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Letters to the Editor

Comment on "Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma"

The authors of this study[1] should be commended on the important and complex undertaking of identifying multiple antigens for chimeric antigen receptor (CAR) T-cell therapy, which is one of several key limitations to the field. There are 2 levels of antigenic heterogeneity at play that require consideration—intrapatient variability, in which the existence of antigen-negative tumor cells may result in eventual recurrence, and interpatient variability, in which different expression patterns across all patients make identification of a single or "universal" therapeutic for treatment difficult, if not impossible. The authors state that by targeting 3 antigens: human epidermal growth factor receptor 2 (HER2), interleukin-13 receptor alpha 2 (IL13Rα2), and ephrin-A2 (EphA2) using a trivalent UCAR, they can "overcome intrapatient and interpatient variability" and that "trivalent T cells were able to approach killing in 100% of tumor cells in nearly all patients modeled." As investigators working in the field of immuno-oncology, we very much wish this to be the case; however, these conclusions are not supported by the data presented in the manuscript.

First, the investigators claim that these 3 antigens are present in most patients and have sufficient expression in each patient to cover all tumor cells. However, their own data specifically demonstrate that at least 20% of patients have at least 10% of glioblastoma (GBM) cells that are negative for all 3 antigens (Figure 1). Furthermore, these data are incongruous with those presented in articles from The Cancer Genome Atlas wherein 56% to 68% of GBM patients fail to express even one of these antigens.[2,3] This is a significant percentage of cells in a significant proportion of patients. The small sample size and the discrepancy with larger published datasets demand further inquiry before stating that targeting these 3 antigens offers a universal treatment that overcomes interpatient heterogeneity.

Second, although the authors include in their study cutting edge modeling of autologous CAR T cells and patients' GBMs, these experiments fall short of addressing interpatient heterogeneity, as 4 patients with *very similar* antigen expression profiles were chosen for in vitro studies. Moreover, the 2 patient xenografts, UPN001 and UPN005, selected for the autologous in vivo xenograft model show an almost identical frequency of expression of the 3 antigens (Figure 1, Supplementary Table S1). Thus, the claim of harnessing the power of autologous patient xenografts for modeling interpatient heterogeneity is overstated.

These investigators also attempt to claim that there is a more robust immunological engagement using immune synapse distance of the UCAR construct relative to monovalent HER2 CAR T cells. However, the immune synapse distance between HER2 CAR T cells and nontransduced cells does not appear to be different (Figure 3). This calls into question whether the HER2 CAR T-cell population is representative of monovalent CAR T-cell function, which was used as the comparator to the UCARs. Inclusion of the IL13Rα2 CAR or EphA2 CAR T cells for this comparison may have been more appropriate.

Finally, the authors state in the abstract that the UCAR T cells "exhibited improved cytotoxicity and cytokine release over the best monospecific and bispecific T cells per patient tumor profile." However, the UCAR T cells have comparable cytotoxicity to monovalent IL13Rα2 and EphA2 CAR T-cell populations (Figure 2). The cytotoxicity "superiority" of the UCAR constructs over biCAR T cells was only demonstrated in a very small sample of autologous tumor patient gliomas (n = 2/4), which is insufficiently statistically powered to support this conclusion.

In summary, although this study does attempt to address an important limitation in the field of targeting glioblastoma antigen heterogeneity using CAR T cells and uses a cutting edge murine model of GBM, the authors undercut the value of the model by choosing patients' xenografts with nearly identical antigenic frequency in vitro and in vivo, and as such, do not really address interpatient variability. In addition, the presentation of the UCAR as "universal" for all GBM patients is misleading when considering the antigen expression profiling of 15 GBM patients, which is incongruent with data from TCGA. Thus, these fundamental flaws in the presented study do not support the interpretation that "trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma" and have the potential to misrepresent the progress in the field.

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Response to the comment on "Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma" by Bielamowicz et al

The use of chimeric antigen receptor (CAR) T cells has shown promising results in preclinical and early clinical trials for glioblastoma (GBM). Antigen escape, the downregulation or loss of target antigens, occurs after CAR T-cell therapy targeting single antigens and culminates in tumor recurrence. We have previously shown a clear advantage to combinatorial targeting of the 2 GBM antigens human epidermal growth factor receptor 2 (HER2) and interleukin-13 receptor subunit alpha-2 (IL-13Rα2), in offsetting antigen escape and enhancing T-cell performance.[1] Our data indicated that the interpatient variability in surface antigen expression hinders the clinical impact of targeting 2 antigen pairs, though. In Bielamowicz et al,[2] we therefore studied whether a CAR T cell targeting a third GBM antigen, ephrin-A2 (EphA2), would broaden the T-cell spectrum enough to overcome this obstacle—thereby increasing the probability of eligibility of GBM patients to a single trivalent product.

To create a probability model we first studied the pattern of surface protein expression of these 3 target antigens in a cohort of 15 serially diagnosed primary GBM surgical samples. Specifically, we assessed the immunoreactivity to HER2, IL-13Rα2, and EphA2 in 100 000–200 000 single cells to interrogate the probability of targeting >95% of cells within individual tumors (an overarching indicator of eligibility for the proposed trivalent product). This study was prospectively powered, wherein data from 12 or more tumors were anticipated to reach statistical significance. Next we performed hierarchical comparisons of the most prevalent single versus most prevalent 2 versus 3 antigens of interest (8 permutations) with an adjusted *P*-value of <0.0001 as a cutoff (these data are detailed in Supplementary Tables S1–S3 in Bielamowicz et al). Accordingly, we built a Boolean "OR" routine of tumor coverage as a function of probability of eligibility to 7 potential cellular products. Figure 1 is a nonparametric probability estimator that shows that bivalent products favorably bundle together above univalent products, yet the trivalent product significantly surpasses their mean probability of eligibility ($P < 0.0001$).

An invaluable alternative for studying targeted therapeutics is The Cancer Genome Atlas (TCGA), an atlas of nucleic acid profiles. We interrogated data from the reports from 2008 (208 GBMs)[3] and 2013 (152 GBMs)[4] cited by Caruso and Heimberger (Supplementary Figure S1). Unfortunately, we found several fold discrepancies between the expression in TCGA of HER2, IL-13Rα2, and EphA2 and their immunoreactivity as assessed by us and as repeatedly reported in the literature.[5–8] There are several potential explanations for such genome/proteome discrepancies, such as (i) epigenetic changes that are rampant in GBM especially after chemoradiation and (ii) posttranslational modifications.[9] Importantly, TCGA databases are derived from tumor bulks and are "internally controlled" using arbitrary cutoffs, which makes the absolute incidence of target expression and the definition of normalcy, such as for HER2, which is overexpressed but not amplified in GBM, very elusive. Equally important, overexpression is not a prerequisite for CAR T-cell–based targeting.[10] For these reasons, we used the data from TCGA to assess "trends" of target expression and coexpression but deemed it unsuitable for assessing the targetability of surface proteins using multivalent CAR T cells. The development of The Human Protein Atlas (THPA) is under way and would represent a more appropriate resource when complete (www.proteinatlas.org).[11,12]

We concluded that trivalent CAR T cells represent a single product that can be used across this cohort as an index sample representing larger cohorts of GBM patients. Further studies using THPA data or building on our strategy above in larger numbers could significantly substantiate our findings. Nevertheless, the trends in the cohort of patients we used justified the creation of a novel trivalent CAR T-cell product that enhanced the effector functions, which were thereafter extensively tested using patient CAR T-cell products against autologous GBM/patient-derived xenografts. Several patient sample sets were used (as biological replicates, rather than to account for heterogeneity). The superiority of the product being tested was demonstrated above the best univalent and bivalent products based on traditional immunoassays, subcellular imaging, and in vivo studies of primary GBM.