**A supported lipid bilayer platform to study the impact of bi-specific T cell engager architecture on T cell activation**

Alexander Leithner, Srinath Kasturirangan, Michael L. Dustin

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Bi-specific T cell engagers (TcEs) are artificial antibodies that represent a promising therapy for cancer. In their basic form, they are fusion proteins of the variable domains (VDs) of two antibodies of different specificity, allowing them to link two molecules of choice. Most commonly, this approach is used to link CD3 on T cells with tumour associated antigens (TAAs), in order to re-direct the cytotoxic activity of T cells to kill tumour cells.

However, while a plethora of different TcE formats exist that differ in the spacing of their VDs or their valency for CD3 or TAA, we lack the systematic understanding of how these architectural features influence their ability to activate T cells on the cellular and molecular level.

To address these questions, we adapted the classic planar lipid bilayer system, developed to study the immunological synapse (IS) of T cells, to present TAA, allowing for imaging in high spatio-temporal resolution. We show that in the presence of TcEs, T cells rapidly cluster TAA and frequently form highly organized synapses. Furthermore, by using a panel of TcEs with different architectures, we show that minimizing the distance between VDs leads to increased TAA clustering and T cell signalling.

We suggest that our system will lead to a deeper understanding of the cellular and molecular events that lead to TcE mediated T cell activation and that it might also serve as a platform to screen large numbers of different TcEs in a fully controlled setup.

- The His-tagged Her2 extracellular domain can be introduced into the supported lipid bilayer system (at different densities) where, in the presence of TcEs, it is clustered by T cells, leading to their activation.
- A comparison of three TcEs with different distance (short, intermediate, long) between their CD3 and Her2 binding arms, shows that minimizing epitope spacing leads to most efficient immune synapse formation on three levels
    - Short format triggers most efficient monofocal synapse formation
    - Short format leads to most efficient Her2 clustering (only at high Her2 densities)
    - Short format leads to most efficient TCR signalling (measured via pPLCgamma1), (only at high Her2 densities)
- In cell-cell assays with resting CD8 T cells and BT-474 tumour cells (high expression of Her2) under a wide range of TcE concentrations (500nm to 0.005pM) we measured T cell activation (CD69 up-regulation after 18-24h) and the induced cytotoxic activity (LDH release assay at 48h)
    - There are only very little differences between the TcEs at the level of CD69 up-regulation (not a good indicator of cytotoxicity); however, this assay revealed the considerable ‘unspecific activity’ (if you can call it like that) of ZM2 (intermediate TcE).
    - However, when looking at killing we see that the shortest TcE format is performing best, especially at low concentrations (5pM, there is a 2-fold difference compared to the other TcEs, 30 vs. 60% killing efficiency)
    - The shortest TcE format keeps its edge over the whole range of concentrations although it becomes less pronounced