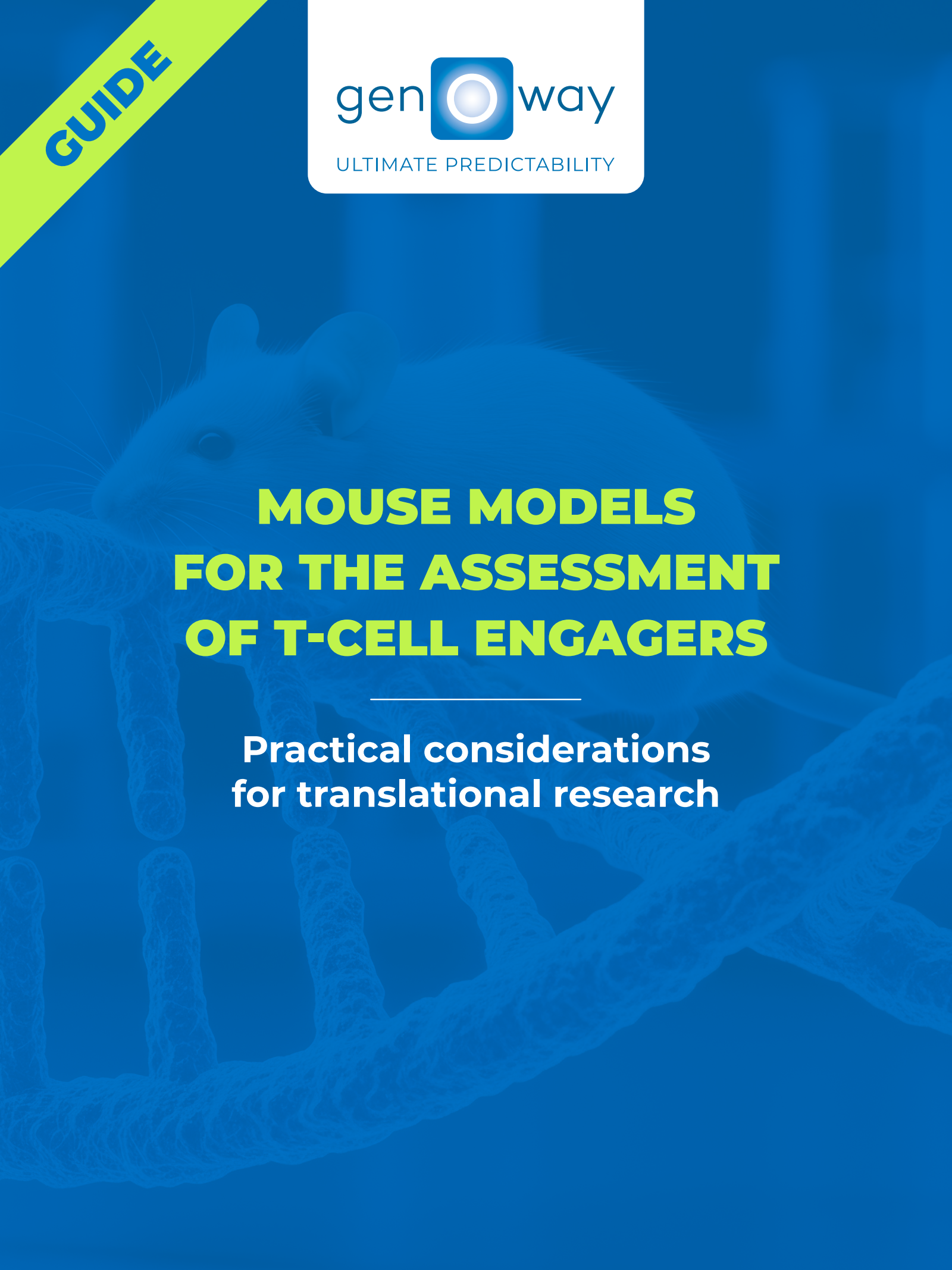
GUIDE

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ULTIMATE PREDICTABILITY



MOUSE MODELS FOR THE ASSESSMENT OF T-CELL ENGAGERS

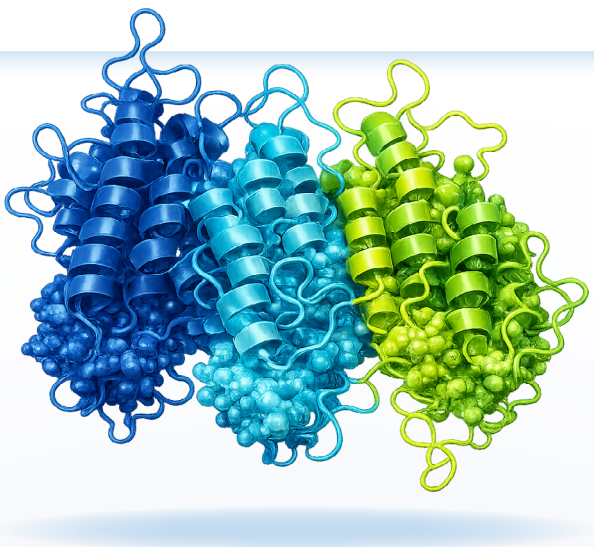
Practical considerations for translational research

Featuring the genO-panCD3 (scan or click)

Featuring the genO-BRGSF-HIS (scan or click)

KEY MESSAGES

- ✓ CD3 engagement is **the core mechanism of action of T-cell engagers** (TCEs) and **must be relevant for humans**
- ✓ Species differences in the CD3 sequence and structure between mice and humans, as well as their limited antibody cross-reactivity, **may result in inaccurate findings** when using wild-type (WT) mouse data
- ✓ **Humanized CD3 mouse** models enable **mechanistic**, **efficacy**, and **safety-relevant TCE assessment**
- ✓ Model choice strongly **impacts T-cell activation strength**, **cytokine release**, and **translational value**
- ✓ Using the wrong model can **lead to false negatives**, **incorrect toxicity**, or **misleading potency ranking**



INTRODUCTION / BACKGROUND

What are T-cell engagers (TCEs)?

T-cell engagers are bispecific or multispecific biologics designed to:

- Bind CD3 on T-cells
- Simultaneously bind a target antigen on tumor or diseased cells
- Trigger target-dependent T-cell activation and cytotoxicity

Common formats include:

- IgG based bispecific antibodies (BiTEs)
- Trispecific antibodies
- Multivalent or Fc-engineered constructs

What is the role of CD3?

CD3 is an essential signaling component of the T-cell receptor (TCR) complex.

- Composed of CD3ε, CD3δ, CD3γ, and CD3ζ chains
- Transduces antigen recognition into intracellular activation signals
- Controls: T-cell activation, cytokine secretion, proliferation, cytotoxic function of IgG half-life

What is the role of CD3 for testing TCEs?

For TCEs, CD3 is:

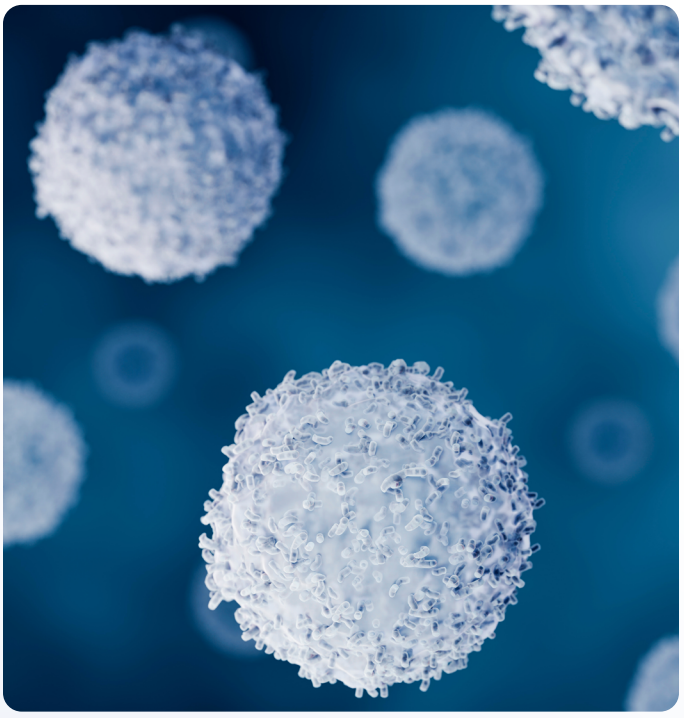
- The primary pharmacological target
- The main driver of: T-cell activation, cytokine release (including CRS risk), therapeutic index (the quantitative measurement of the relative safety of a drug with regard to risk of overdose)

Implication: CD3 expression, structure, and signaling must match human biology to generate interpretable and translational data.

Other types of therapies that can be tested in humanized CD3 mice

Beyond classical TCEs, humanized CD3 mouse models are relevant for:

- CD3 monoclonal antibodies
- Fc-engineered anti-CD3 antibodies
- Antibody fragments and recombinant formats



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Featuring the genO-panCD3 (scan or click)

Featuring the genO-BRGSF-HIS (scan or click)

HOW TO CHOOSE THE CORRECT MODEL FOR A STUDY

Ask yourself these questions first:

1. Is my molecule binding human CD3?
2. Do I need *in vivo* T-cell activation?
3. Is cytokine release a key liability to assess?
4. Am I testing efficacy, safety, or both?
5. Is this data used for candidate ranking or go/no go decisions?



Comparison of mouse models for TCE assessment

MOUSE MODEL	USE WHEN	AVOID WHEN	ADVANTAGES	KEY LIMITATIONS
Wild-type mice	· Very early feasibility · Non-human CD3-binding constructs	· Human CD3-specific TCEs · Translational studies	· Simple · Low cost	· Murine CD3 differs structurally and functionally, limiting translatability · No relevance for CD3-targeting therapeutics · No predictive value for TCE activity
Non-genOway CD3ε humanized mice commercially available	· Confirming binding of human CD3ε-targeting TCEs (SP34 epitope) · Early proof-of-mechanism studies	· Translational efficacy or safety studies · Cytokine risk assessment	· Enables engagement of human CD3ε · More relevant than WT mice for CD3 binding	· Only CD3ε is humanized · Often reduction of T cell number and altered CD4:CD8 ratio compared to WT · Smaller thymus · Limited versatility for TCE optimization
genO-hCD3ε (genOway)	Testing SP34-derived antibodies	Studies with TCEs targeting different CD3 subunits	· Crosstalk between tumor and stroma · Conserved interaction with CD3γ, CD3δ and CD3ζ subunits · Physiological expression of human CD3 · Functional T/B-cell cooperation · Fully functional mouse immune system	· Only CD3ε is humanized · Mouse immune system · Requires the implantation of murine tumor cells · Does not support the study of human tumors · Limited versatility for TCE optimization

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MOUSE MODEL	USE WHEN	AVOID WHEN	ADVANTAGES	KEY LIMITATIONS
Non-genOway CD3εδγ humanized mice commercially available	· Confirming binding of human CD3-targeting TCEs · TCE efficacy studies · *In vivo* T-cell activation assessment	· Studies requiring TCEs safety analysis · Decision making studies	· Humanization of multiple CD3 signaling subunits · Improved CD3 signaling fidelity vs CD3ε-only models	· Expression levels and regulation may be non-physiological · Requires careful functional validation before decision-grade use · Problems in thymus development
genOway's humanized CD3εδγ (genO-panhCD3)	· Translational assessment of efficacy and safety of TCEs or mAbs · Candidate ranking	· Requirement of tumor and stroma cross-talk · Human liquid tumors	· Crosstalk between tumor and stroma · Conserved interaction with CD3γ, CD3δ and CD3ζ subunits · Physiological expression of human CD3 · Functional T/B-cell cooperation · Fully functional mouse immune system · Translationally relevant T-cell activation and cytokine responses	· Mouse immune system · Requires the implantation of murine tumor cells · Does not support the study of human tumors
PBMC-reconstituted mice	· Rapid assessment with human immune cells · Exploratory efficacy	· CRS prediction	· Human T-cells present · Easy implementation	· Xenogeneic activation · High risk of GvHD · Misleading cytokine levels · Short experimental window

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MOUSE MODEL	USE WHEN	AVOID WHEN	ADVANTAGES	KEY LIMITATIONS
Non-genOway HIS mice commercially available	· Rapid assessment with human immune cells · Assessment of efficacy and safety of TCEs or mAbs · Longitudinal immune response studies	· CRS prediction	· Human immune cell repertoire · Supports tumor-immune interactions	· Residual mouse immune environment · Variable and incomplete immune reconstitution · Overexpression of human cytokines / growth factors · Short to intermediate life-span · Potential to develop anemia and myeloid cell hyperactivation syndrome
genO-BRGSF-HIS mice	· Translational assessment of efficacy and safety of TCEs or mAbs · Longitudinal immune response studies · Multi-cellular immune interactions · Combination therapies	· Requirement of tumor and stroma cross-talk	· Functional human myeloid cells · Functional human lymphoid cells · No development of GvHD · Long therapeutic window	· High variability · Higher cost

PRACTICAL EXAMPLES

What happens if I test a TCE in a PBMC-reconstituted model?

Observed effects:

- Strong, non-specific T-cell activation
- Elevated baseline cytokines
- Rapid xenogeneic GvHD

Consequence:

- Cytokine release could be underestimated due to the lack of human myeloid cells
- Difficult to disentangle drug-specific effects from model artefacts

What model should I use if I need to test the safety of a TCE?

PREFERRED APPROACH

Humanized CD3 Knockin mice (genO-panhCD3) with:

- Physiological CD3 expression
- Endogenous T-cell development
- Preserved immune architecture

Why:

- Enables dose-dependent CD3 activation
- Supports meaningful cytokine profiling
- Avoids xenogeneic immune artefacts

ALTERNATIVE APPROACH

genO-BRGSF-HIS mice with a human immune system:

- Functional human myeloid and lymphoid compartments
- Presence of human T-cells
- Physiological CD3 expression

Why:

- Can be engrafted with PDX, enabling the expression of human tumor-associated antigens
- Enables dose-dependent CD3 activation
- Supports meaningful cytokine profiling

FAQ

Which CD3 model is best for T-cell engager studies?

The most appropriate CD3 mouse model for T-cell engager studies is one that expresses human CD3 subunits under physiological regulatory control, such as the **genO-panhCD3**, as this enables human-relevant CD3 engagement, preserves native T-cell development, and supports interpretable *in vivo* T-cell activation and cytokine responses, which are critical determinants of TCE efficacy and safety (Smith-Garvin et al., 2009; Labrijn et al., 2019).

What are the differences between CD3 mouse models?

CD3 mouse models differ primarily in which CD3 subunits are humanized, how their expression is regulated, and whether T-cells develop endogenously or are introduced exogenously (e.g., PBMC-reconstituted mice), with partial humanization (e.g., CD3ε alone) enabling target binding but often failing to recapitulate full TCR-CD3 signaling fidelity, whereas multi-subunit humanization improves signaling relevance but still requires careful functional validation (Goebeler and Bargou, 2020).

Do CD3 models support T-cell activation *in vivo*?

Humanized CD3 mouse models support *in vivo* T-cell activation provided that human CD3 is expressed at physiological levels within an intact TCR complex, allowing CD3 engagement by TCEs to trigger downstream signaling, proliferation, cytotoxicity and cytokine release in a dose-dependent manner that mirrors key aspects of human T-cell biology (Smith-Garvin et al., 2009).

How can CD3-engager activity be optimized to reduce cytokine release?

CD3-engager activity can be optimized to reduce cytokine release by modulating CD3-binding affinity, geometry, and valency, as excessive or non-physiological CD3 signaling is a major driver of cytokine release syndrome, and preclinical studies have shown that lower-affinity or conditionally activated CD3 engagement improves the therapeutic index without fully compromising antitumor efficacy.

How is CD3 expression validated in humanized mouse models?

CD3 expression in humanized mouse models is validated through flow cytometry analysis of T-cell subsets, comparison of surface CD3 expression levels to human peripheral blood T-cells or to WT mice, and functional assays confirming that CD3 engagement induces canonical downstream signaling and activation responses, ensuring that the observed pharmacological effect reflects target biology rather than aberrant expression artefacts (Smith-Garvin et al., 2009; Labrijn et al., 2019).

How do CD3 humanized mice respond to CD3 stimulation?

CD3 humanized mice respond to CD3 stimulation with robust T-cell activation characterized by upregulation of early activation markers and cytokine secretion profiles that are more controlled and human-relevant than those observed in xenogeneic PBMC-based models, which are prone to be exaggerated, non-target-specific activation driven by host-graft incompatibility (Shultz et al., 2012; Walsh et al., 2017).

What is CD3 and its role in T-cell activation?

CD3 is a multi-subunit signaling complex that associates with the T-cell receptor and transduces antigen recognition into intracellular signaling through immunoreceptor tyrosine-based activation motifs (ITAMs), thereby controlling T-cell activation, proliferation, effector function, and cytokine production, all of which are directly exploited by CD3-engaging therapeutic antibodies (Smith-Garvinet al., 2009).

What is the structure and function of the TCR-CD3 complex?

The TCR-CD3 complex consists of an antigen-binding TCRαβ heterodimer non-covalently associated with CD3εδ, CD3εγ, and CD3ζζ signaling dimers, and effective T-cell activation requires precise structural coupling between these subunits to ensure correct signal transduction upon engagement by peptide-MHC complexes or CD3-targeting biologics (Smith-Garvin et al., 2009; Courtney et al., 2018).

What is a CD3εδγ humanized mouse model?

A CD3εδγ humanized mouse model is a genetically engineered mouse in which the CD3ε, CD3δ, and CD3γ chains are replaced by their human orthologs, enabling human-specific CD3 engagement while preserving endogenous T-cell development and providing a more faithful platform for evaluating T-cell engager pharmacology, including activation strength and cytokine liability, than single-subunit or xenogeneic models (Goebeler and Bargou, 2020; Labrijn et al., 2019).

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AN-1512-EN-2611 **Rev A**