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Quantification of NK cell activity using whole blood: methodological aspects of a new test

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Abstract

Natural Killer (NK) cells are essential in the biological fight against cancer and intracellular pathogens, and their level of activity has in many settings been used as a biomarker for a functional immune response. Currently, NK cell activity is measured using either ^{51}Cr-release assays or flow cytometry based assays revealing the cells´ cytotoxic capacity or by stimulating them to produce cytokines. Although very effective, these are cumbersome techniques not suitable for high volume clinical laboratories.

Recently, an assay has been introduced to measure NK cell activity in a simple and standardized manner. Following stimulation of NK cells in whole blood with a recombinant protein, it utilizes the concentration of IFNγ released to the plasma as a surrogate marker for NK cell activity. However, whole blood holds several sources of IFNγ which may blur the results and hamper the interpretation of the test. Therefore, the present study aimed at analyzing how specifically the test is measuring the activity of NK cells.

Intracellular flow cytometry showed that NK cells, T cells, and Natural Killer T (NKT) cells were producing IFNγ in the assay, however when analyzing the distribution of lymphocytes in the IFNγ–expressing subset, the proportion of NK cells far exceeded the percentage of T-, and NKT cells ($p<0.0001$). Hence, our data indicate that the readout of the test was indicative of the NK cells´ ability to mount a response and thus the results may pave the way for the assay to become applicable in the clinical setting as an estimate of NK cell activity for both diagnostic and prognostic purposes.

Keywords: Natural killer cells, Interferon-gamma, cytokine stimulation, flow cytometry, standardization

Abbreviations: APC, Allophycocyanin; APC-H7, Allophycocyanin-Hilite7; BFA, Brefeldin A; ELISA, Enzyme-linked Immunosorbent Assay; FITC, Fluorescein-isothiocyanate; Freq, Frequency; IFNγ, Interferon-gamma; IL, Interleukin; MFI, Median fluorescence intensity; MoAb, Monoclonal antibody; NK, Natural Killer; NKT, Natural Killer T; PE, Phycoerythrin; PE-Cy7, Phycoerythrin-cyanine 7

1. Introduction

Natural Killer (NK) cells were originally discovered and characterized on the basis of their unique ability to kill tumor cells without previous engagement and activation; however this cell type also plays an indispensable role as producer of cytokines and chemokines upon stimulation (Bryceson et al., 2006, Kiessling et al., 1975, Kiessling et al., 1975). When released, these proteins exert a plethora of effects both in terms of orchestrating the immune response against viral infected- and neoplastic cells and by having direct anti-viral and anti-tumor effects on the latter. In this regard, interferon-gamma (IFNγ) is one of the most important players as it promotes Th1 differentiation and activates macrophages. It also increases the expression of major histocompatibility (MHC) class I on infected and transformed cells thus promoting antigen presentation and it impacts both cell proliferation and apoptosis (Boehm et al., 1997, Farrar et al., 1993).

Previous reports have nicely demonstrated the importance of functional NK cells in the immune response towards both infections and tumor development (Biron et al., 1989, Imai et al., 2000). Thus, being a key component, the measurement of NK cell activation makes an appealing biomarker for functional immunity in several entities. Currently, such studies are performed using either ^{51}Cr-release assays or flow cytometry based analyses for revealing the cytotoxic capacity of NK cells or by stimulating these cells to produce IFNγ and tumor necrosis factor alpha by use of interleukin (IL)-2, IL-12, IL-15, IL-18 and/or IL-21 (Alter et al., 2004, Brunner et al., 1968, Nielsen et al., 2016, Valiathan et al., 2012). Although very effective, these methodologies are cumbersome and therefore not suitable in the clinical setting where NK cell activity may be measured for prognostic, predictive, and/or diagnostic purposes.

A new test became commercially available in 2012 claiming to measure NK cell activity in an easy and standardized manner. Specifically, a small volume of whole blood is drawn into a tube holding a patented recombinant protein, Promoca, which allegedly causes the NK cells to produce IFNγ during 20-24 hours of incubation. Thus, the amount of IFNγ in the plasma measured after the incubation period is claimed to be a surrogate marker for the NK cell's ability to mount an immune response. However, besides being produced by NK cells, it is known that IFNγ is released from stimulated CD4+ T cells, CD8+ T cells, and NKT cells, while it is debated whether monocytes may also be producing this cytokine (Kraaij et al., 2014, Schoenborn et al., 2007). Hence, a number of

cell types are potential contributors to the total yield of IFNγ in the plasma in the NK Vue® assay. We performed a study combining measurements of plasma IFNγ and IFNγ producing cells to establish whether NK cell activity was in fact reflected by the IFNγ concentrations found in the plasma.

2. Materials and Methods

2.1 Donors, sampling, and incubation

Flow cytometry was performed on a total of 8 healthy volunteers (male/female ratio 3/5; median age 38.5 years (range 26-60 years)), while for the ELISA analysis 10 healthy donors were included (male/female ratio 3/7; median age 37 years (range 26-61 years)). Informed consent was obtained from all donors. Both analyses were performed on 7 of the donors. Blood was collected in Na-heparin vacuum tubes (Becton Dickinson, Franklin Lakes, NJ) and for each donor 1mL of the whole blood was transferred to each of 12 NK Vue® tubes (ATGen, Seongnam-si, South Korea). There were two tubes for each of 6 periods of incubation; 5-, 10-, 15-, 20-, 24-, and 28 hours. As a control to reveal stimulation caused by handling and incubation, 1mL of whole blood was added to two 15mL CELLSTAR® tubes (Greiner Bio-one, Kremsmünster, AT) and incubated for 24 hours. Finally, 1 mL of whole blood collected in the Na-heparin tubes and centrifuged immediately after the blood draw was used as baseline samples. This blood collection procedure deviated from the manufacturer´s recommendations as the blood sample is intended to be drawn directly into the NK Vue® tubes; however to have uniform blood samples in all tubes, the described approach was used. All tubes were incubated in a CO_2 incubator set to 37°C and 5% CO_2. One of the two duplicates was incubated leaving the tube cap on as recommended by the manufacturer (termed 37°C samples) while the cap was kept off the second tube thus allowing gas exchange during incubation (termed CO_2 samples). In order to accumulate IFNγ intracellularly for flow cytometric detection, 10μg/mL Brefeldin A (BFA) (Sigma-Aldrich, St. Louis, MO) was added to each sample two hours prior to end of incubation.

2.2 Plasma separation

One milliliter of Na-heparinized whole blood centrifuged for 10 minutes at 2000xg served as a baseline sample. The plasma was harvested and stored at -80^{o}C until use. For the incubated samples, plasma lying on top of the sedimented cells was carefully collected and spun at 11,500xg for 1 minute. Ensuring not to get close to the pellet, the plasma was collected and stored at -80^{o}C until use

2.3 Enzyme-linked Immunosorbent Assay (ELISA)

Plasma concentration of IFNγ was quantified in duplicates using the NK Vue® ELISA (ATGen) (n=10). The assay was carried out following the manufacturer´s instructions for a 96-well plate analysis. Samples outside the standard curve were reanalyzed in a 1:10 dilution in the sample diluent provided and if still outside the standard curve they were recorded as values of 20,000pg/mL. The dynamic range of the ELISA was 65-2000pg/mL, the intra-assay variation was <10%, and inter-assay variation was <12%.

2.4 Flow Cytometry

Expression of cell surface markers and presence of intracellular IFNγ was evaluated by flow cytometry both at baseline and in all incubated samples (n=8).

A total of 200μL whole blood was used for the analysis. Surface staining was performed for each sample using titrated concentrations of monoclonal antibodies against CD56, CD14, CD69, and CD3 as described in Table 1. After 15 minutes of incubation at room temperature in the dark, 2mL of BD FACS Lysing Solution x1 (BD Biosciences, San Jose, CA) was added and the samples were incubated for additionally 10 minutes after which they were spun at 500xg. After removing the supernatant, the cells were resuspended in 0.5mL 1x BD FACS Permeabilizing Solution 2 (BD Biosciences), incubated for 10 minutes, and spun at 500xg. Subsequently, the cells were stained intracellularly using anti-IFNγ FITC for 30 minutes (Table 1), washed in phosphate buffered saline with 0.5% bovine serum albumin and added 1% v/v formaldehyde. All samples were analyzed

within 24 hours on a FACSCanto II equipped with a 488nm- and a 633nm laser and a median of 97,517 lymphocytes (range 44,263-101,881) was recorded in each sample. 7-color setup beads were used for daily quality control of the instrument and compensation was performed using BD COMPBeads (BD Biosciences) combined with the antibodies described in Table 1. FlowJo version X (Flowjo, Ashland, OR) was used for data analyses.

Both the percentage of IFNγ+ cells within the different cell subsets and the distribution of NK-, T-, and NKT cells in the IFNγ+ subset were determined (gating strategies in Fig. S.1 and S.2). Objective gating for IFNγ- and CD69 positivity was performed using the unstimulated samples incubated for 24 hours as a control.

2.5 Statistics

The Friedman test and Dunn´s multiple comparisons test as post hoc analysis was employed when testing whether frequency of CD69+ and IFNγ+ cells after incubation differed from the baseline. As only 6 donors were evaluable after 28 hours of incubation, this time point was excluded from the analyses. The same tests were employed when analyzing the relationship between IFNγ concentration in plasma at the different time points.

Wilcoxon matched-pairs signed rank test was employed to test whether percentage of CD69+ cells and IFNγ+ cells at each time point was influenced by the method of incubation. The same test was used to determine whether median fluorescence intensity (MFI) of IFNγ+ subsets as measured by flow cytometry at each time point was influenced by the method of incubation and to test if IFNγ in plasma at each time point was influenced by the method of incubation.

One way ANOVA with Tukey´s multiple comparisons test as post hoc analysis was used to determine if any particular cell type made up a larger percentage of the IFNγ+ cell subset at the different time points.

When analyzing whether the proportion of IFNγ+ lymphocytes that were NK cells differed between the two types of incubation two-way ANOVA was used. The same analysis was performed for T cells and NKT cells.

For all tests a p value below 0.05 was considered significance. Statistical analysis was performed using GraphPad Prism version 7 software (GraphPad Software, La Jolla, CA).

3. Results

3.1 Promoca-induced activation of cell subsets in whole blood

The degree of activation in the NK-, T-, and NKT cell subsets at baseline and after 5-, 10-, 15-, 20-, 24-, and 28 hours of incubation was evaluated by the early activation marker CD69 using the gating strategy depicted in Fig. S.1. Incubation in the NK Vue® tubes caused an upregulation of this activation marker on all three cell types (Fig. 1) (Table S.1) and the frequency of CD69+ cells was significantly different from the baseline sample after 10 hours of incubation ($p \leq 0.0376$ for NK cells, $p \leq 0.0067$ for T cells, and $p \leq 0.0132$ for NKT cells, respectively). As a sign of activation, robust upregulation of CD69 was measured on monocytes as well (data not shown). Hence, the cell subsets investigated were activated in the assay either directly by the recombinant protein Promoca or by cytokines released as a result of Promoca-stimulation thus indicating that all cell types were potential contributors to IFNγ in the plasma.

When measuring IFNγ intracellularly in these cell subsets a clear production of the cytokine was seen in NK-, T-, and NKT cells (Fig. 2 A-C) (Table S.2). No IFNγ was measured in monocytes (data not shown). The frequency of IFNγ producing cells obtained after 10-20 hours of incubation were significantly different from the level in baseline samples ($p \leq 0.0251$ for NK cells, $p \leq 0.002$ for T cells, and $p \leq 0.0106$ for NKT cells, respectively), while this percentage returned to levels comparable to baseline after 24 hours for NK cells incubated in CO_2, for T cells incubated at 37°C, and for NKT cells under both conditions. A statistical analysis of the intracellular levels of IFNγ as measured by median fluorescence intensity (MFI) showed that in the 37°C samples NKT cells had more intracellular IFNγ than NK cells at the 10 hour timepoint ($p=0.0078$). In the CO_2 samples the intracellular levels of IFNγ in NK cells were surpassed by T cells at 10 hours of incubation ($p=0.0156$) and by NKT cells at both the 10 hour timepoint and the 15 hour timepoint ($p=0.0078$ and $p=0.0234$, respectively) (Fig. 2 D-F) (Table S.3). When analyzing whether the conditions during incubation influenced the percentage of IFNγ-producing NK-, T-, and NKT cells, we found no

statistical difference between these subsets in the 37°C samples and the CO_2 samples nor was there a difference in the level of IFNγ expression as measured by MFI of the IFNγ+ cell subsets (Fig. 2 D-F).

3.2 Distribution of lymphocyte subsets among the IFNγ-producing cells

Analysis of the IFNγ+ lymphocytes with respect to CD3 and CD56 allowed us to identify the cell type constituting the largest fraction of the IFNγ-producing cells during stimulation (Gating strategy depicted in Fig. S.2). At all time points and by both methods of incubation, the differences between the percentage of CD3-CD56+ NK cells and the two other subsets, the CD3+CD56- T cells and the CD3+CD56+ NKT cells, were highly significant ($p<0.0001$) (Fig. 3) (Table S.4). When looking at this IFNγ+ subset at the time span between 0-24h thus covering the incubation period recommended by the manufacturer the median ratio of NK cells to T cells was 3.8 (range 2.8-27.1) and of NK cells to NKT cells it was 9.5 (range 7.1-26.6) in CO_2. In the 37°C samples these median ratios were 3.9 (range 3.2-37.5) and 12.1 (range 10.1-26.4), respe ctively. By comparing the fraction of NK cells over time between samples kept at 37°C and CO_2 there was no statistical difference between the two incubation strategies. The same was true for both T cells and NKT cells.

3.3 Increasing concentration of IFNγ in plasma in response to Promoca-stimulation

Measurement of IFNγ in the plasma illustrated that the concentration obtained by both types of incubation plateaued after 15 hours and there was no significant difference between samples incubated between 15 hours and 28 hours (Fig. 4) (Table S.5). Note that samples from one donor kept in CO_2 were above the standard curve of the ELISA in a 1:10 dilution after 15-, 20-, 24-, and 28 hours. These data caused an underestimation of the median value for these time points as these were included in the dataset as having the highest value possible in the assay for a 1:10 dilution (20,000pg/mL). A comparison of the concentration of IFNγ in the plasma over the entire time course demonstrated that at several time points, the cells of the CO_2 samples secreted larger

concentrations of this cytokine than cells in 37°C samples having no gas exchange during incubation (Fig. 4). Specifically, the difference was statistically significant after 10 hours ($p=0.0098$), 15 hours ($p=0.0039$), 20 hours ($p=0.0039$), 24 hours ($p=0.0195$), and 28 hours ($p=0.0098$).

4. Discussion

NK Vue® is a test that has become commercially available claiming to measure NK cell activity in one milliliter of whole blood, in an easy and standardized manner. As NK cell activity has the potential of being a biomarker for immune competence in the clinical setting, it is easy to imagine applications for such a test that is far less laborious and more cost effective than both the ^{51}Cr-release assay and flow cytometry-based analyses. However, as opposed to these standard techniques, the NK Vue® test does not measure the cytotoxic capacity of the NK cells, but focuses on these cells' critical regulatory capacity as producer of IFNγ. While NK cells by far are the only cells to produce this cytokine in whole blood upon stimulation, an essential prerequisite for its use was to verify that the concentration of IFNγ measured in the assay was in fact a reflection of the NK cells´ activity level (Chiappini et al., 2012, Kraaij et al., 2014, Schoenborn et al., 2007).

By using flow cytometry to determine cellular activation levels as revealed by presence of the early activation marker CD69, we were able to show that not only NK cells, but also T cells, NKT cells, and monocytes were stimulated in the NK Vue® assay. This activation of the T cells, NKT cells, and monocytes might have been caused directly by the activator present in the NK Vue® tubes or in response to factors released from stimulated NK cells. In either case, a clear activation of cells with IFNγ producing abilities suggested that all four cell types were potential contributors to IFNγ in the plasma in the NK Vue® assay. This notion was confirmed when intracellular IFNγ in the NK-, T-, and NKT cells was measured as the percentage of IFNγ-producing cells in all three cell types increased until 15 hours of incubation, after which it declined to a level either corresponding to or near the level of the baseline sample. On the other hand, this cytokine was not detected in monocytes, but as to whether this cell type is actually a source of IFNγ is an ongoing topic of debate (Kraaij et al., 2014). Therefore, this outcome was somewhat expected.

The intracellular labeling of IFNγ showed that NK cells, T cells, and NKT cells were producing IFNγ during incubation in the NK Vue® tubes, however analyses of the distribution of cell subsets among the bulk of IFNγ+ cells revealed that the percentage of IFNγ+ NK cells far exceeded the fraction of both T cells and NKT cells producing the cytokine throughout the incubation period. Thus, the NK cells clearly appear to be the biggest contributor of IFNγ in the plasma. However, the results also indicated that the amount of intracellular IFNγ in NK cells was exceeded by T- and NKT cells at 1-2 time points (depending on the method of incubation) hence the T- and NKT cells per se could be secreting larger amounts of IFNγ into the plasma at these given time points. Nevertheless, we believe that our data still point to the NK cells as the primary contributor of plasma IFNγ despite the fact that T cells and NKT cells contribute an unknown level of cytokine as well; Partly because of the significantly greater fraction of IFNγ producing NK cells thoughout incubation and not least due to key fact that the readout of the NK Vue® assay is IFNγ accumulation in plasma over 20-24 hours where levels of IFNγ inside the three cell subsets were comparable for most time points analyzed.

The level of CO_2 in the blood plays a pivotal role as a buffer that in turn allows gases, metabolites, and nutrients to be exchanged without fluctuations in pH, which is why cell cultures are often kept in an optimal CO_2 environment. However, although being cultured blood, the NK Vue® assay is to be performed in sealed tubes. In order to determine whether allowing gas exchange would help NK cell activation and hence the production of IFNγ, we performed parallel studies keeping paired samples in a CO_2 incubator in both open and closed tubes. We found no significant differences when comparing the percentages of cells positive for IFNγ and the intensity of the IFNγ found in each cell type between samples kept at 37°C in 5% CO_2 and at 37°C with no gas exchange (i.e., closed tubes). Furthermore, the kinetics of activation of the three cell types as measured by both CD69 expression and IFNγ production seemed to be comparable and independent of method of incubation. Nevertheless, statistical analyses of plasma concentrations of IFNγ showed that after the 10 hour time point cells kept according to manufacturer's recommendations secreted less IFNγ compared to samples that were allowed exchange of gases. Thus, the measures of plasma concentrations of IFNγ signified that the cells kept under these two different conditions may have differed in their ability to secrete protein. An obvious explanation for this notion is that a hypoxic

environment is built up inside the closed tubes which in turn inhibits the normal function of the cells. Thus, a higher cellular response to Promoca-stimulation may be expected if handling the samples as a standard cell culture.

The procedure for the test described by the manufacturer recommends that samples are to be incubated for 20-24 hours. As the concentration of IFNγ in all samples seemed to reach a plateau after 15 hours, the recommended time for incubation appears reasonable. In further support of this notion, the kinetic studies performed by flow cytometry showed that the percentage of IFNγ-producing NK cells peaked around 15 hours thus implying that incubation for longer than 20-24 hours would not cause further NK cell activation. On the other hand, the results also suggest that 15-20 hours of incubation would bring results equal to that of 20-24 hours, but while handling 15 hours of incubation in a routine clinical laboratory can be cumbersome, it is likely that the incubation of 20-24 hours will be preferable. With regard to these results on the proposed incubation period it should be noted that the use of BFA may have caused minor but unavoidable changes in the plasma concentrations of IFNγ presented here compared to standard NK Vue® analyses. Thus, our results would expectedly be somewhat lower as this fungal metabolite blocked the protein transport between the endoplasmatic reticulum and the golgi apparatus, hence inhibiting protein secretion from the cell membrane and in turn IFNγ was built up in the cytoplasm instead of being released into the plasma during the last two hours of incubation. Also, note that these optimization studies of the incubation period were carried out using material from healthy donors. When using NK cell activity measured by this assay as a diagnostic-, prognostic-, or predictive biomarker, parallel analyses of IFNγ in both plasma and intracellularly equal to ours may be necessary to carry out in order to obtain the optimal incubation period and valid results for a given disease entity.

In summary, by combining intracellular detection of IFNγ with measures of this cytokine secreted into the plasma, we have been able to show that NK cells were likely the predominant source of IFNγ in the NK Vue® assay. On the other hand, while the T- and NKT cells did secrete a fraction of this cytokine as well, it is believed to be in a lower proportion. Thus, the readout of the assay seemingly reflects the overall NK cell activity. Consequently, in terms of using NK cell activity as a biomarker, the NK Vue® assay is an alternative being both less laborious and more standardized

than traditional techniques used for this purpose hence making it applicable to both research and clinical use.

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The contribution of the authors were as follows:. L.N. designed the study. L.N. performed the experiments. L.N. and M.H. analyzed the data. L.N. drafted the manuscript and received input and critical reviews from M.H., T.F.H., and A.J. All authors read and approved the final manuscript. This work was supported by ATGen Co. Ltd.

Declarations of interest

A.J. receives research funding from ATGen Co. Ltd. The company did not interfere with the study design, analysis, and data interpretation, nor the preparation of the manuscript or the submission process.

Table 1. Flow Cytometry Panel

MoAb	Fluorochrome	Clone	Company
α-IFNγ	FITC	25723.11	BD Biosciences
α-CD56	PE	C5.9	DAKO
α-CD14	PE-Cy7	M5E2	BioLegend
α-CD69	APC	L78	BD Biosciences
α-CD3	APC-H7	SK7	BD Biosciences

MoAb: monoclonal antibody, FITC: fluorescein-isothiocyanate, PE: phycoerythrin, PE-Cy7: phycoerythrin-cyanine7, APC: allophycocyanin, APC-H7: allophycocyanin-Hilite7.

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Figure Legends

Fig. 1. Percentage (median and range) of CD69+ cells in NK-, T-, and NKT cell subsets incubated in a CO_2 incubator (solid line) and at 37^{o}C with no exchange of gases during incubation (dashed lines). n=8.

Fig. 2. Illustration of the frequency (freq) of NK cells (A), T cells (B), and NKT cells (C) producing IFNγ when incubated in a CO_2 incubator (solid line) and at 37^{o}C with no exchange of gases during incubation (dashed lines). The median fluorescence intensities (MFI) measured for the IFNγ+ subsets of the NK cells (D), the T cells (E), and the NKT cells (E) are shown. All graphs depict median and range. n=8.

Fig. 3. The distribution of NK-, T-, and NKT cells in the IFNγ+ cell subset during incubation in a CO_2 incubator (solid line) and at 37^{o}C with no exchange of gases during incubation (dashed lines). The graph shows mean ±SD. n=8.

Fig. 4. Concentration of IFNγ in plasma obtained after incubation in a CO_2 incubator (solid line) and at 37^{o}C with no exchange of gases during incubation (dashed lines). Open symbols indicate underestimated values coursed by levels of IFNγ outside the standard curve as measured in a 1:10 dilution (>20,000pg/mL). Grey symbols show IFNγ level in unstimulated control samples. The graph illustrates median and range of 10 donors.

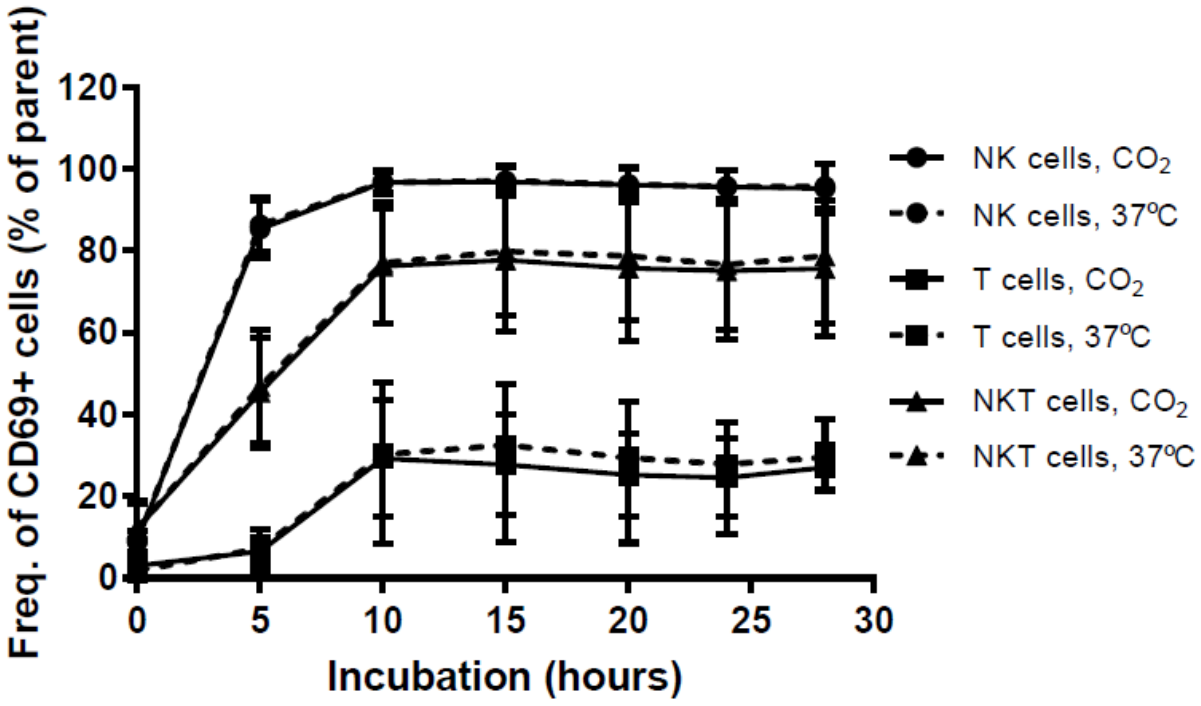

Fig. 1

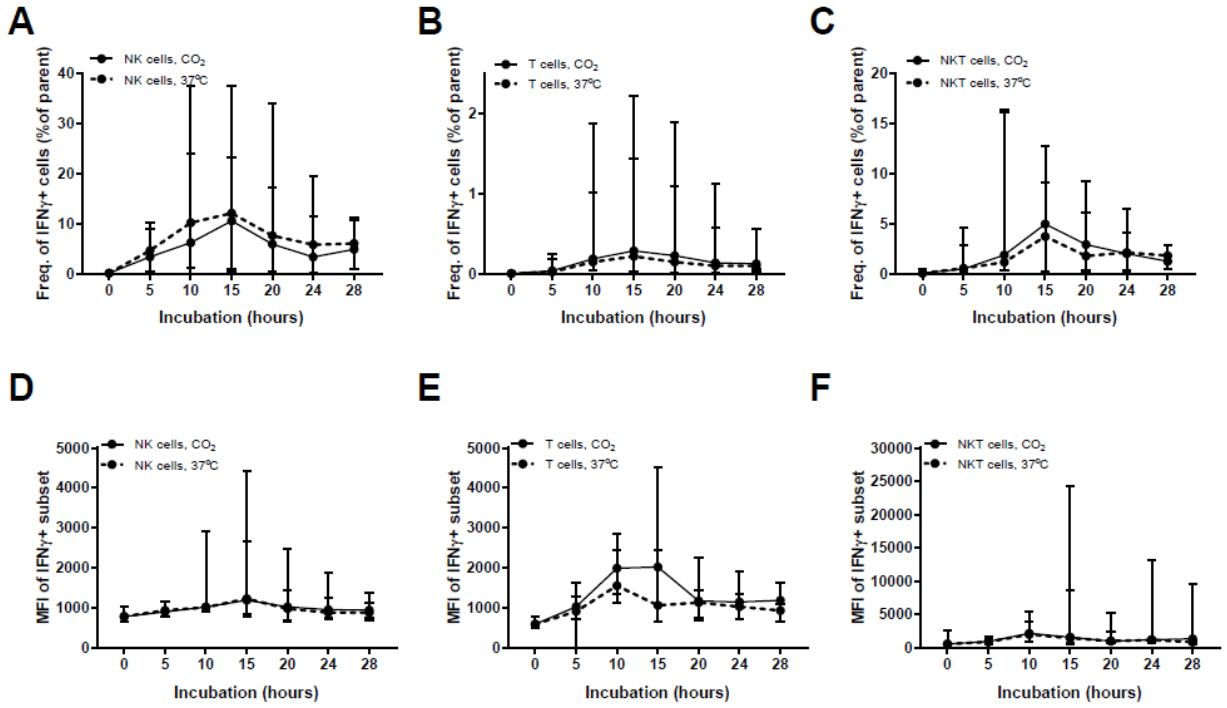

Fig. 2

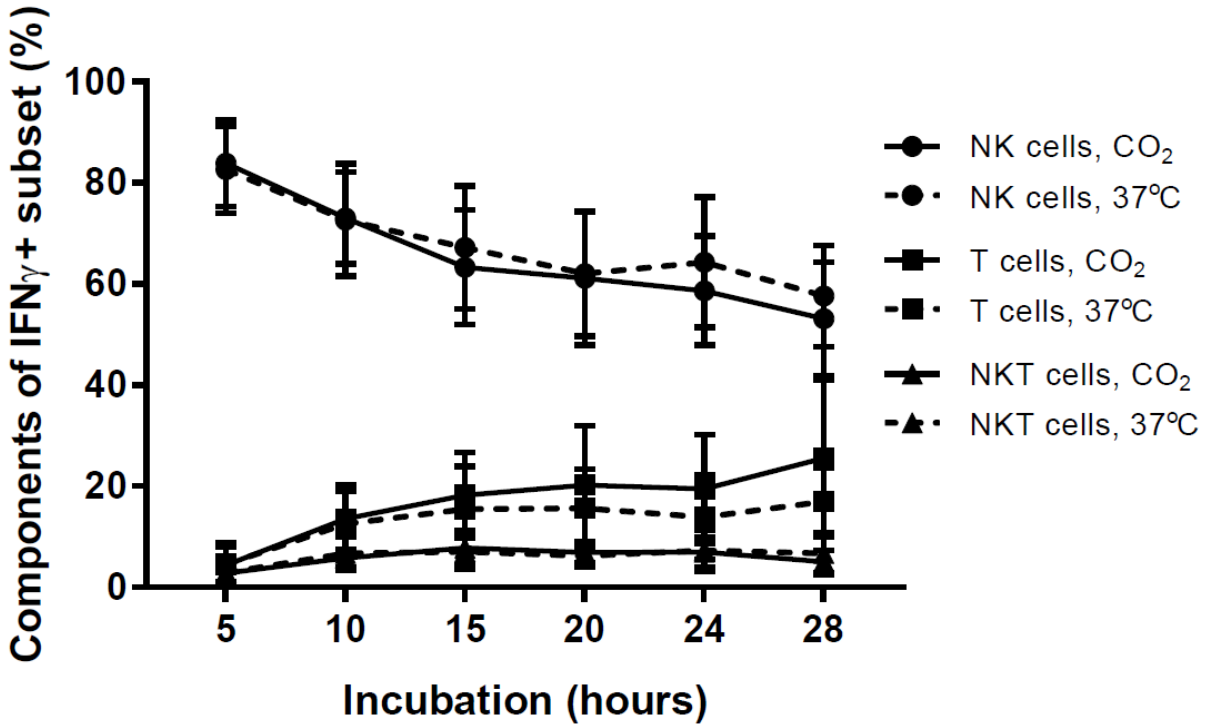

Fig. 3

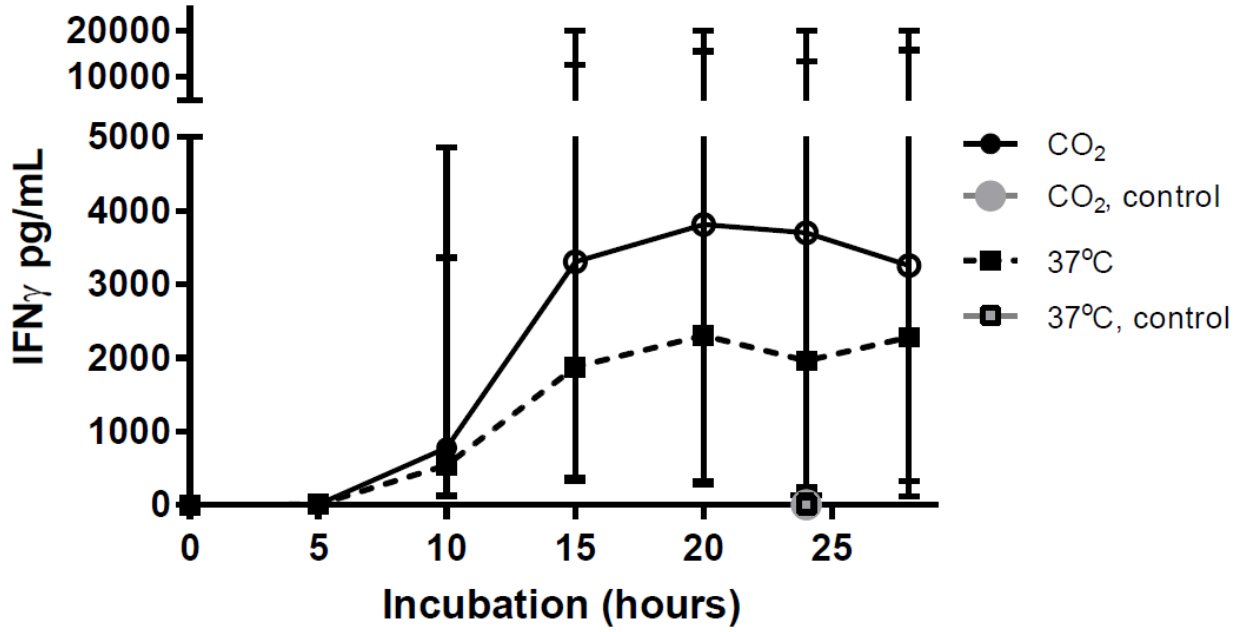

Fig. 4

Highlights

- NK cell activity can be measured in whole blood using the NK Vue® assay
- IFNγ in plasma, which is the test readout, primary originates from NK cells
- Other contributors are T cells and NKT cells
- Incubation in a CO_2 environment increases IFNγ secretion from the cells
- This may ease the way for using NK cell activity as biomarker in clinical settings