Zoonotic Vaccinia Viruses Belonging to Different Genetic Clades Exhibit Immunomodulation Abilities That Are Proportional to Their Pathogenic Potential

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Research

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Abstract

Background

The *Vaccinia virus* (VACV) isolates, Guarani P1 virus (GP1V) and Passatempo virus (PSTV), were isolated from zoonotic outbreaks in Brazil and belong to two different VACV clades, as defined by biological aspects that include virulence in mice and phylogenetic analysis. Considering that information about how vaccinia viruses from different groups elicit immune responses in animals is scarce, we investigated such responses in mice infected by GP1V (group 2) or PSTV (group 1) using VACV Western Reserve strain (WR) as control.

Methods

The severity of the infections was evaluated in BALB/c mice considering diverse clinical signs and defined scores, and the immune responses triggered by GP1V and PSTV infections were analysed by immune cell phenotyping and intra-cytoplasmic cytokines detection.

Results

Infected mice showed significant weight loss and developed spleen lesions as well as liver and lung damage. Mice infected with PSTV, however, developed only moderate clinical signs. We detected a reduction of total lymphocytes (CD3+), macrophages (CD14+) and NK cells (CD3-CD49+) in animals infected with VACV-WR or GP1V. VACV-WR was able to significantly downmodulate cell immune responses upon mice infection, and GP1V-infected animals also showed intense downmodulation in cell responses. Contrarily, PSTV presented little ability to downmodulate mice immune responses.

Conclusions

Our results suggest that VACV immunomodulation *in vivo* is clade-related and is proportional to the strain virulence upon infection. Our data corroborate the classification of the different Brazilian VACV isolates in clades 1 and 2, taking into account not only phylogenetic criteria, but also clinical and immunological data.

Background

Members of the *Poxviridae* family present a double-stranded DNA genome that varies from 140 kbp to 300 kbp in size, depending on the virus species. Their genomes encode more than 150 genes including some involved in immunomodulatory mechanisms [1–3]. In Brazil, *Vaccinia virus* (VACV) has been circulating in rural and wild environments for decades [4]. Brazilian VACV isolates () are classified into two groups: group 1 (less virulent in a murine model of infection), and group 2 (more virulent in a murine model of infection) (Fig. 1). In addition to virulence in mice, this division reflects biological properties of the isolates such as plaque phenotype in BSC-40 cells and genetic differences, including the presence or

absence of an 18 nucleotide deletion in the viral hemagglutinin protein (HA A56R) encoding gene (see inset on Fig. 1) [5].

The Guarani P1 virus (GP1V) and Passatempo virus (PSTV), used in this study, were isolated in Minas Gerais, Brazil, in 2001 and 2003 respectively, from outbreaks in rural properties involving cattle and humans [6, 7]. The infection by these viruses caused lesions on the udder, teats, snout and oral mucosa of cows and calves. Milkers usually develop hand lesions after unprotected contact with sick cows. Clinical signs such as high fever, severe headache, back pain and lymphadenopathy were also reported by individuals handling infected cows [6, 7].

Much of the replicative success of poxviruses is related to their capacity to obstruct, escape or subvert essential elements of their host's antiviral response. It has been proposed that the poxviruses' ability to downmodulate the host's immune response is directly proportional to their virulence *in vivo* [8]. Previous studies have already shown a difference in the modulation of the immune response in mice infected with non-replicating (Modified *Vaccinia virus Ankara*), attenuated (*Vaccinia virus* Lister), and replicative (*Vaccinia virus Western Reserve*) viruses [8, 9].

The evaluation of infections caused by naturally circulating VACV strains GP1V (group 2) and PSTV (group 1) represents an opportunity to analyse immune responses triggered in the host as a result of infections with viruses presenting different virulence patterns. Such studies can, then, be compared to those including either laboratory strains of VACVs or vaccine strains. Here we investigated the immune responses triggered in BALB/c mice infected with the GP1V or PSTV VACV strains, originated from zoonotic outbreaks in Brazil, compared to infections by the prototype of the genus *Orthopoxvirus*, *Vaccinia virus Western Reserve* (VACV-WR). Our results are important not only to better define patterns of immunomodulation *in vivo*, caused by zoonotic vaccinia viruses, but they also corroborate the genetic classification of feral VACVs into two separate clades, which has been lately subject of criticism.

Methods

Viruses

Samples of GP1V and PSTV strains were kindly provided by Dr. Erna Kroon (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) and VACV-WR was gently provided by Dr. Bernard Moss (NIAID/NIH, Bethesda, EUA). The three viral samples were grown and titrated (PFU/ml) in BSC-40 cells through plaque essay and purified in sucrose cushions, as described before [10].

Infection of mice and clinical signs

BALB/c mice used in this study were obtained from UFMG's central animal facility (Belo Horizonte, Brazil) and maintained in our experimental animal facility (Departamento de Microbiologia, Belo Horizonte, Brazil). Animals were kept into ventilated cages with food and water *ad libitum*. All *in vivo* procedures were approved by the Committee of Ethics for Animal Experimentation (CETEA) from UFMG, under

permission 9/2019. Six-week-old male mice were separated into groups infected with GP1V, PSTV, VACV-WR or mock-infected (control). Animals were anaesthetized by intra-peritoneal injection of ketamine and xylazine (70 mg/kg and 12 mg/kg of body weight in phosphate-buffered saline [PBS], respectively). The intranasal (i.n.) route was used to inoculate PBS or 10 μ L of purified viruses diluted in PBS on subgroups of five animals each (for clinical signs and weight loss evaluation) or subgroups of seven animals each (histopathological analysis, splenocyte preparation, immunophenotyping and also detection of intracellular cytokines).

In order to monitor the infection from a clinical perspective, groups of five animals each were inspected daily, starting from the inoculation day. Inoculums of 10^4 , 10^5 , 10^6 PFU of PSTV, VACV-WR or GP1V were used to infect the animals whilst the control group was inoculated with sterile PBS. Weight loss and clinical signs were evaluated for 10 days and registered.

Histopathological analysis

Seven days post inoculation of 10^6 PFU of GP1V, PSTV, VACV-WR or PBS, animals were euthanized and had spleens, lungs and livers harvested for histopathological analysis. Fragments of organs were fixed with formalin for 24 hours and dehydrated with increasing concentrations (from 70 to 100%) of ethanol. Tissue fragments were diaphanized in xylol and embedded in paraffin. The segments were sectioned in a microtome (5 μ m) and stained using Hematoxylin and Eosin. A pathological characterization of these slide preparations was performed considering the presence and distribution of inflammation, edema, pulmonary hemorrhage and inflammation along with hepatic and splenic reactive degeneration, through the attribution of clinical scores [11–13].

Splenocyte preparation, immunophenotyping and detection of intracellular cytokines

In order to evaluate the production of IFN- γ and TNF- α by CD4 + and CD8 + T lymphocytes, mice were inoculated with 10^6 PFU of PSTV, GP1V or VACV-WR through the intranasal route. Splenocytes were obtained through maceration of their spleens. For erythrocyte lysis, cell extracts were resuspended in deionized water and incubated on ice. PBS 10X was used to stop the lysis process. Cell proliferation assays were performed through splenocyte labelling with Bromodeoxyuridine (BrdU). 96-well plates containing 2×10^5 spleen cells per well received stimulus of 10^4 PFU of purified UV-inactivated VACV-WR, concanavalin A (ConA) or just RPMI medium (Gibco, Carlsbad, USA). Plates were incubated at 37°C in a 5% CO₂ atmosphere for 72 hours. The evaluation of cell proliferation was performed according to the BrdU Cell Proliferation Assay kit (Millipore, USA) manufacturer's instructions. For cell immunophenotyping, 96-well plates containing 2×10^5 splenocytes per well were cultivated and incubated for 30 minutes at 4°C in the dark with monoclonal antibodies. Cell surface markers CD4, CD8, CD19, CD3/CD49 and CD14 and also the costimulatory molecules such as CD25, CD28, CD80 and CD86 were evaluated. Plates were washed with PBS, centrifuged and cells were suspended in 200 μ L of Macs Facs Fix (MFF) fixative solution.

For the purpose of detecting intracellular cytokines, 10^7 cells extracted from the macerated spleens were stimulated overnight with UV-inactivated VACV-WR (10^4 PFU) and incubated for 4h at 37°C with Brefeldin A (Sigma, MO, USA) at 1 mg/ml. Then, cells were washed in FACS buffer and stained with anti-CD4 and anti-CD8 antibodies (BD Pharmingen, NJ, USA) for 30 min at 4°C in the absence of light. Cells were permeabilized with FACS buffer containing 0.5% saponin, and then stained with mouse anti-TNF-, -IFN-, -IL-4, and -IL-10 (BD Pharmingen, NJ, USA) for further 30 min at room temperature. A new washing step with FACS buffer containing 0.5% saponin was followed by two steps of FACS buffer only. Cell preparations were stored at 4°C in the absence of light after fixation using FACS fix solution. A FACSCalibur cytometer (Becton, Dickinson, NJ, USA) was used for flow cytometry, and further analyses were performed using FlowJo software, parameters granularity (SSC) versus size (FSC) (TreeStar Inc., OR, USA).

Statistical analysis

The data was compared by analysis of variance (ANOVA) using Tukey post-test and parametric Student's T test. P values under 0.05 were considered significantly different. Statistical analyses were performed using Prism 8 software (GraphPad Software).

Results

Clinical signs in infected mice

All infected mice showed dose-dependent clinical signs typical of VACV infection, including piloerection and weight loss (Fig. 2). The severity of clinical signs in animals infected with 10^6 PFU of PSTV was considered moderated and the maximum weight loss was less than 10% percent of the animals' original weight throughout the experiment (Fig. 2A). Animals infected with 10^6 PFU of GP1V lost up to 28.84% of their initial weight (Fig. 2B). Weight loss in animals infected with 10^6 PFU VACV-WR was close to 30% (Fig. 2C). In addition to piloerection and weight loss, mice infected with VACV-WR developed the most severe clinical signs when compared to animals infected with other VACV strains in this study. Clinical signs included accentuated arched backs, swelling of the face, and conjunctivitis.

Histopathological analysis of liver, spleen and lung

The pathological characterization of the lungs, livers and spleens of mice uninfected or infected with 10^6 PFU of GP1V, PSTV or VACV-WR was performed through the attribution of clinical scores. Scores were given in relation to severity and distribution of the evaluated parameter on the tissue and these values were converted to a total score. For severity and distribution, the scale adopted scores from 0 to 5 or 0 to 4, in which the maximum value corresponds to a greater severity and distribution of the pathological alteration in the studied tissue. Animals infected with GP1V or VACV-WR developed more intense liver inflammation and degeneration when compared to animals of the control group and to those infected with PSTV (Fig. 3). All infected mice showed higher levels of splenic reactivity (hyperplasia of the splenic white pulp) and pulmonary inflammation when compared to mock-infected animals. Greater levels of

oedema and pulmonary haemorrhage were found in the GP1V- and VACV-WR-infected animals compared to the uninfected controls and PSTV-infected animals. The histological findings are consistent with the observed clinical signs.

Splenocyte proliferation after VACV stimulation and subpopulation characterization of immune cells elicited during VACV infection

Spleen cells' proliferation rates were higher in samples of infected mice stimulated with ConA compared to those stimulated with the UV-inactivated VACV-WR (Fig. 4). This increase was similar for splenocytes from GP1V- and PSTV- infected animals and less pronounced for VACV-WR infected animals.

Analyses of the splenic immune cells' subpopulations, including T-helper cells, B lymphocytes, NK cells and monocytes from uninfected mice or animals infected with 10^6 PFU of GP1V, PSTV or VACV-WR were performed (Fig. 5). A reduction in the frequency of CD3 + cells was observed in the animals from the group infected with VACV-WR and GP1V in comparison to the group infected with PSTV and uninfected controls (Fig. 5A). A higher statistical significance was found when PSTV-infected or uninfected animals were compared to VACV-WR than when compared to GP1V. Although no differences were detected in the frequency of CD4 + cells in the evaluated groups (Fig. 5B), CD8 + cells were more frequent in groups infected with GP1V and VACV-WR, respectively, in comparison to those infected with PSTV or uninfected controls (Fig. 5C). There was a decrease in the frequency of CD14 + and CD3-CD49 + cells in the groups infected with VACV-WR and GP1V when compared to the PSTV and the uninfected control group (Fig. 5D-E). CD14 + subpopulation was even less frequent in animals infected with VACV-WR than in animals infected with GP1V. The unique ability of VACV strains to downmodulate subsets of the host immune cells has been described [9], including human infections by zoonotic samples of Brazilian VACV [8].

The activation profile of TCD8 + cells, observed through the analysis of the expression of the CD28 + molecule, suggested that only animals infected with VACV-WR had a significant reduction in the activation profile of these cells (Fig. 6A). The modulation of CD8 + CD28 + cells after infection by the VACV-WR virus in mice has been demonstrated previously, as opposed to infections by VACV Lister and *modified Vaccinia virus Ankara* strains [8]. By analysing B lymphocytes expressing CD80 + we observed that the frequency of these cells' subset decreased similarly in mice infected with VACV-WR and GP1V in comparison to animals inoculated with PSTV (Fig. 6B). On the other hand, animals infected with PSTV presented more CD80 + B cells than uninfected control animals (Fig. 6B). Compared to the control and PSTV groups, the frequency of CD19 + CD86 + cells in animals infected with VACV-WR was lower. Similarly, GP1V group showed a significant reduction in these cells compared to PSTV group (Fig. 6C). This reduction in the frequency of CD80 + and CD86 + B lymphocytes was also reported for individuals affected by bovine vaccinia in Brazil [9].

Production of lymphocytic cytokines during infection by different VACV

We observed a general decrease in the IFN- γ -producing CD4 + T lymphocytes for the infected groups (PSTV, GP1V and VACV-WR) compared to the uninfected controls, in cells stimulated or not with UV-inactivated VACV-WR (Fig. 7A). The same trend was not observed when the IFN- γ -producing CD8 + T cells were analysed (Fig. 7B). The production of TNF- α by CD4 + T lymphocytes was similar for all groups evaluated after virus-antigen stimulation or not (Fig. 7C). Although the levels of TNF- α -producing CD8 + lymphocytes were slightly higher in animals infected with PSTV, compared to all other groups, (Fig. 7D) it was clear that the effect of VACV infection in TNF production by T cells is much more subtle than for IFN- γ production.

Discussion

The importance of innate, cellular and humoral immunity components on fighting Orthopoxvirus' infections has been demonstrated in several studies. The depletion of macrophages in mice results in their inability to control infection by vaccinia virus [14]. Likewise, the decline of NK cells levels in C57BL/6 mice culminates in increased ectromelia virus (ECTV) titers and disease severity [15, 16]. Complement-deficient mice developed more severe disease when infected with cowpox virus [17]. Evaluation of cytokines, such as IFN (I and II) and TNF also confirmed the key role of these molecules in the innate immune response against orthopoxviruses [18–21]. Both cellular and humoral responses are highly coordinated and require the combined activity of B and T lymphocytes. The primary infection of mice by ECTV cannot be controlled exclusively by TCD8 + lymphocytes [22] and production of antibodies by B lymphocytes is also essential in disease control, reinforcing the functional complementarity of the immune response to poxvirus' infections. This interaction between B and T cells is also crucial in subsequent exposures to these viruses [23]. Nonetheless, poxviruses are capable of encoding several proteins that are related to the evasion of the immune response [24, 25]. Indeed, it has been demonstrated that poxviruses infecting humans are able to significantly modulate components of host-specific immune response [26, 27]. Likewise, many studies have demonstrated the immunomodulatory ability of poxviruses in animal infections [8, 16, 25, 28]. Therefore, the viruses' ability to block, escape or subvert the essential elements of the antiviral response is essential for their replicative success in the host [24].

The Brazilian VACV isolates have been divided into two distinct groups. This classification considers characteristics such as the virulence of these isolates in a murine model, which in turn is linked to intrinsic genetic differences in their respective genomes. Analyses on how different zoonotic VACV isolates interact with their hosts, as well as other virological and biological aspects, could reinforce and support their segregation and classification into different genetic groups.

In this study, we showed how VACVs that belong to genetically different groups are able to modulate the immune response in mice in distinct patterns. Infections with VACV can lead to the appearance of clinical signs such as piloerection, weight loss, back arching, and facial edema. Nonetheless, animals infected with different VACV isolates show these signs differently [8, 29]. Ferreira and colleagues have demonstrated that infection by VACV-WR and GP1V in mice led to the appearance of signs such as piloerection, back arching, periocular alopecia and 25% weight loss. In contrast, the same study showed

that animals infected with PSTV and other VACV belonging to group 1, such as the *Araçatuba virus* and GP2V samples, did not exhibit typical clinical signs of the infection and did not experience marked weight loss. We have replicated these experiments and observed that animals infected with GP1V and WR presented the typical symptoms of VACV infection belonging to group 2. On the other hand, mice infected with PSTV did not manifest significant symptoms after virus inoculation.

Poxviruses have an extensive capacity to infect different hosts. However, viral multiplication rates vary according to the host species, considering that it depends on host-specific antiviral mechanisms [30]. The acute infection initiated in the lung after VACV intranasal inoculation can spread to other organs of the host [29]. One hundred percent of the animals inoculated with the PSTV, GP1V and VACV-WR showed chronic interstitial pneumonia. Liver and spleen were also compromised by infection with viral samples, indicating that PSTV, GP1V and VACV-WR multiply initially in the lungs, spreading to other organs and causing systemic disease. We also found that only VACV-WR was able to cause pulmonary haemorrhage in animals. The histopathological evaluation of the samples showed that PSTV is associated with a lower degree of liver and splenic damage when compared to the other studied viruses (as shown on Fig. 3), similarly to what was described by Ferreira and collaborators [29].

As previously reported, both cellular and humoral immunity are important for controlling infections triggered by Orthopoxvirus. Cell proliferation analysis is a parameter to detect the presence of antigen-specific lymphocytes, in order to obtain information about the cellular response induced by the infection. Gomes and collaborators [9] performed cell proliferation experiments carried out with human peripheral blood mononuclear cells (PBMCs) naturally infected with zoonotic VACV. They observed that after mitogenic and antigenic stimulation, individuals naturally infected with VACV showed a significant proliferative cell response compared to uninfected individuals. Similarly, our results showed increased levels of cell proliferation, after stimulation with VACV-WR, in cells from animals infected with the WR and PSTV samples (Fig. 4).

To deceive the cellular and humoral immune response, poxviruses encode several proteins capable of modulating their hosts' immune systems. Gomes and collaborators [9] also showed a lower frequency of CD14 + and an increase in CD8 + in humans infected with VACV zoonotic viruses. The immunomodulation of these subsets of cells suggests that such cells are important in controlling primary infection, preventing viral multiplication in infected cells. Furthermore, several studies have shown that the depletion of CD4 + T lymphocytes, macrophages and NK cells leads to greater disease severity in mice inoculated with VACV [16, 18, 31]. Some authors suggest that the primary VACV infection does not appear to be controlled solely by the activity of CD8 + T lymphocytes [9, 22]. Overall, these viruses have developed specific downmodulation mechanisms for most immune cells that are important to counter the infection. Our data reflect the differences in patterns of immune responses triggered by different VACV strains and different abilities to downmodulate such responses, culminating with distinct patterns of virulence. Infections by the GP1V and WR viruses (VACV Group 2) resulted in a robust T CD8 + response, unlike the animals infected with the PSTV sample (VACV Group 1), which presented a similar immune patterns observed in the mock-infected group. In addition, a reduction in total lymphocytes, NK

cells and macrophages were observed in the group infected with VACV-WR. However, once again the group infected with the sample belonging to the phylogenetic group whose virulence characteristics in mice are milder or non-existent did not show variation in these cell groups, presenting a global profile that was similar to the group of mock-infected animals. The cell activation patterns were also different when different VACV strains were inoculated into mice. VACV-WR- and GP1V-infected animals showed a tendency in CD19 + CD80 + cells downmodulation when compared to the uninfected controls. This was also observed in the study of VACV infections in humans [21]. Antibody production by B lymphocytes is essential to control infections caused by VACV [24]; therefore, it is not surprising that these viruses developed countermeasures that inhibit the activation of such cells. Mice infected with VACV-WR showed lower expression of CD28 in CD8 + T lymphocytes when compared to uninfected controls. Similarly, it has been suggested that this molecule is responsible for enhancing the activation of T cells after infection in mice with this VACV isolate [8].

Cytokines are secreted water-soluble proteins that act as mediators of immune responses, with autocrine and/or paracrine action. VACV produce virokines and viroceptors that mimic the molecules of the host's immune system, mainly affecting IFN, TNF and other cytokines [32, 33]. We also observed a reduction in IFN- γ production by CD4 + T lymphocytes in animals infected with GP1V and VACV-WR after antigen stimulation (Fig. 7A). This cytokine participates in the activation of macrophages, stimulation of inflammation and the mounting of Th1-type responses, all essential for the control of viral infections [34]. The reduction of IFN- γ production by these cells in GP1V- and VACV-WR-infected mice emphasizes the immunomodulatory capacity of these viruses as opposed to the PSTV strain. It corroborates the different virulence patterns found on the literature and observed in a murine model in this work [29].

Our data support a model in which the primary immune responses to acute Orthopoxvirus infection has the involvement of Macrophages/Monocytes and possibly CD4 + T cells, whereas the Lymphocyte-mediated response CD8 + would have a secondary role in infection control. The observation of modulation of those compartments in both humans [9] and mice, reinforces this hypothesis [11]. Finally, we demonstrated here that zoonotic vaccinia viruses belonging to different clades exhibit immunomodulation properties that are proportional to their pathogenic potential. These observations reinforce the idea that the segregation of zoonotic VACVs in two distinct clade/groups reflects not only genetic differences, but distinct virological and biological aspects as well.

Conclusions

Our data suggests that zoonotic VACV belonging to different clades exhibit immunomodulation properties that are proportional to their pathogenic potential.

List Of Abbreviations

BrdU - Bromodeoxyuridine

CETEA: Comitê de Ética em Experimentação Animal

ConA - concanavalin A

ECTV - Ectromelia virus

FACS: Fluorescence-activated single cell sorting

FSC: Forward scatter

GP1V - Guarani P1 virus

GP2V - Guarani P2 virus

HA A26R - Viral hemagglutinin protein encoding gene

IFN- γ - Interferon

IL-10 - Interleukin 10

IL-4 - Interleukin 4

IN: Intranasal

MFF - Macs Facs Fix

NK: Natural killer

PBMC: Peripheral blood mononuclear cell

PBS - Phosphate-buffered saline

PFU - Plaque-forming unit

PSTV - Passatempo virus

SSC: Side-scattered light

TNF - Tumor necrosis factor

UV: Ultraviolet

VACV- Vaccinia virus

VACV WR - Vaccinia virus Western Reserve

VACV-BR: Brazilian Vaccinia viruses

Declarations

Ethics approval

All in vivo procedures were approved by the Committee of Ethics for Animal Experimentation (CETEA) from UFMG, under permission 9/2019.

Consent for publication

Not applicable.

Availability of data and materials

Data and materials are available upon request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

KL performed the experiments and analyzed the data, prepared the original draft. LC performed the experiment and helped with the manuscript writing. LH, RR and FF designed the experiment and helped with the preparation of the final manuscript. All authors have read and approved the final version of the manuscript.

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Phylogenetic analysis of VACV-BR based on nucleotide sequences of the A56R gene (hemagglutinin). VACV-BR group 1 is highlighted with light yellow and group 2 with light blue. White arrows indicate the Brazilian viruses used in this study. The alignment was built using Muscle with standard parameters [1]. The maximum likelihood tree was built with IQ-TREE [2]. The best-fit replacement model selected by ModelFinder was HKY + F + I. The branch supports were estimated using 1000 replicates of standard bootstrap and the tree was visualized with iTOL [3]. Bootstrap values lower than 50 were suppressed. The clade of Variola viruses has been collapsed. The scale bar indicates the number of substitutions per site. A schematic alignment was included (inset) to indicate the genomic signature that divides VACV-BR into two distinct groups, an 18-nt deletion between positions 740 and 757 of the A56R gene. The representation was based on the VACV-WR (AY243312) gene.

Figure 2

Body weight of BALB/c mice uninfected or infected with VACV-BR. Groups of mice (n=5) were inoculated with PBS or 104, 105, 106 PFU of PSTV (A), GP1V (B) or VACV-WR (C) by the intranasal route. panel D represents the comparative weight loss in animals infected with 106 PFU of each of the three viral samples. The mice's weight was determined daily and those that lost more than 30% of their initial weight were euthanized. The percentages of median weight relative to their initial weight were plotted. The error

bars indicate the standard deviations. Significance variations were calculated using the Two-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Pathological analysis of liver, spleen and lung of BALB/c mice uninfected or infected with VACV-BR. Groups of mice (n=7) were inoculated with PBS or 10⁶ PFU of PSTV, GP1V or VACV-WR by the intranasal route. The liver inflammation (A) and degeneration (B), spleen reactivity (C), lung inflammation (D), edema and hemorrhage were determined 7 days post infection. Scores were assigned to severity and distribution of the assessed characteristic on the tissue and these values were converted to a total score. For severity and distribution, the scale adopted scores from 0 to 5 or 0 to 4, in which the maximum value corresponds to a greater severity and distribution of the pathological alteration in the studied tissue. The pathological scores were plotted. The error bars indicate the standard deviations. Significance variations

were calculated using the One-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4

Proliferation of splenocytes from BALB/c mice uninfected or infected with VACV-BR. Groups of mice ($n=7$) were inoculated with PBS or 106 PFU of PSTV, GP1V or VACV-WR by the intranasal route. The spleen cells proliferation was determined 7 days post infection with BrdU Cell Proliferation Assay (Millipore, EUA) by the absorbance at 450nm measuring. For that, spleen cells were cultured in the presence of 2 $\mu\text{g/mL}$ of Concanavalin A (black bars), 104 PFU of UV-inactivated VACV-WR (gray bars) or mock-treated with medium (white bars). The absorbance values were plotted. The error bars indicate the standard deviations. Significance variations were calculated using the One-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5

Immunologic populations from spleen of BALB/c mice uninfected or infected with VACV-BR. Groups of mice (n=7) were inoculated with PBS or 106 PFU of PSTV, GP1V or VACV-WR by the intranasal route. The percentage of T lymphocytes CD3+ (A), CD4+ (B) and CD8+ (C), monocytes CD14+ (D) and NK cells CD3-CD49+ (E) was determined 7 days post infection by flow cytometry and analyzed using FlowJo software, parameters granularity (SSC) versus size (FSC). The error bars indicate the standard deviations. Significance variations were calculated using the One-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6

Activated phenotype in CD8+ T-cell and B lymphocyte from BALB/c mice uninfected or infected with VACV-BR. Groups of mice (n=7) were inoculated with PBS or 106 PFU of PSTV, GP1V or VACV-WR by the intranasal route. The mean fluorescence intensity of CD8 T lymphocytes expressing CD28 (A) surface activation marker and percentage of B lymphocytes CD19 expressing CD80 (B) e CD86 (C) was determined 7 days post infection. The cells stained with monoclonal antibodies linked to fluorochromes against cell surface molecules were detected by flow cytometry. The error bars indicate the standard deviations. Significance variations were calculated using the One-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 7

Interferon-gamma and tumor necrosis factor production from BALB/c mice uninfected or infected with VACV-BR. Groups of mice (n=7) were inoculated with PBS or 106 PFU of PSTV, GP1V or VACV-WR by the intranasal route. The percentage of T lymphocytes CD4+ expressing IFN- γ (A) and TNF- α (C) or CD8+ expressing IFN- γ (B) and TNF- α (D) was determined 7 days post infection by flow cytometry. For that, spleen cells were cultured in the presence of 104 PFU of UV-inactivated VACV-WR (black bars) or mock-treated with medium (white bars) and were labeled with mouse anti-IFN- γ and anti-TNF- α antibodies. The error bars indicate the standard deviations. Significance variations were calculated using the One-way ANOVA post hoc Bonferroni with statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.