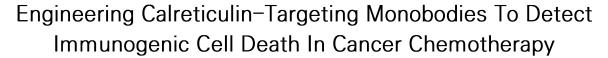


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Background: Cancer cell surface—exposed calreticulin (ecto—CRT) is the primitive form of signal during immunogenic cell death (ICD). It is a well–known candidate to allow "eat—me" signal from dying cells, which further contributes to their perception in directing the immune system. Various forms of anticancer agents and ionizing radiation can facilitate the ICD via ecto—CRT exposure. Ecto—CRT is an immunogenic signal induced in response to treatment with chemotherapeutic agents such as doxorubicin (DOX) and mitoxantrone (MTX), and two peptides (KLGFFKR (Integrin— α) and GQPMYGQPMY (CRT binding peptide 1, Hep–I)) are known to specifically bind CRT.

Methods: To engineer CRT-specific monobodies as agents to detect immunogenic cell death (ICD), we fused these peptide sequences at the binding loops (BC and FG) of human fibronectin domain III (FN3). CRT-specific monobodies were purified from E. coli by affinity chromatography. Using these monobodies, ecto-CRT was evaluated in vitro, in cultured cancer cell lines (CT-26, MC-38, HeLa, and MDA-MB-231), or in mice after anticancer drug treatment.

Results: Monobodies with both peptide sequences (CRT3 and CRT4) showed higher binding to ecto-CRT than those with a single peptide sequence. The binding affinity of the Rluc8 fusion protein-engineered monobodies (CRT3-Rluc8 and CRT4-Rluc8) to CRT was about 8 nM, and the half-life in serum and tumor tissue was about 12 h. By flow cytometry and confocal immunofluorescence of cancer cell lines, and by in vivo optical bioluminescence imaging of tumor-bearing mice, CRT3-Rluc8 and CRT4-Rluc8 bound specifically to ecto-CRT and effectively detected pre-apoptotic cells after treatment with ICD-inducing agents (DOX and MTX) but not a non-ICD-inducing agent (gemcitabine).

Conclusions: Taken together, our data clearly demonstrate the functional properties of engineered CRT-targeting monobodies to detect ICD during cancer chemotherapy. This strategy of engineering novel monobodies using peptides may simplify the process required to generate high-affinity biomolecules for inaccessible or challenging targets.

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