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Molecular Therapy

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Safer CARS

Genetically modifying T cells to express synthetic chimeric antigen receptors (CARs)[1] is an attractive strategy for producing anti-tumor effects. The first-generation CARs usually consisted of the single-chain variable fragment of an antibody specific for tumor antigen linked to the transmembrane and intracellular signaling domains of either CD3ζ or FcRγ. This produces human leukocyte antigen (HLA)-unrestricted activation of the modified cytotoxic T lymphocytes (CTLs) upon encounter with tumor antigen, leading to lysis of tumor cells *in vitro* and eradication of tumor in mouse models. Because CARs bind to target antigens in an HLA-unrestricted manner, they are resistant to many tumor-immune evasion mechanisms, such as downregulation of HLA class I molecules or failure to process or present proteins. However, efficacy was modest in clinical trials in subjects with lymphoma, neuroblastoma, or ovarian or renal cancer[2–4] because of incomplete activation of T cells with limited expansion and persistence *in vivo*. Normal T-cell activation is dependent on receipt of two classes of signal: one through the engagement of the T-cell receptor (TCR) with antigen presented in the context of the major histocompatibility complex and a second through engagement of costimulatory molecules such as CD28, OX40, and CD40L. Tumors often do not express appropriate ligands for costimulatory molecules, and engagement of first-generation CARs in the absence of costimulation leads to anergy and failure of *in vivo* expansion.

To overcome these limitations, second-generation CARs were developed to incorporate the intracellular domains of one or more costimulatory molecules such as CD28, OX40, and 4-1BB within the endodomain, and these improved antigen-specific T-cell activation and expansion.[1,5] Third-generation CARs include a combination of costimulatory endodomains.[6] An alternative approach to providing costimulation is to express CARs in antigen-specific T cells, which will then also be activated and expanded following engagement of their native αβTCR by antigen on professional antigen-presenting cells, with attendant costimulation.[7,8] In a recently reported clinical trial, subjects receiving Epstein-Barr virus (EBV)-specific CTLs engineered with a CAR (CAR-CTLs) specific for the disialoganglioside antigen GD2a on neuroblastoma cells showed longer *in vivo* persistence than unselected T cells engineered with a distinguishable GD2-CAR, due to stimulation of the CAR-CTLs by EBV antigen.[7]

More than 10 clinical trials evaluating second- or third-generation CARs are currently listed on ClinicalTrials.gov, and there is some encouraging preliminary evidence of clinical efficacy[9,10] However, in this issue of *Molecular Therapy*, two recent serious adverse events (SAEs) are reported in subjects enrolled in these trials, raising concerns about these receptors. The first event, described by Morgan and colleagues at the National Institutes of Health (NIH),[11] occurred in a patient with widely metastatic colon cancer who received more than 10^{10} T cells modified with a CAR targeting HER2 containing two costimulatory moieties (CD28 and 4-1BB) after intensive lymphodepletion. The subject developed pulmonary toxicity within 15 minutes in association with very high cytokine levels, followed by cardiac arrest, and died 4 days later. After extensive investigation and analysis of autopsy specimens, the investigators concluded that the toxicity may have been due to targeting of low levels of HER2 on pulmonary endothelium by transgenic T cells.

In the second report, Brentjens and colleagues from Memorial Sloan–Kettering Cancer Center describe a patient with bulky chronic lymphocytic leukemia and extensive previous treatment who received autologous T cells transduced with a CD19-28ζ CAR at a dose of 3×10^7 cells/kg after lymphodepletion with cyclophosphamide.[12] This patient developed fever, hypotension, and dyspnea 20 hours after infusion, which rapidly progressed. Elevated cytokine levels were seen before the T-cell infusion, but an autopsy failed to reveal an obvious cause of death. The investigators concluded that low-grade sepsis was the most likely trigger in this heavily pretreated immunosuppressed patient but also considered the possibility that a cyclophosphamide-induced "cytokine storm" may have enhanced the *in vivo* activation of modified T cells.[12]

Although the adaptive immune response is considered a highly targeted system, it should be noted that "on-target but off-organ" effects have been observed in other studies with both native and genetically

modified T cells. For example, when T cells specific for minor histocompatibility antigens were given to treat leukemic relapse, severe toxicity resulted from the unexpected presence of the target antigens on lung tissue.[13] Similarly, when T cells were genetically modified with MART-1-specific TCRs, impressive regression of melanoma was observed, but it was associated in some cases with toxicities to melanin-expressing cells in the inner ear and the retina.[14] Finally, a CAR targeting carbonic anhydrase IX expressed by renal carcinoma cells produced liver toxicity because the target antigen was also expressed on bile duct epithelium.[4] Morbidity due to bystander inflammation has also been reported during therapeutic responses to EBV-specific T lymphocytes.[15] Notably, however, none of these examples was associated with the rapid and fulminant onset observed with CAR–T-cell infusion described by Morgan *et al.*, and all were either self-limited or responsive to steroids. Indeed, the rapid onset of the CAR-associated SAE at the NIH is most reminiscent of the SAEs reported with a superagonist CD28 antibody.[16]

In addition to these explanations, there is a concern that second- and third-generation receptors may be too easily triggered by low avidity "off-target" binding to produce a potent activation signal that leads to a lethal cytokine storm. It is still unclear whether the combination of lymphodepletion and the presence of costimulatory moieties in the second- and third-generation CARs used might have contributed to the cytokine storm.

Because the increased potency of second- and third-generation CARs has considerable potential benefits for patients with malignant disease, how could the safety profile be enhanced? For many T-cell therapies, such as donor lymphocyte infusion after hematopoietic stem cell transplant, the side effects correlate with the cell numbers infused,[17] so one possibility is to evaluate lower doses of T cells to prevent severe immediate toxicity. Should the subsequent expansion of infused CAR-specific T cells produce delayed but more indolent toxicities, several therapeutic maneuvers are available. These include corticosteroids in doses used to treat graft-versus-host disease (GvHD) or antibodies to T cells such as alemtuzumab (CAMPATH-1H), each of which will deplete the majority of circulating transduced cells. After the fatality in the CD19-28ζ CAR study, the regimen was modified so that cells are infused over 2 days, with one-third given on the first day and two-thirds on the second day.[12] The NIH investigators also concluded that for future first-in-human studies with CARs, a more restricted dose-escalation scheme should be evaluated.[12]

A second approach is to include a safety switch mediated by a second transgene in the construct to allow the cells to be destroyed on exposure to a specific signal. The herpes simplex viral thymidine kinase (*TK*) gene—the product of which phosphorylates nucleoside prodrugs such as ganciclovir and acyclovir to the active moiety, which interferes with DNA polymerase—has been introduced into allogeneic T lymphocytes used as donor lymphocyte infusions following stem cell transplantation. If the infused T cells induce GvHD, administration of the prodrug causes T-cell death and abrogates GvHD. This approach has now reached a phase III clinical trial.[18] Because the *TK* suicide gene is potentially immunogenic and requires a prodrug that would otherwise have its own therapeutic applications, investigators are also developing alternatives such as inducible caspase 9 (icasp9). Because caspase 9 is a naturally occurring component of the caspase pathway, it should be nonimmunogenic. Moreover, icasp9 is highly effective in producing rapid (less than 120 minutes) apoptosis even in nondividing cells.[19] The molecule can be triggered by administration of a small-molecule dimerizer that brings together two nonfunctional icasp9 molecules to form the active enzyme, and the approach is currently being evaluated in the clinic.

A follow-up Recombinant DNA Advisory Committee meeting will be held in June 2010 to focus on how best to develop a safe approach to implementing these promising new therapeutics, and it is a great credit to all investigators involved that they have been so forthcoming in providing detailed reports of SAEs. With their help, we should speedily resolve these remaining concerns so that CARS will become both safe and effective components of cancer therapy.

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