Natural Killer Cell Cytotoxic Activity:

Measurement of the Apoptotic

Inducing Mechanisms

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Abstract

Natural Killer cell cytotoxic activity is vital for the clearance of viral and malignantly transformed cells. The aim of this study was to investigate the apoptotic inducing mechanisms of NK cells to propose an additional measurement for NK cell effector function. 19 healthy controls (age=31±7.2 years) participated in this study. Flow cytometric protocols assessed NK cell cytotoxic activity against tumour K562 cells, lytic proteins, degranulation and interferon gamma production. Perforin release was significantly correlated with cytotoxic activity (r=-0.46, p<0.05) and degranulation (r=-0.60, p<0.05). These results suggest that perforin may be an additional measure for NK cell cytotoxic effector function.

Keywords: Cytotoxic activity, Lytic Proteins, Degranulation

INTRODUCTION

Natural Killer (NK) cells are innate immune cells which lyse infected and malignantly transformed cells by cytotoxic activity [10]. Cytotoxic NK cells contain high numbers of secretory granules which store and release death-inducing proteins such as perforin and granzymes [18]. These lytic proteins are stored in granules surrounded by a lipid bilayer which contain lysosomal associated membrane glycoproteins including CD107a, CD107b and CD63 [25]. The granule secretory pathway is the predominant pathway used for NK cell cytotoxic activity [24]. NK cell recognition of a target cell activates exocytosis of the lytic proteins in a process known as degranulation, releasing perforin and granzymes into the immune synapse [12].

Perforin facilitates the delivery of serine proteases known as granzymes into the target cell by forming a pore either on the endosome or plasma membrane of the target cell [15, 31]. The granzymes released activate three distinct pathways of apoptosis in the target cell. In humans, granzyme B (GrzB) and granzyme A (GrzA) are the most potent activators of apoptosis [5, 14]. GrzB induces apoptosis via activation of a caspase cascade or the mitochondrial pathway which is kinetically slower to activate [4, 15]. Both pathways lead to the activation of deoxyribonuclease (DNase), which fragments the double stranded DNA, leading to rapid cell lysis [5]. GrzA induces target cell apoptosis independent of caspase activation by targeting the endoplasmic reticulum associated complex for proteolysis which activates a DNase causing single stranded nicks in the DNA [4, 15]. The DNase works in combination with 3′ repair exonuclease (Trex1), preventing DNA repair by blocking the ends from re-annealing and leads to cytotoxic lysis of the target cell [5].

NK cells also initiate target cell apoptosis through the death receptor pathway [24]. Tumour necrosis factor (TNF) ligands expressed on NK cells bind to the Fas (CD95/Apo-1) and TNF-related apoptosis inducing ligand (TRAIL) on the target cell to induce apoptosis [29, 32]. The death receptor pathway is augmented by NK cell production of the cytokine interferon gamma (IFN-γ) [28, 29]. IFN-γ increases cell surface expression of the ligands for TRAIL and Fas and also sensitizes the target cell to the cytotoxic effects of the death receptor pathway by acting as transcriptional targets for pro-apoptotic genes [13, 28, 29].

The ability to measure NK cell cytotoxic activity has important implications in the clinical setting where reduced activity is associated with susceptibility to severe infections [30]. Traditional assays that measure NK cell cytotoxic activity include the chromium release assay (CRA) and the flow cytometric based cytotoxic assay which measure target cell lysis induced by NK cell cytotoxic activity [12]. Further investigations into the NK cell cytotoxic activity pathway may provide additional measurements for the NK cell effector functions responsible for inducing apoptosis in the target cell [21]. The aim of this study was to investigate the apoptotic inducing mechanisms of NK cells to determine an additional measurement for NK cell cytotoxic activity. NK cell cytotoxic activity was compared in samples of peripheral blood mononuclear cells (PBMCs) and isolated NK cells to determine if preferentially isolated NK cells have an increased sensitivity to cytotoxic activity.

METHODS

Study Participants
19 healthy volunteers (10 male, 9 female, age: 31±7.2 years) donated whole blood samples. A full blood count (FBC) analysis including a five-part differential and C reactive protein test confirmed participant inclusion for the study.

Cells
Ficoll-Hypaque density gradient centrifugation was used to isolate the PBMCs (GE Health Care, Uppsala). A MACS NK cell negative isolation kit (Miltenyi Biotec, Teterow) separated the NK cells from the PBMCs according to the manufacturer's instructions. After isolation, the PBMCs and isolated NK cell samples were adjusted to a concentration of 1×10^6 cells/ml with RPMI-1640 (Invitrogen Life Technologies, Carlsbad) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad), 1% streptomyocin/penicillin (Invitrogen Life Technologies, Carlsbad), sodium pyruvate solution (Invitrogen Life Technologies, Carlsbad) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (Invitrogen Life Technologies, Carlsbad).

NK Cell Cytotoxic Activity Assay

The ability of NK cells to lyse K562 tumour cells was measured in samples of PBMCs and isolated NK cells on the flow cytometer as previously described [6]. Briefly, the effector cells were labelled with Paul Karl Horan (PKH)-26 (Sigma-Aldrich, St. Louis) and combined with target K562 cells (1×10^5 cells/ml) at three effector to target ratios (25:1, 50:1 and 100:1) to determine the dose response relationship between the number of K562 cells lysed by the NK effector cells. Each sample was plated in duplicate and a control K562 sample was included. The cells were incubated for 4 hours at 37°C with 5% CO_2. Post incubation, 7 amino-actinomycin D (7-AAD) (BD Biosciences, San Jose) and fluorescein isothiocyanate (FITC) Annexin V (BD Bioscience, San Jose) were added to determine the number of K562 cells in apoptosis on the FACS-Calibur flow cytometer (Becton Dickinson [BD] FACSCalibur, San Jose). A total of 10,000 events were analysed and the number of events in each region were used to determine NK cell cytotoxic activity according to methods previously described [6].

Intracellular Staining of the Lytic Proteins

Intracellular staining was used to detect the presence of perforin, Grz A and GrzB in NK cells [17]. Control and K562 cell stimulated (25:1) samples with either PBMCs or NK cells (1×10^6 cells/ml) were plated in duplicate and placed in the incubator at 37°C with 5% CO_2 for 4 hours. Following incubation, fluorochrome-conjugated monoclonal antibodies were added for surface staining of the cluster of differentiation (CD) specific for NK cells. For the perforin and GrzA samples, CD16 FITC was added and CD56 phycoerythrin (PE) was added to the GrzB samples. Monoclonal antibodies, perforin PE, GrzA PE and GrzB FITC (BD Biosciences, San Jose) were added to each respective sample for analysis on the flow cytometer. A total of 10,000 events were analysed for each sample to determine the percentage of gated lymphocytes CD56+/CD16+ and the lytic proteins.

Degranulation and Interferon Gamma Measurement

NK cell expression of CD107a was measured as a marker for degranulation and intracellular staining determined IFN-γ production [2]. CD107a and IFN-γ required the addition of Monensin (BD Bioscience, San Jose) to prevent CD107a degradation and Brefeldin A (BD Bioscience, San Jose) to block the exocytosis of IFN-γ [2, 8]. PBMC and NK cells (1×10^6 cells/ml) were stimulated with K562 cells (1×10^5/ml) at a ratio of 25:1 or 10ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis) and 1μg/ml of ionomycin (I) (Sigma-Aldrich, St. Louis) [1, 2]. CD107a FITC was added to all the samples to detect degranulating NK cells. The samples were plated in duplicate and incubated at 37°C with 5% CO_2 for 6 hours. Following incubation, CD56 PE (BD Bioscience, San Jose) was added and intracellular staining determined IFN-γ production. A total of 10,000 events were collected and analysed on the flow cytometer to determine the percentage of gated lymphocytes positive for CD107a and IFN-γ.

Statistical Analysis

Statistical analysis was completed on GraphPad PRISM (version 6). An independent sample T-test was used to identify significant differences between males and females for the FBC and C-reactive protein test. A repeated measures analysis of variance (ANOVA) for two dependent variables was conducted on the data collected from the K562 lysis, lytic proteins and CD107a/IFN-γ assays. Bonferronii's multiple comparisons identified significance where the P value was less than 0.05. Spearman's correlation identified any significant relationships between the apoptotic inducing mechanisms and cytotoxic activity determined by K562 lysis.

RESULTS

Participant Characteristics

Participant inclusion in the study was confirmed by a FBC and C reactive protein test. Significant increases (p<0.05) were observed in white blood cells, monocytes, red blood cells, haemoglobin, haematocrit and mean corpuscular haemoglobin concentration when the male means were compared to the females (Table 1).

Increased NK Cell Cytotoxic Activity in PBMCs

NK cell cytotoxic activity was determined by the number of apoptotic and lysed K562 cells. In the PBMC samples, increasing the effecter to target ratio resulted in a significant increase (p<0.01) in NK cell cytotoxic activity when the 25:1 ratio was compared with 50:1 and 100:1 (Figure 1[A]). No significant differences were observed for cytotoxic activity in the samples of isolated NK cells (Figure 1 [B]). Comparison of cytotoxic activity at different ratios in the PBMCs and isolated NK cells showed no significant differences (data not shown).

Reduced NK Cell Lytic Proteins in PBMCs

Perforin, GrzA and GrzB in NK cells was determined in samples of PBMCs (Figure 2 [B]) and isolated NK cells (Figure 2 [C]). Lytic proteins were compared in control and K562 stimulated samples and no significant changes were observed. Expression of the lytic proteins was higher in the isolated NK cell samples.

Increased NK Cell Degranulation and IFN-γ Following Stimulation

NK cells were stimulated to degranulate and produce IFN-γ. Comparison of the K562 stimulated cells to a control sample showed no significant difference in the expression of CD107a and IFN-γ (Figure 3[B]). When the results for the PMA/I stimulated cells were compared with a control sample, a significant increase (P<0.05) in the expression of CD107a and IFN-γ was observed in both PBMC and isolated NK cell samples.

Correlations of NK Cell Apoptotic Mechanisms and Cytotoxic Activity
A significant correlation was identified between NK cell perforin and cytotoxic activity at 25:1 (Table 2). In NK cells stimulated with K562 cells, a significant correlation was also observed between lytic proteins (perforin, GrzA and GrzB) and CD107a expression.

DISCUSSION

Cytotoxic activity is an essential process for maintaining health as it ensures the removal of pathogen infected and malignantly transformed cells. The present study examined apoptotic inducing mechanisms in the NK cell cytotoxic activity pathway to determine an additional measurement for NK cell effector function. The results showed that perforin was significantly correlated with cytotoxic activity and degranulation, indicating that it may be an additional measurement for NK cell cytotoxic activity.

Routine health assessments of the participant population identified no confounding variables that may affect NK cell cytotoxic activity measurement. Whilst significant differences in the means between males and females were observed in some of the FBC parameters, gender differences caused by genetic variation and three anaemic females may account for the differences observed [7, 11].

NK cell cytotoxic activity for the healthy population in this study was consistent with the value of 41.1±15% reported in the literature [16]. The dose response relationship for the PBMCs significantly increased as the effector to target ratio increased. This was also associated with a significant increase in cytotoxic activity. In the isolated NK cells, increasing the effector to target ratio had no significant effect on cytotoxic activity. This may be explained by the stringent regulation of the resting NK cells, which prevents autoimmune derived diseases [9].

Lytic proteins are the apoptotic inducing mechanisms released from the cytotoxic granules of NK cells. Compared to the PBMCs samples, a significant increase in perforin, GrzA and GrzB in NK cells were observed in the isolated NK cells. Reduced levels of lytic proteins in the PBMC samples may be explained by the presence of other lymphocytes including macrophages, T cells and B cells [34]. CD56$^+$ NK cells comprise of 15±8% of the total circulating lymphocytes which may explain the reduced levels of lytic proteins in the PBMC samples [23, 33]. Hence, in comparison to the total lymphocyte population in the peripheral blood, there are fewer NK cells available which translates into less lytic protein levels.

NK cells are required to be activated from the resting state to elicit cytotoxic effects. Degranulation is a critical step required for the release of the lytic proteins from the secretory granule in the NK cell [25]. No significant differences were

observed in CD107a expression and IFN-γ production between the PBMC and isolated NK cell samples. Stimulation of NK cells with PMA/I upregulated CD107a and IFN-γ. PMA is a substitute for diacylglycerol (DAG), one of the adaptor proteins required for the activation of protein kinase C [3, 20]. Ionomycin is a selective calcium ionophore which increases intracellular calcium levels [19]. Therefore, the combination of PMA/I facilitates the activation of protein kinase C and an influx of intracellular calcium which are the necessary signalling events for degranulation [3, 33].

Perforin in NK cells was significantly correlated with cytotoxic activity and degranulation. Both the correlations were negative, suggesting that a decrease in perforin was associated with an increase in degranulation and cytotoxic activity. The literature supports the correlation between CD107a expression and perforin release, further highlighting the importance of CD107a expression as a marker of NK cell activation for cytotoxic activity [2, 8]. No correlation was identified between CD107a expression and NK cell lysis of the K562 cells. The literature reports positive correlations between CD107a expression and NK cell cytotoxic activity. NK cell expression of CD107a from this study population may not have been correlated with cytotoxic activity due to the 25:1 ratio used. Lower ratios ranging from 1:1 and 10:1 have been reported as optimal ratios for detecting CD107a expression [1, 2, 22, 26, 27].

The correlation of perforin release with the lysis of tumour cells and degranulation suggests that perforin may be an additional measurement for NK cell effector function. As perforin is a lytic protein released from NK cells to induce apoptosis in the target cell, measurement of perforin may be beneficial in the clinical setting for identifying deficiencies affecting cytotoxic activity.

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Figure Legends

Figure 1: NK cell cytotoxic activity in samples of PBMCs (A) and isolated NK cells (B). The box plots show NK cell lysis of K562 cells at three ratios. The boxes for each ratio represent the interquartile range (IQR), showing the distribution of the data. The middle line in each box represents the median value. *Denotes significance (**$p<0.01$ and ***$p<0.001$).

Figure 2: Lytic proteins in NK cells. Flow cytometry figures from an isolated NK cell sample (A) represent the number of CD56+/CD16+ NK cells expressing perforin, GrzA and GrzB. Lytic proteins in PBMC samples (B) and isolated NK cell samples (C) showed no significant difference following pathogenic stimulation. Data is presented as the mean ± the standard error of the mean.

Figure 3: CD107a and IFN-γ in PBMCs and isolated NK cells. Flow cytometric figures represent NK cells from a PBMC sample expressing CD107a and IFN-γ (A). In the control sample, 1.22% of the gated lymphocytes expressed CD107a and IFN-γ. When the cells were stimulated with K562 cells and PMA/I, the number of NK cells expressing CD107a and IFN-γ increased to 15.84 and 19.60% respectively. The IQR for the PBMC and NK cell samples increases from the control to the K562 and PMA/I stimulated cells (B). The median values for the samples are represented by the line in the centre of the box. *Denotes significance ($p<0.05$).

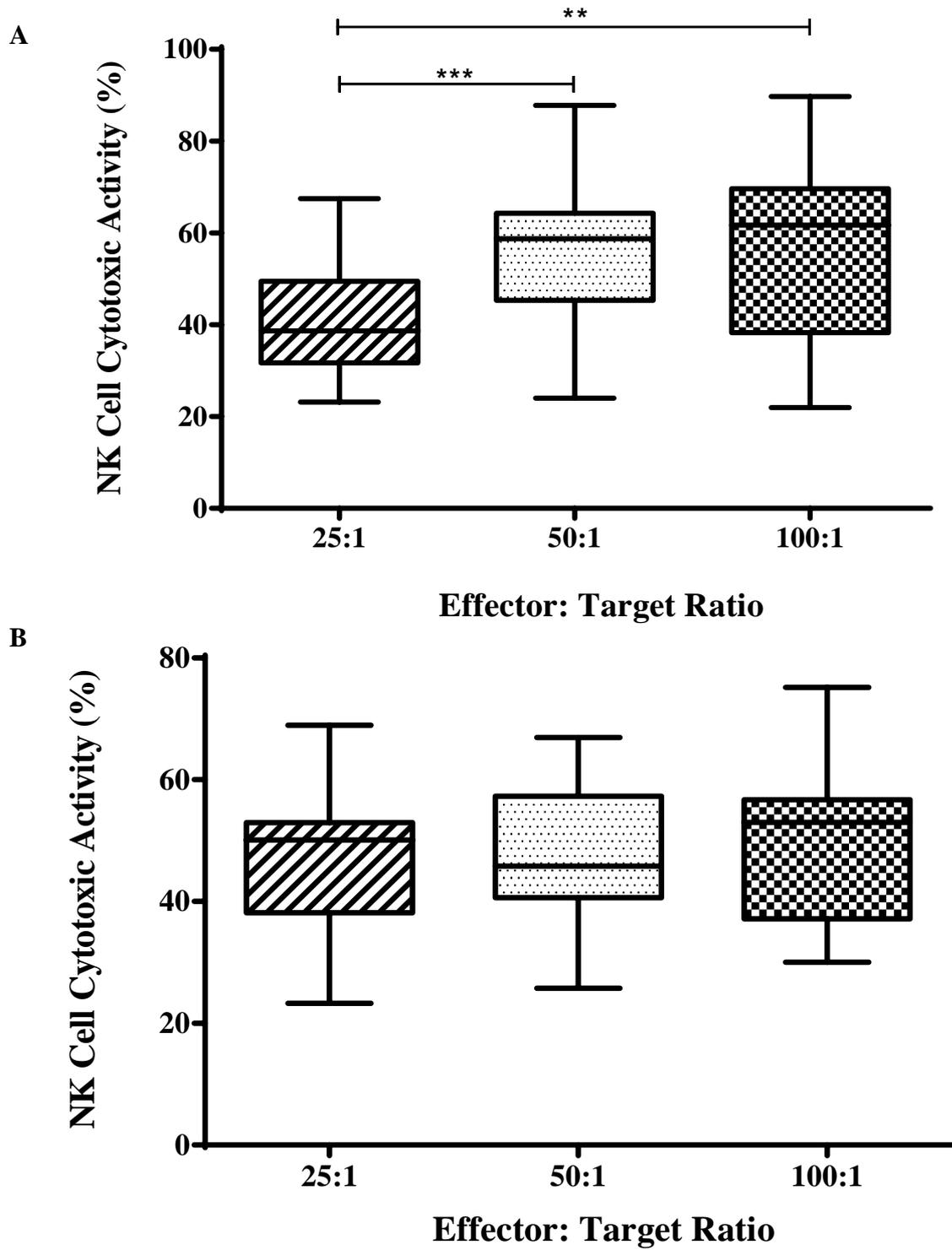Figure 1
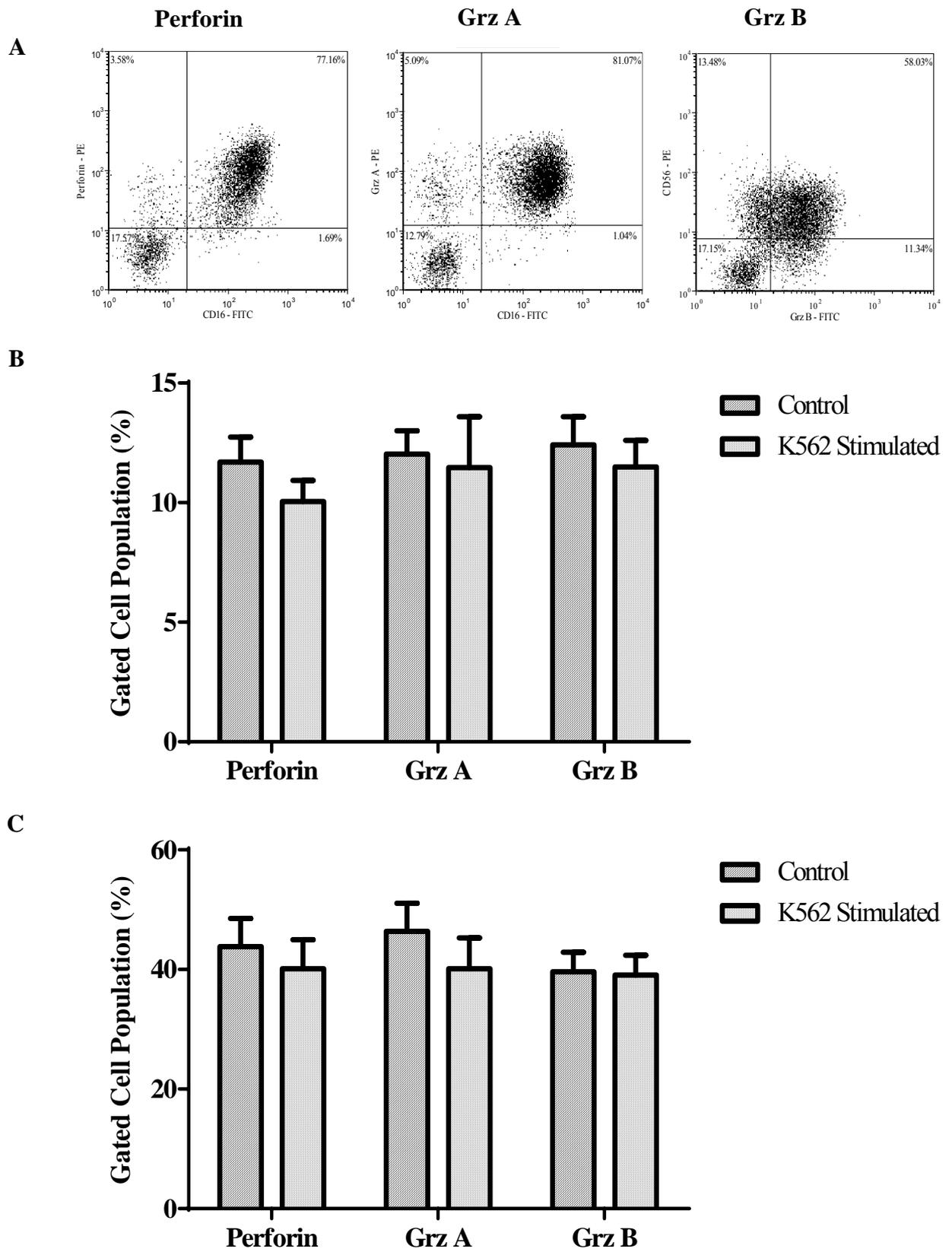
A

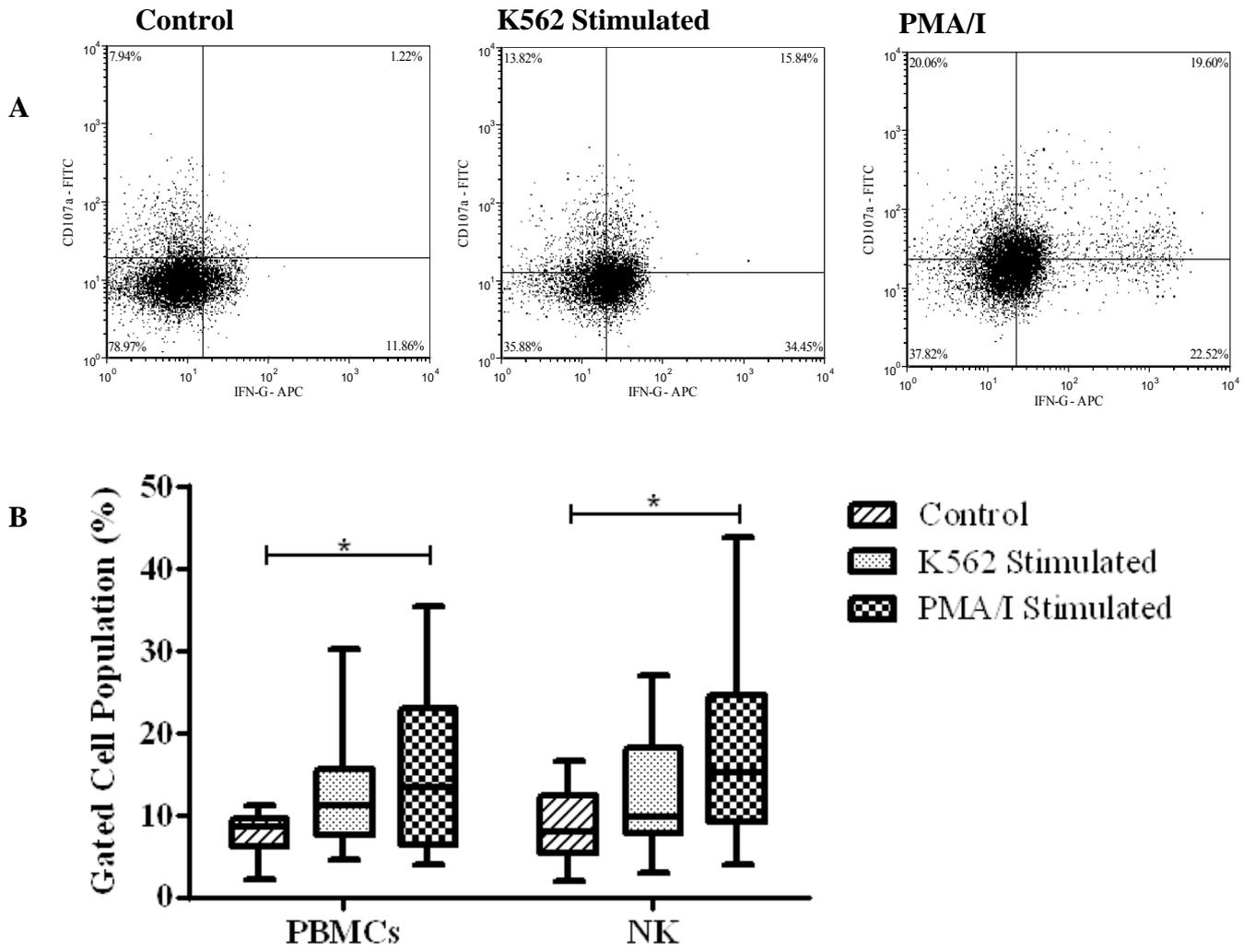| Perforin | Grz A | Grz B |

B

C

Figure 2

Figure 3

Table 1: Participant blood parameters and C reactive protein analysis. The results are presented as means ± standard deviation from a five-part differential analysis of leucocytes, red blood cell counts and haematological parameters. * Denotes significant differences (p<0.05).

	Males	**Females**	**P Value**
Total Participants (N)	10	9	
White blood cells (x10^9/L)	6.641 ± 1.494	5.216 ± 0.653	0.017*
Neutrophils (x10^9/L)	3.683 ± 0.928	2.977 ± 0.955	0.121
Lymphocytes (x10^9/L)	2.028 ± 0.474	1.636 ± 0.470	0.089
Monocytes (x10^9/L)	0.558 ± 0.127	0.357 ± 0.118	0.002*
Eosinophils (x10^9/L)	0.284 ± 0.360	0.160 ± 0.079	0.331
Basophils (x10^9/L)	0.089 ± 0.037	0.158 ± 0.220	0.340
Red Blood Cells (x10^{12}/L)	5.370 ± 0.430	4.763 ± 0.277	0.002*
Haemoglobin (g/L)	156.400 ± 8.181	138.111 ± 11.731	0.001*
Haematocrit (L/L)	0.445 ± 0.026	0.407 ± 0.034	0.013*
Mean corpuscular volume (fL)	83.080 ± 3.889	85.556 ± 6.619	0.328
Mean corpuscular haemoglobin (pg)	29.240 ±1.730	29.078 ± 2.491	0.870
Mean corpuscular haemoglobin concentration (g/L)	351.900 ± 7.475	339.556 ± 6.598	0.001*
Red cell distribution width (%)	11.660 ± 0.353	12.611 ± 1.140	0.039
C reactive protein (mg/L)	0.599 ± 0.324	0.829 ± 0.699	0.384

Table 2: Significant correlations identified between NK cell apoptotic mechanisms and cytotoxic activity. Perforin was significantly correlated with NK cell cytotoxic activity and CD107a expression.

	R	p value
Perforin & Cytotoxic Activity	-0.467	0.044
Perforin & CD107a	-0.571	0.029
GrzA & CD107a	-0.561	0.049
GrzB & CD107a	-0.536	0.042