5. Prochnow C, Bransteitter R, Klein MG, Goodman MF, Chen XS. The APOBEC-2 crystal structure and functional implications for the deaminase AID. Nature. 2007;445:447-451.
6. Larijani M, Petrov AP, Kolenchenko O, Berru M, Krylov SN, Martin A. AID associates with single-stranded DNA with high affinity and a long complex half-life in a sequence-independent manner. Mol Cell Biol. 2007;27:20-30.
7. McBride KM, Barreto V, Ramiro AR, Stavropoulos P, Nussenzweig MC. Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. J Exp Med. 2004;199:1235-1244.
8. Ito S, Nagaoka H, Shinkura R, et al. Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. Proc Natl Acad Sci U S A. 2004;101:1975-1980.

Response

Cautious interpretation of assessment of AID variant activities using cells with endogenous AID expression

In their correspondence, although van Maldegem and colleagues confirmed much of what we reported,[1] they concluded instead that activation-induced cytidine deaminase (AID) splicing variants were nonfunctional. They first used a green fluorescence protein (GFP) reversion assay and demonstrated that AID variants can mutate this construct permitting GFP expression. However, they also found that various anticipated negative controls exhibited activity, prompting them to conclude that AID variants are nonfunctional. Regarding the interpretation of these results, it was not clear to us if they were challenging our results by concluding that AID variants were functionally inactive, or if they were questioning the accuracy of the assay itself. We believe it is difficult to interpret results emerging from experimentation in which the negative controls behave unexpectedly. Indeed, if their assertion is correct, it is reasonable that one should also be skeptical about the functionality of the full-length AID protein (AID-FL). We agree there is merit in use of alternative negative controls provided they are shown to indeed qualify as true negative controls.

The authors also provide evidence that GFP reversion frequency correlates with the expression level of yellow fluorescence protein (YFP), and that YFP also acquired mutations which would be an artifact, possibly contributing to the GFP^+ population. The authors argue this may be further evidence that the GFP assay is not reliable in their hands and further suggest endogenous AID may be active in the NIH 3T3 cells used in their assay. Based on the presence of endogenous AID expression alone, we think the assay used under their conditions appears to be problematic. However, whether this is specific to an NIH 3T3–based assay in general or is unique in this case due to some unexpected contamination remains to be seen. However, our assay uses the mouse pre–B-cell line 70Z/3, and these cells do not express any detectable endogenous AID[2,3] thereby lessening the possibility this was an issue in our system.

The authors also used an in vitro deamination assay that showed bacterially expressed AID variants did not display any detectable activity, whereas AID-FL did. It remains possible that the lack of activity displayed by the variants reflects instead a lack of proper protein folding or alterations in the specificity for the synthetic DNA substrate. Most importantly, although this assay has been used in several publications, its relevance to the in vivo function of AID proteins remains largely unknown. Indeed, other investigators recently set out to specifically address this question and concluded that "DNA deamination activity does not represent the physiological function of AID."[4]

Finally, because intracellular localization of AID may spatially regulate the function of AID protein in cells the authors used localization studies to argue that because AID-ivs3 and AID-dE4 did not undergo nucleocytoplasmic shuttling like AID-FL, this is additional evidence that AID variants are functionally inactive. We believe it is problematic to view AID localization as a surrogate measure of activity because localization and deaminase activity are each dictated by their specific sequences. It is also worth noting that we only minimally tagged FL-AID and its variants with a 10-amino-acid Flag epitope, which differs considerably from the 238-amino-acid GFP protein used by the authors. Actually, the dissonant localization of N-terminally and C-terminally tagged AID or its variants observed by the authors suggests that those proteins are altered by the GFP tag.

Xiaosheng Wu and Diane F. Jelinek

Department of Immunology, College of Medicine, Mayo Clinic, Rochester, MN

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Diane F. Jelinek, Department of Immunology, College of Medicine, Mayo Clinic, 200 First Street SW, Gugg. 4, Rochester, MN 55905; e-mail: jelinek.diane@mayo.edu.

References

1. Wu X, Darce JR, Chang SK, Nowakowski GS, Jelinek DF. Alternative splicing regulates activation-induced cytidine deaminase (AID): implications for suppression of AID mutagenic activity in normal and malignant B cells. Blood. 2008;112:4675-4682.
2. Wu X, Geraldes P, Platt JL, Cascalho M. The double-edged sword of activation-induced cytidine deaminase. J Immunol. 2005;174:934-941.
3. Wang CL, Yang DC, Wabl M. Directed molecular evolution by somatic hypermutation. Protein Eng Des Sel. 2004;17:659-664.
4. Shivarov V, Shinkura R, Honjo T. Dissociation of in vitro DNA deamination activity and physiological functions of AID mutants. Proc Natl Acad Sci U S A. 2008;105:15866-15871.

To the editor:

C/EBPA methylation is common in T-ALL but not in M0 AML

The CCAAT/enhancer binding protein α (C/EBPA) is a stage-specific transcription factor that controls proliferation and differentiation toward a myeloid and against a T-lymphoid fate.[1] Nonconditional targeted disruption of C/EBPA leads to a selective early block to granulocyte maturation,[2] similar to that observed in acute myeloid leukemia (AML), without affecting other hematopoietic lineages. The function of C/EBPA can be inhibited by several mechanisms in AML, including mutation,[3] (post)transcriptional modulation,[4] posttranslational inhibition,[5,6] and, more recently, epigenetic modification of C/EBPA expression.[7] Recently, Wouters

Table 1. C/EBPA^{meth} status of 99 T-ALLs as a function of their stage of maturation arrest

	Total	IM0	IMδ	IMγ	IMβ	TCRγδ	PREαβ	TCRαβ
C/EBPA^{meth}	37	1	3	6	5	8	12	2
Non-C/EBPA^{meth}	62	7	4	6	5	8	16	16

IM indicates immature, cTCRβ-negative T-ALL; TCR, T-cell receptor; and PRE-αβ, cTCRβ expression in absence of a surface TCR.

Table 2. C/EBPA^{meth} status of 99 T-ALLs as a function of their immunophenotype

	CD117	CD2	CD13	CD33	CD34	CD56	CD123	CD1a	CD4	CD8	CD10
C/EBPA^{meth}	17*	53*	12	22	29	9	18	49	58	35	38
Non-C/EBPA^{meth}	3*	84*	18	13	42	14	20	40	51	52	30

Numbers represent the percentage of positive cases for each antibody tested.
*$P < .05$.

et al,[8] using a bisulfite genomic sequencing method, defined a novel specific subgroup of AML with C/EBPA silencing by methylation (*C/EBPA^{meth}*) and T-lymphoid features (expression of cytoplasmic [c] CD3 and CD7 transcripts, T-cell receptor γ [TCRγ] rearrangement, and *NOTCH1* mutation [*NOTCH1^{m}*]).

We initially searched for *C/EBPA^{meth}* by methylation-specific polymerase chain reaction (MSP), as described by Chim et al,[7] in 5 AML with down-regulated *C/EBPA* by transcriptional profiling, and confirmed that the 3 cases (French-American-British [FAB]–M0/M1) with *C/EBPA^{meth}* demonstrated cCD3 (n = 1), CD7 (n = 3), and/or TCRγ (n = 1) rearrangement. The specificity of the MSP was investigated by DNA sequencing, which showed the expected patterns of bisulfite-induced changes, in keeping with the cases described by Wouters et al.[8] To further investigate *C/EBPA^{meth}* in early T and myeloid acute leukemia, we searched for *C/EBPA^{meth}* and mutation and *NOTCH1^{m}* in 99 T-ALL. Diagnosis of T-ALL was based on European Group for Immunophenotyping of Leukemia (EGIL) criteria and expression of cCD3 and CD7. TCR-based classification of T-ALL[9] was performed centrally at Necker-Enfants Malades Hospital (Paris, France), using at least cCD3, CD3, TdT, CD2, CD5, CD7, CD1a, CD4, CD8, TCRαβ, TCRγδ, βF1, CD34, CD117, CD13, CD33, MPO, CD10, CD19, and cCD79a antibodies. No *C/EBPA* mutations were found, but 37/99 (37%) demonstrated *C/EBPA^{meth}* and 47/90 (52%) *NOTCH1^{m}*. The stage of maturation arrest and immunophenotype of T-ALLs as a function of their *C/EBPA^{meth}* status is detailed in Tables 1 and 2, respectively. *C/EBPA^{meth}* was relatively rare in the most immature T-ALL (IM0) with no evidence of TCR rearrangement (12%) and in mature sTCRαβ^{+} cases (11%) compared with all other categories (34/73; 47%). *C/EBPA^{meth}* T-ALL expressed CD117 more frequently ($P = .022$) and CD2 ($P = .001$) less frequently but did not differ significantly from non-*C/EBPA^{meth}* T-ALL with respect to expression of CD13, CD33, CD34, CD56, CD123, CD1a, CD4, CD8, or CD10. Biphenotypic acute leukemia (AL), defined by an EGIL score greater than 2, was seen in 1 *C/EBPA^{meth}* and 1 non-*C/EBPA^{meth}* cases. *NOTCH1^{m}* were seen in 63% of *C/EBPA^{meth}* T-ALLs, compared with 47% of non-*C/EBPA^{meth}* cases ($P = .14$).

In contrast to T-ALL, *C/EBPA^{meth}* was found in only 1 of 49 immature cCD3-negative AML (FAB M0[10]); this case was also CD7-positive. None of the 49 AML M0 expressed *NOTCH1^{m}*.

These data demonstrate that *C/EBPA^{meth}* is common in T-ALL but not in M0 AML. It cosegregates with cCD3 expression and the onset of TCR rearrangements and is common at all stages of maturation arrest other than IM0 and TCRαβ T-ALL. As such it is a marker of neither immaturity nor "myeloid lineage/biphenotypic acute leukemia" and is virtually specific to T-ALL with the context of acute T/myeloid leukemia. Distinction of AML from early T-ALL is essentially based on detection of cCD3, which can be difficult to standardize in a multicenter setting. Detection of *C/EBPA^{meth}* could potentially be used as an alternative, although methylation-specific PCR may not be easier to standardize. Since *C/EBPA^{meth}* was not seen in most T-ALL, other mechanisms of silencing must be operational. These data also raise issues regarding AML versus ALL protocol assignment and the use of demethylating agents in T-ALL. They also suggest that the potentially misleading "biphenotypic leukemia" label be reassessed.

Louis Terriou, Raouf Ben Abdelali, Christophe Roumier, Ludovic Lhermitte, John de Vos, Pascale Cornillet, Olivier Nibourel, Kheira Beldjord, Hervé Dombret, Guy Leverger, Vahid Asnafi, Claude Preudhomme, and Elizabeth Macintyre

This work was supported by the Laurette Fugain Association, the Fondation de France (Comité Leucémie), and the North-West Canceropole (Onco-Hematology Axis).

Contribution: C.P. and E.M. conceptualized and designed the research, analyzed data, and wrote the paper; L.T. and R.B.A. performed the research and wrote the paper; L.L., J.d.V., O.N., K.B., V.A., and C.R. analyzed data; and P.C., H.D., and G.L. provided data and samples.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Elizabeth Macintyre, Laboratoire d'Hématologie, Tour Pasteur, Hôpital Necker, 149-161, rue de Sèvres, 75743 Paris cedex 15, France; e-mail: elizabeth.macintyre@nck.aphp.fr.

References

1. Laiosa CV, Stadtfeld M, Xie H, de Andres-Aguayo L, Graf T. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU. 1 transcription factors. Immunity. 2006;25:731-744.
2. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A. 1997;94:569-577.
3. Preudhomme C, Sagot C, Boissel N, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). Blood. 2002;100:2717-2723.
4. Pabst T, Mueller BU, Harakawa N, et al. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha int(8;21) myeloid leukemia. Nat Med. 2001;7:444-451.
5. Perrotti D, Cesi V, Trotta R, et al. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. Nat Genet. 2005;30:48-58.
6. Helbling D, Mueller BU, Timchenko NA, et al. CBFB-SMMHC is correlated with increased calreticulin expression and suppressesthe granulocytic differentiation factor CEBPA in AML with inv(16). Blood. 2005;106:1369-1375.
7. Chim CS, Wong AS, Kwong YL. Infrequent hypermethylation of CEBPA promotor in acute myeloid leukaemia. Br J Haematol. 2002;119:988-990.
8. Wouters BJ, Jordà MA, Keeshan K, et al. Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced CEBPA and mutations in NOTCH1. Blood. 2007:110:3706-3714.
9. Asnafi V, Beldjord K, Boulanger E, et al. Analysis of TCR, pT(alpha) and RAG-1

in T acute lymphoblastic leukemias improves understanding of early human T lymphoid lineage commitment. Blood. 2003;101:2693-2703.

10. Roumier C, Eclache V, Imbert M, et al. M0 AML, clinical and biologic features of the disease, including AML1 genemutations: a report of 59 cases by the Groupe Francais d'Hematologie Cellulaire (GFHC) and the Groupe Francais de Cytogenetique Hematologique (GFCH). Blood. 2003;101:1277-1283.

Response

CEBPA promoter hypermethylation in a subset of myeloid/T-lymphoid leukemias with a distinct gene expression profile

With great interest we have read the letter by Terriou and coworkers, who have performed an extensive survey of *CEBPA* CpG promoter hypermethylation in T-ALL as well as in a selection of immature acute myeloid leukemia (AML) cases. This study relates to our previous work in which we identified hypermethylation of the proximal *CEBPA* promoter in a small subset of AMLs with low *CEBPA* mRNA.[1] It should be noted that the patient group we described was not primarily defined by *CEBPA* promoter hypermethylation. Instead, those cases were studied because they exhibited a unique gene expression profile. In addition to this most discriminating feature, the leukemic blasts coexpressed CD34 and T-cell antigens with myeloid markers, frequently carried *NOTCH1* mutations, and expressed the transforming gene *TRIB2*.[1,2] Using a predictive gene expression signature in an independent cohort of AML, we identified genetically and immunophenotypically similar cases with silenced *CEBPA*.

In the present study, Terriou et al found *CEBPA* promoter hypermethylation in 4/54 AML cases. These 4 cases all revealed one or more T-cell characteristics (cCD3, CD7, and/or *TCRG* rearrangement) and may therefore resemble the leukemias we described. In T-ALL, the investigators found *CEBPA* promoter hypermethylation more frequently, in 37/99 cases. Importantly, however, only a minority of those T-ALLs with methylated *CEBPA* coexpressed myeloid surface markers (CD13 and/or CD33) and CD34. The findings of Terriou et al therefore indicate that *CEBPA* proximal promoter hypermethylation in combination with an immature myeloid/T-lymphoid immunophenotype is generally rare in acute leukemia, and can be found in a small percentage of AML as well as in a small fraction of T-ALL. It will be important to apply gene expression profiling to these cases to fully assess their relationship with the leukemias that we described previously.

Together, these observations highlight the challenges in classifying particular acute leukemias as AML, ALL, or a unique entity. We believe that studies as the one performed by Terriou et al will be instrumental in obtaining a better understanding of this topic. Gene expression profiling applied to these AML and T-ALL cohorts will clearly be of added value. Combining data from multiple research groups will be a requirement to address important questions regarding prognosis, and should help to evaluate according to which protocols these relatively small patient groups are best treated.

Bas Wouters and Ruud Delwel

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Bas Wouters or Ruud Delwel, Department of Hematology, Erasmus University Medical Center, PO Box 2040, 3000CA, Rotterdam, The Netherlands; e-mail: b.wouters@erasmusmc.nl or h.delwel@erasmusmc.nl.

References

1. Wouters BJ, Jorda MA, Keeshan K, et al. Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced CEBPA and mutations in NOTCH1. Blood. 2007;110:3706-3714.
2. Keeshan K, He Y, Wouters BJ, et al. Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous leukemia. Cancer Cell. 2006;10:401-411.

To the editor:

JAK2-V617F–triggered preemptive and salvage adoptive immunotherapy with donor-lymphocyte infusion in patients with myelofibrosis after allogeneic stem cell transplantation

Primary myelofibrosis is a myeloproliferative disease, and results of conventional treatment remain unsatisfactory.[1,2] Allogeneic stem cell transplantation after dose-reduced conditioning has become a reasonable, curative treatment option.[3,4] Single case reports about successful donor lymphocyte infusion (DLI) for relapsed patients provided evidence of a graft-versus-myelofibrosis effect.[5-7] Here, we report on 17 patients with either myelofibrosis (n = 16) or secondary AML post myelofibrosis (n = 1) and a median age of 52 years (range, 32-63 years) who received DLI from related (n = 5) or unrelated (n = 12) donor, either for clinical relapse (salvage DLI; n = 9) or residual disease monitored by JAK2 mutation level in peripheral blood (preemptive DLI; n = 8). Details are summarized in Table 1. One patient (no. 9) received DLI twice: once for molecular residual disease and once for reappearance of molecular disease. Sixteen patients were JAK2-V617F–positive. The median time from transplantation to first DLI was 269 days (range, 127-1570 days). The median percentage of JAK2V617 mutation level in peripheral blood before first DLI was 6.2% (range, 0.2%-72.8%) and significantly higher in patients with clinical relapse than with molecular relapse (24.7% vs 0.37%; $P = .03$). The median cell dose of the first DLI was 10^6 $CD3^+$ cells per kilogram of body weight (BW; range, 0.5-9 $\times$ 10^6 cells/kg BW). Two patients received as first DLI CD4-selected T cells (5×10^6 $CD4^+$ cells/kg BW). Second and subsequent half-log–increased DLI were given if no graft-versus-host disease (GVHD) and no significant response were observed. The median interval between the first and the second DLI was 103 days (range, 43-661 days). The response to DLI for relapsed patients was determined by the response criteria of the International Working Group,[8] and for residual disease by quantitative real time JAK2-V617F polymerase chain reaction (PCR) performed from genomic DNA from peripheral blood as recently described.[9] The sensitivity