however, NK with a twist, having this atypical phenotype recently described as "adaptive" that resembles T cells in some ways. Unlike the typical mature CD56^{dim} NK cells described above, adaptive NK cells have a particular phenotype (PLZF^{low}, FcεRγ^{-}, SYK^{-}, EAT-2^{-}). In vitro functional studies prove these long-lived NK cells can produce IFN-γ in response to FcR engagement but not following IL-12 and IL-18 stimulation, consistent with previous descriptions of adaptive NK cells.

Corat et al shed yet more light on the mystery of orphan NK cells using the model of paroxysmal nocturnal hemoglobinuria (PNH). Patients with this disease have somatic X-linked mutations in *PIGA* which encode an enzyme essential for the synthesis of glycosylphosphatidylinositol (GPI) cell membrane proteins, including those protecting cells from complement-mediated red cell lysis. Patients can have stable levels of GPI-positive (GPI^{pos}) and GPI-negative (GPI^{neg}) red cells and neutrophils for many years, reflecting ongoing output from both mutated and unmutated HSPCs. In PNH, offspring of mutated and unmutated HSPCs can be identified by flow cytometric quantification of their surface GPI anchors. Knowing neutrophils mature directly from HSPCs, the authors use neutrophil GPI expression as an indirect measure of what is happening at the progenitor level in real time. If NK cells were all produced continuously from the same pool of HSPCs, we would expect the proportion of GPI^{neg} NK cells to be similar to the proportion of GPI^{neg} neutrophils. However, we find that circulating NK populations, particularly adaptive NK, have only small proportions of GPI^{neg} cells and NK cells from clones still normally expressing GPI persist longer than would be expected, suggesting the circulating GPI^{pos} NK cells are propagating independently of HSPCs (see figure). The lower proportions of GPI^{neg} NK cells in the circulation are in fact close to that of T cells which we know can propagate independently, suggesting NK cells do likewise.

The concept of self-renewing NK cells is very recent and characterizing them in humans is novel. These articles also make unique use of naturally occurring stem cell mutations to track progeny, which in these days of bar coding and cell trackers, is elegantly simple. These articles challenge current thinking about NK-cell development.

But what does all of this mean? What is the clinical significance of these longer-lived NK cells? Can they be harnessed to improve longevity of NK cellular therapies? Certainly there is potential for the detailed characterization provided in these articles to be exploited in this way. With a typical 2-week half-life, NK-cell therapy is a short-lived anticancer strategy that can be repeated if necessary. Strategies are under way to improve the proliferation and persistence of adoptively transferred NK-cell therapy using cytokines such as IL-15, but selecting or specifically expanding long-lived self-renewing NK cells to transfer could avoid the potential side effects of this and provide a pool of NK cells ready to respond to relapse. Further questions remain as to whether these new NK cells can kill tumor or virally infected cells and ensure they are not "exhausted" cells. If fully functional, as recent data suggest,[11] the findings presented could potentially open the door to a new era of long-lived NK-cell therapies.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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DOI 10.1182/blood-2016-12-755546

LYMPHOID NEOPLASIA

Comment on Rossi et al, page 1947

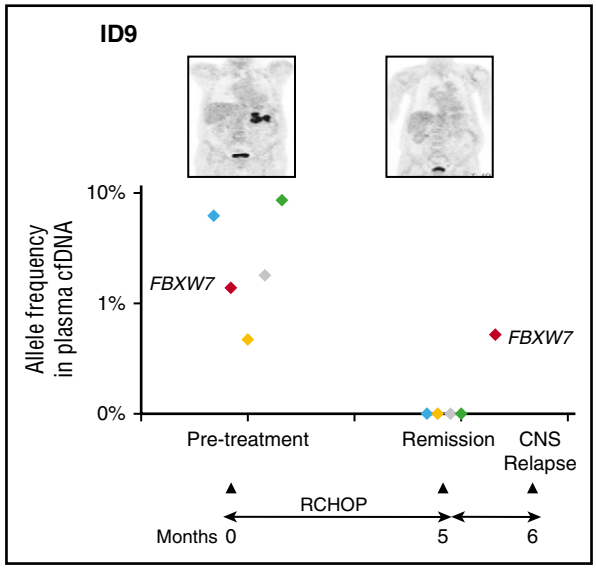
A flood of information from drops of blood

Eric D. Hsi CLEVELAND CLINIC

In this issue of *Blood*, Rossi et al demonstrate that the "liquid biopsy" has a future in the management of patients with lymphoma.[1]

Circulating tumor DNA (ctDNA) is the component of cell-free DNA (cfDNA) released into serum from tumor cells as a result of apoptosis, necrosis, and secretion. Over the last few years, researchers have begun to explore the possibility of diagnosis and monitoring of solid tumors using cfDNA.

In this proof-of-principle study, plasma samples were collected pretreatment, during rituximab-cyclophosphamide-doxorubicin-vincristine-prednisone (R-CHOP) immunochemotherapy, after therapy, and at progression from 30 patients with de novo diffuse large B-cell lymphoma (DLBCL). cfDNA was isolated and subjected to next generation sequencing (NGS) using a target capture strategy designed to test for mutations in 59 lymphoma-associated genes to a depth of at least 1000 times coverage in 80% of gene targets. Germ line DNA from patient granulocytes was also evaluated to ensure mutations detected were not spurious. At



Mutational profile from cfDNA in this patient (ID9) revealed a series of mutations in the pretreatment sample. Interestingly, the *FBXW7* mutation was not detected in the corresponding tumor biopsy. This mutation persisted despite clearance of other mutations from cfDNA and clinical remission. The patient relapsed in the CNS and the mutation was detected at a low level in cells from the cerebrospinal fluid. See Figure 7 in the article by Rossi et al that begins on page 1947.

diagnosis, mutations were found in 67% of patients' serum. Among the most frequently mutated genes were *KMT2D*, *TP53*, and *CREBP* (20%-30% of cases), *PIM1*, *TNFAIP3*, *EZH2*, *STAT6*, *TBL1XR1*, *B2M*, *BCL2*, *CARD11*, *CCND3*, *STAT6*, and *FBXW7* (10%-20% of cases). Importantly, analysis of 6 healthy donors showed no mutations.

To confirm that these mutations were also in the lymphoma tissue, 17 paired frozen samples were also analyzed. Seventy-nine percent of mutations seen in genomic tumor-derived DNA were found in the paired cfDNA. Limiting the tissue mutations to those with >20% variant allele frequency showed 95% of the mutations could be found in cfDNA. Of note, cfDNA samples from 2 of these 17 cases lacked detectable mutations. Thus, a minority of patients did not have abnormalities at diagnosis in cfDNA. Although 1 patient had stage 1 disease, the other had stage 4 disease suggesting that tumor burden is not the only factor. With regard to specificity, there were 16 mutations detected in the cfDNA samples that were not detected in tissue. Given the over 15 million potential nonsynonymous variants in the sequenced regions, this represents a very low potential false-positive rate (specificity of over 99.9%), assuming error is randomly distributed. An alternative consideration, with precedent in other systems, is that these mutations are not false positives but derive from a subclone present at a site other than that which was biopsied. This interpretation is supported by an example case (see figure) in which a *FBXW7* mutation seen in the pretreatment cfDNA sample was not found in the diagnostic biopsy tissue. However, it was present in the central nervous system (CNS) relapse sample and in a remission cfDNA sample before clinical relapse. Finally, cfDNA samples collected during therapy and in follow-up showed clearance of mutations in responding patients but not in refractory patients. New mutations were detected in refractory/relapsing patient ctDNA, potentially providing insight into clonal evolution, possible resistance mechanisms, and therapeutic targets.

This is not the first study examining cfDNA in DLBCL patients and, in aggregate, the evidence is mounting that cfDNA will have a role in diagnosis and management of patients with lymphoma. ctDNA is present at diagnosis in most if not all patients with DLBCL.[2,3] Two NGS strategies for analyzing ctDNA have been used. One has focused on immunoglobulin receptor *IGH VDJ* and *IGK VJ* gene rearrangement, a unique clonal marker for an individual patient's B-cell lymphoma. By sequencing a patient's lymphoma tissue to define the lymphoma-specific *IGH VDJ* and *IGK VJ* sequences (clonotype) and then analyzing cfDNA to search for the lymphoma clonotype,[4] investigators have shown feasibility and promising results. In a small series of DLBCL patients, ctDNA was superior to markers of disease burden such as lactate dehydrogenase in detecting active disease, correlated with mean tumor volume by positron emission tomography/computed tomography, and could detect disease at, or even before, relapse.[5] A larger series showed that a clonotype could be defined from biopsy tissues in 94 of 109 patients (86%) and from pretreatment serum from 54 of 86 samples (63%). Analysis of interim and surveillance serum samples showed that interim positivity predicted time to progression, and surveillance sample status had high positive and negative predictive values for recurrence, with identification of ctDNA up to 200 months before clinical relapse.[6] The other strategy (explored by Rossi et al) involves sequencing a selected set of genes known to be recurrently mutated in DLBCL, with or without simultaneous assessment for clonotype and common gene fusions.[3,7] This approach appears to be applicable in the great majority of cases and can provide rich data on the lymphoma mutational profile, clonal evolution, and resistance mechanisms. Furthermore, it may provide guidance for further therapy. Studies such as these suggest that this strategy also provides prognostic information, correlates with relapse, detects minimal residual disease prior to relapse, and outperforms clonotype analysis alone.[3] Of course, there is greater complexity in assay design, bioinformatic algorithms, and interpretation but preanalytical, technical, and computational advances will overcome these challenges.[8]

There is a pressing need for better methods to assess response, monitor, and identify resistance mechanisms and therapeutic targets in order to bring personalized medicine to fruition for the management of patients with DLBCL and other lymphomas. Feasibility and initial performance characteristics of cfDNA analysis are being established by several groups and provide tantalizing glimpses into the future.[1,3,5-7] Incorporation of these assays into clinical trials to define the role of cfDNA analysis in staging, risk stratification, interim response assessment, monitoring, and therapy selection will be required in order to use the liquid biopsy for routine care. The vast amount and rich content of this information, all derived from a few milliliters of serum, will transform the way in which we diagnose and manage patients with lymphoma.

Conflict-of-interest disclosure: E.D.H. has been a consultant for Seattle Genetics, HTG Molecular Diagnostics, and Alexion within the last year and receives research support from Eli Lilly, AbbVie, and Cellerant Therapeutics. ■

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DOI 10.1182/blood-2017-02-764647

● ● ● MYELOID NEOPLASIA

Comment on Laouedj et al, page 1980

S100 proteins in AML: differentiation and beyond

Jerome Tamburini INSTITUT COCHIN; UNIVERSITÉ PARIS DESCARTES

In this issue of *Blood*, Laouedj and colleagues make a new and important contribution to our understanding of cell differentiation impairment in acute myeloid leukemia (AML) by illuminating the role of S100 proteins and Toll-like receptor (TLR) signaling.[1] Although differentiation blockade has been recognized as a hallmark of AML for decades, direct therapeutic application of this concept has been limited to only a subtype of AML, acute promyelocytic leukemia (APL), in which the PML-RARA fusion protein is targeted by the differentiating agents retinoic acid and arsenic trioxide.[2] The extraordinary clinical outcomes achieved by this strategy in APL have motivated efforts to pharmacologically release the differentiation block in other AML subtypes as well. Encouraging results were observed recently in patients with *FLT3-ITD*–mutated AML who were treated with tyrosine kinase inhibitors,[3] and in preclinical models of *NPM1*- or *IDH1*-mutated AML treated with retinoic acid.[4,5] However, mechanisms of cell differentiation impairment in AML remain largely unknown, and no therapy is yet available to induce differentiation across multiple AML subtypes.

S100A8 and S100A9 belong to the S100 family of low-molecular-weight secreted proteins, which contribute to cellular processes including calcium homeostasis, cell growth, and differentiation by engaging membrane receptors such as RAGE or TLR4 and activating downstream signaling pathways.[6] In normal hematopoiesis, myeloid progenitor cells downregulate S100A9 to undergo macrophage and dendritic cell differentiation.[7] Additional evidence in solid tumor biology suggests that S100 proteins cause myeloid differentiation arrest and accumulation of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment, and that they directly exert pro-oncogenic effects on cancer cells as well.[6] It is therefore striking that the study by Laouedj and colleagues is the first to focus on the role of S100A8/A9 in the biology of AML.

Laouedj et al demonstrate that S100A8/A9 proteins are abundantly produced by AML cells in murine models and in primary patient samples. They unambiguously show that S100A8/A9 proteins are secreted primarily by leukemic cells, not by the microenvironment. Using selective therapeutic S100A8/A9 antibodies and recombinant peptides, they elegantly show that these proteins play critical roles in leukemia progression in an H9M1-overexpressing mouse model of AML. Both S100A8 blockade by an anti-S100A8 antibody and treatment with recombinant S100A9 proteins reduce the leukemic burden and significantly prolong survival in this model, with cytologic and flow-cytometric evidence that these agents induce features of myeloid differentiation. Mechanistically, the authors use a multiplex phosphoflow approach that incorporates specific anti-TLR4 antibodies to show that S100A9 induces TLR4-dependent intracellular signaling pathways such as those involving MAPK or NF-κB. Finally, the authors demonstrate that recombinant S100A9 proteins induce differentiation of primary leukemic blasts of the FAB M4/5, but not the M0/1 subtypes, ex vivo.

These data thus suggest a novel strategy for pharmacologic induction of AML cell differentiation with important therapeutic implications. Considering the precedent set by APL, we hypothesize that differentiating agents may be capable of synergizing with chemotherapy to achieve deep molecular responses and, in some cases, the cure of other AML subtypes (see figure). In fact, unbiased proteomic analysis has revealed that S100A8 expression is associated with poor survival in AML[8]; this finding is consistent with the in vivo antileukemic activity of therapeutic anti-S100A8 antibodies that was observed by Laouedj and colleagues.

Although the present study marks an important advance, multiple barriers to translation of these preclinical observations into clinical therapies remain. Chief among them is the need to determine which component of the S100A8/A9 dimer to target. S100A8 and S100A9 exert unique and sometimes opposing effects; even low doses of S100A8 can abrogate S100A9-induced differentiation. Moreover, the present study is based primarily on murine models. Relatively few primary AML samples were tested ex vivo, and among them, only the AML M4/5