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Research paper

# Characterization of FcγRIIIA effector cells used in *in vitro* ADCC bioassay: Comparison of primary NK cells with engineered NK-92 and Jurkat T cells

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ABSTRACT

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MOA) of several therapeutic antibody drugs and evaluation in ADCC bioassays is important in antibody drug development and maintenance. Three types of effector cells now routinely used in bioassay evaluation of ADCC are natural killer cells from human donors (FcγRIIIA + primary NK), FcγRIIIA engineered NK-92 cells and FcγRIIIA/NFAT-RE/luc2 engineered Jurkat T cells. Engineered effector cells were developed to address need for improved precision and accuracy of classic NK cell ADCC bioassays. The main purpose of our study was to rationalize which of these ADCC effector cells best simulate the expected response in human subjects and to identify which effector cells and assays best fit ADCC bioassay needs during antibody drug development. We characterized differences between the effector cells and compared ADCC biological activities using the well-known humanized IgG1 antibody drug, trastuzumab. The three effector cell types studied expressed either V-158 or F-158 allotype of FcγRIIIA, hence six cell preparations were compared. Our results demonstrate highest surface expression of FcγRIIIA in primary NK and engineered NK-92 (V-158) cells with nearly all expressed on the cell surface. In contrast, expression in engineered Jurkat T cells was low with only a small percentage expressed on the cell surface. Studies evaluating binding of trastuzumab to effector cells demonstrated the highest affinity of FcγRIIIA in primary NK and NK-92 (V-158) cells. ADCC cytotoxicity studies showed greatest trastuzumab potency in primary NK and engineered NK-92 (V-158) cells and negligible cell lysis obtained using engineered Jurkat T cells. In contrast, the engineered Jurkat T (V-158) cells responded as effectively as primary NK (V/V) cells to nuclear factor of activated T cells (NFAT2) activation upon binding of trastuzumab to FcγRIIIA, demonstrating similar ADCC pathway activation in these cells despite the low surface expression of FcγRIIIA and its low affinity for trastuzumab. Dose-response range of trastuzumab in activation of NFAT2 (measured as pNFAT2 dephosphorylation) was very similar to response in classic ADCC assay for primary and NK-92 cells and to response in ADCC reporter assay for Jurkat T effector cells, bridging the assays. Trastuzumab potency in ADCC reporter assay using the engineered Jurkat T cells was close to that seen using either primary NK or engineered NK-92 cells in classic ADCC assay. In summary, all three effector cell systems differentially express FcγRIIIA and provide dose-dependent ADCC pathway activity, yet only primary NK and engineered NK-92 cells are capable of inducing ADCC-mediated cell lysis. Engineered Jurkat T effector cells have value to assure antibody manufacturing consistency and in other applications where accuracy and precision are important. For functional assessment of ADCC activity, primary NK or NK-92 (V-158) cells better reflect the physiologically relevant ADCC mechanism of action. As an engineered cell line, NK-92 cells may behave more reproducibly than primary NK, but this must be balanced with the objective for biological relevance in decisions on which NK cells to use in assay.



*Abbreviations:* mAbs, monoclonal antibodies; Fab, fragment antigen-binding; Fc, fragment crystallizable; NK cells, natural killer cells; ADCC, antibody-dependent cell-mediated cytotoxicity; MOA, mechanisms of action; NFAT, nuclear factor of activated T cells; T cells, thymocyte cells; IgG1, immunoglobulin G1; FcR, Fc receptor; IL-2, interleukin-2; ASN-297, asparagine-297; V-158, valine at amino acid 158; F-158, phenylalanine at amino acid 158; EGFR, epidermal growth factor receptor; AAF-Glo™, alanyl-alanyl-phenylalanyl-aminoluciferin; Her2, human epidermal growth factor receptor 2; PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophcocyanin; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; $EC_{50}$, effective concentration of the antibody to reach 50% of maximum signal; MFI, mean fluorescence intensity; E/T ratio, effector cells:target cells ratio; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRDye, infrared dye; pNFAT2, phosphorylated NFAT2; ITAM, immunoreceptor tyrosine-based activation motif; PBMC, peripheral blood mononuclear cell.

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## 1. Introduction

In recent years monoclonal antibodies (mAbs) have been successfully developed and proved valuable for human health as targeted therapies for a diverse family of inflammatory diseases and human malignancies, especially breast, colon and hematological cancers (Scott et al., 2012; Weiner et al., 2010; Chan and Carter, 2010). Due to their specificity for target antigens and long half-life, mAbs are often the preferred treatment for these diseases. Compared to other treatment options, they also have the benefit of less frequent dosing, established efficacy and fewer off-target effects (Hansel et al., 2010). Numerous mAbs have been approved worldwide, with the number approved per year increasing to nine in 2015 for EU or US approval (Reichert, 2016). The upward trend is expected to continue.

Crystallization and studies on biological activities of therapeutic antibodies have shown the Fab (fragment antigen binding) domain recognizes surface antigens on target cells, while the Fc (fragment crystallizable) domain region has the potential to bind Fc receptors on the surface of immune effector cells. The latter binding event may mediate activation or inhibition of the Fc effector function biological activity of antibodies, dependent on the Fc receptor, and has thus been recognized as an important mechanism of action of therapeutic antibodies. Characterization of this Fc effector function activity is essential during antibody drug development, even for antibodies where this biological activity would be undesirable in the specific therapeutic context. The binding interaction between target cell, antigen-bound antibody and Fc receptor on effector cells results in conformational changes and clustering of activating Fc receptors (Kato et al., 2000; Radaev et al., 2001), followed by a series of intracellular signaling events including signaling through the calcineurin and nuclear factor of activated T cells (NFAT) signaling pathway (Nimmerjahn and Ravetch, 2008; Leibson, 1997). In general, this signaling is initiated by a series of tyrosine phosphorylation events which allow $Ca^{2+}$ to enter through $Ca^{2+}$ channels, thereby increasing cytoplasmic $Ca^{2+}$ concentration. This increase in calcium allows the calcineurin enzyme to dephosphorylate pNFAT2 with subsequent translocation to the nucleus. NFAT2 in the nucleus up-regulates the synthesis of cytotoxic immune-modulators, including interleukin-2 (IL-2) (Rao et al., 1997; Hogan et al., 2003), and granzyme and perforin are released from effector cells and mediate lysis of target cells (Leibson, 1997; Aramburu et al., 1995). This particular immune-mediated effector function is called antibody-dependent cell-mediated cytotoxicity (ADCC) and is usually induced by natural killer cells and other effector cell types (Jiang et al., 2011; Chávez-Galán et al., 2009). ADCC is one of the potential MOA of certain antibodies, and those considered Class I MOA (Jiang et al., 2011) plays an important role in cancer treatments.

The biological processes in human patients treated with mAbs containing ADCC functionality is complex due to the many variations in both antibody and effector cells. Among the subclasses of immunoglobulin G (IgG) antibody (IgG1, IgG2, IgG3 and IgG4), IgG1 and IgG3 antibodies exhibit relatively stronger binding to the major Fcγ receptors (FcγR) required for effector functions (Hansel et al., 2010). Therapeutic mAbs produced from recombinant cell lines contain complex glycan structures, predominantly at asparagine-297 (ASN-297) and this is exquisitely important for Fc effector functions (Jefferis et al., 1998). Certain glycoforms have been shown to impact FcγR binding and ADCC activity. For example, a lack of core fucose in IgG1 N-linked glycans (afucosylated) has been shown to increase binding of antibody to the human activating receptor FcγRIIIA by 50-fold and enhance the ADCC response (Shields et al., 2002). This observation has significantly impacted selection of antibody production cell lines and the design of therapeutic antibodies (Shields et al., 2002; Nimmerjahn and Ravetch, 2005; Yamane-Ohnuki et al., 2004). Other glycan moieties have also been shown to impact antibody effector function, though to a lesser extent, (Chung et al., 2014a; Raju, 2008; Dashivets et al., 2015; Thomann et al., 2016) and research is ongoing to understand the influence of these further.

The crystal structure of the complex of human FcγRIIIA and IgG1, the glycosylation profile of the FcγRIIIA receptor itself, and clinical data on FcγRIIIA polymorphisms have provided important insight into Fc receptors presented on effector cells and information on factors that regulate the extent of ADCC activity (Sondermann et al., 2000; Edberg and Kimberly, 1997; Nimmerjahn and Ravetch, 2008; Cartron et al., 2002). FcγRIIIA is the predominant Fc receptor on NK cells and binds IgG1, but is also expressed on other immune cells, such as monocytes. It is considered a major player in ADCC effector function of therapeutic antibodies. Characterization of affinities of the receptor for IgG1 in NK cells and monocytes, both of which are used in *in vitro* ADCC assays, demonstrated a higher affinity of the receptor in NK cells, and this was shown to correlate with differences in FcγRIIIA receptor glycosylation in the two cell types (Edberg and Kimberly, 1997). Furthermore, crystal structure and other studies have demonstrated the importance of carbohydrate-carbohydrate interactions between receptor and antibody on affinity of the receptor for antibody, in that the strength of binding between ASN-162 of human FcγRIIIA and ASN-297 of human IgG is increased with afucosylation of antibody and explains the increased ADCC activity of afucosylated antibodies over those more fucosylated (Zeck et al., 2011; Ferrara et al., 2011). In addition, more detailed analysis of various FcγRIIIA glycosylation profiles has demonstrated the cell-type specificity and complexity of FcγRIIIA glycosylation and increased understanding on the role of FcγR glycosylation in antibody binding and activity (Hayes et al., 2014; Zeck et al., 2011). Lastly, amino acid differences in the FcγRIIIA receptor due to single nucleotide polymorphisms also affect patient outcomes in treatment with various therapeutic monoclonal antibodies. In clinical studies, non-Hodgkin lymphoma patients carrying FcγRIIIA (valine at amino acid site 158, from a point mutation at nucleotide 559), for example, have been shown to respond better to rituximab treatment, a chimeric mAb targeting CD20 antigen on tumor cells, than patients with F-158 (phenylalanine) allotype, and have improved survival outcomes (Cartron et al., 2002). These FcγRIIIA genetic polymorphisms have also been reported as a prognosis marker for the treatment of cetuximab, an inhibitor of the epidermal growth factor receptor (EGFR), in patients with metastatic colorectal cancer (Rodríguez et al., 2012). FcγRIIIA V-158 on NK cells confers a higher binding affinity for a variety of IgG antibodies than does the F-158 variant and contributes to enhanced ADCC activity (Koene et al., 1997; Chung et al., 2014b; Yamane-Ohnuki et al., 2004).

ADCC studies *in vitro* with various effector cell types containing FcγRIIIA receptors to simulate potential *in vivo* ADCC biological activity are of interest to the manufacturers of mAbs, as well as those developing biosimilars. In the latter case, for example, matching the glycosylation profile in the innovator antibody is an important objective for those mAbs where ADCC is a primary MOA and similarity is evaluated using *in vitro* ADCC assays. *In vitro* ADCC assays play an important part throughout all monoclonal antibody drug development, in studies addressing antibody MOA, characterization, comparability during manufacturing scale-up and transfer, and stability amongst others. Various cell sources, including primary and immortalized cell lines, are available for these evaluations, as are different assay formats. Recently, we have used two different ADCC bioassay formats, one developed to measure the signal directly from dead target cells when using an engineered NK cell line or primary NK cells (classic ADCC assay format) and the other developed to measure reporter gene signal generated by ADCC pathway activation in engineered Jurkat T effector cells (reporter gene ADCC assay format). The latter format is a surrogate ADCC assay measuring relevant pathway activation in the effector cells as an alternative to measuring target cell death (ADCC reporter gene assay; Cheng et al., 2014). For the classic ADCC assay we use CytoTox-Glo™ assay reagent to quantify the ATP molecules released from the lysis of target cells with luminescence signal readout. Conversely, the ADCC reporter gene assay quantifies the signal from the effector cells due to the translocation of NFAT2 protein into the nucleus and the consequent