IN THE SPOTLIGHT

Mass Cytometry: A High-Throughput Platform to Visualize the Heterogeneity of Acute Myeloid Leukemia

Priscilla Do and John C. Byrd

Summary: Behbehani and colleagues use mass cytometry to profile the heterogeneity of acute myeloid leukemia (AML) as compared with normal hematopoiesis. By using this approach and characterizing leukemic stem cells, important differences in response to chemotherapy between AML subsets can potentially be explained and new targeted approaches considered. *Cancer Discov; 5(9); 912–4. ©2015 AACR.*

See related article by Behbehani et al., p. 988 (3).

The ability to measure up to 18 different parameters—surface protein, intracellular protein, cell cycle, viability, and more—at a single-cell level has made traditional flow cytometry an essential tool in the study of multiple diseases. Although traditional flow cytometry maintains unique advantages, a new technology, mass cytometry, has the capacity to measure over 30 parameters simultaneously. Mass cytometry utilizes stable isotopes of nonbiologic metals conjugated to conventional antibodies, negating the issue of spectral overlap as the size of the panel increases (1). One breakthrough study in 2011 by Bendall and colleagues used mass cytometry to map the human immune system, demonstrating the impressive utility of this technique. They provide a holistic view of hematopoiesis, including immunophenotyping via surface antigen expression, intracellular signaling changes, and cell cycle, on one platform, at the level of a single cell (2). Building off of this work, mass cytometry can potentially be used to inform on the pathogenesis of disease as well as assess and guide therapeutic regimens that might not be apparent without analysis of subclones or small cell populations. A model disease where malignant stem cells are believed to be important in pathogenesis, acute myeloid leukemia (AML), is investigated in the current study by using a high-dimensional analysis via mass cytometry (3).

AML describes a group of heterogeneous myeloid malignancies, divided into several subsets based on cytogenetic, mutational, and morphologic distinctions. Response to chemotherapy as the current first-line treatment as reported by Mrózek and colleagues ranged from 39% to 96% complete remission (CR; ref. 4). These response rates are hypothesized to vary widely as a result of the disease heterogeneity, from which several risk factors have been identified to divide patients into prognostic groups based on favorable, intermediate, and unfavorable risk. The favorable-risk group generally responds well to chemotherapy alone, the unfavorable-risk group may be advised for allogeneic stem cell transplantation, given eligibility, and the best course of treatment for the intermediate-risk group is currently unclear. Furthermore, why some favorable risk–group patients respond so well to cell cycle–directed therapy (i.e., cytarabine) is also poorly understood. In the present study, Behbehani and colleagues demonstrate *in vivo* phenotypic differences between patients primarily with AML, other myeloid malignancies, and healthy donor bone marrow. Of interest, core binding factor AML (CBF–AML) of the favorable-risk group and normal karyotype AML (NK–AML) with internal tandem duplications within the fms-related tyrosine kinase 3 gene (*FLT3*-ITD) from the intermediate-risk group are directly compared. The authors propose a mechanism explaining their differential response to chemotherapy as an example of the exploratory inference that this technique can provide, aiding in our understanding of observed clinical biology.

The authors use a single platform, mass cytometry, to elegantly unveil several stages of phenotyping: cell subsets present, their relative quantities, 28 individual markers of abnormal protein expression, cell cycle, intracellular signaling, and *in vivo* response to therapy in a high-dimensional analysis of patients with myeloid malignancy of different subtypes compared with healthy donors. Samples of bone marrow aspirates from 35 AML, 4 acute promyelocytic leukemia (APL), 2 myelodysplastic syndrome, and 5 healthy donors were immediately collected and minimally processed prior to freezing the samples until the time of mass cytometric analysis. Using two panels covering 39 parameters (25 in common between both), samples were bar-coded and run simultaneously. Barcoding provides the advantage of combining the samples into a single staining reaction, eliminating technical variability between samples (5). The results by mass cytometry were compared with clinical flow cytometry data, and the methods were highly consistent. Consistency between these techniques provides validation and suggests an eventual transition of this new technique to clinical diagnostic laboratories where direct decisions can be made based upon the results observed.

Immunophenotypic analysis was completed with 19 surface markers and Spanning-tree Progression Analysis of Density-normalized Events (SPADE) analysis. SPADE analysis enables a system-wide view of cell phenotypes, the relative

Division of Hematology, Department of Medicine, The Ohio State University, Columbus, Ohio.

Corresponding Author: John C. Byrd, The Ohio State University, B304 Starling Loving Hall, 320 West 10th Avenue, Columbus, OH 43210. Phone: 614-293-9321; Fax: 614-293-7526; E-mail: john.byrd@osumc.edu

doi: 10.1158/2159-8290.CD-15-0905

relationships between populations, and relative abundance in a hierarchical diagram of high-dimensional data (6). There was a consistent phenotype across healthy donors and a large (50-fold) increase in immature cells [hematopoietic stem cells (HSC): lin$^-$CD34$^+$ CD38^{lo}CD45RA$^-$CD90$^+$CD33^{lo}, and multipotent progenitor cells (MPP): lin$^-$CD34$^+$CD38^{lo}CD45 RA$^-$CD90$^-$CD33^{lo}] in CBF–AML and adverse karyotype (ARK) AML and a smaller increase (3–10-fold) in NK–AML and APL.

Next, surface marker expression (28 parameters) of AML samples compared with healthy donors revealed aberrations in over 35 immunophenotypic populations. All AML samples had aberrations in every stage of myeloid lineage cells, and for several patients, aberrations were also detected in nonmyeloid cells, including B, T, and natural killer cells. There were distinctions made between subsets of AML in the hematopoietic stem and progenitor cell populations (HSPC; lin$^-$CD34$^+$CD38^{lo}). This finding is of particular interest to the hypothesized pathogenesis of AML. Until now, only indirect evidence *in vitro* and using murine transgenic and xenograft models has been able to support the presence of HSC-like leukemia stem cells (LSC; lin$^-$CD34$^+$CD38$^-$) that are thought to be responsible for relapsed AML (7). In this study, additional *in vivo* evidence of an aberrant lin$^-$CD34$^+$CD38^{lo} population distinct between AML and normal counterparts is provided. The potential to monitor the effects of therapy on this particular population could be a critical step forward in the treatment of AML and for following patients post-therapy for the elimination of these cells. In addition, for differential surface antigen expression, this study identified *FLT3*-ITD$^+$ NK–AML HSPCs to have more CD33 and CD123 expression, providing justification for targeted antibody or antibody-toxin–directed treatment toward this subset.

The authors further characterize the differences among HSPCs using viSNE to represent high-dimensional data. viSNE has the power to visualize several parameters simultaneously in 2 to 3 dimensions. With viSNE analysis, cells can be clustered by phenotype even without the addition of canonical markers. This tool enables one to robustly visualize changes in the phenotypic landscape across samples (8). Distinctions were observed with 19 surface markers across the HSPC populations compared between molecular aberrations derived from different international European LeukemiaNet (9) AML groups and the normal donor HSPCs. Using the same gate determined by the 5 normal donor samples, which mapped HSPCs very similarly, HSPCs from all disease groups compared gave distinctly divergent patterns from the normal donors. In comparison between subsets, such as the CBF–AML and NK–AML *FLT3*-ITD$^+$ samples, viSNE analyses tended to display more similar patterns within a given subset. More specifically, within the CBF–AML subset, similar patterns existed between defined karyotypic abnormalities as demonstrated with 2 patients with inv(16) and 2 of 3 patients with t(8;21). Ten of 11 patients with NK–AML *FLT3*-ITD$^+$ also displayed similar patterns, and within this subset, only 1 of 11 patients had a dissimilar pattern. Interestingly, this patient was later determined to be negative for *FLT3*-ITD at disease relapse. Four APL and 2 of 3 *FLT3*-TKD samples had similar patterns to *FLT3*-ITD$^+$ NK–AML. Within the HSPC compartment, AML differentially expresses surface proteins compared with healthy bone marrow and, furthermore, between different subsets of this disease based on karyotype and genetic groupings. How this information can be harnessed to direct clinical treatment depends on linkage of these aberrations to functional consequences within the hematopoietic landscape.

The authors begin to connect these changes in cell markers to functional consequences by progressing into cell-cycle analysis, intracellular signaling, and response to chemotherapy. With regard to cell cycle, healthy donor bone marrow had the highest percentage of S-phase cells in early committed progenitor populations (myelomonoblasts, promonocytes, and promyelocytes) with the HSPC compartment having a relatively lower percentage of S-phase cells. AML samples on average had a lower percentage of S-phase cells compared with their normal counterparts. Comparing AML subsets, CBF–AML had the highest S-phase fraction of HSPCs, whereas *FLT3*-ITD$^+$ NK–AML HSPCs had a lower percentage of S-phase cells than other AML samples. These data correlate with clinical response to chemotherapy, where CBF–AML tends to have higher CR rates after initial therapy than *FLT3*-ITD$^+$ NK–AML (4). Next, Behbehani and colleagues looked at the aberrant CD34$^+$CD38^{lo} populations for intracellular signaling differences compared with their normal counterparts and found that almost all AML subtypes exhibited higher MAPKAPK2 phosphorylation, and, furthermore, the *FLT3*-ITD$^+$ AML had higher pSTAT5A, a known downstream target of *FLT3*-ITD. *In vivo* evidence for phenotypic abnormalities in specific populations of cells can guide identification of potential therapies to target malignant populations of cells, while potentially sparing normal cells. Finally, the *in vivo* response to therapy (oral hydroxyurea; HU) was assessed in 10 treated patients compared with 23 untreated patients. Myeloid lineage populations were mostly unaffected by HU treatment as measured by percentage of S-phase cells. CD34$^+$CD38^{lo} populations were least affected by HU, and actively cycling cells were unchanged or increased in treated patients. This result is in contrast with the effectiveness against proliferative cells determined using *in vitro* studies, but in line with the lack of efficacy observed as a long-term effect of this agent in treating AML (9, 10).

Behbehani and colleagues have opened doors with the current study. An astonishing amount of information was afforded from one set of bone marrow aspirates. The combination of analyzing a sample directly from the patient and immediate determination of cell subsets present, their specific aberrations in protein expression, signaling, or cell cycle via mass cytometry could potentially be a new method of guiding clinical decisions and will certainly deepen our understanding of disease pathogenesis and response to therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Published online September 2, 2015.

REFERENCES

1. Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. Trends Immunol 2012;33:323–32.
2. Bendall SC, Simonds EF, Qiu P, Amir el-AD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and

drug responses across a human hematopoietic continuum. Science 2011;332:687–96.
3. Behbehani GK, Samusik N, Bjornson ZB, Fantl WJ, Medeiros BC, Nolan GP. Mass cytometric functional profiling of acute myeloid leukemia defines cell-cycle and immunophenotypic properties that correlate with known responses to therapy. Cancer Discov 2015;5:988–1003.
4. Mrózek K, Marcucci G, Nicolet D, Maharry KS, Becker H, Whitman SP, et al. Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. J Clin Oncol 2012;30:4515–23.
5. Behbehani GK, Thom C, Zunder ER, Finck R, Gaudilliere B, Fragiadakis GK, et al. Transient partial permeabilization with saponin enables cellular barcoding prior to surface marker staining. Cytometry A 2014;85:1011–9.
6. Qiu P, Simonds EF, Bendall SC, Gibbs KD Jr, Bruggner RV, Linderman MD. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. Nat Biotechnol 2011;29:886–91.
7. Pollyea DA, Gutman JA, Gore L, Smith CA, Jordan CT. Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. Haematologica 2014;99:1277–84.
8. Amir el-AD, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotechnol 2013;31:545–52.
9. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 2010;115:453–74.
10. Roboz GJ, Rosenblat T, Arellano M, Gobbi M, Altman JK, Montesinos P, et al. International randomized phase III study of elacytarabine versus investigator choice in patients with relapsed/refractory acute myeloid leukemia. J Clin Oncol 2014;32:1919–26.