NovoCyte® Flow Cytometer

Application Note No. 14

ACEA Biosciences, Inc.

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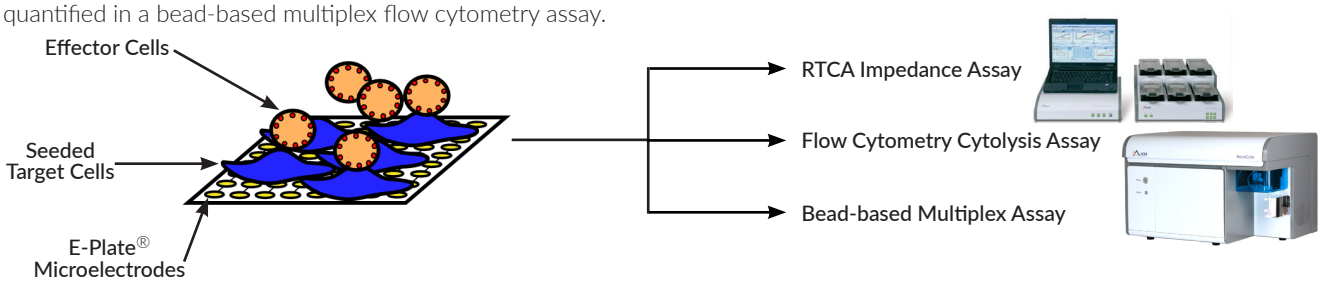
Tumor Cell Killing by T Cells: Quantifying the Impact of a CD19-BiTE Using Real-Time Cell Analysis, Flow Cytometry, and Multiplex Immunoassay

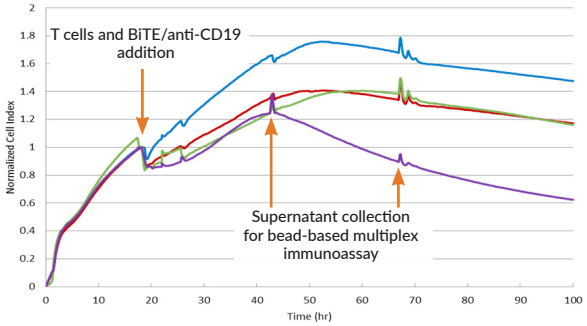
Both the adaptive and innate arms of the immune system play a pivotal role in a host's defense against tumors. CD8+ cytotoxic T lymphocytes (CTL), which are a major component of the adaptive immune response, directly eliminate target tumor cells by releasing cytolytic proteins such as granzymes, perforin, and granulysin, in addition to producing multiple cytokines. The ability to quantitatively correlate T cell biomarker expression/secretion with target cell killing is critical in both basic and applied studies of tumor immunology. Coupling flow cytometry with multiplex bead-based immunoassays enables quantitative measurement of multiple secreted targets in a single sample simultaneously. In this Application Note, we demonstrate a workflow involving the use of an xCELLigence® Real-Time Cell Analysis (RTCA) instrument in conjunction with a NovoCyte® flow cytometer to study both CTL-mediated destruction of tumor cells and the corresponding secretion of cytokines and cytolytic proteins by CTLs.

Representing a promising new class of therapeutics, bispecific T-cell engagers (BiTEs) harness the power of the adaptive immune response by enhancing the ability of CTLs to specifically recognize and eliminate tumors. For example, CD19-BiTEs are designed to simultaneously bind CD3 on CTLs and CD19 on cells of B cell lineage, thereby enhancing the efficiency of CTL effector functions against various B cell derived tumors. Herein, the enhancement effects of CD19-BiTEs have been evaluated using three distinct assays. While target cell death was monitored using an RTCA impedance assay and a flow cytometry-based cytolysis assay, secretion of cytokines and cytolytic proteins was quantified in a bead-based multiplex flow cytometry assay.



CD19-BiTE enhances T cell-mediated target cell killing in an xCELLigence impedance assay

Daudi cancer B cells were immobilized on an xCELLigence electronic microtiter plate (E-Plate®) that had been pre-coated with anti-CD40 antibody. Interaction between the cells and the gold microelectrodes leads to an increase in electrical impedance, the magnitude of which correlates with cell number, size, and cell-substrate attachment quality. 18 hours post Daudi cell seeding, T cells enriched from primary PBMCs (using the EasySep™ Human T Cell Enrichment Kit, StemCell Technologies) were added at a ratio of T/Daudi = 10. At the same time, CD19-BiTE (G&P Bioscience, cat.# FCL770) or anti-CD19 antibody control (G&P Bioscience, cat.# MAB0776) were also added. The impedance signal of the Daudi cell monolayer was recorded every 15 minutes, and is plotted here using the unitless parameter Cell Index. While T cells alone induced some B cell killing, the presence of CD19-BiTE increased killing efficacy dramatically **(Figure 1)**.



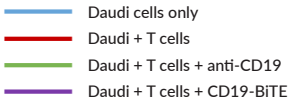


Figure 1. Dynamic monitoring of CD19-BiTE enhancement of T cell-mediated Daudi cell killing in an xCELLigence impedance assay. Daudi target cells were seeded at 50,000 cells/well in a 96-well E-Plate coated with anti-CD40 antibody. 18 hours after Daudi cell seeding, enriched effector T cells were added with or without CD19-BiTE (0.33 µg/mL) or an anti-CD19 antibody as a negative control. Relative to the controls, the presence of CD19-BiTE caused a significant decline in Cell Index, indicative of enhanced Daudi cell killing by effector T cells.

CD19-BiTE enhances T cell-mediated target cell killing in a flow cytometry-based cytolysis assay

The role of CD19-BiTE as a powerful facilitator of T cell-mediated killing of Daudi B cells was further confirmed using flow cytometry. Daudi cells were first labeled with CFSE and cultured with enriched primary T cells with or without CD19-BiTE. After 48 hours, 7-AAD was added to label dead cells prior to analysis on a NovoCyte flow cytometer. While the presence of CTLs increased the proportion of Daudi target cells (CFSE+) that were dead (7-AAD+), the inclusion of CD19-BiTE dramatically increased target cell death **(Figure 2)**.

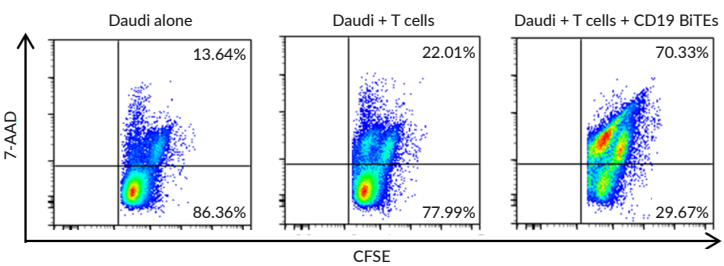


Figure 2. CD19-BiTE enhances T cell-mediated Daudi cell death based on flow cytometry staining. Daudi cells were first labeled with CFSE and seeded at 100,000 cells/well. 18 hours after Daudi cell seeding, enriched T cells were added with or without CD19-BiTE. 48 hours post T cell addition, 7-AAD was added to identify dead cells. The combination of T cells and CD19-BiTEs increased the percentage of dead Daudi cells (CFSE/7-AAD double positive) compared to T cells alone.

CD19-BiTE enhances T cell scretion of cytokines and cytolytic proteins in a bead-based immunoassay

In parallel, to demonstrate the correlation between T cell-mediated target cell killing and cytokine/cytolytic protein secretion, we utilized a commercially available bead-based multiplex immunoassay to examine the supernatant from a co-culture containing primary effector T cells and Daudi B cells. Daudi target cells were seeded as described in Figure 1, and cultured with enriched T cells with or without CD19-BiTE or an anti-CD19 antibody negative control. Cytokine and cytolytic protein production were quantified in the culture supernatant 24 hours **(Figure 3)** and 48 hours (data not shown) after T cell addition using the BioLegend 13-plex Human CD8/NK panel (cat.# 740267). Briefly, a pre-mixed pool of analyte-specific capture beads was added to the supernatant samples. After washing, biotinylated detection antibodies were added to the beads, followed by further washing and addition of streptavidin-PE. Subsequently, the beads were read on a NovoCyte flow cytometer to simultaneously quantify 13 secreted analytes produced under the different co-culture conditions **(Figure 3)**.

Figure 3. CD19-BiTE enhances the cytotoxic activity of T cells in a bead-based multiplex immunoassay. Daudi target cells were seeded at 50,000 cells/well on an anti-CD40 antibody-coated plate. 18 hours later, enriched T cells were added with or without CD19-BiTE (0.33 µg/mL) or anti-CD19 antibody (0.33 µg/mL) as a negative control. 24 hours after T cell addition, supernatants were collected and analyzed by a bead-based multiplex immunoassay. **(A)** The combination of T cells and CD19-BiTEs resulted in enhanced secretion of cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, and TNFα. **(B)** Production of cytolytic proteins such as granzyme A, granzyme B, perforin, soluble FasL, and granulysin was also substantially increased by the presence of CD19-BiTE.

Consistent with our findings from the RTCA- and flow-based cytolysis assays, this bead-based multiplex immunoassay shows that the presence of CD19-BiTE significantly enhances production/secretion of cytokines and effector molecules that mediate and sustain target cell killing. Specifically, the presence of CD19-BiTE enhanced T cell production of IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, and TNFα by factors of 2.8 to 20 **(Figure 3A)**. At the same time, secretion of the cytolytic proteins granzyme A, granzyme B, perforin, soluble FasL, and granulysin increased 2.4- to 11.8-fold in the presence of CD19-BiTE **(Figure 3B)**, consistent with the enhanced cell killing observed in the RTCA and flow assays.

Herein, in a single workflow we've coupled quantitative cell killing assays with biomarker quantitation to provide an in depth view of how CD19-BiTE affects killing of Daudi B cells by CTLs. The continuous monitoring of cell number, size, and attachment quality using xCELLigence enables quantitative and kinetic assessment of the killing process, which is simultaneously corroborated by flow cytometry. Linking this cell killing data with quantitative analysis of cytokine and effector protein production maximizes the information that is extracted from the assay. This type of workflow, which integrates both cellular and molecular phenomena, is becoming increasingly important for both basic and applied studies in the field of cancer immunotherapy.

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