (centrifugation at 1800 rpm for 5 min) twice with phosphate-buffered saline (PBS) and permeabilized by resuspension in 2 ml ice-cold 95% methanol for 10 min. Cells were stored at −20°C before staining for flow cytometry.

PFA-fixed, methanol-permeabilized cells were rehydrated by adding 2 ml PBS and washed (centrifugation at 2500 rpm for 5 min) twice. Cells were resuspended in 500 μl FACS buffer (HBSS containing 4% FBS, HyClone) and incubated at 4°C for 2 hr.

Unconjugated primary antibodies were added at optimized concentrations (p-ERK 1:100 or p-S6 1:50) and incubated at room temperature for 45 min. Samples were washed (centrifugation at 2500 rpm for 5 min) once with FACS buffer. Samples were stained in 100 μl final reaction volumes, using PBS supplemented with 0.5% bovine serum albumin (Sigma, Cat. A2153) as a diluent. Secondary and directly conjugated antibodies were added at optimized concentrations and incubated in the dark at room temperature for 30 min. Samples were washed (centrifugation at 2500 rpm for 5 min) once with PBS and analyzed on an LSR II flow cytometer (BD Biosciences) equipped with 433 nm and 633 nm lasers.

Data Collection and Analysis

Data were collected using DIVA software (BD Biosciences) and analyzed using Cytobank (http://www.cytobank.org/), an open-source flow cytometry storage and analysis application developed in the Nolan and Levy laboratories at Stanford University. Samples that showed a p-ERK response to PMA were deemed viable and used for analysis.

In Figure 1B, the percent of p-STAT5-responsive cells was determined by drawing a gate using SSC and p-STAT5 to identify the percent of p-STAT5+ cells and scaling the response such that the maximum percent of p-STAT5+ cells was equivalent to 100 and the percent of p-STAT5+ cells at unstimulated was equal to 0.

For Figure 3 and Figures S6 and S7, the normalized p-STAT5 (or p-ERK or p-S6) response was calculated by transforming the raw data using the inverse hyperbolic sine, part of the biexponential class of functions used for digital flow cytometry data (Parks et al., 2006), and calculating a change in the 95th percentile of p-STAT5 response between a GM-CSF-stimulated sample and its unstimulated/basal state. The algorithm is outlined as follows:

For each patient, stain and collect samples stimulated with GM-CSF at the following concentrations: unstimulated, 0.04 ng/ml, 0.16 ng/ml, 0.32 ng/ml, 2 ng/ml, and 10 ng/ml.

a. For each sample:
  i. Transform the raw data: $\sinh^{-1}$(raw data/150)
  ii. Identify live cells
  iii. Calculate the 95th percentile of p-STAT5 in the transformed space
  iv. Subtract this value from the 95th percentile of p-STAT5 of the unstimulated GM-CSF sample.

b. For each patient:
  i. Calculate the range of p-STAT5 response to GM-CSF: max 95th percentile p-STAT5 difference − min 95th percentile p-STAT5 difference
  ii. Ignore patient if range of response is < 0.05
  iii. Normalize the change in p-STAT5 such that the unstimulated sample is equal to 0 and the max difference is equal to 100: (difference in p-STAT5/range of response) × 100.

SUPPLEMENTAL DATA

The Supplemental Data include nine figures and two tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/4/335/DC1/.

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