A FLUORESCENT BIOSENSOR METHOD FOR QUANTIFYING ANTIBODY BINDING AND INTERNALIZATION INTO EFFECTOR CELLS AND TARGET CELLS IN A CO-CULTURE SYSTEM

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Background Antibody-mediated delivery of agonists into the tumor microenvironment to selectively activate intracellular targets in immune cells (effector cells) relative to tumor cells (target cells) is not well understood. Here we develop a method using a fluorescent biosensor to quantitatively assess the number of antibodies binding to a cell and frequency of internalized antibodies per cell in both tumor and immune cells in co-cultures by utilizing quantitative spectral flow cytometry.

Methods A hlgG1, anti-HER2 monoclonal antibody (HER2-mAb) or isotype-matched control antibody (Iso-mAb) were conjugated with a fluorescent biosensor consisting of Alexa Fluor 568 (AF568) and Alexa Fluor 647 (AF647) separated by a cathepsin B-cleavable peptide linker. AF568 fluorescence is quenched until the biosensor is cleaved inside cells, while AF647 is fluorescent pre- and post-cleavage. Quantitative flow cytometry calibration curves were prepared to calculate the number of fluorophore molecules per cell and antibody molecules per cell using the measured median fluorescent intensity (MFI).

Murine effector cells, RAW264.7 and RAW309 (macrophage) and MutuDC1940 (dendritic), were plated in 96-well plates 24 hours prior to the addition of target cells expressing variable levels of HER2: JIMT-1, N87, and SKBR3, pre-loaded with pHrodo green cell tracker. Target cells and biosensor-conjugated mAb were added simultaneously to effector cells at 37°C for 0, 1, 4, or 24 hours. At each designated time point, cells were stained with CD11b-BV785, and flow cytometry was conducted with at least three independent runs.

Flow gates were placed on live single cells of either pHrodo green positive tumor cells, CD11b-BV785 positive immune cells, or double-positive cells. The AF568 and AF647 MFI values were converted to the number of fluorophore molecules or antibodies bound per cell allowing for the number of antibody molecules and % internalized antibody per cell to be determined.

Results Target cells expressing HER2 were required for appreciable HER2-mAb binding and internalization into effector cells. Antibody binding on effector cells correlated with increased HER2 expression across the three tumor cell lines. Importantly, the HER2-mAb-biosensor showed rapid and increased internalization into immune cells with 40-60% of antibody internalized by 1 hour in contrast to <10% internalization into tumor cells.

Conclusions This in vitro quantitative flow cytometry allowed determination of both the number of antibodies and the % internalized antibodies per cell, important parameters for modeling delivery of antibody-mediated immunostimulatory agonists. The demonstrated methodology can be applied to in vivo models to optimize therapeutic agonist delivery.