



Consistent Quantitative Gene Product Expression: #2. Antigen Intensities on Bone Marrow Cells Are Invariant between Individuals

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• Abstract

Five reference populations in bone marrow specimens were identified by flow cytometry using specific combinations of reagents in order to define the variation of gene product expression intensities both within and between individuals. Mature lymphocytes, uncommitted progenitor cells, promyelocytes, mature monocytes and mature neutrophils can be reproducibly identified as distinct clusters of events in heterogeneous, maturing bone marrow specimens. Support Vector Machines were used to identify the reference populations in order to reduce subjective bias in manually defining boundaries of these populations since they were not discretely separated from the remainder of the cells. Reference populations were identified in 50 randomly selected bone marrow aspirates obtained over a period spanning 3 years and 6 months from pediatric patients following chemotherapy for acute myeloid leukemia (AML). The quantitative expression of gene products (cell surface antigens) and light scattering characteristics on these stressed specimens were demonstrated to be tightly regulated both within individuals and between individuals. Within an individual most gene products (CD45, CD34, CD14, CD16, CD64, CD33) demonstrated limited variability with a standard deviation of <0.20 log units while CD13 and CD36 exhibited broader variation >0.25 log units. Surprisingly, with the exception of CD33, the variation of the mean intensities of each antigen between individuals was even less than the variation within an individual. These data confirm that the amounts of gene products expressed on normal developing cells are highly regulated but differ in intensities between different lineages and during the maturational pathway of those lineages. The amounts of gene products expressed at specific stages of development of each lineage are a biologic constant with minimal variation within or between individuals. © 2016 International Society for Advancement of Cytometry

• Key terms

flow cytometry; bone marrow aspirates; quantitative antigen expression; support vector machines

INTRODUCTION

HEMATOPOIESIS is a highly regulated, step-wise process in which undifferentiated hematopoietic stem cells undergo commitment to different lineages and develop into functional mature cells found in blood, lymph nodes and other tissues (1,2). The ontology of blood cells present a unique model for understanding the precise nature of cellular maturation since bone marrow specimens include multiple lineages of cells in all stages of development. Although much has been elucidated about how cells regulate turning on and turning off genes, there has been little appreciation for the regulatory mechanisms that precisely control the quantitative amounts of gene

products.

The flow cytometric approach “Difference from Normal”, used to detect response to therapy in patients with leukemia, is based on a precise knowledge of the timing and quantitative expression levels of surface antigens (gene products) on normal regenerating bone marrow cells (3). This approach patterns the normal regenerating bone marrow cells in multidimensional space in order to identify populations or clusters of events that are at least 0.5 log units disparate from the nearest normal counterpart (3,4). Low levels of leukemia cells can thus be identified based on surface gene product expression patterns that are different from those observed in normal hematopoiesis. This technique has been applied to acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, chronic myelogenous leukemia (CML) as well as myelodysplastic syndromes (MDS) (3–8). The success of a “Difference from Normal” approach is predicated on understanding the consistent gene product expression patterns identifiable from individual to individual.

The study of bone marrow specimens is complicated by a lack of lineage specific markers, especially on the developing myeloid cells. The same gene products can be identified on multiple lineages but are often expressed at different intensities, which also can change with cellular maturation (9). As a result of this variability, few distinct cell populations form discrete clusters of events within normal regenerating bone marrow.

Flow cytometry was used to define remission status in the Children’s Oncology Group Study AAML1031 using the “Difference from Normal” flow cytometric approach (clinicaltrials.gov/AAML1031). In this manuscript, we focus on the normal regenerating bone marrow cells, not the residual leukemia. We identify five cell populations that are reproducibly identifiable within regenerating pediatric bone marrow aspirates. We demonstrate that the intensities of most of the key gene products expressed on these reference cell populations are invariant from individual to individual in patients with stressed bone marrow specimens recovering from chemotherapy. These results not only support the technical approach to “Difference from Normal,” but also establish a basic underlying biological principle regarding regulation of amounts of gene products, defining the variation of gene product expression within and between individuals.

MATERIALS AND METHODS

Patient Data Set

Pediatric acute myeloid leukemia patients enrolled on Children’s Oncology Group study AAML1031 ($n > 1,000$) were eligible for this study (clinicaltrials.gov/AAML1031). A total of 77 randomly selected patients from the therapeutic study who were approximately one-month post chemotherapy were selected for the current study. The specimens were required to have excellent specimen quality, limited hemodilution, and no evidence of residual disease (3,10). The data were obtained over a period of three years and six months using

Table 1. Monoclonal antibody combinations

	FITC	PE	PERCP	APC
Tube 1	HLADR	CD11b	CD45	CD34
Tube 2	CD36	CD38	CD45	CD34
Tube 3	CD15	CD13	CD45	CD34
Tube 4	CD14	CD33	CD45	CD34
Tube 5	CD7	CD56	CD45	CD34
Tube 6	CD38	CD117	CD45	CD34
Tube 7	CD36	CD64	CD45	CD34
Tube 8	CD19	CD123	CD45	CD34

The monoclonal antibody combinations used for MDF assessment are listed for each tube. Both CD45 (PerCP) and CD34 (APC) were consistent between all eight antibody combinations.

three separate flow cytometers, multiple reagent lots and processed by multiple technicians.

Flow Cytometry

Bone marrow aspirates were collected in heparin (the preferred anti-coagulant) or EDTA. Specimens were processed as routine clinical bone marrow aspirates as previously described (3). Briefly, 100 μ L of bone marrow was added to cocktails of pre-titrated antibodies at room temperature in the dark. Red blood cells were lysed using 3.5 mL of buffered NH_4Cl (0.83%) at 37°C for 5 minutes before centrifugation at 300G. Cells were then washed with 3 mL of phosphate buffered saline containing 2% fetal calf serum and re-suspended to 0.5 mL in 1% paraformaldehyde for analysis on one of three FACS Calibur instruments (Becton Dickinson Biosciences, San Jose, CA). 200,000 events were collected for each tube. The flow cytometers were cross standardized and calibrated using RCP-30A and RFP-30A beads (Spherotech, Lake Forest, IL) with spectral compensation performed using peripheral blood cells labeled with CD4 (SK3, BD) conjugated to fluorescein (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Eight combinations of antibodies are presented in Table 1. The 8th tube was added after the beginning of the study to identify immature B lymphoid precursors/plasmacytic dendritic cells and basophils, so this reagent combination was only implemented in 45 of the 50 patients in the testing cohort.

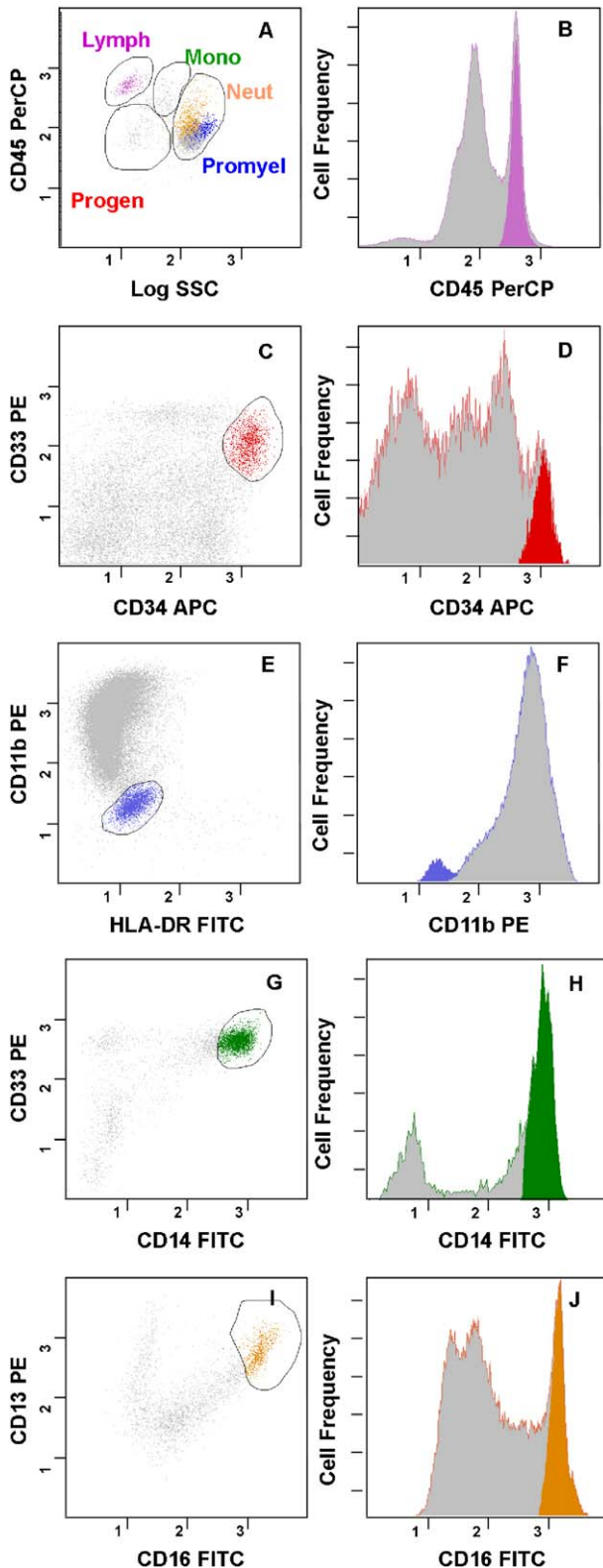
Reference Populations

An eight tube reagent panel was used to pattern the development of hematopoietic cells from the hematopoietic stem cell to mature monocytes, neutrophils, and erythroid cells in the AAML1031 clinical study (Table 1). A total of 5 reference populations were investigated for this study: lymphocytes (Tubes 1–8), uncommitted progenitor cells (Tubes 1–8), promyelocytes (Tube 1), monocytes (Tube 4, Tube 7), and mature neutrophils (Tube 3), Table 1.

Support Vector Machines

Patients were randomly assigned to training ($n = 27$) and testing ($n = 50$) cohorts. An expert-analyst (MRL) classified the 5 reference cell populations using WinList (Verity Software

House, Topsham, ME) for all patients in both the training and testing cohorts. All flow-cytometry data were analyzed in log space, with the exception of forward light scatter (FSC).



Expert-classified cell populations from the cohorts were exported from Winlist into R software (R Software, Vienna, Austria). The key reference populations were identified as having a distinct clustering of cells that could be identified as a separate population even if one or more of the parameters were continuous, i.e. not discrete. In cases where parameters were continuous, populations were defined to approximate symmetrical shaped histograms (Fig. 1). A unique Support Vector machine (SVM) was trained to identify each of the 5 reference populations. Identification of the uncommitted progenitor cell population required 8 separate SVMs, one for each tube. All SVMs demonstrated strong performance in replicating the gate of an expert while eliminating subjective analytical bias (Supporting Information Table 1). The performance of the SVMs to replicate the expert is the basis of a previous companion article (11).

RESULTS

Five Reference Populations in the Bone Marrow

It is difficult to identify homogenous cell populations with identical antigenic expression in heterogeneous bone marrow aspirates. However, by using specific reagent combinations, five key reference cell populations can be distinguished. These reference populations serve as guideposts for the maturational patterns of hematopoietic cells, from the earliest progenitor cells to the most mature cells.

The first reference population, mature lymphocytes (Figs. 1A and 1B), survive standard AML chemotherapies and are identifiable at relatively high frequency after AML treatment. The lymphocytes (purple) are identified by high expression of CD45 and low SSC. The second reference population, uncommitted progenitor cells (red) (Figs. 1C and 1D), include the most immature cells in the bone marrow. These cells have not yet expressed cell surface markers that indicate lineage commitment. This unique cell cluster includes hematopoietic stem cells (HSC) and multipotent progenitor cells, but does not include lineage specific colony forming cells (12–14). The uncommitted progenitor cells are identified by high expression of CD34 and co-expression of CD33 (11,15). The third reference population, promyelocytes (blue) (Figs. 1E and 1F), are the most mature normal-counterpart of AML along the neutrophil pathway. The promyelocytes are identified by high SSC and a lack of CD11b and HLA-DR expression (1,16). The fourth reference population, monocytes (green) (Figs. 1G and 1H), are the most mature normal-counterpart of AML along

Figure 1. Identification of reference populations in stressed bone marrow specimens (all intensity units are in logarithmic scale; all frequency units are in linear scale). **A, B:** Mature lymphocytes were identified based on high expression of CD45 and low expression of log SSC (Purple). **C, D:** Uncommitted progenitor cells were identified by high expression of CD34 and intermediate expression of CD33 (Red). **E, F:** Promyelocytes expressed high levels of SSC (A) without expression of HLA-DR or CD11b (Blue). **G, H:** Mature monocytes were defined based on high levels of CD14 and CD33 (Green). **I, J:** Mature neutrophils expressed high levels of SSC as well as CD16 and CD13 (Gold) (The reference population colors are maintained in subsequent figures).

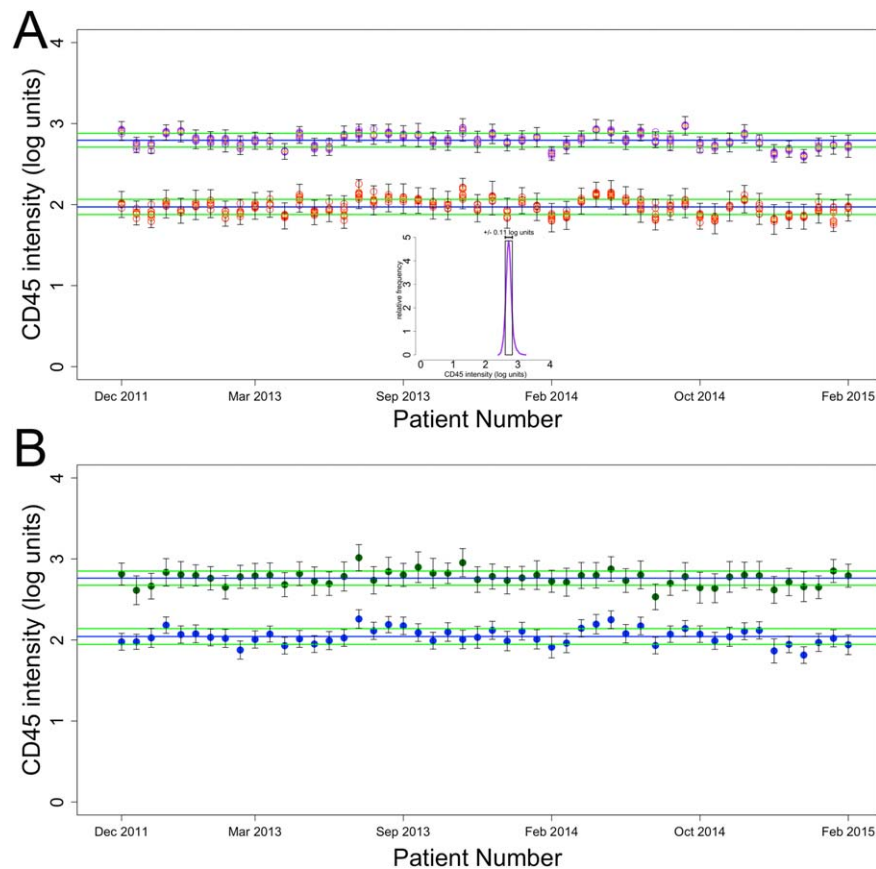


Figure 2. CD45 intensity variation on reference populations for 50 pediatric bone marrow specimens. **A:** Variability of CD45 on lymphocytes (purple) and uncommitted progenitor cells (red). Each patient has 8 replicates for these two populations. The blue line represents the parameter mean averaged over all patients. Within patient variability is depicted by the whiskers (± 1 SD). The average between patient variability (± 1 SD) of the means is shown as the green lines. The histogram insert is added as a reference to visually demonstrate how little variation is observed for CD45 expression on lymphocytes (1 SD = 0.11 log units). **B:** CD45 intensity variability for promyelocytes (blue) and mature monocytes (green) assayed in a single reagent combination. The parameter means are shown as a blue line. Within patient variability (± 1 SD) is demonstrated by the whiskers while the between patient variability (± 1 SD) is depicted by the green lines.

the monocytic pathway. The monocytes are identified by high expression of CD14 with coexpression of CD33 (9,17). The fifth reference population, mature neutrophils (gold) (Figs. 1I and 1J), are important to assess hemodilution and to determine proper maturation to segmented neutrophils (10). The mature neutrophils are identified by high levels of SSC, CD13, and CD16.

Each reference population was identified in the test patient cohort ($n = 50$). Four mean and variation characteristics were computed to assess the regulation of multiple gene products on each reference population, and are described below and in Supporting Information Figure 1:

1. Parameter mean: the mean surface gene product intensity of all cells in a given reference population, averaged across all patients in the test cohort.
2. Within-patient variation: the variation (standard deviation, SD) of surface gene product intensities within one patient (histogram width, whiskers), averaged over the entire testing cohort of 50 patients
3. Between-patient variation: the variation (SD) between the 50 patient mean intensities in the test cohort.

4. Replicate variation: the variation (SD) of mean intensity values between eight replicate assays for the same patient. Replicate variation was only assessed for CD45, CD34, and light scatter characteristics, as these parameters were measured in all eight reagent tubes (Table 1).

Consistency in CD45 Intensity

The intensity of CD45 expression, in combination with log SSC, provides the most information regarding cell lineage and maturation compared to any other single surface gene product in a flow cytometric analysis of bone marrow (18,19). CD45 means and variation characteristics for lymphocyte, uncommitted progenitor cell, promyelocyte, and monocyte reference populations are illustrated in Figure 2. The amount of CD45 is reproducibly expressed at different levels on each of the reference populations (Table 2). Lymphocytes have the highest CD45 expression of the reference populations, followed by monocytes, mature neutrophils, promyelocytes, and lastly uncommitted progenitor cells.

Within each patient, CD45 expression was tightly regulated for each reference population (Fig. 2). Cells belonging to

Table 2. Variability of cellular characteristics for reference populations

PARAMETER	REFERENCE POPULATION	PARAMETER MEAN	BETWEEN PATIENT VARIATION (SD)	WITHIN PATIENT VARIATION (SD)	REPLICATE VARIATION (SD)
A. CD45	Lymphocyte	2.79	0.085	0.11	0.017
	Uncommitted Progenitor Cells	1.97	0.094	0.17	0.032
	Monocytes	2.76	0.088	0.16	NA
	Promyelocytes	2.04	0.097	0.11	NA
	Mature Neutrophils	2.4	0.18	0.29	NA
B. SSC	Lymphocyte	1.24	0.071	0.14	0.02
	Uncommitted Progenitor Cells	1.46	0.09	0.17	0.053
	Monocytes	1.86	0.055	0.15	NA
	Promyelocytes	2.43	0.054	0.14	NA
	Mature Neutrophils	2.32	0.076	0.17	NA
C. CD34	Uncommitted Progenitor Cells	3.14	0.065	0.15	0.032
D. CD14	Monocytes	2.54	0.062	0.18	NA
E. CD64	Monocytes	2.88	0.11	0.18	NA
F. CD16	Mature Neutrophils	3.07	0.11	0.16	NA
G. CD33	Monocytes	2.74	0.25	0.14	NA
	Uncommitted Progenitor Cells	2.13	0.22	0.29	NA
H. CD13	Uncommitted Progenitor Cells	2.26	0.12	0.31	NA
	Mature Neutrophils	2.81	0.15	0.26	NA
I. CD36	Monocytes	2.99	0.21	0.33	NA

Parameter mean, between-patient variation (1 SD), and within-patient variation (1 SD) values are reported for CD45 (A), SSC (B), CD34 (C), CD14 (D), CD64 (E), CD16 (F), CD33 (G), CD13 (H), and CD36 (I) for the reference populations. All values are reported in log units. NA indicates that the replicate variation was not assessed because the reference population was only identified in one combination of reagents.

lymphocyte, uncommitted progenitor cell, promyelocyte, and monocyte reference populations expressed consistent amounts of CD45, and within-patient CD45 variation was small (0.11–0.17 log units) (Table 2). Mature neutrophils had slightly broader, yet still remarkably consistent, CD45 expression within each Patient (0.29 log units) (Table 2).

Surprisingly, the between-patient variation of the mean amount of CD45 for each population was less than the within-patient variation (Fig. 2, Table 2). Lymphocytes, uncommitted progenitor cells, monocytes, and promyelocytes all exhibited particularly tight regulation of mean CD45 intensity between the 50 patients, demonstrating between-patient variations ranging from 0.08 to 0.10 log units (Table 2). Mature neutrophils had a slightly broader, but still low, between-patient variation of 0.18 log units (Table 2).

Since lymphocytes and uncommitted progenitor cells were identified in each of the eight combinations of reagents, the replicate variation of mean CD45 intensity was assessed for these reference populations (Fig. 2). For the 50 test patients, the replicate variation of CD45 between the eight

assays was 0.017 log units for the lymphocytes and 0.032 log units for the uncommitted progenitor cells, indicating a highly reproducible assessment (Table 2).

Consistency of Log SSC

The consistency of log SSC was evaluated for each reference population, with the promyelocytes exhibiting the highest SSC of the reference populations, followed by the mature neutrophils, the monocytes, the uncommitted progenitor cells, and the lymphocytes (19) (Fig. 3, Table 2). Within each patient, cells had consistent levels of granularity (measured by log SSC) for each reference population (Fig. 3). In each lineage studied, the within-patient variation of measured SSC was low (0.14–0.17 log units) (Table 2). Furthermore, the between-patient SSC demonstrated little variation between the 50 test patients, ranging from 0.05 to 0.09 log units for the different reference populations. Finally, replicate variation of SSC on the lymphocytes (0.02 log units) and uncommitted progenitor cells (0.053 log units) again demonstrate a highly reproducible assessment of SSC within the eight assays for

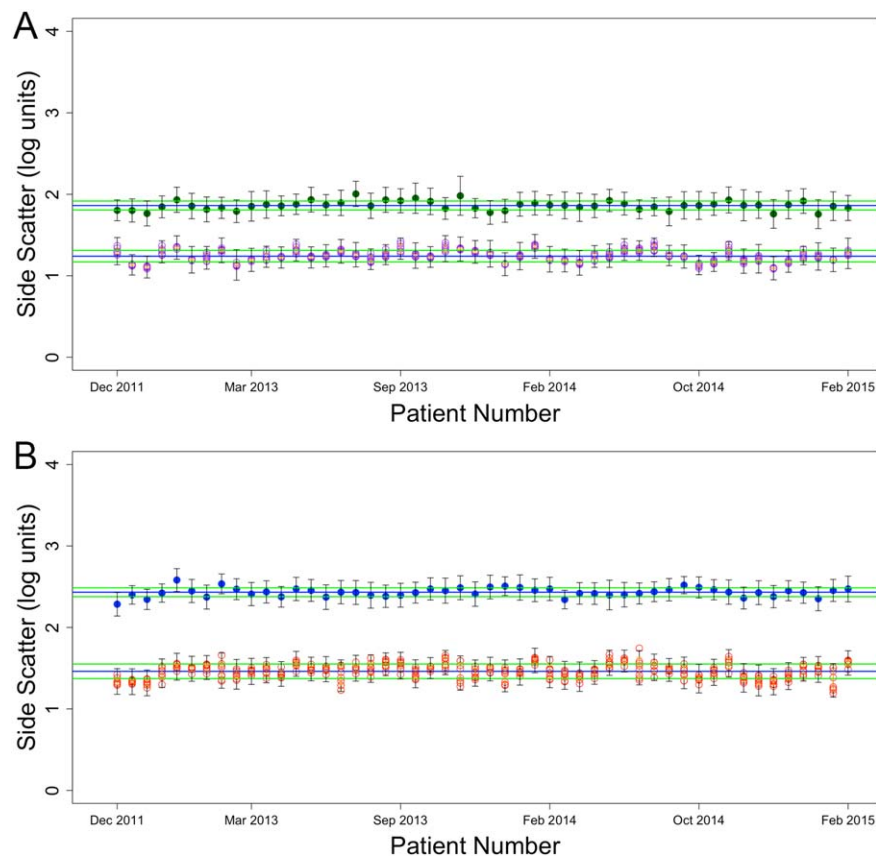


Figure 3. Side scatter (SSC) variation for reference populations in 50 pediatric bone marrow specimens. **A:** Variability of SSC is displayed for lymphocytes (purple, with replicates) and monocytes (green). The parameter means are shown as a blue line. Within patient variability (± 1 SD) is demonstrated by the whiskers while the between patient variability (± 1 SD) is depicted by the green lines. **B:** Variability of uncommitted progenitor cells (red, with replicates) and promyelocytes (blue). The parameter means are shown as a blue line. Within patient variability (± 1 SD) is demonstrated by the whiskers while the between patient variability (± 1 SD) is depicted by the green lines.

each patient. As granularity is a difficult metric to calibrate in setting up of a flow cytometer, the consistency of such SSC measurements is particularly impressive.

Normalization of CD45 and Log SSC

Combining CD45 and log SSC defines the position of the reference populations for 50 normal-but-stressed bone marrow specimens over a greater than three-year period (Fig. 4A). An ellipsoid represents the location of each reference population, and illustrates the variation in mean intensity of CD45 (y-dimension, ± 2 SD) and SSC (x-dimension, ± 2 SD) between patients for each studied lineage. The locations of these clusters are the basis of CD45 and log SSC identification of “Normal Positions” using the CD45 gating procedure (19).

In an attempt to more precisely define the position of each reference population in CD45 vs SSC, a normalization of intensities relative to the included mature lymphocytes was performed. The mean lymphocyte-population intensity for each patient can be translated to a single location. Each corresponding reference population for that patient can then be translated by an identical vector in both the CD45 and SSC dimensions, thereby normalizing the positions of all populations to that of the lymphocytes (Figure 4B). The boundaries

(± 2 SD) of the uncommitted progenitor cells, promyelocytes, monocytes, and mature neutrophils can be visualized before and after normalization. After normalization, the dispersion of the uncommitted progenitor cells and promyelocytes dramatically decrease. The positions of the monocytes and mature neutrophils do not change as dramatically after normalization to the lymphocytes.

Consistency of Other Gene Products (CD13, CD14, CD16, CD33, CD34, CD36, and CD64)

The consistency of gene product expression can be further examined by studying the variability of additional gene products on these reference populations. CD34 intensity on progenitor cells is a particularly important parameter for identifying populations of neoplastic myeloid cells after chemotherapeutic treatment. CD34 intensity was high on the uncommitted progenitor cells, (3.14 log units) (12) (Fig. 5A). Within each patient, the variation in CD34 intensity was low (within-patient variation = 0.15 log units) (Table 2). Again, the variation in mean CD34 intensity between patients (0.065 log units) was less than the within-patient variation. Within the eight replicate assays, the variability in the mean intensity of this antigen averaged 0.032 log units. In contrast to the

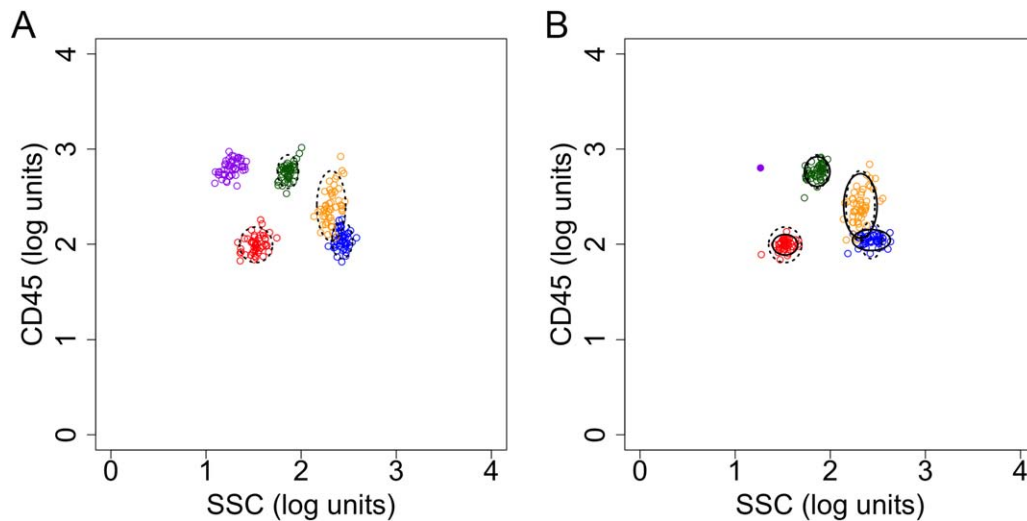


Figure 4. CD45/log SSC relationships for reference populations for 50 pediatric bone marrow specimens. **A:** The CD45 vs. SSC position of mature lymphocytes (purple), uncommitted progenitor cells (red), mature monocytes (green), promyelocytes (blue), mature neutrophils (gold). The dashed ovals represent the between patient variability of CD45 (± 2 SD, y-axis) and SSC (± 2 SD, x-axis). **B:** Normalization of the position of the reference populations to lymphocytes. The lymphocytes (purple) were moved to the same location and the corresponding positions of the other reference parameters were also translocated using the same vector for each patient. The solid ovals represent the variation of the normalized positions in the CD45 dimension (± 2 SD, y-axis) and the SSC dimension (± 2 SD, x-axis).

consistency of quantitative CD34 expression, the proportion of uncommitted progenitor cells was highly variable from individual to individual (Fig. 5A, lower line) and ranged from 0.3% to 5.3% of total events in each specimen.

The intensities of CD14 and CD64 on mature monocytes as well as the intensity of CD16 on mature neutrophils also exhibited a low level of variability (Table 2). Each of these antigens was tightly regulated within each individual Patient (0.16 to 0.18 log units). Likewise, the mean intensity of these antigens was reproducible between patients, with low between-patient variation (0.06 to 0.11 log units).

In contrast, the variability of CD33 intensity on the mature monocytes showed a different pattern of expression compared to CD45, CD34, CD14, CD16, CD64 and log SSC (Table 2). The within patient variability was low (0.14 log units) while the between patient variation was large in comparison (0.25 log units). Five out of the fifty patients in the test cohort had dramatically lower CD33 expression (Fig. 5C). High between-patient CD33 variability was also observed on the uncommitted progenitor cells (Table 2) and the intensities varied in concert with the expression on the monocytes (Supporting Information Fig. 2).

Two other antigens in this study (CD13 and CD36) showed a broader distribution within individuals compared to the other studied gene products. CD13 was quantified on both the uncommitted progenitor cells and the mature neutrophils while CD36 was identified on the mature monocytes (Table 2). The within-patient variation in CD13 intensity was high for the uncommitted progenitor cell population (0.31 log units) and the mature neutrophils (0.26 log units) compared to other studied gene products. However, CD13 demonstrated much lower between-patient variation on the two cell lineages (0.12 log units and 0.15 log units, respectively).

Similarly, the within-patient variation of CD36 on the mature monocytes was 0.33 log units while the between patient variation was 0.21 log units (Table 2). These data indicate that the regulation of amount of CD13 and CD36 within an individual is not as restrictive as the other cell surface gene products, however, the between patient variation remained less than the within patient variation.

DISCUSSION

The detection of abnormal cell populations based on “Difference from Normal” requires a precise knowledge of gene product expression during hematopoiesis (3). Defining normal gene product expression patterns is predicated on both consistent gene product expression from individual to individual and a stable analytic platform in which to detect cellular characteristics on stressed cells. By assaying the quantitative expression of multiple gene products on individuals recovering from chemotherapy over a several year period, it is possible to precisely define the variation observed for each gene product in a routine clinical setting. By knowing the variance within and between patients for the expression of myeloid gene products on normal regenerating cells, the boundaries of what is “abnormal” can be more precisely defined.

The lack of variability of intensity measurements in this study confirms the underlying principle that the amounts of gene products are precisely regulated during normal maturation and that these amounts are invariant from individual to individual. Each gene product is regulated around a mean for each cell type and maturational stage. The within patient variability is a measure of how tightly the cells regulate around the mean for a specific cell population. The between patient variability measures how much this mean changes from

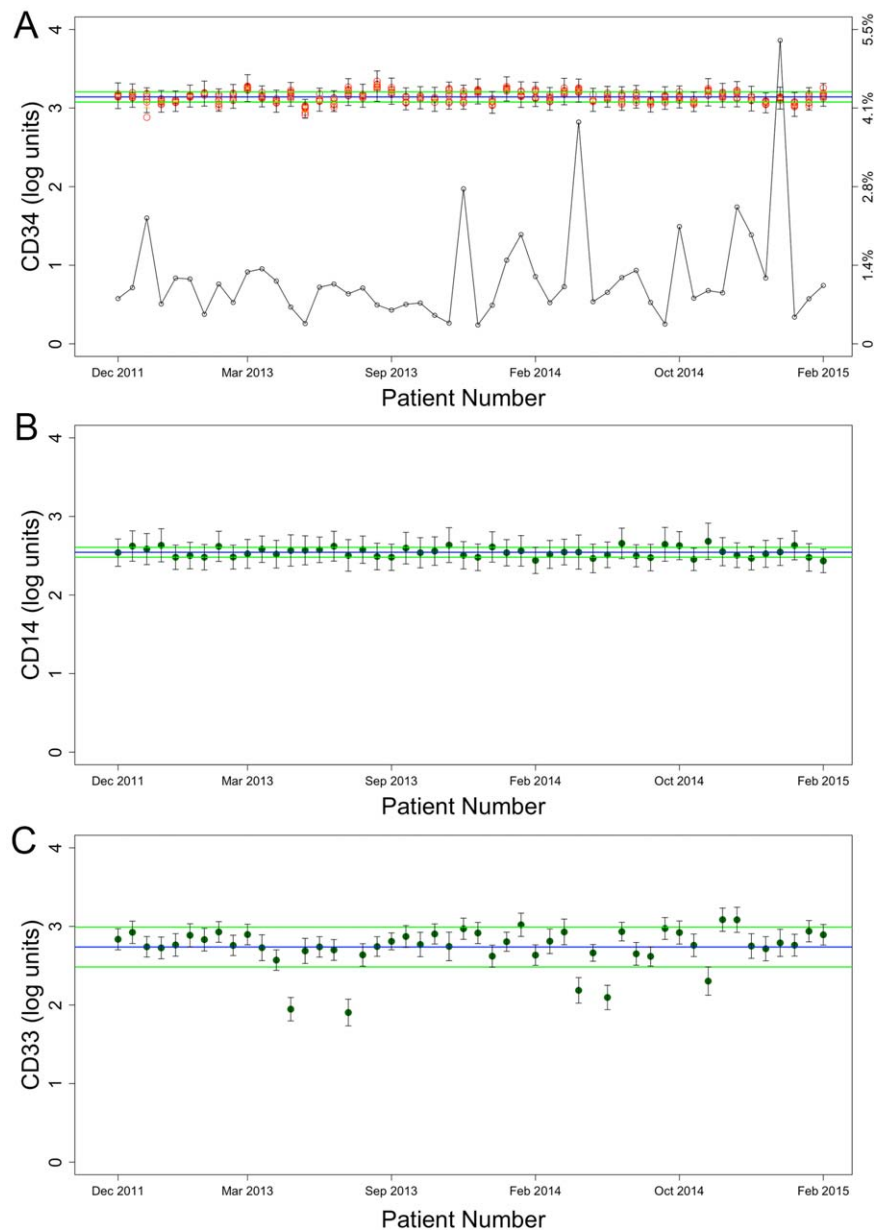


Figure 5. Variation of intensities of CD34, CD14, and CD33 on 50 pediatric bone marrow specimens. **A:** CD34 variation on uncommitted progenitor cells showing 8 replicates, within patient variation (whiskers), between patient variation (green lines) and parameter mean (blue line). The proportion of uncommitted progenitor cells is variable (black lower line). Frequency is depicted on the y-axis on the right side of the plot. **B:** CD14 expressed on mature monocytes showing the within patient variation (whiskers), between patient variation (green line) and parameter mean (blue line). **C:** CD33 expression on mature monocytes shows tight within patient variation (whiskers) but broader between patient variation (green line).

individual to individual. By focusing on this multilevel variation of each of the cellular characteristics, the precision of regulation of these processes can be discerned. Most previous studies of antigenic quantitation have focused on defining the average amount of specific gene products on mature lymphoid and myeloid cells found in blood (17,20–22). These studies have not extensively examined the variance around the means or the consistency from individual to individual. By extending the analysis of variance to myeloid cells in bone

marrow the precise regulation during the developmental process can also be elucidated.

Some gene products are tightly regulated demonstrating a within patient variation of <0.2 log units (CD45, CD34, CD14, CD33, CD16, CD64), while others exhibit a broader variation of >0.25 log units (CD13, CD36). With the exception of CD33, the variation around this mean observed within individuals is greater than the variability of these means from individual to individual. For most gene products the amount

of the proteins expressed on the cell surfaces are a biological constant with precise quantitative regulation.

Both CD45 and log SSC for each reference population was surprisingly constant within and between individuals (22). Normalization of CD45 and log SSC to the mature lymphocytes reduced the variability from individual to individual almost in half for some reference populations. This suggests an even tighter regulation of these two parameters on different cell lineages than the statistics indicate. Slightly higher or lower expression of CD45 or SSC on the lymphocytes is reflected on both the uncommitted progenitor cells and the promyelocytes. Normalization to lymphocytes does not dramatically change the relative positions of the mature monocytes or mature neutrophils in these patients. This finding may indicate that mature, non-dividing cells (mature monocytes and neutrophils) may not exhibit as precise a regulation of these parameters as those more immature forms that are undergoing maturation and cell division.

It is remarkable that the intensity of CD34 of the uncommitted progenitor cells in stressed bone marrows is also invariant from individual to individual. The proportions of these cells, however, do change dramatically from individual to individual. The rate of hematopoietic reconstitution (cell number) post chemotherapy may be variable, however the pathway of cells progressing from the hematopoietic stem cell through the committed progenitor cell to the mature blood cell with respect to quantitative gene product expression remains fixed, permitting the identification of abnormal cells in a background of regenerating normal cells. Each gene product must be properly expressed at each time point for the cells to continue proper maturation.

CD33 was the only antigen studied that demonstrates a higher variability between individuals than within individuals. The amount of CD33 expressed on mature monocytes show a tight distribution, with an average within patient variation of 0.14 log units. The variation of means between individuals is nearly double that value, 0.25 log units, with 5 of 50 individuals expressing this antigen at almost one full log decade in reduced intensity. Interestingly, the intensity of CD33 on uncommitted progenitor cells and mature monocytes fluctuate together (Supporting Information Fig. 2). Although there is an underlying difference in total amount of CD33 for different individuals, the relative amounts (ratios) of this gene product expressed during maturation on different lineages remains constant. CD33 intensity has been previously reported to be variable and to correlate with disease outcome in patients treated with a drug antibody conjugate (23).

Early studies of quantitative gene product regulation were performed on cloned cell lines in which cells with the highest and lowest 10% of cell surface antigen markers were sorted and then placed back into tissue culture (24). Both the bright and the dim cell populations regenerated the entire original fluorescence distribution, indicating the mean intensity and distribution of expression was an inherent property of a population of identical cells. Quantifying the within patient variability for gene product expression on the reference populations parallels these earlier experiments, and

suggests that each individual cell can detect the amount of gene product on the surface and proceed to up or down regulate this amount. For many gene products this variability is minimal while for other gene products (such as CD13 and CD36) the regulation is not as stringent. The current data suggest that the target means change during development, however, they are the same from individual to individual for identical cell populations.

In retrospect, this concept is not surprising since each maturing cell must have the appropriate relationships between multiple gene products in order to either mature properly or to function as a mature cell. Without precise regulation of the amounts of gene products, cells would not be able to develop properly (as observed in acute leukemia) or would not be able to function properly once they are released out of the bone marrow (2,25). The mechanism for maintaining the precise amounts of gene products on these developing cells is unknown. Studies investigating these regulatory mechanisms require examining normal developing cell systems, since tissue culture cell lines derived from neoplastic cells have lost the precise regulation observed in normal maturing cells.

In this manuscript, we present a formal quantification of the amount of surface gene products expressed on normal developing cells in a controlled study of pediatric patients which provides the foundation of the "Difference from Normal" approach for detection of phenotypically aberrant cell populations. In the next companion manuscript, we identify the same reference populations in an adult cohort and show that gene product expression is invariant with age (26).

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