

Inactivated *Parapoxvirus ovis* induces a transient increase in the expression of proinflammatory, Th1-related, and autoregulatory cytokines in mice

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Abstract

The immunostimulatory properties of inactivated *Parapoxvirus ovis* (iPPVO) have long been investigated in different animal species and experimental settings. In this study, we investigated the effects of iPPVO on cytokine expression in mice after intraperitoneal inoculation. Spleen and sera collected from iPPVO-treated mice at intervals after inoculation were submitted to cytokine mRNA determination by real-time PCR (qPCR), serum protein concentration by ELISA, and interferon (IFN)- α/β activity by bioassay. The spleen of iPPVO-treated animals showed a significant increase in mRNA expression of all cytokines assayed, with different kinetics and magnitude. Proinflammatory cytokines interleukin (IL)-1 β , tumor necrosis factor-alpha (TNF- α), and IL-8 mRNA peaked at 24 hours postinoculation (hpi; 5.4-fold increase) and 48 hpi (3- and 10-fold increases), respectively. A 15-fold increase in IFN- γ and 6-fold IL-12 mRNA increase were detected at 48 and 24 hpi, respectively. Increased expression of autoregulatory cytokines (Th2), mainly IL-10 and IL-4, could be detected at later times (72 and 96 hpi) with peaks of 4.7- and 4.9-fold increases, respectively. IFN-I antiviral activity against encephalomyocarditis virus was demonstrated in sera of treated animals between 6 and 12 hpi, with a >90% reduction in the number of plaques. Measurement of serum proteins by ELISA revealed increased levels of IL-1, TNF- α , IL-12, IFN- γ , and IL-10, with kinetics similar to those observed by qPCR, especially for IL-12 and IFN- γ . These data demonstrate that iPPVO induced a transient and complex cytokine response, initially represented by Th1-related cytokines followed by autoregulatory and Th2 cytokines.

Key words: Cytokine; mRNA; ORF virus; Immunostimulant; Innate immune response

Introduction

Parapoxvirus ovis (PPVO or ORF virus) is a member of the genus *Parapoxvirus* within the family Poxviridae (1). PPVO is an important pathogen of sheep and goats distributed worldwide and produces a mucocutaneous, inflammatory, and proliferative disease known as contagious ecthyma, orf, or scabby mouth (1,2). Occasionally, the virus is transmitted to humans, in which it produces vesiculopapular lesions on the hands and fingers (3). The 138-kb double-stranded DNA PPVO genome has been entirely sequenced and encodes more than 130 products, many with unknown functions (4). One interesting feature of PPVO is its ability to repeatedly infect the host in spite of a vigorous immune response (5,6). Several immune-escape

mechanisms and/or gene products have been identified in PPVO, including captured cellular genes [e.g., interleukin (IL)-1 homologue and granulocyte macrophage colony-stimulating factor], an IL-2-inhibiting protein, the vaccinia E3L gene encoding an interferon (IFN)-resistance product (7), and several gene products interfering with the nuclear factor kappa beta (NF- κ B) pathway (8-10). Nevertheless, the immune evasion by PPVO seems to be rather complex to be explained by a single factor or mechanism (6).

The immunomodulatory effects of PPVO have long been recognized and raised a significant interest in veterinary research in the last decades. Inactivated

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PPVO (iPPVO) retains many immunomodulatory properties of the live virus, suggesting that these effects should rely on the structural components of the viral particle. The effects of iPPVO on the innate immune response have been investigated *in vitro* in immune cells of several species and experimental settings. In summary, iPPVO exerts a strong effect on early cytokine secretion in mice and human cells, leading to an autoregulated loop of initial upregulation of inflammatory and Th1-related cytokines, followed by regulatory and Th2-related cytokines (11). Early proinflammatory cytokine secretion includes IL-6, IL-8, and tumor necrosis factor- α (TNF- α) by monocytes and/or by antigen-presenting cells, IL-2, interferon type I (IFN-I) IFN- α/β and Th1 cytokines (IL-12, IL-16, IFN- γ) by T helper lymphocytes. IFN- γ secretion by T lymphocytes or natural killer (NK) cells seems to be an important component of iPPVO activity (12-17). Most studies on immunomodulation by iPPVO have been conducted *in vitro*, yet the immunomodulatory effects of iPPVO have also been demonstrated in a few studies *in vivo*. For example, iPPVO has been shown to induce antiviral activity against genital herpes in a guinea pig model, in a transgenic mouse model of hepatitis B virus, and in mice infected with herpes simplex virus (18). Studies addressing the immunomodulation by iPPVO in horses have demonstrated a balanced and early increase in cytokine production (19-21). Nevertheless, most studies on the effects of iPPVO on the innate immune response and the knowledge derived thereof were performed *in vitro* (13,15,18-21).

In the present study, we investigated the effects of iPPVO on selected aspects of the innate immune response in mice using an *in vivo* approach. Groups of mice were inoculated with iPPVO, and samples collected at different intervals were tested for indicators of the innate immune response. Our previous study demonstrated that iPPVO stimulates phagocytosis, neutrophil oxidative bursts, serum bactericidal activity, and IFN- α/β production in treated mice (Anziliero A, unpublished results). In the present article, we describe a detailed investigation on the cytokine profile following iPPVO administration in mice, by real-time PCR (qPCR), ELISA, and IFN-I biological assays.

Material and Methods

Experimental design

Mice were inoculated with iPPVO [\log_{10} tissue culture infectious dose per mL (TCID₅₀) of 10^7] by the intraperitoneal (*ip*) route. Spleen and blood samples were collected at different times postinoculation [hours postinoculation (hpi) 6, 12, 24, 48, 72, 96, 120] and assayed for cytokine expression. Cytokine expression in the spleen (mRNA) was measured by qPCR, cytokines in sera were assayed by ELISA, and IFN-I activity in serum was investigated by a biological test.

Animals

All experiments used 6- to 8-week-old female Swiss mice (*Mus musculus*), weighing 23-30 g each. Animals were housed in plastic cages under controlled temperature ($20 \pm 2^\circ\text{C}$) with a 12:12-h light-dark cycle and access to food and water *ad libitum*. Ten mice per group were used in all experiments. The study was approved by the Institutional Ethics and Animal Welfare Committee, Universidade Federal de Santa Maria, Brazil (#069/2011).

Viruses and cells

Viruses. Virus stocks used were ORFV IA-82 (passage 5), kindly provided by Dr. Daniel Rock (University of Illinois at Urbana, Champaign, IL, USA). Bovine herpes virus 1 (BoHV-1; Cooper strain) and the vaccinia virus (VACV) Brazilian isolate Pelotas 1 (22) were from our lab collection. The murine encephalomyocarditis virus (EMCV) was kindly provided by Dr. Erna G. Kroon (Universidade Federal de Minas Gerais, Brazil).

Cells. Primary ovine fetal turbinate cells (OFTu) were used to amplify ORFV IA-82, Madin-Darby bovine kidney (MDBK) cells were used for the amplification of BoHV-1, and Vero cells were used to amplify VACV. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Nutricell, Brazil), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin, and maintained at 37°C and 5% CO_2 .

Preparation of iPPVO

The iPPVO inoculum was prepared as follows. Briefly, ORFV strain IA-82, passage 10, was propagated in primary OFTu and harvested when the cytopathic effect reached about 90% of the monolayer. The supernatant was collected and submitted to three cycles of freeze-thaw followed by centrifugation at low speed to remove cell debris. The supernatant was harvested and submitted to virus quantitation by limiting dilution, and virus titers were calculated (23) and reported as TCID₅₀/mL. The viral suspension was inactivated with binary ethylenimine (BEI) for 18-24 h at 37°C . BEI (0.1 M) was added to the virus suspension to a final concentration of 0.1%, and residual BEI was hydrolyzed by the addition of 1 M sterile sodium thiosulfate solution at a final concentration of 1%. Viral particles in the suspension were then concentrated by ultracentrifugation at 116,939 *g* for 2 h at 4°C and stored at -80°C until use. In all experiments, ultracentrifuged supernatant of mock-infected OFTu cells was used as control.

Animal inoculation and sample collection

Groups of mice were inoculated with iPPVO (10^7 TCID₅₀) *ip* in a volume of approximately 100 μL . At different times postinoculation (6, 12, 24, 48, 72, 96, and 120 h, depending on the experiment), blood samples and

spleen tissue specimens were collected for the assays described below. All experiments (qPCR, ELISA, and IFN) included a mock-treated group (placebo) inoculated *ip* with ultracentrifuged supernatant of OFTu cells (5-7 mice/group). In experiments assayed for IFN- γ , control groups included mice inoculated *ip* with inactivated BoHV-1 (iBoHV-1) and inactivated VACV (iVACV). The controls used (BoHV-1 and VACV) were submitted to the same process of inactivation described above for iPPVO.

For sample collection, animals were previously anesthetized with isoflurane by inhalation, followed by cervical dislocation. The animals were then necropsied for tissue collection at different intervals after iPPVO inoculation (12-120 hpi). Spleen specimens were collected rapidly and submitted to determination of amount of material (50 mg/animal). Specimens from individual animals were then placed in RNeasy lysis reagent (Qiagen, USA) and stored at -80°C until RNA extraction. Blood was collected from the cardiac chamber and left to clot overnight at 4°C . The blood was then centrifuged for 20 min at 160 *g* for serum collection and stored at -80°C for cytokine analysis.

Cytokine mRNA expression

RNA isolation and cDNA synthesis. Isolation of total RNA was performed using the RNeasy mini kit from Qiagen, according to the manufacturer's instructions. The RNA concentration and purity were determined by the absorbance ratio at 280 and 260 nm. RNA integrity was assessed by denaturing gel electrophoresis on 1% agarose gel stained with ethidium bromide. All samples were treated with amplification-grade DNase I (Invitrogen, USA) to remove traces of genomic DNA contamination (24). According to RNA concentrations, treated RNA concentration was adjusted for 1 μg RNA per sample.

RNA samples were reverse transcribed (RT) using the SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Briefly, 1 μg DNase-treated RNA was mixed with 1 μL oligo(dT)₂₀ primer (50 μM) and 1 μL dNTP mix (10 mM) and completed up to 10 μL with DNase/RNase-free water. Samples were heated at 65°C for 5 min and subsequently cooled on ice for 1 min. After that, 10 μL cDNA synthesis mix (RT buffer, MgCl_2 , Superscript III RT, DTT, and RNaseOUT) was added to the RNA/primer, mixed gently, and collected by brief centrifugation. The reaction was incubated again at 50°C for 50 min followed by heating at 85°C for 5 min and chilled on ice. To remove RNA template from cDNA, 2 μL RNase H was added to the mixture to a final volume of 22 μL . A work solution was prepared diluting cDNA 1:20 in DNA/RNA-free water. For every reaction set, one RNA sample was performed without Superscript II RT (RT-minus reaction) to provide a negative control in the subsequent qPCR. The cDNA was stored at -80°C until qPCR assays.

Quantification of cytokine mRNA expression by qPCR

Relative gene expression was assayed by qPCR using the iCycler iQ5 RT-PCR system (Bio-Rad, USA) using standard conditions. All samples were analyzed in triplicate. Each reaction contained 12.5 μL Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA), 8 μL water, 1 μL forward primer (20 μM), 1 μL reverse primer (20 μM), and 2.5 μL cDNA. Custom-made specific primers and internal controls for all targets were designed using the Primer3 Input online program (<http://fokker.wi.mit.edu/primer3/input.htm>). The specific annealing of the designed primers to the mRNA targets was previously analyzed by BLAST (25). Primers/amplicons were validated using a melting curve analysis, and two housekeeping genes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin] were evaluated to select the best internal control. The sequences of primers used in this study are listed in Table 1. The reaction was carried out as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 20 s, and 59°C for 1 min. Melting curve analyses were performed immediately after amplification by an additional denaturation at 95°C and continuous melting curve acquisition from 55° to 95°C with a $1^{\circ}\text{C}/\text{s}$ ramp rate to verify product specificity. Changes in cytokine gene expression were calculated by relative quantitation using the $\Delta\Delta\text{Ct}$ (threshold cycle) method (26), where $\Delta\Delta\text{Ct} = \text{iPPVO (cytokine gene}^{\Delta\text{Ct}} \text{ minus housekeeping gene}^{\Delta\text{Ct}}) \text{ minus control (cytokine gene}^{\Delta\text{Ct}} \text{ minus housekeeping gene}^{\Delta\text{Ct}})$. Treatment-induced changes in cytokine gene expression for each individual sample were calculated using $2^{-\Delta\Delta\text{Ct}}$. Results are reported as means \pm SE fold-change in cytokine gene expression from the iPPVO group over the control group. To determine the efficiency of the qPCR assays, 2-fold serial dilutions of cDNA samples were used, with the Ct of each dilution being defined and plotted on a semi-log (log 10) graph for analysis. The slopes of accurate tendency lines ($r^2 \geq 0.98$) were used to determine the efficiency of the reactions ($E = -1 + 10^{(-1/\text{slope})}$). Means \pm SE were calculated for each group, and results are reported as times-fold increase (iPPVO-treated relative to controls).

Cytokine measurement

Serum samples collected from iPPVO-inoculated and control mice at different intervals were submitted to cytokine determination by ELISA. To ensure specificity of the iPPVO effect, control groups included mice inoculated *ip* with iBoHV, iVACV, and ultracentrifuged supernatant of mock-infected OFTu cells. Cytokines related to the innate immune response (IL-1 β , IL-12, TNF- α , IFN- γ , and IL-10) were assayed using a mouse cytokine enzyme immunoassay kit (BD Biosciences, USA). Briefly, 100 μL capture antibodies for each cytokine were diluted in coating buffer (0.2 M sodium phosphate, pH 6.5, 11.8 g Na_2HPO_4 , 16.1 g NaH_2PO_4 , sufficient quantity to 1 liter, pH 6.5), coated on ELISA plates (Nunc, USA), incubated overnight

Table 1. Primer pairs used in qPCR for determination of cytokine mRNAs.

Target	Primer sequence (5'-3')	Product size (bp)
GAPDH	F: CAGCCTCGTCCCGTAGACAA R: ACCCCGTCTCCGGAGTCCATCACAAT	178
β -actin	F: AGGCCAACCGTGAAAAGATGACCCAGAT R: GTAGACCATGTAGTTGAGGTCAATGAA	139
IL-1 β	F : TGGCCTTCAAAGGAAAGAATCTATACCTGTCC R: GTTGGGGAAGTCTGCAGACTCAAACCTCCAC	179
IL-8	F: GACGCCCCCAGGACCCCACTG R: AGCCCAGGCTCCTCCTTTCCAGGTC	162
TNF- α	F: CAGGCCTTCTACCTTCAGACCTTTCCAGAT R: ACACCCCGCCCTTCCAAATAAATACATTTCAT	166
IL-12p40	F: TGCCCCACAGAAGACGTCTTTGATGAT R: GATGGCCACCAGCATGCCCTTGTC	158
IFN- γ	F: GCCAAGACTGTGATTGCGGGGTTGTATCT R: TAAAGCGCTGGCCCGGAGTGTAGACA	178
IL-10	F: AGCTGCAGGGCCCTTTGCTATGGTG R: GATGAAGCGGCTGGGGGATGACAGTA	180
IL-4	F: CACTGAGAATGAAAGGCCCAAGTCTTGA R: AGCCGGGAGGACAGATCTCTGGTGAAG	168

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IL: interleukin; IL-12p40: IL-12, subunit p40; TNF- α : tumor necrosis factor-alpha; IFN- γ : interferon gamma; F and R: forward and reverse primers, respectively.

at 4°C, and then washed 3 times (PBS with 0.05% Tween-20, freshly prepared). Plates were blocked with 200 μ L/well PBS with 10% FBS, pH 7.0, and incubated at room temperature for 1 h. After that, microplates were washed again (3 times) while samples and standard curve dilution were prepared following the manufacturer's recommendations. Then, 100 μ L serum sample and standard curve dilutions for each cytokine were added in duplicate to plates and incubated at room temperature for 2 h. Plates were washed 5 times and blotted to remove any residual content, and 100 μ L working detector solution (detection antibody + streptavidin-horseradish peroxidase reagent) was added to the wells and incubated for 1 h at room temperature. Wells were aspirated and plates were washed again (7 times), and 100 μ L substrate solution (tetramethylbenzidine and hydrogen peroxide) was added. The solution was incubated for 30 min at room temperature in a dark chamber. Finally, the reaction was stopped by adding 50 μ L stop solution, and the absorbance was measured at 450 nm. Absorbance was transformed to cytokine concentrations (pg/mL) using the detection limits assessed from the standard curve for each cytokine. Results are reported as means \pm SE pg/mL. Results were submitted to statistical analysis by the Shapiro-Wilk test to verify normality, followed by the Student *t*-test to compare mean values between groups. Statistical significance was accepted as $P < 0.05$.

IFN-I assays

Serum samples obtained at different times postinoculation (6, 12, 24 hpi) from mice inoculated with iPPVO or controls (iBoHV-1 and iVACV) were assayed for IFN-I

activity against murine EMCV according to a previous study (27). An IFN standard (IFN- β^1) was included in all assays to monitor cell conditions and reproducibility.

Confluent L₉₂₉ cells (2×10^6 cells/mL) grown on 96-well plates were incubated with 2-fold dilutions of mouse sera for 6 h. Plates were then drained, washed 3 times with MEM, inoculated with 100 TCID₅₀ EMCV, and incubated for 2 h at 37°C. The inoculum was removed and cells were washed 3 times and incubated with culture medium containing 2% FBS for 48 h and monitored for the EMCV cytopathic effect. IFN activity was expressed as the reciprocal of the serum dilution that produced an inhibition of the cytopathic effect in 50% of the cell monolayers. Results are reported as log₂ of geometric mean titers.

Antiviral activity of IFN-I was also determined by plaque reduction assay (28) comparing both groups (iPPVO vs control). Briefly, L₉₂₉ cells were seeded onto 6-well plates at a density of 1×10^6 per well on the day before the experiment. On the following day, the medium was removed and cells were washed 3 times with medium and incubated with duplicates of mouse serum diluted 1:10 (MEM) for 6 h. In addition to mock controls, monolayers were inoculated with sera of mice inoculated with iBoHV-1 and iVACV. Cells were then drained, washed 3 times, and inoculated with 100 TCID₅₀ EMCV. After 2 h of adsorption at 37°C, cultures were washed and overlaid with MEM supplemented with 2% FBS and incubated for 48 h at 37°C and 5% CO₂. After that, monolayers were fixed with formalin (10%) and stained with crystal violet (0.3%) for 2 h. The percentage inhibition of plaque formation was calculated as follows: [(mean number of plaques in

control)–(mean number of plaques in sample)] × 100/ (mean number of plaques in control). Viral plaques were counted for each replicate, and results are reported as the percentage of plaque reduction and means ± SE.

Results

Standardization of PCR conditions by conventional PCR

Initially, a conventional PCR was performed using iPPVO and control DNA samples to verify the specificity and functionality of the primers and to optimize the PCR conditions. PCR settings were considered satisfactory when a single well-defined band was observed after electrophoresis on 1% agarose gel. Based on the efficiency of amplification and variability results, GAPDH was selected as the housekeeping control gene for qPCR. During the entire experiment, an adequate PCR amplification efficiency was determined by the slope of the standard curves (between -3.3 and -3.7), and linear regression analysis showed that all standard curves had an r^2 value of ≥ 0.98 (data not shown).

Cytokine mRNA expression upon iPPVO stimulation

Standardized qPCR was then used to measure the expression of cytokine mRNAs in total RNA extracted from the spleen of mice inoculated with iPPVO. qPCR was performed in total RNA extracted from spleens collected from mice at different times postinoculation. For this purpose, cytokines from the proinflammatory route (IL-1 β , IL-8, and TNF- α), Th1 type (IFN- γ , IL-12), and regulatory Th2 (IL-4 and IL-10) were selected. The results of qPCR for cytokine mRNAs are shown by groups of cytokines (IL-1 β , IL-8, and TNF- α), Th1 type (IFN- γ , IL-12), and Th2 (IL-4 and IL-10) in Figures 1A, 2A, and 3A, respectively. Controls included spleen obtained from mice inoculated with the supernatant of iPPVO ultracentrifugation tested at the same intervals.

Increased expression of proinflammatory cytokines (mRNA) was first detected for IL-8 at 12 hpi, with a 4-fold increase over the controls (Figure 1A). At this time, expression of TNF- α and IL-1 β mRNA remained unaltered. At 24 hpi, all three mRNAs were increased (>5 -fold increase for IL-1 β , 2-fold increase for TNF- α , and 4-fold increase for IL-8, Figure 1A). High IL-1 β expression was detected up to 96 hpi, with a progressive reduction in magnitude of expression observed over time (>5 -fold at 24 hpi to <3 -fold at 96 hpi). Expression of TNF- α remained within the range of a 2- to 3-fold increase over the period. IL-8 expression remained high during the period, with a strong peak at 48 h (>10 -fold). Increased expression of these mRNAs could no longer be detected after 96 hpi, indicating a short-term induction.

Enhanced expression of IFN- γ and IL-12 mRNA was first detected at 24 hpi, with 3-fold and <6 -fold increases, respectively. IL-12 expression showed a slight reduction

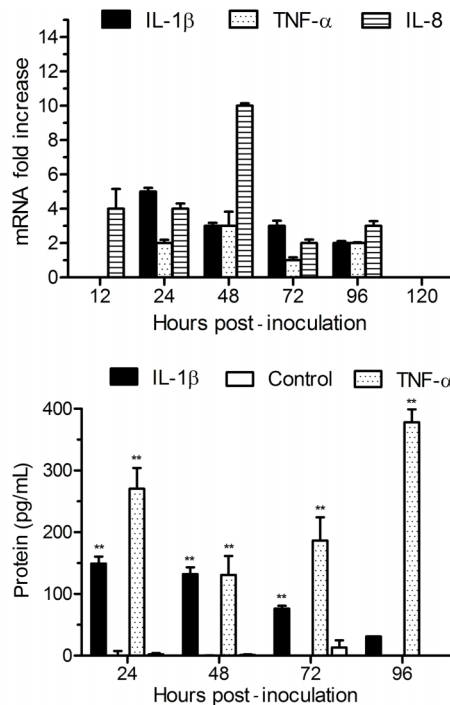


Figure 1. Proinflammatory cytokines measured by qPCR in mice spleen (A) and by ELISA in serum samples (B) at different time points after inactivated *Parapoxvirus ovis* inoculation. IL-1 β mRNA was measured up to 120 hours post-inoculation. IL: interleukin; TNF- α : tumor necrosis factor-alpha. Data are reported as means \pm SE as times-fold increase over the control group (qPCR) and as pg/mL serum protein in ELISA. ** $P \leq 0.01$, iPPVO compared to control (t -test).

by 48 hpi, but remained above the control values (2- to 4-fold) up to 96 hpi (Figure 2A). IFN- γ expression presented a strong peak at 48 hpi (15-fold), returning to lower levels, but still above the controls at 72 and 96 hpi. Both mRNAs returned to base levels at 120 hpi.

mRNA levels of regulatory Th2 cytokines remained within basal levels for the first 48 h after iPPVO inoculation. Then, a strong pulse of expression of IL-4 mRNA was detected at 72 hpi (5-fold), reducing to 2.5-fold at 96 hpi (Figure 3A). IL-10 expression showed an opposite behavior, with a moderate increase at 72 hpi (1.5-fold) and a marked increase at 96 hpi (4.5-fold). The two mRNAs were below detection limits at 120 hpi.

Taken together, these results showed that iPPVO stimulation resulted in a time-dependent, transient, and autoregulatory increase in expression of several classes of cytokines. The kinetics and magnitude of the stimulation effects varied according to the respective cytokine group and was first observed for IL-8 at 12 hpi. Most proinflammatory and Th1 cytokines showed a peak in expression at 24 and 48 hpi, whereas Th2-regulatory cytokines peaked at 72 h and 96 hpi. Expression of all tested cytokines returned to steady state at 120 hpi.

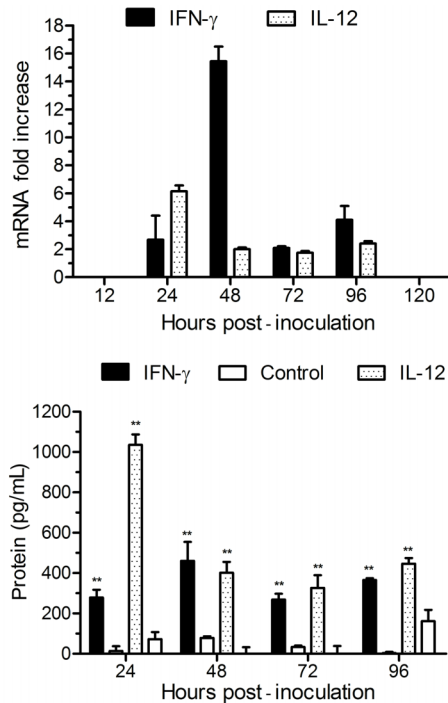


Figure 2. Th1-related cytokines in the spleen of mice measured by qPCR (A) and serum levels of protein assayed by ELISA (B) at different time points after inoculation with inactivated *Parapoxvirus ovis*. IL: interleukin; IFN- γ : interferon gamma. Data are reported as means \pm SE as times-fold increase over the control group (qPCR) and as pg/mL serum protein in ELISA. ** $P \leq 0.01$, iPPVO compared to control (*t*-test).

Cytokine levels in sera

To further investigate the effects of iPPVO in cytokine expression, individual concentrations in sera were measured by ELISA at different time points, except for IL-8 and IL-4. Results were calculated as the mean absorbance for each set of duplicate standards minus the mean zero standard absorbance. The standard curve absorbance values were plotted on a log-log graph to determine cytokine concentrations over sample absorbance. The levels of the respective groups of cytokines in sera at different intervals after iPPVO inoculation are reported in Figures 1B, 2B, and 3B. A marked increase in cytokine levels was detected in iPPVO-treated animals compared to control/placebo groups for all cytokines assayed, with the profile varying according to the respective cytokine. Among proinflammatory cytokines, levels of IL-1 β were high from 24 to 72 hpi, presenting a progressive reduction toward near-basal levels at 96 hpi. TNF- α levels also remained high, with peaks at 24 and 96 hpi. Among Th1-related cytokines, a peak in IL-12 subunit p40 (IL-12p40) was noted at 24 hpi, with levels reducing, yet remaining at relatively high levels up to 96 hpi. IFN- γ levels were also increased during the entire period, with a slight peak at 48 hpi. Among the Th2 cytokines, only IL-10 was measured, presenting increased levels from 48 to 96 hpi,

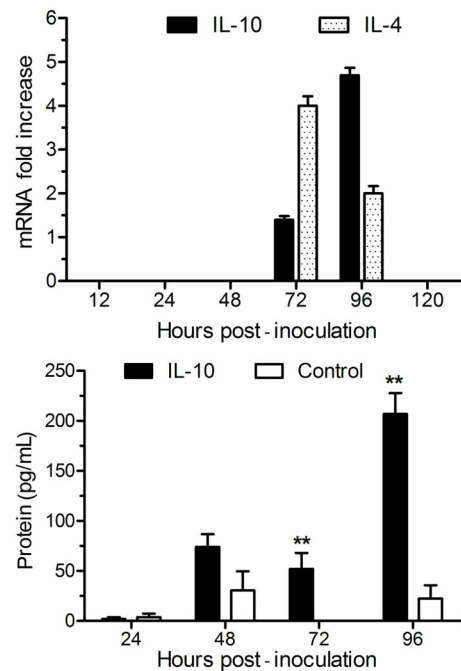


Figure 3. Quantification of mRNA Th2 cytokines in spleen of mice inoculated with inactivated *Parapoxvirus ovis* (iPPVO) at different intervals. Data are reported as means \pm SE as times-fold increase over the control group (A). IL-10 mRNA was measured up to 120 hpi. Panel B reports the levels of IL-10 in the sera of mice after inoculation of iPPVO, measured by ELISA. IL: interleukin. Data are reported as means \pm SE as pg/mL serum protein. ** $P \leq 0.01$, iPPVO compared to control (*t*-test).

with a late peak (96 hpi). Consistent with mRNA findings, measurement of IL-1 β , TNF- α , IL-12, IFN- γ , and IL-10 protein levels in sera confirmed the qPCR results (Figures 1, 2, and 3), indicating a broad spectrum of cytokine response following iPPVO stimulation. With the exception of IL-8, the profile of mRNA expression and cytokine detection in sera were roughly similar. Using the supernatant of iPPVO ultracentrifugation and large viruses (BoHV-1 and VACV) submitted to the same process of preparation and inactivation of iPPVO, no stimulation over the innate immune cytokines could be detected, confirming that the immunomodulation is iPPVO specific.

IFN-I induction

The production of IFN-I by iPPVO-treated mice was assayed by investigating IFN-I activity in a biological assay against EMCV (27,28). Inhibition of EMCV replication was detected in sera collected 6 to 24 h after iPPVO inoculation, with a peak in antiviral activity observed between 6 and 12 hpi. At 24 hpi, sera from 2 of 6 animals exhibited antiviral effects. Murine internal laboratory standard recombinant interferon (IFN γ) provided intra-assay and interassay controls during the experiment. No antiviral activity was detected in sera collected from mice

inoculated with MEM, supernatant from mock-infected OFTu cells and from ultracentrifugation of iPPVO, iBoHV-1, or iVACV. Thus, the inhibitory effect of EMCV replication seems to be specific to iPPVO stimulation. To better characterize the inhibitory IFN activity on EMCV replication, a plaque reduction assay was performed. Again, a marked inhibitory effect on EMCV replication was observed in sera collected at 6 hpi from mice inoculated with iPPVO. The >94% plaque reduction observed at 6 hpi decreased to 28% at 12 hpi and was no longer observed at 24 hpi. No plaque reduction was observed in the sera of mice inoculated with iBoHV-1 or iVACV, indicating an iPPVO-specific effect. These results indicate that iPPVO inoculation leads to a significant and transient increase in IFN-I production, as measured by the IFN-I inhibitory effect on EMCV replication, which seems to be iPPVO specific rather than a general response to inoculation of inactivated viral particles.

Discussion

Our results demonstrated that iPPVO administration leads to a transient and coordinated increase in the expression of several cytokines in mice, as measured by qPCR, biological assays (IFN-I), and ELISA. The kinetics and magnitude of the effects varied according to the cytokine group. Increased expression levels were detected as early as 6 hpi for IFN-I and at 12 hpi for IL-8. The cytokine levels were, in general, maintained above normal limits for up to 72-96 h, returning to basal levels at measurements performed at 120 hpi. Most proinflammatory and Th1 cytokines increased from 24 to 96 hpi, with a peak between 24 and 48 hpi. Regulatory and Th2 cytokines peaked later, at 72 and 96 hpi. These results from *in vivo* iPPVO stimulation confirm and extend previous results from *in vitro* studies, demonstrating a broad stimulatory effect on proinflammatory and Th1- and Th2-related cytokines. These effects would likely contribute to the immunostimulatory properties of iPPVO observed in several animal species. However, a comprehensive understanding of the immunological mechanisms underlying these effects still represents a challenge toward an adequate use of iPPVO as immunostimulant in animal and human infectious diseases.

The immunostimulant properties of iPPVO have long been recognized and paved the way for its use as a commercial stimulator of the innate immune response (Baypamun[®], Compind, Zylexis[®]). Immune modulation by iPPVO has been investigated in several systems and was primarily associated with stimulation of a broad range of cytokines, including proinflammatory and Th1- and Th2-related cytokines (29). This complex cytokine response is also associated with the activation of several cell populations, including monocytes and Th1-like cells, human neutrophils, canine monocytes, and murine bone marrow-derived dendritic cells (BMDCs), among others

(13,15-18,29,30). Most of these studies focused on *in vitro* stimulation of specific cell populations by preparations of iPPVO. In a previous study, we demonstrated that iPPVO administration to mice resulted in increased phagocytosis *in vitro* and *in vivo* by macrophages, enhanced neutrophil oxidative bursts, serum bactericidal activity, and IFN- α/β production (Anziliero A, unpublished results). The present study investigated the effects of iPPVO stimulation on the expression of selected cytokines after *in vivo* exposure.

Our results confirmed previous findings (Anziliero A, unpublished results) and demonstrated a prompt and transient IFN-I response, peaking at 6 hpi and remaining up to 24 hpi. Previous studies have demonstrated that peripheral blood mononuclear cells (PBMCs) and BMDCs exposed to iPPVO *in vitro* produced IFN-I in the range of 6 and 24 hpi (17,19,30). Also confirming earlier *in vitro* studies (13,18, and Anziliero A, unpublished results), the IFN-I-stimulatory effect seems to be iPPVO specific, since it was not detected upon iBoHV-1 or iVACV inoculation. Although transient, IFN-I induction/activity may trigger downstream mechanisms involved in microbial resistance and clearance by the immune system. It is also possible that the cascade of antiviral genes activated by the IFN system contributed to the broad cytokine responses observed at later times after iPPVO stimulation.

An early increase in proinflammatory cytokines was a consistent finding in the present study (Figure 1). Increased IL-8 mRNA expression was detected as early as 12 hpi, followed by increased expression of IL-1 β and TNF- α . In fact, iPPVO immune modulation *in vitro* has been associated with the production of proinflammatory cytokines (IL-6, IL-8, TNF- α) by monocytes or antigen-presenting cells (17,30). Mouse BMDC respond *in vitro* to iPPVO secreting TNF- α and IL-12p40 (17). In addition, local TNF- α induction and increased levels in the blood have been demonstrated in horses 24-48 h after intradermal or intramuscular iPPVO administration, respectively (29). Thus, an early induction of proinflammatory cytokines in immune cells appears to be a common effect of iPPVO stimulation both *in vivo* and *in vitro*.

Cytokines TNF- α , IL-1 β , and IL-8 mediate the initial response of the innate immune system to a challenge, infection, or injury (31-33). TNF and IL-1 β activate endothelial cells, attracting polymorphonuclear cells and monocytes to the site of inflammation and enhancing their movement through the blood. IL-8 is a chemokine, acting as a monocyte chemoattractant, and it is later responsible for IL-10 synthesis (33,34). Antigen-presenting cells secrete IL-1 β , which promotes T cell activation. When activated, Th1-related cells secrete IFN- γ that activates macrophages to secrete more IL-12. In our study, iPPVO treatment resulted in a prompt stimulus on these proinflammatory cytokines during the early response.

Increased expression of Th1-related cytokine mRNA (IL-12 and IFN- γ) was detected from 24 to 96 hpi, with

mRNA peaks at 24 hpi (IFN- γ) and 48 hpi (IL-12). With the use of ELISA, a similar profile was observed, with IL-12 peaking earlier (24 h) and IFN- γ at 48 hpi. These data are consistent with previous studies showing that antiviral activity of iPPVO against genital herpes in guinea pigs and murine hepatitis virus in a mouse model was strongly associated with the Th1-related immune response, especially IL-2, IL-8, and IFN- γ (18). In this model, IFN- γ was defined as the key mediator of antiviral activity. The induction of a Th1-type immune response by iPPVO has been detected in different species, and it has been suggested that this response is elicited by the viral particles themselves (11,15,30). These effects have also been demonstrated *in vivo*, because young horses treated with iPPVO intramuscularly or intradermally showed a transient increase in IFN- γ gene expression in blood cells or locally, respectively, at 24-48 hpi (19). Taken together, these results suggest that IFN- γ synthesis by T lymphocytes and/or NK cells, thus directing a Th1-type response, plays a pivotal role in iPPVO-immunostimulatory activity (32).

The inflammatory and Th1 response observed at early times after iPPVO stimulation are usually limited by

subsequent upregulation of regulatory and Th2 cytokines, namely IL-1RA and IL-10, followed by IL-4 (18,29). The late upregulation of IL-1R (a natural antagonist of IL-1 β), IL-10 (a regulatory cytokine), and IL-4 (a Th2 cytokine and Th1 cytokine antagonist) in PBMCs could explain the absence of notable side effects or tissue damage after iPPVO administration (15,18). Consistent with these findings, in the present study, induction of Th2-regulatory cytokines (IL-10, IL-4) was detected at later times, noticeably at 48-72 hpi (IL-10) and 72-96 hpi (IL-4) (Figure 3).

In summary, our results confirm and extend the previous findings that iPPVO exerts a strong effect on cytokine expression by immune cells, leading to an initial induction of proinflammatory and Th1-related cytokines followed by a Th2 regulatory-cytokine response. An understanding of the regulatory mechanisms and effects is key to be able to manipulate the immune response and use iPPVO effectively as an immunostimulant or therapeutic aid in human and animal infectious diseases that are difficult to treat. In addition, the present study illustrates the usefulness of having a combination of molecular mechanisms, immunoassays, and biological assays to measure cytokine response.

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