Commentary

Cancer Immunology Research

Serial Killers and Mass Murderers: Engineered T Cells Are up to the Task

Carl H. June

It's my job as best friend to make sure he's not a serial killer. Or an English major, not sure which one's worse.

—Shelly Crane

See related articles by Liadi et al., p. 473, and Davenport et al., p. 483

Adoptive cell therapy (ACT) has gained increasing traction based on encouraging results in patients with cancer and chronic infection (1). However, the factors that contribute to successful cellular products for ACT remain incompletely identified. Examples of the "known unknowns" include the ideal composition of the lymphocyte subset, the features of chimeric antigen receptor (CAR) or T-cell receptor (TCR) design, the optimal state of cell differentiation, and the requirement for host lymphodepletion (2).

The emerging data from several trials with CAR-modified T cells (CAR T cells) demonstrate encouraging clinical responses in a variety of hematopoietic malignancies (3–6). Similarly, clinical results from several trials with TCR-modified T cells have brought encouraging results (7). ACT with tumor-infiltrating lymphocytes (TIL) is also promising (8, 9), although the mechanisms involved in tumor elimination appear to be distinct from those related to CAR T cells (10). However, for all forms of ACT, major questions remain regarding the mechanisms of rapid tumor destruction. Information on the mechanisms involved in tumor elimination is limited, due in part to the inability to image infused cells and to observe the tumor microenvironment in clinical settings. Biopsies of tumors after ACT have shown the intratumoral presence of genetically engineered T cells, but whether the CAR T cells are directly cytotoxic for tumor cells or if they promote killing by bystander cells, such as natural killer (NK) cells or macrophages, remains to be defined. Two articles in this issue of *Cancer Immunology Research* examine the mechanisms of tumor elimination after ACT (11, 12).

Davenport and colleagues (11) used a novel transgenic mouse model to examine the structure and function of T cells expressing a transgenic TCR and a CAR. Transgenic mice expressing the MHC class I–restricted OT-1 TCR were bred with mice that express a second-generation CAR specific for c-erbB2/HER2. With this system, these authors were able to study for the first time the function of CTL activated through either the endogenous TCR or the CAR in the same cell. They found that expression of CARs did not affect the stimulation of naïve splenic T cells through the endogenous TCR over a wide range of peptide/MHC stimulation, as assessed by induction of proliferation and differentiation to effector cells. The bispecific CTLs expressing the engineered CAR and the transgenic TCR produced levels of cytotoxic granule proteins similar to those produced by conventional CTLs; however, the level of CAR expression at the cell surface was less than that of TCR expression. Expression of the CAR was driven by a vav promoter, which is relatively weak, and this may account for the difference in cell-surface expression of these receptors. When cytotoxicity of the dual-specific CTLs was assessed against tumor cells expressing either cognate peptide/MHC or HER2, there was no significant difference in the time interval for target recognition to delivery of the lethal hit. Surprisingly, the time interval from target recognition to detachment of the CTL from the dead tumor cell was faster when bispecific CAR cells were ligated via the CAR as compared with recognition through the TCR. Using a novel impedance-based cytotoxicity assay, the authors show that at several different effector-to-target (E:T) ratios, the rate of killing of targets by the CAR or by the TCR was equivalent for up to 8 hours. When a low E:T ratio was tested in a prolonged cytotoxicity assay, the rate of killing by either TCR- or CAR-ligation was similar for the first 20 hours, but the killing by CAR-ligated CTLs slowed markedly beyond this time point. One potential mechanism for this effect was that the dual-specific T cells maintained expression of the TCR while they lost expression of the CAR. Furthermore, time-lapse video microscopy demonstrated that individual bispecific CAR T cells were serial killer cells, as approximately 22% of CAR T cells sequentially delivered a lethal hit to two or three tumor targets. Interestingly, the authors observed the same frequency of serial killing when the CAR CTLs were triggered through their endogenous TCR by peptide/MHC stimulation.

Previous studies have demonstrated serial killing by CTLs (13, 14) and NK cells (15). However, *in vivo* imaging studies reveal that the intratumoral dynamics of CTLs and NK cells differ markedly; whereas CTLs form stable contacts with tumor cells, NK cells tend to make transient contacts (16). Davenport and colleagues (11) are the first to analyze the triggering of TCRs and CARs in the same cell. An important aspect of the study is the use of tumor targets that are well balanced for equivalence of peptide/MHC and CAR targets at the cell surface. A limitation of the study is that these results may not be applicable to studies that use other TCRs or CARs. For example, the OT-1 TCR is often used because it is one of the highest-affinity MHC class I–restricted TCRs, and these results may not reflect results that would occur with lower-affinity TCRs (17, 18). Similarly, whether the HER2 CAR results can be extrapolated to other CARs remains to be determined, because many variables of CAR design exert effects on the efficiency of CAR killing beyond the specificity of the particular CAR. For example, the CAR hinge length, scFv affinity, transmembrane

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domains, and signaling domain can all have major effects on CAR recognition and function (19, 20).

Davenport and colleagues (11) used a second-generation CAR with a CD28 signaling domain. We have found that some CARs with CD28 signaling domains have constitutive ligand-independent activity (21); therefore, it is possible that the results from this study may not be generalizable to other CAR T cells. However, results from most studies have shown that when cytotoxicity is the endpoint, the signaling domain becomes relatively unimportant, as first-generation CAR designs with ζ chain only have equivalent cytotoxicity to second- or third-generation CARs.

In a separate study published in this issue of *Cancer Immunology Research*, Liadi and colleagues (12) used time-lapse imaging microscopy to study the mechanisms of $CD4^+$ and $CD8^+$ CAR T-cell killing *in vitro*. Human T cells endowed with a CD19 CAR were used for these studies in nanowell grids. These authors developed a novel model system by constructing a nanowell array comprising approximately 85,000 wells with cells in 125 pL per well and subjected the cells to repeated imaging. They found that $CD4^+$ CAR T cells are slower than $CD8^+$ CAR T cells in target-cell killing. They also report substantial frequencies of CAR T cells undergoing apoptosis following target-cell killing. This was not spontaneous apoptosis but rather activation-induced cell death triggered by target recognition.

Liadi and colleagues (12) classified killing events as multiplexed or serial killing. Multiplexed killing occurred when the CAR T cells simultaneously engaged multiple targets. They distinguished this type of killing from true serial killing, which happens when the CAR T cell conjugated to the first tumor cell kills, releases, and then repeats the sequence to kill a different tumor cell. They found that the frequency of multiplexed tumor killing is similar to the frequency of true serial killing. At high tumor densities, it is likely that multiplex tumor killing becomes more frequent.

A systems biology approach analyzing six parameters of the CAR T cells revealed three subsets (termed S1, S2, and S3) of $CD4^+$ and $CD8^+$ CAR T cells. S2 was the dominant subgroup and was characterized by poor motility and early target-cell encounter, often followed by apoptosis of the tumor target and the CAR T cells. In contrast, the highly motile S1 subpopulation of CAR T cells killed and detached efficiently, resuming exploration of the local microenvironment. This suggests that the motility of CAR T cells might help identify efficient killers with decreased propensity for apoptosis. When serial killer T cells went out for patrol, the frequency of CAR T-cell and tumor-cell conjugations that resulted in target elimination during the first tumor-cell encounter was not significantly different from the frequency of successful kills upon engagement with the second tumor cell, suggesting that the killing efficiency is sustained. It is not yet known if there are specific functional phenotypes associated with these subgroups of cells, such as distinct cytokine profiles or levels of Granzyme B expression.

A major conclusion of the study by Liadi and colleagues (12) is that both single-killer and multi-killer CAR $CD4^+$ T cells required longer conjugation and showed delayed kinetics of killing in comparison with CAR $CD8^+$ T cells. Individual CAR $CD4^+$ T cells on average required 2 additional hours to induce tumor-cell death. Overall, these results demonstrate that the major difference in CAR $CD4^+$ and $CD8^+$ T cells participating in either single killing or multikilling is the kinetics of tumor-cell death. There is some debate on the optimal subset composition of CAR T cells (22). In mouse models, Moeller and colleagues (23, 24) found that mixtures of $CD4^+$ and $CD8^+$ CAR T cells were optimal for sustained antitumor effects. Brentjens and colleagues (25) used CARs with a second-generation CD28 signaling domain to treat patients with leukemia and reported that effective products contain abundant $CD8^+$ CAR T cells. Results from our studies suggest that the optimal endodomain of the CAR is different for $CD8^+$ and $CD4^+$ CAR T cells (26). The slower rate of $CD4^+$ CAR killing may be due to the Granzyme B content being lower than that in $CD8^+$ CAR cells. However, tumor reduction is not always due to direct cytotoxicity, as for example, $CD4^+$ cell–derived cytokines can induce tumor-cell senescence (27). Other factors identified in the effective destruction of tumors after ACT include vascular disruption and a "field effect" resulting in the bystander elimination of antigen-negative cancer cells (28).

A final conclusion from this study is that the fate of the CAR T cell subsequent to target encounter is dependent on the density of the tumor cells. If CAR T cells are plated at an initial E:T of 1:1 in the nanowell, then the frequency of subsequent apoptosis is higher than when the CAR T cells are plated at an E:T of 1:2–5. These results may have significant implications in clinical trials when CAR T cells are infused in settings of low tumor burden. A further caution is that this study was performed on B-cell tumors, and it remains to be determined if the same results would be obtained with CAR T cells targeting solid tumors.

A strength of the study by Liadi and colleagues (12) is that the experiments used a CD19 CAR T cell that is currently being evaluated in clinical trials (NCT00968760). Further, these CAR T cells were prepared using manufacturing protocols very similar to those used for the clinical production of the CAR T cells. However, this procedure uses a prolonged period of *in vitro* culture (29), and thus some of the results, particularly the high rates of CAR apoptosis that were observed, may not reflect the results with CAR T cells prepared using a short manufacturing process.

There are a number of differences between the immune systems of mice and humans (30, 31). Live cell microscopy with primary human and mouse CTLs and NK cells reveals substantial species differences in the calcium signaling that is required for perforin delivery (32). Perhaps relevant to species differences and CAR T cells, Liadi and colleagues (12) report a substantially higher rate of serial killing with human CD19 CAR T cells, compared with that of the mouse HER2 CAR T cells (11). Finally, Davenport and colleagues (11) have not yet studied the consequences to CAR T cells that encounter tumor targets displaying peptide/MHC and ligands for the CAR on the same cell. Would such double recognition increase killing and/or increase the rate of apoptosis of the effector cell? Would polarity of the granule release be maintained?

Natural T cells are capable of efficient serial killing under some circumstances. For example, bispecific T-cell engagers, recombinant proteins that redirect T cells to tumor antigens, result in activation of T cells that kill multiple tumor targets (33). However, activated human T cells do not always have a penchant to become serial killers. When human CTLs target HIV-infected $CD4^+$ cells, they efficiently kill the first target, but serial killing is rare (34).

Disclosure of Potential Conflicts of Interest

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