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Different combinations of cytokines and activating receptor stimuli are required for human natural killer cell functional diversity

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ABSTRACT

Although cytokine induced NK cell activation protocols are commonly used in many laboratories worldwide, a systematic study of the effect of different cytokines either alone or in combination on NK cell function is lacking.

In this study we performed a comparative evaluation of several cytokines potentially important for NK cell stimulation, focusing particularly on IL21 because of its promising role in anti-tumor therapy. To simulate *in vivo* physiological condition, we evaluated cytokine stimulation in total peripheral blood mononuclear cells (PBMCs), as accessory cells are responsible for the secretion of many soluble factors and can simultaneously trigger multiple activation signals through engagement of NK cell activating receptors. We show here that NK cell responses are finely regulated by several incoming stimuli and that combinations of IL21 + IL2 or IL21 + IL15 strongly induced NK cell function. Cytokine stimulation combined with NK receptor engagement can be helpful in the dissection of NK cell responses in health and disease.

1. Introduction

The ability of NK cells to control tumors and viral infections largely depends on their capacity to respond to the cytokine milieu and to the engagement of different receptors which induce the production of various cytokines, including interferon gamma (IFNγ) and tumor necrosis factor alfa (TNFα) and the activation of the cytolytic pathway. IL2, IL12, IL15, IL18, IL21 and type I IFNs are the cytokines that regulate NK activity [1–3], as they are recognized as powerful activators of NK cell function. Among these, IL21 has been described [4] as an important modulator of NK cell function, being able to enhance cytotoxic activity and proliferation [4] as well as antibody-dependent cell-mediated cytotoxicity (ADCC) [5]. A synergistic interaction between IL21 and IL15 has also been shown both for IFNγ production *in vitro* [6] and in a murine model of cytotoxicity *in vivo* [7,8]. Moreover, a combination of IL21 and IL15 or IL2 have synergistic effects on NK cell proliferation, IFNγ secretion and cytotoxicity against K562 cells [9]. Because of these characteristics, IL21 has been hypothesized to have antitumor activity and, indeed, different studies confirmed the potent effect of IL21 as antitumor agent in animal models *in vivo* [10–13].

In addition to interleukin induced activation, NK cell activity is regulated by a balance between signals received by inhibitory and activating receptors. NKp46, NKp30 and NKp44 (Natural Cytotoxic Receptors, NCRs) and NKG2D are the main activating receptors involved in recognition and killing of tumor or virus infected cells [14]. NK cells also express Fcγ receptor III (CD16) that enables them to exert ADCC [15,16]. NCRs are known to mediate cytotoxicity to a variety of tumor target cells but also to pathogen-specific antigens and they play a crucial role by activating NK cells in the absence of additional stimuli [14].

Although different studies have demonstrated the role of IL21 in the activation of sorted NK cells [5,17–19], only few have analyzed its effect on total human PBMC and none have compared its activity after engagement of NCRs.

In view of the increasingly recognized biological importance of IL21 as a tumor-controlling agent, we compared different NK activation protocols based on IL21 stimulation of NK cells either alone or in combination with IL2, IL12 or IL15, before or after engagement of different NK receptors (NKp46, Fcγ RIII, NKp30, NKG2D).

We show here that optimal cytokine production is strongly induced by IL21 + IL15 combination, while IL21 + IL2 exerts a synergistic effect on degranulation. Moreover, we show that TNFα and IFNγ production are differentially secreted by $CD56^{dim}$ and $CD56^{bright}$ NK cells after CD16 or NKp46 receptor engagement.

Abbreviations: PBMCs, peripheral blood mononuclear cells; NK, natural killer cells; IL, interleukin; ADCC, antibody-dependent cell-mediated cytotoxicity; FITC, fluorescein isothiacyanate; APC, allophycocyanin; PE, phycoerythrin; PC5, Phycoerythrincyanin 5; mAbs, monoclonal antibodies; MFI, mean fluorescence intensity; IFNγ, interferon gamma; TNFα, tumor necrosis factor alfa.

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2. Materials and methods

2.1. Cells and culture conditions

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by standard Ficoll gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden). After centrifugation, PBMC were washed with RPMI 1640 media (RPMI 1640, CellGrow) containing 10% heat-inactivated fetal bovine serum (FBS, HyClone), resuspended in 90% FBS + 10% DMSO solution on ice at a cell concentration $<10^7$/ml and frozen in liquid nitrogen after intermediate cooling at −80 °C in a Mr. Frosty device. The viability of PBMCs after thawing was consistently higher than 85%, as evaluated by flow cytometry with standard propidium iodide incorporation. PBMC were cultured at 10^6/ml in complete medium supplemented with 10% FBS, L-glutamine 2 mM (Gibco), Penicillin G sodium 100 U/ml and Streptomicyn sulfate 100 mg/ml (Gibco). Optimal cytokine concentrations for PBMC activation were as follows: IL2 100 U/ml, IL12 0.5 ng/ml, IL21 100 ng/ml (Biosource), IL15 20 ng/ml. Resting cells were resuspended in complete medium without cytokines. PBMC were incubated for 24–48 h with various cytokine combination. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board and Ethical Committee of Fondazione IRCCS Policlinico San Matteo. The human erythroleukemia cell line K562 and the mouse mastocytoma cell line P815 were maintained in RPMI 1640 or in DMEM 4.5% glucose, respectively, supplemented with 2 mM L-glutamine and 10% FBS.

2.2. Monoclonal antibodies and reagents

The following monoclonal antibodies were used for flow cytometry analysis, redirecting assay and plate coating: anti-CD56 R-phycoeritrin-Cyanin 5.1 (PC5) (mouse IgG1, clone N901, Beckman-Coulter) anti-CD16-AlloPhycoCyanin (APC) (mouse IgM, clone VEP-13, Miltenyi), anti-CD3-Fluorescein IsoThioCyanate (mouse IgG2b, clone HIT3a, BD), anti- IFNγ-APC (mouse IgG2b, clone 25723.11, BD) anti-TNFα APC (mouse IgG1, clone Mab11, BD), anti-CD19-APC (mouse IgG1, clone HIB19, BD) anti-CD20-PhycoErithrin (PE) (mouse IgG2b, clone 2H7, BD), anti-CD107a-PE (mouse IgG1, clone H4A3, BD) anti-CD16 purified (IgM, CLONE MEM168, EXBIO). The following purified monoclonal antibodies were a kind gift of Dr. A. Moretta (University of Genoa, Genoa, Italy): anti-NKp46 (mouse IgM, clone KL247), anti-NKp46 (mouse IgG1, clone BAB281), anti NKp30 (mouse IgG1 clone AZ20), anti-NKG2D (mouse IgG1, clone ON72).

2.3. Coating

Flat-bottom 96-well plates were coated with 10 μg/ml CD16 IgM antibody or 2 μg/ml NKp46 IgM antibody in cold PBS at 4 °C

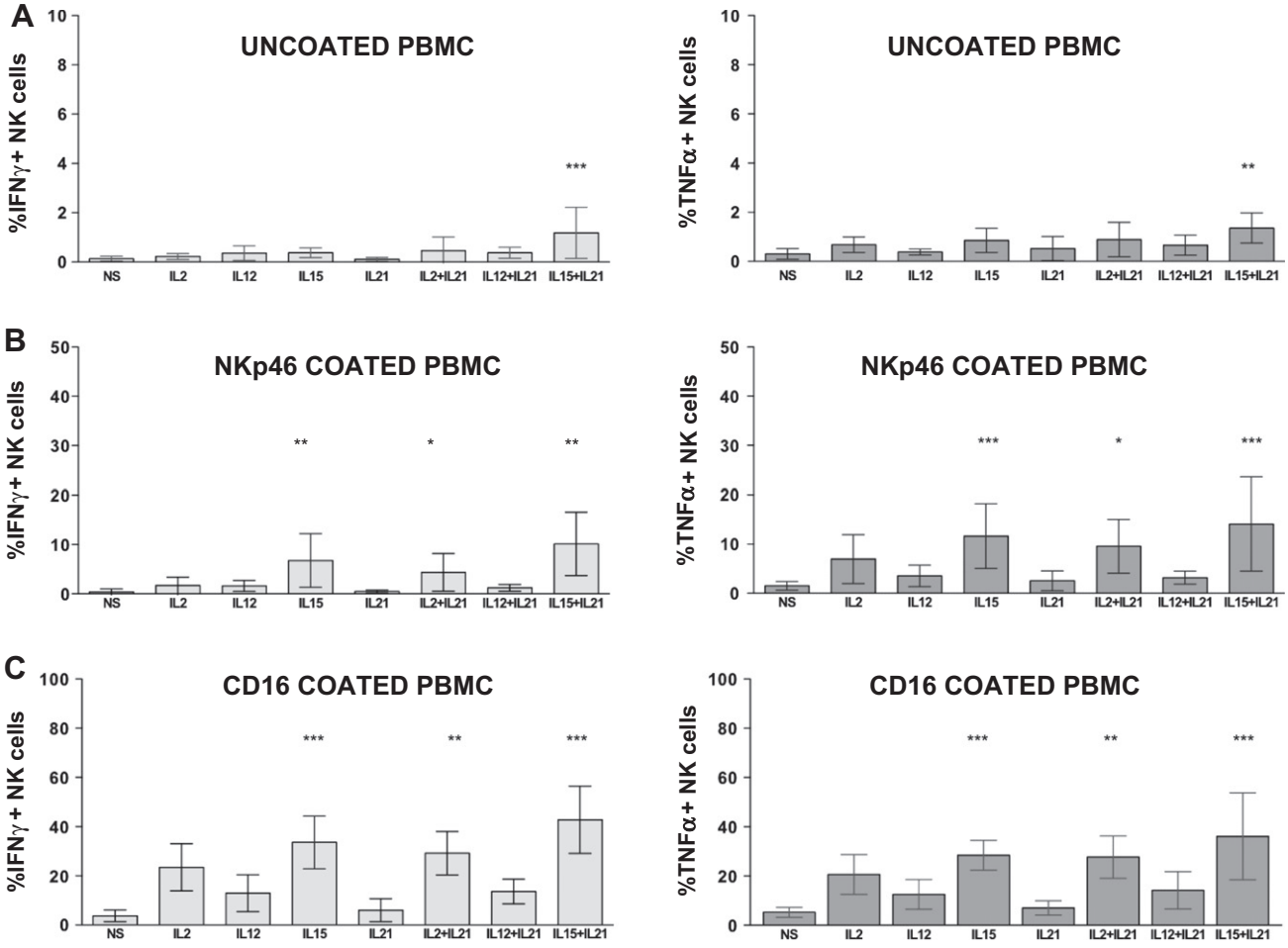

Fig. 1. Different NK stimulation protocols influence cytokine production. Different experimental conditions showed that maximal NK cytokine production was obtained using a combination of IL21 + IL15. PBMC were cultured in uncoated (A) and in NKp46- (B) or CD16- (C) coated wells. Asterisks indicate statistical significance compared to unstimulated (NS) NK cells. Data were analyzed by the Friedman test followed by Dunn's multiple comparison test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.