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Standing at odds: mutated RAS and hematopoietic stem cells

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doi:10.3324/haematol.2019.230029

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by the accumulation of myeloid leukemic blasts unable to complete differentiation. However, AML is a complex disease with variable outcomes and prognoses.[1] Underlying these heterogeneous phenotypes is the fact that each sub-type of AML is defined by a different set of mutations and is controlled by a specific transcriptional and signaling network distinct to that of normal stem and progenitor cells.[2] Genes mutated in AML are involved in gene regulation and include transcription factors, chromatin modifiers / remodelers, splicing regulators, DNA methyltransferases and signaling regulators that control the activity of inducible transcription factors. The result is a profound deviation from the normal differentiation trajectory, with each AML sub-type taking a different path and establishing its own cellular identity.[2,3] Most AML sub-types carry more than one mutation and, with the exception of MLL-translocations (which are a hallmark of pediatric AML[4]), for a number of sub-types it has been shown that the first oncogenic hit is not sufficient to cause overt leukemia. In AML patients, mutations in genes from different functional categories co-exist, and data from sequencing studies as well as mouse models support this notion.[5,6]

The t(8;21) translocation, occurring in 7% of adult *de novo* patients, is one of the most frequent cytogenetic aberrations in AML.[7] This translocation fuses the DNA-binding domain coding region of the hematopoietic master regulator RUNX1 (*AML1*) to the Eight-twenty-One (*ETO*, *RUNX1T1* or MTG8) gene, which encodes a nuclear co-repressor. The result is the formation of the AML1-ETO (alternatively named RUNX1-ETO) chimeric protein, which retains the ability to bind to RUNX1 binding motifs but lacks the transactivation domain of RUNX1.[8,9] Germline expression of full-length AML1-ETO in mice causes embryonic lethality,[10,11] but conditional expression in hematopoietic stem cells (HSC) leads to an initial expansion of myeloid progenitor cells, including HSC and granulocyte-macrophage progenitors (GMP). Such expansion was also seen with *AML1-ETO*-transduced human cord blood-derived HSC *in vitro*.[12] Fusion t(8;21) transcripts have been detected *in utero* and in postnatal blood samples[13] and remain expressed at low levels in blood samples from t(8;21) AML patients in long-term remission.[14] Furthermore, several *AML1-ETO*-expressing mouse models have failed to fully develop t(8;21) AML unless challenged by mutagenesis or aging,[15-18] indicating the necessity of additional secondary mutations. These findings suggest that this chromosomal rearrangement is the driver mutation establishing a pre-leukemic clone. This notion is supported by the finding that t(8;21) patients present with a number of different secondary mutations.[19] The most prominent of these mutations

occur in genes encoding for the growth factor receptors KIT and FLT3,[20,21] rendering these receptors chronically active, together with activating RAS mutations such as the K-RAS (G12D), all of which co-operate with AML1-ETO. Interestingly, K-RAS activating mutations occur late during leukemia development and are rarely detectable within the leukemic stem cell compartment[21] (Figure 1A). In myelodysplastic syndromes (MDS), it was shown that the order of acquisition of mutations makes a profound difference on the disease phenotype,[22] but whether the same stands true for t(8;21) AML is not known.

In this issue of *Haematologica*, Di Genua *et al.*[23] uncovered the mechanistic explanation for the absence of signaling pathway mutations, such as K-RAS activating mutations, within the t(8;21) pre-leukemic HSC compartment by analyzing hematopoietic development and gene expression in conditional murine knock-in models expressing human *AML1-ETO* and *K-RAS(G12D)* individually or in combination, resulting in the generation of an *Aml1*$^{ETO/+}$ (AM), a *K-Ras*$^{G12D/+}$ (KM), and a double-targeted *Aml1*$^{ETO/+}$; *K-Ras*$^{G12D/+}$ (AKM) mouse lines. Prior studies had shown that mice expressing *K-Ras*(G12D) from its endogenous locus develop MDS but not AML.[24] When combined with AML1-ETO in human hematopoietic cells, K-RAS(G12D) promoted leukemic transformation in murine transplantation models.[25] However, here expression levels were likely to be non-physiological and it had not been defined whether the presence of mutated RAS was compatible with the maintenance of *AML1-ETO*-expressing HSC. Di Genua *et al.* addressed these

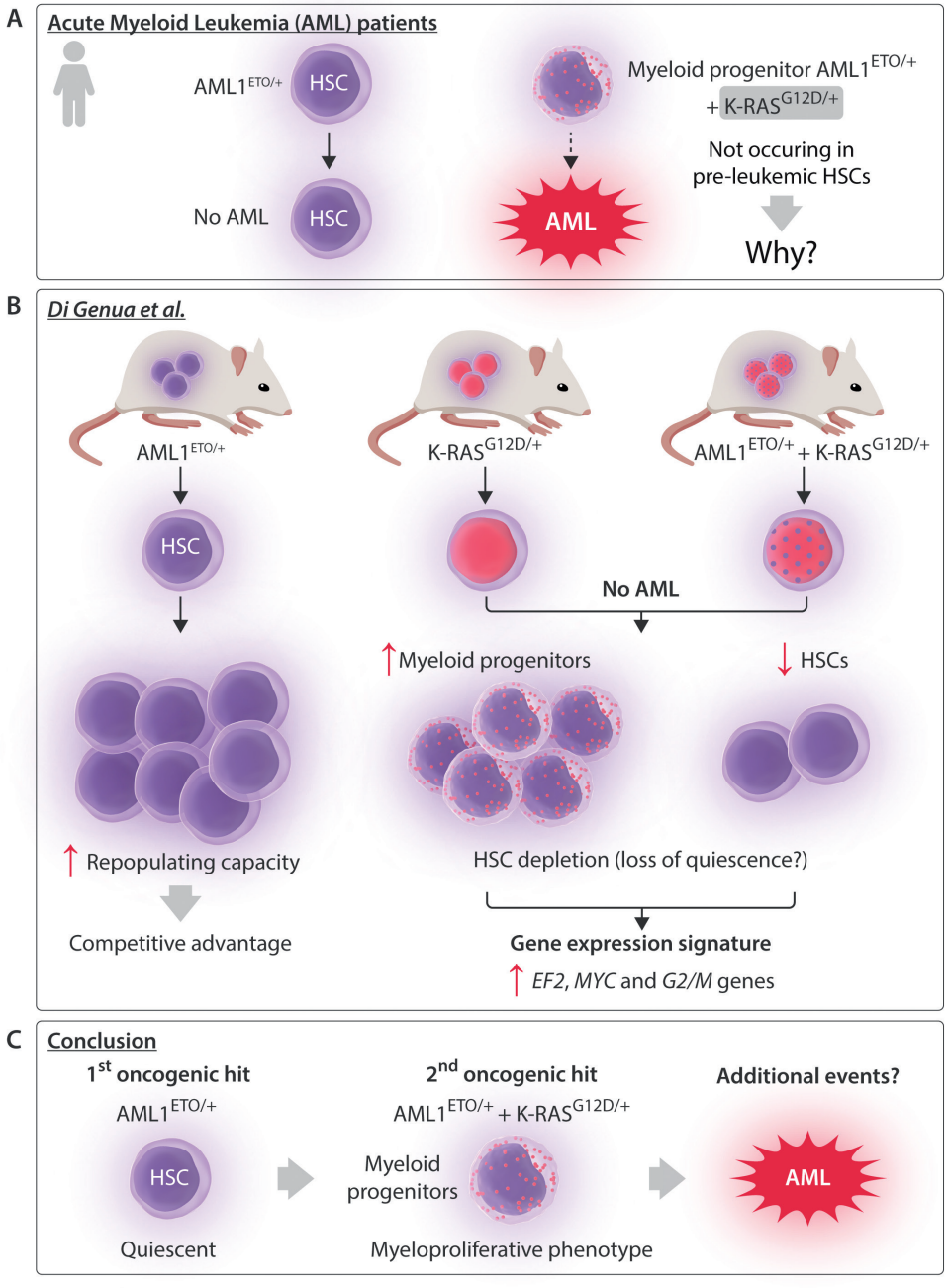

Figure 1. Co-expression of AML1-ETO and mutant RAS is incompatible with hematopoietic stem cell (HSC) maintenance. (A) In acute myeloid leukemia (AML) patients, HSC express *AML1-ETO*, but this is not sufficient to cause overt disease. AML leukemic blasts represent myeloid progenitors harboring the translocation that have undergone secondary oncogenic events, including mutations in signaling pathway genes such as the K-RAS$^{G12D/+}$. However, prior to Di Genua *et al.*,[23] the molecular explanation for the lack of mutations in signaling pathway genes in the pre-leukemic stem cells was unknown. HSC (purple cells), myeloid progenitors (purple cells with pink dots). (B) Di Genua *et al.*[23] generated conditional murine knock-in models expressing human *AML1-ETO* and *K-RAS(G12D)* individually or in combination: *Aml1*$^{ETO/+}$ (purple nuclei), K-*Ras*$^{G12D/+}$ (pink nuclei), and *Aml1*$^{ETO/+}$ *K-Ras*$^{G12D/+}$(pink and dotted nuclei), respectively, and performed competitive transplantation assays to compare wild-type and transgenic stem cell activity and measure global gene expression as indicated. (C) Model of step-wise oncogenesis in t(8;21) patients: the presence of t(8;21) in HSC results in a quiescent phenotype. Acquisition of K-RAS(G12D) occurs at later stages and results in increased proliferation. However, the double oncogenic event is not sufficient to develop overt AML, and the nature and order of acquisition of additional oncogenic events remains unknown.

issues by conditionally introducing both mutant genes from the respective genomic loci in mice in order to reproduce physiological oncogene expression levels. They then subsequently performed competitive transplantation assays to be able to directly compare stem cell activity in wild-type and transgenic cells (Figure 1B).

AML1-ETO expression on its own affected platelet, B- and T-cell development, and led to an increased number of functional HSC together with enhanced myeloid reconstitution in secondary recipients. Expression of mutant K-RAS on its own resulted in a myeloproliferative phenotype, led to a lack of HSC expansion, decreased engraftment and diminished *in vitro* re-plating potential, which, importantly, was seen regardless of the presence of AML1-ETO. Interestingly, co-expression of both oncoproteins led to a milder myeloproliferative phenotype but not overt AML, as seen in patients, and only aggravated the defect in platelet development. The authors suggest that the competitive advantage observed in *AML1-ETO*-expressing HSC was due to an enhanced self-renewal capacity. To further elucidate the mechanisms behind the functional impairment of *K-Ras(G12D)*-expressing HSC, they compared gene expression in HSC from genetically modified and control cells using transplantation assays. The presence of mutant K-RAS conferred increased cell cycle activity as well as upregulation of the expression of checkpoint associated genes, such as *E2F*, *Myc* and G2M-associated genes, as compared to HSC harboring the *AML1-ETO* transgene only. Moreover, the presence of mutated K-RAS protein resulted in the downregulation of gene expression signature associated with self-renewal activity and acquisition of a GMP-associated transcriptional signature. Overall, the transcriptional profiles of K-RAS(G12D)-expressing HSC resembled those of myeloid progenitors. Gene expression changes in double mutant cells were distinct from those of cells carrying the individual mutations. For example, two genes, *Gja1* and *Gzmb*, were up-regulated in the double mutant HSC and the authors suggest that they regulate the p53 pathway and oxidative phosphorylation, respectively, both increasing cell death and apoptosis.

Taken together, Di Genua *et al.*[23] show that expression of a mutant K-RAS is not compatible with a pre-leukemic state of murine AML1-ETO-expressing HSC. Although AML1-ETO alone confers a competitive advantage to HSC, the additional presence of K-RAS(G12D) results in loss of HSC function, most likely by exhaustion. The study shows that this phenomenon is explained by an increase in cell cycle activity leading to a loss of quiescence in HSC co-expressing both mutations. Therefore, they hypothesize that acquisition of mutant K-RAS in t(8;21) AML occurs at the myeloid progenitor stage rather than within pre-leukemic HSC compartment harboring the t(8;21) translocation. Overall, this study demonstrates that the order of appearance of each class of mutations is also relevant for leukemic transformation in AML.

Additional questions arise from this and previous work (Figure 1C). Firstly, Di Genua *et al.* show that, even in the presence of K-RAS(G12D), AML1-ETO is unable to cause an overt leukemic phenotype *in vivo*. The same was found in xenotransplantation experiments with retrovirally transduced human CD34$^+$ cord blood cells expressing *AML1-ETO* together with *N-RAS(G12D)*.[26] This notion ties in with the observation of Cabezas-Wallscheid *et al.*[16] that the development of AML in an AML1-ETO mouse model is a slow process, probably requiring multiple mutations or epigenetic reprogramming events, which were not examined in their study. Moreover, it has been previously shown that expression of *AML1-ETO* in an inducible transgenic mouse model leads to a block in differentiation, but not to an enhanced proliferation.[17] Di Genua *et al.*[23] add to this result that AML1-ETO-expressing pre-leukemic stem cells have a quiescent phenotype, leading to an accumulation of cells with high re-plating activity that are capable of going into cycle in a transplantation setting. Given that the double oncogenic event studied here is not enough to cause AML, the question that is now apparent is which additional events cause AML in patients. This work demonstrates that, even with a type of AML that has been studied in fine molecular detail for decades, the players of selection and clonal evolution in patients still have additional cards up their sleeve.

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Carfilzomib combination treatment as first-line therapy in multiple myeloma: where do we go from the Carthadex (KTd)-trial update?

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doi:10.3324/haematol.2019.228684

The prognosis and treatment of multiple myeloma (MM) patients have substantially changed in the last decade due to a better understanding of the disease and the introduction of novel agents (NA) with new mechanisms of action against malignant plasma cells.[1–3] In parallel with the improved understanding of myeloma biology, the field has witnessed a flood of NA, including immunomodulatory drugs (IMiD: thalidomide, lenalidomide, pomalidomide); proteasome inhibitors (PI: bortezomib, carfilzomib, ixazomib), monoclonal antibodies (mAb: daratumumab, elotuzumab),[4] and histone deacetylase inhibitors, which have substantially improved progression-free survival (PFS) and overall survival (OS). Other NA in clinical trials (selinexor, venetoclax, novel immunotherapeutics, iberdomide, and others) are being intensively tested, and specifically immunotherapeutics beyond mAb, such as bispecific T-cell engager (BITE) molecules and chimeric antigen receptor (CAR)-T cells will expand anti-myeloma therapy options.[1–4] Concomitantly, the application of tools that reliably assess "frailty" of patients is also helping with decision making, given that many patients with MM are elderly and often have significant comorbidities.[5–10] Sustained disease response is crucial in fit and in frail patients, since disease response can significantly improve quality of life and may reduce MM-induced comorbidity. Optimizing tolerability for timely treatment delivery has also proved beneficial.[11] However, this may prove challenging with triplet or quadruplet regimens that are being developed for continued therapy, where adverse events (AE) may lead to treatment interruptions and discontinuation.

After the introduction of the first PI bortezomib (Btz/V), second- and third-generation PI were developed, with the aim of providing therapy that would be potentially more efficacious and less toxic, including an improved polyneuropathy (PNP) side effect profile. Carfilzomib (Cfz/K) is a second-in-class, epoxyketone-based, irreversibly binding PI, which is approved in combination with dexamethasone (Kd) or lenalidomide and dexamethasone (KRd) for the treatment of relapsed/refractory MM (RRMM) patients.[12,13] The ENDEAVOR study compared Kd *versus* Btz plus dexamethasone (Vd) and reported a longer PFS and OS, with lower risk of painful PNP with Kd.[13] The ASPIRE study demonstrated the superiority of KRd over Rd, with unprecedented PFS benefit, as well as OS benefit in RRMM.[14] These studies have established the place of Cfz in treating RRMM.

Dyspnea, hypertension and cardiac toxicities stand out as clinically relevant side effects, and a widening experience of these has led to published guidance for the use of Cfz, as well as a re-appraisal of the baseline cardiovascular morbidity present in this patient group.[15] Such guidance provides a helpful description of expected events, as well as suggestions for subsequent monitoring, detection and management.[16,17] The analysis of cardiovascular adverse events (CVAE) in Cfz-treated patients revealed that, in those with CVAE, 91% had uncontrolled hypertension, with acute coronary syndrome or cardiac arrhythmias each present in 4.5%. Subjects with CVAE also had significantly higher blood pressure, left ventricular mass, and pulse wave velocity at baseline evaluation, compared to those without. Baseline uncontrolled blood