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Myeloid leukemia switch as immune escape from CD19 chimeric antigen receptor (CAR) therapy

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Gardner *et al.* recently reported on two cases of mixed lineage leukemia $(MLL)^+$ acute myeloid leukemia (AML) occurring after CD19 chimeric antigen receptor (CAR) therapy for MLL^+ B-acute lymphoblastic leukemia (ALL) (1). This report provides new insights into mechanisms of immune escape from targeted immunotherapy and strategies to prevent their occurrence with adapted immune interventions.

Adoptive cell therapy utilizing genetically engineered T cells expressing second generation CARs specific for CD19 has shown remarkable efficacy against a range of chemo-refractory and relapsed B cell malignancies, most remarkably in ALL (2). A single infusion of CD19 CAR T cells may induce a complete remission in subjects whose leukemia has developed drug resistance and is unlikely to respond to allogeneic stem cell transplantation. This novel cell-based immunotherapy was highlighted, together with checkpoint blockade, as the basis for selecting cancer immunotherapy as the scientific breakthrough of the year in 2013 (3). CARs recognize tumor antigens irrespective of human leukocyte antigen (HLA) and can thus target tumor cells that have down-regulated HLA expression or proteasomal antigen processing, two mechanisms that contribute to tumor escape from T cell receptor (TCR)-mediated immunity (4). The advent of second generation CARs not only enables to retarget T cells but also to augment their functional capabilities (5).

The two cases recently reported by Gardner *et al.* include a 52-yo woman and an 18-mo girl with an initial diagnosis of MLL-rearranged B-ALL, who presented a lineage switch at relapse after CAR therapy. Several facets of leukemia biology and resistance to CAR therapy need to be taken into account in analyzing these observations.

The *MLL* gene is frequently involved in chromosomal translocations found in human acute leukemias characterized as AML, ALL or biphenotypic (mixed lineage) leukemia (6–8). *MLL* rearrangements are found in >70% of infant leukemias, whether the immunophenotype is that of ALL or AML (9). *MLL* translocations are also found in about 10% of AML in adults, and in therapy-related leukemias that develop in patients previously treated with

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topoisomerase II inhibitors for other malignancies. More than 50 different translocation fusion partners have been identified. Five of them account for 80% of all *MLL*-translocation-bearing leukemias: t(4;11)(q21;q23) or MLL-AF4, as reported here in the adult subject (case 1); t(9;11)(p22;q23) or MLL-AF9; t(11;19)(q23;p13.3) or MLL-ENL; t(10;11)(p12;q23) or MLL-AF10, as reported here in the infant subject (case 2); and t(6;11)(q27;q23) or MLL-AF6 (10,11). Children patients with MLL-rearranged ALL have a particularly poor outcome compared to children with other forms of ALL (12). Adult AML with MLL-AF9 is associated with a more aggressive disease that is more likely to exhibit resistance to chemotherapy (13). Leukemias that bear translocations involving the MLL gene on chromosome 11q21 possess unique clinical and biological characteristics, including the activation of self-renewal properties. MLL rearrangements may direct leukemogenic transformation of committed myeloid progenitors (14), suggesting that myeloid leukemias can originate not only from hematopoietic stem cells (HSCs) but also from committed myeloid progenitors lacking self-renewal capabilities. Some MLL fusion proteins direct a partial stem cell gene expression program in committed progenitors, coinciding with a gain of self-renewal properties. This program has been designated a self renewal-associated signature (15). Given that MLL fusions modulate chromatin structure through histone modification (through the loss of H3K4 methyltransferase activity), it is likely that the process of reactivation of self-renewal can be initiated through modulation of the epigenetic state of a cell.

Given these features of MLL^+ leukemias, the first question to address is whether the MLL^+ cases reported by Gardner *et al.* represent a true lineage switch at relapse or biphenotypic/bilineal leukemias at diagnosis. The authors excluded the second possibility based on the following phenotypic characterization:

I. Case #1

- **i.** Diagnosis: $MLL^+CD45^+CD19^+CD22^+CD38^+$HLA $DR^+CD15^+CD33^+CD13^{dim}TdT^{dim}$lymphoblasts;
- **ii.** Relapse: MLL^+CD19^-HLA-$DR^+CD64^+CD15^+CD3$ $3^+CD13^{dim}CD71^+MPO^+$ monocytic cells.

II. Case #2

- **i.** Diagnosis: $MLL^+CD45^+CD19^+CD38^+CD58^+CD22^{dim}$H LA-$DR^+CD34^+$lymphoblasts;
- **ii.** Relapse: $MLL^+CD45^+CD19^-CD4^+CD56^+CD64^+$CD $13^+CD33^+CD38^+$HLA$DR^+CD34^+CD71^+$myeloblasts.

Although specific lineages can be identified for most leukemias, there are instances in which both lymphoid- and myeloid-lineage markers, or T-cell and B-cell markers, coexist (16). The diagnosis of biphenotypic (divergent morphologic and immunophenotypic features uniformly present in one blast population)/bilineal (distinct blast populations in a single patient) leukemia was excluded by the authors according to the WHO classification which describes the mixed phenotype acute leukemia (MPAL) based on the expression of strictly specific T-lymphoid (cytoplasmic CD3) and myeloid (MPO) antigens, the latter shown by

either flow cytometry or cytochemistry and/or clear evidence of monocytic differentiation. B-cell lineage assignment in MPAL relies on the strong expression of CD19 together with another B cell-associated marker or, in cases with weak CD19, on the expression of at least three B-lineage markers. The European Group for the Immunological Classification of Leukemia (EGIL) defines MPAL by a scoring system based on the number and specificity degree of lymphoid and myeloid markers expressed by leukemic cells, with point values are greater than 2 for myeloid and 1 for lymphoid lineages. 2: B-lymphoid (CD79a, CD22, cyIgM) T-lymphoid (CD3) myeloid (MPO); 1: B-lymphoid (CD10, CD19), T-lymphoid (CD2, CD5), myeloid (CD13, CD33); 0.5: B-lymphoid (TdT), T-lymphoid (TdT, CD7), myeloid (CD14, CD15, CD11b, CD11c). Therefore, the reported cases seemingly represent a lineage switch at relapse, a phenomenon that is occasionally observed (17). By definition, this occurs when acute leukemias that meet the standard FAB criteria for a lineage (lymphoid or myeloid) at the time of initial diagnosis meet the criteria for the opposite lineage upon relapse. A lineage switch is an uncommon type of mixed leukemia, observed in 6–9% of relapsed acute leukemia (18,19). In ALL, most AML relapses occur during treatment within the first year of diagnosis, as is the case in this report.

The second question is how to explain the lineage switch at relapse. A lineage switch may represent either a relapse of the original clone with heterogeneity at the morphologic level or high plasticity (capacity of changing cell fate without altering genotype), or the emergence of a new leukemic clone (20). The relapsed disease maintained the MLL rearrangement in both cases. Lineage switching has been reported to occur more frequently in children than adults with most cases involving the conversion of ALL to AML (17). Changes in cell potentials can be explained by mechanisms operating at different levels: (I) at the cell-intrinsic level, possibly defined by epigenetic cues (which MLL fusions are capable of mediating); and (II) at the cell/environment interface including therapeutic pressure.

Kawamoto and Katsura have suggested that a myeloid potential is retained in erythroid, T-, and B-cell branches even after these lineages have segregated from each other (21,22). MLL-positive B-ALL show expression profiles consistent with early hematopoietic progenitors, raising the possibility that early bipotential or oligopotential progenitor cells may serve as a substrate for leukemogenic translocations and account for lineage switching events (23). Alternatively, the MLL translocation might induce a stem/progenitor cell phenotype, irrespective of the cell lineage targeted by the translocation, enabling the cellular environment to promote lineage conversions (18). Therapy may also facilitate or induce a subsequent lineage switch. Clonal selection induced by therapy might suppress or eradicate the leukemic clone that is apparent at the time of diagnosis, favoring expansion of a subclone with a different phenotype and/or disrupting the hematopoietic environment to add further selective pressure. In a human-based MLL leukemia mouse model, the microenvironment has been shown to be critical to lineage outcome, with manipulation of the in vivo cytokine milieu influencing the commitment of both lineage-restricted and multipotent leukemia initiating cells (24). The severe cytokine release syndrome (CRS) observed in the reported patients with high levels of IL-6 might have contributed to environmental changes that ultimately supports a lineage switch in a MLL^{+} clone (25). An elegant model of MLL-AF9-induced AML showed the importance of the microenvironment in providing instructive signals for leukemic lineage fates (26). It is possible that the human microenvironment and

the fusion protein cooperate in promoting the lineage phenotype arising from a primitive leukemic stem cell.

A lineage switch from $CD19^+$ to $CD19^-$ may thus account for resistance to CD19 CAR therapy. This mechanism stands in contrast to two other previously described immune escape mechanisms, based on either post-transcriptional editing or mutational loss of CD19 itself.

Jacoby *et al.* reported that murine E2a:PBX1-driven pre-B ALL $CD19^+$ cells treated with CD19 CAR T cells may undergo early and late relapse following CD19 CAR therapy. Early post CAR relapses retained a pre-B phenotype with isolated loss of CD19 extracellular expression by flow cytometry, loss in CD19 exon1 and 2 mRNA but intact mRNA for all other CD19 exons. In contrast, late relapses demonstrated complete loss of CD19 protein and mRNA expression with concomitant loss of the major B cell transcription factors PAX5 and EBF1 suggesting loss of the B-cell developmental program. Late post CD19 CAR relapses typically gained myeloid, stem cell or T cell phenotypic markers, consistent with a lineage switch, which was confirmed by RNA-seq of multiple late relapse samples. The authors demonstrated intermediate phenotype of post-CD19 CAR relapse in vivo with co-expression of both myeloid (Gr1, CD11b) and B cell markers (B220, CD22) on the same cells, suggesting a differentiation rather than a selection process. Leukemic relapse with CD19 expression loss that retain a B-cell program rapidly regain CD19 upon in vivo passage in the absence of CD19 CAR pressure. However, relapses due to lineage reprogramming retained a stable myeloid phenotype upon serial passage without regain of CD19 or other B cell markers (27).

Another example of CD19 post-transcriptional editing involves the clustering of nonsense and missense mutations in exon2 of CD19. Frameshift mutations clustered in the non-constitutive exon2 eliminate full-length CD19, but allow expression of the isoform that is mostly cytosolic (hidden from T cells) and its membrane fraction does not trigger killing by CART-19. However this isoform rescues defects in cell proliferation and pre-BCR signaling associated with CD19 loss (28). A case report of lineage switch from $CD19^+$ALL to $CD19^-$AML was also described following CD19-targeted therapy with blinatumomab (29).

In general, acquired resistance of tumor cells to immunotherapy may proceed as a Darwinian selection for genetic or epigenetic heritable traits that pre-exist in the tumor mass before a therapeutic intervention. This outcome is likely the consequence of genomic and epigenetic instability of transformed cells (*MLL* translocation for instance) as well as some therapeutic interventions that affect mutational load or tumor microenvironmental conditions. In the case of immunotherapy, tumor cells may also alter their gene expression in response to interactions with immune cells, such as PDL1 up-regulation in response of IFN-gamma, which may alter the risk of selecting for resistant clones.

The multiplicity of mechanisms accounting for antigen escape calls for adapted modifications to CAR therapy. The use of CD19 CARs targeting CD19 essential exons has been previously suggested (30), but this would only address a subset of CD19 losses. A more promising approach entails the targeting of a second, independently expressed antigen.

Thus, most CD19-negative relapses appear to retain expression of CD22, for which CARs have now been developed (31). For the cases reviewed here, this approach would require the identification of suitable AML targets. Four AML CAR targets have been reported to date. Lewis (Le)-Y, a difucosylated carbohydrate antigen, was targeted in a phase I study in four patients with relapsed AML. Infusion of a second generation CD28-based CAR resulted in stable/transient remission of three patients, who ultimately progressed despite T cell persistence (32). CD123 is the high-affinity interleukin-3 receptor α-chain. A partial remission was induced in a patient with FLT3-ITD$^+$ AML treated with a third generation CD123-CD28/CD137/CD27/CD3z CAR (33). Preclinical studies have however revealed significant myeloablation attributed to CD123 expression in early hematopoietic stem/progenitor cells (34). CD33 is a myeloid-specific sialic acid-binding receptor, that has been targeted by gentuzumab ozogamicin (35), which has shown survival benefit (36,37). Preclinical activity of CD33 CAR$^+$ CIK cells resulted in slowing disease progression (38) and CD33 CAR$^+$ T showed significant effector functions *in vitro* and *in vivo* with reduction of myeloid progenitors in mice (39). One AML patient treated with CD33 CAR T cells at the Chinese PLA General Hospital, showed transient efficacy and mild fluctuations in bilirubin (40). Folate receptor-β is another myeloid-lineage antigen, that has also been proposed as a target in AML (41,42). None of these CARs has yet been thoroughly evaluated in clinical trials, and all pose varying concerns owing to their expression in normal tissues. The quest for suitable AML targets is still awaiting its champions.

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