APPLICATION NOTE

Simultaneous measurement of chemokine/cytokine release and antibody-dependent cellular cytotoxicity upon binding with rituximab.

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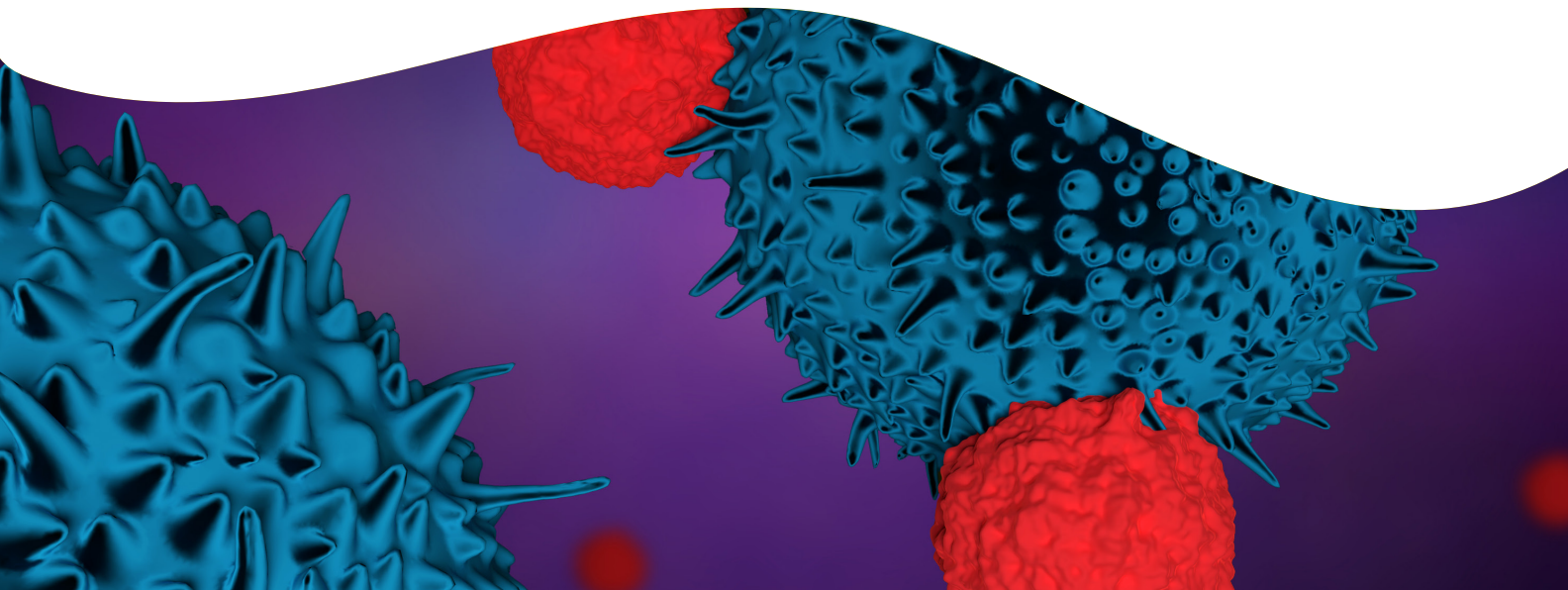
Introduction

Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism of cell-mediated immune response wherein an effector cell actively lyses an antibody-bound target cell. Cells expressing tumor or pathogen-derived antigens on their surface are targeted by therapeutic antibodies through the Fab region of the antibody while the Fcγ receptor (primarily FcγRIIIa/CD16) on immune effector cells binds to the Fc region of the antibody to form a complex of effector and target cell. Once binding is established and the complex is formed, a series of signaling events occur between the two cell types and the immune effector cells become activated. Effector cell activation triggers a cascade of cell signaling events releasing chemokines (i.e. MIP-1α, MIP-1β, RANTES, IL-8) and cytokines (i.e. TNFα, IFNγ) into the tumor microenvironment along with cytotoxic molecules perforin and granzymes whic combine to lyse the target cells.[1] Some of the secreted factors do not act directly on the target cells but rather they act as chemo-attractants to other immune types to help eradicate the targeted tumor cells.[2] Cell signaling factors present during an ADCC experiment can be effectively measured *in vitro* through the use of AlphaLISA™ technology. Amplified luminescent proximity homogeneous assay (Alpha), is a no wash, bead-based assay format used to measure an analyte in cell supernatant or lysates as well as other complex matrices (serum, plasma, urine, etc.). Figure 1 depicts the general AlphaLISA principle.



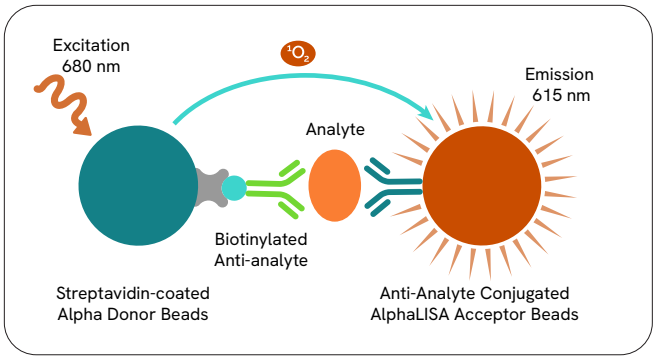


Figure 1: AlphaLISA Assay Principle. A biotinylated anti-analyte antibody is bound to the Streptavidin-coated AlphaLISA Donor Beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor Beads. In the presence of analyte, the Donor and Acceptor Beads come into close proximity. Excitation of the Donor Beads at 680 nm provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in the nearby Acceptor Beads, resulting in maximum emission at 615 nm. AlphaLISA signal is proportional to the amount of analyte present in the sample.

Additionally, the availability of a non-radioactive *in vitro* assay to specifically measure cell death in target cells during an ADCC experiment is critical. DELFIA™ (dissociation-enhanced lanthanide fluorescence immunoassay) EuTDA Cytotoxicity Reagents offer a time-resolved fluorescence (TRF) assay format that can be used effectively to specifically label the targeted tumor cells with a ligand prior to performing co-culturing assays. Figure 2 describes the assay principle in the context of an ADCC experiment. Target cells are loaded in a rapid and gentle method with a fluorescent enhancing ligand (BATDA) which crosses the cell membrane passively. Once inside the cell, the ligand is immediately hydrolyzed by cellular esterases to generate a hydrophilic molecule (TDA) that can no longer penetrate the cell membrane. Activation of the immune effector cells leads to cytolysis of the target cells which then release TDA into the supernatant. TDA forms a highly fluorescent lanthanide chelate (EuTDA) when europium solution is added. The cytotoxic effect on the target cells is proportional to the amount of fluorescent signal generated by the final EuTDA product.

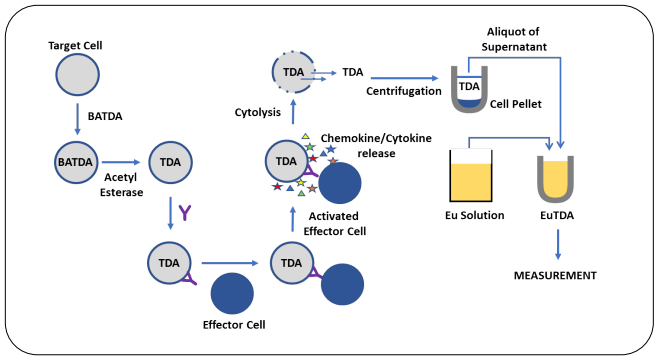


Figure 2: DELFIA EuTDA Cytotoxicity Assay for ADCC Determination. Target cells are first loaded with BATDA reagent which is hydrolyzed inside the cells to create TDA. Next, the target cells are combined with antibody and co-cultured with the chosen immune effector cell type. The antibody binds antigen on the target cell surface while the effector cells bind the Fc region of the antibody to form a complex with the target cells. Complex formation activates the immune effector cells which proceed to release cytotoxic molecules perforin and granzymes as well as chemokines and cytokines. The resulting molecules cytolyze the target cells releasing TDA into the supernatant. The assay plate is centrifuged and then supernatant is transferred to a new assay plate and europium solution added to form the active lanthanide (EuTDA). Fluorescent signal is detected using DELFIA TRF settings on a compatible plate reader (excitation 340 nm and emission at 615 nm).

Here we performed an ADCC experiment with rituximab, an anti-CD20 monoclonal therapeutic antibody, as well as two modified versions of rituximab known to increase or decrease ADCC activity. Rituximab is effective against a variety of B-cell malignancies such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL); its effectiveness is driven in part through mediation of ADCC.3 The target cell line used in the co-culture was the CD20 expressing Raji (human, Burkitt'slymphoma) and the immune effector cell type chosen were Natural Killer (NK) cells which represent 5-15% of the peripheral blood mononuclear cell (PBMC) population. NK cells are a subset of cytotoxic innate lymphoid cells which can kill tumor cells and help drive adaptive immunity by secretion of cytokines.4 Supernatants were taken at the time points noted and tested by AlphaLISA for NK cell activation as evidenced by chemokine and cytokine release. In parallel, target cell death was measured by DELFIA TRF demonstrating the effectiveness of the activated NK cells in the ADCC assay.

Materials and methods

Recents and consumables

- DELFIA EuTDA Cytotoxicity Reagents (Revvity, #AD0116)
- Raji cells (ATCC, #CCL-86)
- Natural Killer cells, CD56+/CD3-(Discovery Life Sciences)
- RPMI media (ATCC, #30-2001)
- Fetal bovine serum (FBS), heat inactivated (Thermo Fisher, #10082-147)
- Phosphate buffered saline (PBS), (Thermo Fisher, #10010- 023)
- HEPES 1M solution (Thermo Fisher, #15630-080)
- Triton™ X-100 (Sigma Aldrich, #T8787)
- StorPlate™-96V (Revvity, #6008299)
- CellCarrier™ Spheroid ULA 96-well Microplates (Revvity, #6055330)
- AlphaPlate™-384, light gray (Revvity, #6005350)
- Rituximab, anti-hCD20-hIgG1 (InvivoGen, #hcd20-mab1)
- Isotype control, anti-β-gal-hIgG1 (InvivoGen, #bgal-mab1)
- Rituximab, anti-hCD20-hIgG1fut, non-fucosylated (InvivoGen, #hcd20-mab13)
- Rituximab, anti-hCD20-hIgG1NQ, non-glycosylated (InvivoGen, #hcd20-mab12)
- Falcon® 5 mL round-bottom polystyrene tubes (VWR, #60819-295)
- AlphaLISA kit – IL-8, biotin-free (Revvity, #AL328)
- AlphaLISA kit – IFNγ, biotin-free (Revvity, #AL327)
- AlphaLISA kit – TNFα, biotin-free (Revvity, #AL325)
- AlphaLISA kit – TNF5α (Revvity, #AL257)
- AlphaLISA kit - MIP-1β (Revvity, #AL258)
- AlphaLISA kit - MCP-1 (Revvity, #AL244)
- AlphaLISA kit – RANTES (Revvity, #AL289)

ADCC assay protocol

Effector cell handling: Purified NK cells were negatively selected from a PBMC population and characterized as CD56+/CD3- and ≥85% pure by the provider. Frozen purified NK cells (5 x 10^6 cells/vial) were removed from liquid nitrogen storage, thawed rapidly in a 37°C water bath, washed one time in 10 mL cell culture media and then prepared at the appropriate plating density to match the effector-to-target cell ratio (5-to-1 and 15-to-1). Media alone (control wells) or 100 µL of NK cells at each required density was added to the assay plate.

Target cell loading: Raji cells were harvested from a T75 flask, washed one time with PBS and counted. Cell number was adjusted to 1 x 10^6 cells/mL in Loading Buffer (PBS + 10% FBS + 20 mM HEPES buffer) in a Falcon® round-bottom tube. Next, 3 µL of BATDA reagent was added to 2 mL of cells, mixed gently, and incubated for 15 minutes at 37°C with 5% CO_2 in the cell culture incubator. After loading was complete, cells were centrifuged and washed 3X with Wash Buffer (PBS + 20 mM HEPES buffer). At each wash step the cell pellet was carefully resuspended by gently flicking the tube before adding wash buffer. After the final wash, the cell pellet wasresuspended in 1 mL of cell culture media and cell density counted again. Cells were adjusted to 2 x 10^5 cells/mL in order to plate 10,000 cells/well in 50 µL volume.

Antibody addition: Antibody dilutions were generated at 4X desired concentration in cell culture media and 50 µL was added to each appropriate well to reach a final concentration of 1 µg/mL.

Final assay volume in the 96-well CellCarrier Ultra Low Attachment (ULA) U-bottom shaped assay plate was 200 µL. The assay plate was centrifuged briefly after all components were present to promote contact between the cell types and facilitate the formation of the ADCC complex. The U-bottom shape of the plate and low attachment surface also promoted settling of the effect or and target cells together. Similarly, sterile V-bottom polystyrene assay plates could be used for the same effect of increasing cell to cell contact in a co-culture assay format.

Data collection and analysis

Cell supernatant was carefully sampled from the assay plate so as not to disturb the cells at 1, 2, and 4 hours post setup of the complete assay plate (target cells + antibody + effector cells or appropriate controls). Collected supernatant samples were centrifuged to remove any unwanted cell carryover. DELFIA TRF was measured immediately while remaining supernatant was split into 25 µL aliquots in StorPlate-96V plates and kept frozen at -20°C prior to AlphaLISA testing to avoid freeze thaw cycles.

AlphaLISA: All AlphaLISA assays were performed following Revvity's recommended protocol for each kit. Each assay required 5 µL of sample in a 50 µL reaction. A fresh aliquot plate was thawed on the day of testing. Since the biotin level in RPMI media can interfere with the Streptavidin-coated Donor Beads in many kits, biotin-free kits were substituted when available. AlphaLISA signal was measured on a Revvity EnVision™ 2105 multimode plate reader using default values for Alpha detection.

DELFIA cytotoxicity: 20 µL of cleared supernatant was mixed with 200 µL of europium solution in the provided DELFIA strip plate. The plate was incubated for 15 minutes at room temperature with shaking on a DELFIA Plateshake on the low speed setting. Fluorescence was measured on a Revvity EnVision 2105 multimode plate reader using default values for DELFIA TRF detection.

Definitions of background, spontaneous and maximum release of TDA:

- **Background (= sample at time zero):** Aliquots of the loaded target cells were removed immediately after loading and plating density adjustment. Aliquots were centrifuged to pellet cells, and then 50 µL of supernatant was removed and pipetted into the assay plate well with 150 µL of cell culture media to reach the final 200 µL assay total volume.
- **Spontaneous Release (= target cells without effector cells present, sampled at each time point):** Loaded target cells and antibody mix (100 µL combined) were incubated with 100 µL cell culture media instead of effector cells during the assay time frame. Supernatant was collected at specified time points.
- **Maximum Release (= lysed target cells):** Loaded target cells (50 µL) were incubated with 150 µL of cell culture media supplemented with lysis buffer (0.5% final concentration of Triton X-100).

Formula Calculation

%Specific Release

(Experimental Release – Spontaneous Release) /
(Maximum Release – Spontaneous Release) x 100

%Spontaneous Release-

(Spontaneous Release – Background) /
(Maximum Release – Background) x 100

Results

ADCC response is effectively measured by DELFIA TRF
Spontaneous release was calculated as described in the Data Analysis section using the isotype control antibody condition in the absence of NK cells as representative for the experiment. Percent Spontaneous Release remained low in the loaded Raji cells throughout the 4-hour assay window with 7.9, 13.7, and 21.1% calculated at the 1, 2, and 4-hour time points, respectively. Percent Specific Release was calculated, and the 4-hour time point is shown in Figure 3. After accounting for spontaneous release, cytolysis approaches 30-40% at the 15-to-1 effector-to-target cell ratio for the active antibodies. Adding more NK effector cells to the co-culture increased ADCC activity (15-to-1 > 5-to-1); this was most likely due to increased contact with the Raji target cells in the assay plate. Additionally, there is a striking difference between the modified rituximab antibodies. Directed changes can be made to the N-linked glycans on the Fc core of a monoclonal antibody (mAb). It has been reported that non-glycosylated rituximab loses its ADCC activity, and when it has a non-fucosylated core there is a marked increase in ADCC response due to an increased affinity for the FcγRIIIa on the effector cells.[5,6] Figure 3 shows no measurable ADCC activity for the non-glycosylated form of rituximab and an increase in ADCC activity with non-fucosylated rituximab relative to unaltered rituximab as expected based on reported literature. This result corroborates the idea that beneficial changes to an antibody structure can improve ADCC activity and increase the efficacy of therapeutic antibodies.

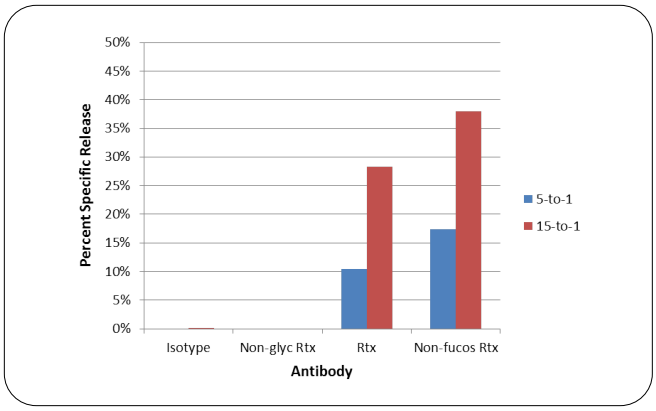


Figure 3: Percent Specific Release at four Hours. Increased cytolysis is achieved by increasing effector-to-target cell ratio to 15-to-1 in the presence of active versions of rituximab. The non-glycosylated rituximab (Non-glyc Rtx) is inactive and performs similarly to the isotype control showing no specific release. The non-fucosylated rituximab (Non-fucos Rtx) results in ~10% more cytolysis than rituximab (Rtx) at the 15-to-1 effector-to-target cell ratio with a modest increase seen at the 5-to-1 effector-to-target cell ratio

Chemokine and cytokine release from natural killer cells

Seven different targets including five chemokines (MIP-1α, MIP-1β, RANTES, IL-8 and MCP-1) and two cytokines (TNFα, and IFNγ) were tested using AlphaLISA technology on the supernatants of the co-culture ADCC model. Targets were selected based on literature that showed them to be released from NK cells in a co-culture setting.[7] Addition of rituximab or non-fucosylated rituximab led to activated NK cells as observed by the DELFIA TRF results above. Four targets were detected by AlphaLISA in response to NK cell activation (MIP-1α, MIP-1β, RANTES, and TNFα) while three others remained undetectable up to the longest time point in the experiment (IFNγ, MCP-1, and IL-8). Figure 4 shows the four-hour time point for MIP-1α (Figure 4A), MIP-1β (Figure B), RANTES (Figure 4C), and TNFα (Figure 4D) and demonstrates the effect of increasing NK cell number per well. The 15-to-1 effector-to-target ratio resulted in a two to three-fold increase above the 5-to-1 condition in all four targets detected as expected from the increased number of NK cells present.

Figure 5 reflects the expression profile over time of the NK cells used in this ADCC experiment based on the panel of targets tested at the 15-to-1 effector-to-target ratio. The regulation of NK cell chemokine and cytokine released after activation can be seen with each of the four expressed targets. MIP-1α (Figure 5A) and MIP-1β (Figure 5B) are chemokines that are upregulated and begin to be released in the first hour of NK cell activation and continue with strong secretion from the NK cells at two hours and beyond. The chemokine RANTES also shows an early response to NK cell activation but with a gradual release over time (Figure 5C).

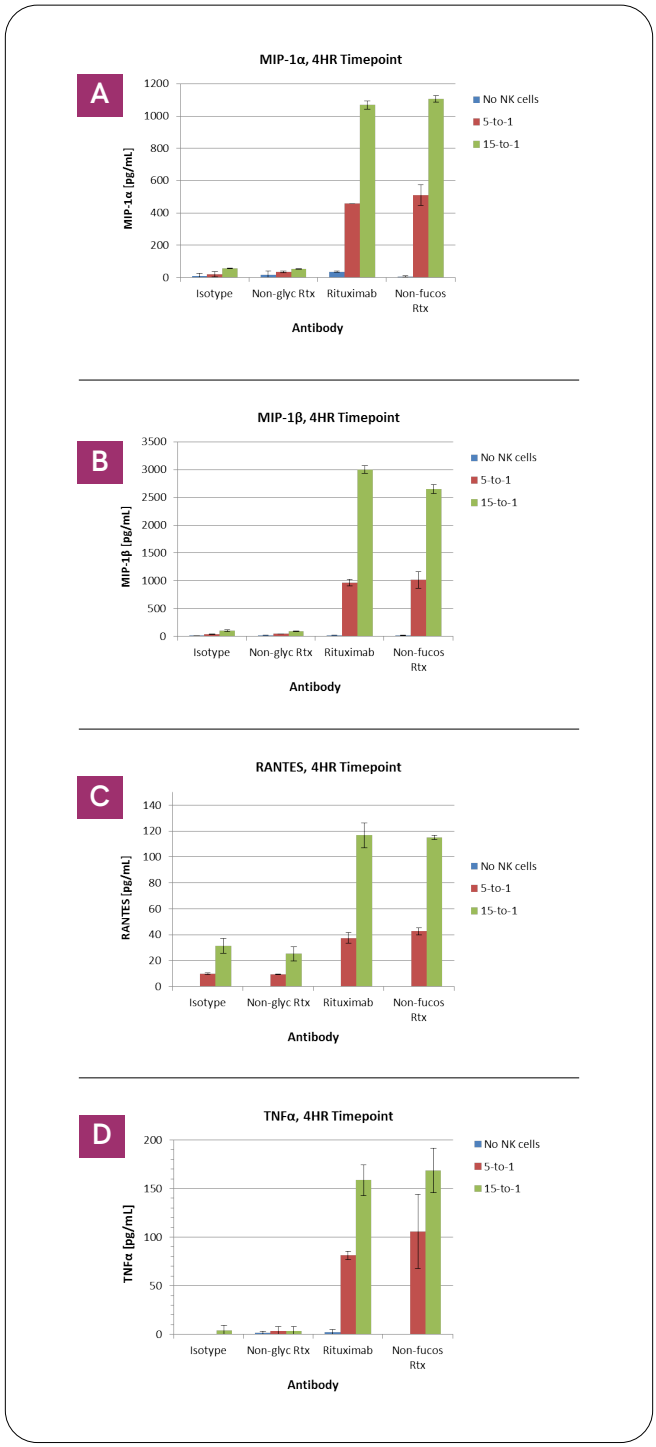


Figure 4: Chemokine and Cytokine Release at four Hours. Results represent pg/mL amount in a 5 µL supernatant sample for MIP-1α (A), MIP-1β (B), RANTES (C), and TNFα (D). Rituximab and non-fucosylated rituximab (Non-fucos Rtx) activate NK cells in the co-culture setting and result in increased chemokine and cytokine release over baseline levels seen with the isotype and inactive non-glycosylated rituximab (Non-glyc Rtx).

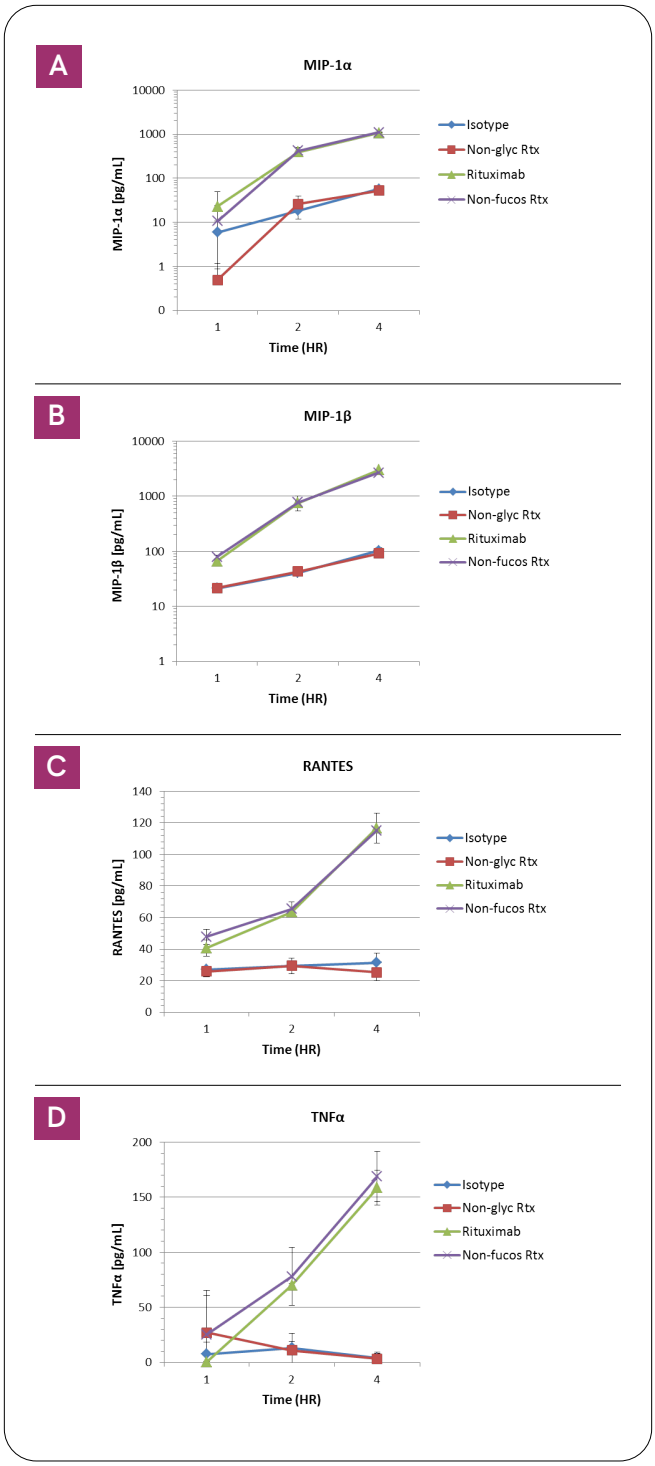


Figure 5: AlphaLISA Results for Chemokine and Cytokine Targets Released into the Supernatant. Data shown is from an effector-to-target ratio of 15-to-1. The nonglycosylated rituximab (Non-glyc Rtx) and the isotype control antibody do not result in activated NK cells. Rituximab and the non-fucosylated rituximab (Non-fucos Rtx) form the ADCC complex which activates the NK cells and stimulates the release of chemokines and cytokines into the supernatant. MIP-1α and MIP-1β (A and B, respectively) exhibit the strongest increase in secretion over time. RANTES (C) and TNFα (D) increase to a lesser degree over the four-hour time frame sampled. Results are reported as pg/mL in a 5 µL sample volume.

The level of TNFα (Figure 5D) is below the lower limit of detection (LDL) at the one-hour time point and increases gradually at two hours and beyond suggesting a higher threshold of signaling between activated NK cells and the target cells is required to trigger release of the cytokine TNFα from NK cells. Meanwhile chemokine production is an early response of NK cells and is triggered with relatively weak signals requiring less stimulation than is needed for cytokine production.[7] Despite the phenotypic difference in the DELFIA ADCC result between the non-fucosylated rituximab and rituximab (Figure 3), there was no significant difference in chemokine or cytokine release by the antibodies at the time points tested in this experiment at 1 µg/mL concentration of each antibody. This suggests that the increased effect on cytolysis seen by DELFIA TRF may not be due to a differential effect on the chemokine or cytokine targets measured in this experiment at the time points chosen for analysis.

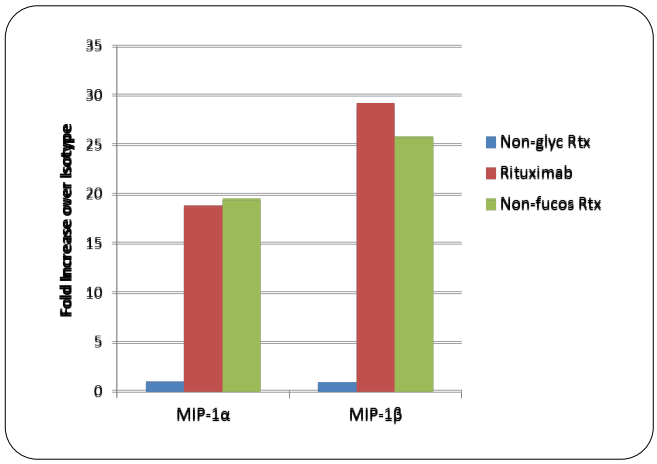


Figure 6: Fold Increase of Chemokine Release over Isotype Control at four Hours. A low level of chemokine release was seen in the absence of activating antibodies. Secretion of MIP-1α and MIP-1β were both strongly induced by rituximab and nonfucosylated rituximab above baseline levels nearly 20-fold and 25-fold respectively.

Additionally, there was a low level of release over time in the isotype and non-glycosylated rituximab samples for MIP-1α and MIP-1β as seen in Figure 5A and 5B. This is likely due to constitutive secretion of these two chemokines in the absence of an activating antibody. In both instances however, the addition of rituximab or non-fucosylated rituximab significantly increases the release over baseline levels. Figure 6 shows the fold increase over isotype antibody for rituximab and the two modified versions at the four-hour time point.

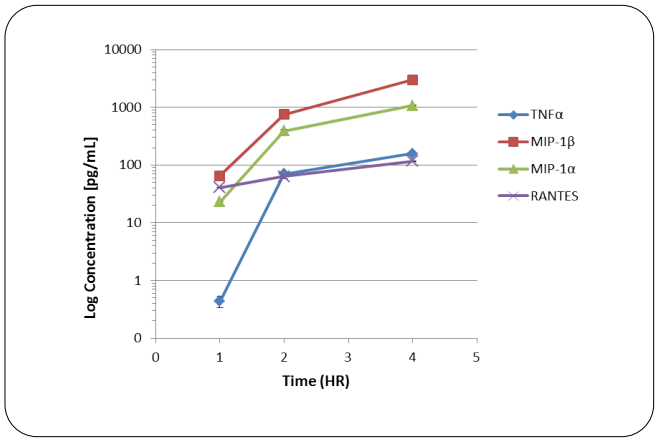


Figure 7: AlphaLISA Time Course Results. Data shown is from 1 µg/mL rituximab antibody addition and 15-to-1 effector- to-target cell ratio. Chemokine release from MIP-1β and RANTES is increased above baseline levels at one hour. MIP-1α and the cytokine TNFα are below the LDL for each assay at one hour. At the two-hour time point TNFα secretion has increased and is on par with RANTES levels while MIP-1α and MIP-1β continue to be strongly released into the supernatant by the activated NK cells.

Activation of NK cells is known to have a proinflammatory profile of chemokine and cytokine release and kinetic studies have shown a rapid release of chemokines relative to cytokine secretion.7 Figure 7 represents the 1 µg/mL rituximab response over time at the 15-to-1 effector-to-target cell ratio and shows that at one hour the secreted levels of the chemokines MIP-1α, MIP-1β and RANTES are higher than that of the cytokine TNFα. At later time points, TNFα is released at the same level as RANTES whereas MIP-1α and MIP-1β continue to rise strongly. These results support a temporal progression of chemokine followed by cytokine production and release from NK cells based on the subset of targets tested in this experiment.

Summary

We have clearly shown here that simultaneous detection of ADCC activity and release of chemokines and cytokines from activated NK cells is possible via the combined use of DELFIA TRF and AlphaLISA technologies. The DELFIA EuTDA cytotoxicity reagents performed as expected in the four-hour assay window by displaying specific release from the target cells over the relatively low level of spontaneous release in the Raji cell line. AlphaLISA showcased its sensitivity as illustrated by quantifying four of the seven targets tested from 5 µL of supernatant sample from the 200 µL total assay volume. The remaining three targets (IFNγ, MCP-1 and IL-8) were either not expressed in the fourhour assay window or perhaps were not released from NK cells activated by rituximab or non-fucosylated rituximab. Both technologies were able to discriminate between active and inactive (non-glycosylated) forms of rituximab when tested at 1 µg/mL; however only the DELFIA ADCC data displayed a difference between rituximab and the non-fucosylated antibody (Fig. 3) suggesting the modification did not affect the level of release for the chemokines and cytokines tested by AlphaLISA in this experiment at the time points sampled. The kinetics of chemokine compared to cytokine release is well studied and herein we show a progression of chemokine release (MIP-1β, and RANTES) at the early one-hour time point relative to the later release of TNFα (Figure 7). IFNγ was not detected at the four-hour time point with levels remaining below the LDL; however, it is possible it could be detected if the experiment were run longer. Spontaneous release of TDA from the target cells generally limits the DELFIA TRF EuTDA cytotoxicity assay to approximately four hours which represented the end point for this experiment. In summary, this application note demonstrates that AlphaLISA and DELFIA TRF technologies can be performed in parallel from the same supernatant to simultaneously detect cell signaling events occurring in the context of a co-culture ADCC assay.

References

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