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Research paper

# Assessment of the antiviral capacity of primary natural killer cells by optimized *in vitro* quantification of HIV-1 replication

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ABSTRACT

Despite a growing number of studies investigating the impact of natural killer (NK) cells on HIV-1 pathogenesis, the exact mechanism by which NK cells recognize HIV-1-infected cells and exert immunological pressure on HIV-1 remains unknown. Previously several groups including ours have introduced autologous HIV-1-infected $CD4^+$ T cells as suitable target cells to study NK-cell function in response to HIV-1 infection *in vitro*. Here, we re-evaluated and optimized a standardized *in vitro* assay that allows assessing the antiviral capacity of NK cells. This includes the implementation of HIV-1 RNA copy numbers as readout for NK-cell-mediated inhibition of HIV-1 replication and the investigation of inter-assay variation in comparison to previous methods, such as HIV-1 p24 Gag production and frequency of $p24^+$ $CD4^+$ T cells. Furthermore, we investigated the possibility to hasten the duration of the assay and provide concepts for downstream applications. Autologous $CD4^+$ T cells and NK cells were obtained from peripheral blood of HIV-negative healthy individuals and were separately enriched through negative selection. $CD4^+$ T cells were infected with the HIV-1 strain JR-CSF at an MOI of 0.01. Infected $CD4^+$ T cells were then co-cultured with primary NK cells at various effector:target ratios for up to 14 days. Supernatants obtained from media exchanged at days 4, 7, 11 and 14 were used for quantification of HIV-1 p24 Gag and HIV-1 RNA copy numbers. In addition, frequency of infected $CD4^+$ T cells was determined by flow cytometric detection of intracellular p24 Gag. The assay displayed minimal inter-assay variation when utilizing viral RNA quantification or p24 Gag concentration for the assessment of viral replication. Viral RNA quantification was more rigorous to display magnitude and kinetics of NK-cell-mediated inhibition of HIV-1 replication, longitudinally and between tested individuals. The results of this study demonstrate that NK-cell-mediated inhibition of HIV-1 replication can be reliably quantified *in vitro*, and that viral RNA quantification is comparable to p24 Gag quantification via ELISA, providing a robust measurement for NK-cell-mediated inhibition of viral replication. Overall, the described assay provides an optimized tool to study the antiviral capacity of NK cells against HIV-1 and an additional experimental tool to investigate the molecular determinants of NK-cell recognition of virus-infected cells.



## 1. Introduction

Natural killer (NK) cells represent a subset of lymphocytes that is critically involved in the control of viral infections (Jost and Altfeld, 2013; Orange, 2002; Vidal et al., 2011). The function of NK cells is governed by multiple inhibitory and activating receptors balancing self-tolerance and effective responses recognizing cells with "non-self" or "altered-self" phenotypes. NK-cell activation is accompanied by the release of cytotoxic granules (degranulation) to eliminate virus-infected cells and the production of various pro-inflammatory and anti-viral cytokines. Accumulating evidence strongly indicate a role of NK cells in HIV-1 pathogenesis (Carrington and Alter, 2012; Jost and Altfeld, 2012). Epidemiological studies have identified several members of the killer-cell immunoglobulin-like receptor (KIR) family expressed by NK cells to be involved in the control of HIV-1 infection (Martin et al., 2002, 2007). Furthermore, it has been shown that NK cells are able to produce cytokines upon stimulation that compete with HIV-1 co-receptor CCR5 (Fauriat et al., 2010; Oliva et al., 1998). However, it

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is still not fully understood how NK cells recognize HIV-1-infected cells and which cellular host factors would enable efficient NK-cell-mediated control of HIV-1 infection.

The function of NK cells in response to cellular targets has been studied extensively mostly using MHC class I devoid cell lines such as 721.221 (Burlingham et al., 1989) and K-562 (Lisovsky et al., 2015a). However, given the divergent origin of these cells, they display certain limitations with respect to HIV-1 research. This includes the lack of MHC class I molecules that serve as important ligands for activating and inhibitory NK-cell receptors and therefore are potent regulators of NK-cell activation (Nash et al., 2014). Thus, results and conclusions on NK-cell function drawn from experiments with these models should be used with caution in the setting of HIV-1 infection. In contrast, autologous $CD4^+$ T cells are the natural target for HIV-1. The abundant expression of ligands for inhibitory NK-cell receptors, including MHC class I molecules, and low expression of ligands for activating receptors render autologous $CD4^+$ T cells initially not susceptible to NK-cell-mediated killing. However, *in vitro* HIV-1 infection and subsequent HIV-1-mediated alterations of the cellular phenotype make HIV-1-infected autologous $CD4^+$ T cells a suitable model for HIV-1-specific target-cell recognition by NK cells. Several groups as well as ours have developed assays for the assessment of direct and indirect antiviral functions of NK cells (Bonaparte and Barker, 2003; Ward et al., 2007; Fogli et al., 2008; Davis et al., 2011; Lisovsky et al., 2015b; Norman et al., 2011; Alter et al., 2007; Oliva et al., 1998; Bernstein et al., 2004). This includes the assessment of the ability of NK cells to produce antiviral cyto- and chemokines, to lyse infected target cells or to inhibit HIV-1 replication.

Based on a previous approach of Alter et al. (Alter et al., 2007), we re-evaluated and optimized a standardized *in vitro* assay that allows the assessment of the antiviral capacity of primary NK cells. The presented approach uses HIV-1-infected autologous $CD4^+$ T cells as target cells and quantification of NK-cell-mediated inhibition of HIV-1 replication as a measure for the ability of NK cells to control HIV-1 infection *in vitro*.

## 2. Materials and methods

### 2.1. Study subjects

A total of 22 HIV-1-negative healthy subjects were recruited for this study. Subjects were enrolled at the Massachusetts General Hospital in Boston, USA and at the Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany. The study was approved by the respective local Institutional Review Boards. All individuals gave written informed consent for participation in this study. Study participants have been randomly selected for each individual experiment.

### 2.2. Sample processing and isolation of peripheral blood mononuclear cells (PBMC)

50 ml ACD-treated venous peripheral blood was obtained through phlebotomy from all participants. PBMC were isolated by density-gradient centrifugation within 2 h of sample collection and subsequently resuspended in complete medium (RPMI-1640 medium (Sigma) supplemented with 10% (*v*/v) fetal bovine serum (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (All from Corning Cellgro). PBMC yield and viability was assessed using a NucleoCounter (NC-200, ChemoMetec). Recovery of PBMC ranged between 50–100 million cells with an average viability >99%.

### 2.3. Enrichment of autologous $CD4^+$ T cells and primary NK cells

PBMC were divided at a 1:10 ratio for enrichment of $CD4^+$ T cells and primary NK cells respectively. Negative-selection strategy was applied using $CD4^+$ T cell or NK-cell enrichment kits (Stemcell Technologies) according to the manufacturer's protocol. Purity of the enriched cell populations was verified by multi-parameter flow cytometry using the following antibodies: CD4-APC (clone RPA-T4), CD3-PacificBlue (clone UCHT1), CD56-APC-Cy7 (clone HCD56), CD8-FITC (clone HIT8a, all BD) and CD16 BV510 (clone EG8, Biolegend). Average cell purity for $CD4^+$ T cells was 97.2% and 94.5% for NK cells respectively with <0.3% contamination of $CD8^+$ T cells. $CD4^+$ T cells were resuspended in complete media at $5 * 10^6$ cells/ml and stimulated with 100 IU/ml human recombinant IL-2 (hrIL-2; NIH) and 1 μg/ml phytohaemagglutinin (PHA, Fisher) overnight. NK cells were resuspended in complete media supplemented with 1 ng/ml human recombinant IL-15 (hrIL-15; R&D systems) overnight.

### 2.4. In vitro infection and long-term NK/$CD4^+$ T cell co-culture

Overnight cultured $CD4^+$ T cells were infected with the laboratory HIV-1 strain JR-CSF at a multiplicity of infection (MOI) of 0.01 in 100 ul of complete media supplemented with 100 IU/ml hrIL-2 for 4 h at 37 °C. Following infection $CD4^+$ T cells were washed twice with 14 ml of complete media to remove free viral particles. $CD4^+$ T cells were then plated in a 96 round-bottom well plate ($5 * 10^4$ cells/well) in the absence or presence of NK cells. NK cells were added at effector:target ratios of 0.1:1, 1:1 and 10:1 in a total volume of 300 ul of complete media supplemented with 50 IU/ml hrIL-2 and 1 ng/ml hrIL-15 for 14 days. Each condition was plated at least in duplicate.

### 2.5. Sample collection and assessment of HIV-1 replication

Culture supernatants were collected every 3 or 4 days for subsequent batch analysis of HIV-1 p24 Gag concentration and copy numbers of HIV-1 RNA. A total of 150 ul of supernatant was collected from each well, briefly centrifuged (5 min, 350 RCF) to remove residual cells and then stored at −20 °C. Replicates for each condition were pooled prior to freezing. In addition, residual cells were used to assess the frequency of HIV-1-infected $CD4^+$ T cells after 14 days of co-culture.

### 2.6. Quantification of HIV-1-infected $CD4^+$ T cells by flow cytometry (p24 ICS)

The frequency of HIV-1-infected $CD4^+$ T cells was measured by intracellular detection (**I**ntra**c**ellular **s**taining, ICS) of the HIV-1 p24 capsid protein using multi-parameter flow cytometry. After removal of culture supernatants, cells of identical replicates were pooled and washed once with PBS. Surface staining for viability and lineage markers was performed by resuspension of cells in 100 ul PBS supplemented with 2% (*v*/v) FBS followed by 30 min incubation at room temperature with the respective antibodies and reagents: CD3-Pacific Blue (BD), CD4-APC (BD), LIVE/DEAD Fixable blue dye (Invitrogen). Subsequent intracellular staining was conducted using anti-p24-FITC (clone KC57, Beckman Coulter) and a commercially available cell permeabilization and fixation kit (Invitrogen) according to the manufacturer's protocol. Labelled cells were washed with PBS and fixed in 1% (*w*/*v*) paraformaldehyde (PFA) solution (Affymetrix) until flow cytometric acquisition.

### 2.7. Quantification of p24 Gag protein in viral supernatants by ELISA (p24 ELISA)

For the assessment of HIV-1 p24 Gag concentration in culture supernatants a commercially available HIV-1 p24 ELISA kit (Perkin Elmer) was used in accordance with the manufacturer's instructions. Samples were read in duplicate on a TECAN Sunrise ELISA Plate Reader and analysed by Magellan Software Version 6.5. Analytical sensitivity of the ELISA was 4.3 pg/ml; reproducibility within the assay was C.V.: 5.5%.

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