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Profile of natural killer cells after a previous natural *Vaccinia virus* infection in an *in vitro* viral re-exposure

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ABSTRACT

The present study compares the profile of NK cells in an *in vitro* re-exposure by *Vaccinia virus* (VACV), in groups that have had a previous vaccination or natural infection. Our data suggests that stimulation with VACV triggers a cytotoxic response by NK cells marked by an increase of NCRs: NKp30, NKp44, and NKp46 in infected (vaccinated and unvaccinated) subjects and in non-infected vaccinated patients, when compared with non-infected unvaccinated individuals. However, the degranulation and secretion processes are inhibited in infected (vaccinated and unvaccinated) subjects and in the non-infected vaccinated patients, when compared with non-infected unvaccinated individuals. We demonstrated that stimulation with VACV downregulates the percentage of expression of Perforin, Granzyme A, and CD107a, but upregulate CD94 in infected (vaccinated and unvaccinated) subjects and in non-infected vaccinated patients, when compared with non-infected unvaccinated individuals. Furthermore, the percentage of IFN-γ^+ NK cells was significantly lower in non-infected unvaccinated subjects, when compared with infected (vaccinated and unvaccinated) and non-infected vaccinated individuals. Our results also show that the percentage of TNF-α^+ NK cells was significantly higher in infected (vaccinated and unvaccinated) subjects and in non-infected vaccinated patients, when compared with non-infected unvaccinated individuals, after *in vitro* stimulation with UV-inactivated VACV. Our data suggest that the expression of NCRs NKp30, NKp44, NKp46 and cytokines by NK cells are important in the innate response against VACV.
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1. Introduction

Vaccinia virus (VACV) is the causative agent of a zoonotic infection that affects cattle and humans in many regions of Brazil and since 1969, VACV outbreaks have been reported in different regions of the country (Medeiros-Silva et al., 2010). Despite the fact that the number of human cases is still increasing and new epidemic foci have been reported, little is known about the human immunological response against VACV natural infection, but it is clear that both the innate and adaptative responses are important (Lewis-Jones, 2004).

Natural killer (NK) cells constitutes an important element of the innate immune response against *Poxvirus* (Bukowski et al., 1983; Stitz et al., 1986). It has been described that VACV infection induces activation, proliferation, and accumulation of NK cells at the site of infection (Bukowski et al., 1983; Daniels et al., 2001; Dokun et al., 2001; Natuk and Welsh, 1987). It has been demonstrated that infection with *Orthopoxvirus* induces a marked increase in the susceptibility of target cells to lysis by NK cells and it seems that the natural cytotoxicity receptors NKp30, NKp44, and NKp46 are the most important NK cell receptors for the recognition of the VACV-infected target cell (Chisholm and Reyburn, 2006). Other NK cell receptors are well known to be involved in the immune response against *Poxvirus*. Among them are the surface molecules, CD161, that is involved in the inhibition process and is one of the earliest markers of NK cells (Rosen et al., 2005; Aldemir et al., 2005; Germain et al., 2011) and CD94 that has an essential role in the resistance of B6 mice to *Mousepoxvirus* (Fang et al., 2011).

Several new families of activators and inhibitory receptors have been recently identified on NK cells that have their activity determined by the balance of the expression of these receptors (Konjević et al., 2009). Although this has been shown to be critical there are no

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studies that evaluate the role of the different receptors expressed by NK cells against *Vaccinia virus* in human infection. In the present study, we evaluated the phenotype of NK cells expressing inhibitory markers (CD161), activation markers (CD94), natural cytotoxicity receptors (NKp30, NKp44, and NKp46), cytokines (IFN-γ and TNF-α), Granzymes (Granzyme A and B) and Perforins, after *in vitro* stimulation with UV-inactivated VACV following a natural *Vaccinia virus* infection after a zoonotic outbreak that occurred in Brazil in 2005.

2. Patients, materials, and methods

2.1. Study population

In 2005, a zoonotic outbreak of *Vaccinia virus* infection occurred in different areas in the state of Minas Gerais in Brazil. During the outbreak, blood from the affected individuals and from the cattle were collected and sent to Laboratory of Virus of the Universidade Federal de Minas Gerais (UFMG) and to the Immunology Laboratory in the Centro de Pesquisas Rene Rachou, FIOCRUZ-Minas to confirm the infection by VACV. As an extension of the studies during the outbreak, this investigation was initiated to evaluate the immune responses 5 year post exposure. The same individuals were recruited by our research group and blood collected at the 5-year time point to investigate the NK cells phenotype after *in vitro* stimulation with the virus. A total of 42 individuals were included in this study (Table 1). Out of the 42 individuals, 22 were previously infected by VACV and 20 were not affected by the disease. Important to note that out of the 22 infected individuals, 12 were vaccinated against smallpox and 10 were unvaccinated. Out of the 20 non-infected individuals, 10 were previously vaccinated and 10 were normal healthy individuals unvaccinated (negative control). Infections and/or vaccinations were confirmed with Plaque-reduction neutralization tests and/or IgG ELISA tests. Vaccinated individuals were identified by their vaccination card and/or vaccination scar. All study participants provided written informed consent following the guidelines of the Human Research Ethics Committee of the Universidade Federal de Minas Gerais.

2.2. Viruses and cells

Vero cells were used for virus replication, titration and UV inactivated viral test (Campos and Kroon, 1993). A thin layer of viral stocks, at a distance of 15 cm, were exposed 5 to 10 min to UV radiation at a wavelength of 280 nm. UV irradiated viruses were then tested for virus infectivity. Viruses that were unable to form plaques were considered to be UV inactivated. The infectious and cytopathic nature of VACV live virus limits its use in some applications, because it causes the host cell's death. UV inactivated VACV were used because it has been demonstrated that treatment of VACV with UV light results in the inactivation of viral replication without the abolition of viral transcription under early viral promoters. Furthermore, the antigenicity of the UV-inactivated VACV was found indistinguishable from that of the replicating VACV, both for restimulating the VV-primed cytotoxic T lymphocytes and serving as the cytotoxic T-lymphocyte target *in vitro* (Tsung et al., 1996). The cytopathic effect (CPE) of infection with VACV in particular includes the induction of early cell rounding, damage to the host genome and RNA, inhibition of host protein synthesis, and eventually, death of the infected cells (Bablanian, 1975).

2.3. Cell phenotype analysis

In vitro short-term cultures of whole blood samples were performed. Whole blood cells were stimulated *in vitro* with UV-inactivated VACV in RPMI 1640 media for 6 h at 37 °C. Approximately 1×10^4 PFU of *Vaccinia virus* strain WR were added per 1×10^6 leukocytes. Control cultures were maintained in culture media for the same period of time. Cultured cells were washed in FACS buffer and stained with monoclonal antibodies against CD3 (FITC, BD-Pharmingen, clone HIT3a), CD16 (PerCp, EXBIO, clone LNK16) and CD56 (APC, BD-Pharmingen, clone B159). The same cells were labeled simultaneously with antibodies against CD94 (PE, BD-Pharmingen, clone HP-3D9), CD107a (PE, BD-Pharmingen, clone H4A3), NKp30 (PE, BD-Pharmingen, clone P30-15), NKp44 (PE, BD-Pharmingen, clone P44-8.1), NKp46 (PE, BD-Pharmingen, clone 9E2/Nkp46). Cell preparations were fixed in FACS fix solution and stored at 4 °C in the dark. A total of 100,000 events/tube were acquired using a FACScalibur flow cytometer (Becton Dickinson). CELLQuest^{TM} software was used for data acquisition and the FLOW JO Version 7.5.5 software for analysis.

2.4. Intracellular cytokine staining (ICCS)

Whole blood was stimulated *in vitro* with UV-inactivated VACV as described above. During the last 4 h of culture, Brefeldin A was added. Cultured cells were washed twice in FACS buffer and stained with monoclonal antibodies specific for the different cell-surface markers, as described above. The cells were then permeabilized in saponin buffer and stained intracellularly with monoclonal antibodies against IFN-γ (PE, BD-Pharmingen, clone 4S.B3), TNF-α (PE, BD-Pharmingen, clone 6401.1111), Perforin (PE, BD-Pharmingen, clone δG9), Granzyme A (PE, BD-Pharmingen, clone CB9) and Granzyme B (PE, BD-Pharmingen, clone GB11). A total of 100,000 events/tube were acquired using a FACScalibur flow cytometer (BD Biosciences, USA). The samples were analyzed by using FlowJo software (Tree Star—Version 7.5.5).

2.5. FACS analysis of surface markers and intracellular cytokine

NK cells were analyzed for their intracellular cytokine expression patterns and frequencies as well as for cell surface markers using the FlowJo software (Tree Star—Version 7.5.5), as described in Supplementary Fig. 1. (A) First we performed the identification of peripheral lymphocytes population in diagram of FSC × SSC (R1) (Fig. S1A). Second we made dot plots of FSC × FL-1, displaying the frequency of $CD3^+$ and $CD3^-$ cells. Thus, $CD3^-$ cells were selected (R2) (Fig. S1B). Third, dot plots of FL-3 × FL-4 were made displaying the frequency of $CD3^-CD16^-CD56^+$, $CD3^-CD16^+CD56^-$, $CD3^-CD16^+CD56^+$ populations (R3) (Fig. S1C). Finally, we made dot plots of FSC × FL-2, displaying the frequency of NK cells, co-expressing the marker CD107a (R4) (Fig. S1D). The same strategy was used to select the frequency of NK cells, co-expressing NKp30, NKp44, NKp46, CD161, CD94, IFN-γ, TNF-α, Granzyme A, Granzyme B and Perforin (R4) (figure not shown). Limits for the quadrant markers were always set based on negative populations and isotype controls.

2.6. Statistical analysis

Analyses were performed using GraphPad Prism version 4.0 software (GraphPad Software Inc, USA). The nonparametric Mann–Whitney *U* test was performed to compare stimulated and non-stimulated cultures in each of the four groups. Kruskal–Wallis test was used to compare the four clinical groups, followed by Dunn's test to compare all pairs of experiments. Differences were considered significant when the p value was less than 0.05.

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