Preclinical CAR-T cell target safety, biodistribution, and tumor infiltration analysis using the RNAscope® ISH assay

Helly Pimentel, Helen Jarnagin, Hailing Zong, Courtney Todorov, Courtney Anderson, Bingqing Zhang, Christopher Bunker, Xiao-Jun Ma
Advanced Cell Diagnostics Inc., 7707 Gateway Blvd, Newark, CA, USA 94560

Highlights

In this report we illustrate a way to detect activated CAR-T cells in the tissue context, as well as low expression of CAR target antigen, using the RNAscope® in situ hybridization (ISH) technology. CAR-T cells are activated by target antigen engagement even at very low levels of target expression. In order to avoid adverse events resulting from "on-target/off-tumor" toxicity, a highly sensitive and specific gene expression assay is necessary for preclinical target safety assessment. The RNAscope® assays can be incorporated into preclinical CAR-T safety assessment and clinical pharmacokinetic workflows to:

- Detect low levels of gene expression across all normal tissues in animal models and human samples
- Assess cell-specific target expression in normal tissues and expression heterogeneity in tumor tissues
- Visualize and quantify CAR-T cell infiltration and activation in tumor and off-tumor tissues
- Simultaneously detect CAR/TCR vector and cytokines, T cell markers, or other cell-type markers

Chimeric antigen receptor (CAR)-T cell therapy has proven to be highly effective in treating hematologic malignancies, and major efforts are being made to achieve similar efficacy in solid tumors. The greater potency of CAR-T cells compared to antibody therapeutics demands a more stringent CAR-T target safety assessment to avoid adverse events resulting from "on-target/off-tumor" activity. Furthermore, it is critical to track and monitor the pharmacokinetics of CAR+ T cells within the context of intact tissue and tumor to understand the mechanisms underlying off-tumor toxicity and efficacy in tumor killing. However, current methods are either not sensitive enough to detect low levels of expression or not capable of detecting CAR+ T cells in the tissue context.

The high specificity and single-molecule sensitivity of the RNAscope® *in situ* hybridization (ISH) technology for the detection of gene expression in the tissue context make it a key tool for the stringent assessment of "on-target/off-tumor" gene expression. The RNAscope® assay detects mRNA in routine formalin-fixed paraffin-embedded (FFPE) tissues by utilizing a unique probe design and signal amplification strategy that enables visualizing individual RNA molecules as discrete dots[1]. The key

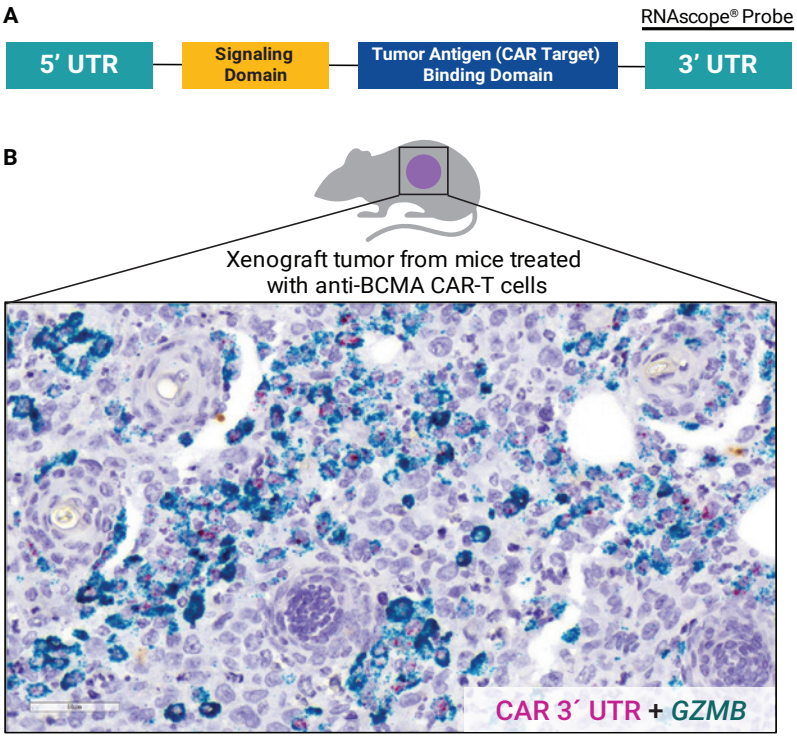

FIGURE 1. RNAscope® assay for detection. (A) RNAscope® probes were designed to target the 3′ UTR of the CAR vector. (B) Activated CAR+ T cells traffic to the tumor site. The RNAscope® 2.5 LS Duplex Assay simultaneously detected the 3′ UTR from the CAR vector (red) and granzyme B (*GZMB;* green) in a tumor xenograft from RPMI-8226 mice treated with anti-BCMA CAR-T cells.

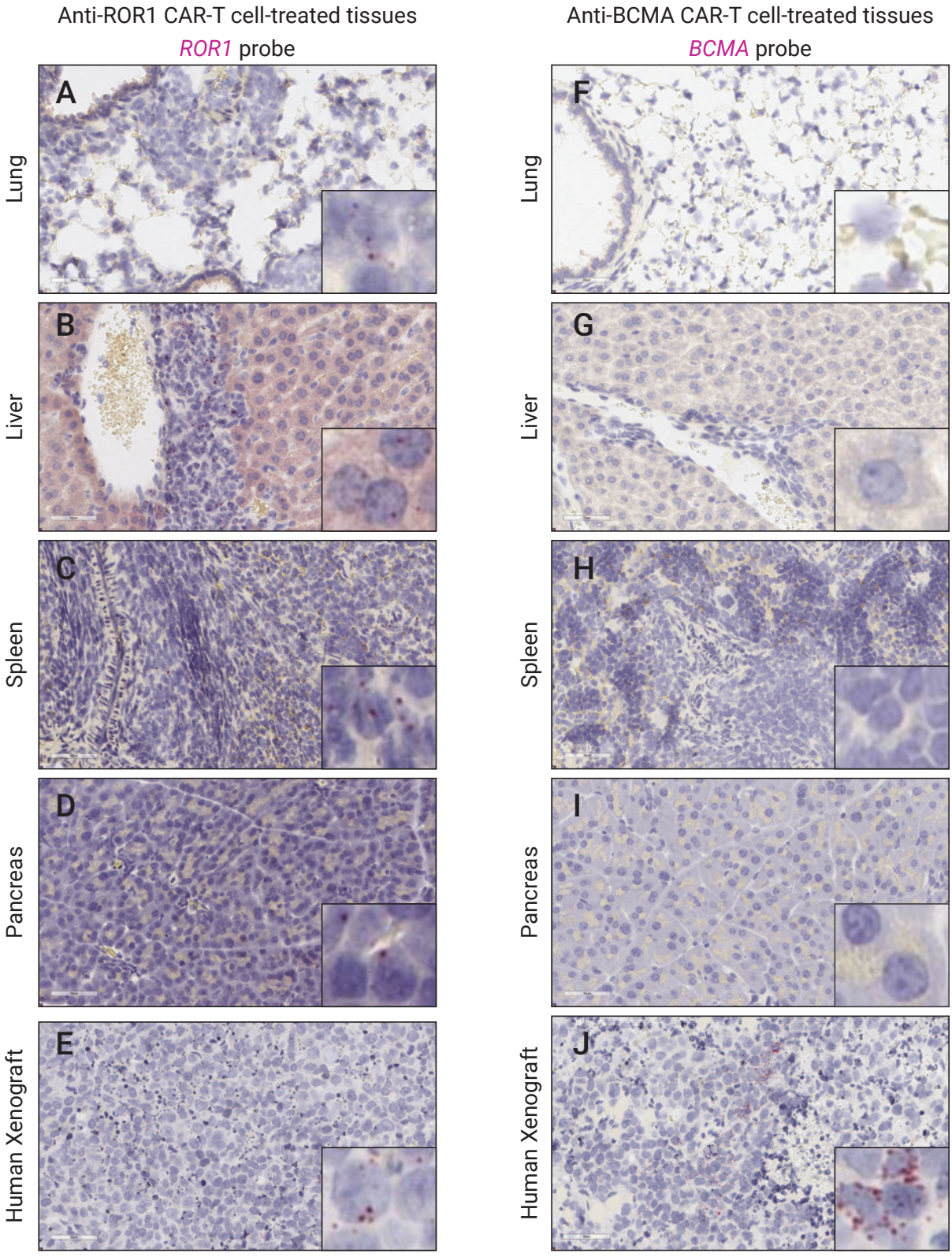

FIGURE 2. Target expression pattern for safety assessment. The RNA expression pattern of the CAR-T cell target antigens *ROR1* and *BCMA* was assessed using the RNAscope® 2.5 LS Red Assay. (A–E) Tissues and xenograft tumors from RPMI-8226 mice treated with anti-ROR1 CAR-T cells were assessed for *ROR1* expression using probes for either Mm-Ror1 (A–D) or Hs-ROR1 (E). (F–J) Tissues and xenograft tumors from RPMI-8226 mice treated with anti-BCMA CAR-T cells were assessed for *BCMA* expression using probes for either Mm-Bcma (F–I) or Hs-BCMA (J).

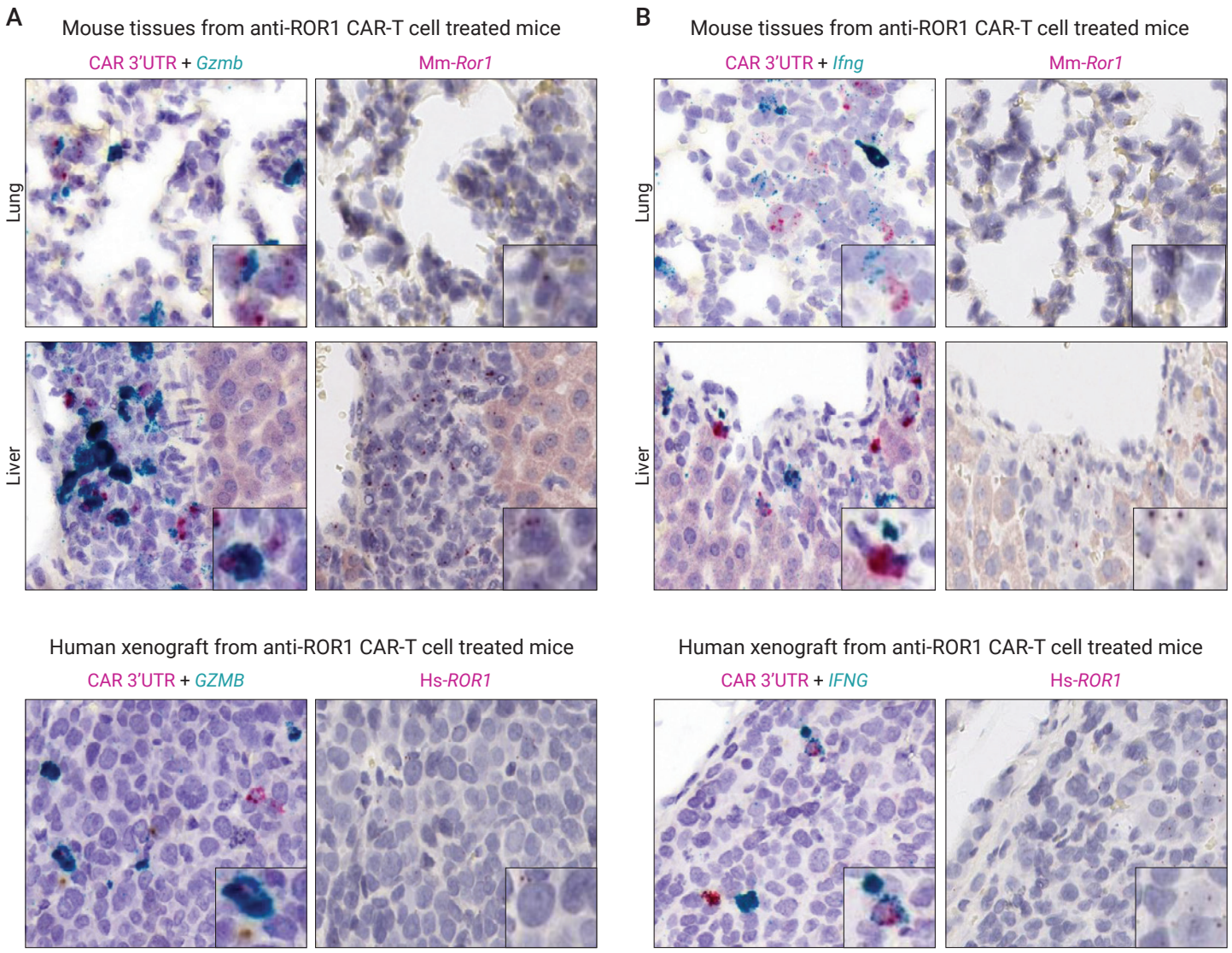

FIGURE 3. Activated anti-ROR1 CAR-T cells detected in lung, liver, and xenograft tumor, revealing on-target/off-tumor activity. The RNAscope® 2.5 LS Duplex Assay was used to simultaneously detect the 3' UTR from the CAR vector (red) and either *GZMB* (A) or *IFNG* (B) (green) in liver, lung, and tumor xenograft from RPMI-8226 mice treated with anti-ROR1 CAR-T cells. Expression of *ROR1* was examined in a serial section, revealing *ROR1* expression in lung, liver, and tumor xenograft where activated CAR-T cells are present.

advantage of the RNAscope® ISH assay over IHC for CAR detection is its ability to target the viral untranslated regions (UTRs) of the CAR transcript, which can be used as unique sequence tags for CAR+ T cell detection in both animal and human tissues. The RNAscope® assay can also be multiplexed for simultaneous detection of CAR, cytokines, T cell markers and other markers. Cell-based whole-slide image analysis of multiplexed RNAscope® data can provide greater insight into CAR-T cell trafficking and activation. Taken together, the RNAscope® technology provides an unparalleled sensitive and specific method for cell and tissue-specific assessment of preclinical CAR-T targets as well as tumor infiltration of activated CAR-T cells.

Several studies have applied the RNAscope® technology for CAR-T assessment. Researchers at the University of Pennsylvania and Novartis used the RNAscope® assay to determine the extent of CAR-T-EGFRvIII infiltration into the tumors of patients with glioblastoma[2]. Using a probe against the 3' UTR of their CAR vector as well as a probe for *IFNG* they demonstrated that activated CAR-T-EGFRvIII cells traffic to the tumor site 2 weeks post-infusion. In another study from the same institutions, researchers found that despite delayed tumor growth following CAR-T cell treatment against the FR-α antigen in mice, tumors eventually grew exponentially[3]. To understand why the tumors escaped CAR-T cell therapy, the RNAscope® Duplex assay was used to examine expression of FR-α and CD3. The authors found several areas of the tumors that expressed FR-α and contained large numbers of CD3+ T cells, suggesting that, despite successfully infiltrating the tumors, the FR-α CAR-T cells had become hypofunctional.

In this study we employed the RNAscope® ISH assay to assess CAR-T target expression specificity for two candidate CAR-T targets, *BCMA* and *ROR1*, in xenograft and host tissues using the RPMI-8226 xenograft mouse model. We also utilized the RNAscope® Duplex and Multiplex assays to track CAR-T cell distribution and activation.

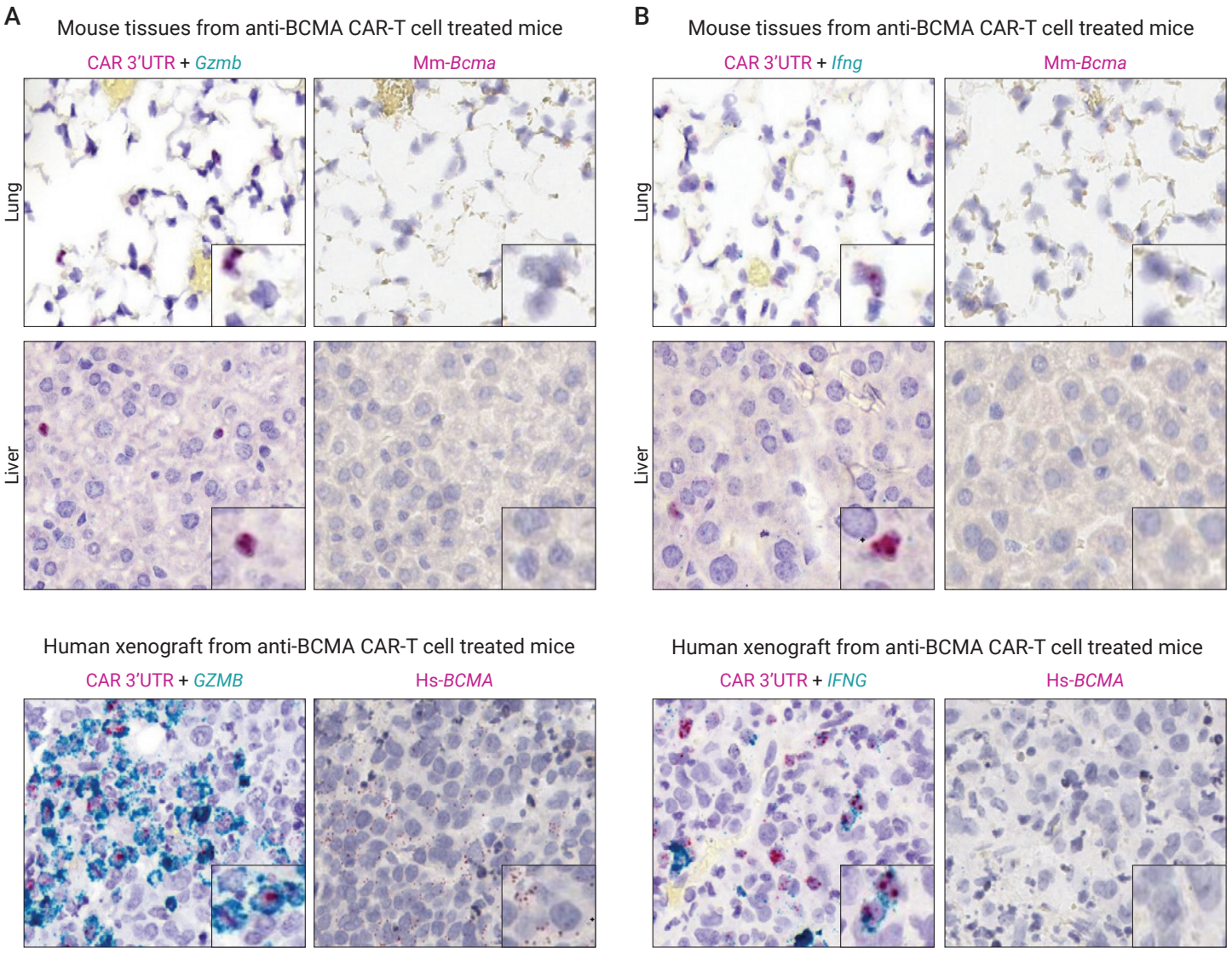

FIGURE 4. Activated anti-BCMA CAR-T cells detected only in xenograft tumor, demonstrating on-target/on-tumor activity. The RNAscope® 2.5 LS Duplex Assay was used to simultaneously detect the 3' UTR from the CAR vector (red) and either *GZMB* (A) or *IFNG* (B) (green) in liver, lung, and tumor xenograft from RPMI-8226 mice treated with anti-BCMA CAR-T cells. Expression of *BCMA* was examined in a serial section, revealing *BCMA* expression only in the tumor xenograft where activated CAR-T cells are present.

Results

CAR-T TARGET ANTIGEN EXPRESSION AND DISTRIBUTION

For the CAR-T cell target candidates, *BCMA* and *ROR1*, RNAscope® ISH revealed that *BCMA* was only expressed in the xenograft tumor and in no mouse organs, while *ROR1* was found to be expressed at low levels in mouse lung and liver as well as in the xenograft tumor (Figure 2).

CAR-T CELL CONTRIBUTION

To detect CAR+ T cells in the tissue context, we designed an RNAscope® probe that targets the 3' UTR of the CAR vector utilized in this study (Figure 1A). RPMI-8226 xenograft mice were then treated with the CAR-T cells for up to 7 days and tissue samples were collected. The anti-ROR1 CAR-T cells used in this study recognized both the mouse and human proteins whereas the anti-BCMA CAR-T cells recognized only the human protein. Duplex RNAscope® ISH assays with probes targeting the CAR 3' UTR and either *IFNG* or *GZMB* allowed for highly sensitive and specific detection of CAR-T cells and their activation state in both tumor and normal tissues from vehicle, anti-BCMA CAR-T cell, or anti-ROR1 CAR-T cell treated mice. Activated anti-BCMA CAR-T cells expressing *GZMB* or *IFNG* were found only in the xenograft tumor, where BCMA is expressed (Figure 4). In contrast, activated anti-ROR1 CAR-T cells were found almost exclusively in mouse lung and liver, with very few anti-ROR1 CAR-T cells found in the xenograft tumor (Figure 3).

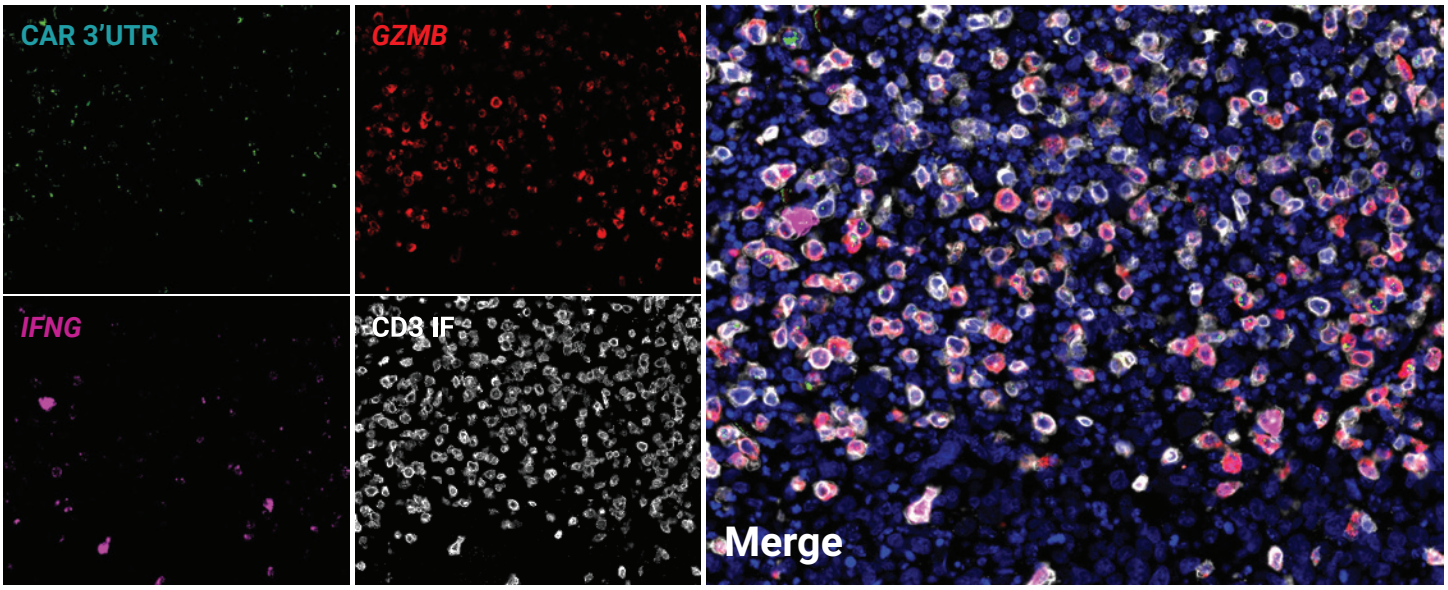

FIGURE 5. Trafficking of activated CAR-T cells to the tumor site. The RNAscope® LS Multiplex Fluorescent assay was combined with IF to visualize tumor infiltration of activated anti-BCMA CAR-T cells. RNAscope® ISH for the 3' UTR of the CAR vector (green), *GZMB* (red), and *IFNG* (pink) was followed by IF for CD3 (white) in xenograft tumors from anti-BCMA CAR-T cell treated RPMI-8226 mice.

CAR-T CELL ACTIVATION

Lastly, a multiplex RNAscope® ISH-IF approach confirmed the presence of activated anti-BCMA CAR-T cells in the xenograft tumor through simultaneous detection of the CAR 3' UTR, *IFNG*, *GZMB*, and CD3 (Figure 5).

Conclusion

Given the high sensitivity of CAR-T cells to very low densities of target antigen, it is critical to employ a highly stringent and sensitive assay for preclinical target safety assessment. In this study we illustrate the ability of the highly sensitive RNAscope® assay to detect on-target but off-tumor expression of *ROR1*. Anti-ROR1 CAR-T cells were active in off-tumor tissues, suggesting potential toxicity of Target Y. Conversely, *BCMA* was only expressed in the tumor and anti-BCMA CAR-T cells were active only in the tumor. These data demonstrate how the RNAscope® assay can be utilized for CAR-T cell efficacy and safety/toxicity assessment in preclinical models by detecting very low levels of target antigen expression in off-tumor tissues, as well as for monitoring CAR-T cell pharmacodynamics and activation in tumor models. Furthermore, the technology has demonstrated unique utility for both CAR-T and TCR-T cell pharmacokinetic and pharmacodynamic analysis in post-treatment patient biopsies. For more information please visit us online at www.acdbio.com/genetherapy.

References

1. Wang F, et al. RNAscope®: A novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn.* 2012; 14(1):22–9.
2. O'Rourke DM, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med.* 2017; 9(399): pii: eaaa0984.
3. Wing A, et al. Improving CART-cell therapy of solid tumors with oncolytic virus-driven production of a bispecific T-cell engager. *Cancer Immunol Res.* 2018; 6(5):605–16.

T cell therapy like you've never seen it before: Tissue-based cell therapy analysis by ACD Pharma Assay Services

- Utilize ACD's expertise in the RNAscope® technology for recommendations on a controlled study design
- Rely on ACD's scientific experts for accurate data interpretation
- Visualize the distribution of CAR-T or TCR-T cells in the tumor microenvironment
- Evaluate co-expression of the CAR-T or TCR-T cell vector with cell-type markers
- Assess safety/toxicity by screening for target expression in non-tumor tissues
- Results are delivered in 4–5 weeks on average
- Learn more about ACD's Pharma Assays Services: www.acdbio.com/pas

Learn more about the RNAscope® technology for CAR-T cell efficacy and safety assessment at **www.acdbio.com/genetherapy**

ACD™
a bio-techne brand

California, USA