



CD4 and CD8 T cells participate in the immune memory response against *Vaccinia virus* after a previous natural infection[☆]



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ABSTRACT

The present study evaluates the immune response of memory CD4⁺ and CD8⁺ T cells from patients following a natural *Vaccinia virus* (VACV) infection. A total of 42 individuals were involved in the study being: 22 previously infected individuals (vaccinated or not against smallpox) and 20 non-infected individuals (vaccinated or not). A short-term *in vitro* stimulation with UV-inactivated VACV of whole blood cells was performed. Our study showed that previously infected individuals have a lower percentage of CD4⁺ T cells expressing lymph-node homing receptors (CD4⁺CD62L⁺CCR7⁺) and higher percentage of memory CD4⁺ T cells subsets (CD4⁺CD45RO^{high}) when compared with non-infected subjects, after *in vitro* viral stimulation. We also showed that infected individuals presented higher percentages of CD4⁺ and CD8⁺ memory T lymphocytes expressing IFN- γ when compared to non-infected individuals. We verified that the percentage of CD4⁺ and CD8⁺ T memory cells expressing TNF- α was higher in infected and non-infected vaccinated subjects when compared with non-infected unvaccinated individual. We also observed that previously infected individuals have higher percentages of CD8⁺ T cells expressing lymph-node homing receptors (CCR7⁺ and CD62L⁺) and that the memory T cells expressing IFN- γ and TNF- α were at higher percentages in the whole blood cells from infected and non-infected vaccinated individuals, when compared to unvaccinated non-infected subjects. Thus, our findings suggest that CD4⁺ and CD8⁺ T cells are involved in the immune memory response against *Vaccinia virus* natural infection.

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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, since 1969 [1]. The occurrence of these outbreaks is interesting as some individuals have no immunity against this infection and there are no immunological studies on the duration of immunity after natural human VACV infection. Most of the studies have evaluated the duration of immunity after smallpox vaccination [2–8], but there is little information about the immunity against a natural *Smallpox* and VACV human infection [7,9].

Smallpox vaccine induces strong CD4⁺ and CD8⁺ T cell responses that peak at 2–4 weeks post-immunization and decreasing to form a stable memory population of T cells thereafter [5,10]. Previous studies demonstrated the importance of the CD4⁺ T cell compartment and have shown that long-term cellular memory to VACV predominantly involves CD4⁺ Th1 IFN- γ producing cells [3,11,12] and have observed a reduction over time of VACV-specific memory T cells in previously vaccinated individuals that retained a predominant CD4⁺ memory T cell response [5,11]. The CD8⁺ T cell memory population seems to decline faster than memory CD4⁺ T cells [3]. These studies investigated the CD4⁺ and CD8⁺ memory T cells against the *Orthopoxvirus* in some individuals after *smallpox* infection [9,13] or in vaccinated individuals [5,9,11,12]. In humans, two broad memory T cell subsets (CD4⁺ or CD8⁺) have been delineated: central memory (T_{CM}) and effector memory (T_{EM}) T cells [14–16]. T_{CM} are CD45RO⁺ memory cells that constitutively express CCR7 and CD62L (T_{CM}CD45RO⁺CCR7⁺CD62L⁺), home to the lymph nodes, produce low levels of IFN- γ and have limited effector function, but they proliferate and become effector cells upon secondary stimulation [17–22]. By contrast T_{EM} are memory cells that

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have lost the constitutive expression of CCR7, are heterogeneous for CD62L ($T_{EM}CD45RO^+CCR7^-CD62L^{-/+}$), home to peripheral tissues, and can rapidly produce effector cytokines such as IFN- γ upon antigenic stimulation [17–19,22]. T memory cells are not classified just by the expression of the surface markers CD45RO, CD62L and CCR7. It is also necessary to evaluate the production of effector cytokines, such as IFN- γ . T_{EM} expresses high levels of activation markers (such as CD45RO), lose expression of the chemokine receptor CCR7, and acquire the capacity to produce IFN- γ and to kill target cells. In contrast, T_{CM} cells retain CCR7 expression, and fail to acquire cytotoxic function, although produce low levels of IFN- γ [17–22].

The importance of the role of CD4⁺ and CD8⁺ T cells in the control of VACV infection, in the induction and maintenance of long-term memory is well known [23], but the phenotype of these T cell subsets was not described. In the present study, we evaluated the phenotype of CD4⁺ and CD8⁺ T cells expressing activation markers (CD45RO), lymph node-homing receptors L-selectin (CD62L) and CCR7 and production of cytokines (IFN- γ and TNF- α) 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. Participants, materials, and methods

2.1. Study population

In 2005 a zoonotic outbreak of VACV infection occurred in different areas in Minas Gerais state, Brazil. During the outbreak, blood from these individuals and from cattle were collected and sent to the Virus Laboratory of the Federal University of Minas Gerais (UFMG) and to the Immunology Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ) as a part of the outbreak investigation to confirm the infection with *Vaccinia virus*. For confirmation of the infection some experiments were performed as previously described by virus isolation, phylogenetic analysis, plaque-reduction neutralization test and IgG ELISA [24]. As an extension of the outbreak investigation, this study was initiated to assess immune responses 5 years post-exposure. The same individuals were recruited and specimens collected to investigate the profile of CD4 and CD8 memory T cells. A total of 42 individuals were considered in these analyses. The studied group consisted of 22 infected human patients and 20 non-infected and healthy individuals. Importantly, out of 22 individuals previously infected, 12 were vaccinated against smallpox in the past, as confirmed by visualization of a typical vaccination scar in their left arm. Of the 20 non-infected individuals, 10 were vaccinated against smallpox in the past, as confirmed by visualization of a typical vaccination scar in their left arm and by data on the vaccination card; 10 were unvaccinated (control group) (Table 1). All patients were previously examined by a physician and those presenting apparent clinical signs of any other disease, infectious or not, were not included in the study. All study participants provided a written informed consent following the guidelines of the Ethics Committee of the Federal University of Minas Gerais.

2.2. Viruses and cells

Vero cells were used for virus replication, titration and UV inactivated viral test [25]. A thin layer of viral stocks, at a distance of 15 cm, was exposed for 5–10 min to UV radiation at a wavelength of 280 nm. UV irradiated viruses that were unable to form plaques were considered to be UV inactivated.

2.3. Cell phenotype analysis

In vitro short-term cultures of whole blood samples were performed. Whole blood was stimulated *in vitro* with UV-inactivated

VACV for 6 h at 37 °C and 5% CO₂. Approximately 1×10^4 PFU of *Vaccinia virus* strain WR was added per 1×10^6 leucocytes. 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 CD4, CD8, CD45RO, CCR7, and CD62L. Cell preparation was incubated in the dark for 30 min at room temperature. Following incubation, erythrocytes were lysed, the cells washed and fixed in FACS fix solution. A total of 100,000 events/tube were acquired using a FACScalibur flow cytometer (Becton Dickinson). The 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 (10 mg/mL) was added to the cultures. Cultured cells were washed and stained with monoclonal antibodies against the different cell-surface markers, as described above. The cells were then fixed in FACS fix solution. Finally, the cells were incubated and stained intracellularly with monoclonal antibodies against IFN- γ and TNF- α PE. A total of 100,000 events/tube were acquired using a FACScalibur flow cytometer (BD Biosciences, USA). Samples were analyzed using the FlowJo software (Tree Star – Version 7.5.5).

2.5. FACS analysis of surface markers and intracellular cytokine

Lymphocytes 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. S1. First we performed the identification of peripheral lymphocytes population in diagram of FSC \times SSC (Fig. S1A). Second we made dot plots of FL-1 \times FL-4, displaying the frequency of CD4⁺CD45RO⁺ cells. The CD4⁺CD45RO^{Low} (R1) and CD4⁺CD45RO^{High} (R2) populations were sorted using the indicated sorting gates (Fig. S1B). The same strategy was used to select the CD4⁺CD62L⁺ subpopulations. Dot plots of FL-1 \times FL-4 were made displaying the frequency of CD4⁺CD62L⁺ cells. The CD4⁺CD62L^{neg} (R1), CD4⁺CD62L^{Low} (R2) and CD4⁺CD62L^{High} (R3) populations were sorted using the indicated sorting gates (Fig. S1C). Finally, we made dot plots of FL-4 \times FL-2, displaying the frequency of CD4⁺CD45RO^{Low}IFN- γ ⁺, CD4⁺CD45RO^{High}IFN- γ ⁺, CD4⁺CD62L^{neg}IFN- γ ⁺, CD4⁺CD62L^{Low}IFN- γ ⁺ and CD4⁺CD62L^{High}IFN- γ ⁺ (Fig. S1D). 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 used to compare stimulated and non-stimulated cultures in each of the four groups. The Kruskal-Wallis test was used to compare the four clinical groups, followed by Dunn's test to compare all stimulated and non-stimulated cultures. Differences were considered significant when a *p* value of less than 0.05 was obtained.

3. Results

3.1. Previous vaccination leads to a mild form of VACV infection

Some of the infected individuals were previously vaccinated against smallpox, so we evaluated the clinical differences that occurred between the vaccinated and unvaccinated individuals. For this purpose we applied and analyzed a questionnaire previously validated by our group. In unvaccinated individuals, the mean age was

Table 1
Characterization of the population involved in the study.

Groups	Vaccination status	Number of individuals studied	Mean age (min–max)	Gender (n)	
				Male	Female
Previously infected ^a	Vaccinated ^b	12	56 (42–68)	12	0
	Unvaccinated	10	24.5 (18–34)	10	0
Non-infected	Vaccinated ^c	10	46.4 (39–62)	7	3
	Unvaccinated	10	29.2 (28–31)	6	4

^a These individuals were infected with *Vaccinia virus* during outbreaks that occurred in 2005 in different farms in the state of Minas Gerais, Brazil.

^b Importantly, out of 22 individuals previously infected, 12 were vaccinated against smallpox in the past, as confirmed by visualization of a typical vaccination scar in their left arm.

^c Of the 20 individuals non-infected, 10 were vaccinated against smallpox in the past, as confirmed by visualization of a typical vaccination scar in their left arm and data from vaccination card.

24.5 years with a minimum of 18 years and a maximum of 34. However, in patients previously vaccinated, the mean age was 56 years, with a minimum of 42 and a maximum of 68. All subjects studied were male milkers and the average time worked in this function was 18 years in the unvaccinated individuals and 32 in vaccinated patients. None of these individuals reported having prior infection by VACV. It was observed that the clinical symptoms (fever, headache, muscle pain, nausea, lymphadenopathy, headache, and others) occurred in a milder form in the vaccinated individuals. Analysis of the clinical symptoms showed that in unvaccinated patients the mean was six symptoms, ranging from 4 to 7; in the vaccinated individual the mean was 2.5 symptoms, ranging from 1 to 7. As for the features of the lesions, the vaccinated individuals had an average of 2.5 injuries and the unvaccinated individuals an average of eight lesions. In relation to the place of occurrence of these lesions, in the vaccinated group they concentrated largely on the hands (100%) and small events in other regions (17%) (arms, nose and legs), while in the unvaccinated, the primary site of involvement were the hands (100%), however, it also occurred in other regions (40%) (face, neck and legs). Assessing the days of leave of absence due to illness it was found that 25% of the vaccinated group had an average of 10 days off and in the unvaccinated 60% of individuals with an average of 15 days of sick leave.

3.2. Previously infected individuals have lower percentages of CD4⁺ T cells expressing lymph-node homing receptors cells but higher percentages of CD4 effectors T cells and CD8 T cells expressing lymph-node homing receptors

The mean percentage of T lymphocytes (CD3⁺) and its subsets (CD4⁺ and CD8⁺) (Table 2) was not different when the four groups were compared. In order to evaluate the CD4 and CD8 T cells phenotype, we used lymph node-homing receptors, L-selectin (CD62L) and CCR7 and activation markers (CD45RO, IFN- γ , TNF- α). Our data showed that the expression of CCR7 on CD4⁺ T cells, was significantly lower in the infected groups – vaccinated ($p < 0.05$) and unvaccinated ($p < 0.01$) – after *in vitro* stimulation (Fig. 1A) when compared with non-infected (vaccinated and unvaccinated). Despite this observation, the percentage of CCR7 negative CD4 T cells (CD4⁺ CCR7⁻) was significantly higher, in the infected groups, vaccinated ($p < 0.05$) and unvaccinated ($p < 0.01$), after *in vitro* stimulation (Fig. 1B), when compared with non-infected (vaccinated and unvaccinated). The results also showed that previously infected individuals had higher percentage of CD4⁺ T memory cells (CD4⁺ CD62L^{neg} CCR7⁻) when compared with non-infected subjects (vaccinated and unvaccinated) (Fig. 1C). Our data also showed that the expression of CD45RO^{High} by CD4⁺ T cells (memory lymphocytes) was statistically higher in the infected groups – vaccinated ($p < 0.01$) and unvaccinated ($p < 0.05$) – after *in vitro* stimulation with VACV, when compared to non-infected individuals (vaccinated and unvaccinated). In the non-infected individuals, there were no increases in the percentage of CD4 memory T cells (Fig.

1D).

On the other hand the expression of CCR7 in CD8⁺ T cells was higher in the infected groups (vaccinated and unvaccinated) when compared with non-infected (vaccinated and unvaccinated) subjects, after viral stimulation (Fig. 2A). The results also show that infected (vaccinated and unvaccinated) individuals have a higher percentage of CD8⁺ T cells expressing lymph node-homing receptors (CD8⁺ CD62L⁺ CCR7⁺) when compared with non-infected (vaccinated and unvaccinated) individuals (Fig. 2B). Furthermore, when we analyzed the subpopulation of CD8⁺ CD45RO^{High} memory T cells, we observed that there were a statistically higher percentage of these cells only in the infected groups (vaccinated and unvaccinated) ($p < 0.05$) after *in vitro* stimulation with VACV, when compared with non-infected (vaccinated and unvaccinated) patients.

3.3. *Vaccinia virus* exposure leads to higher percentages of IFN- γ and TNF- α expression by CD4⁺ T cells and its subsets (CD4⁺ CD45RO⁺ and CD4⁺ CD62L^{neg})

As it is well established that *Vaccinia virus* infection induces strong cellular responses capable of secreting effectors cytokines such as IFN γ and TNF- α , we evaluated the expression of these markers. In this study the expression levels of IFN- γ and TNF- α on CD4⁺ T lymphocytes were evaluated and the results showed that expression of IFN- γ and TNF- α on CD4⁺ T cells were significantly higher in the infected (vaccinated and unvaccinated) individuals and in the non-infected vaccinated subjects, after *in vitro* stimulation with VACV, when compared with non-infected unvaccinated patients (Fig. 3A and B).

Following the identification of CD4⁺ T cells expressing IFN- γ and TNF- α , the intracytoplasmatic presence of these cytokines in CD4⁺ CD45RO⁺, CD4⁺ CD62L⁻ (activated cells) and CD4⁺ CD62L^{Low} T cells was evaluated in the four groups, in the presence of *in vitro* stimulation by UV-inactivated VACV. The mean percentage T CD4 lymphocytes co-expressing CD45RO and IFN- γ showed that only the subjects who had been previously infected – both vaccinated ($p < 0.001$) and unvaccinated ($p < 0.01$) – presented a significantly higher percentages of CD4⁺ CD45RO⁺ and CD4⁺ CD45RO^{High} T memory cells expressing IFN- γ (Fig. 4A and B), when compared with non-infected (vaccinated and unvaccinated) individuals. We compared in Fig. 4C and D the production of IFN- γ by CD4 T cells that lost the selectin, CD62L, to those cells that appear to regain the marker (CD62L^{Low}), those called T_{CM} cells. Expression levels of IFN- γ were measured on CD4⁺ CD62L^{neg} T cells, and a significant increase of these cells was detected only in the infected individuals – vaccinated ($p < 0.01$) and unvaccinated ($p < 0.01$), after culture in the presence of UV-inactivated VACV, when compared with non-infected (vaccinated and unvaccinated) patients (Fig. 4C). Analysis of IFN- γ on CD4⁺ CD62L⁺ and CD4⁺ CD62L^{Low} T lymphocytes showed that there were significantly higher percentages of this CD4⁺ T cell subset only in the infected

Table 2

Mean percentage of T cells (CD3⁺, CD4⁺, CD8⁺) on the whole blood from individuals infected (vaccinated and unvaccinated) and noninfected (vaccinated and unvaccinated) by *Vaccinia virus*, after *in vitro* stimulation with UV-inactivated VACV.

Groups	CD3 ⁺ (%) ^a	Cell phenotype	
		CD4 ⁺ (%) ^a	CD8 ⁺ (%) ^a
Non-infected	Unvaccinated	71.62 ± 6.31	42.9 ± 5.36
	Vaccinated	65.18 ± 8.19	45.66 ± 8.78
Infected	Unvaccinated	76.28 ± 7.61	40.9 ± 6.33
	Vaccinated	72.18 ± 5.63	45.95 ± 7.08

^a There were no statistically significant differences in the mean percentage of T cells (CD3⁺, CD4⁺, CD8⁺) when the four groups were compared.

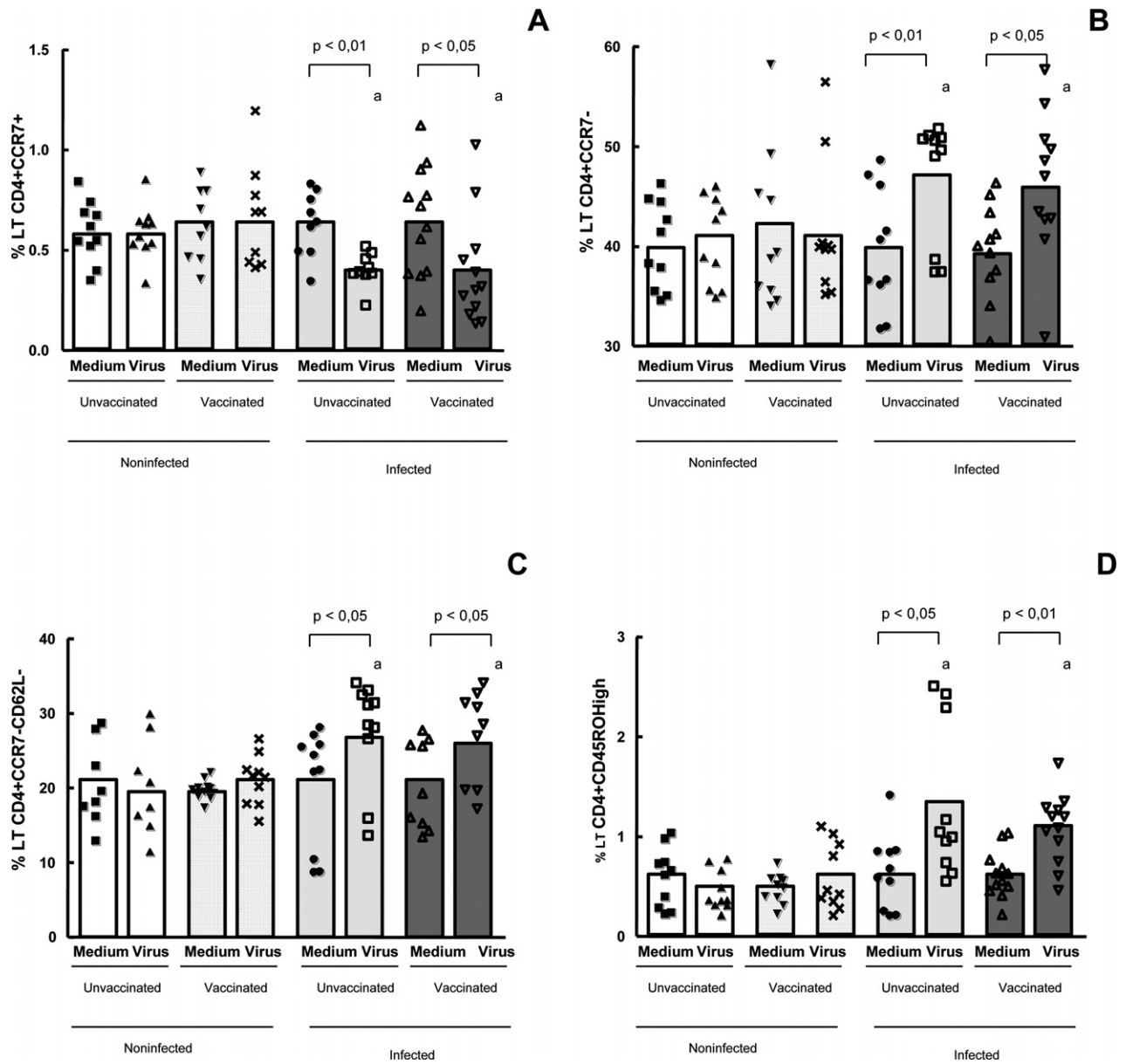


Fig. 1. Evaluation of memory and naive TCD4⁺ cells in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV or mock-treated (medium). After stimulus, cells were labeled with mouse anti-human CD4, CD62L, CCR7 and CD45 antibodies. The percentages of CD4 + CCR7 + (A); CD4 + CCR7 - (B); CD4 + CCR7 - CD62L - and CD4 + CD45RO^{High} T cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

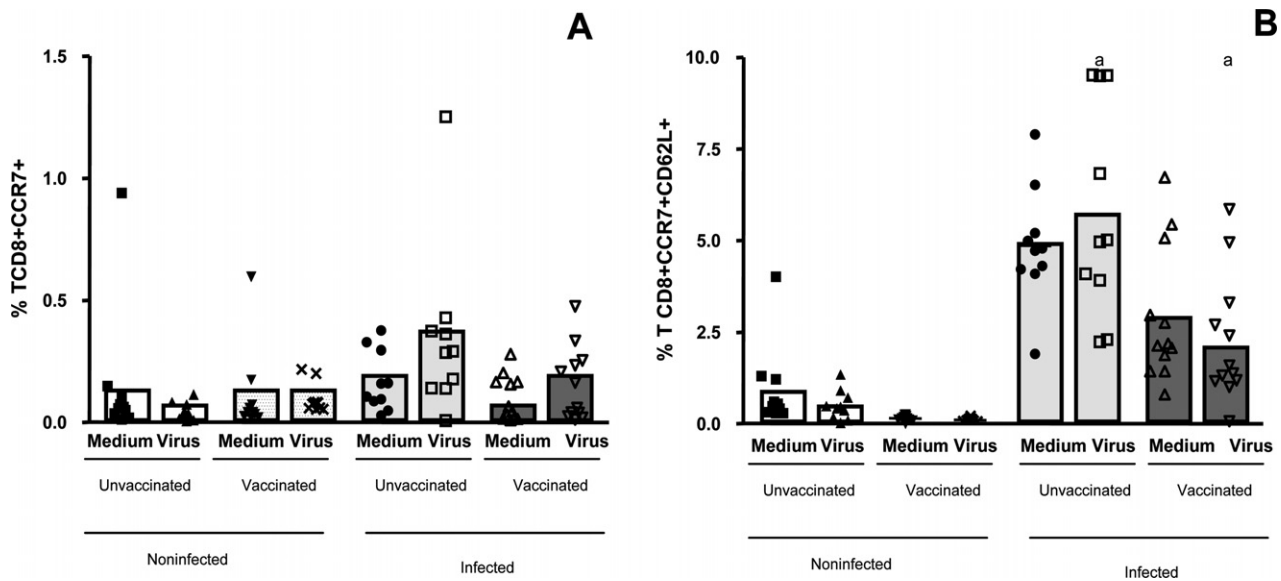


Fig. 2. Evaluation of naïve and memory TCD8 + cells in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV or mock-treated (medium). After stimulus, cells were labeled with mouse anti-human CD8, CD62L, CCR7 antibodies. The percentages of CD8 + CCR7 + (A) and CD8 + CCR7 + CD62L + (B) T cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

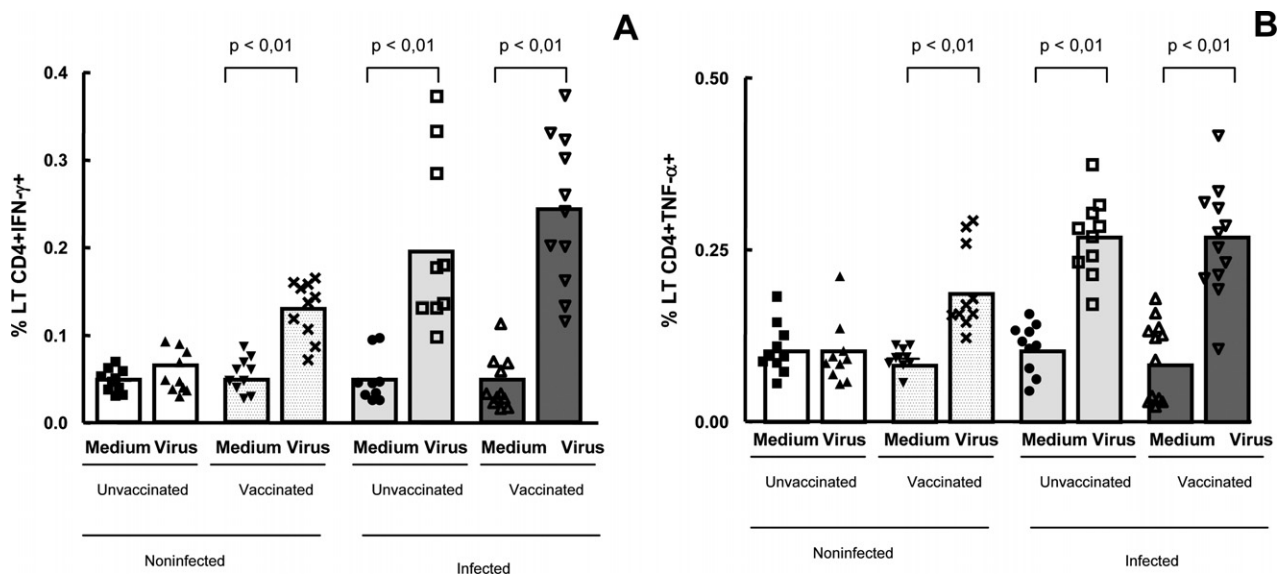


Fig. 3. Intracellular IFN- γ and TNF- α detection in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV or mock-treated (medium). After stimulus, cells were labeled with mouse anti-human CD4 antibodies and intracellular staining was used to measure the percentage of positive IFN- γ and TNF- α CD4 T cells. The percentages of CD4 + IFN- γ + (A) and CD4 + TNF- α + (B) T cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

(vaccinated and unvaccinated) groups, after *in vitro* stimulation with VACV, when compared with non-infected (vaccinated and unvaccinated) patients (Fig. 4D).

Analysis of the expression of TNF- α on CD4⁺CD45RO⁺ and CD4⁺CD45RO^{Low} T memory cells showed that there were significantly higher percentages of these cells in the non-infected vaccinated individuals ($p < 0.05$) and in the infected (vaccinated and unvaccinated) subjects ($p < 0.01$), when compared with non-infected unvaccinated patients (Fig. 5A), after viral stimulation. The mean percentage of CD45RO^{High}CD4⁺TNF α ⁺ T memory cells was lower in the non-infected unvaccinated group ($p < 0.01$) and were not different when we compared the other groups (Fig. 5B).

In this study we compared the expression of TNF- α by CD4 effector T cells that lost the selectin, CD62L, to those cells that appear to regain the marker. Expression levels of TNF- α on CD4⁺CD62L^{neg} and CD4⁺CD62L^{Low} T effector cells showed a significantly higher ($p < 0.05$) percentage of these cells in the infected (vaccinated and unvaccinated) groups and in the non-infected vaccinated individuals, after culture in the presence of UV-inactivated VACV, but not in the non-infected unvaccinated group (Fig. 5C and D).

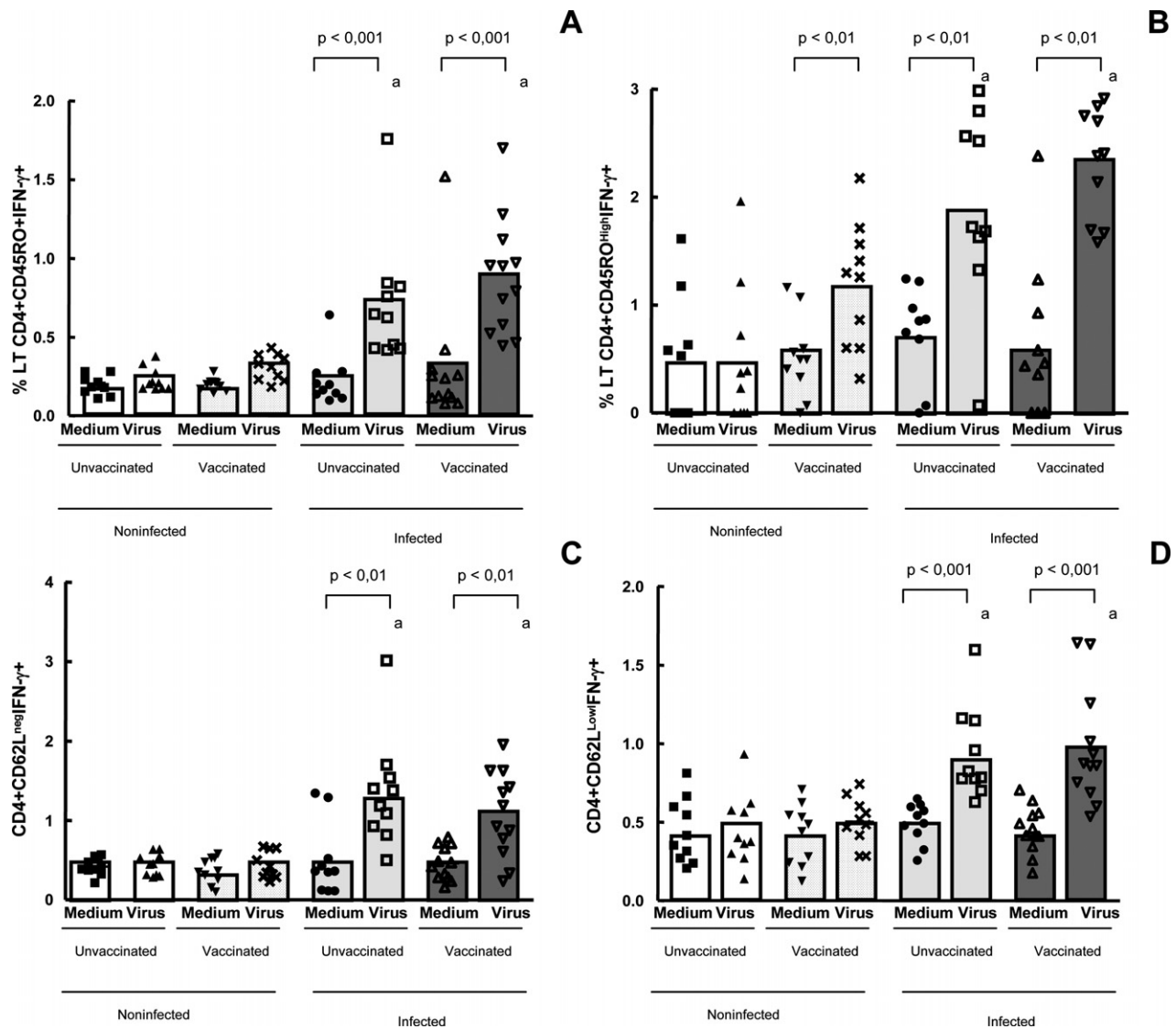


Fig. 4. Intracellular IFN- γ detection in CD4⁺ T memory cells in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV or mock-treated (medium). After stimulus, cells were labeled with mouse anti-human CD4, CD45RO and CD62L antibodies and intracellular staining was used to measure the percentage of positive IFN- γ cells. The percentages of CD4 + CD45RO + IFN- γ + (A); CD4 + CD45RO^{High}IFN- γ + (B); CD4 + CD62L^{neg}IFN- γ + (C) and CD4 + CD62L^{Low}IFN- γ + T cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

3.4. Previous *Vaccinia virus* exposure leads to higher percentages of IFN- γ and TNF- α by CD8⁺ memory (CD8⁺CD45RO⁺) and lymph-node homing (CD8⁺CD62L⁺) T cells

Following the identification of CD8⁺ T cells expressing lymph-node homing receptors and activations markers, we evaluated the intracytoplasmic expression of the effectors cytokines IFN- γ and TNF- α by these cells, in the groups of non-infected and infected individuals (vaccinated and unvaccinated), after *in vitro* stimulation by UV-inactivated VACV.

The data showed that only the infected individuals – both vaccinated ($p < 0.001$) and unvaccinated ($p < 0.01$) – presented a significantly higher percentage of IFN- γ producing activated CD8⁺ T cells (CD8⁺CD45RO⁺IFN- γ ⁺ and CD8⁺CD45RO^{Low}IFN- γ ⁺), after *in vitro* stimulation (Fig. 6A and B). Analysis of CD8⁺CD45RO^{High} T cells expressing IFN- γ did not show any significant difference when we compare the percentage of these cells in all four groups, after *in vitro* stimulation (data not shown). We also analyzed the CD8⁺CD62L⁺ expressing IFN- γ and we observed a significantly higher percentage (p

< 0.05) of these cells in the infected subjects and in the non-infected vaccinated individuals, after culture in the presence of UV-inactivated VACV, but not in the non-infected unvaccinated group (Fig. 6C). Analysis of CD8⁺CD62L⁺ and CD8⁺CD62L^{High} T cells expressing TNF- α showed that a significantly higher ($p < 0.05$) percentage of these cells was observed in the infected subjects and in the non-infected vaccinated individuals, after viral stimulation, but not in the non-infected unvaccinated group (Fig. 7A and B). Furthermore the analysis of CD8⁺ activated cells showed that the CD8⁺CD45RO⁺ T effectors cells expressing TNF- α were at higher percentages only in the infected individuals, when we compared the stimulated cultures (Fig. 7C).

4. Discussion

In this study the phenotype of CD4⁺ and CD8⁺ memory T cells was described following a natural *Vaccinia virus* infection and compared with the profile of CD4⁺ and CD8⁺ T memory cells induced by smallpox vaccination. Some differences were detected comparing CD4 and CD8 T cells, but the data clearly indicated that these cells

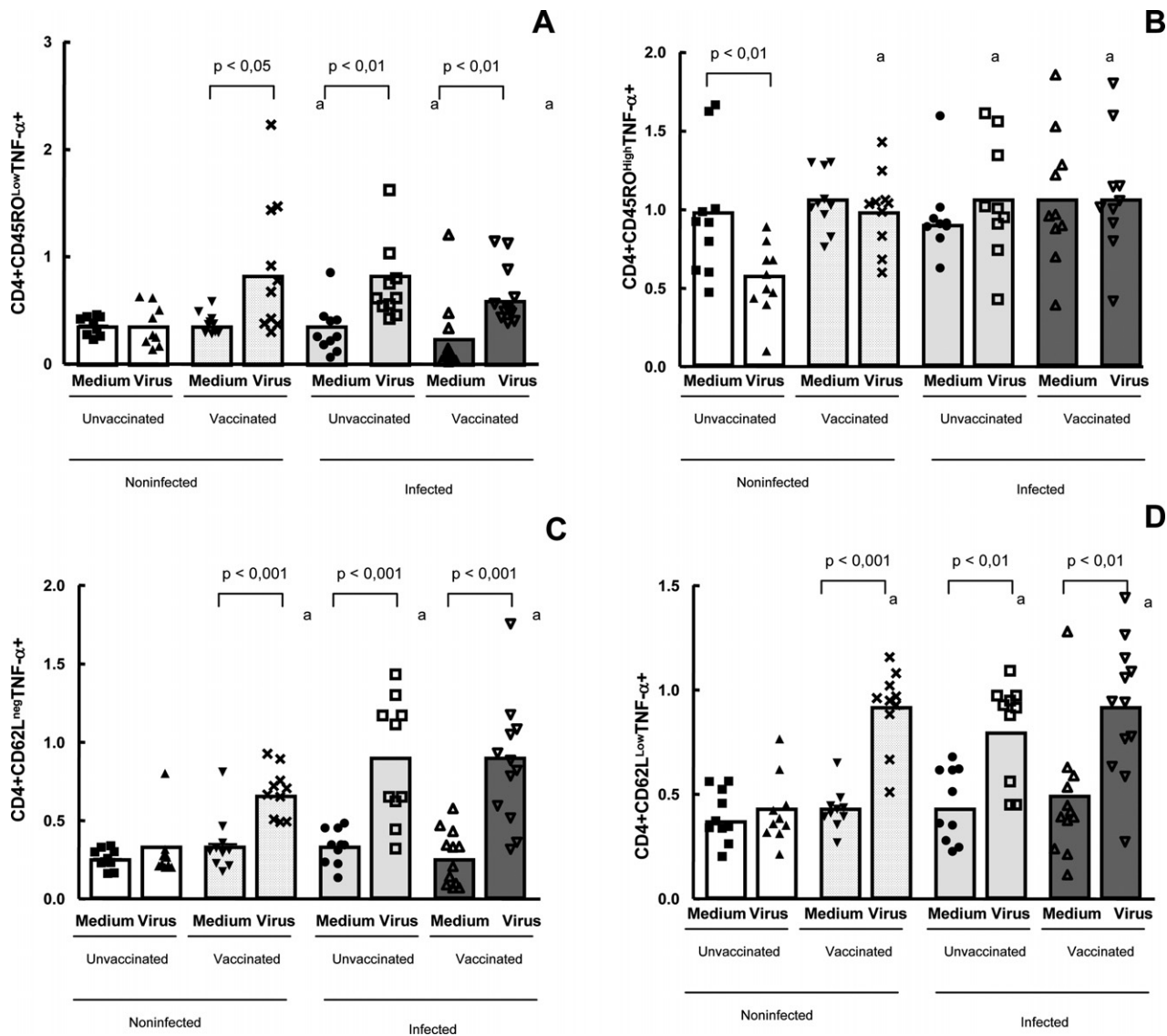


Fig. 5. Evaluation of CD4⁺ TNF-α⁺ T memory cells, using intracellular staining in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV or mock-treated (medium). After stimulus, cells were labeled with mouse anti-human CD4, CD45RO and CD62L antibodies and intracellular staining was used to measure the percentage of positive TNF-α⁺ cells. The percentages of CD4+CD45RO^{low}+ TNF-α⁺ (A); CD4+CD45RO^{high}TNF-α⁺ (B); CD4+CD62L^{neg} TNF-α⁺ (C) and CD4+CD62L^{low}TNF-α⁺ T-cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

are involved in the immune response to the VACV infection. In the present study, 12 (54%) of the 22 individuals previously infected by *Vaccinia virus* received a previous (childhood) smallpox vaccination. Despite their vaccination status, they acquired the VACV infection, but developed a milder form. A possible explanation for this fact is that *Vaccinia virus* infection occurred long after the vaccination, at least 30 years before the recent infection, suggesting that previous exposure to the antigen induced partial protection that was reflected on a milder symptoms. However, at this point it is not possible to elucidate what mechanisms are involved in this process. Karem and colleagues correlated the absence of complete smallpox vaccine-derived protection during the *Monkeypox* outbreak to the longer interval in time between vaccination and the outbreak [26]. 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 [27].

In this present study, we used lymph node-homing receptors, L-selectin (CD62L) and CCR7, and activation markers (CD45RO^{high}) to describe the lymph-node homing capability and the effectors functions of CD4⁺ and CD8⁺ T-cells. Several markers have been identified to allow for the differentiation of naive and memory T cells. Human naive cells are CD45RO⁻, CCR7⁺ and CD62L^{high} while memory cells (T_{EM}) are CD45RO⁺, CCR7⁻ and CD62L⁻. However, some memory cells appear to regain CD62L and CCR7, especially T_{CM} cells [17–22,28,29]. Here the CD4⁺ T cell memory subsets were evaluated and the data indicated that infected individuals (vaccinated or not) have higher percentages of T memory cells (CD4⁺ CCR7⁻ CD62L⁻ and CD4+CD45RO^{high}) when compared to non-infected individuals (both vaccinated and unvaccinated). The percentage of CD4⁺ T lymph-node homing T cells (CD4⁺ CCR7⁺ CD62L⁺) did not differ between the four groups analyzed. However, when we analyzed the CD8⁺ T cells, our results showed that previously infected individuals (vaccinated or not) have higher percentage of CD8⁺ naive and/or T_{CM} cells (CD8⁺ CCR7⁺ and CD8⁺ CCR7⁺ CD62L⁺) when compared

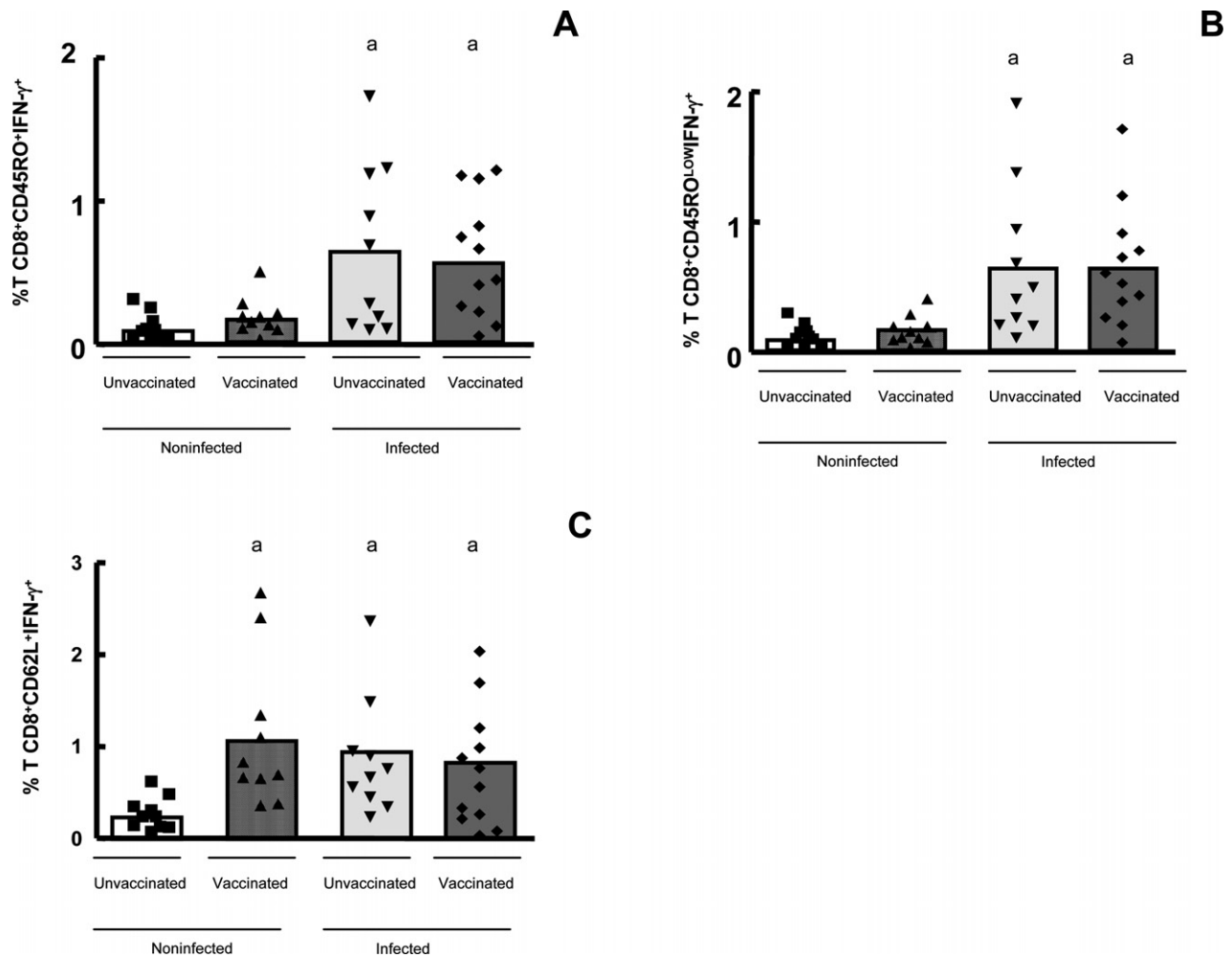


Fig. 6. Intracellular IFN- γ detection in CD8⁺ memory and naïve T cells in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*, after viral stimulation. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV. After stimulus, cells were labeled with mouse anti-human CD8, CD45RO and CD62L antibodies and intracellular staining was used to measure the percentage of positive IFN- γ cells. The percentages of CD8 + CD45RO + IFN- γ + (A); CD8 + CD45RO^{Low}IFN- γ + (B); and CD8 + CD62L + IFN- γ + (C) T cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

with non-infected individuals (vaccinated and unvaccinated). It is also important to note is that these cells co-express IFN- γ . Similarly, a recent study identified antigen-specific CD8⁺ T cells with a central memory phenotype. These cells were CD44^{High} and CD62L⁺ virus-specific CD8⁺ T cells that constituted approximately 10% of all antigen-specific T cells and expressed high levels of CCR7 [30]. Our data suggest that effector memory is predominantly composed by CD4⁺ T cells and these cells are at higher levels only in previously infected individuals, since this increase was not observed in non-infected vaccinated and unvaccinated patients.

In order to further study the population of CD4⁺ and CD8⁺ effectors memory T cells, *in vitro* stimulation was performed with UV-inactivated VACV and intracellular cytokine staining to identify virus-specific CD4⁺ and CD8⁺ T cells that expressed either IFN- γ or TNF- α . We observed that the percentage of CD4⁺ T cells expressing IFN- γ was higher in individuals previously infected by *Vaccinia virus* (vaccinated and unvaccinated) and in non-infected vaccinated when compared with non-infected unvaccinated subjects. Puissant-Lubrano and colleagues also found that the frequency of VACV-specific IFN- γ -producing cells and of VACV-specific proliferating cells in previously vaccinated volunteers was significantly higher than that of unvaccinated healthy volunteers [12]. In the present

study, it was noted that the CD4⁺ IFN- γ -producing T cells were composed of CD4⁺ CD45RO⁺ (CD4⁺ CD45RO^{Low} and CD4⁺ CD45RO^{High}), CD4⁺ CD62L^{Low} and CD4⁺ CD62L⁺ T cells. It was also detected that just the infected individuals (vaccinated and unvaccinated) presented higher percentages of CD4⁺ CD62L⁺ and CD4⁺ CD45RO^{High} T cells co-expressing IFN- γ -which are the effector-memory T cells (T_{EM}). In the non-infected individuals (vaccinated and unvaccinated), this increase in the mean percentage of CD4 memory lymphocytes expressing IFN- γ was not observed. Our data reinforce the idea that cellular memory to VACV predominantly involves CD4⁺ Th1 IFN- γ producing cells, as previously described [9,31,32]. In our studies, we also observed that recent infection by VACV induced a more significant CD4 effector memory response when we compared previously infected individuals (vaccinated and unvaccinated) to non-infected vaccinated patients.

Similarly, an increase on the percentage of the CD8⁺ memory T cells (CD8⁺ CD45RO^{High}) was detected only in individuals previously infected by *Vaccinia virus* (vaccinated and unvaccinated), but not in the non-infected (vaccinated or not) subjects. Similar results were observed in the CD4 compartment where an increase in the percentage of the CD4⁺ CD45RO⁺ memory T cells expressing IFN- γ was identified only in the previously infected individuals (vaccinated and unvaccinated), after *in vitro* stimulation with UV-inactivated VACV, when compared with non-infected (vaccinated and unvaccinated)

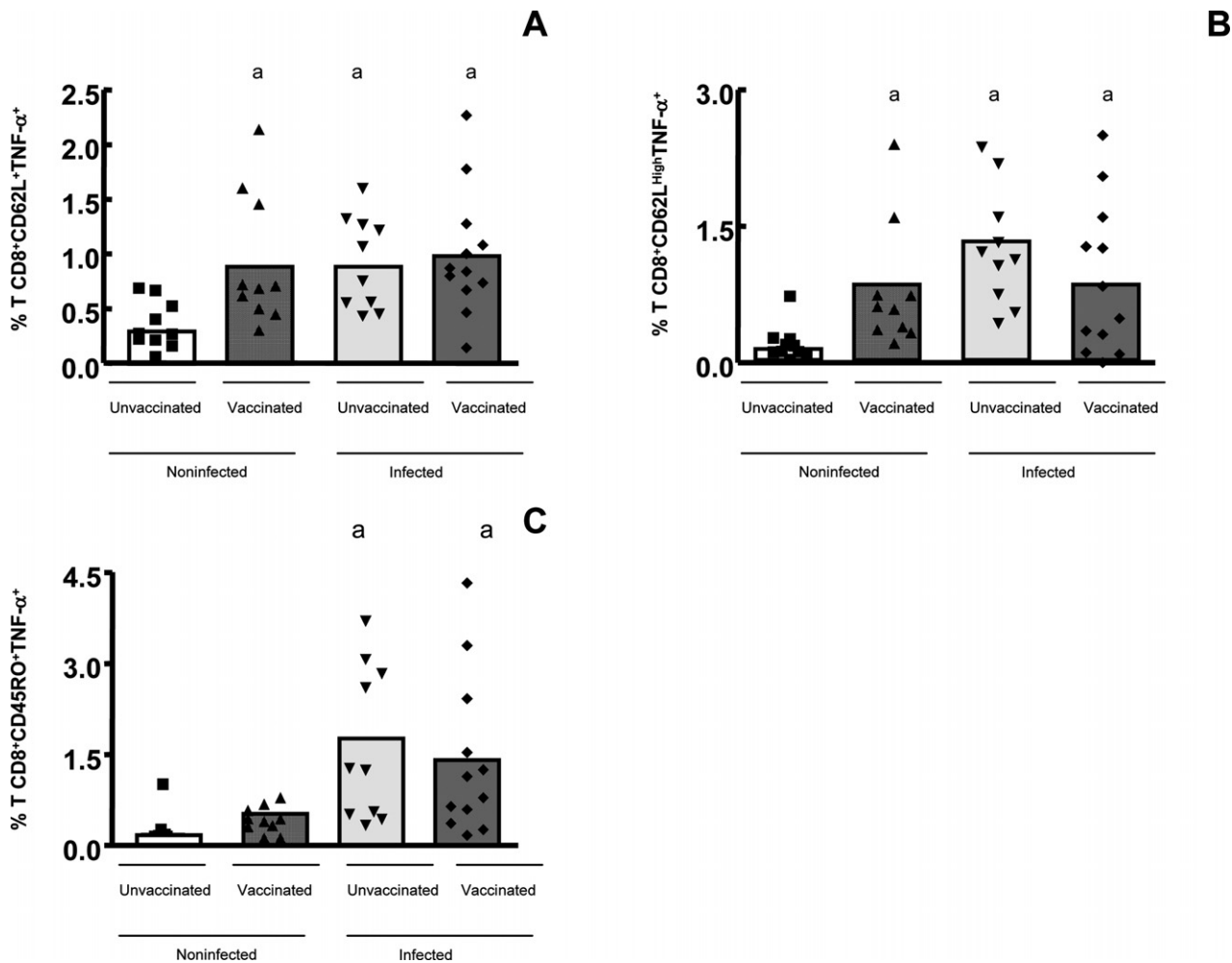


Fig. 7. Intracellular TNF- α ⁺ detection in CD8⁺ naïve and memory T cells in the whole blood cells from individuals previously infected or not by zoonotic *Vaccinia virus*, after viral stimulation. Whole blood cells from patients infected or not with zoonotic *Vaccinia virus* were stimulated in the presence of UV-inactivated VACV. After stimulus, cells were labeled with mouse anti-human CD8, CD45RO and CD62L antibodies and intracellular staining was used to measure the percentage of positive TNF- α ⁺ cells. The percentages of CD8 + CD62L + TNF- α ⁺ (A); CD8 + CD62^{Low}TNF- α ⁺ (B) and CD8 + CD45RO + TNF- α ⁺ (C) T-cell subsets were determined by flow cytometry. Statistical significance (*P* values) is presented on the graphs. *a means different from non-infected unvaccinated, stimulated cultures.

patients. Analysis of T CD8⁺ CD62L⁺ memory cells expressing IFN- γ showed an increase in the percentage of these cells in individuals previously exposed to *Vaccinia virus* (infected vaccinated or not and non-infected vaccinated), but not in the non-infected unvaccinated group, after viral stimulation. In the present study, it was observed that the CD8⁺ IFN- γ -producing T cells were composed of CD8⁺ CD45RO⁺, CD8⁺ CD45RO^{Low}, and CD8⁺ CD62L⁺ T cells. These results suggest that previous smallpox vaccination during childhood does not lead to a complete protection against VACV infection, but there is still a residual immunity. We also noted that previous infection leads to a more significant CD8 memory response when we compared previously infected individuals (vaccinated and unvaccinated) to non-infected vaccinated patients.

Moreover, it was demonstrated that CD4⁺ T cells from individuals who were once exposed to *Vaccinia virus* (either by smallpox vaccination or *Vaccinia virus* infection) were able to produce TNF- α . Our data showed that the *Vaccinia*-specific TNF- α producing T cells were composed mainly of CD4⁺ CD45RO^{Low}, CD4⁺ CD62L⁻ and CD4⁺ CD62L^{Low}. Other studies have already demonstrated that smallpox vaccination induces TNF- α expression by CD4⁺ memory T cells [5,12]. Similar results were observed when CD8⁺ T memory cells (CD8⁺ CD62L⁺ and CD8⁺ CD62L^{High}) co-expressing TNF- α were analyzed. The results showed an increase in the percentage of these

cells in individuals previously exposed to VACV (either by infection or vaccination) but not in the non-infected unvaccinated group. Our data also demonstrated an increase in the percentage of the CD8⁺ CD45RO⁺ TNF- α ⁺ memory T cells only in the previously infected individuals (vaccinated and unvaccinated), when compared with non-infected (vaccinated and unvaccinated) individuals. It is important to note that in this study both CD4⁺ and CD8⁺ T memory cells were expressing TNF- α . One study, using cell cultures and ICCS, demonstrated that in response to VACV infection, the large majority of the cytokine secreting cells produce only TNF- α (52–95% depending on the clone) [23]. Our results corroborates with this study, since we detected that CD4 and CD8 T memory cells are expressing TNF- α , an important molecule involved in the resistance against VACV infections.

Finally, our findings suggest that vaccinated individuals, even long after vaccination for smallpox, maintain a residual beneficial immune response to the virus. It induces partial long-term protection that in our study was clearly reflected on the fact that milder symptoms were developed and on the presence of memory cells. Our study, however, has some limitations such as the size of the analyzed group, which comprises 42 individuals, being 22 *Vaccinia virus* infected individuals and 20 non-infected individuals. As we previously described in another study the notification of human *Poxvirus* infections is not

mandatory in Brazil, and identification of patients is done through active random search [24]. This fact impaired our ability to enroll a larger number of patients and we cannot elucidate at this time the mechanisms involved in the immune memory response against *Vaccinia virus* infection.

5. Conclusions

In this study we observed that previous smallpox vaccination does not lead to a complete protection against VACV infection, since previously vaccinated individuals acquired the infection; however it lead to partial protection that is reflected on a milder clinical form. This fact is probably related to the fact that only previously infected individuals had higher percentages of CD4 and CD8 T memory cells when compared to non-infected subjects, vaccinated or not. Furthermore, it was found that CD4⁺ and CD8⁺ T memory cells expressing IFN- γ ⁺ are at higher percentages only in previously infected individuals that may lead to faster elimination of the virus.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rinim.2013.10.002.

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