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When and How to Evaluate the Natural Killer Cell Function

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Authors' contributions

This work was developed in collaboration by the both authors, who contributed equally to the literature review and writing of the manuscript. Both authors read and approved the final manuscript.

Review Article

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ABSTRACT

NK cell dysfunction is observed in a variety of physiological and pathological conditions and cytotoxic assays allow to evaluating in vitro the lytic activity of NK cells against tumors or transformed target cells. Since the earliest cytotoxic tests based on the direct visualization of effector/target cell conjugates and the use of trypan blue to exclude non-viable target cells using a light microscope, a variety of cytotoxic assays have been developed. The 51chromium release assay was the most widely used for a long time, although it has several significant drawbacks, the major disadvantage being the use of radioactive compounds. To overcome this problem, several non-radioactive methods have been described, but none is broadly accepted. Among them, flow cytometry has a potential for providing information about the ability of the NK cells to lyse their targets. We review the clinical conditions associated with NK cell dysfunction as well as the role of the NK cells in immunotherapy and describe the available assays for measuring the activity of NK-cells with emphasis on flow cytometry.

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Keywords: Natural killer cells; natural killer cell function; natural killer cell based immunotherapy; cytotoxicity assays; flow cytometry.

ABBREVIATIONS

7-AAD: 7- amino-actinomycin D; ADCC: Antibody dependent cell citoxicity; AM: Aceto-methyl esters; BCECF: 2':7'-bis(carboxyethyl)-5:6-carboxyfluorescein; CAM: Calcein acetoxymethyl ester; 51Cr: 51chromium; CCA: Colorimetric cytotoxic assay; CDCC: Complement dependent cell citoxicity; CFSE: carboxyfluorescein succinimidyl ester; CTL: Cytotoxic T lymphocytes; CRA: Chromium release cytotoxic assay; CTO: Cell Tracker Orange; DiO or DiOC18: Dioctadecyloxacarbocyanine; E/T ratio: Effector / target cells ratio; EGFP; Enhanced green fluorescent protein; ERA: Europium release assay; F18: Octadecylamine-fluorescein isothiocyanate; FCCA: Flow cytometry cytotoxic assay; FHL: Familial hemophagocytic lymphohistiocytosis; FITC: Fluorescein isothiocyanate; FSC: Forward light scatter; GFP: Green fluorescent protein; GvHD: Graft-versus-host disease; HSC: Hematopoietic stem cells; HSCT: Hematopoietic stem cell transplantation; IPSC: Induced pluripotent stem cells; HLA: Human Leukocyte Antigen; HL: Hemophagocytic lymphohistiocytosis; HS: Hemophagocytic syndrome; IFN: Interferon; IL: Interleukin; KIR: Killer cell immunoglobulin like receptors; KLR: Killer cell lectin type receptors; LAK: Lymphokine activated killer; LAMP-1, Lysosome-associated membrane protein 1 (CD107a); LGL: Large granular lymphocytes; LU: Lytic units; mAb: Monoclonal antibody; MCA: Modified micro cytotoxicity assay; MHC: Major Histocompatibility Complex; MTG-FM: MitoTracker Green FM; MTT: 3-(4:5-dimethylthiazol-2-yl)-2:5-diphenyl tetrazolium bromide; NCR: Natural cytotoxic receptors; NK: Natural killer; PBMC: Peripheral blood mononuclear cells; PHA: Phytohemaglutinin; PI: Propidium iodide; SSC: Side light scatter.

1. INTRODUCTION

Natural killer (NK) cells have long been regarded as an important component of immune response based on their cytotoxic activity against virus-infected and tumor cells [1-3], thereby playing an important role in various clinical conditions [4]. They are identified as CD3- and CD16/CD56+ large granular lymphocytes (LGL) and they kill the targets using either perforin-granzyme or Fas-Fas ligand based mechanisms, both pathways leading to activation of caspase cascades responsible for target cell apoptosis [5,6]. The lytic process is characterized by the binding of the NK cell to their targets, target cell recognition, activation of the lytic machinery and target cell dead. Physical interaction between NK cells and their targets is a critical event and the machinery that controls NK cell behavior is much more complex than it was thought before, being now evident that the NK activity is tuned by the opposing activity of stimulatory and inhibitory receptors [7,8].

Different approaches have been used to directly or indirectly evaluate NK cells, using both *Ex vivo* and *In vitro* studies, and analyzing various parameters [9-11]. The accurate measure of cytotoxic function is critical to investigate a number of immunodeficiency states, infections and cancer and to evaluate vaccine and immunotherapy effects. New techniques for measuring NK cytotoxic activity by flow-cytometry have been developed, offering an alternative to the standard chromium (51Cr) release assay [12,13].

We review the clinical conditions associated with NK cell dysfunction as well as their role in cell based and antibody based immunotherapies, and describe the available methods for measuring the cytotoxic activity of NK-cells with emphasis on flow cytometry.

2. CLINICAL CONDITIONS ASSOCIATED WITH NK CELL DYSFUNCTION

NK-cell cytotoxicity is impaired in various primary and secondary immunodeficiencies, with or without a decrease in relative and absolute NK cell counts [14-17]. Of the inherited causes, most occur as part of a more generalized immunodeficiency syndrome and involve abnormalities of genes codifying for proteins important in cytolysis, whereby perforin and granzymes are delivered to induce target cell apoptosis (Table 1) [18-30].

Table 1. Primary immunodeficiency diseases that affect NK cell function

Disease	Genes	NK cell number	NK cell cytotoxicity	Ref.
Familial hemophagocytic lymphohistiocytosis	PFP1, UNC13D	Normal	Decreased	[18]
Chediak– Higashi syndrome	LYST	Normal	Decreased[a]	[19,20]
Griscelli syndrome	RAB27A	Normal	Decreased[b]	[21-23]
X-linked lymphoproliferative syndrome	SH2D1A	Normal	Decreased[c]	[24]
NEMO deficiency	IKBKG	Normal	Decreased[b]	[25]
Bare lymphocyte syndrome	TAP1, TAP2	Normal	Decreased[a,d]	[26]
Leukocyte adhesion deficiency-I	ITGB2	Augmented	Decreased[e]	[27]
Wiskott–Aldrich syndrome	WASP	Augmented	Decreased	[28]
Severe combined immunodeficiency	IL2RG, JAK3, ADA	Decreased	Decreased	[29][30]

Adapted from [4]. Abbreviations: ADCC, Antibody dependent cell cytotoxicity. a: ADCC / CD16 mediated cell killing is also impaired; b: ADCC/CD16 mediated cell killing is normal; c: NK-cell mediated cytotoxicity was induced with anti-2B4 antibodies; d: NK-cell mediated cytotoxicity is partially restored by IL-2 stimulation; e: NK-cell mediated cytotoxicity was induced with anti-DNAM antibodies

Hemophagocytic syndrome (HS) or hemophagocytic lymphohistiocytosis (HL) comprises a heterogeneous group of disorders characterized by deregulated T- and NK-cell activation causing an aberrant cytokine release that results in uncontrolled macrophage proliferation and organ infiltration by activated histiocytes, ultimately leading to multiple organ dysfunctions. Common features include fever, splenomegaly, cytopenias, hemophagocytosis in the bone marrow, spleen, and/or lymph nodes, liver dysfunction, coagulopathy, hypofibrinogenemia, high lactate dehydrogenase, hypertriglyceridemia, high serum ferritin and CD25 levels and absent or low NK-cell activity [31,32]. It can be either primary, i.e. due to an underlying genetic defect, or secondary, associated with malignancies, autoimmune diseases or infections. Infectious agents are frequently herpes viruses, the Epstein Barr virus being the most common [33].

The primary form of HL, also known as familial hemophagocytic lymphohistiocytosis (FHL) is an autosomal recessive disorder [34]. Five disease subtypes (FHL1, FHL2, FHL3, FHL4 and FHL5) were described, mutations of the perforin (PRF1; MIM170280) or of the cytotoxic proteins MUNC13-4, Syntaxin 11 and MUNC18-2 genes defining the FHL2, FHL3 FHL4 and FHL5 subtypes, respectively [25,35-37]. Related autosomal recessive defects of secretory cytotoxic lysosomes, including LYST 1 (Chediak-Higashi syndrome), Rab27A (Griscelli syndrome), and X-linked lymphoproliferative disorder also carry a high risk of HL, as the

defective proteins also participate in the events occurring at the NK cell immunological synapse [25,27,28,38,39]. Clinical manifestations usually arise during infancy and the prognosis is usually fatal, whereas that for secondary HL is reported to vary. FHL is difficult to diagnose when the patient is a first child or lacks an affected sibling. However, several clinical findings can support the diagnosis, including an age of less than 2 years, central nervous system involvement, and defective NK cell activity, which has been observed in a majority of FHL patients, although the number of circulating NK cells is normal [40].

Although most patients with HL have defective NK cell cytotoxicity using a 4-hour NK cytotoxicity test against K562 targets, the defect is heterogeneous and in some cases it can be restored by increasing the incubation time and/or by stimulating NK-cells with mitogens, such as phytohemaglutinin (PHA), or interleukins (IL), such as IL-2. Thus, Schneider et al. proposed to classify the cytotoxicity deficiency in four subtypes: Type 1, if the NK function can be restored either by stimulating NK cells with PHA (but not with IL-2) or by prolonging the incubation time from 4 to 16 hours; Type 2, if the recovery is achieved only by adding IL-2, i.e., lymphocytes with and without PHA stimulation in vitro mediated-lysis at 4h and 16h showed low values, but lymphokine activated killer (LAK) cells generated in vitro showed normal lysis rates of K562 cells in 4 and 16 hours killing assays; type 4, if it can be increased only by prolonging the incubation time, i.e., cytolytic activity with and without stimulation of PHA and IL-2 is low or absent as determined in the 4 hours killing assay, but normal in the 16 hours assay; and, type 3, if they have a lack of cellular cytotoxicity independently of assay variations, i.e., cellular cytotoxicity was absent, and neither PHA or IL-2 stimulation nor prolongation of the incubation time of effector and target cells could restore the deficient cytolytic activity [41].

Finding a low or absent NK cell cytotoxicity in patients with HL is a high-risk indicator and type 3 patients have a poor prognosis [42,43]. Comparison of NK cell activity among the FHL2, FHL3, and non-FHL2/FHL3 subtypes did not reveal significant differences, as most of the cases have deficient or low cytotoxic activity [18]. However, FHL2 patients showed any recovery after chemotherapy, in contrast to the FHL3 and non-FHL2/FHL3 subgroups in which a partial recovery may be observed [18]. Moreover, in secondary HL, NK cell activity has been reported to normalize during remission [18,43]. Thus, recognition of the cytotoxic abnormalities associated with HL may be clinically helpful.

Decreased NK cell mediated cytotoxicity has been also observed in autoimmune disorders, such as systemic lupus erythematous [44], rheumatoid arthritis [45] and Sjögren's syndrome [46]. A depression of NK cell function has also been described in patients with disseminated infections [47] and advanced cancer [48]. Pregnant women, especially those in a late stage of gestation, have been shown to have low NK cytotoxicity [49,50] and this state prolongs during the post-partum [51]. Other pathological conditions associated with diminished NK cell cytotoxicity include the chronic fatigue syndrome [52] and the obesity states [53].

In contrast, an enhanced NK cell activity has been observed during viral infections [54,55]. Moreover, women with recurrent spontaneous abortion have both higher numbers of activated circulating NK-cells [56] and high levels of NK cell cytotoxicity [57], although these parameters apparently do not correlate to each other [58]. In addition, various cytokines, including IL-2, IL-12, IL-15, IL-21 and interferons do augment the cytotoxic activity both *In vitro* [59-63] and *In vivo* [64,65], with therapeutic implications [66]. Drugs that modulate NK cell function offer therapeutic options in various clinical conditions and some methods have been proposed for NK cell assays in immunotoxicity testing and drug evaluation [67,68].

Previous studies have indicated that fetal and neonatal cytotoxic activity is lower, whereas that observed in children is similar, comparatively to that observed in adults [69-71]. There are conflicting data on the functional activity of NK cells during ageing, which was reported to remain unchanged [72,73], to decrease [74-76] or to increase [77]. Other factors that may influence the NK-cell activity are the nutritional and the feeding status [78-80], the smoke [81,82], the circadian rhythm [83] and the stress [84,85].

3. NATURAL KILLER CELLS IN IMMUNOTHERAPY

In recent years, antibody- and cell-based therapies offered alternatives for the treatment of patients with cancer, being now recognized in addition to surgery, chemotherapy, radiotherapy and other conventional treatments [86]. Cells used for immunotherapy include dendritic cells, cytotoxic T lymphocytes (CTL), and NK cells [86].

Natural killer cells discriminate between healthy self cells from those that are transformed or infected by a delicate balance of inhibitory and activating signals [87]. Killer cell immunoglobulin like (KIR) and lectin type (KLR) receptors act as inhibitory receptors for the NK cells by interacting with self Major Histocompatibility Complex (MHC) class I molecules, and tumor and viral infected cells that express low levels of these molecules are the ideal targets for NK cells [88]. Activation molecules on NK cells include the natural cytotoxicity receptors (NCR), NKp46 (CD335), NKp44 (CD336), and NKp30 (CD337), as well as NKG2D (CD314) and some activating co-receptors such as 2B4 (CD244) and DNAM-1 (CD226) [89]. Stress-inducible molecules (e.g. MICA, a MHC class I homolog) that acts as ligands for activating receptors), are frequently expressed in tumor cells [90]. These molecules involved in NK cell regulation can be exploited in NK-cell based immunotherapies.

The NK cells used for adoptive cellular immunotherapy can be derived from several sources including autologous or allogeneic NK cells, NK cell lines, genetic modified NK cells, hematopoietic stem cells (HSC), and induced pluripotent stem cells (IPSC) [91,92].

The expansion and activation of the NK cells prior to the adoptive transfer are critical steps [93]. Interleukin-2 has longer being used to transform NK cells into LAK cells exhibiting greater cytotoxic activity [94-96] and other cytokines, such as IL-12, IL-15 and IL-18 also activate NK cells [97]. The functional and genetic manipulation of the NK cells have been used to increase tumor-cell killing efficiency [98,99]. Some of the strategies already tested include the transgenic cytokine expression [100], induction of chemokine receptor expression [101], up-regulation of activating receptors using the appropriate cytokines [102], inactivating inhibitory receptors with monoclonal antibodies [103], and redirecting NK cells via chimeric tumor-antigen specific receptors [104,105]. For example, antibody blocking KIR significantly promote NK cell cytotoxicity responses against tumor cells [106] and novel therapies targeting NKG2D ligands expressed on tumor cells are currently under investigation [107].

Antitumor NK cell-based immunotherapy has been tested in various hematological malignancies, such as leukemia, lymphoma, and myeloma, not only in the field of allogeneic hematopoietic stem cell transplantation (HSCT) but also in non-transplanted patients, as well as in non-hematological tumors [91,108]. In general, these NK cell-based immunotherapeutic approaches have generating more promising results in hematological cancers [109-111] than in solid tumors [112]. Several studies have shown that in patients with acute leukemia given allogeneic HSCT, part of the therapeutic effect lies on the anti-

tumor effect displayed by NK cells and CTL. In particular, donor-derived NK cells play a crucial role in the eradication of cancer cells in patients given an allograft from a Human Leukocyte Antigen (HLA)-haploidentical relative, especially when there is a KIR-KIR ligand mismatch in the donor-recipient direction [113]. Alloreactive donor-derived NK cells have been also demonstrated to kill recipient antigen-presenting cells and CTL, thus preventing graft-versus-host disease (GvHD) and graft rejection [114].

In addition to be used in cell based therapies, NK cells are important players in antibody based treatments for hematological cancers, solid tumors, allergy and autoimmune diseases [115-119], as the mechanisms of action of the monoclonal antibodies (mAbs) directed against antigens expressed in cell surface molecules include complement-dependent cellular cytotoxicity (CDCC) and antibody-dependent cellular cytotoxicity (ADCC) [120-123]. Therefore, developing tests for evaluating NK cell mediated ADCC and CDCC would be important not only to elucidate the mechanisms of action of the available mAbs, but also for monitoring the effects of the therapy [124,125].

4. ASSAYS TO MEASURE THE NK-CELL MEDIATED CYTOTOXICITY

Different approaches have been used to directly or indirectly evaluate NK cells, using both *Ex vivo* and *In vitro* studies, and the simultaneous analysis of various parameters allow a comprehensive view of the NK cell function [10,11,126-140] (Table 2).

Natural killer cell-mediated cytotoxicity was measured for a long time using radioactive 51chromium (51Cr) release assays (CRA), which were extensively tested. However, CRA are not easy to perform in clinical laboratories because of difficulties with management and disposal of radioactive materials, short reagent half-lives, high cost, limited sensitivity, nonspecific background and inter-laboratory variability, with difficult standardization. These disadvantages led investigators to design alternative non-radioactive assays.

4.1 Radioactive Assays

Radioactive 51Cr release assays has been considered the standard test to study cell-mediated cytotoxicity *In vitro*. In this method, target cells are loaded with 51Cr, which passively enters cells and binds to intracellular proteins. Upon target cell lysis, 51Cr is released into the supernatant and the amount of 51Cr is quantified using a beta or a gamma counter [141].

Using CRA, the NK-cell mediated cytotoxic activity is usually evaluated by co-incubating the effector cells and 51Cr labeled targets cells at different effector/target (E/T) ratios. The assay is traditionally performed with peripheral blood mononuclear cells (PBMC) (containing the effector cells), although whole blood tests were also developed [11,142]. The spontaneous and maximal levels of 51Cr release are determined by adding the target cells (but not effector cells) to wells containing medium alone and to wells containing a detergent that acts as a membrane permeabilizing solution, respectively. After the incubation period (usually 4 hours), an aliquot of supernatant is collected and read in a scintillation gamma counter, the percentage of specific 51Cr release being calculated as (cpm experimental release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) x 100, where cpm indicates counts per minute. In some studies, data on NK mediated cytotoxicity are expressed in terms of lytic units (LU40) per 10^7 cells, calculated on the basis of a dose-

response curve, one LU40 corresponding to the number of effector cells necessary to lyse 40% of the targets [72,143].

Table 2. Available strategies to directly or indirectly evaluate NK-cell function

Strategy used	References
Quantification of NK cells and NK cell subsets with different immunophenotypic and functional properties.	[126,127]
Evaluation NK-cell activation, either *Ex. vivo* or *In vitro*, under the influence of different stimuli, taking in account characteristic immunophenotypic profiles or the expression of specific activation related markers.	[128-130]
Cell surface molecules involved in NK-cell chemotaxis and homing that regulate NK-cell migration into inflamed tissues or lymphoid organs and tissues.	[131,132]
Adhesion molecules and other molecules involved in NK-cell – target cell interactions leading to conjugate formation	[133,134]
Expression of killer immunoglobulin like (KIR), killer lectin type (KLR), leukocyte immunoglobulin like (LIR), natural killer (NCR) and costimulatory receptors and their ligands.	[135]
Analysis of signal transduction.	[136]
Intracellular expression of cytotoxic granule proteins (e.g. granzymes, perforin, granulysin, etc.) involved in target cell killing.	[137,138]
Translocation of molecules expressed on the membrane of the intracellular granules to the cell surface (e.g. CD107a).	[10,139]
Cytokine production after *In vitro* stimulation with different activators	[11,140]
Ability of NK-cells to form effector/target cell conjugates or capacity to kill target cells.	Reviewed herein

4.2 Non-Radioactive Assays

Non-radioactive assays are based on luminescence, colorimetric or fluorometric methods. Among them, flow cytometry-based cytotoxicity assays (FCCA) are probable the most sensitive and biologically informative, being performable in most clinical hematology and immunology laboratories; nevertheless, FCCA are also time consuming and expensive tests [144,145]. Several comparative studies between FCCA and CRA have been done, flow cytometry being now considered to allow a reliable measure of in vitro cellular cytotoxicity, eliminating the use of radioactive material [144-151].

4.1.1 Flow cytometry based cytotoxic assays

In the first FCCA proposed, effector and target cells were distinguished from each other only based on their light scatter properties and dead target cells were identified by staining with propidium iodide (PI), a nucleic acid probe that penetrates only in cells with damage membranes [144,145]. Since then, the methodology has been progressively improved. In accordance, different approaches have been tested for the labeling of the target and effector cells, as well as for the identification of E/T conjugates and apoptotic and/or dead targets. More recently, FCCA have been used to simultaneously measure the phenotype of effectors, the formation of E/T conjugates and the viability of the targets [146], and single platform, no-wash multicolor assays were developed in order to quantify NK cell cytotoxicity against

leukemia cells [152]. FCCA were also optimized for the functional screening of natural compounds modulating NK cell activity, using IL-2 stimulated samples as a positive control [69], as well as for testing the effect of new drugs on tumor cells using human and animal models [67,153].

Nowadays, the phenotype of effectors, the formation of E/T cell conjugates and the viability of the targets are measured by flow cytometry based on light side (SSC) and forward (FSC) scatter and fluorescence signals, using fluorescent probes and fluorochrome conjugated mAbs. In addition, kinetic studies (usually from 1 to 24h of incubation) at different E/T cell ratios (usually 50:1 to 5:1) either in the absence or in the presence of stimulators (e.g. IL-2) can be performed.

4.1.1.1 Effector cells

In most FCCA, as in other cytotoxic assays, PBMC are used to as effector cells, although in some cases they were depleted from monocytes by adherence from 1h to an overnight incubation [78,154]. In other cases, cytotoxic assays are performed either in whole blood [152] or with sorted NK-cells, using either flow or electromagnetic sorters [53,155,156]. These conditions should be considered when interpreting and comparing the results. In fact, there is evidence that monocytes significantly decrease the NK-cell cytotoxic activity [79]. Moreover, it has been shown that although cells most effective in mediating cytolysis against K562 target cells are NK cells, a substantial degree of cytotoxic activity is detected within the cytotoxic T cells [155]. This is because CTL also display a certain degree of spontaneous non-MHC-restricted cytotoxicity against K562 targets [157,158], although some advise that stimulation with IL-2 or IL-15 is first necessary [159].

4.1.1.2 Target cells

When evaluating NK-cell cytotoxicity by FCCA, as by other assays, most use standard NK-sensitive MHC class I deficient cell lines, such as K562 cells, an erythroleukemia cell line derived from a patient with chronic myeloid leukemia in blast crisis that has been considered the reference-standard target [160], or Daudi cells, a lymphokine-sensitive malignant B-lymphoblastoid cell line, derived from a Burkitt' lymphoma [161] In other cases, NK cells are tested against leukemia cells, or against non-hematological, such as neuroblastoma, gastric, breast and ovarian carcinoma and Wilms' tumor cells [153,156]. For CTL evaluation, autologous tumor cells, Human Leukocyte Antigen (HLA)-matched allogeneic cell lines or dendritic cells loaded with the antigen of interest are used as targets.

4.1.1.3 Effector cell labeling

The percentage of NK-cells among lymphocytes is usually quantified in whole blood and/or in the PBMC before testing cytotoxicity, often using the combination of anti-CD3, anti-CD16 and anti-CD56 mAbs, in order to distinguish NK cells from CTL. However, effector cells are not specifically labeled in most FCCA, as the aim of the study is only to observe the cytotoxic effect on their targets. If however the purpose is to go further on the cells involved on the cytotoxic effect and/or to detect E/T conjugates, co-staining for NK cells and T cells should be performed in cell cultures.

4.1.1.4 Effector cell stimulation

Two different experimental conditions have been used to test the NK cell mediated cytotoxic activity: the assessment of the natural (spontaneous) NK cell activity (unstimulated cell cultures) and the evaluation of LAK cell cytotoxicity (stimulated cell cultures, under the influence of cytokines). For some target cells (e. g. neuroblastoma cell lines and Daudi cells) stimulation is mandatory, whereas for others (e.g. K652 cells) is facultative. As the cytotoxicity values really depends on using (or not) a stimulus, as well as on its type and intensity, this is also a major factor to be considerer when comparing the results. In the majority of the studies where the LAK cell activity was evaluated, IL-2 was the cytokine used, with a concentration ranging from 20 to 200U/ml [162-164], although some other cytokines, such as IL-12 and/or IL-15 [165] or interferon gamma (IFN-γ)) [61] have also been tested. A few studies have used specific stimulators such as tool like receptor ligands [81,166,167] or antibodies against the NK receptors [24,27].

4.1.1.5 Target cell labeling

Discrimination of target and effector cells is critical in FCCA and in most cases these cells can be reasonably separated based on their light scatter and fluorescence profiles. For instance, K562 cells are reasonably distinguished from mononuclear cells based on the higher SSC and green auto-fluorescence, but not based on the FSC [145,154,168]. If light scatter properties are not enough informative and/or if a better discrimination is desired, the approach is to label the target cells with a suitable fluorescent dye.

Different dyes have been tested for labeling of target cells, including green or red fluorescent probes, which is usually achieved by incubating target cells with the dye just before the assay (Table 3) [61,78,146,149,151,152,164,169-177]. Fluorogenic esterase substrates, including calcein and 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein (BCECF) aceto-methyl esters (AM) and various fluorescein diacetate derivatives can be passively loaded into cells. Once inside the cells, these non-fluorescent substrates are converted by intracellular esterases into fluorescent products that are retained by cells with intact plasma membranes. In contrast, both the un-hydrolyzed substrates and their products rapidly leak from cells with compromised membranes, even when they retain some residual esterase activity. Alternatively, cells lines transfected with reporter enzymes or fluorescent proteins can also be used with success [78,162]. This is the case of GHINK-1 cells, obtained by transfection of HFWT cells (an anchorage-dependent NK-cell sensitive Wilms' tumor cell line) with the green fluorescence protein (GFP) gene. After being co-cultured with effector cells, GHINK-1 target cells release GFP into the medium and the intensity of the fluorescence from the released GFP can be used to measure the cytotoxic activity [164].

When choosing a dye to label target cells several factors should be considered: The dye should able to integrate stably in the target cell membrane, allowing for short or long co-incubation periods without leakage to neighboring cells in order to avoid unspecific staining; the vitality of the targets and cytotoxicity of the effector cells should not altered by staining; The spectral emission wavelength of the dye should be compatible with those of probes used to detect apoptotic and/or dead targets and with fluorochrome conjugated mAbs used to stain target and/or effector cells. Some investigators expressed concern about dyes leaking into the medium during co-incubation (due to target cell membrane disruption) and labeling of other cells in the environment [162,178,179]. In alternative, they advise the use of fluorochrome conjugated mAbs (e.g. anti-CD33 for K562 and anti-CD19 for Daudi cell lines) for target cell labeling, as mAbs does not pose such a disadvantage [179].

Table 3. Fluorescent probes that have been tested for target cell labelling in flow cytometry based cytotoxic assays

Group	Fluorescent probe	References
Fluorescein and derivatives	F18,FITC,BCECF,CFSE	[149,152,169-171]
Long-chain carbocyanines and derivatives[a]	DiOC18	[61,172-174]
Mitochondrion-selective probes	MTG-FM[b]	[152]
Calcein acetoxymethyl ester	CAM	[152,175,176]
Green fluorescent protein and derivatives	GFP, EGFP	[78,151,164]
PKH fluorescent dyes[c]	PKH26	[170,177]
Cell trackers	CTO	[146]

Abbreviations: F18, Octadecylamine-fluorescein isothiocyanate; FITC, Fluorescein isothiocyanate; BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; CFSE, Carboxyfluorescein succinimidyl ester; DiO or DiOC18, Dioctadecyloxacarbocyanine; MTG-FM, MitoTracker Green FM; CAM, Calcein acetoxymethyl ester; GFP, Green fluorescent protein; EGFP; Enhanced green fluorescent protein; CTO, Cell Tracker Orange; PKH26;a: Amphiphilic probes comprising a charged fluorophore and lipophilic aliphatic "tails" that anchor the probe to the membrane. The most widely used carbocyanine probes are the octadecyl indocarbocyanines DiIC18, also referred by the acronym DiI, and oxacarbocyanines DiOC18, also referred as DiO; b: MitoTracker Green FM is green-fluorescent mitochondrial stain which appears to localize to mitochondria regardless of mitochondrial membrane potential. The dye stains live cells; c: PKH (from the author who developed these dyes: Paul Karl Horan) are vital lipophilic, fluorescent, membrane intercalating dyes. They contain two long alkyl chains, which allow a strong anchorage in the lipid bilayer. When labelled cells divide, the resulting daughter cells receive half the label, reducing the fluorescence intensity to one-half that the parent cells. As a consequence, the proliferation of labelled cells correlates to a decrease in fluorescence. Three different PKH dyes are used for labelling cells: PKH2, PKH26, and PKH67. PKH2 and PKH67 are green fluorescent dyes, whereas PKH26 emits red fluorescence

4.1.1.6 Identification of apoptotic/dead target cells

Another critical step in cytotoxic assays is the identification of dead targets. Morphological alterations that occur in dead cells usually produce changes in their light scattering properties. In the first FCCA assays the only parameter used to separate dead and viable targets cells was the FSC, at the same time the SSC distinguish between effectors and targets [168].

In order to improve this methodology, orange (e. g. PI) or green (e.g. 7- amino-actinomycin D, 7-AAD) nucleic acid dyes are usually added at the end of the incubation period in order to quantify apoptotic / dead target cells that have lose their membrane integrity. Labeling with fluorochrome conjugated annexin V (that preferentially binds to negatively charged phospholipids like phosphatidylserine in the presence of Ca2+) [180] or co-labeling with fluorochrome conjugated annexin V and nucleic acid fluorescent probes (such as PI or 7-AAD) [178,181,182] have also been used to identify apoptotic / dead target cells. Since phosphatidylserine is translocated to the cell surface in the earlier stages of apoptosis, this approach would theoretically make possible to identify earlier the non-viable targets. Simultaneous labeling with PI, annexin V and cytoplasmic non-lipophilic membrane-impermeable dyes, such as BCECF, allow to identifying sequential apoptotic steps on target cells, characterized by PI incorporation, annexin staining and BCECF release [183]. Alternatively, caspase activation can be measured within target cells using cell permeable fluorescent caspase substrates [184-188]. This assay reliably detects, by flow cytometry or fluorescence/confocal microscopy, antigen-specific CTL and NK cells, providing an alternative sensitive and physiological method, as it measures a biological indicator of apoptosis within target cells.

Results are usually expressed as percentage of NK cytotoxic activity calculated as follows: (% dead K562 target cells in the sample - % spontaneously dead K562 target cells) / (100 - % spontaneously K562 dead target cells) × 100. In order to better measure the NK cell activity, other concepts were introduced such as the maximal target cell lysis and the slope of the cytolytic curve [189].

4.1.1.7 Identification of effector/target conjugates

Flow cytometry has also been used for a long time to evaluate cell-to-cell interactions conditioning the formation of aggregates [190], and the same strategy has been successfully employed to measure the binding of NK cells to targets [154,168,191-197]. By quantifying conjugate formation, the earliest steps of E/T cell interaction can be evaluated and the role of the adhesion molecules and cytokines can be explored [198].

The first condition to identify E/T cell conjugates is to label effector cells with fluorochrome conjugated mAbs. If fluorescent dyes are simultaneously used to detect non-viable targets, conjugate formation and target cell death can be measured at the same time [78,146,162,179]. However, the formation of conjugates may occur early during the course of incubation, been reported to reach a maximum after 40–60 min when LAK cells are used as effectors, whereas the maximum of target cell killing is usually observed after 3 to 4 hours [78,154]. Thus, kinetic studies are necessary in order to define the best conditions to detect both E/T conjugate formation and target cell dead.

4.1.2 Other cytotoxic assays

For the last years various non-radioactive assays have been developed, providing alternative methods to evaluate cell-mediated cytotoxicity (Table 4) [156,199-217].

Bioluminescence assays are based on the production and emission of light (measured by luminometers) by living cells as the result of a chemical reaction. The luciferase/luciferin system, which depends on the ATP, has been the preferred enzyme/substrate system due to its high sensitivity. Colorimetric assays rely on the measurement of the absorbance of light of a particular wavelength using a spectrophotometer or an ELISA reader, which is directly proportional to the concentration of the substance in solution. When the substance by itself does not absorb light, reagents to produce colored compounds have to be employed. Fluorometric assays are based on the use of fluorescent probes or fluorochrome conjugated mAbs. They are performed with fluorescence microscopes, fluorometers, fluorescence microplate readers or flow cytometers. In general, all these assays are alternatives to the CRA for measuring cytotoxicity, avoiding the problems associated with the use of radioactivity and most are rapid and more amenable to standardization. However, some of them perform better in specific applications.

Although some of these methods have been extensively used for specific purposes (e.g. MTT assay for cytotoxic drug sensitivity and resistance studies), the majority of them have been used only for research and are not validated for use in clinical laboratories.

Table 4. Methods for testing cytotoxicity other than flow-cytometry based assays

Assay	Type of assay	Main characteristics	Comments	References
ATP release assay	Luminescence assay.	Bioluminescent assays in which ATP is the limiting reagent for the luciferin / luciferase reaction.	Unsuitable to evaluate cytotoxicity because the lytic signal is indirect.	[199]
GAPDH release assay	Luminescence assay.	Based on the measurement of the GAPDH released from damaged cells. Highly general, since all known cells express GAPDH, and, unlike other enzymes, GAPDH is very readily released from the cytoplasm upon cell lysis. The release of GAPDH is coupled to the activity of the enzyme PGK to produce ATP, which is detected via the luciferase / luciferin bioluminescence methods.	Useful for measuring cytotoxic activity of cells, complement and other lytic agents. Commercially available. Not extensively used.	[200-204]
EuTDA assay or Europium release assay (ERA)	Fluorimetric assay.	The EuTDA assay is based on loading target cells with an acetoxymethyl ester of the fluorescence enhancing ligand (BATDA). The ligand penetrates the cell membrane quickly and within the cell the ester bonds are hydrolyzed to form a hydrophilic ligand (TDA) which no longer passes the membrane. Once target and effector cells are co-incubated, TDA is released from damaged target and reacts with Europium in solution, forming a highly fluorescent and compound (EuTDA), which is measured by time-resolved fluorescence. The measured fluorescence signal correlates directly with the amount of damaged cells.	Sensitive and specific assay that performs well using non-adherent target cells. Needs fewer target and effector cells comparatively to CRA, but some targets can not be used due to high spontaneous europium release (ex. Daudi cells). Commercially available. Not extensively used.	[205-210]
LDH release assay	Colorimetric and fluorimetric assays.	Based on the measurement of the LDH released from damage cells, using enzymatic reactions. The amount of color / fluorescent product is proportional to the amount of LDH, which is in turn is proportional to the number of dead or damaged cells. LDH participates in a reaction which converts a yellow tetrazolium salt into a red, formazan dye which is measured by absorbance at 492 nm. Alternatively, LDH is measured with an enzymatic assay that results in the conversion of resazurin into fluorescent resorufin.	Commercially available. Not extensively used.	[156,211,212]

Assay	Type of assay	Main characteristics	Comments	References
MTT assay	Colorimetric and fluorimetric assays.	Relies on the evaluation of the redox potential of viable cells, measured by the reduction of tetrazolium salts (yellow) to insoluble colored (purple) formazan crystals. Towards the end of the incubation period of target and effector cells in microplates, an acid / alcohol solution is added to dissolve formazan crystals. After a new incubation, the plate is read on an ELISA reader, at 570 nm. Optical density values of test wells (targets + effectors) are compared with that of the control wells (targets) and results are presented as percentage of cell survival, using a calibration curve. A similar redox-based assay has also been developed using the fluorescent dye, resazurin.	Applied in the assessment of cytotoxic drug therapy (drug sensitivity and resistance studies). Commercially available.	[213-215]
Modified micro cytotoxicity assay (MCA)	Optical assay	Performed in microwells with adherent tumor target cells. Following to exposure to effector cells, death target cells became non-adherent and are removed by washing. Remaining adherent (viable) target cells are fixed, stained and optically counted. Solid tumor cell lines (such as BT-20 breast cancer), which are resistant to granzyme / perforin mediated killing but sensitive to killing by cell membrane bound TNF family ligands, are killed using MCA (but not using CRA).	Developed specifically for measuring perforin/granzyme-independent NK cell-mediated apoptotic killing against adherent target cells. Not extensively tested.	[216,217]

Abbreviations: CRA, Chromium release assay; GADPH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactic dehydrogenase; PGK, 3-Phosphoglyceric Phosphokinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

5. CURRENT STATE AND FUTURE PERSPECTIVES

Multicolor flow cytometry is now being used to quantify and characterize the NK cells for the expression of cytotoxic molecules (e. g. granzymes, perforin), receptors with important biological functions (e. g. KIR, KLR, NCR, chemokine receptors) and adhesion (e.g. CD56, CD11a-CD11c/CD18) and activation-related (e.g. CD63) molecules, as well as to evaluate the cytokine production (e. g. IFN-γ), degranulation process (e. g. CD107a surface expression) and cytotoxic activity of the NK cells at a single cell level [140]. These studies have been performed in various disease models and are contributing for the characterization of the immunological abnormalities in various pathological conditions, as well as to a better understanding of the role of the NK cells in the disease etiopathogeny [218]. They are also being used to characterize the immunomodulatory mechanism of drugs that are currently used in clinical practice [204,219], as well as to investigate the mechanism involved in transplant rejection [220]. For instance, new flow cytometry approaches combining both cytotoxicity and antibody binding have been developed in order to detect complement fixing anti-HLA antibodies that have been associated with an increased risk of early antibody-mediated graft rejection in kidney transplants [221]. Cytotoxicity assays against virus infected cells have also been described, allowing to measuring the ability of NK cells to kill target cells infected with different viruses, or expressing different viral proteins [222]. In addition, flow cytometry based methods for evaluation of the ADCC activity using NK cells as effectors have been developed in order to evaluate the efficacy of antibody therapeutics [124,125]. Flow cytometry based assays have also been used to test the cytotoxicity of NK cells expanded by co-culture with tumor cell lines representative of pediatric solid tumors, in order to be used for NK cell based immunotherapy [223].

Among the methods described to assess the function of the NK cells, degranulation assays deserve attention. Secretory lysosomes containing perforin and granzymes are indispensable for NK-cell cytotoxicity because their release results in the induction of target-cell apoptosis. Previous studies have shown that expression of the lysosome-associated membrane protein (LAMP) 1 (CD107a) on the surface of the transport vesicles is important for perforin trafficking to lytic granules and for the deliver of the apoptosis-inducing granzyme B to target cells [224], having also an important role in protecting the cytotoxic cells from self-destruction [225]. CD107a is up-regulated on the surface of activated NK cells and degranulation assays based on CD107a expression correlates with both cytokine secretion and NK cell-mediated lysis of target cells [10,139], allowing for a rapid and reliable classification of patients with genetically determined HL [226].

6. CONCLUSION

Natural killer cells are able to kill target cells directly through the secretion of cytotoxic granules or through binding to death receptors. Measuring the ability of NK cells to kill target cells, is important to evaluate the immune response in various clinical conditions, as well as to study the effects of new drugs in the NK response. Although chromium release assay is recognized as 'gold standard' for measuring NK cell activity, it has disadvantages like use of radioactive compounds and standardization problems. Various non-radioactive assays have been developed to evaluate cell-mediated cytotoxicity. Among them, flow cytometry based assays provide a friendly, biologically informative, and sensitive approach to evaluate the cytotoxic response in both clinical and research settings. They include, among others, cytotoxic and degranulation assays, as well as evaluation of cytokine production by the

activated NK cells, and they are expected to contribute for a better understanding of the role of the cytotoxic cells in various physiological and pathological conditions.

COMPETING INTERESTS

The authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

REFERENCES

1. Cheent K, Khakoo S. Natural killer cells: integrating diversity with function. Immunology. 2009;126(4):449-457. DOI: 10.1111/j.1365-2567.2009.03045.x. PMID: 19278418.
2. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27(45):5932-43. doi: 10.1038/onc.2008.267. PMID: 18836474.
3. Yokoyama WM, Altfeld M, Hsu KC. Natural killer cells: tolerance to self and innate immunity to viral infection and malignancy. Biol Blood Marrow Transplant. 2010;16(1Suppl):S97-S105. DOI: 10.1016/j.bbmt.2009.10.009. PMID: 19835969.
4. Orange JS, Ballas ZK. Natural killer cells in human health and disease. Clin Immunol. 2006;118(1):1-10. PMID: 16337194.
5. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: how do they pull the trigger? Immunology. 2009;128(1):7-15. DOI: 10.1111/j.1365-2567.2009.03123.x. PMID 19689731.
6. Hoves S, Trapani JA, Voskoboinik I. The battlefield of perforin/granzyme cell death pathways. J Leuk Biol. 2010;87(2):237-43. DOI: 10.1189/jlb.0909608. PMID: 19915166
7. Biassoni R, Ugolotti E, De Maria A. NK cell receptors and their interactions with MHC. Curr Pharm Design. 2009;15(28):3301-10. PMID: 19860679.
8. Biassoni R. Human natural killer receptors, co-receptors, and their ligands. Current Protocols in Immunology/edited by John E Coligan [et al.], 2009. Chapter 14:Unit 14.10.
9. Andre P, Anfossi N. Clinical analysis of human natural killer cells. Methods Mol Biol. 2008; 415:291-300. PMID: 18370161.
10. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. Cellular Immunology. 2009;254(2):149-54. DOI: 10.1016/j.cellimm.2008.08.007. PMID: 18835598.
11. Claus M, Greil J, Watzl C. Comprehensive analysis of NK cell function in whole blood samples. J Immunol Methods. 2009;341(1-2):154-64. DOI: 10.1016/j.jim.2008.11.006. PMID: 19056395.
12. King MA. Detection of dead cells and measurement of cell killing by flow cytometry. J Immunol Methods. 2000;243(1-2):155-66. PMID: 10986413.
13. Fischer K, Andreesen R, Mackensen A. An improved flow cytometric assay for the determination of cytotoxic T lymphocyte activity. J Immunol Methods 2002;259(1-2):159-69. PMID: 11730851.
14. Orange JS. Human natural killer cell deficiencies. Curr Opin Allergy Clin Immunol. 2006.6(6):399-409. PMID: 17088643.
15. Brenner BG, Dascal A, Margolese RG, Wainberg MA. Natural-Killer Cell-Function in Patients with Acquired Immunodeficiency Syndrome and Related Diseases. J Leuk Biol. 1989;46(1):75-83. PMID: 2659714.

16. O'Connor GM, Holmes A, Mulcahy F, Gardiner CM. Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. Clin Immunol. 2007;124(3):277-83. PMID: 17611161.
17. Pachlopnik Schmid J, Côte M, Ménager MM, Burgess A, Nehme N, Ménasché G, et al. Inherited defects in lymphocyte cytotoxic activity. Immunol Rev. 2010;235(1):10-23. DOI: 10.1111/j.0105-2896.2010.00890.x. PMID: 20536552.
18. Ishii E, Ueda I, Shirakawa R, Yamamoto K, Horiuchi H, Ohga S, et al. Genetic subtypes of familial hemophagocytic lymphohistiocytosis: correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions. Blood. 2005;105(9):3442-8. PMID: 15632205.
19. Haliotis T, Roder J, Klein M, Ortaldo J, Fauci AS, Herberman RB. Chédiak-Higashi gene in humans I. Impairment of natural-killer function. J Exp Med. 1980;151(5):1039-48. PMID: 6154765.
20. Klein M, Roder J, Haliotis T, Korec S, Jett JR, Herberman RB, Katz P, Fauci AS. Chédiak-Higashi gene in humans. II. The selectivity of the defect in natural-killer and antibody-dependent cell-mediated cytotoxicity function. J Exp Med. 1980;151(5):1049-58. PMID: 6966316.
21. Virelizier JL, Griscelli C. Selective defect on interferon secretion associated with impaired natural killing activity. Arch Fr Pediatr. 1981;38(2):77-81. PMID: 6165331.
22. Klein C, Philippe N, Le Deist F, Fraitag S, Prost C, Durandy A, Fischer A, Griscelli C. Partial albinism with immunodeficiency (Griscelli syndrome). J Pediatr. 1994;125(6 Pt 1):886-95. PMID: 7996360.
23. Gazit R, Aker M, Elboim M, Achdout H, Katz G, Wolf DG, Katzav S, Mandelboim O. NK cytotoxicity mediated by CD16 but not by NKp30 is functional in Griscelli syndrome. Blood. 2007;109(10):4306-12. PMID: 17255357.
24. Tangye SG, Phillips JH, Lanier LL, Nichols KE. Cutting edge: Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. J Immunol. 2000;165(6):2932-2936. PMID: 10975798.
25. Orange JS, Brodeur SR, Jain A, Bonilla FA, Schneider LC, Kretschmer R, et al. Deficient natural killer cell cytotoxicity in patients with IKK-gamma/NEMO mutations. J Clin Invest. 2002;109(11):1501-9. PMID: 12045264.
26. Zimmer J, Donato L, Hanau D, Cazenave JP, Tongio MM, Moretta A, de la Salle H. Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). J Exp Med. 1998;187(1):117-22. PMID: 9419217.
27. Shibuya K, Lanier LL, Phillips JH, Ochs HD, Shimizu K, Nakayama E, et al. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. Immunity. 1999;11(5):615-23. PMID: 10591186.
28. Orange JS, Ramesh N, Remold-O'Donnell E, Sasahara Y, Koopman L, Byrne M, et al. Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. Proc Natl Acad Sci USA. 2002;99(17):11351-6. PMID: 12177428.
29. Notarangelo LD, Giliani S, Mazza C, Mella P, Savoldi G, Rodriguez-Pérez C, et al. Of genes and phenotypes: The immunological and molecular spectrum of combined immune deficiency. Defects of the gamma(c)-JAK3 signaling pathway as a model. Immunol Rev. 2000;178:39-48. PMID: 11213805.

30. Buckley RH, Schiff RI, Schiff SE, Markert ML, Williams LW, Harville TO, et al. Human severe combined immunodeficiency: Genetic, phenotypic, and functional diversity in one hundred eight infants. J Pediatr. 1997;130(3):378-87. PMID: 9063412.
31. Henter JI, Elinder G, Ost A. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. Semin Oncol. 1991;18(1):29-33. PMID: 1992521.
32. Henter J-I, Horne A, Aricó M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatric Blood & Cancer. 2007;48(2):124-31. PMID:16937360.
33. Maakaroun NR, Moanna A, Jacob JT, Albrecht H. Viral infections associated with haemophagocytic syndrome. Rev Med Virol. 2010;20(2):93-105. DOI: 10.1002/rmv.638. PMID: 20127750.
34. Sieni E, Cetica V, Hackmann Y, Coniglio ML, Da Ros M, Ciambotti B, Pende D, Griffiths G, Aricò M. Familial Hemophagocytic Lymphohistiocytosis: When Rare Diseases Shed Light on Immune System Functioning. Front Immunol. 2014;(16)5:167. eCollection 2014. PMID: 24795715.
35. Göransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, Söderhäll C, Samuelsson A, Janka G, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Human Genetics 2001;68(3):590-7. PMID: 11179007.
36. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003;115(4):461-73. PMID: 14622600.
37. Stepp S, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science 1999;286(5446):1957. PMID: 10583959.
38. Nichols KE, Ma CS, Cannons JL, Schwartzberg PL, Tangye SG, et al. Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. Immunol Rev 2005;203:180-99. PMID: 15661030.
39. Filipovich AH. Hemophagocytic lymphohistiocytosis and related disorders. Hematology Am Soc Hematol Educ Program. 2009;127-31. DOI: 10.1182/asheducation-2009.1.127. PMID: 20008190.
40. Filipovich AH. Hemophagocytic lymphohistiocytosis: A lethal disorder of immune regulation. J Pediatr. 1997;130(3):337-8. PMID: 9063402.
41. Schneider EM, Lorenz I, Müller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. Blood. 2002;100(8):2891-2898. PMID: 12351400.
42. Imashuku S, Hyakuna N, Funabiki T, Ikuta K, Sako M, Iwai A, et al. Low natural killer activity and central nervous system disease as a high-risk prognostic indicator in young patients with hemophagocytic lymphohistiocytosis. Cancer. 2002;94(11):3023-31. PMID: 12115393.
43. Ishii E, Ueda I, Shirakawa R, Yamamoto K, Horiuchi H, Ohga S, et al. Genetic subtypes of familial hemophagocytic lymphohistiocytosis: correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions. Blood. 2005;105(9):3442-8. PMID: 15632205.

44. Green MRJ, Kennell AS, Larche MJ, Seifert MH, Isenberg DA, Salaman MR. Natural killer cell activity in families of patients with systemic lupus erythematosus: Demonstration of a killing defect in patients. Clin Exp Immunol 2005;141(1):165-73. PMID: 15958083.
45. Aramaki T, Ida H, Izumi Y, Fujikawa K, Huang M, Arima K, et al. A significantly impaired natural killer cell activity due to a low activity on a per-cell basis in rheumatoid arthritis. Mod Rheumatol. 2009;19(3):245-52. DOI: 10.1007/s10165-009-0160-6. PMID: 19283441.
46. Izumi Y, Ida H, Huang M, Iwanaga N, Tanaka F, Aratake K, et al. Characterization of peripheral natural killer cells in primary Sjogren's syndrome: Impaired NK cell activity and low NK cell number. J Lab Clin Med 2006;147(5):242-9. PMID: 16697772.
47. Krishnaraj R, Svanborg A. Low natural killer cell function in disseminated aspergillosis. Scand J Infect Dis. 1993;25(4):537-41. PMID: 8248758.
48. Kadish AS, Doyle AT, Steinhauer EH, Ghossein NA. Natural cytotoxicity and interferon production in human cancer: deficient natural killer activity and normal interferon production in patients with advanced disease. J Immunol. 1981;127(5):1817-22. PMID: 6170674.
49. Gregory CD, Shah LP, Lee H, Scott IV, Golding PR. Cytotoxic Reactivity of Human Natural-Killer (NK) Cells during Normal-Pregnancy - A Longitudinal-Study. J Clin Lab Immunol. 1985;18(4):175-81. PMID: 3879503.
50. Opsahl M, Hansen K, Klein T, Cunningham D. Natural killer cell activity in early human pregnancy. Gynecol Obstet Invest. 1994;37(4):226-8. PMID: 8050724.
51. Groer M, El-Badri N, Djeu J, Harrington M, Van Eepoel J. Suppression of Natural Killer Cell Cytotoxicity in Postpartum Women. Am J Reprod Immunol. 2010;63(3):209-13. DOI: 10.1111/j.1600-0897.2009.00788.x. PMID: 20055786.
52. Fletcher MA, Zeng XR, Maher K, Levis S, Hurwitz B, Antoni M, et al. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. PLoS One. 2010;5(5):e10817. PMID: 20520837.
53. O'Shea D, Cawood TJ, O'Farrelly C, Lynch L. Natural Killer Cells in Obesity: Impaired Function and Increased Susceptibility to the Effects of Cigarette Smoke. PLoS One. 2010;5(1):e8660. DOI: 10.1371/journal.pone.0008660. PMID: 20107494.
54. Echevarria S, Casafont F, Miera M, Lozano JL, de la Cruz F, San Miguel G, et al. Interleukin-2 and natural killer activity in acute type B hepatitis. Hepatogastroenterology. 1991;38(4):307-10. PMID: 1937376.
55. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology. 2009;137(3):1151-60. 1160 e1-7. DOI: 10.1053/j.gastro.2009.05.047. PMID: 19470388.
56. Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. Human Reproduction. 2001;16(5):855-61. PMID: 11331628.
57. Hadinedoushan H, Mirahmadian M, Aflatounian A. Increased natural killer cell cytotoxicity and IL-2 production in recurrent spontaneous abortion. Am J Reprod Immunol. 2007;58(5):409-14. PMID: 17922693.
58. Gilman-Sachs A, DuChateau BK, Aslakson CJ, Wohlgemuth GP, Kwak JY, Beer AE, et al. Natural killer (NK) cell subsets and NK cell cytotoxicity in women with histories of recurrent spontaneous abortions. Am J Reprod Immunol. 1999;41(1):99-105. PMID: 10097793.

59. Herberman RR, Ortaldo JR, Bonnard GD. Augmentation by interferon of human natural and antibody-dependent cell-mediated cytotoxicity. Nature. 1979;277(5693):221-3. PMID: 95205.
60. Henney CS, Kuribayashi K, Kern DE, Gillis S. Interleukin-2 augments natural killer cell activity. Nature. 1981;291(5813):335-8. PMID: 6164929.
61. Kane KL, Ashton FA, Schmitz JL, Folds JD. Determination of natural killer cell function by flow cytometry. Clin Diagn Lab Immunol 1996;3(3):295-300. PMID: 8705672.
62. Ozdemir O, Savaşan S. Combinational IL-2/IL-15 induction does not further enhance IL-15-induced lymphokine-activated killer cell cytotoxicity against human leukemia/lymphoma cells. Clin Immunol. 2005;115(3):240-9. PMID: 15893691.
63. Strbo N, de Armas L, Liu H, Kolber MA, Lichtenheld M, Pahwa S. IL-21 augments natural killer effector functions in chronically HIV-infected individuals. AIDS. 2008;22(13):1551-60. DOI: 10.1097/QAD.0b013e3283089367. PMID: 18670213.
64. Deehan DJ, Heys SD, Ashby J, Eremin O. Interleukin-2 (IL-2) augments host cellular immune reactivity in the perioperative period in patients with malignant disease. Eur J Surg Oncol. 1995;21(1):16-22. PMID: 7851545.
65. de Castro FA, Palma PV, Morais FR, Simões BP, Carvalho PV, Ismael SJ, et al. Immunological effects of interferon-alpha on chronic myelogenous leukemia. Leuk Lymphoma. 2003;44(12):2061-7. PMID: 14959848.
66. Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J Clin Invest. 1993;91(1):123-32. PMID: 7678599.
67. Cederbrant K, Marcusson-Ståhl M, Condevaux F, Descotes J. NK-cell activity in immunotoxicity drug evaluation. Toxicology. 2003. 185(3):241-250. PMID: 12581699.
68. Li Q. NK cell assays in immunotoxicity testing. Methods Mol Biol. 2010;598:207-19. DOI: 10.1007/978-1-60761-401-2_15. PMID: 19967516.
69. Hallberg A, Malmström P. Natural killer cell activity and antibody-dependent cellular cytotoxicity in newborn infants. Acta Paediatr Scand. 1982;71(3):431-6. PMID: 7136657.
70. Georgeson GD, Szony BJ, Streitman K, Kovács A, Kovács L, László A. Eur J Pediatr. 2001;160(8):478-82. Natural killer cell cytotoxicity is deficient in newborns with sepsis and recurrent infections. PMID: 11548185.
71. Imashuku S, Hibi S, Sako M, Ishii T, Kohdera U, Kitazawa K, Ooe K, Naya M, Sawada H, Kawakami T. Heterogeneity of immune markers in hemophagocytic lymphohistiocytosis: comparative study of 9 familial and 14 familial inheritance-unproved cases. J Pediatr Hematol Oncol. 1998;20(3):207-14. PMID: 9628431.
72. Sansoni P, Cossarizza A, Brianti V, Fagnoni F, Snelli G, Monti D, et al. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. Blood. 1993;82(9):2767-73. PMID: 8219229.
73. Krishnaraj R. Immunosenescence of human NK cells: effects on tumor target recognition, lethal hit and interferon sensitivity. Immunol Lett. 1992;34(1):79-84. PMID: 1478709.
74. Mariani E, Monaco MC, Cattini L, Sinoppi M, Facchini A. Distribution and lytic activity of NK cell subsets in the elderly. Mech Ageing Dev. 1994;76(2-3):177-87. PMID: 7885064.
75. Ogata K, Yokose N, Tamura H, An E, Nakamura K, Dan K, Nomura T. Natural killer cells in the late decades of human life. Clin Immunol Immunopathol. 1997;84(3):269-75. PMID: 9281385.

76. Miyaji C, Watanabe H, Minagawa M, Toma H, Kawamura T, Nohara Y, Nozaki H, Sato Y, Abo T. Numerical and functional characteristics of lymphocyte subsets in centenarians. J Clin Immunol. 1997;17(5):420-9. PMID: 9327342.
77. Kutza J, Murasko DM. Effects of aging on natural killer cell activity and activation by interleukin-2 and IFN-alpha. Cell Immunol. 1994;155(1):195-204. PMID: 7513259.
78. Allegra S, Deleine C, Michael-Jubely R, Gryson C, Boirie Y, Kantakamalakul W, Vasson MP. Implementation of the EGFP-K562 flow cytometric NK test: determination of NK cytotoxic activity in healthy elderly volunteers before and after feeding. Cytometry A. 2006;69(9):992-8. PMID: 16969797.
79. Ravaglia G, Forti P, Maioli F, Bastagli L, Facchini A, Mariani E, Savarino L, Sassi S, Cucinotta D, Lenaz G. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged >/=90 y. Am J Clin Nutr. 2000;71(2):590-8. PMID: 10648276.
80. Mariani E, Ravaglia G, Forti P, Meneghetti A, Tarozzi A, Maioli F. Vitamin D, thyroid hormones and muscle mass influence natural killer (NK) innate immunity in healthy nonagenarians and centenarians. Clin Exp Immunol. 1999;116(1):19-27. PMID: 10209500.
81. Mian MF, Lauzon NM, Stämpfli MR, Mossman KL, Ashkar AA. Impairment of human NK cell cytotoxic activity and cytokine release by cigarette smoke. J Leukoc Biol. 2008;83(3):774-84. PMID: 18055568.
82. Ferson M, Edwards A, Lind A, Milton GW, Hersey P. Low natural killer-cell activity and immunoglobulin levels associated with smoking in human subjects. Int J Cancer. 1979;23(5):603-9. PMID: 457307.
83. Arjona A, Boyadjieva N, Sarkar DK. Circadian rhythms of granzyme B, perforin, IFN-gamma, and NK cell cytolytic activity in the spleen: Effects of chronic ethanol. J Immunol. 2004;172(5):2811-7. PMID: 14978081.
84. Dopp JM, Miller GE, Myers HF, Fahey JL. Increased natural killer-cell mobilization and cytotoxicity during marital conflict. Brain Behav Immun. 2000;14(1):10-26. PMID: 10729214.
85. Wang T, Delahanty DL, Dougall AL, Baum A. Responses of natural killer cell activity to acute laboratory stressors in healthy men at different times of day. Health Psychol. 1998;17(5):428-35. PMID: 9776001.
86. Qian X, Wang X, Jin H. Cell Transfer Therapy for Cancer: Past, Present, and Future. J Immunol Res. 2014;2014:525913. PMID: 24741604.
87. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood Rev. 2006;20:123–37. PMID: 16364519.
88. Ljunggren HG, Karre K. In search of the “missing self”: MHC molecules and NK cell recognition. Immunology Today. 1990;11(7):237–244. PMID: 2201309.
89. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J. 2004;23:255–9. PMID: 14685277.
90. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285(5428):727-9. PMID: 10426993.
91. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol. 2013;10(3):230-52. PMID: 23604045.
92. Knorr DA, Bachanova V, Verneris MR, Miller JS. Clinical utility of natural killer cells in cancer therapy and transplantation. Semin Immunol. 2014;26(2):161-172. PMID: 24618042.

93. Childs RW, Berg M. Bringing natural killer cells to the clinic: ex vivo manipulation. Hematology Am Soc Hematol Educ Program. 2013;2013:234-46. PMID: 24319186.
94. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. Journal of Experimental Medicine. 1982;155(6):1823–1841. PMID: 6176669.
95. Rosenberg SA. Immunotherapy of cancer by systemic administration of lymphoid cells plus interleukin-2. Journal of Biological Response Modifiers. 1984;3(5):501–511. PMID: 6389777.
96. Law TM, Motzer RJ, Mazumdar M, Sell KW, Walther PJ, O'Connell M, et al.Phase III randomized trial of interleukin-2 with or without lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma. Cancer. 1995;76(5):824–832. PMID: 8625186.
97. Ni J, Miller M, Stojanovic A, Cerwenka A. Toward the next generation of NK cell-based adoptive cancer immunotherapy. Oncoimmunology. 2013;1,2(4):e23811. PMID: 23734329.
98. Pegram HJ, Kershaw MH, Darcy PK. Genetic modification of natural killer cells for adoptive cellular immunotherapy. Immunotherapy. 2009;1(4):623-30. PMID: 20635990.
99. Shook DR, Campana D. Natural killer cell engineering for cellular therapy of cancer. Tissue Antigens. 2011;78(6):409-15. PMID: 22077621.
100. Zhang J, Sun R, Wei H, Zhang J, Tian Z. Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy. Haematologica. 2004 Mar;89(3):338-47. PMID: 15020274.
101. Somanchi SS, Somanchi A, Cooper LJ, Lee DA. Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. Blood. 2012;119(22):5164-72. PMID: 22498742.
102. Boyiadzis M, Memon S, Carson J, Allen K, Szczepanski MJ, Vance BA, et al. Up-regulation of NK cell activating receptors following allogeneic hematopoietic stem cell transplantation under a lymphodepleting reduced intensity regimen is associated with elevated IL-15 levels. Biol Blood Marrow Transplant. 2008;14(3):290-300. PMID: 18275895.
103. Romagné F, André P, Spee P, Zahn S, Anfossi N, Gauthier L, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. Blood. 2009;114(13):2667-77. PMID: 19553639.
104. Müller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, et al. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. Cancer Immunol Immunother. 2008;57(3):411-23. PMID: 17717662.
105. Altvater B, Landmeier S, Pscherer S, Temme J, Schweer K, Kailayangiri S, et al. 2B4 (CD244) signaling by recombinant antigen-specific chimeric receptors costimulates natural killer cell activation to leukemia and neuroblastoma cells. Clin Cancer Res. 2009;15(15):4857-66. PMID: 19638467.
106. Binyamin L, Alpaugh RK, Hughes TL, Lutz CT, Campbell KS, Weiner LM. Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. J Immunol. 2008;180(9):6392-401. PMID: 18424763.
107. Spear P, Wu MR, Sentman ML, Sentman CL. NKG2D ligands as therapeutic targets. Cancer Immun. 2013;13:8. PMID: 23833565.

108. Ames E, Murphy WJ. Advantages and clinical applications of natural killer cells in cancer immunotherapy. Cancer Immunol Immunother. 2014 Jan;63(1):21-8. DOI: 10.1007/s00262-013-1469-8. PMID: 23989217.
109. Locatelli F, Merli P, Rutella S. At the Bedside: Innate immunity as an immunotherapy tool for hematological malignancies. J Leukoc Biol. 2013;94(6):1141-57. PMID: 24096380.
110. Locatelli F, Moretta F, Brescia L, Merli P. Natural killer cells in the treatment of high-risk acute leukaemia. Semin Immunol. 2014;26(2):173-179. PMID: 24613727.
111. Chouaib S, Pittari G, Nanbakhsh A, El Ayoubi H, Amsellem S, Bourhis JH, et al. Improving the Outcome of Leukemia by Natural Killer Cell-Based Immunotherapeutic Strategies. Front Immunol. 2014;5:95. eCollection 2014. PMID: 24672522.
112. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. Clin Cancer Res. 2011;17(19):6287-97. PMID: 21844012.
113. Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood. 2011;118(12):3273-9. PMID: 21791425.
114. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295(5562):2097-100. PMID: 11896281.
115. Cheney CM, Stephens DM, Mo X, Rafiq S, Butchar J, Flynn JM, et al. Ocaratuzumab, an Fc-engineered antibody demonstrates enhanced antibody-dependent cell-mediated cytotoxicity in chronic lymphocytic leukemia. MAbs. 2014;6(3):748-54. PMID: 24594909.
116. Petricevic B, Laengle J, Singer J, Sachet M, Fazekas J, Steger G, et al. Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent in both adjuvant and metastatic HER2/neu breast cancer patients. J Transl Med. 2013;11:307. PMID: 24330813.
117. Jandl T, Revskaya E, Jiang Z, Bryan RA, Casadevall A, Dadachova E. Complement-dependent cytotoxicity of an antibody to melanin in radioimmunotherapy of metastatic melanoma. Immunotherapy. 2013;5(4):357-64. PMID: 23557419.
118. Mauerer R, Gruber R. Monoclonal antibodies for the immunotherapy of solid tumours. Curr Pharm Biotechnol. 2012;13(8):1385-98. PMID: 22339220.
119. Ghazi A, Trikha A, Calhoun WJ. Benralizumab-- A humanized mAb to IL-5Rα with enhanced antibody-dependent cell-mediated cytotoxicity--a novel approach for the treatment of asthma. Expert Opin Biol Ther. 2012;12(1):113-8. PMID: 22136436.
120. Alderson KL, Sondel PM. Clinical cancer therapy by NK cells via antibody-dependent cell-mediated cytotoxicity. J Biomed Biotechnol. 2011;2011:379123. PMID: 21660134
121. Fernández-Marrero Y, López-Requena A. Lonely killers: effector cell- and complement-independent non-proapoptotic cytotoxic antibodies inducing membrane lesions. MAbs. 2011;3(6):528-34. PMID: 22123064.
122. Hubert P, Amigorena S. Antibody-dependent cell cytotoxicity in monoclonal antibody-mediated tumor immunotherapy. Oncoimmunology. 2012;1(1):103-105. PMID: 22720225.
123. Boross P, Leusen JH. Mechanisms of action of CD20 antibodies. Am J Cancer Res. 2012;2(6):676-90. PMID: 23226614.

124. Schnueriger A, Grau R, Sondermann P, Schreitmueller T, Marti S, Zocher M. Development of a quantitative, cell-line based assay to measure ADCC activity mediated by therapeutic antibodies. Mol Immunol. 2011;48(12-13):1512-7. PMID: 21570725.
125. Chowdhury F, Lode HN, Cragg MS, Glennie MJ, Gray JC. Development of immunomonitoring of antibody-dependent cellular cytotoxicity against neuroblastoma cells using whole blood. Cancer Immunol Immunother. 2014 Mar 22. PMID: 24658837.
126. Lima M, Teixeira MA, Queirós ML, Leite M, Santos AH, Justiça B, Orfão A. Immunophenotypic characterization of normal blood CD56+lo versus CD56+hi NK-cell subsets and its impact on the understanding of their tissue distribution and functional properties. Blood Cells Mol Dis. 2001;27(4):731-43. PMID: 11778657.
127. Verneris MR, Miller JS. The phenotypic and functional characteristics of umbilical cord blood and peripheral blood natural killer cells. Br J Haematol. 2009;147(2):185-91. Doi: 10.1111/j.1365-2141.2009.07768.x. PMID: 19796267.
128. Lima M, Almeida J, dos Anjos Teixeira M, Queirós ML, Justiça B, Orfão A. The "ex vivo" patterns of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR expression identify acute/early and chronic/late NK-cell activation states. Blood Cells Mol Dis. 2002;28(2):181-90. PMID: 12064914.
129. Lima M, Almeida J, Teixeira MA, Santos AH, Queirós ML, Fonseca S, et al. Reactive phenotypes after acute and chronic NK-cell activation. J Biol Regul Homeost Agents. 2004;18(3-4):331-4. PMID: 15786700.
130. Giustiniani J, Bensussan A, Marie-Cardine A. Identification and characterization of a transmembrane isoform of CD160 (CD160-TM), a unique activating receptor selectively expressed upon human NK cell activation. J Immunol. 2009;182(1):63-71. PMID: 19109136.
131. Inngjerdingen M, Damaj B, Maghazachi AA. Expression and regulation of chemokine receptors in human natural killer cells. Blood. 2001;97(2):367-75. PMID: 11154210.
132. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. J Immunol. 2006;177(11):7833-40. PMID: 17114454.
133. Montoya MC, Sancho D, Vicente-Manzanares M, Sánchez-Madrid F. Cell adhesion and polarity during immune interactions. Immunol Rev. 2002;186:68-82. PMID: 12234363.
134. Dustin ML, Long EO. Cytotoxic immunological synapses. Immunol Rev. 2010;235(1):24-34. doi: 10.1111/j.0105-2896.2010.00904.x. PMID: 20536553.
135. Lankry D, Gazit R, Mandelboim O. Methods to identify and characterize different NK cell receptors and their ligands. Methods Mol Biol. 2010;612:249-73. DOI: 10.1007/978-1-60761-362-6_17. PMID: 20033646.
136. Chini CC, Leibson PJ. Signal transduction during natural killer cell activation. Curr Protoc Cytom; 2001. Chapter 9:Unit 9.16. DOI: 10.1002/0471142956.cy0916s14. PMID: 18770753.
137. Maher KJ, Klimas NG, Hurwitz B, Schiff R, Fletcher MA. Quantitative fluorescence measures for determination of intracellular perforin content. Clin Diagn Lab Immunol. 2002;9(6):1248-52. PMID: 12414757.
138. Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, et al The cytolytic enzymes granzyme A, granzyme B, and perforin: Expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. J Leukoc Biol. 2009 Jan;85(1):88-97. DOI: 10.1189/jlb.0208107. PMID: 18820174.

139. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294(1-2):15-22. PMID: 15604012.
140. Bryceson YT, Fauriat C, Nunes JM, Wood SM, Björkström NK, Long EO, Ljunggren HG. Functional analysis of human NK cells by flow cytometry. Methods Mol Biol. 2010;612:335-52. DOI: 10.1007/978-1-60761-362-6_23. PMID: 20033652.
141. Garovoy M, Carpenter C. Lymphocyte-mediated cytotoxicity. Manual of Clinical Immunology, 2nd edition. Washington, DC: American Society for Microbiology. 1980:290–296.
142. Bromelow KV, Galea-Lauri J, O'Brien ME, Souberbielle BE. A highly sensitive whole blood natural killer cell assay. J Immunol Methods. 1998; 217(1-2):177-84. PMID: 9776587.
143. Pross HF, Baines MG, Rubin P, Shragge P, Patterson MS. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. J Clin Immunol. 1981;1(1):51-63. PMID: 7334070.
144. Zarcone D, Tilden AB, Cloud G, Friedman HM, Landay A, Grossi CE. Flow-Cytometry Evaluation of Cell-Mediated Cytotoxicity. J Immunol Methods. 1986;94(1-2):247-55. PMID: 3782814.
145. Papa S, Vitale M, Mariani AR, Roda P, Facchini A, Manzoli FA. Natural killer function in flow cytometry. I. Evaluation of NK lytic activity on K562 cell line. J Immunol Methods. 1988;107(1):73-8. PMID: 3343519.
146. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: comparisons to a 4h 51Cr-release assay. J Immunol Methods. 2007;325(1-2):51-66. PMID: 17617419.
147. Wierda WG, Mehr DS, Kim YB. Comparison of fluorochrome-labeled and 51Cr-labeled targets for natural killer cytotoxicity assay. J Immunol Methods. 1989;122(1):15-24. PMID: 2760476.
148. Chang L, Gusewitch GA, Chritton DB, Folz JC, Lebeck LK, Nehlsen-Cannarella SL. Rapid flow cytometric assay for the assessment of natural killer cell activity. J Immunol Methods. 1993;166(1): 45-54. PMID: 8228287.
149. Kantakamalakul W, Jaroenpool J, Pattanapanyasat K. A novel enhanced green fluorescent protein (EGFP)-K562 flow cytometric method for measuring natural killer (NK) cell cytotoxic activity. J Immunol Methods. 2003;272(1-2):189-97. PMID: 12505723.
150. Cholujova D, Jakubíková J, Kubes M, Arendacká B, Sapák M, Ihnatko R, et al. Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays. Immunobiology. 2008;213(8):629-40. PMID: 18765168.
151. Motzer SA, Tsuji J, Hertig V, Johnston SK, Scanlan J. Natural killer cell cytotoxicity: A methods analysis of 51chromium release versus flow cytometry. Biol Res Nurs. 2003;5(2):142-52. PMID: 14531218.
152. Zimmermann SY, Esser R, Rohrbach E, Klingebiel T, Koehl U. A novel four-colour flow cytometric assay to determine natural killer cell or T-cell-mediated cellular cytotoxicity against leukaemic cells in peripheral or bone marrow specimens containing greater than 20% of normal cells. J Immunol Methods. 2005;296(1-2):63-76. PMID: 15680151.

153. Kloss S, Bochennek K, Huenecke S, Zimmermann SY, Kuçi S, Müller T, et al. A novel five-colour flow cytometric assay to determine NK cell cytotoxicity against neuroblastoma and other adherent tumour cells. J Immunol Methods. 2007;325(1-2):140-7. PMID: 17663991.
154. Zamai L, Mariani AR, Zauli G, Rodella L, Rezzani R, Manzoli FA, Vitale M. Kinetics of in vitro natural killer activity against K562 cells as detected by flow cytometry. Cytometry. 1998;32(4):280-285. PMID: 9701396.
155. Srour EF, Leemhuis T, Jenski L, Redmond R, Jansen J. Cytolytic activity of human natural killer cell subpopulations isolated by four-color immunofluorescence flow cytometric cell sorting. Cytometry. 1990;11(3):442-6. PMID: 2187659.
156. Szkaradkiewicz A, Karpiński TM, Drews M, Borejsza-Wysocki M, Majewski P, Andrzejewska E. Natural killer cell cytotoxicity and immunosuppressive cytokines (IL-10, TGF-beta1) in patients with gastric cancer. J Biomed Biotechnol. 2010;2010:901564. PMID: 20445748.
157. Schmidt RE, Murray C, Daley JF, Schlossman SF, Ritz J. A Subset of Natural-Killer-Cells in Peripheral-Blood Displays a Mature T-Cell Phenotype. J. Exp. Med. 1986;164(1):351-356. PMID: 3088199.
158. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol. 1986;136(12):4480-6. PMID: 3086432.
159. Dunne J, Lynch S, O'Farrelly C, Todryk S, Hegarty JE, Feighery C, Doherty DG. Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15. J Immunol. 2001;167(6):3129-38. PMID: 11544298.
160. Lozzio CB, Lozzio BB. Human Chronic Myelogenous Leukemia Cell-Line with Positive Philadelphia Chromosome. Blood. 1975;45(3):321-334. PMID: 163658.
161. Dupuis M, Ramoni C, Yassen A, Samoggia P, Tombesi M, Caserta M, Cianfriglia M. The over-expression of P-glycoprotein in K-562 and DAUDI cells, is associated with a high susceptibility to NK and LAK cells. J Biol Regul Homeost Agents.1991;5(4):137-41. PMID: 1687102.
162. Godoy-Ramirez K, Franck K, Gaines H. A novel method for the simultaneous assessment of natural killer cell conjugate formation and cytotoxicity at the single-cell level by multi-parameter flow cytometry. J Immunol Methods. 2000;239(1-2):35-44. PMID: 10821945.
163. Bruunsgaard H, Pedersen AN, Schroll M, Skinhøj P, Pedersen BK. Decreased natural killer cell activity is associated with atherosclerosis in elderly humans. Exp Gerontol. 2001;7(1):127-36.
164. Harada H, Saijo K, Ishiwata I, Ohno T. A GFP-transfected HFWT cell line, GHINK-1, as a novel target for non-RI activated natural killer cytotoxicity assay. Hum Cell. 2004;17(1):43-8. PMID: 15369136.
165. Lin SJ, heng PJ, Huang YJ, Kuo ML. Evaluation of cytotoxic function and apoptosis in interleukin (IL)-12/IL-15-treated umbilical cord or adult peripheral blood natural killer cells by a propidium-iodide based flow cytometry. Pediatr Allergy Immunol. 2004;15(1):79-85. PMID: 14998386.
166. Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: Induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci U S A. 2004;101(27):10116-21. PMID: 15218108.

167. Lauzon NM, Mian F, MacKenzie R, Ashkar AA. The direct effects of Toll-like receptor ligands on human NK cell cytokine production and cytotoxicity. Cell Immunol. 2006;241(2):102-12. PMID: 17049504.
168. Vitale M, Neri LM, Comani S, Falcieri E, Rizzoli R, Rana R, Papa S. Natural killer function in flow cytometry. II. Evaluation of NK lytic activity by means of target cell morphological changes detected by right angle light scatter. J Immunol Methods. 1989;121(1):115-20. PMID: 2754257.
169. Chung HJ, Park CJ, Lim JH, Jang S, Chi HS, Im HJ, Seo JJ. Establishment of a reference interval for natural killer cell activity through flow cytometry and its clinical application in the diagnosis of hemophagocytic lymphohistiocytosis. Int J Lab Hematol. 2010;32(2):239-47. doi: 10.1111/j.1751-553X.2009.01177.x. PMID: 19614711.
170. Radosević K, Garritsen HS, Van Graft M, De Grooth BG, Greve J. A simple and sensitive flow cytometric assay for the determination of the cytotoxic activity of human natural killer cells. J Immunol Methods. 1990;135(1-2):81-9. PMID: 2273268.
171. Lecoeur H, Février M, Garcia S, Rivière Y, Gougeon ML. A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. J Immunol Methods. 2001;253(1-2):177-87. PMID: 11384679.
172. Mattis AE, Bernhardt G, Lipp M, Förster R. Analyzing cytotoxic T lymphocyte activity: A simple and reliable flow cytometry-based assay. J Immunol Methods. 1997;204(2):135-42. PMID: 9212830.
173. Piriou L, Chilmonczyk S, Genetet N, Albina E. Design of a flow cytometric assay for the determination of natural killer and cytotoxic T-lymphocyte activity in human and in different animal species. Cytometry. 2000;41(4):289-97. PMID: 11084614.
174. Höppner M, Luhm J, Schlenke P, Koritke P, Frohn C. A flow-cytometry based cytotoxicity assay using stained effector cells in combination with native target cells. J Immunol Methods. 2002;267(2):157-63. PMID: 12165437.
175. Papadopoulos NG, Dedoussis GV, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M. An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. J Immunol Methods. 1994;177(1-2):101-11. PMID: 7822816.
176. Neri S, Mariani E, Meneghetti A, Cattini L, Facchini A. Calcein-acetyoxymethyl cytotoxicity assay: Standardization of a method allowing additional analyses on recovered effector cells and supernatants. Clin Diagn Lab Immunol. 2001;8(6):1131-5. PMID: 11687452.
177. Hatam L, Schuval S, Bonagura VR. Flow cytometric analysis of natural killer cell function as a clinical assay. Cytometry. 1994;16(1):59-68. PMID: 8033735.
178. Johann S, Blümel G, Lipp M, Förster R. A Versatile Flow Cytometry-Based Assay for the Determination of Short-Term and Long-Term Natural-Killer-Cell Activity. J Immunol Methods. 1995;185(2):209-16. PMID: 7561131.
179. Ozdemir O, Ravindranath Y, Savaşan S. Cell-mediated cytotoxic evaluation using monoclonal antibody staining for target or effector cells with annexinV/propidium iodide colabeling by fluorosphere-adjusted counts on three-color flow cytometry. Cytometry A. 2003;56(1):53-60. PMID: 14566939.
180. Goldberg JE, Sherwood SW, Clayberger C. A novel method for measuring CTL and NK cell-mediated cytotoxicity using annexin V and two-color flow cytometry. J Immunol Methods 1999;224(1-2):1-9. PMID: 10357200.
181. Aubry J, Blaecke A, Lecoanet-Henchoz S, Jeannin P, Herbault N, et al. Annexin V used for measuring apoptosis in the early events of cellular cytotoxicity. Cytometry A. 1999;37(3):197-204. PMID: 10520200.

182. Derby E, Reddy V, Kopp W, Nelson E, Baseler M, Sayers T, et al. Three-color flow cytometric assay for the study of the mechanisms of cell-mediated cytotoxicity. Immunol Lett. 2001;78(1):35-9. PMID: 11470149.
183. Kasatori N, Ishikawa F, Ueyama M, Urayama T. A differential assay of NK-cell-mediated cytotoxicity in K562 cells revealing three sequential membrane impairment steps using three-color flow-cytometry. J Immunol Methods 2005;307(1-2):41-53. PMID: 16246359.
184. Chahroudi A, Silvestri G, Feinberg MB. Measuring T cell-mediated cytotoxicity using fluorogenic caspase substrates. Methods. 2003;31(2):120-6. PMID: 12957569.
185. Jayaraman S. Intracellular determination of activated caspases (IDAC) by flow cytometry using a pancaspase inhibitor labeled with FITC. Cytometry A. 2003;56(2):104-12. PMID: 14608638.
186. Jerome KR, Sloan DD, Aubert M. Measurement of CTL-induced cytotoxicity: The caspase 3 assay. Apoptosis. 2003;8(6):563-71. PMID: 14574062.
187. Telford WG, Komoriya A, Packard BZ, Bagwell CB. Multiparametric analysis of apoptosis by flow cytometry. Methods Mol Biol. 2011;699:203-27. DOI: 10.1007/978-1-61737-950-5_10. PMID: 21116985.
188. Liu L, Packard BZ, Brown MJ, Komoriya A, Feinberg MB. Assessment of lymphocyte-mediated cytotoxicity using flow cytometry. Methods Mol Biol. 2004;263:125-40. PMID: 14976364.
189. Sephton SE, Kraemer HC, Neri E, Stites DP, Weissbecker I, Spiegel D. Improving methods of assessing natural killer cell cytotoxicity. Int J Methods Psychiatr Res. 2006;15(1):12-21. PMID: 16676682.
190. Segal DM, Stephany DA. The measurement of specific cell: cell interactions by dual-parameter flow cytometry. Cytometry. 1984;5(2):169-81. PMID: 6370631.
191. Storkus WJ, Balber AE, Dawson JR. Quantitation and sorting of vitally stained natural killer cell-target cell conjugates by dual beam flow cytometry. Cytometry. 1986;7(2):163-70. PMID: 3512197.
192. Garcia-Peñarrubia P, Koster FT, Bankhurst AD, Galvez J. Effect of conjugate size on the kinetics of cell-mediated cytotoxicity at the population level. J Theor Biol. 1989;138(1):93-115. PMID: 2626066.
193. Cavarec L, Quillet-Mary A, Fradelizi D, Conjeaud H. An improved double fluorescence flow cytometry method for the quantification of killer cell/target cell conjugate formation. J Immunol Methods. 1990;130(2):251-61. PMID: 2197333.
194. Vitale M, Zamai L, Neri LM, Manzoli L, Facchini A, Papa S. Natural killer function in flow cytometry: identification of human lymphoid subsets able to bind to the NK sensitive target K562. Cytometry. 1991;12(8):717-22. PMID: 1838975.
195. Vitale M, Zamai L, Papa S, Mazzotti G, Facchini A, Monti G, Manzoli FA. Natural killer function in flow cytometry. III. Surface marker determination of K562-conjugated lymphocytes by dual laser flow cytometry. J Immunol Methods. 1992;149(2):189-96. PMID: 1534339.
196. Renò F, Papa S, Vitale M, Zamai L. Assays of natural killer (NK) cell ligation to target cells. Curr Protoc Cytom. 2001;9. Unit 9.10. DOI: 10.1002/0471142956.cy0910s04. PMID: 18770748.
197. Bonavida B, Lebow LT, Bradley TP. Mechanism of cell-mediated cytotoxicity at the single cell level. VI. Direct assessment of the cytotoxic potential of human peripheral blood non-lytic effector-target cell conjugates. J Immunol. 1984;132(2):594-8. PMID: 6606675.

198. Quillet-Mary A, Cavarec L, Kermarrec N, Marchiol-Fournigault C, Gil ML, Conjeaud H, Fradelizi D. Target lysis by human LAK cells is critically dependent upon target binding properties, but LFA-1, LFA-3 and ICAM-1 are not the major adhesion ligands on targets. Int J Cancer. 1991;47(3):473-9. PMID: 1704356.
199. Crouch SP, Kozlowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods. 1993;160(1):81-8. PMID: 7680699.
200. Corey MJ, Kinders RJ, Brown LG, Vessella RL. A very sensitive coupled luminescent assay for cytotoxicity and complement-mediated lysis. J Immunol Methods. 1997;207(1):43-51. PMID: 9328585.
201. Ogbomo H, Hahn A, Geiler J, Michaelis M, Doerr HW, Cinatl J Jr. NK sensitivity of neuroblastoma cells determined by a highly sensitive coupled luminescent method. Biochem Biophys Res Commun. 2006;339(1):375-9. PMID: 16297863.
202. Ogbomo H, Geiler J, Leutz A, von Kietzell K, Michaelis M, Doerr HW, Cinatl J Jr. Measurement of cytotoxic T lymphocyte activity of human cytomegalovirus seropositive individuals by a highly sensitive coupled luminescent method. Med Microbiol Immunol. 2009;198(4):257-62. PMID: 19813020.
203. Ogbomo H, Michaelis M, Geiler J, van Rikxoort M, Muster T, Egorov A, Doerr HW, Cinatl J Jr. Tumor cells infected with oncolytic influenza A virus prime natural killer cells for lysis of resistant tumor cells. Med Microbiol Immunol. 2010;199(2):93-101. PMID: 20012989.
204. Ogbomo H, Michaelis M, Altenbrandt B, Doerr HW, Cinatl J Jr. A novel immunomodulatory mechanism of ribavirin in suppressing natural killer cell function. Biochem Pharmacol. 2010;79(2):188-97. PMID: 19665449.
205. Blomberg K, Granberg C, Hemmilä I, Lövgren T. Europium-labelled target cells in an assay of natural killer cell activity. I. A novel non-radioactive method based on time-resolved fluorescence. J Immunol Methods. 1986;86(2):225-9. PMID: 3456003.
206. Blomberg K, Granberg C, Hemmilä I, Lövgren T. Europium-labelled target cells in an assay of natural killer cell activity. II. A novel non-radioactive method based on time-resolved fluorescence. significance and specificity of the method. J Immunol Methods. 1986;92(1):117-23. PMID: 3745921.
207. Blomberg K, Ulfstedt AC. Fluorescent europium chelates as target cell markers in the assessment of natural killer cell cytotoxicity. J Immunol Methods. 1993;160(1):27-34. PMID: 8450237.
208. Pacifici R, Di Carlo S, Bacosi A, Altieri I, Pichini S, Zuccaro P. Modified procedure for labelling target cells in a europium release assay of natural killer cell activity. J Immunol Methods. 1993;161(1):135-7. PMID: 8486925.
209. Patel AK, Boyd PN. An improved assay for antibody dependent cellular cytotoxicity based on time resolved fluorometry. J Immunol Methods. 1995;184(1):29-38. PMID: 7622867.
210. von Zons P, Crowley-Nowick P, Friberg D, Bell M, Koldovsky U, Whiteside TL. Comparison of europium and chromium release assays: Cytotoxicity in healthy individuals and patients with cervical carcinoma. Clin Diagn Lab Immunol. 1997;4(2):202-7. PMID: 9067656.
211. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods. 1983;64(3):313-20. PMID: 6199426.
212. Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods. 1988;115(1):61-9. PMID: 3192948

213. Pieters R, Huismans DR, Leyva A, Veerman AJ. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. Cancer Lett. 1988;41(3):323-32. PMID: 3165705.
214. Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk Lymphoma. 2003;44(11):1957-62. PMID: 14738150.
215. Sargent JM. The use of the MTT assay to study drug resistance in fresh tumour samples. Recent Results Cancer Res. 2003;161:13-25. PMID: 12528795.
216. Vujanovic NL, Nagashima S, Herberman RB, Whiteside TL. Nonsecretory apoptotic killing by human NK cells. J Immunol. 1996;157(3):1117-26. PMID: 8757616.
217. Wahlberg BJ, Burholt DR, Kornblith P, Richards TJ, Bruffsky A, Herberman RB, et al. Measurement of NK activity by the microcytotoxicity assay (MCA): A new application for an old assay. J Immunol Methods. 2001;253(1-2):69-81. PMID: 11384670.
218. Scrivo R, Morrone S, Spadaro A, Santoni A, Valesini G. Evaluation of degranulation and cytokine production in natural killer cells from spondyloarthritis patients at single-cell level. Cytometry B Clin Cytom. 2011;80(1):22-7. PMID: 20687202.
219. Jungkunz-Stier I, Zekl M, Stühmer T, Einsele H, Seggewiss-Bernhardt R. Modulation of natural killer cell effector functions through lenalidomide/dasatinib and their combined effects against multiple myeloma cells. Leuk Lymphoma. 2014;55(1):168-76. PMID: 23573828.
220. Shin BH, Ge S, Mirocha J, Karasyov A, Vo A, Jordan SC, Toyoda M. Regulation of anti-HLA antibody-dependent natural killer cell activation by immunosuppressive agents. Transplantation. 2014;97(3):294-300. PMID: 24342979.
221. Alheim M. Flow cytometry-based cytotoxicity and antibody binding assay. Curr Protoc Cytom. 2013;66:Unit 6.34. PMID: 24510725.
222. Aicheler RJ, Stanton RJ. Functional NK cell cytotoxicity assays against virus infected cells. Methods Mol Biol. 2013;1064:275-87. PMID: 23996265.
223. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D. Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res. 2010;16(15):3901-9. PMID: 20542985.
224. Krzewski K, Gil-Krzewska A, Nguyen V, Peruzzi G, Coligan JE. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. Blood. 2013;121(23):4672-83. PMID: 23632890.
225. Cohnen A, Chiang SC, Stojanovic A, Schmidt H, Claus M, Saftig P, et al. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. Blood. 2013;122(8):1411-8. PMID: 23847195.
226. Bryceson YT, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. Blood. 2012;119(12):2754-63. PMID: 22294731.

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