Understanding NK cell cytotoxicity against tumor cells using flow cytometry

Immuno-oncology applications for the BD FACSCelesta™ flow cytometer

Features

- Quantitatively assess multiple indicators of targeted tumor cell death at the single-cell level
- Simultaneously assess degranulation and cytokine production of NK cells in the same panel
- Detect both cell surface and intracellular indicators of activation and toxicity
- Multiplex markers of multiple cell processes to identify the mechanisms of cytotoxicity
- Discriminate tumor cells from NK cells in co-culture

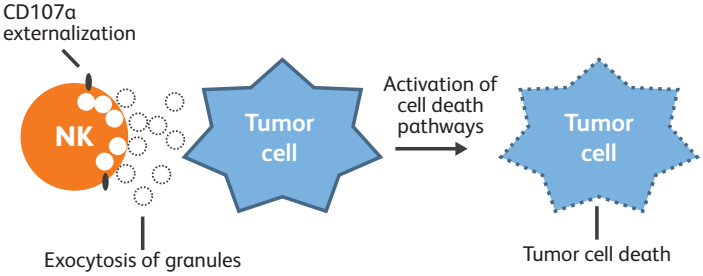

Figure 1. Model assay to evaluate NK cell activation and cytotoxicity against tumor cells

NK cells are activated and then co-cultured with tumor cells. Activation of NK cells is shown by degranulation (measured by cell surface expression of CD107a) and cytokine production and release (measured by intracellular staining). Cytotoxicity against tumor cells is shown by analysis of cell death processes including externalization of phosphatidylserine (PS, measured using BD Horizon™ BV605 Annexin V staining), caspase activation (measured using caspase probes) and loss of mitochondrial membrane potential (measured using BD Pharmingen™ MitoStatus dyes).

When cytotoxic natural killer (NK) cells are attacking tumor cells, it is important to measure both activation of the NK cells and their toxicity against the tumor cells. Conventionally, radioactive (chromium) and colorimetric (MTT or calcein) assays have been used to measure NK-mediated target cell death. However, these bulk assays measure only a single parameter at a time, thus providing only limited insights about the activation of effector cells and the mechanisms involved in the cytotoxic process.

Multicolor flow cytometry can supplement these assays by offering a more comprehensive understanding of these mechanisms, and by further illuminating NK cell function while accurately discriminating the NK cells from the target cell population. On the BD FACSCelesta™ flow cytometer, with up to three lasers and 12 fluorescence parameters, you can not only simultaneously assess NK cell activation and tumor cell death, but also, by exploring the processes and time lines involved, extract deep scientific insights from a single sample.

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The experimental model is shown in Figure 1. When NK cells are activated—by a sensitive target tumor cell line, for example—they secrete inflammatory cytokines and release their lytic granule contents (granzymes and perforin) onto the surface of the target cell. The released contents cause irreversible cellular damage, including loss of mitochondrial membrane potential ($\Delta\Psi_m$), and initiate apoptotic cell death. You can multiplex analysis of these cellular processes with the BD FACSCelesta.

Figure 2 shows a cell surface analysis of K562 chronic myelogenous leukemia cells that were cultured with activated NK cells for four hours. Before co-culturing, the K562 cells were labeled with PKH67, a green fluorescent lipophilic dye. In Figure 2A, plotting PKH67 vs side scatter (SSC) resulted in a clear separation between the K562 and NK cells, which was used to gate the cells for further analysis.

The remaining panels of Figure 2 contain the results for three different measures of cytotoxicity for the co-cultured PKH67$^+$ K562 tumor cells, compared with K562 cells cultured alone. Figure 2B, analyzing externalization of phosphatidylserine (PS) via BD Horizon™ BV605 Annexin V and BD Pharmingen™ 7-AAD, shows a progression among co-cultured cells from viable (lower left quadrant) to early apoptotic (lower right) and then to late apoptotic (upper right). Figure 2C, analyzing a live caspase probe, shows that this progression was correlated with an increase in the activity of effector caspases in co-cultured K562 cells. Finally, Figure 2D, analyzing $\Delta\Psi_m$ via BD Pharmingen™ MitoStatus Red, a dye that can be sequestered by active mitochondria, shows that co-cultured K562 cells were more likely to lose $\Delta\Psi_m$ than cells cultured alone.

All measures showed more rapid apoptosis and cell death among K562 cells co-cultured with a 4:1 ratio of NK cells than with a 1:1 ratio. Cells cultured at a 1:1 ratio demonstrated a bimodal $\Delta\Psi_m$ distribution (Figure 2D), indicating that some but not all cells have lost potential by the four-hour mark.

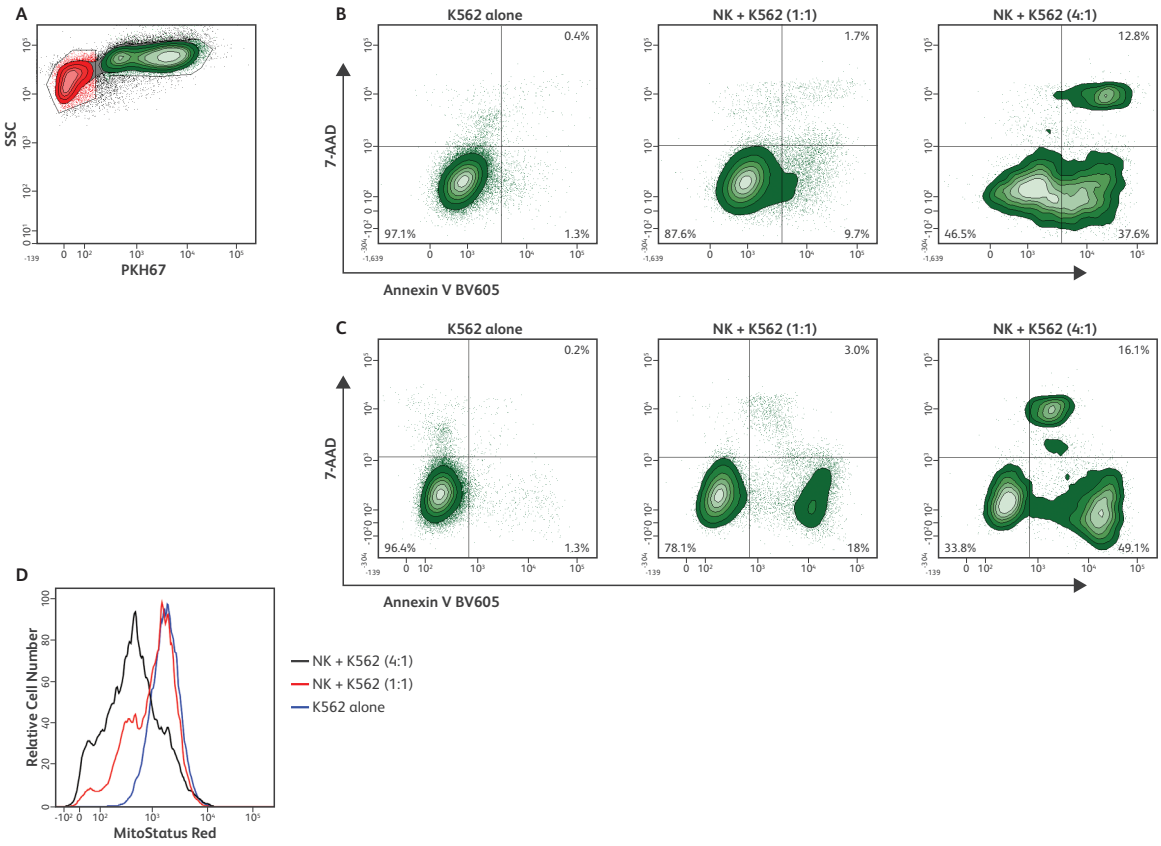

Figure 2. Analysis of K562 tumor cell death after exposure to activated NK cells

NK cells from the peripheral blood of a normal donor were purified using the BD IMag™ Human NK Cell Enrichment Set – DM (Cat. No. 557987), activated with NK Activation and Expansion Set (Miltenyi Biotec) and cultured in media with BD Pharmingen™ Recombinant Human IL-2 (Cat. No. 554603). K562 cells (human chronic myelogenous leukemia, ATCC CCL-243) were labeled with PKH67 Green Fluorescent Cell Linker (Millipore Sigma). Activated NK cells were harvested after five days, co-incubated with PKH67-labeled K562 target cells at an effector-to-target ratio of either 4:1 or 1:1 and acquired on the BD FACSCelesta Blue/Violet/Red (BVR) configuration after four hours. **Results: A.** Side scatter combined with PKH67 fluorescent signal provided a clear separation of NK cells from PKH67$^+$ K562 cells. **B.** BD Horizon BV605 Annexin V and BD Pharmingen™ 7-AAD staining (Cat. Nos. 563974 and 559925) on gated PKH67$^+$ K562 cells showed increased percentages of early apoptotic (Annexin V$^+$) and late apoptotic cells (Annexin V$^+$7-AAD$^+$) in the co-cultures with NK cells compared to K562 cells alone. **C.** BD Pharmingen™ Violet Live Cell Caspase Probe (Cat. No. 565521) detected high levels of caspase activation on intact PKH67$^+$ K562 cells, confirming that these cells were undergoing apoptosis. **D.** BD Pharmingen™ MitoStatus Red (Cat. No. 564697) was highest in PKH67$^+$ K562 cells cultured alone, signifying cell health, while K562 cells co-incubated with NK cells failed to take up the dye, indicating lost mitochondrial membrane potential. K562 cells co-cultured at a 1:1 ratio showed a bimodal distribution compared with those co-cultured at a 4:1 ratio, indicating that only a fraction of these cells lost $\Delta\Psi_m$.

Figure 3 adds cell surface and intracellular analyses of the activation of intact NK cells after four hours of co-culture. These cells, initially gated as PKH67^{-} (co-cultured cells that were not tagged as K562), were further characterized by staining with antibodies to the NK marker CD56. The cells were also labeled with a BD Horizon™ Fixable Viability Stain to identify live cells (FVS510^{-}) before fixation and permeabilization for intracellular analysis.

Figure 3A shows by cell surface analysis that, as expected, after exclusion of dead cells, more NK cells externalized CD107a in the NK-K562 co-cultures (right plot) than in the NK cultures alone (left plot). Only NK (CD56^{+}) cells externalized CD107a.

Figure 3B shows that, again as expected, intact NK cells in co-cultures released more of the inflammatory cytokine tumor necrosis factor (TNF) than unstimulated NK cells. Again, only NK (CD56^{+}) cells released TNF.

Finally, Figure 3C confirms (using intracellular staining) that the co-cultured K562 cells were more likely to progress through apoptosis and cell death than K562 cells cultured alone, as measured by caspase activation.

The use of flow cytometry to assess cytotoxicity allows you to answer important immuno-oncological questions. The BD Accuri™ C6 Plus personal flow cytometer, as explained in our companion product information sheet, can assess immune cell activation and tumor cell death simultaneously on a benchtop. By adding a third laser and additional fluorescence channels, the BD FACSCelesta can further illuminate the processes, mechanisms and time lines involved in immune cell activation and cytotoxicity against tumor cells.

Combining innovations in instrumentation with optimization for bright new reagents and BD's unparalleled service and support, the BD FACSCelesta flow cytometer is designed to help you extract a deeper level of biological information from your cell types of interest. By enabling simultaneous analysis of multiple parameters, and by supporting both cell surface and intracellular investigations, the BD FACSCelesta helps you gain new understanding and insights into immuno-oncological processes.

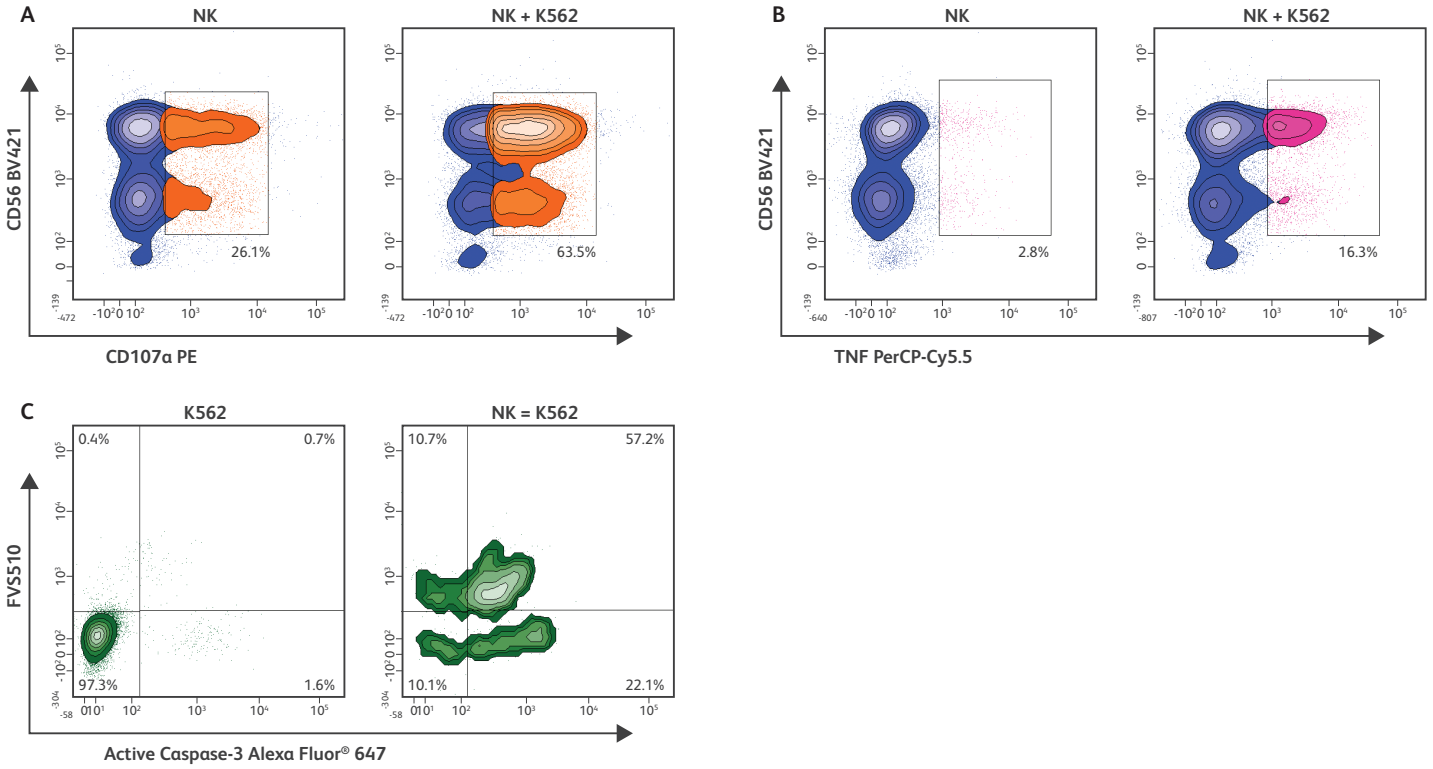

Figure 3. Cell surface and intracellular analysis of co-cultured activated NK cells and K562 tumor cells

NK cells were purified and co-incubated at a 1:1 or 4:1 ratio with PKH67-labeled K562 target cells as described in Figure 2. BD Pharmingen™ PE Mouse Anti-Human CD107a (Cat. No. 555801) was added to the culture media, as recommended in the literature, for detection of cell surface expression of CD107a. After four hours of culture, the cells were harvested and stained with BD Horizon™ BV421 Mouse Anti-Human CD56 (Cat. No. 562751), an NK cell marker. Surface stained cells were further labeled with BD Horizon™ Fixable Viability Stain 510 (Cat. No. 564406), fixed with BD Cytofix™ Fixation Buffer (Cat. No. 554655) and permeabilized with BD Perm/Wash™ Perm/Wash Buffer (Cat. 554723). The cells were then stained intracellularly with BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human TNF (Cat. No. 560679) and BD Pharmingen™ Alexa Fluor® 647 Rabbit Anti-Active Caspase-3 (Cat. No. 560626) and acquired on the BD FACSCelesta BVR configuration system. **Results: A.** Analysis of PKH67^{-} intact cells (FVS510^{-}Active Caspase-3^{-}) revealed higher percentages of CD56^{+} (NK) CD107a^{+} cells in NK-K562 co-cultures than in NK cells cultured alone. **B.** Intracellular analysis of PKH67^{-} intact cells showed increased percentages of CD56^{+} (NK) cells expressing TNF in the NK-K562 co-cultures than in NK cells alone. **C.** Intracellular analysis of active caspase-3 on PKH67^{+} K562 cells was used to simultaneously assess NK-mediated cytotoxicity in permeabilized cells. Confirming the results of Figure 2, there were more apoptotic (FVS510^{-}Active Caspase-3^{+} or FVS510^{+}Active Caspase-3^{+}) K562 tumor cells in NK-K562 co-cultures than in K562 cells alone.

Ordering information: Systems and software	
Description	Cat. No.
BD FACSCelesta™ Flow Cytometer, BVR Configuration	660344
BD FACSCelesta™ Flow Cytometer, BVYG Configuration	660345
BD FACSCelesta™ Flow Cytometer, BVUV Configuration	660346
BD FACSCelesta™ Flow Cytometer, BV Configuration	660343

Ordering information: Reagents	
Description	Cat. No.
BD IMag™ Human NK Cell Enrichment Set – DM	557987
BD Pharmingen™ Recombinant Human IL-2	554603
BD Horizon™ BV605 Annexin V	563974
BD Pharmingen™ 7-AAD	559925
BD Pharmingen™ Violet Live Cell Caspase Probe	565521
BD Pharmingen™ MitoStatus Red	564697
BD Pharmingen™ PE Mouse Anti-Human CD107a	555801
BD Horizon™ BV421 Mouse Anti-Human CD56	562751
BD Horizon™ Fixable Viability Stain 510	564406
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human TNF	560679
BD Pharmingen™ Alexa Fluor® 647 Rabbit Anti-Active Caspase-3	560626

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