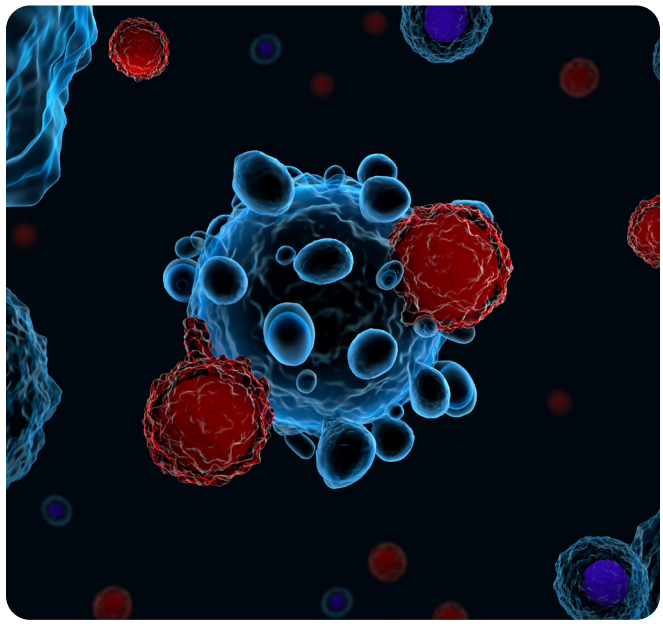
CASE STUDY

# A novel mouse model using optical imaging to detect on-target, off-tumor CAR-T cell toxicity

## Introduction

Cancer remains the second leading cause of death globally, despite the recent explosion of cancer immunotherapeutics that have significantly improved patient outcome for certain tumor types. Reprogramming patient T cells to recognize specific tumor associated antigens using chimeric antigen receptors (CARs) has revolutionized the field of cancer immunotherapy. Autologous CAR-T cell therapy has demonstrated impressive response rates in certain hematological malignancies, including curative results in up to 90% of acute B-lymphoblastic leukemia (ALL) patients.[1] However, the solid tumor microenvironment (TME) poses many challenges for T cell-based therapeutics. Once infused, engineered T cells must traffic to the TME and battle a multitude of obstacles including physical barriers to tumor entry, immunosuppressive cytokines, and T cell checkpoint pathway blockade. Consequently, despite remarkable success in hematological malignancies, CAR-T cell approaches have failed to achieve meaningful efficacy in solid tumors.[2]



Dr. Mauro Castellarin has extensive experience in the field of CAR-T cell therapy, including his work in the lab of CAR-T cell pioneer, Carl June. Dr. Castellarin's focus is on creating novel immune cell therapies with enhanced cytotoxicity and safety with the goal of improving treatment for cancer patients in the clinic. Recently, Dr. Castellarin's research has contributed to a novel universal CAR targeting multiple tumor antigens to treat relapsed malignancies due to antigen loss. The case study herein illustrates how Dr. Castellarin and colleagues used preclinical optical imaging to develop a mouse model of on-target, off-tumor CAR-T cell toxicity by means of controlled expression of human tumor antigen in normal mouse tissues. In this novel mouse model, Revvity's IVIS™ Spectrum imaging system was used to measure antigen expression in normal tissue concomitant with tumor response to CAR-T cell treatment.[3]

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## Our challenge

*"To create a mouse model that expresses human, tumor-associated antigens in normal mouse tissue so that we can study off-tumor CAR-T cell toxicity. We wanted our model to have the capability to monitor both tumor burden and immune cell trafficking in real-time."*

**Dr. Mauro Castellarin**

## The challenge

Success of T cell-based therapy in solid tumors is limited by identification of appropriate tumor antigens that, when targeted, carry limited toxicity while effectively eliminating tumor cells. Selection of an ideal tumor-associated antigen requires high expression on tumor cells combined with minimal expression on nonessential normal tissue, as target antigen expression on normal tissue can result in severe adverse effects commonly referred to as 'on-target, off-tumor toxicity'. One such attractive target is HER2, which is highly expressed in a variety of solid tumors. Aberrant expression of HER2 is extensively linked to malignant transformation of tumors as well as tumor aggressiveness and persistence.4,5 Given its frequent overexpression in tumors and low expression in normal tissues, HER2 is an appealing target for immunotherapy. Indeed, numerous clinical trials have sought to take advantage of this target using antibodies, recombinant immunotoxins, and CAR-T cells.5 Given the clinical success of HER2-targeted monoclonal antibodies such as trastuzumab, a CAR-T therapy was designed based on the trastuzumab sequence and used to treat a patient with colorectal cancer. Unfortunately, in this patient on-target, off-tumor toxicity resulted in irreparable damage to the cardiopulmonary system and lethal toxicity, which was not predicted based on pre-clinical animal experiments.

Prediction of human responses to cellular therapies based on testing in pre-clinical models is challenging, given variability in cross-species reactivity to target antigens. Current mouse models lack the ability to predict on-target, off-tumor toxicity, highlighting the need for an animal model that expresses human targets in normal tissue.6 One method under investigation to improve the specificity of CAR-T cells and reduce this on-target, off-tumor toxicity is genetically tuning the affinity of a CAR by altering the antigen-binding region. Affinity-tuning of CARs allows for detection of high-density target antigen on tumor cells while limiting reaction to low-density antigen on normal cells.7 However, testing of on-target, off-tumor toxicity requires a mouse model that contains human antigen targets on both the tumor and in normal tissue.

To assess the therapeutic efficacy and on-target, off-tumor toxicity of affinity-tuned CARs, Dr. Castellarin and colleagues designed a mouse model expressing the target antigen, human HER2 (hHER2), on both tumor cells and in normal mouse liver tissue. Optical imaging using the IVIS Spectrum was used to assess hepatic expression of target antigen, trafficking of affinity-tuned CAR-T cells to tumor or liver sites, as well as tumor response to CAR-T cell treatment.

## What is IVIS and why use it in CAR-T cell studies?

*"Revvity's IVIS imaging platform provides high sensitivity, non-invasive imaging of bioluminescent and fluorescent cells simultaneously. Evaluation of novel CAR T cell therapeutics in preclinical models relies on reproduction of the human disease state, often with orthotopic tumors that cannot be measured by calipers, such as blood malignancies. In these disease models, the IVIS platform is especially useful for monitoring tumor burden while concurrently evaluating the therapeutic activity of CAR T cells over time."*

**Dr. Mauro Castellarin**

## Viewing the full picture

Optical imaging is frequently used for non-invasive longitudinal monitoring of cancer progression and therapeutic efficacy in preclinical models. The IVIS optical imaging platform facilitates high sensitivity imaging of both bioluminescence and fluorescence in vivo, allowing for collection of numerous biological endpoints simultaneously and providing further insight into underlying molecular events at the site of disease. The IVIS Spectrum imaging system was used in these studies to monitor disease progression, CAR-T cell trafficking, and target gene expression patterns in living animals.

In order to deliver hHER2 to normal murine hepatocytes, Dr. Castellarin and colleagues tested two methods:1) Intravenous (i.v.) adeno-associated virus serotype 8 (AAV8) gene delivery and 2) Hydrodynamic delivery using the piggyBac transposon system. Fluorescent imaging of murine livers ex vivo on the IVIS system demonstrated both methods mediated successful delivery of fluorescent cargo to hepatic cells in a dose-dependent manner, indicating these gene delivery systems can be used to regulate the level of hHER2 expression in vivo. Non-invasive IVIS imaging of luciferase was also used as a surrogate marker for hHER2 antigen delivery and expression using the piggyBac transposon system. The versatility of this system allowed for stable expression of hHER2 and luciferase at pre-determined levels in vivo using different plasmid concentrations, which was easily monitored with weekly bioluminescence imaging to assess kinetics of hepatic antigen expression (see complete manuscript for data).

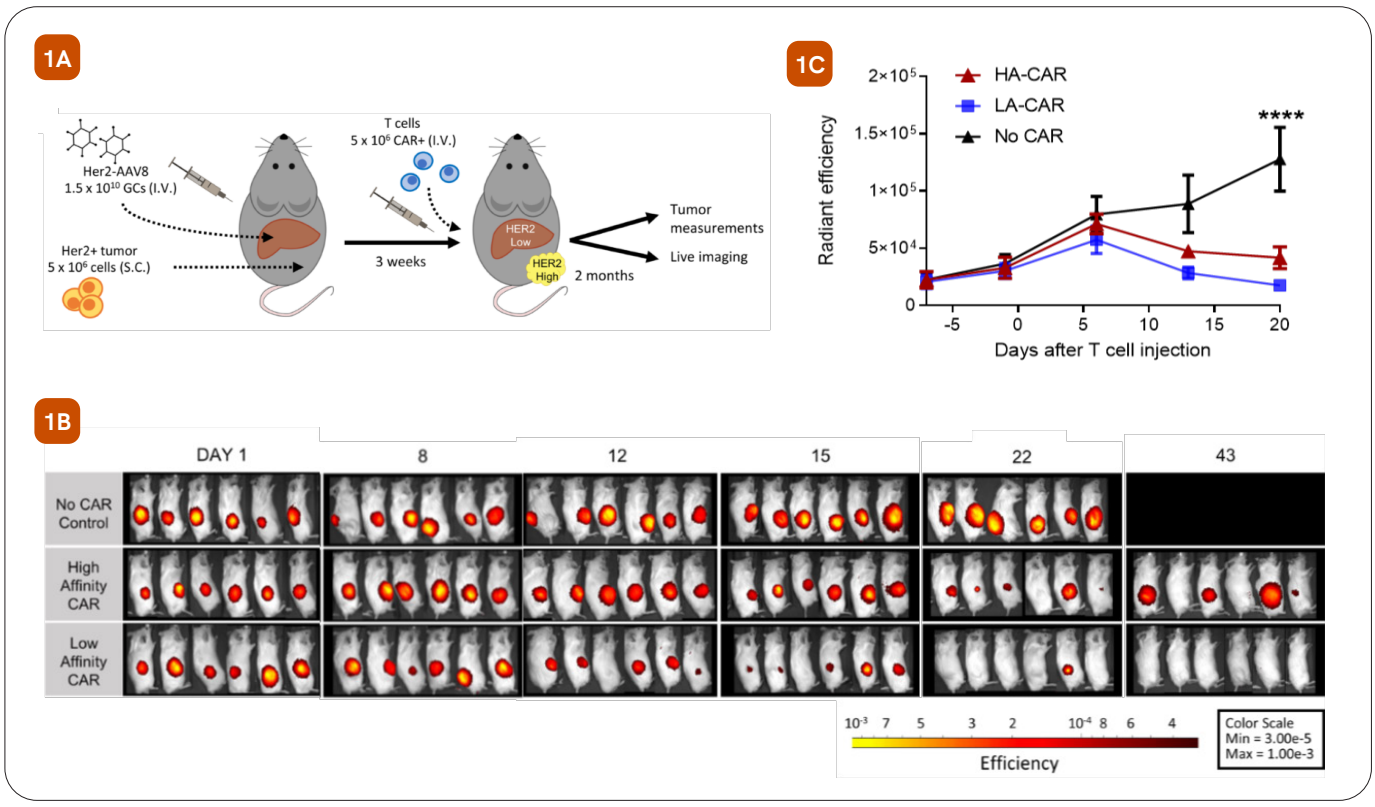


Figure 1: Low-affinity anti-HER2 CAR-T cells exhibit superior tumor control than high-affinity CAR-T cells in mice with concurrent hepatic expression of hHER2. (A) Mice received 1.5e+10 genome copies of hHER2 via intravenous AAV8 delivery and were implanted subcutaneously with 5 x 10^6 iRFP720-expressing SKOV3 tumor cells, which overexpress the hHER2 antigen. After three weeks, mice were treated with 5 x 10^6 affinity-tuned CAR-T cells expressing luciferase and tumor burden was assessed using fluorescence imaging on the IVIS Spectrum (n=6 mice/group). (B/C) Mean tumor burden ± SEM measured by fluorescence. ****P < 0.0001.

To assess whether hepatic hHER2 antigen expression delivered with AAV8 could induce on-target, off-tumor toxicity by anti-HER2 CAR-T cells, mice were infused (i.v.) with luciferase-expressing HER2 CAR-Ts with high (HA) or low affinity (LA) antigen-binding moieties (Figure 1A). Bioluminescent imaging demonstrated the hepatic hHER2 antigen acted as a target for HER2-targeted CAR-T cells, and subsequent liver toxicity was reduced with lower hepatic antigen levels or by use of a LA CAR. To evaluate differential anti-tumor efficacy of HA and LA CAR-T cells in mice expressing hepatic hHER2 antigen, mice were implanted with HER2+ SKOV3 tumor cells expressing the iRFP720 fluorescent reporter protein. Importantly, fluorescent imaging of tumor burden demonstrated treatment with LA CAR-T cells resulted in superior tumor control compared to those treated with HA CAR-T cells when antigen is also present in normal mouse hepatic tissue, suggesting the off-tumor targeting of HA CAR-T cells impedes antitumor efficacy (Figure 1B/C).

To assess if attenuation of anti-tumor efficacy was due to a delay in recruitment or retention of HA CAR-T cells to the tumor, HA and LA CAR-T cells were genetically labeled with luciferase, and bioluminescence imaging was used to non-invasively monitor T cell trafficking (Figure 1A). While whole-body bioluminescence showed similar abundance of LA and HA CAR-T cells over time (Figure 2B), there were striking differences in the trafficking pattern of the HA and LA CAR-T cells. Quantification of T cells in the liver and tumor on days eight and twelve post-treatment revealed the LA CAR-T cells had much more effectively homed to the site of the tumor while HA CAR-T cells remained in the liver (Figure 2C). Thus, the augmented anti-tumor efficacy of LA CAR-T cells may be at least partially attributed to the superior ability of these cells to distinguish between low-antigen healthy tissue and high-antigen tumor tissue and quickly traffic to the tumor.

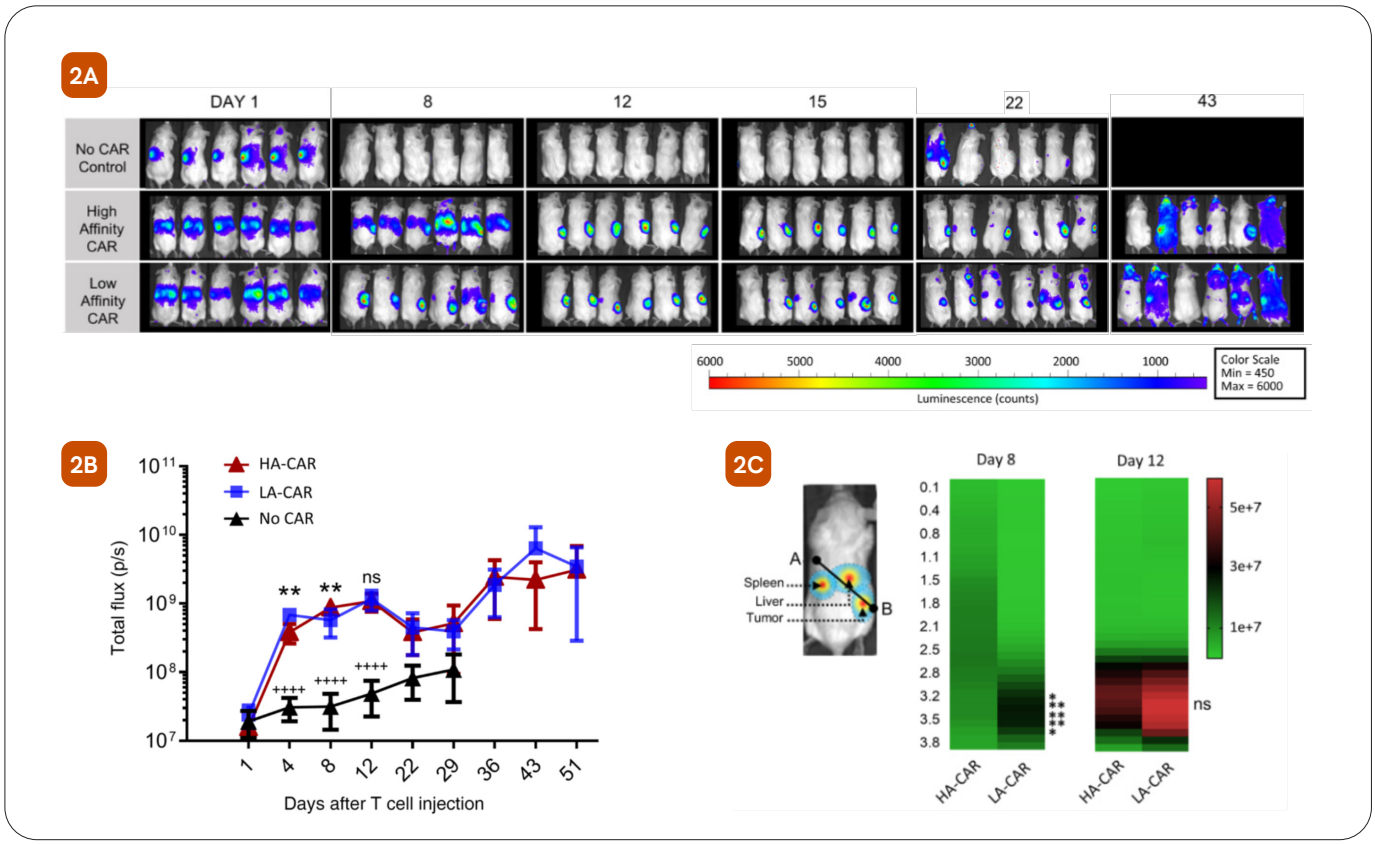


Figure 2: Low-affinity anti-HER2 CAR-T cells home to tumor more efficiently than high-affinity CAR-T cells in mice with concurrent hepatic expression of hHER2. (A) As in Figure 1, mice received 1.5e+10 genome copies of hHER2 via i.v. AAV8 delivery and were implanted s.c. with 5 x 10^6 iRFP720-expressing SKOV3 tumor cells, which overexpress the hHER2 antigen. After three weeks, mice were treated with 5 x 10^6 affinity-tuned CAR-T cells expressing luciferase and T cell trafficking was assessed by bioluminescence of T cell luciferase (n=6 mice/group). (B) Quantification of T cell abundance ± SEM by whole-body bioluminescence. (C) Spatial luciferase expression was measured using a line profile from point A to B, crossing through the spleen, liver and tumor from start to end. Mean luminescence along the line was compared between groups by two-way repeated measures ANOVA with Bonferroni's multiple comparisons test. Statistical significance is denoted as **P < 0.01 and ****P < 0.0001.

## Conclusion

Clinically, selection of CAR-T cell targets is restricted to antigens expressed on tumor cells with limited expression on nonessential normal tissues to avoid on-target, off-tumor toxicity. However, the use of mouse models to assess on-target, off-tumor CAR-T cell toxicity doesn't properly assess potential toxicities, due to variation in species-specific antigen reactivity. Dr. Castellarin and colleagues have developed a novel mouse model which expresses human antigen in normal mouse tissues. These studies used bioluminescence and fluorescence imaging on the IVIS platform to longitudinally monitor off-tumor antigen expression, tumor progression, and CAR-T cell trafficking in live animals. Importantly, this imaging revealed enhanced anti-tumor activity and safety of LA CAR-T cells when antigen is also expressed on normal tissues, indicating affinity-tuning of CAR-T cells may be an effective strategy to evade the dose-limiting toxicity that has remained a challenge for CAR-T cell therapeutics.

## The IVIS Spectrum offers:

- High-sensitivity 2D and 3D in vivo bioluminescence and fluorescence imaging
- High throughput (5 mouse) and high-resolution (20 microns in 3.9 cm field of view) options for both in vivo and ex vivo/in vitro applications
- 3D diffuse tomographic reconstruction allowing for pinpoint localization of optical signal
- Ability to automatically co-register CT or MRI images to provide anatomical context for your optical data

## Acknowledgements

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## The future of IVIS imaging for CAR-T cell research

Dr. Castellarin envisages that the Revvity's IVIS imaging platform will become an even more powerful tool for preclinical studies given the recent and rapid development of fluorescent and bioluminescent reporters to illuminate CAR-T cell trafficking and activity in vivo. The unique ability of the IVIS Spectrum system to co-register optical signal in 2D or 3D from numerous reporters as well as CT or MRI will provide additional anatomical and functional insight into the anti-tumor activity of CAR-T cell therapeutics.

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