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A novel method for measuring cellular antibody uptake using imaging flow cytometry reveals distinct uptake rates for two different monoclonal antibodies targeting L1

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ABSTRACT

Monoclonal antibodies (mAbs) have emerged as a promising tool for cancer therapy. Differing approaches utilize mAbs to either deliver a drug to the tumor cells or to modulate the host's immune system to mediate tumor kill. The rate by which a therapeutic antibody is being internalized by tumor cells is a decisive feature for choosing the appropriate treatment strategy. We herein present a novel method to effectively quantitate antibody uptake of tumor cells by using image-based flow cytometry, which combines image analysis with high throughput of sample numbers and sample size. The use of this method is established by determining uptake rate of an anti-EpCAM antibody (HEA125), from single cell measurements of plasma membrane versus internalized antibody, in conjunction with inhibitors of endocytosis. The method is then applied to two mAbs (L1-9.3, L1-OV52.24) targeting the neural cell adhesion molecule L1 (L1CAM) at two different epitopes. Based on median cell population responses, we find that mAb L1-OV52.24 is rapidly internalized by the ovarian carcinoma cell line SKOV3ip while L1 mAb 9.3 is mainly retained at the cell surface. These findings suggest the L1 mAb OV52.24 as a candidate to be further developed for drug-delivery to cancer cells, while L1-9.3 may be optimized to tag the tumor cells and stimulate immunogenic cancer cell killing. Furthermore, when analyzing cell-to-cell variability, we observed L1 mAb OV52.24 rapidly transition into a subpopulation with high-internalization capacity. In summary, this novel high-content method for measuring antibody internalization rate provides a high level of accuracy and sensitivity for cell population measurements and reveals further biologically relevant information when taking into account cellular heterogeneity.

1. Introduction

Engineered therapeutic antibodies binding to tumor-specific targets have emerged as a powerful tool for tumor imaging and diagnosis but most importantly, for specific cancer therapy (Reichert, 2012). While all therapeutic antibodies have a specific and strong binding of their antigen in common, different modes of action are possible depending on the desired function of the antibody. The most important concepts involve either a direct action of the antibody (e.g. receptor blockade, drug delivery) or a more indirect action by stimulating immune-mediated cell killing (e.g. antibody-dependent cellular cytotoxicity (ADCC) (Scott et al., 2012)). These distinct concepts demand different pharmacokinetic and pharmacodynamic properties of the targeting antibody. When the antibody is to be used for delivering a conjugated payload such as a toxin, high rates of cellular uptake of the antibody are needed. While on the other hand, when aiming at making use of immune-mediated tumor cell killing a sustained presence of the antibody on the cell surface is necessary (Scott et al., 2012).

Here we describe a novel method to quantify cellular antibody uptake using image-based flow cytometry. Monoclonal antibodies are conjugated to the fluorophore Alexa-488, added to the medium and incubated with live cells for different time points. The cells are subsequently fixed in order to prevent further internalization during the process. The internalized antibodies are visible in intracellular vesicles as confirmed using high-resolution laser scanning confocal microscopy (LSCM). In addition to the portion of antibody that is internalized, a substantial amount remains bound at the cell surface at any time point. In order to determine the amount of internalized antibody by conventional flow-cytometry, cell surface fluorescence must be eliminated. Previous methods used acid wash to remove cell surface-bound antibodies (Kameyama et al., 2007). A further improved method makes use of an Alexa-488 specific quenching antibody (Göstring et al., 2010; Schmidt et al., 2008). Both methods have considerable drawbacks. While the acid wash of the cells harms their structural integrity, using the quenching antibody requires splitting up each sample into two (total

Abbreviations: mAbs, monoclonal antibodies; EpCAM, epithelial cell adhesion molecule; ADCC, antibody-dependent cellular cytotoxicity; LSCM, laser-scanning confocal microscopy; L1, L1 cell adhesion molecule; WGA, wheat-germ agglutinin; ISX, ImageStreamX.

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fluorescence and cell-surface quenched) so that the relative fraction of internalization can be determined. Another drawback which both methods have in common is the incomplete removal of the cell-surface associated antibody and a resulting reduction of sensitivity.

The use of imaging flow cytometry enables image-based analysis of highly-sampled cell populations. High-resolution images are submitted to image segmentation, which based on fluorescent intensity identifies regions of interests, defined as masks. Masks are then used to extract high-content data from original fluorescent images, e.g. here we report internalized antibody fraction. The workflow to prepare the samples is identical to other methods except that after fixation of the cells the cell-surface bound fraction of antibody is detected using a secondary Alexa-647-conjugated antibody which binds to the antibody of interest (Fig. 1A). The secondary antibody allows for the discrimination of the fraction of non-internalized antibody by generating a mask of the plasma membrane-localized antibody (extracellular mask). In case that too little non-internalized antibody remains on the cell surface due to high internalization rates, an unspecific marker targeting the cell surface may be used instead to identify and generate the "extracellular mask" (e.g. wheat germ agglutinin). Subsequently, applying to the total intensity primary antibody mask a Boolean AND NOT "extracellular mask" function we obtain the "intracellular mask", corresponding to internalized antibodies (Fig. 1B). Together, this method provides a high-throughput of sample numbers along with high sensitivity and reproducibility on a single cell basis. Below, we establish and validate this approach using a well-characterized, antibody-activated EpCAM uptake response. Subsequently, we perform high-content, population-based analysis of the internalization profiles for two distinct monoclonal mouse antibodies (mAbs) targeting the neural cell adhesion molecule L1 (L1/L1CAM).

Originally identified as a vital factor during development of the nervous system (Rathjen and Schachner, 1984), mounting evidence suggests that L1 plays as well a prominent role in malignant human tumors of different entities (Fogel et al., 2003b; Gavert et al., 2007; Kiefel et al., 2010). Many studies in the past decade associated L1 expression with a variety of tumor-promoting functions such as cell motility and invasion, thus contributing to cancer progression and metastasis (Meier et al., 2006; Sebens Müerköster et al., 2007; Zeimet et al., 2013). Consequently, overexpression of L1 in different human carcinomas has been linked to poor prognosis, more aggressive phenotype and advanced stages (Fogel et al., 2003a; Huszar et al., 2006). Taken together, L1 is an attractive target for antibody-based specific cancer therapy (Novak-Hofer et al., 2008).

We previously generated a panel of nine anti-L1 mAbs and characterized these thoroughly (Wolterink et al., 2010). Out of these nine mAbs two, L1-9.3 and L1-OV52.24 were chosen to be further developed for therapeutic use. Here we report that these two antibodies display differing rates of uptake by the human ovarian carcinoma cell line SKOV3ip. While L1 mAb OV52.24 is rapidly taken up by the cells, L1 mAb 9.3 is stably retained at the cell surface. These findings will be taken into account when developing these antibodies for therapeutic usage.

2. Results

2.1. Measuring endocytosis of EpCAM

We first chose the epithelial cell adhesion molecule EpCAM to validate the assay since it is one of the best-studied targets of human tumors and several antibodies targeting EpCAM have been developed and examined in clinical studies (Baeuerle and Gires, 2007; Moldenhauer et al., 2012; Münz et al., 2010; Trzpis et al., 2007). It is well-documented that EpCAM undergoes rapid internalization upon treatment with antibodies

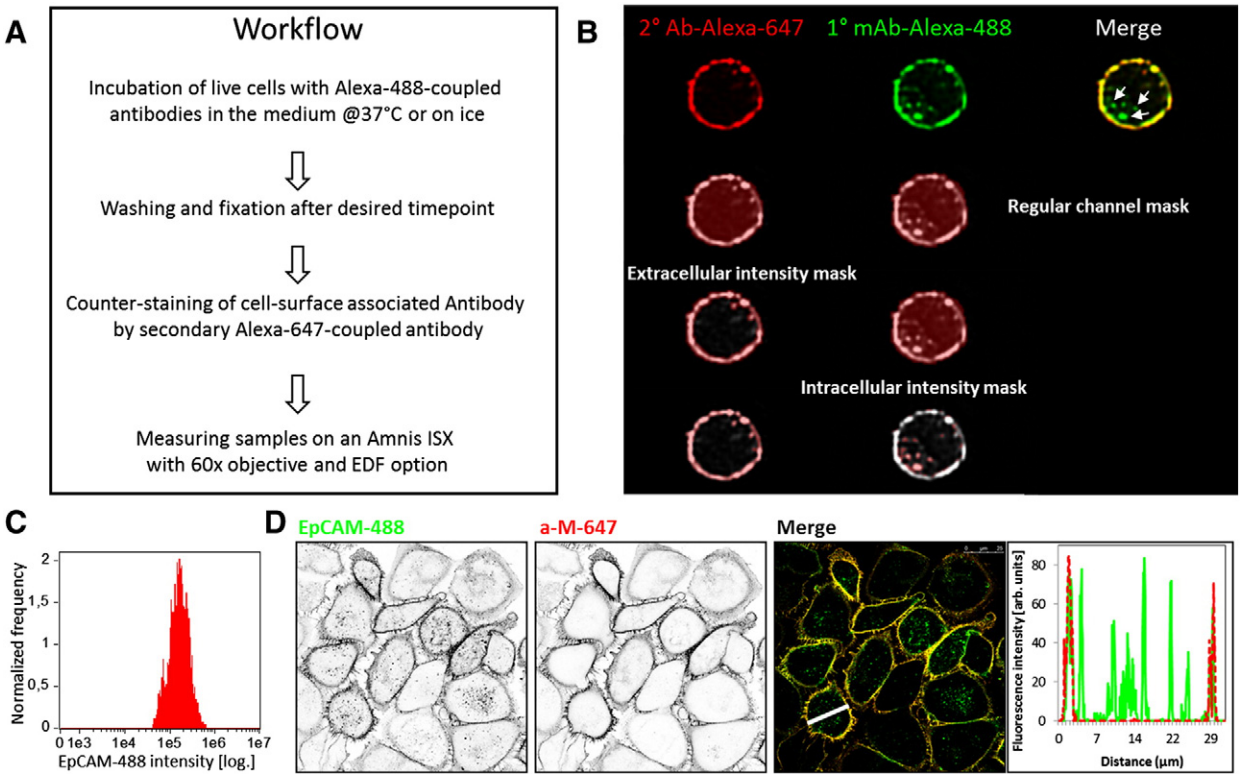

Fig. 1. Workflow and validation of cellular EpCAM uptake by LSCM. Depicted is the general workflow for carrying out this assay (A). An example for generating the mask to measure the intracellular intensity based on a mask identifying extracellular intensity is shown exemplary (B). SKOV3ip cells were detached and stained with an Alexa-488 conjugated EpCAM mAb (HEA-125) on ice for 45 min. Cells were subsequently washed and measured on an Amnis ImageStreamX using a 488 nm laser. 10 000 cells were acquired and analyzed using the Amnis IDEAS software (C). SKOV3ip cells were incubated for 90 min with an Alexa-488 conjugated EpCAM mAb. Samples were subsequently washed and fixed and cell surface bound antibody was detected using a secondary goat-anti-mouse antibody coupled to Alexa-647. Samples were then visualized on a Leica SP5 II confocal laser scanning microscope. Z-slices were acquired in similar z-positions. A line scan was generated using ImageJ and the graph is shown on the right. The experiments were repeated thrice, shown is a representative result.