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

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A B S T R A C T
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 adaptive 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
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 re-stimulating 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-Pharzmening, clone HIT3a), CD16 (PerCP, EXBIO, clone LNK16) and CD56 (APC, BD-Pharzmening, clone B159). The same cells were labeled simultaneously with antibodies against CD94 (PE, BD-Pharzmening, clone HP-3D9), CD107a (PE, BD-Pharzmening, clone H4A3), Nkp30 (PE, BD-Pharzmening, clone P30-15), Nkp44 (PE, BD-Pharzmening, clone P44-8.1), Nkp46 (PE, BD-Pharzmening, 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 FACS caliber flow cytometer (Becton Dickinson). CELLQuest™ 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-Pharzmening, clone 4S.B3), TNF-α (PE, BD-Pharzmening, clone 6401.1111), Perforin (PE, BD-Pharzmening, clone 6G9), Granzyme A (PE, BD-Pharzmening, clone CB9) and Granzyme B (PE, BD-Pharzmening, clone GB11). A total of 100,000 events/tube were acquired using a FACS caliber 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.
3. Results

3.1. Previous vaccination against smallpox leads to a milder form of VACV infection

In this study some infected individual were previously vaccinated against smallpox. Despite their vaccination status they acquired VACV infection. In order to evaluate the clinical differences between vaccinated and unvaccinated infected individuals was previously validated by a questionnaire applied to all volunteers. It was observed that the mean age was 24.3 years in unvaccinated individuals and 56 years in the vaccinated group. All subjects studied were male, milkers and the average time worked in this function was 18 years among the unvaccinated individuals and 32 years in the vaccinated group. None of these individuals reported having a prior infection by VACV. It was observed that the number and type of symptoms (fever, generalized pain, lymphadenopathy, headache, and others) presented by unvaccinated and vaccinated individuals were significantly different. The average of symptoms was of 6, ranging from 4 to 7 symptoms, in unvaccinated individuals and 2.5, ranging from 1 to 7 in vaccinated patients, with mild features. In relation to the features of the lesions, the vaccinated individuals had an average of 2.5 injuries and unvaccinated individuals had an average of 8 lesions. In relation to the place of occurrence of the lesions in vaccinated subjects they concentrated largely on the hands (100%) and small events in other regions (17%) (nose, neck, and legs), while in unvaccinated individuals, the primary site of involvement was also on the hands (100%), but its occurrence in other regions was more significant (40%). Analysis of the days of leave of absence due to illness was 25% of the vaccinated group, which had an average of 10 days off, and in unvaccinated subjects an average of 15 days of sick leave for 60% of the individuals. There were two hospitalizations due to symptoms developed after treatment. There were no lethal cases. We had reports that family members had also contracted the infection by VACV in both groups analyzed.

3.2. Natural cytotoxicity receptors (NCR) are expressed at higher percentages in individuals who had been previously exposed to Vaccinia virus

In order to evaluate NK cells phenotype, after in vitro stimulation with UV-VACV, triple and fourth labeling were used. Initially the cells were stained simultaneously against CD3, CD16 and CD56. The same cells were then labeled against NK cell receptors, CD161, CD94, CD107a, NKP30, NKP44 and NKP46 to evaluate the expression of these molecules in the NK cell surface. The NK cells phenotype was defined based on the relative expression of the CD16 (or FcRIIIA, low-affinity receptor for the Fc portion of immunoglobulin G) and CD56 (adhesion molecule mediating homotypic adhesion) markers in five NK cell subpopulations: (1) CD56<sup>bright</sup>CD16<sup>+</sup> (50–70% of CD56<sup>bright</sup>), (2) CD56<sup>bright</sup>CD16<sup>dim</sup> (30–50% of CD56<sup>bright</sup>), (3) CD56<sup>dim</sup>CD16<sup>-</sup>, (4) CD56<sup>dim</sup>CD16<sup>bright</sup>, and (5) CD56<sup>-</sup>CD16<sup>bright</sup> (Cooper et al., 2001; Mavilio et al., 2005; Caligiuri, 2008).

The results did not show any significant difference in the mean percentage of natural killer cells in the four groups, after in vitro stimulation with UV-inactivated VACV when compared to control cultures (Table 2). The analysis of NK cells subpopulations did not show any significant difference (data not shown).

Analysis of the expression of natural cytotoxicity receptors NKP30, NKP44, and NKP46 showed a significant increase in the mean percentages of NK cells co-expressing NKP30 and NKP44 in the infected individuals (vaccinated and unvaccinated) and in non-infected vaccinated subjects, when stimulated and control cultures were compared (Fig. 1A–D). This was not observed in the non-infected unvaccinated group. Interestingly, the expression of NKP44 receptor, was lower in the non-infected unvaccinated group, when stimulated and control cultures were compared (Fig. 1C). It was also observed that NK cells co-expressing NKP30 or NKP44 were at higher percentages only in infected (vaccinated and unvaccinated) and in non-infected vaccinated patients, when compared to non-infected unvaccinated subjects, after stimulation with UV-inactivated VACV (Fig. 2A and B). However, our data showed that expression of NKP46 by NK cells was significantly higher only in the infected individuals (vaccinated and unvaccinated) when compared control and stimulated cultures (Fig. 1E and F). Previously infected subjects (vaccinated or not) had higher percentages of NK cells co-expressing NKP46, when compared with non-infected individuals (vaccinated or not) (Fig. 2C).

3.3. In vitro stimulation with UV-inactivated VACV upregulates CD161 and downregulates the CD107a

In order to study the inhibitory receptors expressed by NK cells, we analyzed CD161 molecule, which is one of the earliest markers of NK cells (Di Santo, 2006), firstly designated as an activating receptor (Azzoni et al., 1998), however the expression of this marker by NK cells has also been shown to have inhibitory activity (Rosen et al., 2005; Aldemir et al., 2005; Germain et al., 2011). Our results showed an increase on the percentage of NK cells co-expressing CD161 in the infected (vaccinated and unvaccinated)
Fig. 1. NCR’s evaluation of NK cells in the whole blood cells from individuals previously infected or not by zoonotic Vaccinia virus, before and after viral stimulation. Whole blood cells from patients infected or not with zoonotic Vaccinia virus were stimulated in the presence of UV-inactivated VACV or medium. The percentages of NK cells expressing Nkp30, Nkp44, Nkp46 and CD161 in control and stimulated cultures from infected and non-infected groups were evaluated by flow cytometry. (A and B) Percentage of Nkp30⁺ NK cells. (C and D) Percentage of Nkp44⁺ NK cells. (E and F) Percentage of Nkp46⁺ NK cells. (G and H) Percentage of CD161⁺ NK cells. The differences between the groups are considered significant at \( p \) less than 0.05 and are represented by * \( p \), 0.05, ** \( p \), 0.01 and *** \( p \), 0.001.
and in non-infected vaccinated subjects, when control and stimulated cultures were compared. No significant difference was observed in the non-infected unvaccinated group (Fig. 1G and H). When the four groups were compared after viral stimulation, our data showed that CD161 positive NK cells were at higher percentages only in the groups that had previous contact with VACV, either through vaccination or natural infection, when compared to non-infected unvaccinated subjects. Furthermore, previously infected individuals (vaccinated and unvaccinated) had higher percentages of this cells when compared to non-infected vaccinated subjects, after stimulation with UV-inactivated VACV (Fig. 2D).

Since the NK cells of the studied individuals were observed to be activated as measured by NCRs staining, the degranulation process was evaluated using the CD107a molecule. CD107a is a degranulation marker of NK cell and its expression correlates with both cytokine secretion and NK cell-mediated lysis of target cells (Alter et al., 2004). Our results showed that CD107a expression on the surface of NK cells was significantly decreased in all four groups studied, when control and stimulated cultures were compared (Fig. 3A and B). No differences were observed between the four groups, when stimulated cultures were compared (data not shown).

3.4. VACV induced a decrease in the expression of Granzyme A and Perforin by NK cells but did not affect the percentage of NK cells co-expressing Granzyme B

Labeling of peripheral blood NK cells with antibodies against Granzyme A, B, and Perforin was performed to evaluate the process of cytotoxicity of natural killer cells. Expression of Perforin and Granzyme A by NK cells were significantly lower when control and stimulated cultures were compared in all four groups (Fig. 3C–F). Our data did not show any significant difference on the percentage of NK cells co-expressing Perforin and Granzyme A among the four groups when stimulated cultures were compared (data not shown). A decrease in the percentage of NK cells expressing Granzyme B in non-infected unvaccinated group was observed when stimulated and control cultures were compared. Nonetheless, the percentage of these cells was higher in the infected (vaccinated and unvaccinated) and in the non-infected vaccinated individuals, when compared to noninfected unvaccinated group, after stimulation with UV-inactivated VACV (Fig. 3G and H).

3.5. VACV stimulation upregulates CD94 expression

CD94 is another surface molecule involved in the development and maturation of NK cells (Carretero et al., 1997; Lazetic et al., 1996; Phillips et al., 1996). CD94 receptors have been proposed to be important in NK cell tolerance to self, and contribute to NK cell-mediated immunity to viral infections (Orr et al., 2010). In this study, the mean percentage of CD94 positive NK cells were higher in infected (vaccinated and unvaccinated) and in non-infected vaccinated individuals, when control and stimulated cultures were compared (Fig. 4A and B). No significant difference between non-infected and infected groups was observed after viral stimulation (Fig. 4C).
Fig. 3. Evaluation of the percentage of NK cells expressing CD107a, Perforin, Granzyme A and Granzyme B from infected and non-infected individuals, before and after viral stimulation. The percentages of CD107a+, Perforin+, Granzyme A+ and Granzyme B+ NK cells were evaluated by flow cytometry. (A and B) CD107a+ NK cells. (C and D) Perforin+ NK cells. (E and F) Granzyme A+ NK cells. (G and H) Granzyme B+ NK cells. The differences between the groups are considered significant at p less than 0.05 and are represented by *, p, 0.05, **, p, 0.01 and ***, p, 0.001.
3.6. Previous exposure to Vaccinia virus leads to higher percentages of NK-TNF-α positive cells but not the number of NK-IFN-γ positive cells

Our results showed that the percentage of IFN-γ+ NK cells was significantly lower in the non-infected unvaccinated group, when control and stimulated cultures were compared (Fig. 5A and B). No significant differences between non-infected and infected groups were observed, when the stimulated cultures were compared (Fig. 5E). Our data showed that the percentage of TNF-α+ NK cells was significantly higher in infected (vaccinated and unvaccinated) and in non-infected vaccinated individual, when control and stimulated cultures were compared (Fig. 5C and D). Analysis of stimulated cultures of the four groups demonstrated a higher percentage of TNF-α positive NK cells only in the infected individuals (vaccinated and unvaccinated), when compared to non-infected (vaccinated and unvaccinated) subjects (Fig. 5F).

4. Discussion

In the course of VACV infection, the activation, proliferation, and recruitment of NK cell at the site of infection has been shown to be necessary for virus clearance (Bukowski et al., 1983; Daniels et al., 2001; Dokun et al., 2001; Natuk and Welsh, 1987). Furthermore, NK cells have long been proposed to mediate an important element in the protective response against different Poxvirus (Bukowski et al., 1983; Stitz et al., 1986). Previous work indicates that 6 h are sufficient for NK cells to be activated at the primary site of infection (Prlíc et al., 2005). These previous works had demonstrated the importance of NK cells against VACV, using murine models or individuals vaccinated against Smallpox. In this study the phenotype of NK cells were described following a natural Vaccinia virus infection and compared with the profile of NK cells induced by smallpox vaccination. Thus, 22 previously infected individuals and 20 non-infected individuals were selected. Out of the 22 previously infected individuals, 12 were vaccinated against smallpox and 10 were unvaccinated. From 20 non-infected individuals, 10 were vaccinated and 10 unvaccinated. It is interesting to note that in the previously infected group, 12 individuals were vaccinated against smallpox and despite their vaccination status, they acquired the VACV infection, but developed a milder form.

We demonstrated in a precedent work that previously infected individuals (vaccinated or not) have higher percentages of T memory cells when compared to non-infected individuals (both vaccinated and unvaccinated). It was also detected that just the infected individuals (vaccinated and unvaccinated) presented higher percentages of effector-memory T cells (TEM), capable to produce IFN-γ and TNF-α. In the non-infected individuals (vaccinated and unvaccinated), this increase in the mean percentage of T
CD4 memory lymphocytes expressing IFN-γ was not observed (Orr et al., 2010).

In our studies, we also observed that recent infection by VACV induced a more significant CD4 and CD8 effector memory response when we compared previously infected individuals (vaccinated and unvaccinated) to non-infected vaccinated patients (Orr et al., 2010). Once we demonstrated that CD4 and CD8 T cells were involved in the immune memory response following a natural infection with *Vaccinia virus*, we developed this work since it is well known that both the innate and adaptive responses are important to clear the infection (Lewis-Jones, 2004). We aimed to describe the phenotype of NK cells in the whole blood cells from previously infected and non-infected individuals, to better understand the human immunological response against VACV natural infection.

It is well known that different receptor–ligand interactions are responsible for NK-cell activation upon interaction with target cells.

In this study, we determined the profile of activation, degranulation and inhibition of NK cells following a natural *Vaccinia virus* infection and compared it to NK cells phenotype of non-infected individuals, previously vaccinated or not against smallpox. It has been described that natural cytotoxic receptors (NCRs) are involved in NK-cell activation upon interaction with target cells. In our work, we demonstrated an increase in the expression of these natural cytotoxic receptors (NCRs) NKP30, NKP44 and NKP46 by NK cells, after viral stimulation, only in individuals that were previously exposed to VACV, either by vaccination against smallpox or recent natural infection. Our data also showed that the percentage of NK cells expressing NKP44 was lower in the non-infected unvaccinated individuals, when control and stimulated cultures were compared. A significant increase on the percentage of NK cells expressing NKP46 were observed when we compared control and stimulated cultures of infected individuals (vaccinated and unvaccinated), but not in the non-infected groups (vaccinated and unvaccinated).
unvaccinated). When we analyzed the stimulated cultures of the four groups, we observed higher percentages of Nkp46+ cells in previously infected individuals compared with non-infected subjects (vaccinated or not). We also observed a higher percentage of Nkp46+ cells in non-infected vaccinated individuals compared to non-infected unvaccinated patients. Our data suggest that NCRs are involved in VACV recognition after a previous exposure to antigen and probably participate in the immune response to clear the infection. A precedent work demonstrated that pre-treatment of NK cells with a cocktail of MABs specific for all three NCRs completely blocked NK recognition of VACV-infected cells (Chisholm and Reyburn, 2006). This demonstrates the importance of NCR's in viral recognition and clearance.

Another surface marker analyzed in this work was the CD94 molecule. Our data demonstrated an increase on the percentage of NK cells expressing this marker in the infected (vaccinated and unvaccinated) and non-infected vaccinated individuals, when compared with non-infected unvaccinated subjects, after viral stimulation. Previous work with murine models demonstrated that CD94 has an essential role in resistance of infected mice with *Ectromelia virus* (Fang et al., 2008, 2010, 2011). So, we can speculate that the up-regulation of this molecule detected in our work is probably associated with the resistance against *Vaccinia virus* as it has been demonstrated in previous works with *Ectromelia virus* (Fang et al., 2008, 2010, 2011). However, our data showed that only infected (vaccinated and unvaccinated) and non-infected vaccinated individuals, had higher percentages of NK cells expressing CD94, when compared with non-infected unvaccinated subjects, after viral stimulation, suggesting that previous exposure may affect the expression of this markers by NK cells leading to faster elimination of the virus.

The finding of higher percentages of NK cells expressing NCR's (Nkp30, Nkp44 and Nkp46) suggests it is important role in these cells. However, it is also known that the degranulation and secretion processes of NK cells may also be inhibited after viral stimulation. Therefore, in order to determine whether inhibition of degranulation was occurring, we NK cells were intracellular staining for Perforin, Granzyme A and B and CD107a. Our results showed that after *in vitro* stimulation with *Vaccinia virus* the percentages of Perforin+, Granzyme A+ and CD107a+ NK cells were lower, in previously infected (vaccinated and unvaccinated) and non-infected vaccinated individuals, when compared to non-infected unvaccinated subjects. Moreover, VACV induced an increase in the percentage of NK cells expressing CD161, an inhibitory molecule (Rosen et al., 2005; Aldemir et al., 2005), and Granzyme B, in these same groups of individuals. This was not observed in non-infected unvaccinated individuals. This downregulation of molecules that are involved in the degranulation and secretion processes associated with the upregulation of CD107a, an inhibitory molecule, found in our work, could lead to an impaired NK cell immune response against *Vaccinia virus*, in these individuals, but the mechanisms of this process must be studied.

Experiments in murine models suggested that Perforin-mediated cytolysis was not required for resistance to VACV, Vesicular stomatitis virus, or Semliki Forest virus (Kagi et al., 1995). Furthermore, mice of the highly EV-resistant C57BL/6 background have an increased loss of resistance to EV when mutant mice deficient in GrA, GrB, or GrA and GrB were evaluated (Mullbacher et al., 1999). These studies demonstrated that mice lacking Perforin or Granzymes were unable to control primary EV infection and suggested an important role for these serine proteases in resistance to poxviruses. Our results do not show similar data but they suggest that in human infection the balance between Perforin and Granzymes is necessary for the immune response against VACV infection.

We also evaluated the expression of intracellular cytokines by NK cells and the data showed that the percentage of IFN-γ+ NK cells was significantly lower in non-infected unvaccinated group when control and stimulated cultures were compared. We observed that infected (vaccinated and unvaccinated) and non-infected vaccinated subjects had higher percentages of IFN-γ+ NK cells when compared with the non-infected unvaccinated individuals, after viral stimulation. The importance of IFN-γ in host defense against VACV is well established (Reading and Smith, 2003). Previous studies with CD4 memory T cells demonstrated that VACV infection induces the production of IFN-γ and that the frequency of IFN-γ producing cell is higher in previously vaccinated volunteers when compared with unvaccinated healthy volunteers (Combadiere et al., 2004; Puissant-Lubrano et al., 2010). Our data also demonstrated a higher percentage of NK cells expressing IFN-γ by groups that have been previous exposed to VACV. Analysis of TNF-α+ NK cells demonstrated a higher percentage of these cells in infected (vaccinated and unvaccinated) and non-infected vaccinated individuals, when compared to non-infected unvaccinated, after *in vitro* stimulation with UV-inactivated VACV. The role of TNF-α in protection against VACV infection has been previously explored and it was demonstrated that Smallpox vaccination induces TNF-α expression by CD4+ memory T cells (Puissant-Lubrano et al., 2010; Hammarlund et al., 2003, 2010). Although our work analyzed the TNF-α+ NK cells population it is important to notice that this population is significantly increased in those individuals who had a history of VACV infection or smallpox vaccination but not in non-infected unvaccinated subjects. Our results showed that NK cells are also an important source of TNF-α, a cytokine involved in the mechanisms of viral clearance.

In this work NK cells phenotype from individuals previously infected with VACV (vaccinated and unvaccinated) following a natural VACV infection was described and compared with the profile of NK cells induced by smallpox vaccination (non-infected vaccinated individuals). A negative control using non-infected unvaccinated individuals was used to compare the data presented by infected (vaccinated and unvaccinated) and non-infected vaccinated subjects. In this study we did not observe any difference in NK cells phenotype when we compared infected (vaccinated and unvaccinated) individuals to non-infected vaccinated patients (except with marker Nkp46). However, we observed significant differences when comparing infected (vaccinated and unvaccinated) and non-infected vaccinated individuals to the unvaccinated non-infected group, after stimulation with UV-inactivated VACV.

It seems that previous exposure to VACV (either by infection or vaccination) leads to a more robust NK cell immune response in a second Ag exposure, since we demonstrated that NK cells from previously infected individuals (vaccinated and unvaccinated) and non-infected vaccinated subjects expressed higher percentages of NCR's(Nkp30, 44 and 46), Granzyme B, INF-γ and TNF-α (molecules involved in lysis of target cells) when compared to non-infected unvaccinated individuals, after stimulation with UV-inactivated VACV.

In addition, our data showed that previous exposure to the antigen (either by natural infection or vaccination) induced partial protection that leads to a milder form of VACV infection, demonstrated by our clinical data. In previous work of our group we suggested that this fact occurred due to the long time interval between *Vaccinia virus* infection and vaccination, at least 30 years before the recent infection. So, previous exposure to the antigen induced partial protection that was reflected on a milder symptoms (Medeiros-Silva et al., 2013). Corroborating with the data presented in this work, previous studies with human *Monkeypox* infection indicated that smallpox vaccination was not able to prevent the infection, but it seems that *Monkeypox* infection in humans that received a smallpox vaccine manifested in a milder form (Heymann Goldman et al., 2004).
et al., 1998). Another study also suggests that receipt of smallpox vaccination more than 3–4 decades ago may not provide current protection. Furthermore, this study demonstrated that, vaccinia-specific CD4+ and CD8+ T cell responses obviously decline with age (Hsieh et al., 2004). In our work, the vaccinated groups are significantly older than the control group and this could interfere in the NK cells responses, although we did not detect any significant difference when we compared the previously vaccinated individuals (infected and non-infected) with the unvaccinated infected subjects. We had demonstrated that there were some significant differences in the phenotype of NK cells when we compared the infected (vaccinated and unvaccinated) and non-infected vaccinated individuals with the unvaccinated non-infected subjects. However we can speculate that this difference is not just due to the age of the individuals, but it is also associated with the previous exposure to the VACV. Our hypothesis is reinforced by another study that a proliferative memory responses to VACV persisted in 72% of the population and were not influenced by age (Combadiere et al., 2004). We suggest that previous exposure to VACV, either by recent infection or smallpox vaccination, leads to a more robust immune response mediated by NK and T memory cells, as we previously described (Medeiros-Silva et al., 2013), but at this moment we cannot identify what mechanisms are involved in this process.

It is necessary to further explore the mechanisms involved in the activation, degranulation and inhibition of NK cells to fully elucidate the roles of the distinct aspects of the immune response against a primary and secondary response to VACV infection.

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Appendix A. Supplementary data
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References