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Regular paper

CD25 (IL-2R) expression correlates with the target cell induced cytotoxic activity and cytokine secretion in human natural killer cells*

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Cytotoxic activity is one of the major functions of Natural Killer (NK) cells and is a critical effector mechanism of innate immune responses against infected or cancer cells. A variety of assays have been developed to determine NK cell cytotoxic activity, however a receptor-based screening tool is still lacking. Here, we propose the CD25 receptor as a candidate for NK cell cytotoxicity marker. We have verified that there is a correlation between classic target cell induced cytotoxicity markers and the CD25 expression on NK cells. Non-adherent lymphocyte fractions pre-stimulated with *Escherichia coli* O55:B5 lipopolysaccharide were co-cultured with settled HeLa targets in a four hour long cytotoxic assay. The cytotoxic effect was evaluated by MTT reduction assay and quantification of soluble cytotoxicity markers (granzyme B, FasL, caspase-8, IFN-γ and IL-2) was done by ELISA. Lymphocytes were stained with anti-CD3-Cy-5, anti-CD56/CD16/Nkp46-FITC and anti-CD25-PE antibodies and analyzed by flow cytometry. We observed that the CD25 expression exclusively on the $CD3^{-}CD56^{+}CD25^{+}$ NK cells was positively correlated with their cytotoxic function evaluated by the MTT test (r=0.68), the upregulation of granzyme B (r=0.89), IL-2 (r=0.78) and IFN-γ (r=0.57), however, it was not positively correlated with FasL and caspase-8. We conclude that the CD25 expression might serve as an *in vitro* receptor-based screening tool for NK cell activity.

Key words: natural killer cells, CD25, cytotoxic activity, granzyme, caspase, cytokines

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INTRODUCTION

Natural Killer (NK) cells are large granular lymphocytes, distinguishable from T and B cells by the lack of antigen specific cell surface receptors, ability to rapidly produce cytokines and mediate spontaneous cytotoxic activity against broad range of target cells (Lehman *et al*., 1999; Kopeć-Szlęzak *et al*., 2007; Fauriat *et al*., 2013). In healthy adults, the NK cells constitute 5-15% of total peripheral lymphocytes, but they also reside in organs and tissues such as Payers patches, liver, lymph nodes, mucosa-associated lymphoid tissue (MALT) and uterus (Carrega & Ferlazzo, 2012). NK cells are major members of the innate lymphoid cell family, which develop from CD34 hematopoietic cells in the bone marrow and undergo terminal maturation in secondary lymphoid tissues (Kopeć-Szlęzak *et al*., 2007; Caliguri, 2008). NK cells were found to participate in the early control of the virus and intracellular bacterial infections as well as in allogeneic and antitumor responses (Kapetanovic & Cavaillon, 2007; Vivier, 2008; Caliguri, 2008; Vivier & Ugolini, 2011). Recently the role for NK cells in regulating placentation, leptin-dependent obesity and pathogenesis of systemic lupus erythematosus has been considered (Laue *et al*., 2015; Moffett & Shreeve, 2015; Spada *et al*., 2015). The importance of NK cells in clearing infections is evident from studies describing individuals lacking the NK cells, although exhibiting normal adaptive immunity function, who suffered from persistent infections with viruses such as *Herpes simplex*, papilloma and cytomegalovirus (Ballas *et al*., 1990; Orange, 2013). Since the NK cells have been found to express toll-like receptors (TLR) such as TLR2, TLR4 and TLR5 recognizing bacterial lipoproteins, lipopolysaccharides (LPS) and flagellin, respectively, they constitute a potent responders not only to viral but also bacterial stimuli (Hornung *et al*., 2002; Lauzon *et al*., 2007; Souza-Fonseca-Guimaraes *et al*., 2012). In humans, NK cells are identified by the absence of the T-cell receptor complex (TCR) and the presence of neural cell adhesion molecule CD56 as well as an IgG Fc receptor – FcγRIIIA (CD16), although with a variable intensity. Based on the density of CD56 receptors, NK cells are divided into two subpopulations: $CD56^{bright}$ NK cells with high expression of CD56 molecules and $CD56^{dim}$ NK cells with relatively low density of CD56 (Caliguri, 2008; Wilk *et al*., 2008). The $CD56^{bright}$ cells exhibit weaker cytotoxic activity and are potent producers of cytokines: interleukin (IL)-5, 10, 13, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, whereas

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Abbreviations: Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; ApoL, Apo ligand; ATP, adenosine triphosphate; ATCC, American Type Culture Collection; CD, cluster of differentiation; DD, death domain; DNA, deoxyribonucleic acid; DR, death receptor; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FSC, forward-scattered light; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IFN-g, interferon gamma; Ig, immunoglobulin; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; mAb, monoclonal Ab; MALT, mucosa-associated lymphoid tissue; MFI, mean fluorescence index; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCR, natural cytotoxicity receptors; NK, natural killer cell; OD, optical density; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll; PRR, pattern recognition receptors; SSC, side-scattered light; TCR, T-cell receptor; TLR, toll-like receptor; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1

the CD56^{dim} NK cells, considered as more mature subpopulation, demonstrate strong cytotoxic properties and co-express the CD16 receptor involved in the antibody dependent cell-mediated cytotoxicity (ADCC) (Batoni *et al.*, 2005; Lunemann *et al.*, 2009; Fauriat *et al.*, 2010). Despite this functional division, the CD56^{bright} NK cells are also considered to possess immunoregulatory properties, since they might release anti-inflammatory cytokines such as IL-10, that act not only locally but induce systemic or tissue specific tolerance such as during pregnancy (Sharma, 2014). Apart from CD56 and CD16, NK cell express a variety of other surface molecules such as natural cytotoxicity receptors (NCRs): NKp30, NKp44, and NKp46, important mediators of NK cell cytotoxicity which trigger an immune response upon recognition of cognate cellular, viral and bacterial ligands (Sochacka, 2008; Joyce & Sun, 2011). Due to high variability of NK cells, their identification is problematic. Previously, their recognition was based on the CD3$^-$CD56$^+$ or CD3$^-$CD16$^+$ markers, however, recently it has been postulated that NKp46 receptor is the most adequate and precise marker for all human and mice NK cells (Walzer *et al.*, 2007; Moretta *et al.*, 2014).

Three distinct pathways of NK cell activation are now recognized (Horowitz *et al.*, 2012; Rudnicka *et al.*, 2011). In the direct "*missing self*" pathway, NK cell activation results from the interaction with the major histocompatibility complex (MHC) receptors which lack class I ligands for inhibitory receptors on NK cells. Second, direct activation pattern was recently described that relays on a direct recognition of microbial compounds by TLR receptors expressed on NK cells especially in the context of TLR4 interaction with LPSs of Gram-negative bacteria (Chalifour *et al.*, 2012; Chaushu *et al.*, 2012; Souza-Fonseca-Guimaraes *et al.*, 2012). Third pathway involves transmembrane receptor-mediated interactions. These include death receptors that are members of the TNF receptor gene superfamily such as FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Locksley *et al.*, 2001; Rubio-Moscardo *et al.*, 2005). Fourth is an indirect mechanism that depends on myeloid accessory cells that respond to pattern recognition receptors (PRR) by production of cytokines such as IL-2, IL-12, IL-15, IL-18 or IFN-γ (Horowitz *et al.*, 2012; Qi *et al.*, 2014; Fauriat *et al.*, 2013). Particularly, the CD-56^{bright} subpopulation which constitutively expresses the high affinity IL-2 receptor (IL-2R, CD25) can be driven to activation and proliferate in response to IL-2 and IL-15 (Baume *et al.*, 2012; Carson *et al.*, 2007; Bachmann & Oxenius, 2007).

Prior to activation, NK cells are capable of two main immune functions. The first and best described is the ability to mediate contact-dependent killing of target cells. This involves mobilization of the lytic granules that contain perforin, the pore-forming molecule, and death-inducing enzymes, such as granzymes (Shafer-Weaver *et al.*, 2003; Ida *et al.*, 2005). Once a killing cascade is initiated in an NK cell, the lytic granules are translocated to the effector-targeted cell milieu and their contents are secreted. This function of cytotoxicity can be accessed by NK cell activation receptors, a direct NK cell stimulation, as an innate immune defense or by recognition of IgG-opsonized cells through CD16 to enable the ADCC pathway (Ida *et al.*, 2005). The second function of NK cells is the production of soluble factors that may act in an auto- or paracrine matter, further enhancing the activation of NK cells or inducing the activity of other immune cells. The repertoire of factors secreted by activated NK cells includes a wide variety of cytokines, chemokines, and other soluble mediators such as IL-2, IL-10, IL-12, TNF-α and predominantly IFN-γ (Fauriat *et al.*, 2013; Horowitz *et al.*, 2012). NK cells may both, respond to an activating signal from IL-2 (by binding it with CD25 receptor) and constitute a source of this cytokine (Malek *et al.*, 2010).

Taking into consideration the activation pathways and the consequences of NK cell activation there are various ways to measure the activity of NK cells (Fig. 1). The

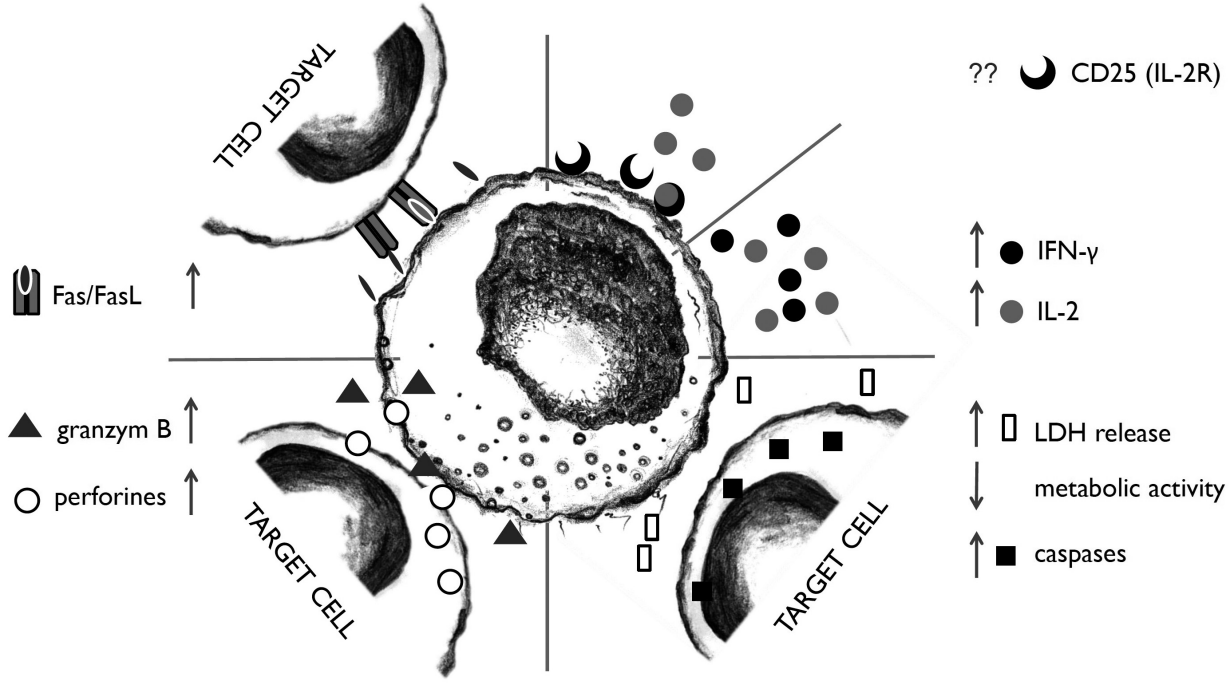

Figure 1. The indicators of NK cell activation used in target-cell based cytotoxicity assays.
Abbreviations: FasL, Fas ligand; IL, interleukin; IFN, interferon; LDH, lactate dehydrogenase.

majority of them are based on the cell-contact dependent cytotoxicity assays, where target cells are co-cultured with NK cells for four hours and the results of NK cell activation is then measured on the basis of: the percentage of dead target cells after cytotoxic assay (e.g. MTT reduction assay, analysis of the DNA content and the integrity of target cell membranes using fluorescent assays, lactate dehydrogenase release, drop in ATP content), indirectly by quantification of soluble target cell death markers (e.g. caspases, death receptors and their ligands such as Fas/FasL), evaluation of degranulation indicators (granzyme A, granzyme B, perforins), or by quantification of cytokines released upon NK activation (eg. IFN-γ, IL-12, IL-2) (Baran *et al*., 2001; Weyermann *et al*., 2005; Blom *et al*., 2009; Wang *et al*., 2010).

Although many other tests are available to monitor cytotoxic activity of NK cells, the classic cell cytotoxic assay is still used as a reference procedure (Al-Hubeshy *et al*., 2011; Clausen *et al*., 2002; Wang *et al*., 2010). However, several examples of the deficiencies of the MTT assay for use in evaluating cytotoxicity have been reported in the literature (Weyermann *et al*., 2005; Wang *et al*., 2010). For instance, it was suggested that certain soluble compounds released upon cell stimulation enhance the formazan formation resulting in false negative results (Pagliacci *et al*., 1993). Furthermore, since the cytotoxic test involves a co-culture of the target together with effector cells, it is possible that the formazan is created by effector cells that possess higher ability to attach to target cells due to previous stimulation, rather than the target cells themselves. Similarly, soluble markers of cytotoxicity released by target cells upon apoptosis, such as caspases, that are used for evaluation are not target cell specific and might be secreted as a result of the effector cell death caused for instance by previous stimuli.

There are also various approaches to the purification of NK cell fraction prior cell cytotoxic assay. The fact that NK cells in humans comprise 5–15% of peripheral blood lymphocytes, isolation of sole NK fraction requires large amounts of blood sample from one donor and the isolation technique which often involves the use of monoclonal antibodies leads to non-specific activation of NK cells (Meinhardt *et al*., 2012). On the other hand, since NK cells may indirectly respond to stimuli, the *in vitro* presence of other accessory cells (lymphocytes, monocytes) might be crucial for their proper activation (Øren *et al*., 2005). Although CD69 was established as a promising marker of NK cell activation, it remains on the surface of NK cells only for a few hours after activation, and in contrast to CD25 it is not recycled back and exposed on the surface of activated NK (Clausen *et al*., 2003). Due to this, the CD69 marker did not suit our study approach where the stimulation period lasted for 24 h. Taking into consideration all the drawbacks of classic target cell-induced cytotoxic assay and the imperfection of the CD69 marker of activation, especially in long-term *in vitro* stimulation protocols, we aimed to evaluate a simple method that would enable to predict the NK cell cytotoxic activity based on the surface expression of the CD25 receptor.

MATERIALS AND METHODS

Cell isolation and stimulation. Forty healthy volunteers (19 male and 21 female; mean age 37; age range, 22–61 years) were included in the study. Volunteers were selected on the basis of medical interview which excluded preexisting acute and chronic diseases. The research was conducted according to the principles expressed in the Declaration of Helsinki and gained an approval of the Local Ethical Committee. Blood samples were obtained with written informed consent from all volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from all individuals by Histopaque 1077 gradient centrifugation (Sigma, St Louis, MI, USA), washed twice, adjusted to the density of 2.5×10^5 cells/ml and stimulated overnight with standard *Escherichia coli* (*E. coli*) LPS of O55:B5 type (Sigma) in a final concentration of 25 ng/ml (positive control) or incubated with no stimuli (negative control). Following stimulation, supernatants were harvested, aliquoted and kept at –80°C for further quantification of cytokines. The viability of leukocytes from each donor was assessed before and after stimulation and it remained in the range of 98–100%. Following stimulation, non-adherent fractions (lymphocytes) were divided into two batches: one used for target cell induced cytotoxicity assay and quantification of soluble cytotoxicity markers and the second used for flow cytometric immunophenotyping.

Cytotoxicity assay. The Henrietta Lacks cervical cancer epithelial cell line (HeLa) purchased from the European Cell Culture Collection (Salisbury Wiltshire, Great Britain) was used as a source of target cells in cytotoxic assay. The cells were incubated for 3–4 days, 37°C, 5% CO_2 in a complete RPMI-1640 medium containing 10% of heat inactivated fetal calf serum (FCS) and standard antibiotics, and then treated with 0.25% trypsin, centrifuged and washed twice with a culture medium. The target cells were adjusted to the density of 2×10^5 cells/ml and distributed (100 μl/well) into 96-well plates. Prior to the cytotoxic assay, the cells were incubated for 24 h, at 37°C, 5% CO_2 in order to obtain the monolayer of adherent cells. Effector cells, untreated or preincubated with *E. coli* LPS were added to settled target cells in a ratio of 100:1 (adjusted experimentally) and incubated for 4 h, at 37°C, 5% CO_2. Then, the lymphocytes were removed and the remaining cells were washed out. Washing effectiveness was assessed under an inverted microscope. The wells containing HeLa cells alone (without lymphocytes) were used as controls for target cell viability. The cytotoxic activity of the lymphocytes was estimated on the basis of the viable target cell ability to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt) using the TACS MTT Cell Proliferation Assay (R&D Systems, Minneapolis, MN, USA), as recommended by the manufacturer. The intensity of MTT reduction was estimated spectrophotometrically at 570 nm.

Quantification of cytokines. The concentrations of IFN-γ and IL-2 were estimated in the PBMC culture supernatants after the stimulation using a commercially available specific enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol, with the detection limits of 7 pg/ml for both cytokines (Quantikine ELISA, R&D Systems, Minneapolis, USA).

Quantification of soluble cell cytotoxicity markers. Following cytotoxic assay, the granzyme B, Fas ligand (FasL) and caspase-8 were quantified by ELISA in supernatants from mixed target/effector cell cultures as recommended by the manufacturers. The detection limits for granzyme B (Diaclone, Gen-Probe, San Diego, CA, USA), FasL (Diaclone, Gen-Probe) and caspase-8 (Affymetrix, eBioscience, Santa Clara, CA, USA), were 20 pg/ml, 8 pg/ml and 0.1 ng/ml, respectively.

Staining of Natural Killer cell surface markers and flow cytometry. Prior to staining, the prestimulated lymphocytes were incubated with 4% autologous serum

in the FACS buffer: phosphate-buffered saline (PBS) supplemented with FCS for 30 min., at 4°C. Surface staining of CD markers was performed on ice, in the dark, for 30 min. in the presence of the experimentally adjusted amount of monoclonal antibodies (mAb) conjugated with fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP). The following labeled mAbs were used: anti-CD3-PerCP, anti-CD56-FITC, anti-Nkp46-FITC, anti-CD16-FITC or anti-CD25-PE (Affymetrics, eBioscience, Santa Clara, CA, USA). After staining, all samples were washed twice and suspended in 300 µl PBS. To ensure the proper identification of positive versus negative antigen expression, and to exclude non-specific binding, additional internal negative controls were used: unstained cells for the detection of autofluorescence and isotype control corresponding to the immunoglobulin G (IgG) clone used in the analysis (IgG1, IgG2a or IgG2b). The acquisition and immunophenotypic analysis were performed within one hour on a LSR2 Flow Cytometer (Becton Dickinson) and the FlowJo Software was used for cell analysis. Approximately 80 000 to 100 000 events were acquired and lymphocyte gating was set by a forward (FSC) and a side scattered light (SSC). A sequential gating strategy was employed to detect NK $CD25^+$ populations: $CD3^-CD56^+CD25^+$, $CD3^-CD16^+CD25^+$ and $CD3^-Nkp46^+CD25^+$. Briefly, a gate was set on viable lymphocytes (forward versus side scatter) (Fig. 2a), and the cells were further analyzed *versus* CD3 on a forward scatter (Fig. 2b). CD-negative population was then gated and further analyzed for the CD56 or Nkp46 or CD16, and CD25 co-expression (Fig. 2c). In order to evaluate the percentage of $CD25^+$ NK cells the percentages of $CD25^+$ cells among NK subpopulations were calculated.

Data analysis and statistics. The differences between *E. coli* LPS stimulated and non-stimulated values were tested using U-Mann-Whitney's U test for impaired data preceded by the evaluation of normality and homogeneity of variances. Results were considered statistically significant when $P<0.05$. The correlation was evaluated by Pearson's test where the correlation was considered positive when $r>0.5$ and $P<0.05$, and negative when $r>-0.5$ and $P<0.05$. For statistical analysis the STATISTICA 5.5 PL software was used (Stat Soft, Poland).

RESULTS

Target cell dependent cytotoxic activity and degranulation

The cytotoxic activity was expressed as a percentage of dead target cells co-cultured with unstimulated (natural cytotoxic activity) or stimulated (*E. coli* LPS) lym-

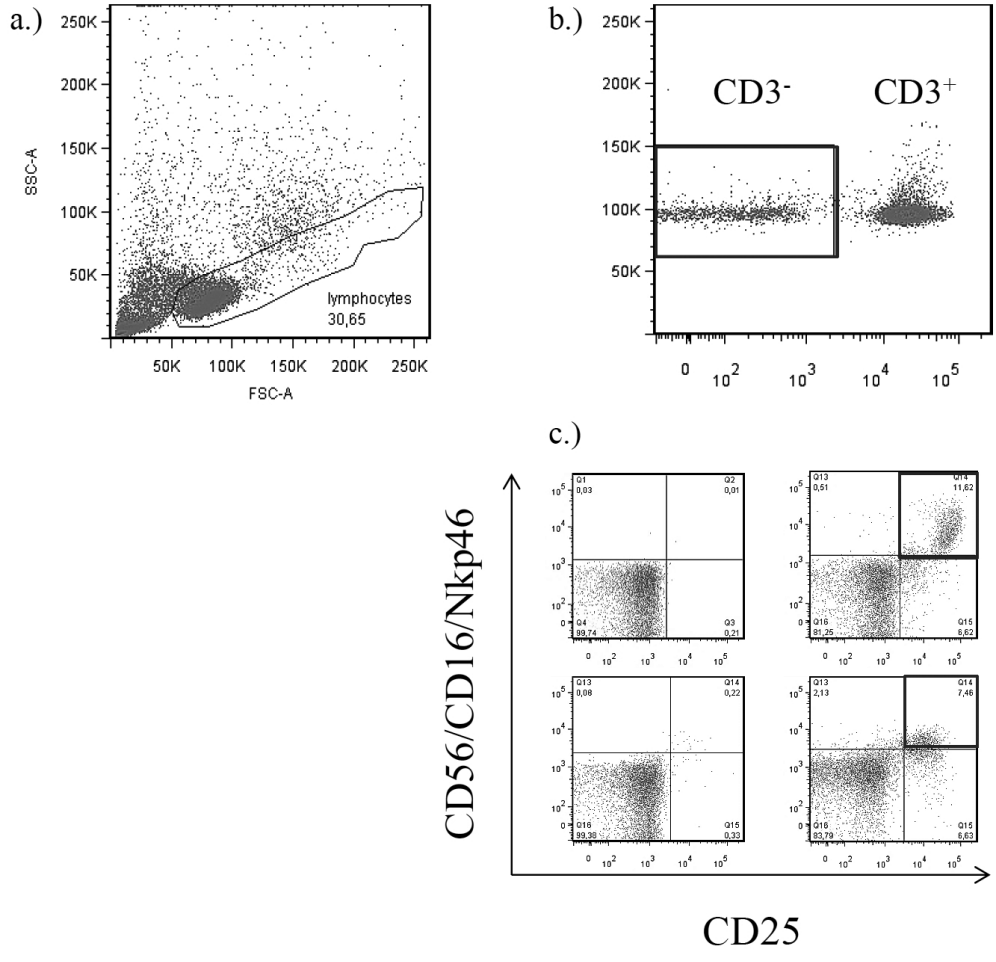

Figure 2. Gating strategy to evaluate NK cell populations.
The lymphocyte gate was set in forward (FSC) *versus* side scattered light (SSC) (**a**), then the gated lymphocytes were analysed for the presence/absence of CD3 (**b**), the $CD3^-$ cells were further analysed for the co-expression of CD56 or CD16 or Nkp46 and CD25 (**c**). Upper right quadrat on dot plot analysis represents $CD3^-CD56^+CD25^+$, $CD3^-CD16^+CD25^+$ and $CD3^-Nkp46^+CD25^+$ NK cell population. **Abbreviations**: NK, Natural Killer; FSC, forward scattered light; SCC, side scattered light.

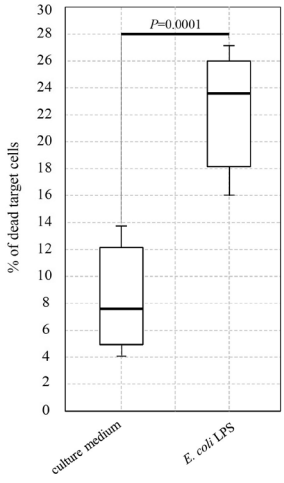

Figure 3. Cytotoxic activity of non-stimulated (culture medium) or *E. coli* LPS stimulated lymphocytes, evaluated in the cell cytotoxic assay towards HeLa target cells in MTT reduction test.
Presented values are equal to percentage of dead target cells after co-incubation with non-stimulated (culture medium) or stimulated (LPS of *E. coli*) lymphocytes. The box plots show the range of cytotoxic activity, with upper line indicating the highest value, the middle line showing the mean value, and the bottom line equal to the lowest value. **Abbreviations**: *E. coli, Escherichia coli*; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

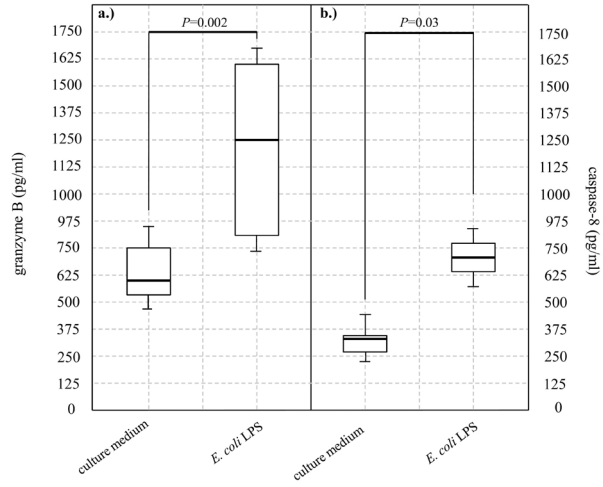

Figure 4. Concentration of granzyme B (a) and caspase-8 in culture supernatants from mixed lymphocyte-HeLa cell cultures after cytotoxic assay performed with non-stimulated or *E. coli* LPS-stimulated lymphocytes, measured by ELISA.
The box plots show the range of cytotoxic activity, with upper line indicating the highest value, the middle line showing the mean value, and the bottom line equal to the lowest value. **Abbreviations**: ELISA, enzyme-linked immnosorbent assay; *E. coli, Escherichia coli*; LPS, lipopolysaccharide.

phocytes calculated in relation to the target cells cultured alone (100% viability). All of the non-stimulated cells incubated in the culture medium alone exhibited a certain level of natural cytotoxic activity towards target cells. Box plots in Fig. 3 show the range of cytotoxic activity expressed as a percentage of dead target cells, with upper line indicating the highest value, the middle line showing the mean value and the bottom line equal to the lowest value. The mean natural cytotoxic activity was equal to $7.6 \pm 2.9\%$ peaking up to $23.3 \pm 6.8\%$ in cultures stimulated with the LPS of *E. coli*. Natural cytotoxic activity remained in the range of 5.2–12.1%, raising to the values of 18.2–25.5% in LPS-induced cultures. Altogether, the LPS of *E. coli* significantly enhanced the cytotoxic function of lymphocytes towards the target cells as measured in the MTT reduction assay ($P = 0.0001$). The upregulation of cytotoxic functions by LPS of *E. coli* and a loss of target cell viability measured in MTT reduction assay was accompanied by the degranulation of lymphocytes. Figure 4a shows the concentration of granzyme B and caspase-8 released by effector and target cells, respectively, after the cytotoxic assay.

Granzyme B concentration in the *E. coli* LPS prestimulated co-cultures was significantly higher (1274.5 ± 92.3 pg/ml) than the mean physiological level of granzyme B in non-stimulated co-cultures (605.3 ± 32.9 pg/ml) ($P = 0.002$). Interestingly, we have observed a highly variable concentrations of granzyme B (811.9–1605.4 pg/ml), although only in the cultures stimulated with LPS of *E. coli*. Moreover, it was shown that caspase-8 was produced more intensively in the cultures of target cells containing lymphocytes stimulated with *E. coli* LPS ($P = 0.03$). There was no recognizable variability in the concentration of caspase-8 neither in stimulated nor non-stimulated cultures (Fig. 4b).

Cytokine activity

The lymphocytes from all donors produced IL-2 and IFN-γ spontaneously (culture medium) with concentrations reaching the ranges of 15.6–25.4 pg/ml and 5.2–25.1 pg/ml, respectively (Fig. 5). The mean concentration of IL-2 in response to *E. coli* LPS was more than 20-times higher (228.3 ± 12.5 pg/ml) as compared to non-stimulated cultures (19.6 ± 4.8 pg/ml), $P = 0.0001$. The enhancement of cytotoxic activity induced by *E. coli* LPS was also accompanied by the increase in IFN-γ secretion reaching the mean concentration of 114.3 ± 11.2 pg/ml, $P = 0.003$. We have also observed that the *E. coli* LPS-mediated induction of lymphocytes to produce IL-2 and IFN-γ was highly variable depending on the individual response to stimuli, reaching the ranges of 176.2–275.2 pg/ml and 95.4–148.2 pg/ml, respectively,

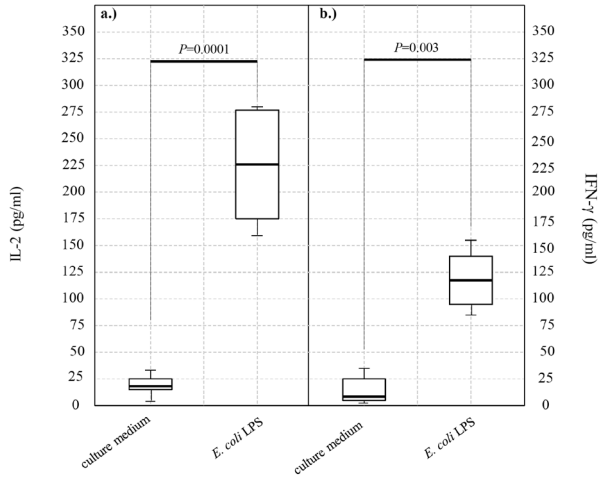

Figure 5. Concentration of IL-2 (a) and IFN-γ in culture supernatants from lymphocyte-HeLa cell mixed cultures after cytotoxic assay performed with non-stimulated or *E. coli* LPS-stimulated lymphocytes, measured by ELISA.
The box plots show the range of cytotoxic activity, with upper line indicating the highest value, the middle line showing the mean value, and the bottom line equal to the lowest value. **Abbreviations**: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; IL-2, interleukin-2; LPS, lipopolysaccharide.

Table 1. The distribution of CD25 receptor on the surface of NK cell subsets after stimulation with *E. coli* LPS in the context of non-stimulated culture.

$CD25^+$ NK cell subsets	culture medium		*E. coli* LPS		*P* value
	[%]*	MFI	[%]*	MFI	
$CD3^-CD56^+CD25^+$	9.8±4.7	128±34	33.3±11.9	892±42	*P*=0.009
$CD3^-CD16^+CD25^+$	8.7±1.8	111±17	7.7±2.6	104±29	*P*=0.782
$CD3^-Nkp46^+CD25^+$	5.6±1.6	98±32	7.5±4.8	102 ±44	*P*=0.314

Abbreviations: NK, Natural Killer; MFI, mean fluorescence index; LPS, lipopolysaccharide. *In total lymphocyte fractions

however this high variability was more pronounced in case of IL-2.

CD25 expression on NK cells

In order to address the question whether the cytotoxic effect observed in target cell-induced cytotoxic assay is a result of NK cell activity and the co-expression of CD25 molecule, in the second stage of the study we have analysed the influence of *E. coli* LPS on the CD25 expression on three different NK cell subsets. We have demonstrated that the enhancement of cytotoxic activity induced by *E. coli* LPS is accompanied by the upregulation of CD25 expression exclusively on $CD3^-CD56^+CD25^+$ NK cells, with no influence on the expansion of $CD3^-CD16^+CD25^+$ or $CD3^-Nkp46^+CD25^+$ (Table 1).

The untreated PBMCs contained small percentages of $CD3^-CD56^+CD25^+$ NK cells (9.8±4.7%), whereas in cultures stimulated with the LPS of *E. coli,* 33.3±11.9% of $CD3^-CD56^+$ NK cells expressed the CD25 receptor (*P*=0.009). As it is presented on Fig. 6, the increase in the percentage of dead target cells corresponds with the upregulation of CD25 expression on $CD3^-CD56^+$ cells able to exhibit target cell induced cytotoxic activity (Fig. 6). The intensity of CD25 expression on $CD3^-CD56^+$ cells differs to some extent between donors, however, the extend of variability was similar in non-stimulated and LPS induced cultures.

Correlation between target cell induced cytotoxic activity and CD25 expression

Comparative analysis of cytotoxic activity markers: percent of dead target cells, granzyme B, caspase-8 and FasL, as well as IL-2 and IFN-γ release, were compared to the variations in the CD25 expression on NK cells after stimulation with the LPS of *E. coli.* Based on the results obtained by the Persons test presented in Table 2, CD25 expression was positively correlated with the cytotoxic activity measured by the percentage of dead target cells (Fig. 7a), degranulation monitored by the granzyme B release after cytotoxic assay (Fig. 7b), production of IL-2 (Fig. 7d) and IFN-γ (Fig. 7e), however, it was not positively correlated with caspase-8 (Fig. 7c) and FasL (Fig. 6f) concentrations. The parameters with the strongest association with increased CD25 expression on $CD3^-CD56^+$ cells include granzyme B release (*r*=0.89; *P*=0.0008) and IL-2 production (*r*=0.78; *P*=0.0009). As shown in Fig. 7b, an increase in CD25 expression was almost accurately correlated with granzyme B release (Fig. 7b). Similarly, the higher the expression of CD25, the higher the production of IL-2 (Fig. 7d). To a low-

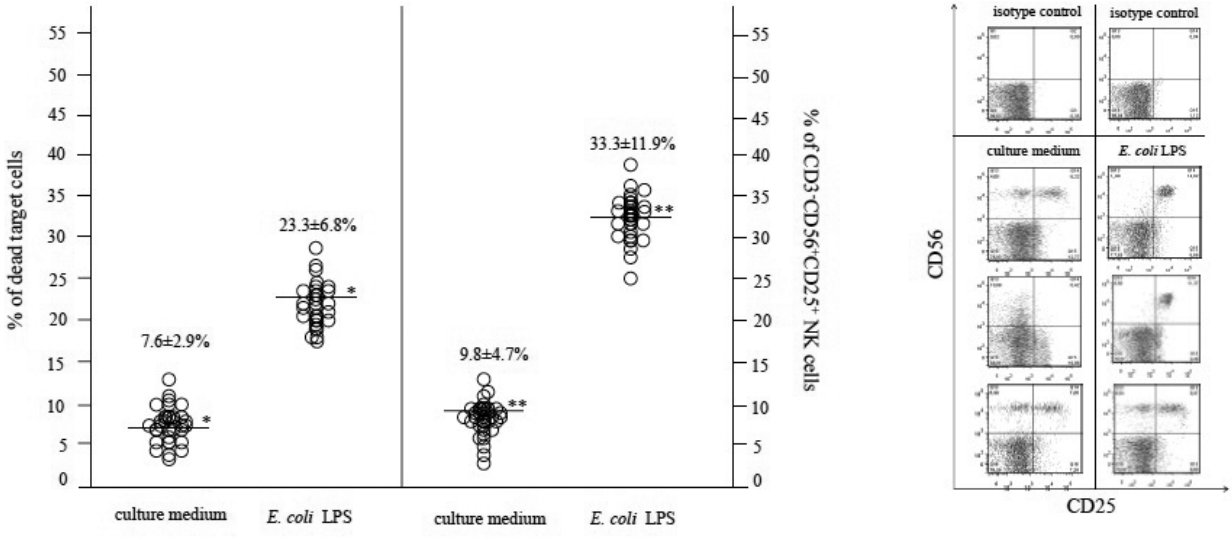

Figure 6. The association of target cell induced cytotoxic activity and expression of CD25 on $CD3^-CD56^+$ NK cells, non-stimulated and treated with the LPS of *E. coli*.
The dot plots show representative samples of non-stimulated and *E. coli* LPS stimulated lymphocytes gated on $CD3^-$ cells and analysed for the co-expression of CD56 and CD25 receptors (upper right quadrats). **Abbreviations**: *E. coli, Escherichia coli*; NK, Natural Killer; LPS, lipopolysaccharide.

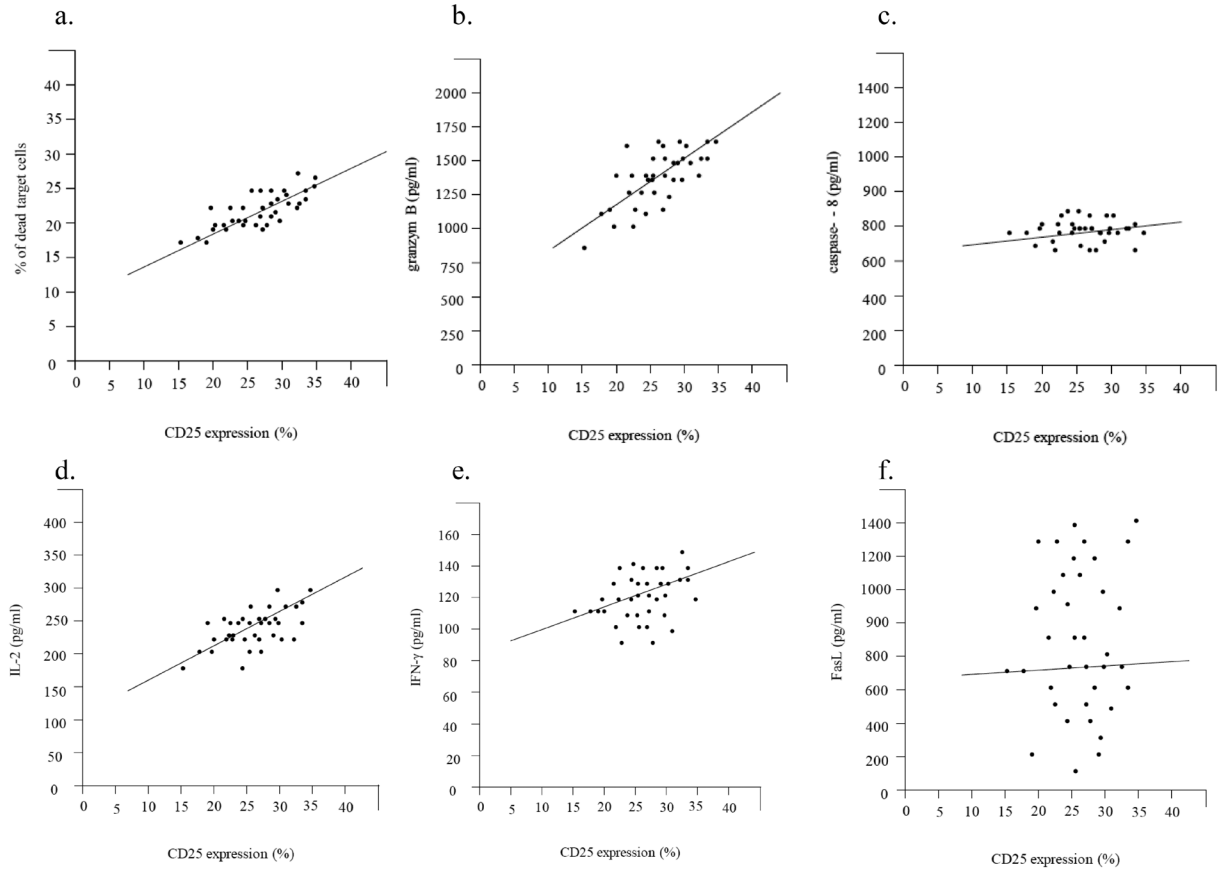

Figure 7. Correlation of CD25 expression on CD3$^-$CD56$^+$ NK cells with classic cell cytotoxicity markers: (**a**) percentage of dead target cells after cytotoxic assay measured by MTT reduction test, quantification of soluble cytotoxicity markers: granzyme b (**b**), caspase-8 (**c**), and FasL (**d**), as well as cytokine production: IL-2 (**e**) and IFN-γ (**f**), by ELISA. **Abbreviations**: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; IL-2, interleukin-2; LPS, lipopolysaccharide.

er extent, the CD25 expression was also positively correlated with the classic target cell induced cytotoxicity measured by MTT reduction assay (r=0.68; P=0.004) (Fig. 7a).

A weak association was found for the IFN-γ production (r=0.57; P=0.007) quantified by ELISA (Fig. 7d). Furthermore, we have observed no correlation (P>0.05) between CD25 expression and caspase-8 release. As shown in Fig. 7c, higher expression of CD25 is not accompanied by any subsequent elevation of caspase-8 –its concentration remained stable, regardless of the increase in CD25 expression (Fig. 7c). Also, no association was observed for the FasL levels – they were highly variable and not associated with CD25 expression patterns (Fig. 7f).

Table 2. The correlation between CD25 expression on CD3$^-$CD56$^+$ NK cells and the release of soluble cytotoxicity markers (granzyme B, caspase-8 and FasL) as well as cytokine production (IL-2, IFN-γ).

CD25 expression on CD3$^-$CD56$^+$ NK cells	assay	source	*r* value*
% of dead target cells	MTT	T	r=0.68**
granzyme B	ELISA	E	r=0.89*
caspase-8	ELISA	T	r=0.18***
FasL	ELISA	E	r=0.13***
IL-2	ELISA	E	r=0.78*
IFN-γ	ELISA	E	r=0.57**

Abbreviations: NK, Natural Killer; E, effector cell; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; IFN-γ, interferon-γ; IL-2, interleukin-2; LPS, lipopolysaccharide; T, target cell.*P<0.001, **P<0.01, ***P>0.05

DISCUSSION

Cytotoxic activity is one of the major functions of NK cells and it is a critical effector mechanism of innate immunity against infected or cancer cells. NK cells respond to viral, bacterial and tumor compounds by cytotoxic activity against target cells and rapid production of cytokines (Caligiuri, 2008; Moretta *et al.*, 2014). Cytotoxic activity is manifested by the release of granzymes and perforins, target cell lysis or TNF-receptor based activation pathway. Due to the expression of NCR receptors and TLRs, including TLR4, NK cells might also respond to bacterial antigens such as lipopolysaccharides (Chalifour *et al.*, 2004; Marcenaro *et al.*, 2008; Souza-Fonseca-Guimaraes *et al.*, 2012; Chaushu *et al.*, 2012).

A variety of assays has been developed to determine NK cell cytotoxic activity. Majority of them are based on target cell induced cytotoxic tests, where settled target cells (L929 fibroblasts or HeLa epithelial cells) are incubated with pretreated effector cells-lymphocytes. Following a four incubation, the viability of target cells is

estimated by standard MTT reduction assay or evaluated indirectly by soluble markers of cytotoxicity, released by target cells (caspases) or effector cells (perforins and granzymes), however, a receptor-based method for NK cell cytotoxic activity still has not been established yet (Baran *et al*., 2001; Das *et al*., 2001; Shafer-Weaver *et al*., 2003; Blom *et al*., 2009; Al-Hubeshy *et al*., 2011; Meinhardt *et al*., 2012).

We have developed a receptor-based tool to evaluate cytotoxic activity of human NK cells within unfractionated peripheral blood leukocytes. In our *E. coli* LPS-stimulated model, we have shown that the monitoring of CD25 expression on $CD3^{-}CD56^{+}$ cells enables to predict target cell induced cytotoxic function, degranulation and proinflammatory cytokine release. Activation of NK cells is manifested in various manners: exhibition of cytotoxic activity and induction of target cell death (mainly apoptosis), degranulation (production of perforins and granzymes) and rapid release of proinflammatory cytokines such as IFN-γ and IL-2. In order to establish whether the CD25 surface receptor might become a marker of activated NK cells, in the first stage of this study we have aimed to show that the stimulator used in the study — the *E. coli* LPS, induces all of the fundamental functions of NK cells, and in the next stage, if the occurrence of all these features is accompanied by the upregulation of CD25.

In vitro stimulation of PBMCs with *E. coli* LPS resulted in a significant enhancement of lymphocyte cytotoxic activity which was manifested by higher percentage of dead targets as evaluated with the MTT reduction test. Apart from the direct evidence of target cells being killed by activated cytotoxic cells, we have shown that the enhanced cytotoxic activity mediated by *E. coli* LPS was associated with the degranulation and the significant upregulation of granzyme B in the cell culture supernatants after cytotoxic assay. The activation of NK cells by *E. coli* LPS was also associated with their cytokine function: IL-2 and IFN-γ detected after LPS stimulation. Interestingly, upon *E. coli* LPS stimulation we have observed a high individual variability in granzyme B and IL-2 production. This heterogeneity in immune response might be a result of genetic predisposition of a host to respond to bacterial lipopolysaccharides. Due to that, human population might be divided into "*responders*" rapidly and intensively responding to LPS stimulation, and "*non-responders*" whose immune cells are less susceptible to LPS stimulation (Cavaillon & Adib-Conquy, 2006; Gioannini & Weiss, 2007). The effect generated by bacterial LPSs greatly depends on their structure, especially in relation to lipid A and sugar O-chains (Chmiela *et al*., 2014; Maeshima & Fernandez, 2013; Lerouge & Vanderleyden, 2002). Previously, we have shown that due to its unusual structure the LPS of *Helicobacter pylori* (*H. pylori*) downregulates cytotoxic, proliferative and cytokine responses of NK cells (Rudnicka *et al*., 2012, 2015).

The lack of FasL association with LPS-mediated activation of NK cells might be explained by the involvement of molecules other than FasL as triggers of target cell apoptosis. FasL is bound by Fas receptor and it triggers the receptor trimerization and consequent aggregation of the intracellular death domains (DDs) which then recruit caspase-8 (Shalini *et al*., 2015; Rudnicka *et al*., 2011). Since we observed the upregulation of caspase-8 and the lack of FasL increase, it is possible that some other death inducer than FasL is involved in this process. Knockout data indicate that besides FasL, tumor necrosis factor receptor 1 and death receptor 3 might be involved in apoptosis initiation. In addition, a direct, non-death-receptor pathway inducing caspase-8 dependent apoptosis was also described (Reytoma *et al*., 1999; Liu *et al*., 2000; Kruidering & Evan *et al*., 2000).

To fully address the question whether the NK cell activation might be monitored by the CD25 marker, we have evaluated the expression of this receptor on three NK cell subsets after LPS stimulation by flow cytometry. The data generated indicates that the enhancement of cell cytotoxicity was accompanied by the expansion of $CD3^{-}CD56^{+}CD25^{+}$ and had no influence on $CD3^{-}CD16^{+}CD25^{+}$ or $CD3^{-}Nkp46^{+}CD25^{+}$ NK subsets.

The exclusive domination of $CD3^{-}CD56^{+}$ cells with the co-expression of CD25 marker is probably due to the unique, although complex nature of this NK cell subset. In healthy individuals the $CD56^{bright}$ population constitutes 10% of all peripheral blood NK cells and their basic cytotoxic activity is lower than that of $CD56^{dim}CD16^{bright}$. However, the $CD56^{bright}CD16^{-}$ cells are more potent producers of cytokines such as IFN-γ, TNF-α, IL-10 and IL-13, they exhibit higher expression of activation receptors, and constitutively express IL-2R. Upon stimulation with cytokines such as IL-2 or IL-12, the cytotoxic activity of all NK cell subsets (including $CD56^{brigh}$ population) dramatically increases.

In our experiments, the expansion of $CD3^{-}CD56^{+}CD25^{+}$ cells was accompanied by IL-2 release and since only $CD56^{bright}$ NK cells constitutively express IL-2 receptor they might first respond, activate and finally degranulate. This explains our observation that the higher cytotoxic activity (induced by LPS of *E. coli*) is accompanied by the upregulation of CD25 marker exclusively on $CD3^{-}CD56^{+}$ cells and not on $CD3^{-}CD16^{+}$ or $CD3^{-}Nkp46^{+}$ cells. Previously, in our study we have demonstrated that the LPS of *E. coli* induced the expansion of $CD56^{bright}$ cells and the propagation of NK cells with intracellular localization of IFN-γ and IL-2 (Rudnicka *et al*., 2015). Since cytotoxic function of NK cells might occur via different pathways, it is possible that in our study the mechanism involving CD16 (FcγRIII) - a receptor for Fc fragment of Igs, is not associated with LPS induced target cell cytotoxicity, and since there is no upregulation in CD25 expression on $CD3^{-}CD16^{+}$ cells, the occurrence of ADCC pathway might be excluded. In this case, the direct recognition of *E. coli* LPS by TLR4 on NK cells might be considered. Marcenaro and colleagues have shown that NK cells from healthy donors can directly respond to products *via* TLR2 (Marcenaro *et al*., 2008). Similarly the NKp46 receptor, belonging to NCRs, has been shown to recognize the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus, and is probably involved in a direct recognition of viruses rather than LPSs of Gram-negative bacteria (Mandelboim *et al*., 2001). Probably due to that we did not observe the activation and responsiveness of the NKp46+ in the NK cell subset.

To summarize, although in general the $CD56^{bright}$ and $CD56^{dim}$ NK subsets have their distinct functions and phenotypes, their role strongly depends on the environment, localization in the human body and a nature of stimulus triggering the response. For example, peripheral $CD56^{bright}$ NK cells are involved in the pathogenesis of endotoxic shock and upon LPS stimulation rapidly release IFN-γ and TNF-α (Chiche *et al*., 2012), but on the other hand uterus (u) NK cells, despite having a whole cytotoxic machinery, release anti-inflammatory IL-10 and maintain tolerogenic homeostasis between trophoblast and uterus (Sharma *et al*., 2014).

Finally, to answer the question if the CD25 marker can be used for preliminary screening of NK cell cyto-

toxic activity, we performed a comparative analysis to assess the correlation between the CD25 expression on $CD3^-CD56^+$ NK cells and other parameters included in the study. Our findings revealed that CD25 expression was positively correlated with the cytotoxic activity measured by the percentage of dead target cells, degranulation monitored by the granzyme B release after cytotoxic assay, production of IL-2 and IFN-γ, however, it was not positively correlated with caspase-8 or FasL concentrations. The highest correlation rate and most reliable association was observed for CD25 vs granzyme B and CD25 *vs* IL-2 concentrations. This shows that the upregulation of CD25 on NK cells is strictly associated with their cytotoxic (degranulation) function as well as cytokine activity. Since CD25 constitutes the receptor for IL-2, it is reasonable that the presence of IL-2 upregulates IL-2R expression. It was also shown that CD69 receptor might serve as a marker of NK cell activation, however, its expression disappears a few hours after stimulation, whereas the CD25 prior binding IL-2 is internalized and recycled back on the surface of NK cells, which makes it a more reliable marker of long-term activation, even when contact with the stimuli will be limited (Clausen *et al*., 2003). The lack of correlation between CD25 expression and FasL might be explained by the involvement of molecules other than FasL in the process of apoptosis. Since caspase-8 is the first to be activated and is rapidly degraded by caspase-9, we suspect that downstream caspases should be further evaluated in the context of CD25 expression.

The obtained results support our hypothesis that the presence of CD25 on NK cells is not only a sign of their activation but is also a functional marker of active degranulation and subsequent target cell killing. In conclusion, our results indicate that the CD25 receptor quantification can be used for preliminary screening of NK cell cytotoxic activity in unfractionated PBMCs cultures previously stimulated with bacterial compounds. Considerably high variability in response to bacterial LPS occurred in regard to granzyme B production and IL-2 secretion. For this reason, it is important to use CD25 evaluation on $CD3^-CD56^+$ cells in combination with one of these methods.

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