Supplementary Figure S5A). Furthermore, *Maff*- and *Hey1*-transduced cells exhibited a significantly higher myeloid contribution (Mac-1^{+}) in PB differentiation 16 weeks after transplantation (Supplementary Figure S5B). Moreover, *Maff*-transduced cells also exhibited significantly higher reconstitution in secondary recipients, whereas *Hey1*-transduced cells exhibited similar engraftment compared with the control (Supplementary Figure S5C). In addition, *Maff*-transduced donor cells but not *Hey1*-transduced donor cells exhibited a higher myeloid contribution in PB 12 weeks after secondary transplantation (Supplementary Figure S5D). Taken together, these results indicate that *Maff* and *Hey1* are both able to enhance the function of HSCs.

In summary, our study provides a new strategy to explore novel regulators of hematopoiesis or HSCs as well as valuable resources for future studies on hematopoiesis in the context of diseases such as leukemia. By using leukemic stress, we not only identified some known functional genes (*Egr1*, *Hes1*, *Nr4a2*, so on), but also defined several novel regulators for hematopoiesis and HSCs (*Maff*, *Hey1* and *Egr3*). Although *Maff* and *Hey1* were both important for the emergence of HSCs during embryonic development, they appear to have distinct roles in adult HSCs. As determined by CFU assay and transplantation, *Hey1* was able to enhance ST-HSC function, whereas the action of Maff was more specific to LT-HSCs. The candidates also contain many unstudied genes (*Nr4a3*, *pou2af1*, *Thbs1*, so on), some of which may have a functional role and others may regulate other genes on that list and are therefore worthy of future study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A recurrent immunophenotype at diagnosis independently identifies high-risk pediatric acute myeloid leukemia: a report from Children's Oncology Group

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Risk stratification of therapy for pediatric acute myeloid leukemia (AML) has been a focus of clinical protocol design to maximize treatment for high-risk groups while reducing therapeutic intensity for lower-risk groups. Molecular and cytogenetic markers have been used to define risk groups before therapy; however, 20% of pediatric cases lack all known markers[1] and ~60% of cases lack markers that stratify outcome.[2,3] The detection of measurable

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residual disease (MRD) by multidimensional flow cytometry (MDF) in the remaining 'standard risk' group has permitted the stratification of patients who lack genetic abnormalities linked to therapy outcomes.[4] However, discrimination between good and poor-risk groups based on the immunophenotype at diagnosis has not been sufficiently robust to routinely stratify patients. Previous reports have largely focused on the relationship between outcome and the expression of a single antigen.[5] Multidimensional immunophenotypes have been reported,[6–8] but none clearly identify risk cohorts.

By combining the quantitative expression of multiple antigens, not just their presence or absence, we have identified a previously uncharacterized multidimensional immunophenotype in a cohort of pediatric patients from Children's Oncology Group (COG) clinical trial AAML0531. We sought to determine the clinical, biological and outcome characteristics of patients with this phenotype.

Of the 1022 newly diagnosed pediatric patients with *de novo* AML (patients with acute promyelocytic leukemia and patients with Down syndrome were excluded) who enrolled in pediatric AML protocol COG-AAML0531, those that submitted a specimen for MDF at diagnosis and consented to MRD testing were eligible for this analysis ($N = 821$). Details of the COG-AAML0531 protocol have been previously described.[9] The initial diagnosis of AML was completed at each contributing institution; however, all immunophenotypic analysis for patients consenting to biological studies was performed centrally. The trial was conducted in accordance with the Declaration of Helsinki and registered at www.clinicaltrials.gov as NCT00372593.

The diagnostic immunophenotype for each of the 821 eligible patients was assessed, and 19 patients were identified with four unique, different-from-normal immunophenotypic features: bright CD56 expression (at minimum 2 log10 units greater than normal myeloid progenitors), dim-to-negative expression of CD45 and CD38, and lack of HLA-DR. A representative patient is shown in Supplementary Figure S1. This immunophenotype was initially observed in a non-COG protocol patient, who was identified to have MRD after day 100 post hematopoietic stem cell transplant (hSCT). This patient was monitored for MRD and the population persisted and expanded into morphologic relapse that resulted in disease-related death. The phenotype was named after the patient's initials (RAM) with documented informed consent. The 19 patients with the defining immunophenotypic features comprise the RAM cohort.

To determine the clinical, biological and outcome characteristics of this reoccurring phenotype, RAM cohort patients were compared with non-RAM patients ($N = 802$). As the RAM phenotype has remarkably high expression of CD56, a surface antigen previously associated with poor outcome in AML,[10,11] the non-RAM cohort was subdivided into a CD56-positive (CD56+ non-RAM) cohort ($N = 166$) and a CD56-negative (CD56 −) cohort ($N = 636$). Sub-analysis compared the RAM cohort, the CD56+ (non-RAM) cohort and the CD56 − cohort to further evaluate the prognostic significance of CD56 expression. Evaluation of the surface gene product expression of CD56, HLA-DR, CD38 and CD45 revealed that the RAM cohort has a multidimensional phenotype distinct from both the CD56+ (non-RAM) and CD56 − cohorts (Figure 1). Further, immunophenotypic analysis combined with morphologic, genetic and clinical features suggest that this phenotype is a unique entity distinct from previously reported CD56+ leukemias, including natural killer/myeloid and plasmacytoid dendritic cell leukemias.[8,12]

Patients in the RAM cohort had a median age at diagnosis of 1.26 years (range 0.75–16.9), which was significantly younger compared with 10.1 years (range 0.01–29.8) in non-RAM patients

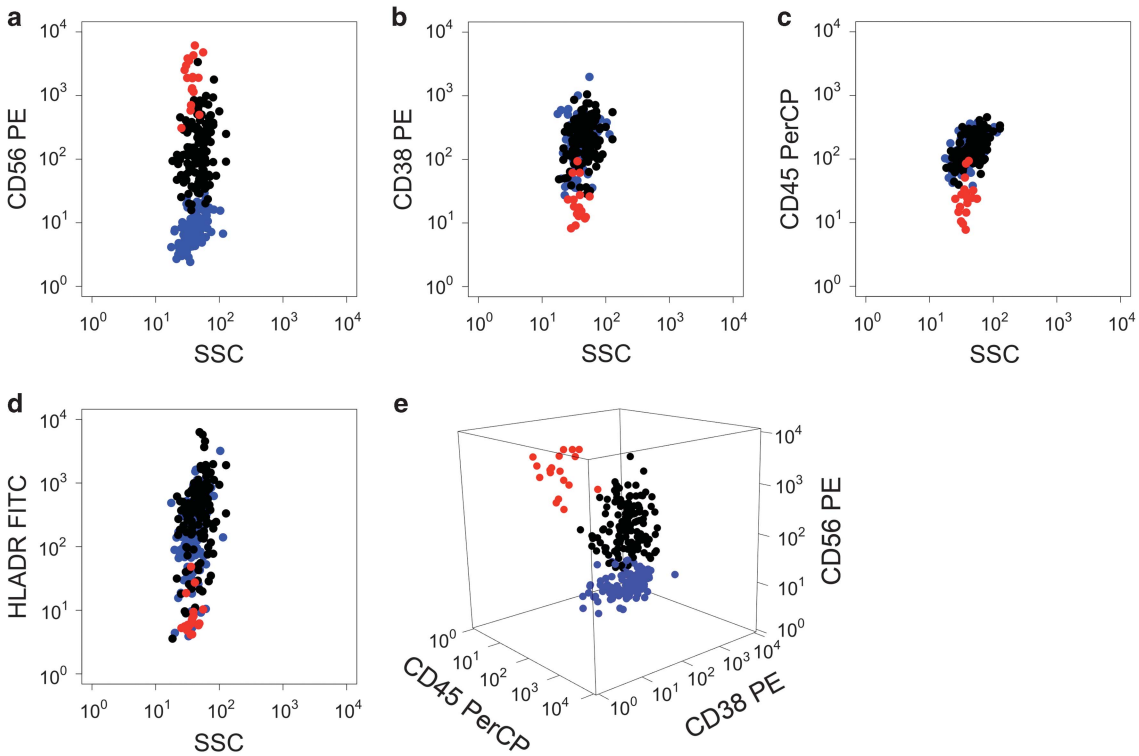

Figure 1. Comparison of RAM, CD56+ (non-RAM) and CD56 − immunophenotypes. The mean fluorescence intensities of CD56, HLA-DR, CD38, CD45 and side scatter parameters were computed for the leukemic cells of each patient. These mean fluorescence intensities of each antigen are plotted (**a**–**d**), where one dot corresponds to one patient. Phenotypes were compared between the 19 patients in the RAM cohort (red), 100 randomly selected patients in the CD56+ cohort (black) and 100 randomly selected patients in the CD56 − cohort (blue). None of CD56 (**a**), HLA-DR (**b**), CD38 (**c**) nor CD45 (**d**) parameters independently identify patients in the RAM cohort. However, collectively, a three-dimensional plot of CD56, CD38 and CD45 (**e**) reveals the distinct multidimensional phenotype of patients in the RAM cohort.

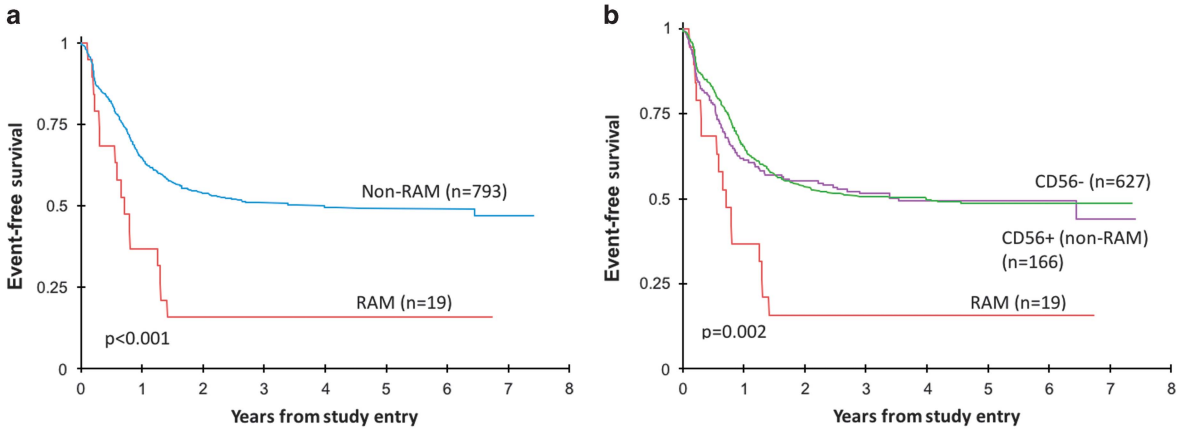

Figure 2. Three-year event-free survival (EFS) of patient cohorts. (**a**) compares the EFS of the RAM cohort (red) versus the non-RAM cohort (blue; $P < 0.001$). (**b**) compares the EFS of the RAM cohort (red), the CD56+ (non-RAM) cohort (purple) and the CD56− cohort (green; $P = 0.002$).

($P < 0.001$). The CD56+ and CD56− cohorts demonstrated similar age distributions to the non-RAM cohort (Supplementary Table S1). No significant trends were observed for differences in WBC, BM blast %, platelet count or hemoglobin levels between RAM and non-RAM cohorts (Supplementary Table S1).

Comparison of cytogenetic and molecular markers demonstrated that all RAM patients had intermediate-risk cytogenetics and lacked molecular risk features (*FLT3*-ITD, *CEBPA* or *NPM1* mutations; Supplementary Table S1; Supplementary Table S2). Of patients in the RAM cohort, 7 had normal cytogenetics, 1 had trisomy 8 and 11 had other cytogenetic abnormalities not associated with prognostic subgroups. Sub-analysis of the CD56+ (non-RAM) and CD56− cohorts demonstrated heterogeneous cytogenetic risk. The CD56+ (non-RAM) cohort had a higher prevalence of *t*(8;21) (31% vs 0%, $P = 0.004$) and 11q23 (35% vs 0%, $P = 0.002$) compared with RAM and the CD56− cohort (*t*(8;21): 31% vs 10%, $P < 0.001$; 11q23: 35% vs 17%, $P < 0.001$). Out of 133 patients with *FLT3*/ITD mutations, 125 had a CD56− immunophenotype (Supplementary Table S2).

Overall risk stratification revealed that 100% of RAM patients were standard risk. The non-RAM cohort was stratified as 48% standard risk ($P < 0.001$, compared with RAM cohort), 38% low risk ($P < 0.001$, compared with RAM cohort) and 14% classified as high risk. The CD56+ (non-RAM) and CD56− cohorts demonstrated a similar risk stratification breakdown in comparison to the non-RAM cohort (Supplementary Table S1).

The RAM cohort had a higher prevalence of the French-American-British (FAB) M7 subtype (38%) compared with the non-RAM (5%, $P < 0.001$), CD56+ (non-RAM; 2%, $P < 0.001$) and CD56− cohorts (5%, $P < 0.001$). Of patients in the CD56+ (non-RAM) cohort, 36% were classified as M2 and 39% were classified as M5. The CD56− cohort demonstrated heterogeneity of FAB classification (Supplementary Table S2).

Complete remission (CR) was defined by morphologic response with blasts identified at <5% and was performed at the submitting institution. The CR rate after initial induction for the RAM cohort (58%) was lower, but not significantly, compared with non-RAM patients (73%, $P = 0.137$; Supplementary Table S3). There were no significant differences in CR rates between the CD56+ (non-RAM) and CD56− cohorts.

Response to therapy was also assessed by MDF (Supplementary Table S3). The diagnostic RAM phenotype was detected at end of induction #1 (EOI) in 16/19 cases (84%) at a level of 0.02–41% (median 0.3%). The MRD-positive rate of the RAM cohort (84%) was significantly higher than the non-RAM cohort (33%, $P < 0.001$). In sub-analysis, the RAM cohort had a much higher MRD-positive rate than the CD56+ (non-RAM) cohort (29%, $P < 0.001$) and the CD56− cohort (33%, $P < 0.001$).

Evaluation of clinical outcome demonstrated that the RAM cohort had a 3-year event-free survival (EFS) of 16% compared with 51% for the non-RAM cohort ($P < 0.001$; Figure 2a). Within sub-analysis, the 3-year EFS of RAM was notably worse than the CD56+ (non-RAM) cohort (52%, $P = 0.003$) and CD56− (51%, $P < 0.001$) cohort (Figure 2b). In addition, the RAM cohort had a worse overall survival (OS) compared with non-RAM patients (26% vs 69%, $P < 0.001$). In sub-analysis the OS of the RAM cohort was markedly worse than the CD56+ (non-RAM) cohort (26% vs 66%, $P < 0.001$) and the CD56− cohort (26% vs 70%, $P < 0.001$; Supplementary Table S3).

Of the RAM patients who achieved a morphologic CR, the cumulative incidence of relapse (RR) was 82%, which was significantly higher than non-RAM patients (36%, $P < 0.001$). Sub-analysis revealed that the RAM cohort has a higher RR compared with the CD56+ (non-RAM) cohort (82% vs 37%, $P = 0.003$) and CD56− cohort (82% vs 36%, $P = < 0.001$; Supplementary Table S3).

To define the clinical significance of RAM phenotype in the context of other prognostic markers, we performed univariable and multivariable Cox regression analyses that included age and FAB class (Supplementary Table S4). In both univariable and multivariable analysis, identification of the RAM phenotype at diagnosis is an independent prognostic factor for OS (univariable hazard ratio (HR) = 3.06, $P < 0.001$; multivariable HR = 3.51, $P < 0.001$), RR (univariable HR = 3.48, $P < 0.001$; multivariable HR = 3.39, $P = 0.012$) and disease-free survival (univariable HR = 3.72, $P < 0.001$; multivariable HR = 4.28, $P < 0.001$). A goodness-of-fit test (using the −2 log likelihood) was performed to compare the fit of such multivariate models with and without the RAM phenotype. Adding RAM to the multivariate models significantly improved model fits ($P < 0.05$) for OS and EFS at study entry, and OS, RR and disease-free survival after the first course of chemotherapy, providing further evidence that the RAM phenotype is predictive of response independent of known risk factors.

Because all RAM patients were standard risk at study entry, an adjustment for cytogenetic definitions, molecular definitions, and risk classifications were not included in these analyses. In addition, these analyses did not adjust for MRD-positive status by MDF after EOI1, as this is a response to therapy indicator and not assessed at study entry. Furthermore, these analyses were not adjusted for hSCT as seven patients withdrew in an earlier course. Only 1 of 12 patients who completed the protocol received hSCT.

Prior studies have implicated CD56 expression with clinical outcome.[10,11] However, in this study, CD56 expression as a single

measure was not useful for predicting patient outcomes. CD56+ non-RAM patients had a similar outcome to CD56− patients, suggesting that it is not the mere expression of CD56 antigen, but its complementary antigen expression that confers the poor response to therapy. Of note, the RAM phenotype is primarily restricted to infants and very young patients, suggesting that this phenotype is a pediatric entity.

In this report, we present a unique diagnostic immunophenotype (RAM phenotype) that identifies otherwise standard risk pediatric patients with high induction failure rate and extremely poor outcome. Clinical outcome of patients with the RAM phenotype is comparable to the worst prognostic features in *de novo* AML (*FLT3*-ITD and high-risk cytogenetics).[13–15] Analysis of this phenotype in the ongoing COG-AAML1031 trial will further validate these findings.

CONFLICT OF INTEREST

LEB, AJM, LP, APV and MRL are employed by Hematologics, Inc. MRL is an equity owner of Hematologics, Inc.

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Effect of measurable ('minimal') residual disease (MRD) information on prediction of relapse and survival in adult acute myeloid leukemia

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The likelihood of therapeutic resistance (that is, failing to achieve complete remission (CR) or relapsing from CR) varies widely in adult acute myeloid leukemia (AML). Conceivably, accurate identification of patients who will have poor outcomes with standard therapies would enable their assignment to investigational treatments and facilitate interpretation of trial results. Yet, our previous studies indicated significant limitations in our ability

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