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To conclude, more stringent and consistent molecular profiling of tumor samples[9] along with careful patient stratification and continued in-depth CAR T-cell product characterization[10] are needed in future clinical trials to help select the right CAR T-cell product for individuals with LBCL and to improve existing CAR T-cell products. The 5-year follow-up of axi-cel in R/R LBCL provided by Neelapu and colleagues in this issue of *Blood* is reassuring and confirms that current and future efforts in the CAR T-cell field are paving the road to success for LBCL treatment. In other words, time to put the pedal to the metal.

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GENE THERAPY

Comment on Calabria et al, page 2316

Intrathymic AAV gene delivery

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In this issue of *Blood*, Calabria et al show that intrathymic (IT) delivery of adeno-associated virus (AAV) vectors results in a site-specific integration within T-cell receptor (TCR) genes close to DNA breaks created by recombination-activating gene (RAG) enzymes during the variable, diversity, and joining [V(D)J] recombination.[1]

With tremendous advances in molecular diagnostic techniques, an increasing number of causative gene defects have been discovered in human diseases. Gene therapy (GT), which aims to fix gene defects, promises a potential cure for these diseases. AAV vectors are the leading platforms for in vivo GT. They have a high transduction efficiency, a broad range of target tissues, and a good safety profile. Because AAV vectors present predominantly episomal, they are not useful in treating highly proliferative cells such as hematopoietic and lymphoid cells owing to the dilution effect.[2,3]

Pouzolles et al injected AAV-ZAP-70 into the thymi of ZAP-70-deficient mice and found that IT injection of AAV-ZAP-70 transduced up to 5% of thymocytes, resulting in the rapid development of functional T lymphocytes and rapid reconstitution of the thymic medulla (within 10 days). The gene-corrected T lymphocytes persisted in the periphery for more than 40 weeks after treatment.[4] Genomic integration was detected in the treated mice. In this issue of *Blood*, the same research team published the results of their study on the AAV integration profile in lymph nodes, spleens, livers, and brains of ZAP-70-deficient mice treated with IT AAV-ZAP70 injection.[1] This study also included some wild-type mice with AAV-GFP IT injection and treated MeCP2 deficiency mice. They found that more than 90% of integrations in T lymphocytes were clustered within the TCR α, β, and γ genes. The insertion sites were mapped to DNA breaks created by enzymatic activity of RAGs during V(D)J recombination. In contrast, AAV integrations in the liver and brain were distributed across the entire mouse genome.[1] This characteristic integration profile is likely related to the tendency of AAV vectors to integrate at DNA breakage sites,[5] which may not occur with other types of vectors.[6]

These findings demonstrate that IT AAV delivery can take advantage of the physiological DNA breaks generated during TCR V(D)J gene recombination, overcome the limitation of AAV vectors, deliver therapeutic transgenes into the genome of T lymphocytes, and achieve a long-term therapeutic outcome (see figure). If the integration interferes with TCR gene rearrangement, transduced thymocytes with no functional TCR will most likely die instead of becoming malignant. Therefore, insertional mutagenesis and genotoxicity are not likely to be an issue in this gene delivery approach. Because transduced thymocytes go through the selection process in the thymus and may develop tolerance to the antigens of the AAV capsid and transgene, immunogenicity seems less likely to be a problem. IT AAV gene

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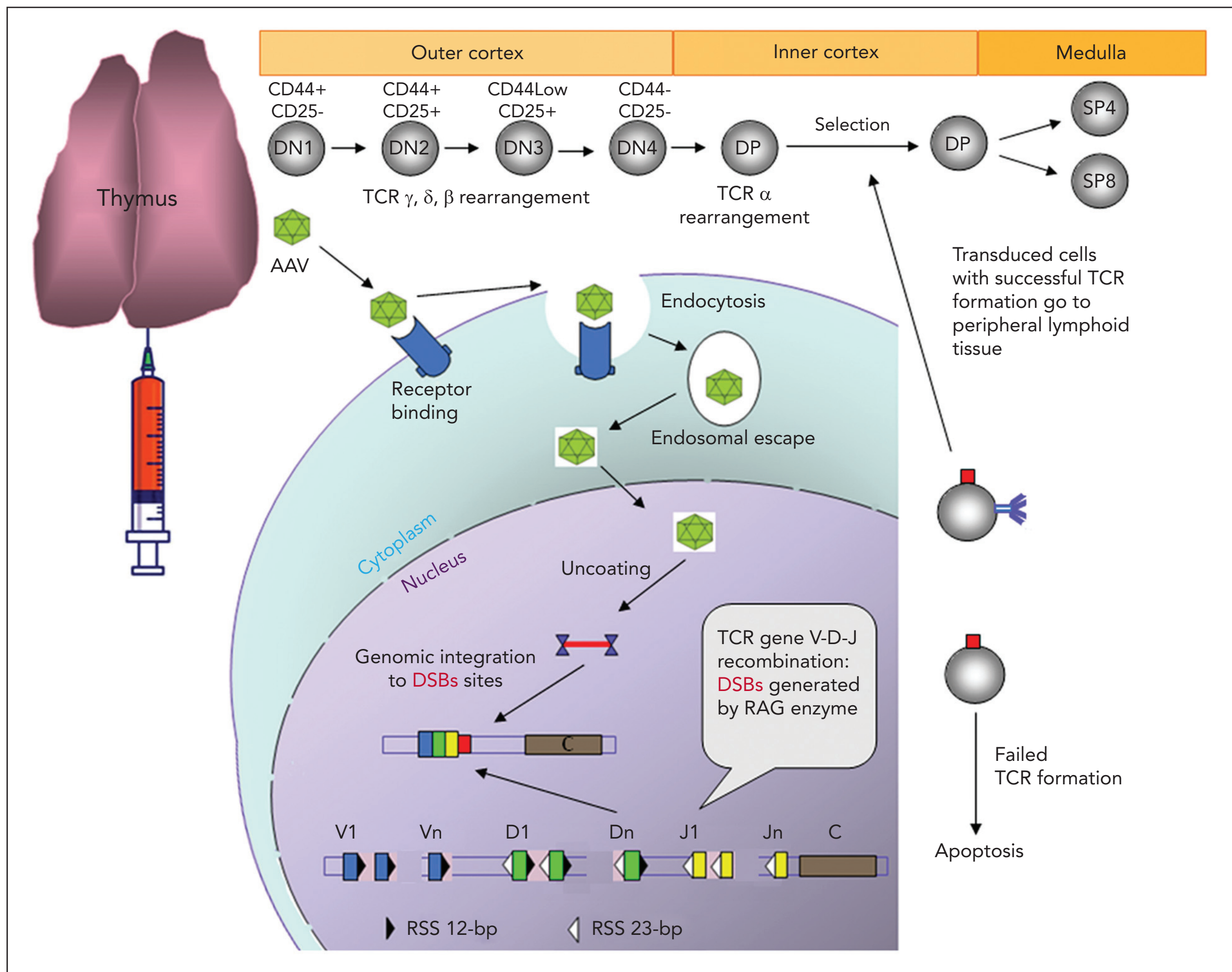

Schematic demonstration of IT AAV gene delivery. In the cortex of a thymus, thymocytes develop from CD4/CD8 double-negative 1 (DN1, early T-cell precursor) to CD4/CD8 double-positive (DP) stage, and these immature thymocytes have double-strand breaks (DSBs) created by RAG enzymes in TCR genes during TCR V(D)J recombination. AAV vectors enter thymocytes through receptor-mediated endocytosis, and the released AAV and transgene DNA in the nucleus is integrated into TCR genes close to the DSBs. The thymocytes with unsuccessful TCR formation owing to the AAV transgene integration will die. The transduced thymocytes with a functional TCR will undergo the selection process, and the positively selected thymocytes will develop into CD4 or CD8 single-positive (SP4 or SP8) T cells in the medulla and eventually be released into the peripheral lymphoid tissue.

delivery may be particularly useful for treating inborn errors of immunity (IEIs) with a gene defect predominantly affecting T lymphocytes. It also provides an ideal method for generating in vivo chimeric antigen receptor (CAR) T cells to treat cancer. Because of the progressive involution of the thymus after birth, this IT GT strategy may not be effective for older children and adults with thymic atrophy. Since a similar gene rearrangement process occurs during B-cell development in the bone marrow (BM), similar integration may occur in the immunoglobulin genes of B lymphocytes if AAV vectors are injected into the BM. It would be interesting to examine peripheral B lymphocytes from animal models treated with an intra-BM injection of an AAV construct.

IEIs are a heterogeneous group of inherited immune disorders often associated with significant morbidity and mortality. Allogeneic hematopoietic stem cell transplantation is the standard treatment for most severe IEIs. However, allogeneic hematopoietic stem cell transplantation requires a suitable donor and carries the risk of graft failure, graft rejection, graft-versus-host disease, and complications related to pretransplant myelosuppressive and posttransplant immunosuppressive agents. GT can overcome most or all of these disadvantages, and has been used in the treatment of some IEIs with decent clinical outcomes. The current GTs for IEIs are ex vivo GT, genetically modifying patients' hematopoietic stem cells ex vivo using gammaretroviral vectors or lentiviral vectors.[7,8] The procedure of ex vivo GT is complex and requires an advanced infrastructure, which is not widely available. In addition, lymphodepletion preconditioning for ex vivo GT has side effects and complications. It will be of great benefit to correct genetic defects in vivo. Given the findings of Calabria et al, IT AAV GT can be a good approach for treating IEIs primarily due to defects in T lymphocytes, such as CD40 ligand deficiency, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome, and cytotoxic T-lymphocyte antigen 4 insufficiency.

CAR T-cell therapy is the most successful and innovative cellular cancer treatment in modern oncology. It genetically modifies a patient's own T cells ex vivo

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to generate CAR T cells that express a synthetic receptor that binds to a tumor antigen. CAR T cells are infused back into patients to attack and kill cancer cells. Good therapeutic responses and clinical outcomes have been demonstrated in CAR T-cell treatments for B-cell malignancies.[9] However, the high cost and complex procedure to generate CAR T cells ex vivo limit its wide application. The generation of in vivo CAR T cells may simplify the process and make CAR T-cell therapy easier to perform and less expensive. Nawaz et al found that infusing AAV-CAR into a humanized tumor mouse model of human T-cell leukemia could generate enough functional in vivo CAR T cells to cause tumor regression.[10] Given the features and advantages of IT AAV gene delivery discussed above, IT delivery of AAV-CAR could be a more effective way to generate in vivo CAR T cells. Because the targeted cells are immature thymocytes, CAR T cells generated by IT AAV-CAR delivery may last longer in the body than the traditionally generated CAR T cells.

It is too early to predict the clinical use of IT AAV gene delivery. However, as an intriguing idea with the support of promising preclinical study results, IT AAV GT deserves further study and may eventually become a useful treatment for certain human diseases.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Noori et al, page 2330

Deciphering genetic uncertainty in familial HLH

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In this issue of *Blood*, Noori et al describe a novel in vitro approach to model germline variants in genes associated with familial hemophagocytic lymphohistiocytosis (fHLH). This approach allows for analysis of variants of uncertain significance (VUS), shedding light on the functional consequences of these variants.[1]

fHLH, also known as primary HLH, comprises a group of rare hyperinflammatory disorders characterized by germline biallelic loss-of-function (LOF) mutations in the genes *PRF1*, *UNC13D*, *STXBP2*, or *STX11*, each of which is required for CD8 T cell– and natural killer (NK) cell–mediated cytotoxicity. In patients with fHLH, CD8 T and NK cells become activated following little to no immune trigger and are unable to eradicate targets, such as pathogen-infected cells and activated antigen-presenting cells. As a consequence, patients with fHLH develop exuberant immune cell activation with release of proinflammatory cytokines that mediate fatal multiorgan failure if untreated.[2] Although medications that induce effector cell apoptosis or inhibit cytokine activity can temporarily control hyperinflammation, definitive treatment requires stem cell transplantation.[3]

Optimizing outcomes in fHLH relies on making a rapid and accurate diagnosis, which currently necessitates identification of LOF mutations in 1 of the fHLH genes. fHLH is associated with genetic diversity, and sequencing frequently identifies novel, rare, or even relatively common VUS, which contribute little to clinical decision making.[4] To facilitate interpretation of sequencing results, several assays have been developed to evaluate how germline variants impact cell function. One such assay measures the translocation of CD107a (also known as LAMP-1), a protein contained within cytotoxic granules, to the surface of patient-derived effector cells following stimulation, serving as a surrogate for degranulation. Although decreased CD107a expression can accurately identify patients with biallelic mutations in *UNC13D*, *STX11*, and *STXBP2*, CD107a expression is normal in cells from patients harboring LOF mutations in *PRF1*.[5] A complementary assay exists to examine the ability of patient-derived NK cells to kill target cells in vitro. This assay, however, is subject to poor specificity because hyperinflammation can suppress NK cell killing,[6] resulting in an abnormal result even in the absence of LOF mutations. The performance of both assays depends on obtaining sufficient numbers of CD8 T or NK cells, which are often reduced in patients with active HLH. Thus, it can be challenging to carry out these assays and interpret their results.[7]

The genetic complexity of fHLH and the caveats of existing functional assays pose diagnostic challenges. To address this gap, Noori et al developed a novel method for functional analysis of variants

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