Technical Overview

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Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells

Authors

Jiaming Zhang, Grace Yang, and Peifang Ye
Agilent Biosciences Co. Ltd.
Hangzhou, China

Nancy Li, Yama Abassi, and Brandon J. Lamarche
Agilent Technologies Inc.
San Diego, CA, USA

Introduction

Harnessing the exquisite potency and molecular specificity of the immune system, and directing these towards cancer cell destruction, immunotherapy in its various forms has permanently changed the landscape of clinical oncology. Among the frontrunners that have helped to usher in this new paradigm are adoptive cell therapies such as chimeric antigen receptor (CAR) T cells. CARs are engineered proteins that consist of an extracellular antigen-binding domain that is linked, via a hinge region and transmembrane domain, to intracellular activation domains that drive T cell activation, proliferation, and target cell killing.[1] A few decades of CAR engineering efforts have made it clear that merely linking these protein domains together, like "beads on a string", is insufficient for generating an effective CAR; subtle differences in how the domains are linked to each other can greatly impact functionality.[1,2] While today's third-generation CARs are improved relative to their predecessors, a universally efficacious CAR architecture has not been identified and efforts to design CARs with novel or refined functionality continue to require empirical optimization on a case-by-case basis.[2]

During CAR optimization, biochemical/biophysical assays can be used to study critical quality attributes such as the antigen affinity of the extracellular domain[3] or the cytokine profile of CAR T cells stimulated by soluble ligands.[4] Although these reductionistic approaches are useful, their results do not necessarily correlate with cancer-killing efficacy within a more physiologically relevant context.[3] To rigorously compare different CAR constructs and identify unwanted behavior such as tonic (antigen-independent) signaling, it is widely acknowledged that CAR function should be evaluated within the complex milieu of the immune synapse, using bona fide cancer cell killing as the readout. For this purpose, release assays (^{51}Cr, LDH) have historically been the gold standard, but suffer from the fact that they require significant "hands-on" time and only provide end-point data. Requiring just target cancer cell seeding and a subsequent CAR T cell addition step, this study used the Agilent xCELLigence RTCA eSight to continuously monitor CAR T cell-mediated killing of cancer cells over the course of multiple days. Providing a direct and objective assessment of target cell number, cell size, and cell-substrate attachment strength, impedance biosensors embedded within the base of eSight microplates quantitatively track the continuum of target cell killing, spanning from early (reduced cell-substrate adhesion strength) to late (lysis) events.

Concurrently, eSight captures live-cell images in brightfield and three fluorescence channels (red, green, and blue), providing an orthogonal readout of the killing process. By combining the strengths of real-time impedance monitoring (simplicity, analytical sensitivity, and objectivity) with that of live-cell imaging (specificity of the readout), eSight increases the information richness of the CAR T cell killing assay without increasing the workload.

Monitoring CAR T cell killing activity by live-cell imaging

When left untreated for 48 hours, the RFP-expressing HEK-293-CD19 cells proliferate to the point of confluence (Figure 1A). However, after 48 hours of exposure to CAR T cells there is a very clear reduction in the number of target cells present. As expected, this killing response is dose-dependent, with the highest E:T ratios causing the most pronounced killing. As the E:T ratio is increased, the unlabeled/grey CAR T cells become more prominent in the field of view, and clustering of these T cells (which is a characteristic of activation) becomes more robust. Note that at late time points these T cell clusters contain a large number of red target cells which, because they display rounding/detachment and cytoplasmic shrinkage, appear to be progressing through apoptosis. Finally, when the assay is repeated using a fixed E:T of

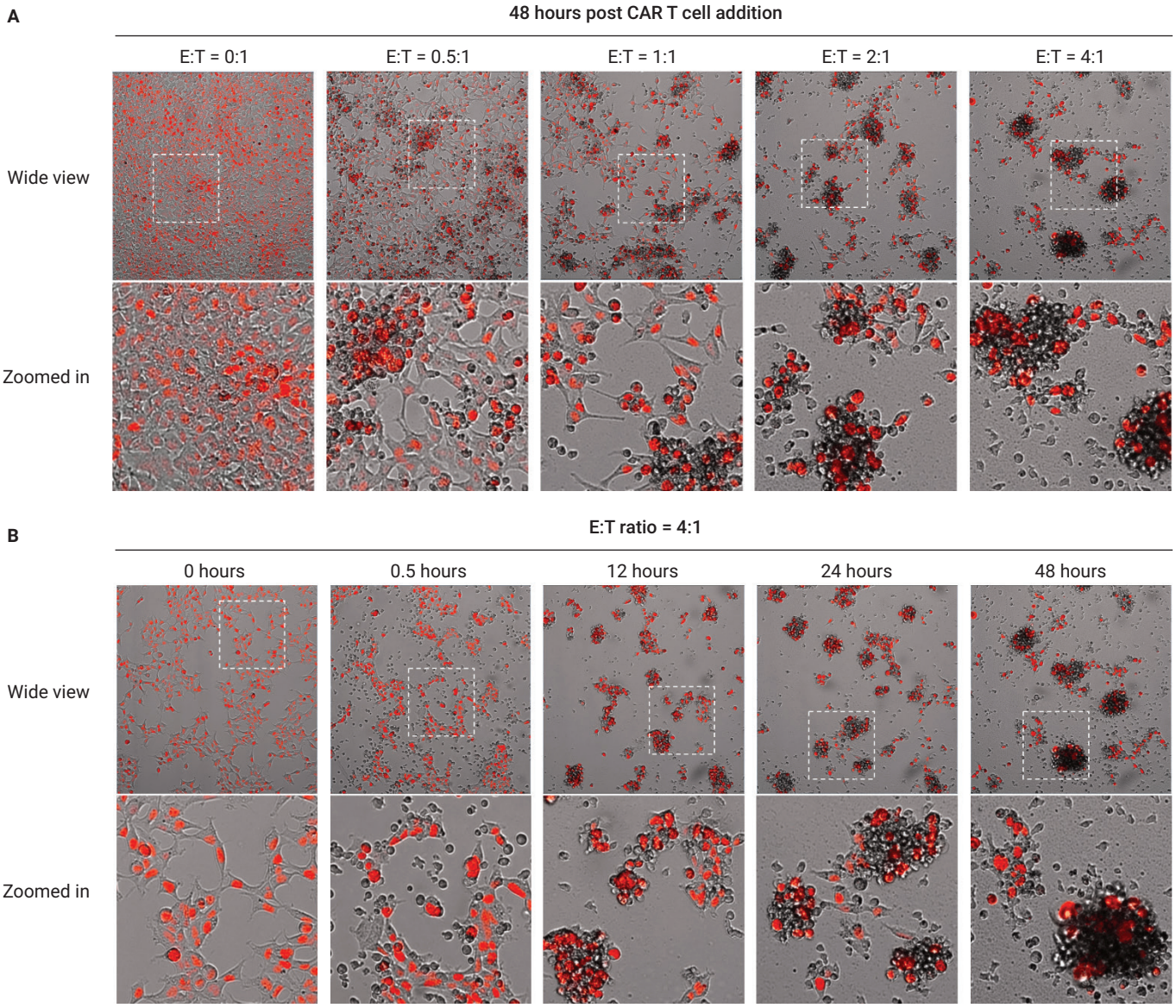

Figure 1. Killing of RFP-expressing HEK-293-CD19 cells by CD19 CAR T cells. (A) Comparison of different E:T ratios 48 hours post CAR T cell addition. The white squares in the upper panels denote the regions that are blown up in the lower panels. The unlabeled CAR T cells are grey. (B) Comparison of different time points for a constant E:T ratio of 4:1.

4:1, the time dependence of the killing response is clearly evident (Figure 1B).

Quantifying CAR T cell killing efficacy

The number of red target cells is plotted as a function of time in Figure 2A. Note that this plot excludes the first 18 hours of target cell proliferation; the zero-hour time point corresponds to the moment that CAR T cells were added to the well. At this time point, each well contains ~15,000 target cells, which is consistent with the published HEK-293 doubling time of 24 to 34 hours and the fact that 10,000 target cells were initially seeded. In the absence of CAR T cells, the target cells continue proliferating until the 50-hour time point (Figure 2A, black data trace). When CAR T cells are added at the very low E:T of 0.06:1, a killing response is not observed until the 30-hour time point (Figure 2A, orange data trace). Progressively increasing the E:T ratio causes the killing response to manifest at earlier time points and utlimately results in a greater number of target cells being destroyed.

Tracking the killing response using impedance (Figure 2C) produces cytolysis curves that are largely similar to the image-based curves in terms of their time- and dose-dependency. One salient difference between the two readouts is the fact that at E:T = 4:1, the impedance response drops to zero, whereas the number of target cells never drops below ~5,000. This persistence of target cells even at late time points is consistent with the photos shown in Figure 1. That the impedance signal concurrently falls to zero suggests that these lingering target cells are no longer strongly adhered to the well bottom.

Consistent with this, nearly all of the red cells that are visible after 48 hours of exposure to CAR T cells at E:T = 4:1 are rounded and appear to be loosely resting on the well bottom (Figure 1).

Using the simple equations shown in the materials and methods section, both the impedance data and the image-based data were converted into % cytolysis plots (Figures 2B and D).

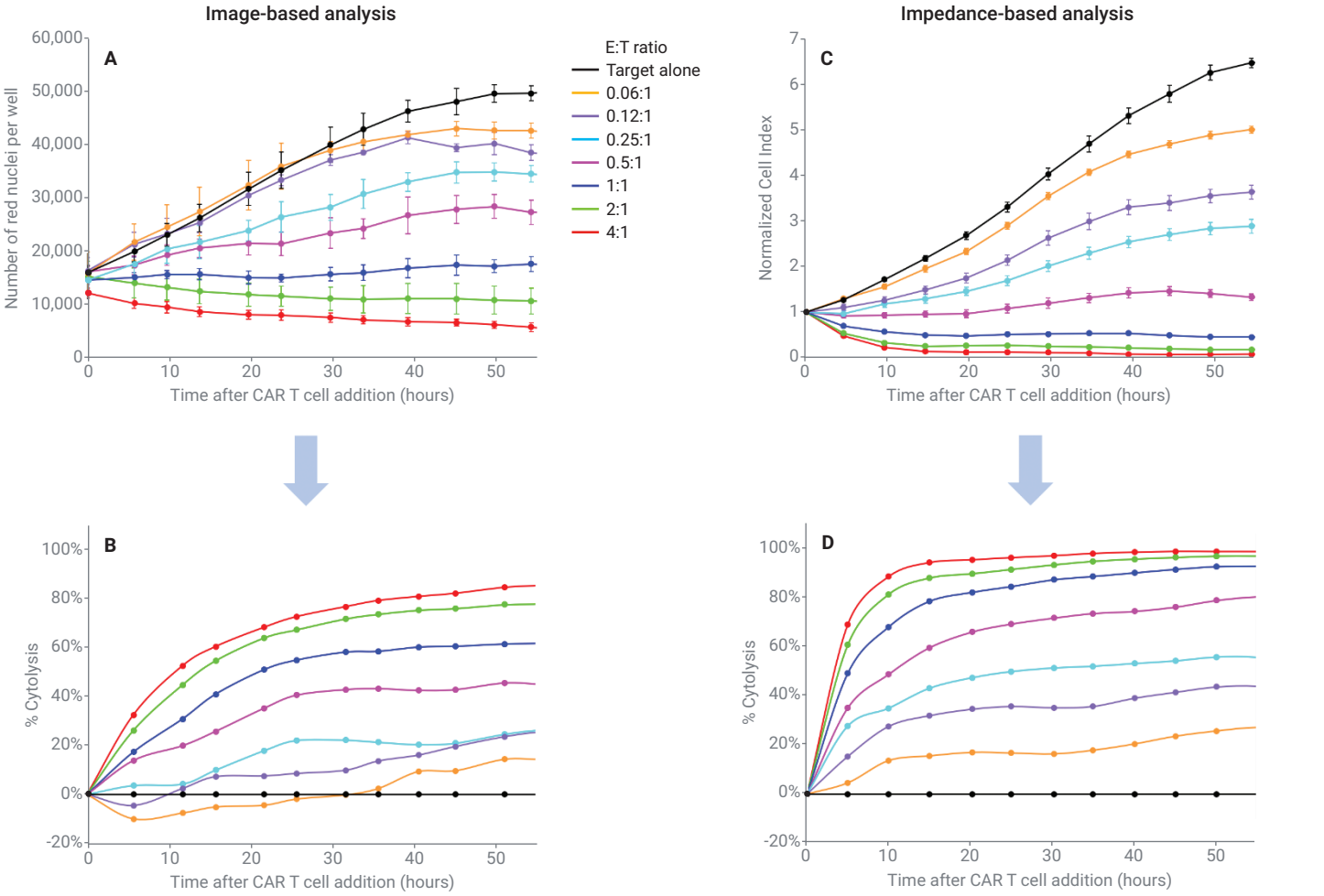

Figure 2. Time courses for HEK-293-CD19 killing by CD19 CAR T cells as measured by imaging (A,B) and impedance (C,D). While upper panels display the primary data, lower panels display % cytolysis, calculated as described in the materials and methods section. Assays were run in duplicate; error bars represent standard deviation.

When plotted this way, the kinetics of the killing responses are strikingly different between the two data sets. Even though both the impedance and imaging data were collected from the exact same population of cells (i.e., cells in the same well), for a given time point, the impedance signal universally gives a higher % cytolysis than the image-based readout.

Discussion

Beyond the benefit of having two independent measurement techniques, it is important to note the objectivity of the impedance readout, which is reported directly, without any processing or input from the user. Conversely, for eSight and all other imaging-based instruments, the raw image files get converted to outputs (such as number of red target cell nuclei) by user-informed algorithms where the expected size range, eccentricity, and brightness of cells must be defined. Although potential problems associated with this approach, such as interuser variability, can be minimized through proper training and consistent usage of the same segmentation parameters, having impedance as an objective comparator helps build confidence in the assay's results.

Although not used in this study, eSight can image cells in three different fluorescent channels. In addition to labeling target and effector cells with different colors, the third channel can be used to track cell death in general or apoptosis specifically through the use of annexin V- or caspase 3-specific reagents.

Conclusion

The xCELLigence RTCA eSight couples the simplicity, analytical sensitivity, and objectivity of real-time impedance monitoring with the highly specific readout of live-cell imaging to characterize CAR T cell killing efficacy with unparalleled ease and information richness.

References

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