Journal of Immunological Methods 350 (2009) 89–96

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Quantification of IFN-γ produced by human purified NK cells following tumor cell stimulation: Comparison of three IFN-γ assays

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ARTICLE INFO

Article history:
Received 28 April 2009
Received in revised form 25 August 2009
Accepted 26 August 2009
Available online 4 September 2009

Keywords:
NK cells
IFN-γ
ELISA
Intracellular cytokine staining
ELISPOT
K562

ABSTRACT

Interferon (IFN)-γ released by natural killer (NK) cells has become a subject of major interest, given its importance in bridging the innate and adaptive immune system. Interestingly, reports concerning tumor cell stimulation of NK cells show divergent data on which stimuli induce IFN-γ production. Here, the question remains whether tumor cell recognition is sufficient to trigger IFN-γ or whether a second signal is required such as type I IFN. While IFN-γ detection methods are abundantly used with peripheral blood mononuclear cells or purified T cell fractions as responder populations, only limited data is available about comparison of these assays with purified NK cells. In this study, we assessed the relationship between stimulation of human purified resting peripheral blood NK cells with one (tumor cell or IFN-α) and two (tumor cell + IFN-α) signals by measuring IFN-γ using three different assays. We performed the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining (ICS) assay in parallel per donor and determined whether there was a correlation between these assays.

Our results show that two-signal stimulation of human resting NK cells induces significantly more IFN-γ as compared to one-signal stimulation, readily picked up by all assays. Moreover, statistical analysis points towards a positive correlation between these assays for IFN-γ produced following two-signal stimulation. Importantly, we show that tumor cell stimulation alone is enough to trigger secretion of IFN-γ, but this finding was only evidenced by ELISPOT. These results reveal that the choice of IFN-γ detection method can markedly influence the outcome regarding induction of NK cell IFN-γ by tumor cells.

1. Introduction

Interferons (IFNs) are key effector cytokines involved in innate and adaptive immune responses. Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) have emerged as central regulators in promoting antitumor immunity (extensively reviewed by Brassard et al., 2002; Dunn et al., 2006; Ikeda et al., 2002). IFN-γ is predominantly produced by CD4 and CD8 T cells, NKT cells and NK cells. Quantification of IFN-γ expression by T cells is a well-established surrogate test for assessing cellular-mediated immune responses, for example for monitoring T cell responses in cancer vaccination trials (review Clay et al., 2001). In addition, IFN-γ released by NK cells has recently been studied intensively, since its importance in shaping both innate and adaptive immunity has recently been invigorated by numerous reports (Ikeda et al., 2002; Strowig et al., 2008; Wallace and Smyth, 2005). Several groups (Costello, 2009; Ferlazzo and Munz, 2004; Lion et al., 2009; Mailliard et al., 2003; Strowig et al., 2008; Wallace and Smyth, 2005; Walzer et al., 2005; Zamai et al., 2007; Smits et al., in press) postulated that NK cells should be harnessed

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doi:10.1016/j.jim.2009.08.014

for immunotherapy not only because of their cytolytic functions towards tumor cells, but also for their ability to mobilize and polarize adaptive immune responses via dendritic cells (DC), thereby bridging innate and adaptive immune pathways. Noteworthy, it has been put forward that evaluation and monitoring of NK cell responses in DC-based immune/vaccine therapies is promising and could prove useful in predicting clinical outcome (Menard et al., 2009; Osada et al., 2007; Strowig et al., 2008; Zamai et al., 2007).

Several IFN-γ detection methods have been developed and are commercially available, all characterized by specific discriminating features. In particular the enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining (ICS) assay are in widespread use. In ELISA, bulk cytokine secretion is quantified in the cell culture supernatant. In general, this assay uses antibodies for capture and detection of the cytokine of interest following measurement of the release of a substrate's color reaction product (Cox et al., 2006). Because of its bulk detection nature, it renders no information about individual cells and cannot be used to enumerate reactive cells (Clay et al., 2001). The ELISPOT assay is mainly based on the traditional ELISA technique, but rather than measuring the amount of cytokine released in supernatant, the ELISPOT assay relies on capturing the substrate's color reaction product on a solid surface, using immobilized antibody, as the 'footprint' of the cytokines secreted by individual cells. In this way, ELISPOT allows the quantitative measurement of the frequency of cytokine-secreting cells at the single-cell level (Cox et al., 2006). However, the exact phenotype of the cytokine-secreting cells cannot be determined and the ELISPOT response can therefore not be ascribed to one single cell type (Desombere et al., 2005). With ICS followed by flow cytometry, produced cytokines are retained in the cell by the use of Golgi complex inhibitors. After fixing and permeabilizing the cells, both the intracellularly trapped cytokines and membrane markers can be stained with fluorochrome-labeled monoclonal antibodies for flow cytometric determination. In this way, ICS has the major advantage of a highly multiparametric read-out that allows for precise phenotyping of the responding cell populations and subset analysis (Maecker et al., 2005).

Since these assays show distinct differences as to when and how IFN-γ is captured, it is important to evaluate whether their results can be correlated. Reports on the comparison of these IFN-γ detection methods are abundant, albeit using total PBMC or purified T cell fractions (Asemissen et al., 2001; Karlsson et al., 2003; Letsch and Scheibenbogen, 2003; Sun et al., 2003; Tassignon et al., 2005; Whiteside et al., 2003) as responder populations. However, scarce information is available about the use of these assays with purified resting peripheral blood NK cells. Previous reports of our group (Lion et al., 2009) and others (Mailliard et al., 2003) have demonstrated that purified resting NK cells can produce high levels of IFN-γ *in vitro* upon two-signal stimulation with specific tumor cells in the presence of IFN-α. These results were all obtained with the IFN-γ ELISA method. However, our own observations as well as reports by other groups show that NK cells are able to already produce IFN-γ when stimulated with tumor cells only (Alter et al., 2004; Anfossi et al., 2006). In these experiments IFN-γ production was measured by ICS and NK cells were examined in total PBMC populations. Taken together, these data seem contradictory as to the type of stimulus needed to trigger NK cell IFN-γ production. Therefore, sensitive detection of secreted cytokines is advocated on defined cell populations. In order to evaluate secreted NK cell IFN-γ at a single-cell level, the IFN-γ ELISPOT assay can be applied. This assay is known to be highly sensitive and should therefore be able to demonstrate minor differences in IFN-γ secretion upon one- or two-signal stimulation.

To our knowledge, this is the first report on the direct comparison of ELISA, ICS and ELISPOT using purified peripheral blood resting NK cells. In this study, we compared ELISA, ICS and ELISPOT using purified resting (i.e., no preactivation with cytokines or other stimuli) NK cells. The assays were performed in batch using NK cells purified from frozen PBMC from thirteen healthy donors. We studied the IFN-γ-producing capacity of NK cells in short-term cultures in the presence of K562 cells with or without addition of IFN-α and determined whether there was a correlation between the three assays for one- or two-signal stimulation.

2. Materials and methods

2.1. Purification of human NK cells

Peripheral blood of thirteen healthy donors was obtained from buffy coats kindly provided by the Antwerp Blood Transfusion Centre. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus gradient separation (Amersham Biosciences, Uppsala, Sweden), frozen in 90% fetal bovine serum (FBS; Perbio Science, Erembodegem, Belgium) supplemented with 10% dimethyl sulphoxide (DMSO; Sigma-Aldrich, Steinheim, Germany) and stored at −80 °C. Purified resting CD56 + CD3-NK cells were obtained from frozen PBMC by using the human negative selection NK cell isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Routinely, a purity of 94 ± 2 % viable CD56 + CD3-NK cells was obtained, without contamination by CD3+ T cells.

2.2. Source of cell lines

The human NK-sensitive K562 cell line (chronic myelogenous leukemia in blast crisis) was obtained from the American Type Culture Collection (Rockville, MD, USA). K562 cells were cultured in complete medium consisting of Iscove's modified Dulbecco's medium (IMDM; Lonza, Verviers, Belgium) with L-glutamine (584 mg/L) and 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES; 25 mM) supplemented with gentamicin (10 mg/L; Invitrogen, Merelbeke, Belgium), amphotericin B (1 mg/L Fungizone; Invitrogen) and 10% FBS (Perbio Science). Cells were maintained in logarithmic phase growth at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

2.3. Cocultures with purified resting NK cells

Three IFN-γ detection techniques – ELISA, ICS and ELISPOT – were performed in parallel at the same time for each donor. For all assays, purified NK cells were cultured in IMDM supplemented with 10% FBS and stimulated at 37 °C with IFN-α (1000 IU/mL; Biosource, Nivelles, Belgium), K562 cells at