When and How to Evaluate the Natural Killer Cell Function

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Authors’ contributions

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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 citotoxicity; AM: Aceto-methyl esters; BCECF: 2',7'-Bis(carboxyethyl)-5,6-carboxyfluorescein; CAM: Calcein acetoxy-methyl ester; 51Cr: 51chromium; CCA: Colorimetric cytotoxic assay; CDCC: Complement dependent cell citotoxicity; CFSE: carboxyfluorescein succinimidyl ester; CTL: Cytotoxic T lymphocytes; CRA: Chromium release cytotoxic assay; CTO: Cell Tracker Orange; DIO or DiOC18: Diocadecylxocarbocyanine; 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 coding 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

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

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-CD24 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, hypofibrinogenaemia, high lactate dehydrogenase, hypertriglyceridaemia, 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 erythematosus [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 been 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 also been 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, non-specific 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 10⁷ 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

<table>
<thead>
<tr>
<th>Strategy used</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantification of NK cells and NK cell subsets with different immunophenotypic and functional properties.</td>
<td>[126,127]</td>
</tr>
<tr>
<td>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.</td>
<td>[128-130]</td>
</tr>
<tr>
<td>Cell surface molecules involved in NK-cell chemotaxis and homing that regulate NK-cell migration into inflamed tissues or lymphoid organs and tissues.</td>
<td>[131,132]</td>
</tr>
<tr>
<td>Adhesion molecules and other molecules involved in NK-cell – target cell interactions leading to conjugate formation Expression of killer immunoglobulin like (KIR), killer lectin type (KLR), leukocyte immunoglobulin like (LIR), natural killer (NCR) and costimulatory receptors and their ligands.</td>
<td>[133,134]</td>
</tr>
<tr>
<td>Analysis of signal transduction. Intracellular expression of cytotoxic granule proteins (e.g. granzymes, perforin, granulysin, etc.) involved in target cell killing.</td>
<td>[136]</td>
</tr>
<tr>
<td>Translocation of molecules expressed on the membrane of the intracellular granules to the cell surface (e.g. CD107a).</td>
<td>[137,138]</td>
</tr>
<tr>
<td>Cytokine production after In vitro stimulation with different activators Ability of NK-cells to form effector/target cell conjugates or capacity to kill target cells.</td>
<td>[139]</td>
</tr>
</tbody>
</table>

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.
4.1.4 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 depend on using (or not) a stimulus, as well as on its type and intensity, this is also a major factor to be considered 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].

4.1.5 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluorescent probe</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein and derivatives</td>
<td>F18, FITC, BCECF, CFSE</td>
<td>[149, 152, 169-171]</td>
</tr>
<tr>
<td>Long-chain carbocyanines and derivatives(^a)</td>
<td>DiO18</td>
<td>[61, 172-174]</td>
</tr>
<tr>
<td>Mitochondrion-selective probes</td>
<td>MTG-FM(^b)</td>
<td>[152]</td>
</tr>
<tr>
<td>Calcein acetoxyethyl ester</td>
<td>CAM</td>
<td>[152, 175, 176]</td>
</tr>
<tr>
<td>Green fluorescent protein and derivatives</td>
<td>GFP, EGFP</td>
<td>[78, 151, 164]</td>
</tr>
<tr>
<td>PKH fluorescent dyes(^c)</td>
<td>PKH26</td>
<td>[170, 177]</td>
</tr>
<tr>
<td>Cell trackers</td>
<td>CTO</td>
<td>[146]</td>
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</tbody>
</table>

Abbreviations: F18, Octadecylamine-fluorescein isothiocyanate; FITC, Fluorescein isothiocyanate; BCECF, 2,7'-bis(carboxyethyl)-5,6-carboxyfluorescein; CFSE, Carboxyfluorescein succinimidyl ester; DIO or DIO18, DiO18, Dioctadecylxocarbocyanine; MTG-FM, MitoTracker Green FM; CAM, Calcein acetoxyethyl 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 phosphatidyserine 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 phosphatidyserine 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: 
\[
\frac{\text{(% dead K562 target cells in the sample} - \text{ % spontaneously dead K562 target cells})}{(100 - \text{ % spontaneously K562 dead target cells})} \times 100.
\]
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

<table>
<thead>
<tr>
<th>Assay</th>
<th>Type of assay</th>
<th>Main characteristics</th>
<th>Comments</th>
<th>References</th>
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<tr>
<td>ATP release assay</td>
<td>Luminescence assay.</td>
<td>Bioluminescent assays in which ATP is the limiting reagent for the luciferin / luciferase reaction.</td>
<td>Unsuitable to evaluate cytotoxicity because the lytic signal is indirect.</td>
<td>[199]</td>
</tr>
<tr>
<td>GAPDH release assay</td>
<td>Luminescence assay.</td>
<td>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.</td>
<td>Useful for measuring cytotoxic activity of cells, complement and other lytic agents. Commercially available. Not extensively used.</td>
<td>[200-204]</td>
</tr>
<tr>
<td>EuTDA assay or Europium release assay (ERA)</td>
<td>Fluorimetric assay.</td>
<td>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.</td>
<td>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.</td>
<td>[205-210]</td>
</tr>
<tr>
<td>LDH release assay</td>
<td>Colorimetric and fluorimetric assays.</td>
<td>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.</td>
<td>Commercially available. Not extensively used.</td>
<td>[156,211,212]</td>
</tr>
<tr>
<td>Assay</td>
<td>Type of assay</td>
<td>Main characteristics</td>
<td>Comments</td>
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<tr>
<td>MTT assay</td>
<td>Colorimetric and fluorimetric assays.</td>
<td>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.</td>
<td>Applied in the assessment of cytotoxic drug therapy (drug sensitivity and resistance studies). Commercially available.</td>
<td>[213-215]</td>
</tr>
<tr>
<td>Modified micro cytotoxicity assay (MCA)</td>
<td>Optical assay</td>
<td>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).</td>
<td>Developed specifically for measuring perforin/granzyme-independent NK cell-mediated apoptotic killing against adherent target cells. Not extensively tested.</td>
<td>[216,217]</td>
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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


