T Cell Activation Standardization for Therapeutic Assay Development

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Abstract

Introduction: Several cancer immunotherapy strategies rely on T-cell activation [1]. While T-cell activation mechanisms are well established, there is a dearth of quantified standardization of these activation pathways. Standardization with quantified end-points [induced by known activators and pathways] allows the development of useful assays to evaluate emerging immunotherapies. Our initial work has focused on T-cell activation.

Materials and Methods: Activation of the Jurkat T-cell line was induced by the combined action of Ionomycin and PMA (phorbol 12-myristate 13-acetate) [2]. Jurkat cells were cultured in 24 well plates in RPMI 1640 media (with 10% FBS and 1% Penicillin/Streptomycin) at a density of 10^6 cells/ml. We looked at a wide concentration range for both activators (250 - 2000 ng/ml for Ionomycin and 10 - 100 ng/ml for PMA), in order to identify their most synergistic combination for T-cell activation as quantified by biomarker expression (i.e. IL-2 ELISA, CD69 Flow cytometry). The negative controls were 0.3% v/v DMSO (used for solubilizing the activators), and PBS (phosphate buffered saline). For a specific combination of 2000 ng/ml ionomycin and 50 ng/ml PMA, the designed time points were 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 32 h, 49 h and 54 h. The cells and supernatant were preserved at the end of these time-points. The IL-2 production was detected using ELISA (Enzyme-Linked ImmunoSorbent Assay). The CD69 expression was detected using Flow Cytometry.

Results: The optimal activating concentrations for PMA and Ionomycin in combination are 50 pg/ml and 2,000 ng/ml respectively. Both IL-2 production and CD69 expression showed the same trends as a function of experimental conditions. In addition to identifying the optimal activating concentrations of ionomycin and PMA, the obtained results characterized the time-course of maximal Jurkat cell activation over a 54 h period. During this 54 h period, the IL-2 levels and CD69 expression levels progressively increased before starting to plateau. The production of IL-2 started increasing at 4 h from 154.06 pg/ml and continued to go up before plateauing at the 24 h and higher time-points (the 32 h time-point displaying the maximal IL-2 production at 8,785.8 pg/ml).

Discussion and Conclusions: Our work establishes a standardized basis to determine the extent and the time-course of maximal T-cell activation through a given mechanism - in this case through the mechanism of calcium ionophore induced (Ionomycin) and phorbol ester induced (PMA) T-cell activation [3]. Such quantified standardization and maximization of T-cell activation *in turn* presents a cell-culture model (or "assay") to evaluate candidate therapeutics designed to work through the same activation pathway. In addition to T-cells, we are working on the quantified standardization of the activation of NK-cells [4] and dendritic cells [5], with activation measured by the end-points of biomarker expression profiling (ELISA / Luminex) and microscopy and flow cytometry (cell-surface marker expression profiling).

Introduction and Background

Standardization of immune cell activation and deactivation *in vitro* will be a useful tool for the evaluation of emerging cancer immunotherapies. However, despite immune activation and deactivation mechanisms being well-established, there is a dearth of standardized assays for the quantification of immune cell activation and deactivation.

The envisaged standardized cell-culture based assays with immune cells (such as T-cells, NK-cells, Dendritic cells etc.) potentially offer high robustness, low variability as well as time-efficient and sensitive endpoint quantification.

Given that several cancer immunotherapy strategies rely on T-cell activation or the prevention of T-cell deactivation [1], our initial work focused on activation induced in Jurkat cells by a combination of 2 known activators i.e. Ionomycin and PMA. We evaluated disparate activation end-points i.e. biomarker expression (Interleukin 2, IL-2) and cell-surface receptor up-regulation (CD69). On the basis of these quantified endpoints, we were able to optimize (a) the concentration combination of the two activators Ionomycin and PMA and (b) the time-course of Jurkat cell activation - therefore resulting in a standardized, robust, time-efficient and sensitive cell-culture assay for T cell activation.

Materials and Methods

Cell culture: The Jurkat human T cell-line was obtained from the University of Massachusetts (Worcester, MA). These Jurkat suspension cells were cultured in RPMI 1640 media (with 10% FBS and 1% Penicillin/Streptomycin), and passaged when they reached a density of greater than 10^6 cells/ml. For experimental evaluation, these Jurkat cells were cultured in 24 well plates at a starting density of 10^6 cells/ml and subsequently exposed to various experimental conditions including:
[A] over a 24 hour period, wide concentration ranges of two activators used in combination i.e. Ionomycin (250 - 2000 ng/ml) and PMA (phorbol 12-myristate 13-acetate, 10 - 100 ng/ml) - with the purpose of identifying the *optimal synergistic combination* of Ionomycin and PMA for T-cell activation, and
[B] the optimal combination of 2000 ng/ml Ionomycin and 50 ng/ml PMA over various time-points i.e. 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 32 h, 49 h and 54 h - with the purpose of identifying the *optimal time-point* for a *rapid yet sensitive* assay for T-cell activation

Experimental controls included: treatment with each activator by itself i.e. 2000 ng/ml Ionomycin and 100 ng/ml PMA separately, as well as the negative control treatments i.e. 0.3% v/v DMSO (used for solubilizing the activators), and DPBS (Dulbecco's phosphate buffered saline).

ELISA: The human IL-2 (Interleukin 2) ELISA ((Enzyme-Linked ImmunoSorbent Assay) kit was obtained from BD Biosciences (San Jose, CA), the ELISA kit containing an anti-human IL-2 capture antibody, a biotinylated anti-human IL-2 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 IL-2 standards within the concentration range of 7.8 - 500 pg/ml. On the basis of this linearly regressed calibration curve, the cell-culture supernatant samples from the various experimental groups were quantified for IL-2 concentration (upon adequate dilution in order to fall within the IL-2 concentration range of 7.8 - 500 pg/ml).

Flow Cytometry: The PE (phycoerythrin) conjugated mouse anti-human CD69 antibody was obtained from BD Biosciences (San Jose, CA). Cells from various experimental groups were incubated for 30 minutes with the antibody in dark, following which they were washed and then evaluated using a Becton Dickinson Accuri C6 flow cytometer, for fluorescence (excitation at 488 nm; emission at 585 nm).

RESULTS

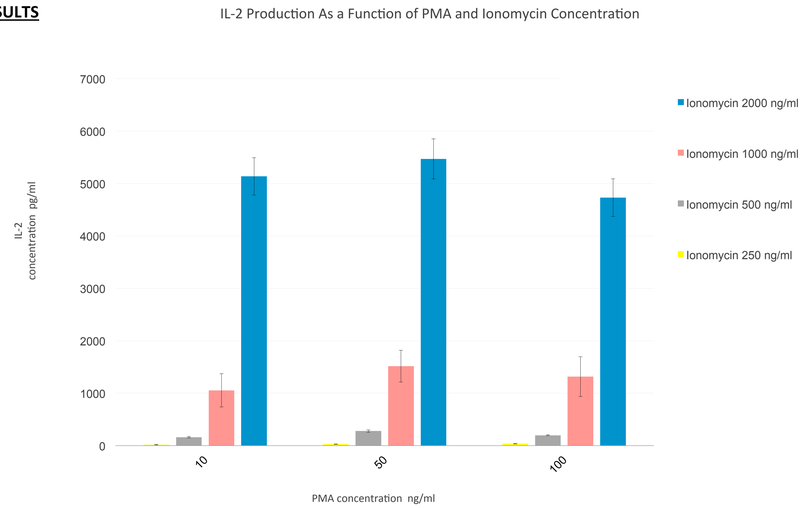

Figure 1A: Jurkat cell activation (measured by IL-2 production) under different concentration combinations of PMA and Ionomycin. PMA (10, 50, 100 ng/ml), in combination with Ionomycin (250, 500, 1000, 2000 ng/ml) activated IL-2 production to different levels after 24 h incubation. Controls (not shown) were 100 ng/ml PMA alone [IL-2 level 3.886 ± 1.709 pg/ml] and 2000 ng/ml Ionomycin [IL-2 level undetectable] alone respectively. Negative controls (not shown) were 0.3% v/v DMSO (used as solvent) [IL-2 level undetectable] and DPBS [IL-2 level undetectable]. All controls showed little or no IL-2 production. Bars indicate standard error.

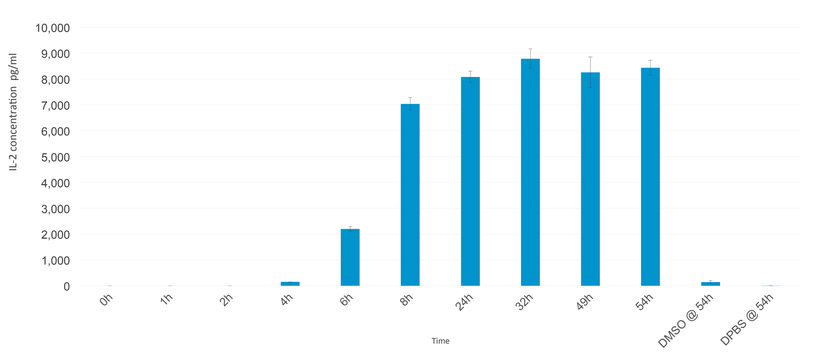

Figure 2A: IL-2 production as a function of time was measured by ELISA. 50 ng/ml PMA and 2000 ng/ml ionomycin were used to activate the Jurkat cells. The activation time points were 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 32 h, 49 h and 54 h. Negative controls were 0.3% v/v DMSO (used as solvent for PMA and Ionomycin) and DPBS treated cells for 54 h. Bars indicate standard error.

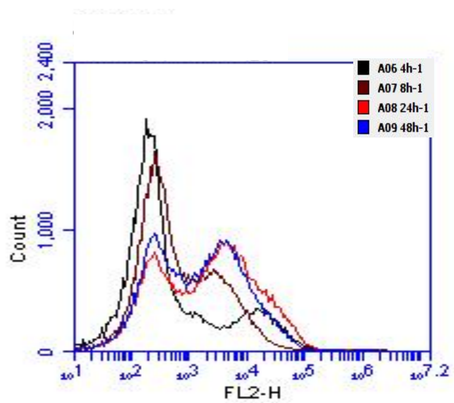

Figure 2B: CD69 expression was tested as a function of time by flow cytometry. 4 time point groups were observed with treatment of 50 ng/ml PMA in combination with 2000 ng/ml Ionomycin. In Figure 2B, the 4 groups are (i) 4 h, (ii) 8 h, (iii) 24 h and (iv) 48 h. The negative control (not shown) was non-treated cells with antibody and showed no activation effect. The CD69 expression levels for these 4 groups were consistent with the relative trends for these same 4 groups for IL-2 expression.

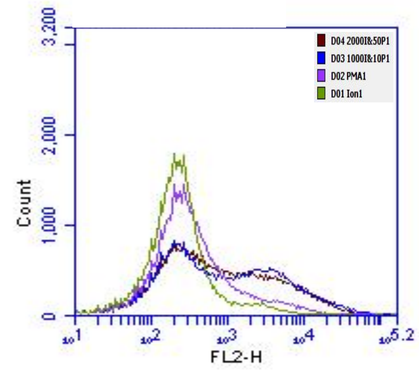

Figure 1B: CD69 expression was tested as a function of PMA and Ionomycin combination for two treatment groups observed with the highest IL-2 expression in Figure 1A i.e. (i) 50 ng/ml PMA in combination with 2,000 ng/ml Ionomycin and (ii) 10 ng/ml PMA in combination with 1,000 ng/ml Ionomycin. The control treatments were were (iii) 100 ng/ml PMA alone, and (iv) 2000 ng/ml Ionomycin alone respectively. The CD69 expression levels for these 4 treatment groups were consistent with the relative trends for these same 4 groups for IL-2 expression

Discussion and Conclusions

A key goal of our assay development effort was the optimization of experimental conditions for the maximal activation of Jurkat cells through the mechanism of calcium ionophore induced (Ionomycin) and phorbol ester induced (PMA) T-cell activation. While the combination of Ionomycin and PMA has been reported previously in the published literature [6,7] on T-cell activation, there is a dearth of published information on the *ideal* or optimized combination of these two activators to achieve T-cell activation.

Our assay development efforts relied primarily on the quantification of the expression levels of the IL-2 biomarker, and the CD69 cell surface marker, with the key data observations summarized below:

- Based on our experimental results (Figure 01A), the relative IL-2 expression levels induced by various concentration combinations of PMA and Ionomycin consistently demonstrated the highest IL-2 expression levels induced by the combination of 50 pg/ml PMA and 2,000 ng/ml Ionomycin. It was also observed that the IL-2 expression induced by PMA and Ionomycin was significantly (i.e. several-fold) higher when the Ionomycin concentration was 2,000 ng/ml rather than 1,000 ng/ml (or lower). In other words, the highest observed assay "signal window" based on IL-2 expression is potentially offered by the combination of 50 pg/ml PMA and 2,000 ng/ml Ionomycin, and that this assay "signal window" would be much smaller if the ideal concentration combination of Ionomycin and PMA was not used.
- Based on the optimal combination of 50 pg/ml PMA and 2,000 ng/ml Ionomycin, our experimental results (Figure 2A) demonstrated the 8 hour time-point consistently offering rapid yet sensitive endpoint evaluation of IL-2 biomarker expression levels. The 8 hour time-point is significantly more time-efficient compared to 24 h (and later) time points, yet at 8 hours, >80% of the maximal IL-2 biomarker expression levels (observed at 32 h) are already achieved. In other words, the 8 h time-point offers excellent balance between time-efficiency, sensitivity, and large assay "signal window" of IL-2 biomarker expression.
- Based on our experimental results (Figures 1B and 2B), the CD69 cell surface marker expression levels between various treatment groups were *consistent* with the corresponding IL-2 relative expression profiles observed with the same treatment groups illustrated in Figures 1A and 2B respectively. In other words, the flow cytometry end point measurements of CD69 cell surface marker expression levels offers robustness and redundancy *in addition to* the IL-2 end point.

Our work establishes a standardized basis to determine the extent and the time-course of maximal T-cell activation through a given mechanism, in this case through the mechanism of calcium ionophore induced (Ionomycin) and phorbol ester induced (PMA) T-cell activation [3].

In conclusion, we have successfully demonstrated the optimization of a cell-culture based *in vitro* method for the evaluation for T-cell activation via the combined calcium ionophore and phorbol ester mechanism. These results offer the potential for a standardized, robust, time-efficient and sensitive assay for the evaluation of relevant cancer immunotherapies designed to work through the same mechanism of T-cell activation. In addition to T-cells, we are working on the quantified standardization of the activation of NK-cells[4] and dendritic cells [5], with activation measured by the end-points of biomarker expression profiling (ELISA / Luminex), proliferation (MTT assay), microscopy and flow cytometry (cell-surface receptor expression profiling).

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