Myelodysplasia and transgene inactivation in X-CGD gamma retroviral gene therapy: the usual suspects and new players

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32 X-linked Chronic Granulomatous Disease (X-CGD) is an inborn error of immunity in which
33 phagocytic cells are unable to generate sufficient reactive oxygen species (ROS) to fight
34 bacterial and fungal infections due to mutations in the CYBB gene encoding the $gp91^{phox}$ -
35 subunit of the NADPH oxidase complex. Hematopoietic stem cell (HSC) gene therapy is now a
36 promising therapeutic option for this disorder. Several clinical trials have reported clear clinical
37 benefits, but also highlighted the difficulties in obtaining sustained correction of neutrophils
38 over time. Sadly, early clinical trials using spleen focus forming virus (SFFV)-derived γ-retroviral
39 vectors were overshadowed by the high incidence of insertional mutagenesis driving the
40 emergence of myelodysplasia.

41 In this issue of *Molecular Therapy*, Uchiyama and coworkers describe the emergence of
42 another case of myelodysplasia following loss of transgene expression, this time in an X-CGD
43 patient who underwent gene therapy with an Moloney murine leukaemia virus (MoMLV)-
44 derived γ-retroviral vector in 2014.[1] Intriguingly, the authors attribute loss of expression to
45 transgene hypermutation mediated by deaminating enzymes derived from virus producer
46 cells. The patient experienced initial clinical benefits, followed by loss of transgene
47 expression within 6 months of therapy and myelodysplasia at month 32. Upon investigation
48 of the genetic cause of the leukemia, the authors observed that a single myeloid-biased
49 clone had dominated from month 12 onward, in which a provirus containing an inactive
50 CYBB gene was inserted at the MECOM locus. Unexpectedly, the provirus was very highly
51 mutated, with over 100 G to A point mutations, consistent with the activity of a cytidine
52 deaminase acting on the minus-sense retrotranscribed ssDNA during vector integration. This
53 was attributed to APOBEC3C packaged into the capsids by the producer cell line. APOBEC3C
54 activity was not considered to be a potential factor for wider mutagenesis and leukemic
55 progression. The emergence of blast cells was instead linked to the insertion site, at which

56 viral enhancer-mediated Evi1 upregulation occurred, leading to a proliferative advantage and
57 clonal dominance. This was followed by a biallelic WT1 tumor suppressor knockout. The
58 patient ultimately underwent autologous HSC transplantation and remained in remission for
59 another 5 years.
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61 MECOM insertion, Evi1 upregulation, subsequent clonal expansion and eventual
62 myelodysplasia have previously been reported after γ-retroviral gene therapy, both in the
63 context of correction of X-CGD and of other diseases.[2,3] In this study, the initial MECOM-
64 inserted dominant clone persisted for many months with a normal karyotype and
65 hematopoiesis, suggesting that Evi1 upregulation alone was insufficient for tumorigenesis.
66 The eventual blast cell transformation was associated with a subsequent biallelic deletion of
67 the WT1 tumor suppressor by large-scale chromosomal rearrangements. Overexpression of
68 the Evi1 transcript has previously been shown to cause genomic instability[3,4] and was
69 associated with monosomy 7 in a previous γ-retroviral trial.[3] Another feature in common with
70 the previous γ-retroviral trial for X-CGD is the inactivation of the CYBB transgene. While loss
71 of transgene expression occurred by methylation of the viral SFFV promoter in the previous
72 study,[3] it was attributed to APOBEC3 hypermutation in this study. APOBEC3 proteins A-H are
73 a family of cytidine deaminases with varying inhibitory activities against viruses and are
74 packaged into the viral capsid bound to the viral RNA genome before acting to deaminate the
75 nascent DNA during retrotranscription in the recipient cell.[5] APOBEC3C, although less potent
76 than other forms, has been found to deaminate MLV viruses.[6] Consistent with this mechanism,
77 the authors report that hypermutation was not observed in the producer cells or viral particles
78 and that APOBEC3C was present in viral particles. Moreover, knockdown of APOBEC3C in the
79 producer cells reduced CYBB hypermutation, whereas knockdown of APOBEC3G in recipient

80 CD34+ cells did not. The observation that G-A mutated proviruses were present in CD34+ cells
81 early after transduction (albeit only detected with far fewer mutations than the dominant
82 clone) is inconsistent with the involvement of tumor-driven APOBEC3 activation. What is
83 perhaps most surprising is the relatively high fraction (7.8% immediately post-transduction of
84 CD34+ cells) of proviruses with deaminating mutations. To our knowledge, this has not been
85 previously observed in a retroviral or lentiviral gene therapy trial and warrants further
86 investigation. It may be informative to compare early-stage provirus sequencing from other
87 gene therapy protocols; perhaps highly mutated proviruses are immediately selected out or
88 fail to integrate in other contexts. Alternatively, the involvement of APOBEC3 hypermutation
89 may potentially be an issue idiosyncratic to the producer cell, the disease context, or the
90 transgene.

91 The loss of CYBB expression strengthens the idea of selective pressure against CYBB
92 expression in the HSC compartment in the context of X-CGD patients. This may be specific to
93 this disorder and/or transgene; in a study in which nine Wiskott-Aldrich Syndrome patients
94 were treated with a γ-retroviral vector containing a WAS transgene, two patients experienced
95 the emergence of a dominant MECOM-inserted clone leading to myeloid malignancy, but
96 transgene expression remained stable throughout the study.[2] Compared to wild-type cells, X-
97 CGD HSCs exhibit a chronic inflammatory phenotype[7] and undergo increased cell cycle entry
98 and more rapid expansion.[8] CYBB expression normally occurs in late stage of myeloid
99 differentiation; expression in the HSC compartment under a constitutive promoter could lead
100 to additional ROS production, which strongly inhibits the repopulating ability of HSCs.[9] In the
101 present study, transgenic expression of CYBB increased apoptosis and DNA damage in CD34+
102 cells *in vitro.* Overall, it is plausible that CYBB inactivation could have relieved the inhibition of
103 proliferation and facilitated the engraftment and expansion of the MECOM-clone. Arguably, if

104 selected for, loss of expression may eventually emerge stochastically by any mechanism;
105 mutations and epigenic changes are constantly generated during normal replication. However,
106 the up-front generation of an estimated 7.8% of APOBEC3C-hypermutated CYBB sequences at
107 the initial transduction generated an immediate and abundant pool for selection, which would
108 expedite the dominance of the clone.

109 This study further underscores the high oncogenic risk of γ-retroviral vectors and the
110 requirement for regulated, tissue-specific transgene expression to avoid selection against the
111 transgene in HSCs. The promising results of a recent clinical trial using a myeloid-specific
112 promoter in the context of a lentiviral vector are an important step in this direction.[10] Ongoing
113 developments in gene editing approaches could also contribute to the goal of regulated,
114 sustained transgene expression without loss of function or the risk of vector-mediated
115 oncogenesis.

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117 Declaration of interests

118 The authors declare no competing interests.
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