

Supplementary Material

Using protein geometry to optimize cytotoxicity and the cytokine window of a ROR1 specific T cell engager

Xueyuan Zhou¹⁺, Felix Klaus Geyer²⁺, Dominic Happel², Jeffrey Takimoto¹, Harald Kolmar^{2,3*}, Brian Rabinovich^{1*}

¹Fuse Biotherapeutics, Woburn, MA, USA

²Institute for Organic Chemistry and Biochemistry, Technical University of Darmstadt, Peter-Grünberg-Strasse 4, D-64287 Darmstadt, Germany

³Centre for Synthetic Biology, Technical University of Darmstadt, Darmstadt, Germany

*** Correspondence:**

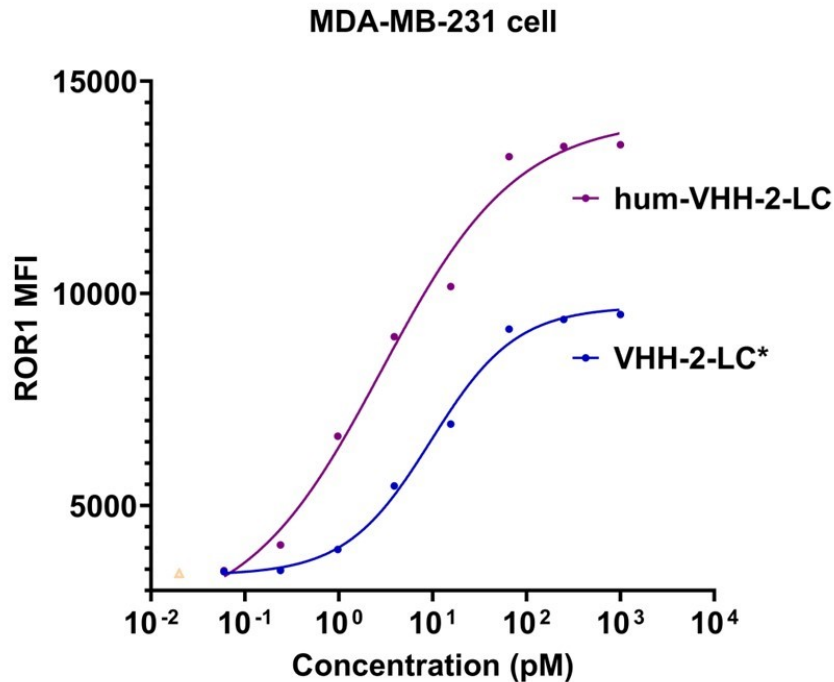
Brian Rabinovich

brabinovich@fusebiotx.com

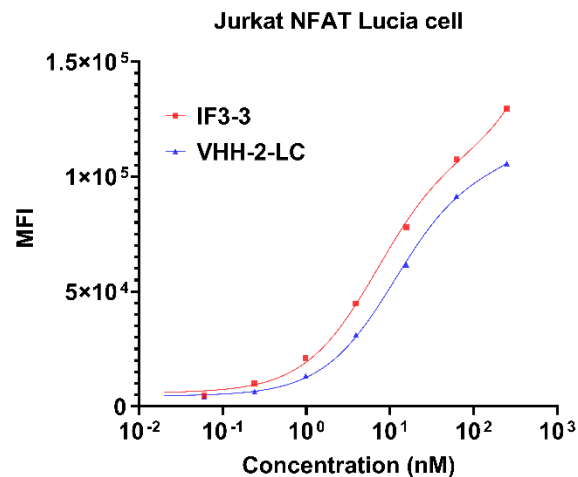
Harald Kolmar

Harald.Kolmar@TU-Darmstadt.de

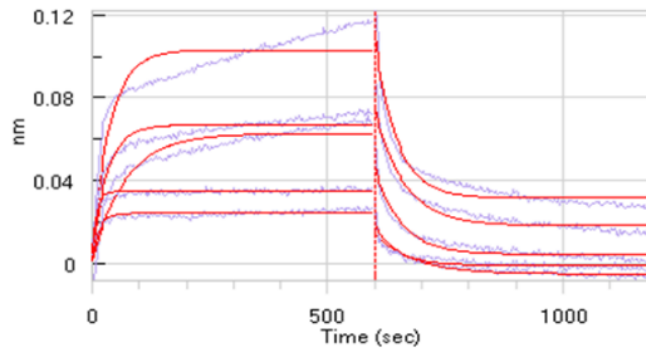
⁺ These authors have contributed equally to this work and share first authorship.



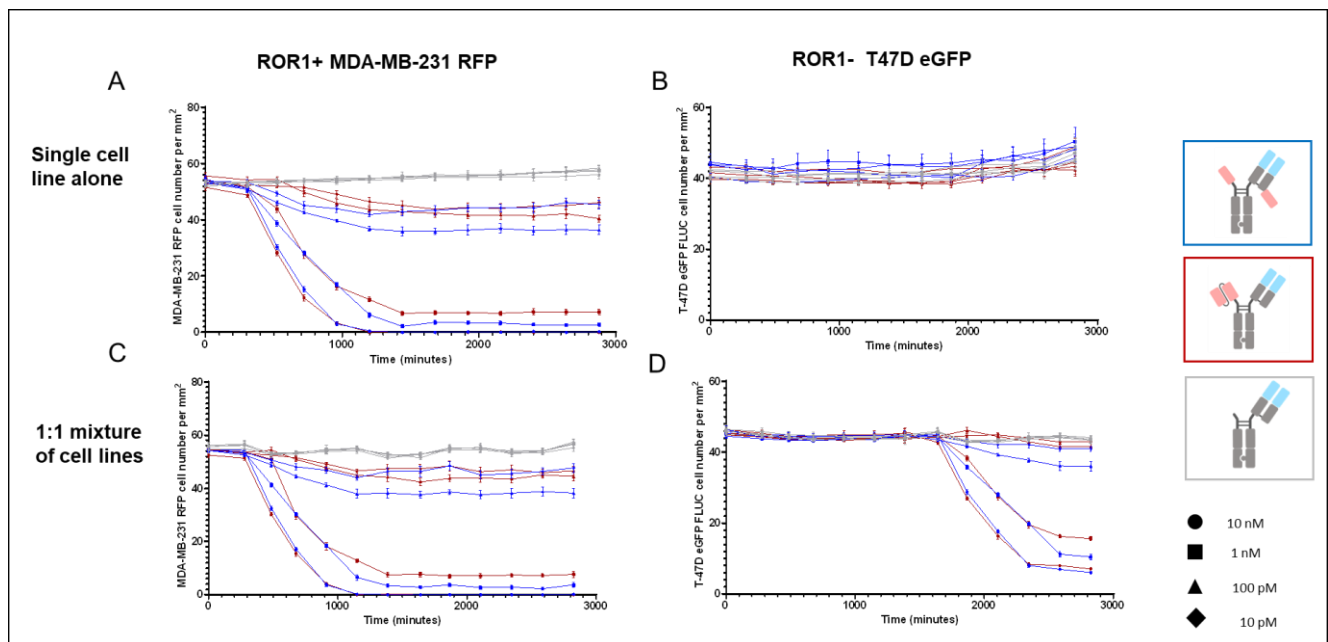
Supplementary figure S1: Apparent affinity determination of 5A1 and humanized 5A1 by flow cytometry. Shown is a non-linear x-y plot of the magnitude of binding to ROR1 on MDA-MB-231 (MFI) of hum-VHH-2-LC (purple closed circles; EC₅₀ ~3 nM) and VHH-2-LC (blue closed circles; EC₅₀ ~20 nM) as a function of test article concentration.



Supplementary Figure S2. Apparent affinity determination of CD3 binder IF3-3. Binding to Jurkat NFAT Lucia cells was analyzed. For IF3-3 alone an EC₅₀ value of 6.9 nM was determined. For VHH-2-LC consisting of one IF3-3 moiety it was 11.4 nM.

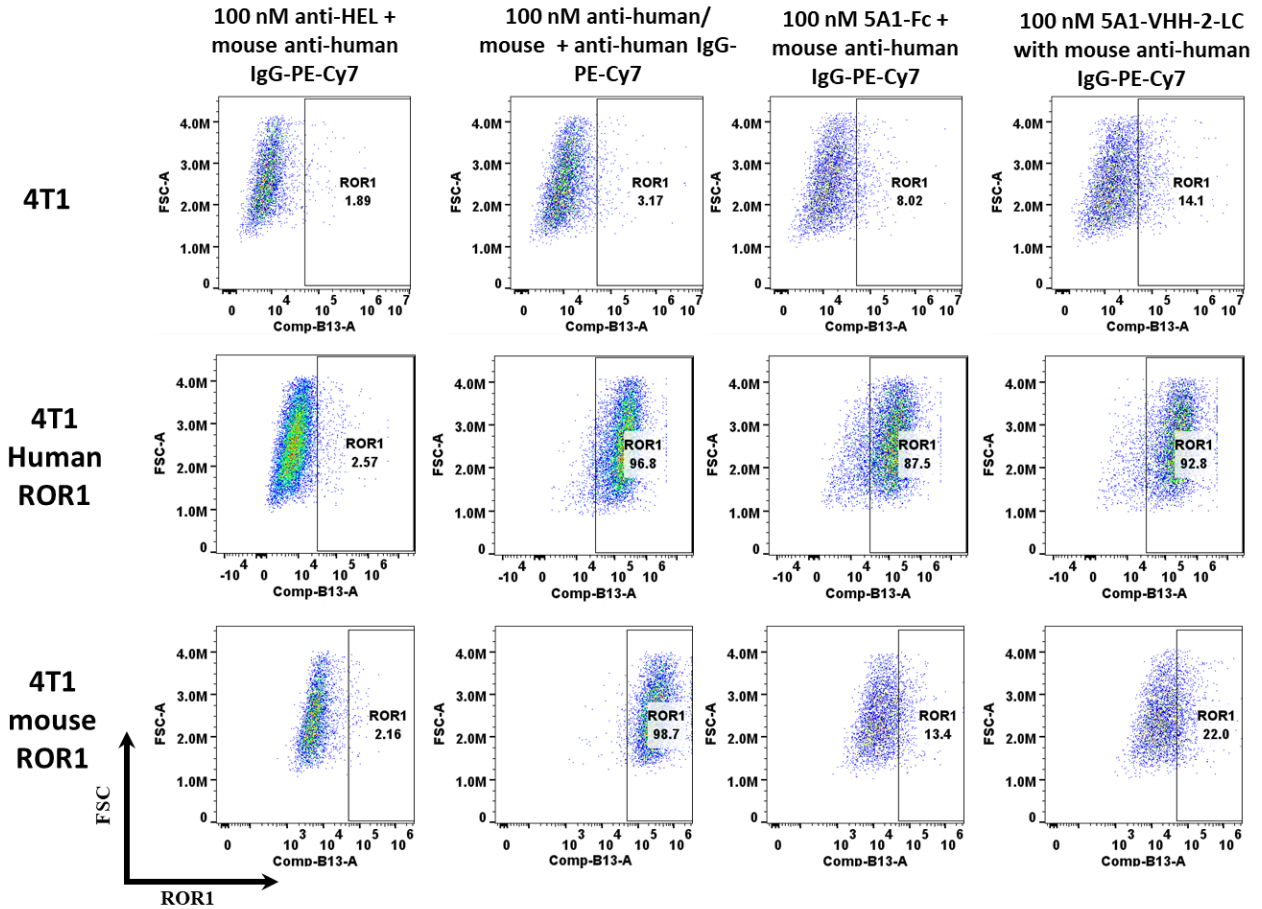


Supplementary figure S3: Binding kinetics of monovalent hum_VHH 5A1 to human ROR1 was examined by BLI measurement. The calculated K_D is approximately 110 nM. The kinetic parameters are $k_{on} = 4.35 \cdot 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{off} = 5.02 \cdot 10^{-3} \text{ s}^{-1}$.

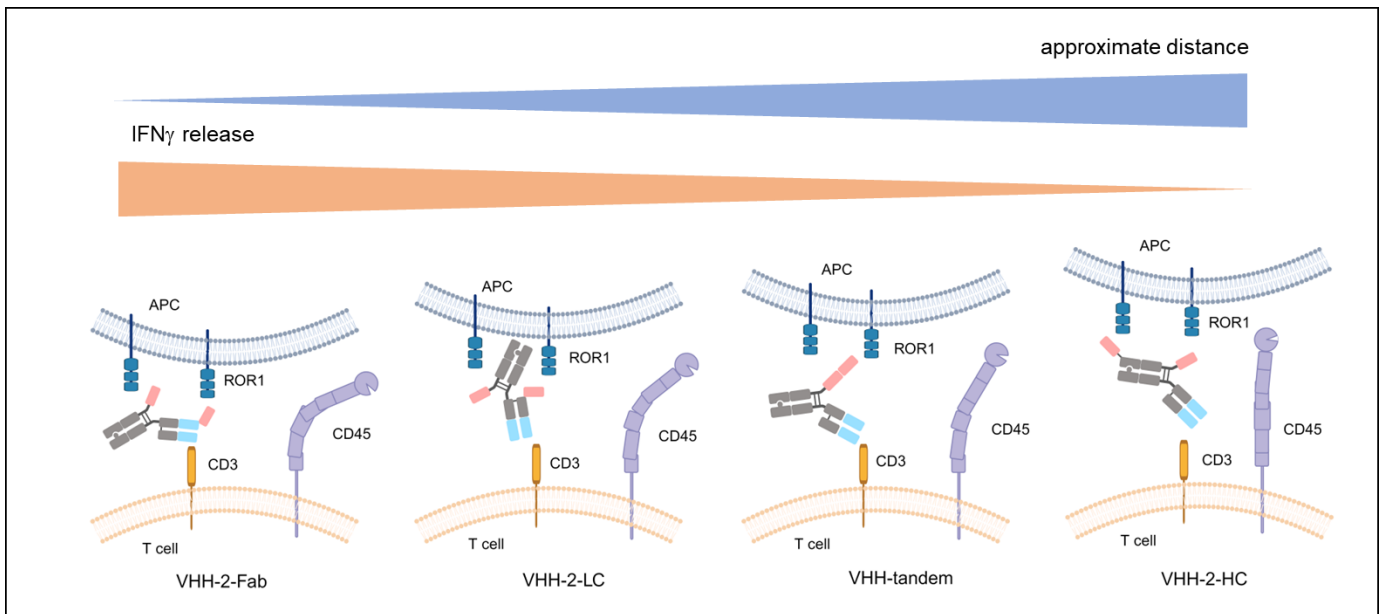


Supplementary Figure S4. Kinetic analysis of TCE induced bystander killing of ROR1 negative tumor cells in a heterogenous cell mixture at different concentrations of TCEs. Kinetic measurements were performed at a construct concentration of 10 nM, 1 nM, 100 pM, and 10 pM. Expanded T cells were used as effector cells at an E:T ratio of 5:1. A Similar concentration-dependent killing of ROR1 positive was observed for R11-scFv-1 and hum-VHH-2-LC. B When ROR1- T-47D tumor cells were co-cultured with expanded T cells, no killing of tumor cell was observed with the presence of TCEs. C When expanded T cells were used as effector cells in co-culture with a 1:1 mixture of ROR1+ MDA-MB-231 and ROR1- T-47D tumor cells, similar killing of ROR1+ tumor cells was observed in comparison to the cultivation with the ROR1+ cell line alone. A ratio of 5:1:1 was used for T cell, ROR1+ MDA-MB-231 cell and ROR1 T-47D cell. D In the co-culture of ROR1+ and ROR1- tumor cells, ROR1- tumor cells were killed in a concentration-dependent manner in the presence of hum-

VHH-2-LC and R11-scFv-1In all assays the negative control CD3-ctrl showed no cell killing. Measurements were made in duplicate wells and four read areas per well.



Supplementary figure S5: Cytometric flow analysis showed binding of the VHH 5A1 to 4T1 cells engineered to express human ROR1 but not to those expressing mouse ROR1.



Supplementary Figure S6: Illustrative figure displaying interaction of different bispecific antibody formats with target ROR1 on APC and CD3 on T cell.