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Effects of inactivated *parapoxvirus ovis* on cellular and humoral events of the innate immune response in mice

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ABSTRACT

The immunostimulating properties of inactivated *parapoxvirus ovis* (iPPVO) have long been demonstrated *in vivo* and *in vitro*, yet the biological and molecular mechanisms involved remain largely unknown. We herein report that intraperitoneal inoculation of iPPVO in mice results in stimulation of several events of the innate immune response. Increased interferon I (IFN-I) activity was demonstrated in sera of mice treated with iPPVO at 6 and 12 h post-inoculation (hpi), and enhanced expression of IFN-γ (15-fold increase) and IL-12 (6-fold) mRNA was detected in the spleen of treated mice at 24 and 48 hpi, respectively. A significant increase in neutrophil activity ($p < 0.01$) was observed at 6 hpi in the blood of iPPVO treated mice. In addition, increased phagocytic activity by peritoneal macrophages of iPPVO-treated mice ($p < 0.01$) was detected *in vivo* (from 24 to 72 hpi) and *in vitro* (12 to 96 hpi). Bactericidal activity of sera mice treated with iPPVO against *Escherichia coli* was also increased ($p < 0.05$) at 24 and 72 hpi. Taken together, these results demonstrate that iPPVO administration leads to a transient stimulation of selected innate immune mechanisms, likely contributing to the immunostimulant effects observed against viral and bacterial infections *in vivo*.

1. Introduction

Parapoxvirus ovis (PPVO) or *orf* virus (OV or OrfV) is a member of the genus *Parapoxvirus* (PPV), family *Poxviridae* [1]. PPVO infection in sheep and goats – its natural hosts – results in a mucocutaneous, debilitating disease known as contagious ecthyma or contagious pustular dermatitis. The disease is characterized by inflammatory, proliferative and scabby lesions in the mucocutaneous junction of the lips, labial commissure, muzzle and, less frequently, in the udder and coronary bands. Human infection may occasionally occur and courses with self-limiting vesicular and pustular lesions in the hands and fingers [2]. Contagious ecthyma is endemic in most sheep and goat raising countries and leads to important economic losses [2]. In several countries, vaccination has been used with relative success to reduce the losses associated with the disease [3].

An interesting aspect of PPVO biology is the ability to re-infect the hosts in spite of a strong immune response [4]. Neutralizing antibodies are barely – if so – detected and seem not to confer protection to reinfection [3]. Upon infection, PPVO strongly stimulates many events of the innate immunity, including phagocytosis, NK cell activity and production of several cytokines (IFN-α, β; TNF-α, GM-CSF) [5]. On the other hand, a number of immune escape mechanisms have been demonstrated for this agent [2–4]. The 138 kilobases (kb) PPVO genome encodes a series of products interfering with the host innate response, including an interferon resistance gene [6], inhibitors of GM-CSF and IL-2 [7,8], a Bcl-2-like apoptosis inhibitor [9], an IL-10 homologue [10], a vascular endothelial growth factor (VEGF) [11] and inhibitors of the NF-KB signaling pathway [12]. The survival strategy of PPVO seems to rely upon a balance between immune stimulatory and escape mechanisms [5].

In addition to the complex interactions with the immune system during infection, inactivated PPVO (iPPVO) has long been recognized to stimulate the innate immune response. Administration of inactivated PPVO was first demonstrated to reduce the mortality of mice challenged with *Pseudomonas aeruginosa* [13]. Then, a number of studies investigated the immunostimulatory effects of iPPVO in different pathological conditions [5,14]. The promising effects of iPPVO as immunostimulant led to the

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development of a commercial product called Baypamun [13], whose immunostimulatory effects were subsequently demonstrated in infectious diseases and stress-mediated disorders of several animal species [15–20].

Trying to elucidate the mechanisms behind the immunostimulating effects, a number of studies *in vitro* focused on the effects of iPPVO on early events of the innate response, noticeably phagocytosis, NK cell activity and IFN-α production [21–24]. PBMC stimulation and proliferation resulting in IL-2, IFN-α and IFN-γ release were observed in iPPVO-stimulated swine blood cells [25]. iPPVO also exerted a strong influence on cytokine secretion by human immune cells, leading to release of inflammatory and Th-1 cytokines (IFN-γ, IL-2, IL-6, IL-8, IL-18) and anti-inflammatory and Th-2 cytokines (IL-4, IL-10) [26]. Upregulation of tumor necrosis factor-alfa (TNF-α), IL-2 and GM-CSF were also demonstrated upon *in vitro* iPPVO stimulation of immune cells [19].

In spite of some promising results from early clinical studies and the identification of putative immune mechanisms *in vitro*, the mechanisms mediating the immunomodulatory effects of iPPVO remain largely unknown. A number of *in vivo* findings have been questioned and, in some cases, some results have been difficult to reproduce [27–29]. Likewise, some *in vitro* findings are controversial, discrepant and difficult to reconcile with *in vivo* observations [25,29–31]. Thus, it has become progressively clear that findings from *in vitro* assays could not be simply transferred to *in vivo* situations and vice versa, and that the immune mechanisms mediating iPPVO immunostimulation are rather complex and far from being completely understood.

The experiments reported herein were designed to identify events/mechanisms associated with the immunomodulatory activity of iPPVO in mice, trying to provide new insights into this complex virus-host interaction. Contrasting with most previous studies we performed *in vivo* stimulation followed by investigation of the biological effects both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Experimental design

Groups of mice were inoculated with inactivated PPVO (iPPVO/10^7TCID$_{50}$) by the intraperitoneal route (*ip*) in a volume of approximately 100 μL. At different times post-inoculation (6, 12, 24, 48, 72, 96, 120 and 144 hpi, depending on the experiment) peritoneal cells, spleen and blood samples were collected for the assays described below. All experiments included a mock-treated group (placebo) inoculated *ip* with the supernatant of ultracentrifuged iPPVO and minimum essential medium (MEM eagle) (5–7 mice/group). In assays for type I interferon (IFN-I) and cytokines IL-12 and INF-γ, control groups included mice inoculated *ip* with inactivated bovine herpesvirus 1 (iBoHV-1) and vaccinia virus (iVACV).

2.2. Animals and ethics

All experiments used six to eight-weeks-old, female Swiss mice (*Mus musculus*), weighing 23–30 g each. Animals were housed in plastic cages under controlled temperature (20 ± 2 °C) with a 12 h light–dark cycle and access to food and water *ad libitum*. The experiments were approved by the Institutional Ethics and Animal Welfare Committee (Comitê de Etica e Bem-estar Animal, UFSM, approval # 069/2011).

2.3. Viruses, cells, yeast and bacteria

Parapoxvirus ovis (PPVO) strain Iowa IA-82 (passage # 5) was provided by Dr. Daniel Rock (University of Illinois at Urbana/Champaign, Illinois, USA). Bovine herpesvirus 1 (BoHV-1 Cooper strain), Brazilian VACV isolate Pelotas 1 (P1 V) were from our lab collection [32]. Murine encephalomiocarditis virus (EMCV) was kindly provided by Dr. Erna G. Kroon (UFMG, Belo Horizonte, MG, Brazil).

Ovine fetal turbinate cells (OFTu) were used to amplify PPVO IA-82; MDBK cells were used for multiplication of BoHV-1 and Vero cells were used to amplify VACV. L_{929} murine fibroblast cells were used for EMCV multiplication and IFN-I assays. Cells were grown in MEM supplemented with 10% fetal calf serum (Nutricell, Brazil), 100 U/mL of penicillin and 100 μg/mL of streptomycin and maintained at 37 °C and 5% CO_2.

A local isolate of *Candida albicans* was used as target in phagocytosis assays. Cells were cultivated in brain heart infusion (BHI) broth at 37 °C for 18 h, centrifuged and washed three times with PBS (pH 7.4). Yeast cells were inactivated at 56 °C for 30 min, counted in a hematocytometer and adjusted to 3×10^8 cells/mL. *Escherichia coli*/ATCC-25922 was kindly provided by Dr. Luiz Carlos Kreutz (UPF, Passo Fundo, RS, Brazil). Bacteria were cultivated in BHI broth at 37 °C for 18 h, centrifuged and washed three times with PBS (pH 7.4) and adjusted to a final concentration of 0.4 OD_{600nm} in PBS.

2.4. Interferon

To provide internal reference standard, recombinant murine interferon-β (IFN-β^r) was provided by Dr. Paulo Cezar Pelegrino Ferreira (UFMG, Belo Horizonte, MG, Brazil). The standard IFN was used in a titer equivalent to 400 for an intra-assay and inter-assay control.

2.5. Preparation of iPPVO

PPVO strain IA-82, passage # 10, was inoculated in 80–90% confluency OFTu cells and harvested when the cytopathic effect (ECP) reached around 90% of the monolayer. The supernatant was submitted to three freeze–thaw cycles followed by centrifugation at 220*g* for 10 min at 4 °C to remove cell debris and then submitted to virus quantitation by limiting dilution. Virus titers were calculated according to standard protocols [33] and expressed as $\log_{10}$ median tissue culture infectious doses per milliliter (TCID$_{50}$/mL). The viral suspension was inactivated with binary ethilenimine (BEI) 0.1% for 18–24 h at 37 °C. Viral particles in the supernatant were concentrated by ultracentrifugation at 68,000*g* for 2 h at 4 °C and stored at −80 °C. The viruses used as controls (BoHV-1 and VACV) were submitted to the same process of inactivation.

2.6. Assays for type I interferon (IFN-I) and expression of cytokine mRNA

2.6.1. Interferon assay

Serum samples obtained at different times post-inoculation (6, 12, 24 hpi) from mice inoculated with iPPVO or controls (MEM, or inoculated with iBoHV-1 or iVACV) were assayed for IFN-I activity against murine encephalomyocarditis virus (EMCV) by plaque reduction assay according to protocol described by [34]. Viral plaques were counted for each replicate and results were expressed as the percentage of plaque reduction and showed as mean ± SEM.

2.6.2. Assays for expression of cytokine mRNA

2.6.2.1. RNA isolation and cDNA synthesis. Isolation of total RNA was performed using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions followed by determination of the absorbance ratio at 280_{nm} and 260_{nm}. All samples were treated