Application Note

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# Deciphering the Impact of Antigen Density on Tumor Targeting Therapeutics


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## Abstract

Receptor expression levels are a critical factor influencing the efficacy and safety of targeted immuno-oncology therapeutics, including immune cell engagers and CAR-T therapies. However, there is a notable paucity of in vitro systems designed to elucidate the relationship between target antigen density and the performance metrics of these therapeutic modalities.

In this application note, Antibody Analytics (Motherwell, UK) integrates IndEx-2, an advanced in vitro cell-based platform, engineered to modulate the expression of one or two target antigens across a range of biologically relevant levels, with the Agilent xCELLigence Real-Time Cell Analyzer (RTCA), Agilent NovoCyte Flow Cytometer, and cytokine release measurement protocols. The aim is to determine the antigen density thresholds required for the activation of potential immunotherapeutic candidates. This comprehensive approach caters to a diverse array of functional readouts, spanning from cytotoxic responses to immune cell activation.

## Introduction

Leveraging the immune system as a powerful anticancer strategy, but achieving acceptable clinical safety remains challenging. One of the key barriers to developing effective immune activating/targeted therapeutics, such as immune cell engagers, chimeric antigen receptor (CAR) engineered cell therapies, and antibody drug conjugates, is the identification of a suitable cancer-associated antigen. In most cases, the antigen (or antigens) targeted are overexpressed by neoplastic cells, but also expressed, albeit to a lower level, in healthy tissues.

A further complication is the heterogeneity of antigen expression level, particularly in solid tumors. Often, this renders therapies ineffective due to low expression of the targeted receptor by a subset of cancer cells. Conversely, when the targeted antigen is expressed in healthy cells, the primary consideration is the development of "on-target, off-tumor" toxicities (OTOT), which arise when nonmalignant tissues are targeted. Severe OTOT toxicities have been observed in various CAR-T clinical trials involving subjects with solid tumors. They constitute a significant safety hurdle for the successful development of these modalities, to the extent that there are FDA guidelines instructing the consideration of these side effects during the preclinical evaluation phase.[1] The considerations also extend to other advanced therapeutics, including immune cell engagers and ADCs, for which similar toxicities have been reported.

Current approaches assessing the impact of antigen density on therapeutic efficacy and safety rely on cell lines with varying antigen expression levels. However, establishing a large panel of such cell lines is logistically and methodologically difficult due to licensing costs, limited availability, and uncertainty around specific antigen expression levels. Additionally, the varied backgrounds of these cell lines can confound data. This is because inherent genetic and epigenetic differences between these cell lines can render them susceptible, or resistant, to a potential therapeutic strategy that is independent of target antigen expression levels. This highlights the need for improved in vitro systems to evaluate the effect of target antigen density on therapeutic efficacy and safety, earlier in the development process.

With the introduction of the FDA Modernization Act 2.0[2], advocating for reduced animal model use, there is further impetus for the deployment of more cost-effective human in vitro models that can be implemented earlier in the drug discovery process. Agile decision making is easier at the preclinical stage, and the ability to pivot is more probable, having reduced cost implications in comparison to a clinical stage therapeutic candidate.

## Antibody Analytics' solution: IndEx-2

The IndEx-2 system is a comprehensive approach for evaluating the influence of antigen expression levels on the efficacy and safety of therapeutic modalities, such as immune cell engagers, CAR-based cell therapies, conventional monoclonal antibodies, and ADCs. The utility of the system is characterized by three key attributes:

**Inducible expression of antigens:** A cell line system that allows the expression of one or two antigens of interest, in a finely titratable manner and with large dynamic range of expression.

**Functional assays:** A suite of functional assays using high content readouts, employing a well-characterized donor pool or even subject material.

**Threshold determination:** An analysis methodology that enables the determination of activation thresholds.

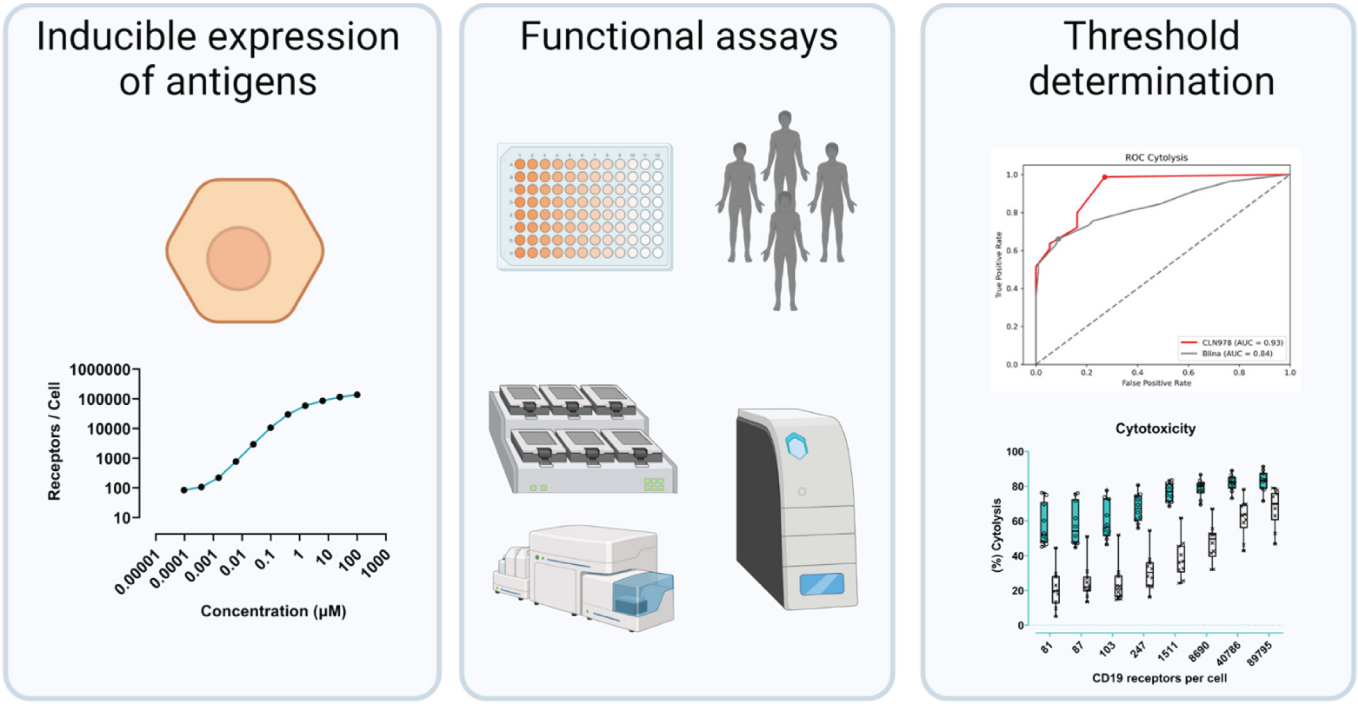


**Figure 1.** The IndEx-2 assay system. The schematic represents the key defining features of the IndEx-2 system: 1. Inducible target antigen expression, 2. Incorporation into functional cell-based assays and 3. Threshold of activation determination using an analysis methodology.

At the core of the IndEx-2 assay lies a cell line system that uses two Chemical Induced Proximity (CIP) systems to provide orthogonal control of the expression of two distinct target antigens of interest (TAOI). CIP systems use membrane-permeable, small molecule inducers to control dimerization between two proteins of interest. By fusing a DNA transactivation domain to one protein, and a DNA binding domain to the other, transcriptional activation can be conditionally controlled.

In the presence of the small molecule inducer, the DNA-binding domain is brought into proximity with the transactivator. This results in a functional complex that drives transcription from a corresponding novel DNA-recognition motif placed upstream of the TAOI. By employing two separate CIP systems, and novel DNA-recognition motifs, IndEx-2 cell lines can be produced, with independent expression control of two TAOIs.

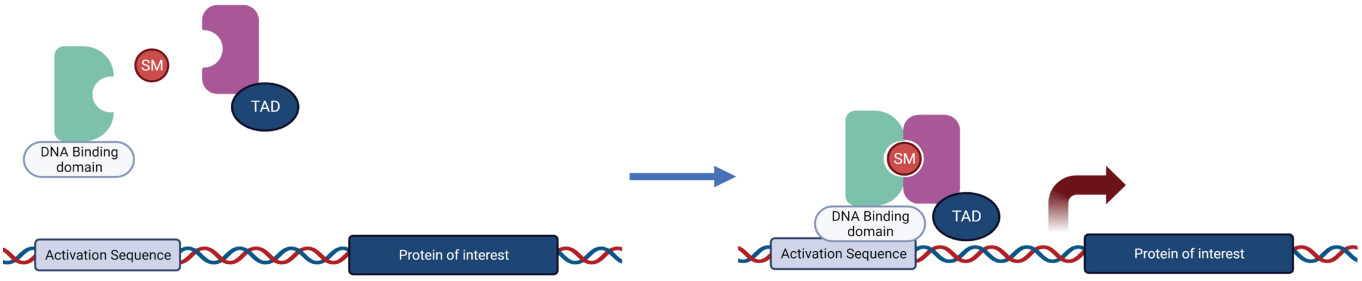


**Figure 2.** Assembly of the active transcription complex. In the presence of the small molecule inducer (SM), the two halves of the CIP system are brought together, bringing the transactivation domain (TAD) into proximity with the DNA binding domain. The latter facilitates binding to the activation sequence, upstream of the sequence of the protein of interest (or TAOI), permitting initiation of transcription via the TAD.

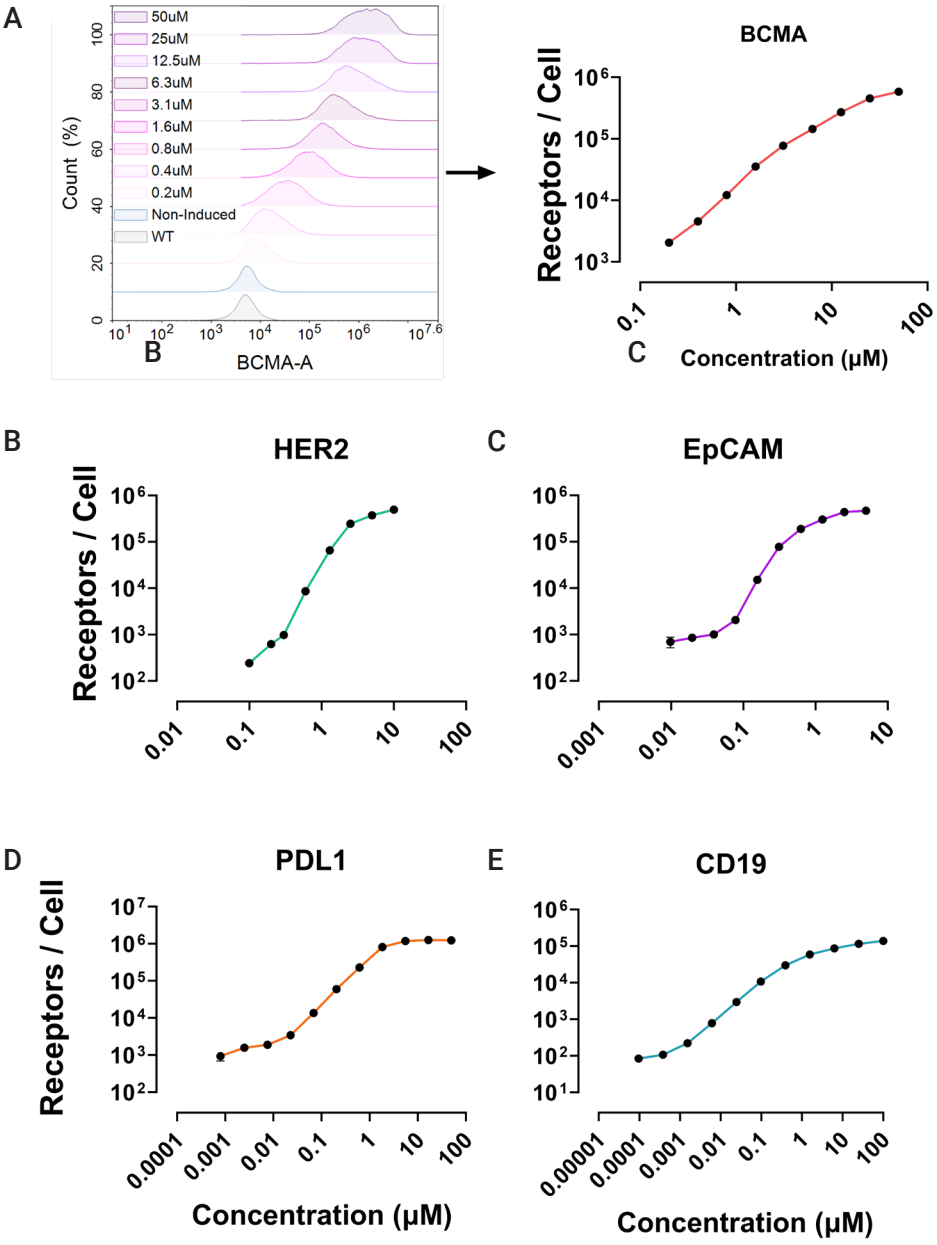


**Figure 3.** (A) Example histograms showing BCMA expression used for receptor quantification by quantitative flow cytometry with the Agilent Novocyte flow cytometer and fluorescence quantitation beads. The high-sensitivity and 7.2-log dynamic range of the Agilent Novocyte Quanteon's silicon photomultipliers enable precise quantification at all levels of receptor expression levels. IndEx-2 cell lines expressing therapeutically relevant targets were generated in a CHO-K1 background for the expression of (B) HER2, (C) EpCAM, (D) PDL1, and (E) CD19. Highly titratable expression ranges from hundreds to hundreds of thousands of receptors per cell were observed.

## CD19-directed T cell engager workflow

Hematological malignancies are prime candidates for targeted treatment with immune cell engagers and CAR-T cell therapies due to the existence of targets, such as CD19, CD20, and CD22, which can be safely targeted to eradicate the problematic tumor cell population. This is only possible because the ablation of the healthy B cell compartment is considered tolerable within the context of an otherwise dismal prognosis. However, a frequent tumor escape mechanism is loss or downregulation of target antigen expression – or 'antigen escape'. This mechanism allows the tumor to circumvent the targeted treatment, resulting in resurgence and proliferation of a resistant compartment. In subjects treated with CD19-targeted CAR-T cell therapies, antigen escape is a frequently reported relapse mechanism.[3] Likewise, there have been reports of relapse in Blinatumomab-treated Acute Lymphoblastic Leukemia of the B lineage (B-ALL) subjects, where tumor regrowth emerges as CD19 low/negative.[4]

Developing CD19-targeting therapeutics that retain efficacy, even at low CD19 expression levels, would be beneficial to reduce the risk of resistance. This would be particularly advantageous for CD19-targeted T cell engagers (TCE), which offer more clinical convenience in comparison to CAR-T therapies. The biological efficacy of these molecules can be modulated by optimizing the affinities of the CD19 and CD3 binding moieties. To identify CD19 TCEs that maintain efficacy at low CD19 expression levels, two CD19 TCEs (blinatumomab and CLN978) with similar architectures, but differing affinities for the CD3 and CD19 binding domains, were assessed. A CD19 IndEx-2 cell line was employed to induce eight discrete levels of CD19 expression. The activity of the two CD19 TCEs across this expression range was analyzed to determine the threshold of receptor expression required for biological activity. The two molecules were compared at a single concentration (225 pM), employing a cohort of 10 healthy human T cell donors as the effector population.

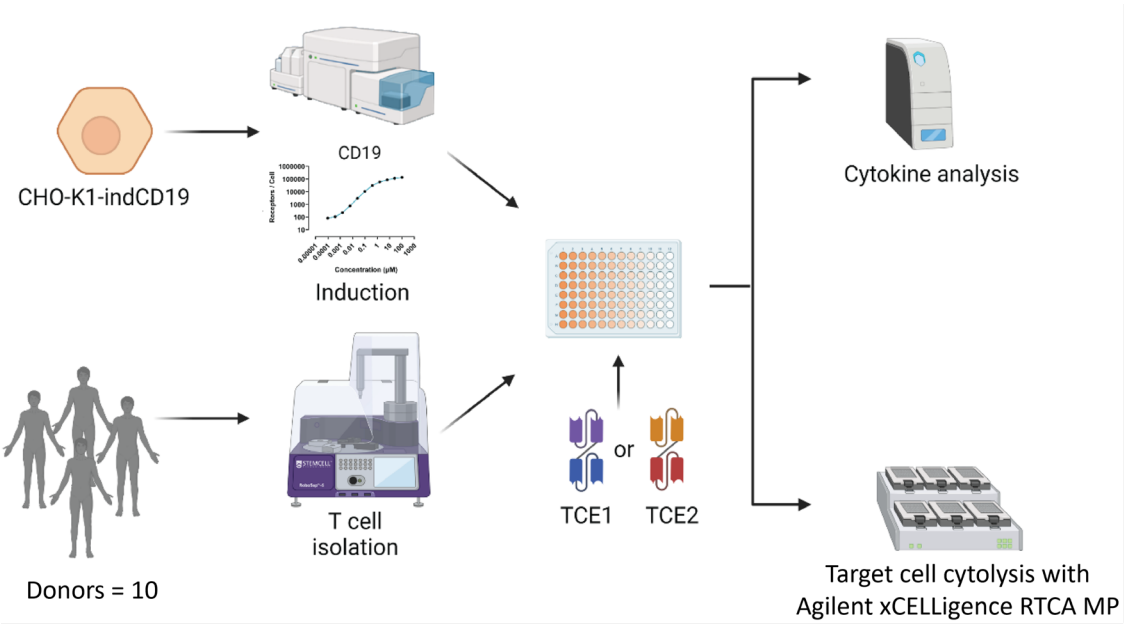


**Figure 4.** An experimental scheme. CD19 inducible IndEx-2 cells (CHO-K1-indCD19) were treated with a dose range of the small molecule inducer, to induce varying levels of CD19 receptors per cell. The CD19-expressing cells were treated with two CD19 TCEs (blinatumomab or CLN978) in the presence of isolated effector T cells, from 10 healthy human donors. Cytolysis was measured using the Agilent xCELLigence RTCA MP and IFNγ production by ELISA.

## Methods

CHO-K1 cells (ATCC) were transduced with lentivirus (VectorBuilder Inc), carrying an induction cassette that expresses a chemically induced proximity activation system. This allows titratable expression of CD19 upon treatment with the chemical inducer, abscisic acid (ABA), enabling expression levels of CD19 on CHO-K1 cells, ranging from 78 to 185,223 copies of CD19 per cell after 24 hours. CD19 expression was quantified by staining with PE anti-CD19 (BioLegend), followed by analysis on an Agilent NovoCyte Quanteon flow cytometer. Fluorescence intensities were compared to a standard curve, generated using a Quantibrite PE receptor quantification kit (BD Biosciences).

For T cell-dependent cellular cytotoxicity (TDCC) studies with inducible CHO CD19 cells, CHO-K1 ABA CD19 cells were seeded in Agilent xCELLigence Biosensor E-Plates and treated with a seven-point concentration range (2.4 nM to 10 µM) of ABA for 24 hours to induce CD19 expression. Effector T cells were isolated from a panel of 10 healthy donors using the EasySep  Human T Cell Isolation kit and the RoboSep-S (STEMCELL Technologies). The purity of the isolated population was confirmed by flow cytometry. The isolated T cells were then cocultured with the target cells in an xCELLigence RTCA MP (Agilent) at a 10:1 E:T ratio in the presence of 225 pM of either blinatumomab (ProteoGenix) or CLN978 (Cullinan Oncology Inc).

Agilent xCELLigence technology enables label-free, real-time monitoring of cell health, function, and behavior. Cytolysis was measured through impedance signal loss due to detachment of the target cells from the base of the E-Plates. IFN-γ production by the effector cells was measured through ELISA from supernatant. The supernatants from the xCELLigence RTCA MP coculture were harvested and stored at –20 °C. When performing the IFN-γ, the supernatants were thawed at room temperature, and the ELISA was performed using the Human IFN-gamma DuoSet ELISA (Bio-techne), as per the manufacturer's instructions. The absorbance was then measured at 450 nm with a correction of 540 nm using the HIDEX Sense Plate Reader (HIDEX).

## Results and discussion

The efficacy of the TCEs across a range of CD19 expression levels was analyzed by evaluating T cell-mediated target cell cytolysis using the xCELLigence RTCA MP and IFN-γ production by ELISA. Wild Type (WT) CHO-K1 cells, which do not express any human CD19 receptors, served as a benchmark control. Comparing the two TCEs, it was evident that CLN-978 had retained its efficacy in both target cell cytolysis and IFN-γ production, even at the lowest CD19 expression level. This was consistent with CLN-978's higher CD19 affinity, relative to blinatumomab (Figures 5A and B).

To estimate a receptor threshold for TCE activity initiation in the 10-donor cohort, we employed the Receiver Operating Characteristic (ROC) analysis. This analysis method is widely used for determining thresholds during diagnostic test development, and requires binary classification of the data as positive or negative. For each donor and each CD19 expression level, a response was considered positive if it was above 3 standard deviations (SD) from the benchmark control mean (activity observed against the WT CHO-K1 cells). This facilitated ROC curve generation for each readout and TCE. By employing a single metric of the ROC curve, termed the Youden's J statistic, we determined the receptor threshold for each readout. The threshold at which cytolysis and IFN-γ production were observed for CLN-978 in the 10-donor cohort was 81 CD19 receptors per cell, the lower limit of detection. In contrast, blinatumomab required 843 CD19 molecules per cell for cytolysis activity and 8,690 CD19 molecules per cell for IFN-γ secretion. This data allowed us to speculate that subjects treated with CLN-978 may be less likely to relapse due to antigen escape. While we do not claim that this can be predicted by this in vitro model alone, it will be interesting to see if these observations translate clinically.

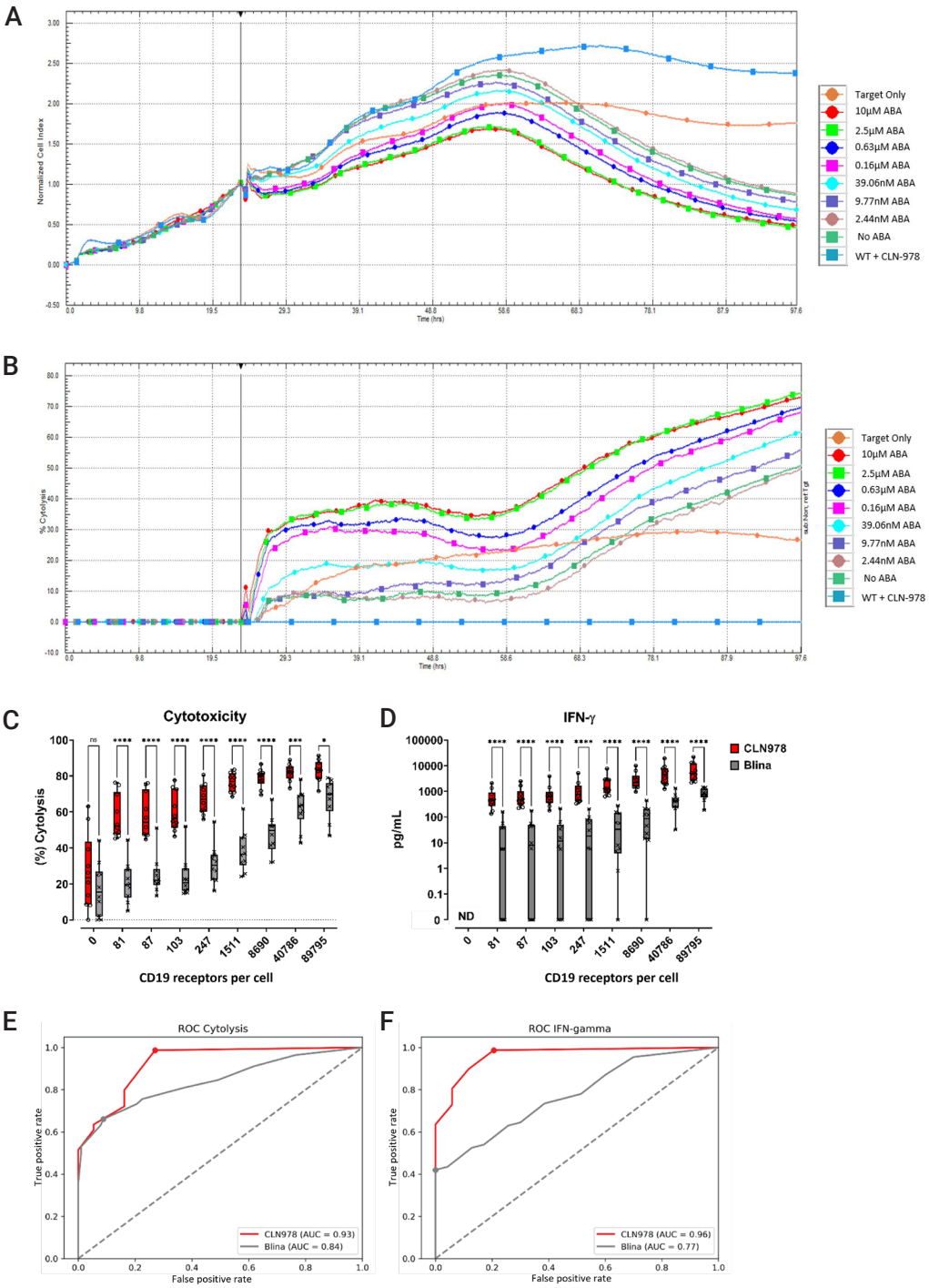


**Figure 5.** CLN978 retains its activity at low CD19 concentrations. (A, B) Representative normalized Cell Index and percentage cytolysis data for CLN-978, across a range of ABA-inducer concentrations from a single donor (determined by kinetic cytotoxicity assessment using the xCELLigence RTCA MP system). (C, D) Percentage cytolysis and IFN-γ production (ELISA) for both TCEs for the 10 donors assessed. The data is represented as a function of CD19 expression level (receptors per cell), quantified using Quantibrite beads (top graphs). 0 receptors is the effect against the WT CHO-K1 cells (benchmark control). The data is represented as a box and whiskers with minimum/maximum responses. The data was analyzed with multiple Mann-Whitney tests, including multiple comparison correction s (Holm-Sidak). **** p<0.0001, ***p<0.001, * p<0.05. (E, F) ROC analysis of each data set, with Youden's J index used to determine the point with least probability of a false negative, indicating the minimum number of receptors for true activation, or the activation threshold for each TCE molecule.

## Conclusion

The IndEx-2 assay system is a robust in vitro model for evaluating the influence of antigen expression levels on a targeted therapeutic's efficacy and safety. This versatile tool enables the controlled expression of one or two antigens of interest, and uniquely allows for the estimation of antigen expression thresholds required for therapeutic activation. As demonstrated in a CD19-targeting therapeutics case study, it can aid in identifying candidates with specific activity characteristics in relation to target antigen expression, facilitating the progression of optimal leads to the clinic. IndEx-2 is uniquely suited as an assay system for the preliminary assessment of on-target, off-tumor toxicities due to its ability to allow estimation of receptor thresholds. Within the IndEx-2 system, the Agilent xCELLigence RTCA MP and Agilent NovoCyte Quanteon flow cytometer are integral components to determine cytolysis and target expression levels for immunotherapeutic development.

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