Characterizing NK Cell Activation in Response to Cytokine Combinations

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Abstract

Introduction: Cancer immunotherapies provide new opportunities for novel combinations [1], with Natural Killer (NK) cells providing an ideal platform given their centrality in both innate and adaptive immune system functions [2]. Combination cytokine treatments of NK cells was evaluated for interleukin (IL) 2 and IL12, given the synergy between the two for NK cell production of IFNγ [3] - a cytokine which plays a central role in coordinating immune responses to tumors and Granzyme B - a protease for mediating apoptosis in target cells.

Materials and Methods: NK92 (ATCC) cells were first cultured in RPMI 1640 media with 20% FBS, 1% Penicillin/Streptomycin and 100 U/ml IL2. Following IL2 starvation for 12 h, cells were plated at a density of 1.5×105 cells / well in 24-well plates, then exposed over 24 h to two cytokines (0-1000 U/ml for IL2 and 0-100 U/ml for IL12). For 1000 U/ml IL2 and 10 U/ml IL12, a time-course study was pursued over 0, 1, 2, 4, 6, 8, 24, 32, 48 and 56 h. IFNγ levels in cell supernatants, and Granzyme B levels in cell-lysates were detected using ELISA (Enzyme-Linked ImmunoSorbent Assay).

Results: The NK92 cell activating combination of 1000 U/ml IL2 and 10 U/ml IL12 yielded an optimal concentration combination for maximal IFNγ production over 24 h, with ~90% of the highest IFNγ levels with less IL12 required. The time-course data suggests plateauing of IFNγ levels between 24 h and 56 h, and also suggests a rapid increase in IFNγ levels between 8 h and 24 h. In comparison to the IFNγ expression, intracellular Granzyme-B expression levels reached their plateau at ~32 h rather than 24 h under the same conditions (1000 U/ml IL2 and 10 U/ml IL12).

Discussion: Our work establishes a standardized cell-culture based assay for NK cell activation, which can be leveraged for evaluating cytokine combination therapies and also for optimizing individualized cytokine dosages for NK cell based personalized immunotherapy regimen.

Introduction and Background

Cancer immunotherapies provide new opportunities for novel combinations [1], with Natural Killer (NK) cells providing an ideal platform given their centrality in both innate and adaptive immune system functions [2]. Combination cytokine treatment of NK cells was evaluated for interleukin (IL)-12 and IL-2, given the synergy between the two for NK cell production of Interferon Gamma (IFNγ) [3] - a cytokine which plays a central role in coordinating immune responses to tumors. The expression levels of Granzyme-B were also evaluated as an outcome of IL-12 and IL-2 synergy, given the relevance of Granzyme-B in the cytotoxicity function of NK cells.

The objective of the study was to employ IL-12 and IL-2 as a prototypical cytokine combination to evaluate and quantify NK-92 cell activation on the basis of the expression levels of IFNγ and Granzyme-B. The overarching goal was the standardization of a cell-culture assay for NK cell activation, in order to evaluate new therapeutics and determine individualized cytokine dose regimens for personalized NK cell based immunotherapy.

Materials and Methods

Cell culture: NK92 (ATCC) cells were first cultured in RPMI 1640 media with glutamine (Gibco, Waltham, MA) with 20% FBS (Gibco, Waltham, MA), 1%(v/v) Penicillin/Streptomycin (Sigma, St. Louis, MO) and 100 U/ml IL-2 (R&D Systems, Minneapolis, MN) at 37 ℃ with 5% CO2. Subsequently, NK92 cells were starved i.e. cultured without IL-2 for 12 h before subjecting them to various experimental treatments. After starvation, the cells were first counted, then collected after centrifugation at 3000 rpm for 3 min. The cells were then exposed over 24 h to two cytokines at different concentration combinations i.e. 0-1000 U/ml for IL-2 (R&D Systems, Minneapolis, MN) and 0-100 U/ml for IL-12 (R&D Systems, Minneapolis, MN). The choice of these concentration ranges for IL-2 and IL-12 was based on previously published concentration ranges for these two cytokines to evaluate NK cell responses (Ye et al, 1995 and D'Anna et al, 1995). Cells were plated at a density of 3×105 cells/ml in 24-well plates for IFNγ concentration experiment and a lower density of 1×105 cells/ml for time course experiment. For Granzyme-B concentration experiment, the density was 0.5×105 cells/ml and 1×105 cells/ml for the time course experiment. Negative controls were starved NK92 cells treated with (a) deionized water alone (used to dissolve cytokines) and (b) Dulbecco's Phosphate Buffered Saline (DPBS) alone. To collect cell lysates, cells from each well of the 24 well plate were centrifuged at 3000 rpm for 3 min, washed by DPBS, centrifuged again at 3000 rpm for 3 min, lysed with 500 µl lysis buffer (150 mM NaCl, 20 mM Tris and 1% (v/v) Triton X-100) on ice for 10 min, and then centrifuged yet again at 3000 rpm 10 min for cell lysate collection. For the specific cytokine treatment concentration combination of 1000 U/ml IL-2 and 10 U/ml IL-12, a time-course study was pursued over 56 h. At the chosen time points of 0, 1, 2, 4, 6, 8, 24, 32, 48 and 56 h, both IFNγ and Granzyme-B levels in cell supernatants and cell lysates respectively were detected using ELISA (Enzyme-Linked ImmunoSorbent Assay).

ELISA: The human IFNγ ELISA kit and Granzyme-B Elisa kit were obtained from BD Biosciences (San Jose, CA) and R&D Systems (Minneapolis, MN) respectively. The ELISA kits contained an anti-human IFNγ or Granzyme-B capture antibody, a biotinylated anti-human IFNγ or Granzyme-B detection antibody, and streptavidin-conjugated horseradish peroxidase (HRP) enzyme. For each 96-well ELISA plate, the calibration curve was set up with linear regression using IFNγ or Granzyme-B standards within the concentration range of 0 - 300 pg/ml or 0 - 2500 pg/ml respectively. On the basis of this linearly regressed calibration curve, the cell-culture supernatant samples from the various experimental groups were quantified for IFNγ and Granzyme-B concentration (upon adequate dilution of the sample in order to fall within the IFNγ concentration range of 0 - 300 pg/ml and 0 - 2500 pg/ml for Granzyme-B, corresponding to the concentration analytical-measurement range of the calibration curve).

RESULTS

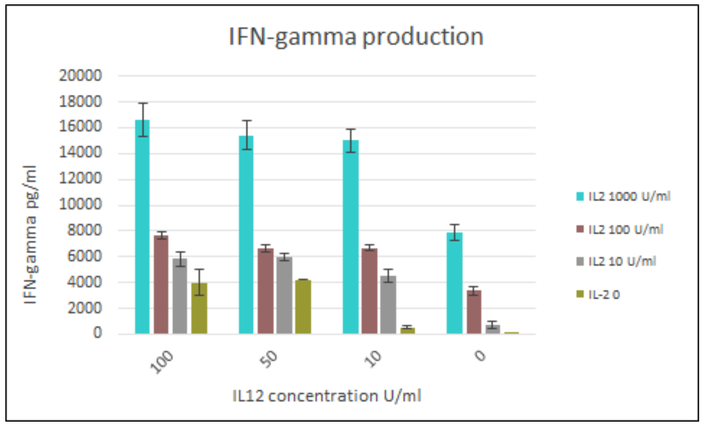

Figure 1A: NK92 cell activation (defined as IFNγ expression) upon exposure to various combinations of IL-2 and IL-12. IL-2 (1000, 100, 10, 0 U/ml), in combination with IL-12 (100, 50, 10, 0 U/ml) activated IFNγ secretion at different levels over a 24 h period. Negative controls were deionized water (used to dissolve cytokines) and DPBS, which showed little or no IFNγ secretion. Bars indicate standard error.

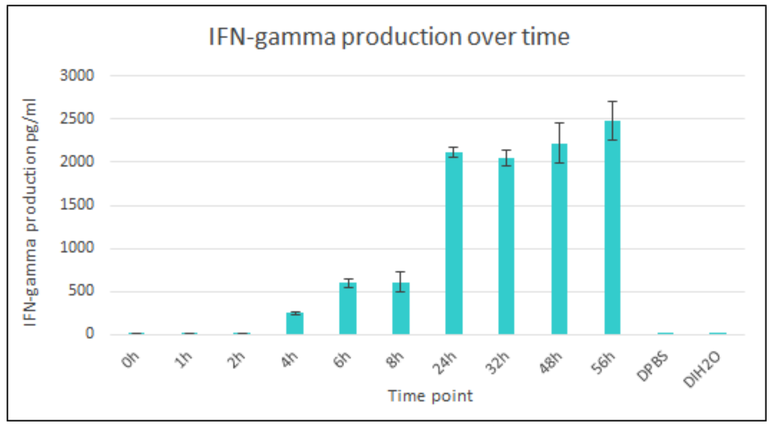

Figure 1B: NK92 cell activation (defined as IFNγ expression) upon exposure to 1000 U/ml IL-2 and 10 U/ml IL-12 over a 56 h time course. Selected time points were 0, 1, 2, 4, 6, 8, 24, 32, 48, 56 h. Negative controls were deionized water (used to dissolve cytokines) and DPBS, which showed little or no IFNγ secretion. Bars indicate standard error.

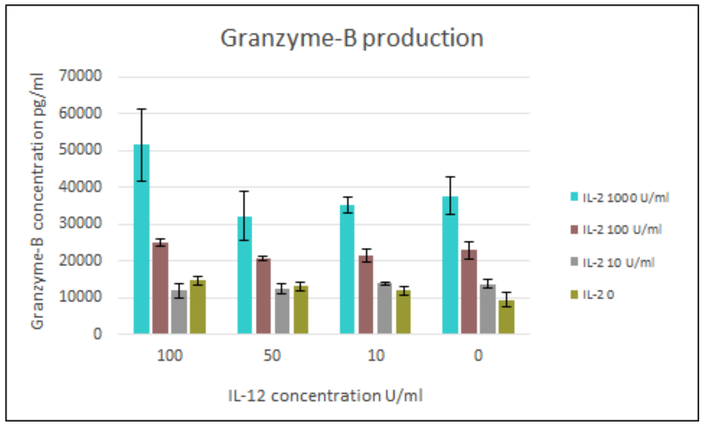

Figure 2A: NK92 cell activation (defined as intracellular Granzyme-B expression) upon exposure to various combinations of IL-2 (1000, 100, 10, 0 U/ml) with IL-12 (100, 50, 10, 0 U/ml) activated intracellular Granzyme-B expression to different levels over a 24 h period. Negative controls were deionized water (used to dissolve cytokines) and DPBS, which showed relatively low levels of Granzyme-B secretion (similar to 0 U/ml IL-2 and 0 U/ml IL-12). Bars indicate standard error.

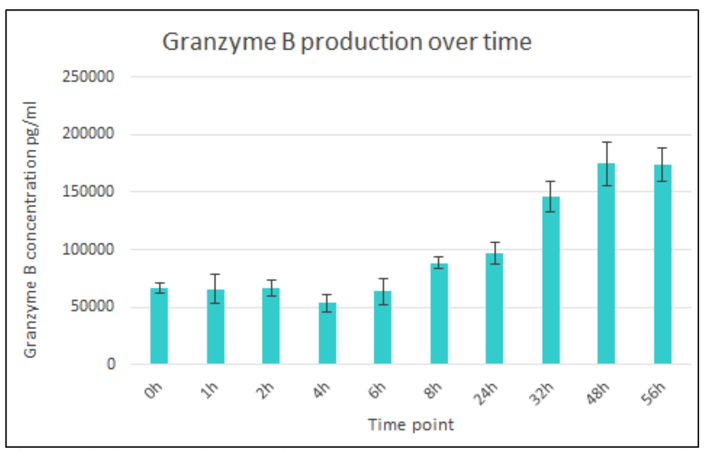

Figure 2B: NK92 cell activation (defined as intracellular Granzyme-B expression) upon exposure to 1000 U/ml IL-2 and 10 U/ml IL-12 over a 56 h time course. Selected time points were 0, 1, 2, 4, 6, 8, 24, 32, 48, 56 h. Negative controls were deionized water (used to dissolve cytokines) and DPBS, which showed relatively low levels of Granzyme-B secretion (similar to 0 U/ml IL-2 and 0 U/ml IL-12). Bars indicate standard error.

Discussion and Conclusions

A key goal of our assay development effort was the optimization of experimental conditions for the maximal activation of NK92 cells through the mechanism of IL-2 and IL-12 acting in combination and synergistically to induce NK-cell activation. For the purpose of this work, NK-92 is a very relevant cell line, given that it is the only one amongst 7 known NK cell lines to have been incorporated into human clinical trials with demonstrable benefits, and being amenable to genetic manipulation to recognize tumor antigens [4].

The choice of cytokine activators of NK cells for the purpose of this study (i.e. IL-2 and IL-12) was driven out of the centrality of these cytokines in immune response signaling, including NK cell mediated signaling as well as NK cell function. In addition, the growing profile of cytokine therapy (Adrolino et al, 2015) as well as NK cell centric therapeutic approaches (Suck, 2006) to treat cancer underlays the motivation to evaluate and quantify the synergy between IL-2 and IL-12 for NK cell activation. The quantification of the synergy between IL-2 and IL-12 for NK cell activation was based on the expression profiles of two disparate biomarkers, i.e. IFNγ and Granzyme-B. The choice of these two biomarkers spans the spectrum of the key functions of NK cells, namely direct cytotoxicity [for which Granzyme-B plays a central role [5]] as well as NK cell initiated downstream signaling to other immune cells [for which IFNγ plays a central role].

Our assay development efforts relied primarily on the quantification of the expression levels of biomarkers central to NK cell function i.e. IFNγ and Granzyme-B biomarkers with the key data observations summarized below:

- For IFNγ expression, the influence of IL-12 seems to plateau at around 10 U/ml in the presence of IL-2 (Figure 1A). At each of the three concentration levels of IL-2 evaluated (i.e. 10, 100, and 1000 U/ml), the addition of IL-12 at or above 10 U/ml only adds incrementally to IFNγ production. For example, the IFNγ production levels at the fixed IL-2 concentration of 10 U/ml is about the same when IL-12 is added at 3 different concentrations (10, 100, and 1000 U/ml). This is also true for the IFNγ production levels at the other two IL-2 concentration levels. Based on IFNγ production levels (Figure 1A), the optimal concentration combination seems to be 10 U/ml of IL-12 with 1000 U/ml of IL-2, since this combination yielded ~90% of the highest observed IFNγ levels in the study. The time course of IFNγ production levels by NK92 cells at this specific optimal concentration combination (Figure 1B) suggests a plateau at 24 hours and beyond.
- For Granzyme-B (Figure 2A), a clear dose-dependent response was observed for IL-2 but not as much for IL-12. The time course experiment (Figure 2B) indicated Granzyme B plateauing at 32 hours and beyond.

Taken collectively, these results suggest that at the optimal concentration combination of 10 U/ml of IL-12 with 1000 U/ml of IL-2, and the optimal time-point of 32 hours, both IFNγ production levels and Granzyme-B expression levels by NK-92 cells can serve as barometers i.e. positive controls for NK cell activation.

With a similar experimental approach, the optimal combinations of other cytokines (e.g. IL-15, IL-18 etc.) can be evaluated for NK cell function. These experimental results also provide the basis of an objective comparison between NK-92 cells and the autologous NK cells from a patient who might be undergoing cytokine therapy, in order to identify the individualized cytokine treatment regimen. Likewise, the relative effectiveness of therapeutics designed for influencing NK cell function could be determined in comparison to well-characterized positive controls such as IL-02 and IL-12 evaluated in this study.

In conclusion, our work establishes a standardized cell-culture based assay for NK cell activation, which can be leveraged for evaluating candidate therapeutics, cytokine combination therapies, and also for optimizing individualized cytokine dosages for NK cell based personalized immunotherapy regimen.

References
1. Morrissey et al, 2016. Clinical and translational science, 9(2), pp.89-104.
2. Guillerey et al, 2016. Targeting natural killer cells in cancer immunotherapy. Nature immunology, 17(9), pp.1025-1036.
3. Weiss et al, 2007. Expert opinion on biological therapy, 7(11), 1705-1721.
4. Klingemann, H., Boissel, L. and Toneguzzo, F., 2016. *Frontiers in immunology*, *7*.
5. Trapani, J.A. and Sutton, V.R., 2003. *Current opinion in immunology*, *15*(5), pp.533-543.
6. Showalter, A., et al, 2017. Cytokines in immunogenic cell death: Applications for cancer immunotherapy. *Cytokine*, *97*, pp.123-132.