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Effects of cryopreservation on effector cells for antibody dependent cell-mediated cytotoxicity (ADCC) and natural killer cell (NK) activity in ⁵¹Cr-release and CD107a assays

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Abstract

Freshly isolated PBMC are broadly used as effector cells in functional assays that evaluate antibody-dependent cell mediated cytotoxicity (ADCC) and NK activity; however, they introduce natural-individual donor-to-donor variability. Cryopreserved PBMC provide a more consistent source of effectors than fresh cells in cytotoxicity assays. Our objective was to determine the effects of cryopreservation of effector PBMC on cell frequency, and on the magnitude and specificity of ADCC and NK activity. Fresh, frozen/overnight rested and frozen/not rested PBMC were used as effector cells in ⁵¹Cr-release and CD107a degranulation assays. Frozen/overnight rested PBMC had higher ADCC and NK activity in both assays when compared to fresh PBMC; however, when using frozen/not rested PBMC, ADCC and NK activities were significantly lower than fresh PBMC. Background CD107a degranulation in the absence of target cell stimulation was greater in PBMC that were frozen/not rested when compared to fresh PBMC or PBMC that were frozen overnight and rested. The percentages of CD16⁺CD56^{dim} NK cells and CD14⁺ monocytes were lower in PBMC that were frozen and rested overnight than in fresh PBMC. CD16 expression on CD56^{dim} NK cells was similar for all PBMC treatments. PBMC that were frozen and rested overnight were comparable to fresh PBMC effectors. PBMC that were frozen and used immediately when evaluating ADCC or NK activity using either a ⁵¹Cr-release assay or a CD107a degranulation assay had the lowest activity. Clinical studies of antibodies that mediate ADCC would benefit from using effector cells that have been frozen, thawed and rested overnight prior to assay.

Keywords

cryopreservation; PBMC; NK cells; ADCC; degranulation; CD107a

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1. Introduction

Natural killer (NK) cells and antibody-dependent cell-mediated cytotoxicity (ADCC) are functions of innate immune effector cells. NK cells respond immediately to virus infected cells and tumor cells; ADCC also requires participation of virus or tumor specific antibodies (Baum et al., 1996; Alpert et al., 2012; Kute et al., 2012; Yoshida et al., 2012; Mata et al., 2013). Both NK and ADCC activity are critical to defense against pathogens. Measuring NK and ADCC activity against viral pathogens and tumors is an important component of evaluation of the effectiveness of new vaccines. The standardization of the effector cells in assays evaluating these activities is critical for understanding the correlates of immune protection.

The ^{51}Cr -release assay has been the standard assay used to evaluate NK and ADCC activity, however, newer assays have been developed that use flow cytometry to measure cytotoxicity against fluorescent-labeled targets or degranulation or granzyme B loss by effector cells following target stimulation (Alter et al., 2004; Kantakamalakul et al., 2006; Aktas et al., 2009; Davis et al., 2009; Pollara et al., 2011; Yamada et al., 2011). These assays vary with respect to incubation times and stimulation conditions. There has also been a focus on the development of new target cell lines and new monoclonal antibodies that are currently being used for immunotherapy (Manches et al., 2003; Borghaei et al., 2009). Identifying a consistent source of effector cells for these assays, however, continues to be a challenge.

Human NK cell lines such as NK-92 and NK3.3 (Klingermann 2010, Dr. Kornbluth, Department of Pathology, Saint Louis University) have been developed in order to standardize effector cell function. Although this is an attractive option, IL-2 stimulation and/or irradiation are required for their maintenance and use, which could alter the specificity and magnitude of NK and ADCC activity. Because of these limitations, the source of effector cells in functional assays that assess ADCC and NK activity are usually freshly isolated peripheral blood mononuclear cells (PBMC) or cryopreserved PBMC. Fresh cells introduce a source of variability based on differences in effector function between individual donors and although the variability is less if the same donor is used repeatedly, there is individual variability from day to day. Freezing larger numbers of effector cells from one healthy individual for use in several experiments would eliminate both of these sources of variability. Even though the results would be more consistent a better understanding of the effects of cryopreservation on these effector cells would enable us to optimize the response and achieve consistency. In 1984, Pross and Maroun reported that cells that are thawed and rested in media for at least 5 hr are better NK effector cells in ^{51}Cr -release assays (Pross and Maroun, 1984). Even though the importance of NK and ADCC are more fully appreciated 30 years later, there has not been another study to expand this observation to include ADCC or CD107a assays.

CD107a (LAMP-1) is a marker for NK degranulation and cytotoxicity (Alter et al., 2004; Aktas et al., 2009) that has recently been shown to be required for perforin trafficking and granule movement (Krzewski et al., 2013). An assay that measures the expression of CD107a on the cell surface, the CD107a degranulation assay, is now also used to evaluate cytotoxicity. In this study, we assessed the effects of cryopreservation on the phenotype of

effector cells by evaluating changes in cell frequency; and evaluated ADCC and NK function of fresh and cryopreserved PBMC, with or without resting after thawing using the ^{51}Cr -release and CD107a degranulation assays since they have comparable target stimulation and incubation times. Our goal was to compare the sensitivity and specificity of the response with fresh, frozen/thawed, and frozen/thawed/rested effector cells on each of these commonly used assays.

2. Materials and Methods

2.1. Cell lines and antibodies

The CEM.NK_R cell line was used as the target cell in all ADCC assays. As previously described, they have less than 5% NK background activity and more than 90% of the target cells bind recombinant HIV-1 gp120 when incubated together for 1 hr (Baum et al., 1996). The MHC-negative cell line K562, was used to evaluate NK activity. Both cell lines were grown at 37°C/5% CO₂ with 95% humidity in RPMI-1640 culture media (Cellgro, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine and penicillin-streptomycin (Gemini Bio-Products, West Sacramento, CA). The cultures were kept in log phase and passed a day before each experiment. The antibody source for the ADCC studies was pooled IgG from asymptomatic HIV-1 seropositive individuals, (HIVIg, donated by NABI). HIVIg was used at its previously determined optimal dilution for ADCC activity, 1:4,000.

2.2 Donor screening and selection

The ADCC activity of 31 healthy, HIV-1 negative effector PBMC donors was evaluated using HIVIg as source of antibody (Figure 1A). Eight of the individuals who had ADCC activity equal to or greater than the mean ADCC activity of all screened donors were used for this study. The initial ADCC activity and NK background (no antibody) using fresh PBMC from the donors selected after screening is shown in Figure 2B. The initial ADCC activity of each donor was comparable to the donor's individual ADCC activity observed on assay days (data not shown).

2.3 Isolation and cryopreservation of PBMC

PBMC were isolated by ficoll gradient centrifugation from heparinized blood. The lymphocyte band was washed once with PBS (1mM EDTA+ 0.1% BSA) to remove platelets. Cells were washed again in complete media and then adjusted to the cell concentrations required to obtain the optimal effector:target (E:T) ratios: 40:1 for ^{51}Cr -release assays and 20:1 for CD017a degranulation assays (data not shown).

For cryopreservation, PBMC were aliquoted at a concentration of 1×10^7 cells per cryogenic vial in culture media that contained 20% dimethyl sulfoxide (DMSO). The cells were placed in a cryo 1°C freezing container (Thermo Fisher Scientific, Rochester, NY) in a -80°C freezer for 24 hr and then transferred into a liquid nitrogen freezer until use. The PBMC were used within 2 months of the date they were frozen to minimize phenotypic changes induced by the length of cryopreservation (Tollerud et al., 1991; Seale et al., 2008). Frozen PBMC were thawed in a 37°C water bath until a small amount of frozen material was left,

then washed in complete media. Depending on experimental conditions, the cells were used immediately, or incubated overnight at 37°C with 5% CO₂. Upon thawing, 80% of the frozen cells were recovered as viable cells, as determined by trypan blue exclusion, upon thawing. Following overnight incubation viable recovery was approximately 70% of the cells originally frozen. Cryopreserved PBMC from each donor were compared to that donor's own fresh cells.

2.4 ⁵¹Chromium Release Assay

CEM.NK_R and K562 target cells were incubated with 100μCi of Na⁵¹CrO₇ (Perkin Elmer, Downers Grove, IL) for 1 hr at 37°C. Following incubation, cells were washed three times to eliminate excess ⁵¹Cr. 1×10⁶ ⁵¹Cr-labeled cells were rotated for 1 hr at room temperature (RT) with or without 0.225μg of HIV rgp120 (Protein Sciences Corporation, Meriden, CT). 1×10³ of rgp120-coated and uncoated CEM.NK_R cells were added in duplicates to wells of a 96-well conical plate in the presence of antibody (HIVIg) to evaluate ADCC, and in absence of antibody to determine background NK activity against the ADCC target cell. After a 10 min incubation of CEM.NK_R cells with antibody, effectors cells were added at the desired E:T ratios. K562 target cells were plated with effectors at corresponding E:T ratios to measure NK activity. 100μL of 0.1% triton-X or complete media was added to the targets to determine maximum and spontaneous lysis, respectively. After effector cells were added, the reaction was incubated for 3.5 hr in a humidified, 37°C, 5% CO₂ atmosphere. Following incubation, 100μL of supernatant was harvested from each well and gamma-irradiation in counts per minute (cpm) was measured using a 2470 wizard gamma counter (Perkin Elmer, Torky, Finland). cpm values were converted to percent specific lysis (% SL) using the following formula:

$$\% \text{ SL} = [(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})] \times 100$$

2.5. Immunophenotyping

Using a 7-color flow cytometry assay, 1×10⁶ fresh, frozen/not rested and frozen/rested overnight PBMC were analyzed for phenotype and the activation marker, CD69. To determine viability, PBMC were washed twice with PBS, resuspended and incubated in 1μL of Aqua amine-reactive viability dye (Molecular Probes, Inc. Eugene, OR) for 20 min at RT in the dark, washed and incubated with 10μL of Fc block (Miltenyi Biotec Inc., Auburn, CA) for another 20 min at RT in the dark. The cells were washed with FACS buffer (0.2% BSA+0.1% NaN₃ in PBS) and incubated for 20 min in the dark at 4°C with anti-CD3-eFluor 450, anti-CD19-APCeFluor 780 (both from eBioscience), anti-CD56-PE/Cy7, anti-CD14-APC, anti-CD16-FITC and anti-CD69-PE/Cy5 (Biolegend, San Diego, CA). After incubation, cells were washed in FACS buffer and fixed in 1% paraformaldehyde. Duplicate sets of PBMC: fresh, frozen/thawed and frozen/thawed/rested overnight, were incubated overnight with or without IL-2 and were stained with identical cocktails or in the absence of anti-CD69 (supplemental Figure 1) for fluorescence minus one (FMO) control (Supplemental Figure 1). Samples were analyzed using an LSRII flow cytometer and FlowJo software (TreeStar Inc., Ashland, OR). Dead cells were excluded from analysis by gating on Aqua amine-reactive viability dye negative cells.

2.6. CD107a degranulation assay

2×10^5 effector cells and $1 \mu\text{L}$ of CD107a-PE antibody (Biolegend) were added to each well of a 96-well v-bottomed microtiter plate. For ADCC, 1×10^3 rgp120-coated or uncoated CEM.NK_R targets were added to the corresponding wells with or without of $50 \mu\text{L}$ of HIVIg antibody. For NK activity, K562 targets were added to duplicate wells. The final E:T ratio for both ADCC and NK wells was 20:1. Duplicate wells that contained effector cells of each treatment with or without target stimulation, were left without CD107a-PE antibody for FMO control. To determine baseline CD107a expression, media was added to wells in the absence of targets. The plate was centrifuged for 5 min at $20 \times g$ and incubated at 37°C in a 5% CO_2 atmosphere for 4 hr. Following incubation, the plate was centrifuged again for 5 min at $400 \times g$. After supernatant was removed from the wells, the cells were washed twice with $200 \mu\text{L}$ of FACS buffer; then, $10 \mu\text{L}$ of Fc block was added to each well for 20 min. Samples were then transferred from the plate into FACS tubes and washed again with FACS buffer. Cells were next stained with anti-CD3-eFluor 450, anti-CD19-APCeFluor780, anti-CD56-PE/Cy7, anti-CD14-APC and anti-CD16-FITC antibodies for 20 min in the dark on ice, and then washed and fixed in 1% paraformaldehyde. Samples were analyzed by flow cytometry. To determine CD107a expression on CD16⁺CD56^{dim} NK cells, PBMC were first gated on live, CD3⁻, CD14⁻ and CD19⁻ cells using FlowJo software; a complete gating strategy for CD107a analysis is shown in Figure 2. The CD107a⁺ cells were determined by gating above the CD107a expression of each subject at each PBMC treatment in the absence of targets (effectors alone); this gate was applied to the target stimulated conditions of that subject (Figure 2H–J).

2.7. Statistics

Data in tables were presented using Microsoft Excel 2011. Data from ^{51}Cr -release and CD107a assays were presented as mean + standard error (SE) and analyzed for statistical differences using GraphPad Prism (GraphPad Software, La Jolla, CA). Wilcoxon paired tests or repeated measures of one-way ANOVA with Dunn's post-test were used when comparing the three PBMC treatments. Differences were considered significant when p values were < 0.05 .

3. Results

3.1. Cryopreserved cells that are rested overnight are a better source of ADCC and NK effector cells than freshly thawed cells

We compared ADCC activity (in presence or absence of CEM.NK_R-gp120 and HIVIg) and NK activity (in presence or absence of K562) for fresh effector cells, frozen/rested overnight effectors, and frozen/not rested effectors to evaluate the effects of cryopreservation on specificity when using the ^{51}Cr -release assay and the CD107a degranulation assay.

Figure 3A shows that ADCC and NK activity evaluated by ^{51}Cr -release assay are specific responses regardless of the cryopreservation treatment of effector cells; that is, non-specific activity was less than 8.0 %SL for all treatments. The nonspecific background activity of cells that were frozen/rested overnight (7.7% SL) was higher than that of the other two groups, which had a background of less than 2 %SL ($p = 0.02$). However, ADCC activity of

fresh PBMC (29.0 %SL) increased to 35.4 % SL when PBMC were frozen, thawed and rested overnight. The NK activity remained similar whether using fresh PBMC (38.6 %SL) or cells that were frozen, thawed and rested overnight (37.0 %SL). The ADCC and NK activity of fresh PBMC and was not statistically different than cells that were frozen, thawed and rested overnight. In contrast, cells that were frozen and not rested had significantly lower ADCC (9.7 %SL) and NK activity (16.0 %SL) than fresh PBMC ($p = 0.008$ for both ADCC and NK).

In the absence of target stimulation, non-specific background activity is seen when fresh cells are used as effectors in CD107a degranulation assays (Chung et al., 2009). We, therefore, evaluated the effects of cryopreservation and overnight recovery on target cell specific degranulation and non-specific background activity for CD16⁺CD56^{dim} cells in the CD107a assay (Figure 3B). In order to control for the effects of each PBMC group on baseline expression of CD107a, we set individual gates for each donor at each condition. By doing this, we were able to eliminate the impact of significant non-specific degranulation when using cryopreserved cells for this assay.

Figure 3B shows that ADCC and NK activity evaluated by the CD107a degranulation assay are also specific if appropriate gates are set, regardless of the cryopreservation treatment of effector cells. Results for this assay were comparable to those seen using the ⁵¹Cr-release assay in that for both NK and ADCC, the frozen/rested overnight condition had higher activity than when effector cells were not rested. Using this assay for ADCC, frozen/rested overnight cells had 14.0 %SL as opposed to 11.0 %SL for fresh PBMC ($p = 0.08$) and 3.5 %SL for frozen/not rested cells ($p = 0.008$). Similarly, the NK activity against K562 was also higher in frozen/rested overnight cells, where 20.0 %SL was observed as opposed to 13.0 %SL for fresh PBMC ($p = 0.06$) and 7.8 %SL for frozen/not rested PBMC ($p = 0.008$).

By setting individual gates for CD107a assay for each treatment, we corrected for the high CD107a background observed in the frozen/not rested cells in this assay. The extent of the background activity can be more fully appreciated by looking at the multilayer overlay of CD107a flow histograms. Figure 3C shows that CD107a in frozen/not rested CD56^{dim} cells without target stimulation is upregulated (the grey peak is shifted to the right) when compared to fresh and frozen/overnight rested treatments; however, unlike fresh and frozen rested effectors, their CD107a expression changes little when stimulated by target cells.

3.2. Phenotypic and activation changes induced by cryopreservation

We wanted to determine, within each effector cell treatment group, if activation and/or differential survival of selected cell subpopulations contributed to the difference in ADCC and NK activity we observed in the ⁵¹Cr-release and CD107a degranulation assays. To achieve this, at the time of the assay, separate aliquots of fresh, frozen/overnight rested and frozen/not rested PBMC in the absence of cytokines and target stimulation were analyzed for expression of CD3, CD19, CD16, CD56, CD14 and CD69 using flow cytometry as described earlier. Table 1 shows the percent of total live cells of each subset before and after cryopreservation with and without resting in 6 healthy subjects. Gating on total live cells, we observed a decrease in the percentage of total of Fc receptor bearing CD16⁺CD56^{dim} NK cells and CD14⁺ monocytes in PBMC that were frozen/thawed/overnight rested when

compared to fresh; but these differences did not reach statistical significance. Interestingly, when compared to fresh PBMC, percent of total CD14⁺ monocytes from frozen/not rested PBMC was significantly higher ($p=0.03$). Even though all PBMC were handled carefully, an increase in activation for both CD16⁺CD56^{dim} NK cells and CD14⁺ monocytes from frozen/overnight rested PBMC as measured by CD69 expression was observed, but it did not reach statistical significance. Overall, the distribution of cell subpopulations in the three treatment groups remained very similar (Table 1).

3.3 Effects of cryopreservation on CD16 expression

Since ADCC is mediated by cells that express the FcR, CD16, we were especially interested in the effects of cryopreservation on expression of this receptor. We wanted to determine whether the expression of CD16 is compromised after cryopreservation, and therefore could have contributed to the difference in NK and ADCC activity under each PBMC treatment observed in Figure 3A and 3B. We therefore evaluated expression of CD16 in Fc receptor bearing cell populations, CD56^{dim} NK cells and CD14⁺ monocytes from PBMC that were fresh, frozen/overnight rested and frozen/not rested without cytokine or target stimulation. Figure 4A shows that CD16 expression on CD56^{dim} NK cells is similar among fresh, frozen/overnight rested and frozen/not rested treatment groups.

In contrast, Figure 4B shows that CD16 expression on CD14⁺ monocytes was upregulated in cells that were frozen/rested overnight. This was true to varying degrees for all donors. While there is an apparent upregulation of CD16 expression on monocytes, did not contribute to the CD107a assay results shown in Figure 3B because the monocytes were gated out of the CD107a analysis (Figure 2D). The monocytes also did not contribute to the ⁵¹Cr-release results (Figure 3A) since monocytes require longer than the 4 hour incubation time to kill the targets (Boot et al., 1989).

4. Discussion

Previous studies with short or no incubation after thawing frozen PBMC show decreased cytotoxic function of NK cells (Callery et al., 1980; Strong et al., 1982; Hirszen et al., 1983; Ichino and Ishikawa, 1985) and changes in the phenotype of T cells (Costantini et al., 2003; Disis et al., 2006). However, frozen/thawed lymphocytes allowed to rest in media for 5 hr, recover cytotoxic function (Pross and Maroun, 1984), which suggests that effector cells may need to rest to recover cell function. We evaluated ADCC and NK activity, including the specificity of each of these activities in PBMC that were freshly isolated, frozen/thawed with overnight resting, and frozen/thawed without resting. We compared cytotoxicity using two different assays; the standard ⁵¹Cr-release assay and another that uses flow cytometry to quantitate CD107a expression on activated effector cells. We confirmed that frozen and overnight rested effector cells were comparable to fresh NK effector cells in ⁵¹Cr-release assays, and extended this observation to include ADCC activity. We observed similar results for the CD107a assay for both NK and ADCC activity. We also evaluated the specificity of cryopreserved effectors, which impacts the results of clinical studies, particularly ones using newly developed assays to measure cytotoxicity.

The ^{51}Cr -release assay is a measure of direct lysis of target cells; while CD107a assay measures CD107a (LAMP-1) expression on the effector cell, which is usually the CD56^{dim} NK cell subpopulation. CD107a expression correlates with and it is needed for the release of perforin and other cytolytic granules that induce death of target cell (Alter et al., 2004; Aktas et al., 2009; Krzewski et al., 2013). Although we were able to modify gating in the CD107a assay to correct for nonspecific degranulation, cryopreserved effectors that were thawed and used without resting had much higher CD107a expression than freshly isolated effectors or cryopreserved, thawed and overnight rested effectors in the absence of cytokine or target cell induced stimulation (Figure 3C).

CD16 is down-regulated when NK effector cells are stimulated with antibody coated P815 targets or MHC-negative targets, K562 (Grzywacz et al., 2007; Liu et al., 2009). This can potentially be used as a marker of NK cytotoxicity. In our study, CD16 was down regulated in fresh and cryopreserved/thawed/overnight incubated CD56^{dim} cells that were stimulated with gp120-coated CEM.NK_R or K562 target cells. However, CD16 down-regulation was less prominent in frozen/not rested PBMC (Supplemental Figure 2) under the same conditions. This and the somewhat lower percentage of total of NK cells in this group (Table 1) could account for the lower level of ADCC activity in frozen/not rested effectors in our assays in the presence of target stimulation in both assays.

The CD16^{low}CD56^{bright} cells, are thought to be predominantly cytokines producers, are not usually thought to be cytotoxic (Cooper et al., 2001) and are more affected by HIV infection (Mantegani et al., 2009), had higher baseline expression of CD107a after freezing and thawing than the CD56^{dim} subset (data not shown). A new ADCC assay recently developed by (Helguera et al., 2011) may be useful in evaluating cytotoxic function for this NK subset since it would determine target lysis simultaneously to CD107a expression on the effectors. This information may be useful when designing studies to compare cytotoxicity or cytokine production for these subsets in response to HIV infection.

ADCC mediated killing of HIV infected target cells is usually directed against envelope glycoproteins present on the surface of infected cells (Ziegner et al., 1992; Zolla-Pazner, 2004; Johansson et al., 2011). Studies that explore effector cell function by their response to activating antibodies against envelope antigens or cellular receptors generally use assays like CD107a degranulation since activating antibodies do not always trigger killing (Chung et al., 2009). Activated NK or ADCC effector cells may directly kill target cells or may produce cytokines that regulate other cells which contribute to either innate or acquired immunity.

Although the major focus of this paper has been to identify the best source of effector cells for ADCC assays against HIV targets, the finding presented here could also be used to study interpersonal differences in NK and ADCC cell function in HIV infected patients. This area is gaining a broader interest as we learn more about specific NK receptors that contribute to host defense against HIV and study the loss of the populations that carry these receptors as HIV progresses (Parsons et al., 2012).

The ^{51}Cr -release assay and the CD107a assay are both valid assays; a number of considerations should contribute to deciding which assay to use. First, whether the investigator wants to measure activation of effector cells or killing of specific target cells. A second consideration is the number of samples to be assayed. If a limited number of samples will be assayed the CD107a assay may be more appropriate. If the study requires a large number of samples, a ^{51}Cr -release assay is more practical, less expensive and provides comparable results. CD107a assays have to be read using a flow cytometer, while hundreds of samples can easily be counted on a gamma counter. Although the use of radioactivity is a concern, the half-life of ^{51}Cr is relatively short (28 days) and several hundred samples can be assayed using as little as 100 μCi of ^{51}Cr .

Recent interest in the potential protective benefit of ADCC against HIV, and the interest in looking at the impact of new vaccines on generation of ADCC activity against HIV make the information provided in this study important. It will help individuals planning to measure NK or ADCC activity make informed decisions about how to handle the effector cells for their assays to obtain the best possible results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PBMC	peripheral blood mononuclear cells
ADCC	antibody-dependent cell mediated cytotoxicity
NK	natural killer cells

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Figure 1. Selection of PBMC donors

Antibody dependent cell-mediated cytotoxicity (ADCC) of freshly isolated PBMC of healthy donors was evaluated in a standard ^{51}Cr -release assay with pooled IgG from HIV infected donors (HIVIg). ADCC activity and NK background (no antibody) are shown as % specific lysis (% SL) against gp120-coated CEM.NK_R target cells. A) All healthy donors (n= 31). B) Study participants selected for use, based on screening and availability (n= 8). Lines on the dot plots represent the mean % SL $\pm$ standard error (SE).

Figure 2. Gating strategy and effects of cryopreservation on baseline CD107a expression
Representative flow dot plots from one donor showing the gating strategy for CD107a expression. Sequentially: A) cells were gated using SSC-A and FSC-A, B) SSC-W and FSC-A gate was used to identify single cells, C) AQUA amine-reactive dye and CD3 were used to gate out dead cells and T cells, respectively, D) CD14 and CD19 were used to gate out monocytes and B cells respectively, E) CD16 and CD56 were used to select NK cells. F–G left) the baseline CD107a expression (no targets) and F–G right) CD107a expression after 4hr stimulation with targets and antibody (ADCC) are depicted for F) fresh, G) frozen, thawed and overnight rested and H) frozen and thawed without resting. A gating strategy that used an individual donor's baseline CD107a expression for each PBMC treatment was employed.

Figure 3. Effects of cryopreservation on specific lysis against HIV-1 gp120

The ^{51}Cr -release and CD107a degranulation assays were evaluated in parallel to determine for effects of cryopreservation on the specificity of effector cell functions: NK activity and antibody dependent cell-mediated cytotoxicity (ADCC). Bar graphs represent the mean NK and ADCC activity of 8 donors $\pm$ standard error (SE). (A) % Specific lysis (%SL) for each effector cell treatment using a standard ^{51}Cr -release assay under different target stimulations. (B) % CD107a expression of $\text{CD}16^+\text{CD}56^{\text{dim}}$ NK cells determined by flow cytometry assay for each effector cell treatment under different target stimulations (C) Overlays of representative flow cytometry histograms from one donor showing expression of CD107a as % of maximum number of cells (% of Max) under each PBMC treatment

condition in the presence or absence of target stimulation. Statistical differences were determined using Wilcoxon paired tests.*= p value >0.05, **= p value >0.001, NS= not significant.

Figure 4. Effects of cryopreservation on regulation of CD16

Histograms of 4 representative healthy donors showing CD16 expression in freshly isolated PBMC, frozen/thawed/overnight rested PBMC and frozen/thawed without resting PBMC with no target or cytokine stimulation. PBMC under each treatment were stained with fluorochrome-conjugated antibodies and analyzed for CD16, shown as % of maximum number of cells (% of Max) in A) CD56^{dim} NK cells and B) CD14⁺ Monocytes.

Table 1

Phenotypic changes in PBMC caused by cryopreservation

Freshly isolated, frozen/thawed/overnight rested and frozen/thawed without resting PBMC from healthy donors (n = 6) were stained with fluorochrome-conjugated antibodies and analyzed for viability and expression of CD3, CD14, CD19, CD56 and CD16. Activation through expression of CD69 in CD56^{dim} and CD56^{bright} NK cells under each treatment was also assessed using appropriate corresponding fluorescence-1 (FMO) control. Aqua amine-reactive dye was used to gate out dead cells. Numbers shown represent the percentage of total cells (mean and SE) after FSC-A/SSC-A, SSC-A/SSC-W (single cells) and exclusion of dead cells (Aqua⁺) gates were applied. Viability = % of total Aqua⁺ cells.

	PBMC Treatment					
	Fresh		Frozen/Overnight Rested		Frozen/Not Rested	
Viability (% of total)	Mean	± SE	Mean	± SE	Mean	± SE
Subpopulation (% of total live)						
T cells (CD3 ⁺)	99.8	0.2	88.0	4.6	99.1	0.8
B cells (CD19 ⁺)	62.6	3.5	65.3	4.9	59.4	3.5
Monocytes (CD14 ⁺)	12.4	2.6	16.9	5.0	13.1	2.6
NK ^{dim} (CD16 ⁺ CD56 ^{dim})	7.0	2.1	5.3	1.4	11.7	2.3
NK ^{bright} (CD16 ^{-low} CD56 ^{bright})	7.0	2.0	5.6	1.0	5.1	0.8
	0.3	0.1	0.4	0.1	0.4	0.1
Activation of Fc receptor-bearing cells (% CD69 ⁺ of total live)						
Monocytes (CD14 ⁺)	0.6	0.4	1.5	1.3	0.6	0.4
NK ^{dim} (CD16 ⁺ CD56 ^{dim})	1.1	0.6	2.0	1.3	0.7	0.4
NK ^{bright} (CD16 ^{-low} CD56 ^{bright})	3.4	2.8	2.2	1.3	1.3	0.9