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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

EFEITOS DO PARAPOXVIRUS OVIS INATIVADO SOBRE EVENTOS DA RESPOSTA IMUNE INATA DE CAMUNDONGOS

TESE DE DOUTORADO

Deniz Anziliero

Santa Maria, RS, Brasil
2013

EFEITOS DO PARAPOXVIRUS OVIS INATIVADO SOBRE EVENTOS DA RESPOSTA IMUNE INATA DE CAMUNDONGOS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
Doutor em Medicina Veterinária

Orientador: Prof. Rudi Weiblen

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Universidade Federal de Santa Maria
Centro de Ciências Rurais
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EFEITOS DO PARAPOXVIRUS OVIS INATIVADO SOBRE EVENTOS DA RESPOSTA IMUNE INATA DE CAMUNDONGOS

Elaborada por
Deniz Anziliero

Como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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RESUMO

Tese de Doutorado
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EFEITOS DO *PARAPOXVIRUS OVIS* INATIVADO SOBRE EVENTOS DA RESPOSTA IMUNE INATA EM CAMUNDONGOS
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Santa Maria, 8 de novembro de 2013

As propriedades imunoestimulatórias do *Parapoxvirus ovis* inativado (iPPVO) têm sido verificadas em diferentes espécies animais e condições experimentais. No presente trabalho foram investigados os efeitos da administração do iPPVO sobre eventos da resposta inata de camundongos. Ativação de neutrófilos, atividade fagocítica de macrófagos, atividade bactericida do soro, indução e atividade antiviral do interferon tipo I (INF-I) e expressão de várias classes de citocinas foram investigados em diferentes intervalos após inoculação de *Mus musculus* pela via intraperitonial com iPPVO (dose 10^7 $TCID_{50}$). O soro de animais tratados com iPPVO apresentou atividade de INF-I frente ao vírus da encefalomiocardite murina (EMCV) entre 6 e 12 horas pós inoculação (hpi), como demonstrado pela redução significativa de formação de placas virais. Uma significativa ativação dos neutrófilos circulantes foi observada pela técnica de redução do NBT em animais tratados com o iPPVO às 6 hpi. Macrófagos peritoneais de camundongos tratados com iPPVO demonstraram um aumento significativo ($p<0,01$) na atividade fagocítica frente a *Candida albicans* tanto *in vivo* (entre 12 e 96 hpi) quanto *in vitro* (24 e 72hpi). Camundongos tratados com iPPVO apresentaram aumento na atividade bactericida do soro frente à *Escherichia coli* ($p<0,05$) em dois períodos avaliados (24 e 72 hpi). Um segundo estudo avaliou a expressão de citocinas em resposta à inoculação do iPPVO. Para isso, amostras de baço e soro foram coletados de camundongos tratados com iPPVO em diferentes intervalos após a inoculação e submetidas a quantificação de RNA mensageiro (RNAm) por PCR em tempo real (qRT-PCR) e detecção/quantificação de citocinas no soro por ELISA. A quantificação de RNAm permitiu detectar um aumento significativo e transitório da expressão de várias citocinas, com magnitude e cinética variáveis. A expressão de RNAm das citocinas pró-inflamatórias (IL-1β, TNF-α e IL-8) atingiu o pico às 24 hpi (aumento de 5,4 vezes), 48 hpi (3 e 10 vezes, respectivamente). Um aumento de 15 vezes na expressão gênica do INF-γ, e de 6 vezes para a IL-12 foi observado às 48 e 24 hpi, respectivamente. Um incremento na expressão das citocinas auto-regulatórias (Th2), principalmente IL-10 e IL-4, foi detectado em períodos mais tardios (72 e 96 hpi) com picos de 4,7 e 4,9 vezes, respectivamente. A determinação da concentração das citocinas séricas por ELISA revelou um aumento nos níveis de IL-1β, TNF-α, IL-12, INF-γ e IL-10, com uma cinética similar à observada pela técnica de qPCR, especialmente para IL-1β e INF-γ. Em resumo, esses resultados demonstram que o tratamento com iPPVO estimula de forma significativa e transitória uma série de eventos celulares e humorais ligados à resposta imune inata. Esses efeitos, se considerados em conjunto, provavelmente contribuem para o aumento da magnitude da resposta imunológica a certos patógenos observada em animais tratados com o iPPVO.

Palavras chave: Ectima contagioso, vírus da orf, imunoestimulante, resposta imune inata.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EFFECTS OF INACTIVATED *PARAPOXVIRUS OVIS* IN EVENTS OF THE INNATE IMMUNE RESPONSE IN MICE

AUTHOR: DENIZ ANZILIERO
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Santa Maria, November 8th, 2013.

The immunostimulatory properties of inactivated *parapoxvirus ovis* (iPPVO) have been investigated in different animal species and experimental settings. This study investigated the effects of administration of iPPVO on selected events of the innate response in mice. Neutrophil activation, phagocytic activity of macrophages, serum bactericidal activity, induction and antiviral activity of interferon type I (IFN - I) and expression of several classes of cytokines were assayed following intraperitoneal inoculation of *Mus musculus* with iPPVO (10^7 $TCID_{50}$). Serum from iPPVO-treated animals showed IFN-I activity against murine encephalomyocarditis virus (EMCV) between 6 and 12 hours post infection (hpi), as shown by plaque reduction. A significant activation of neutrophils at 6hpi was observed by NBT reduction test in animals treated with the iPPVO. Peritoneal macrophages from mice treated with iPPVO demonstrated a significant increase ($p<0.01$) in phagocytic activity against *Candida albicans* both *in vivo* (between 12 and 96 hpi) and *in vitro* (24 and 72 hpi). iPPVO treated mice showed increased serum bactericidal activity against *Escherichia coli* ($p<0.05$) at two periods (24 and 72 hpi). A second study evaluated the expression of cytokines in response to inoculation of iPPVO. For this, spleens and serum samples were collected from mice treated with iPPVO at different intervals after inoculation and subjected to quantification of messenger RNA (mRNA) by real time PCR (qRT-PCR) and detection/quantification of serum cytokines by ELISA. Quantification of mRNA identified a significant and transient increase in the expression of various cytokines, with variable magnitude and kinetics. mRNA expression of proinflammatory cytokines (IL-1β, TNF-α and IL-8) peaked at 24 hpi (5.4 times increase) and 48 hpi (3 and 10 times, respectively). A 15-fold increase in expression of INF-γ and 6-fold for IL-12 was observed at 48 and 24 hpi, respectively. An increase in the expression of self-regulatory cytokines (Th2) cells, especially IL-10 and IL-4 was detected at later periods (72 and 96 hpi) with peaks of 4.7 and 4.9 fold, respectively. The determination of the concentration of serum cytokines by ELISA showed an increase in IL-1β, TNF-α, IL-12, IFN-γ and IL-10 with kinetics similar to that observed by qPCR, especially for IL-1 and INF-γ. In summary, these results demonstrate that inoculation with iPPVO stimulates transiently a number events associated with cellular and humoral innate immune responses. If taken together, these effects would likely contribute for the enhanced resistance to certain pathogens observed in animals treated with iPPVO.

Key words: contagious ecthyma virus, orf virus, immunostimulant, innate immune response.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Analysis of Variance
APC	Antigen-presenting cell
ATCC	American type culture collection
BE	Binary Etilenoimina
BEI	Inactive Binary Etilenoimina
BLAST	Basic local alignment tool
BMDC	Bone-marrow derived plasmacytoid cells
BHI	Brain heart infusion
BPV-1	Bovine parapoxvirus
BPSV	Bovine popular stomatitis virus
BCPV	Bovine pseudocowpoxvirus
BoHV-1	Bovine herpesvirus 1
Ca^{++}	Calcium ion
CBP	Chemokine binding protein
CCR	Centro de Ciências Rurais
$CD5^{+}$	Cluster of Differentiation 5
cDNA	Complementary DNA
CEUA	Comitê de Ética em Uso Animal
CFU	Colony-forming Unit
COBEA	Colégio Brasileiro de Experimentação Animal
CPE	Cytopathic effect
CT	Threshold cycle
DC	Dendritic Cells
ºC	Degrees Celsius
ΔCT	Delta Cycle Threshold
dNTP	Deoxyribonucleotide triphosphate
DMVP	Departamento de Medicina Veterinária Preventiva
DNA	Deoxyribonucleic acid
Dr.	Doutor
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
EMCV	Murine Encephalomiocarditis Virus
F	Forward
FITC	Fluorescein Isothiocyanate
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-GFS	Granulocyte-Macrophage Colony-Stimulating Factor
GMT	Geometric Mean Titer
h	Hours
HANK'S	Hank's Balanced Salt Solution
HEPES	(N-(2-hidroxietil) piperazina-N'-(2-ácido etanosulfônico)
hpi	Hours post inoculation
iBHV-1	Inactivate Bovine herpesvírus 1
IgG	Immunoglobulin class G

IgM	Immunoglobulin class M
IL	Interleukin
IFNs	Interferons
IFN-γ	Gamma Interferon
IFN-I	Type I Interferon
ip	Intra-peritoneal
iPPVO	Inactivated Parapoxvirus ovis
iv	Intravenous therapy
iVACV	Inactivated Vaccinia Virus
kb	kilobases
Kg	Kilogram
log	Logarithm
LPM	Large Peritoneal Macrophage
M	Molar
MDBK	Madin Darby Bovine Kidney
MEM	Minimum Essential Medium
mg	Milligram
Mg^{++}	Magnesium ion
mg/l	Milligram to liter
mg/ml	Milligram to milliliter
min	Minutes
ml	Milliliter
mM	Millimolar
MMA	Mastitis-metritis-agalactia
MØ	Macrophages
mRNA	Messenger RNA
n	Number of replicates
nm	Nanometers
nº	Number
NBT	Nitroblue tetrazoliun salt
NF-kβ	Factor nuclear kappa B
NK	Natural killer cell
nm	Nanometer
OD	Optical Density
OFTu	Ovine fetal turbinate cells
ORFV	Orf virus
OV	Orf virus
PA	Phagocytic Activity
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
%	Percentage
pH	Power of Hydrogen
PhD.	Philosophy Doctor
PPGMV	Programa de Pós-graduação em Medicina Veterinária
PPVO	Parapoxvirus ovis
PRA	Plaque Reduction Assay
R	Reverse
qPCR	Quantitative Real Time PCR
Real-time PCR	Real-Time PCR

RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institut – culture medium
RS	Rio Grande do Sul
RT	Room temperature
SEM	Standard Error of the Mean
SPM	Small Peritoneal Macrophage
SV	Setor de Virologia
TCID	Tissue culture infectious dose
Th-1	T helper cell $CD8^+$
Th-2	T helper cell $CD4^+$
TLR	Toll Like Receptors
TMB	3,3′,5, 5′-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
U	International Units
UFSM	Universidade Federal de Santa Maria
μg	Microgram
UPF	Universidade de Passo Fundo
USA	United States of America
μl	Microliter
VERO	Cells derived from the kidney of an African green monkey
v/v	Weight of solute per volume of solution
x g	g-force

SUMÁRIO

1. INTRODUÇÃO

O vírus do ectima contagioso (*Parapoxvirus ovis*, PPVO) é também conhecido como vírus da *orf* (ORFV) do gênero *Parapoxvirus,* da família ***Poxviridae.*** O PPVO é o agente do ectima contagioso, uma enfermidade contagiosa debilitante que afeta principalmente ovinos e caprinos, além de ser transmissível para humanos (HAIG & MERCER, 1998). A infecção pelo PPVO possui distribuição mundial e é responsável por importantes perdas na ovinocultura e caprinocultura. A infecção ocorre por meio de abrasões na pele e/ou na mucosa oral e a doença caracteriza-se por lesões proliferativas e exsudativas, geralmente na pele ao redor da boca, comissura labial, focinho, tetas e mucosa oral. A replicação viral é frequentemente restrita aos queratinócitos, e a recuperação clínica ocorre em 4 a 6 semanas. No entanto, casos de infecção persistente tem sido sugeridos como um fator importante para a persistência do agente no ambiente (HAIG & McINNES, 2002; FLEMING & MERCER, 2007; LATEEF et al., 2010). A mortalidade resultante da infecção é baixa, porém a morbidade é elevada, podendo atingir mais de 90% dos animais de um rebanho (HAIG & MERCER, 1998). A disseminação do vírus entre os animais é rápida, podendo ocorrer pelo contato direto com animais infectados e/ou com as crostas das lesões que, quando eliminadas no solo contaminam as pastagens (FLEMING & MERCER, 2007). Apesar de uma intensa resposta inflamatória primária durante a infecção natural, o PPVO apresenta a capacidade de reinfectar o hospedeiro em um curto período de tempo, o que provavelmente esteja relacionado aos mecanismos de evasão da imunidade e ao tipo de resposta imunológica do hospedeiro (HAIG, 2006). A resposta imunológica à infecção pelo PPVO é caracterizada por uma resposta imune inata de caráter intensamente inflamatório, seguido pelo desenvolvimento da imunidade adquirida, aparentemente, de curta duração (HAIG, 2006).

O genoma do PPVO consiste de uma molécula DNA linear de fita dupla, com aproximadamente 138 kb e codifica aproximadamente 130 produtos (DELHON et al., 2004). Os poxvírus são conhecidos por modular vários aspectos da resposta imunológica do hospedeiro. Estes vírus codificam diversos fatores imunorregulatórios que agem em diferentes vias de sinalização celular. Alguns alvos da regulação incluem a via dos interferons (gene EL3), interleucina-1, IL-2, IL-18, IL-10, inibidor de IL-12, fator de necrose tumoral (TNF), fator estimulador de colônia de granulócitos e macrófagos (GM-CSF), proteínas ligadoras de quimiocinas (CBL), gene inibidor da apoptose (Bcl-2 like), fator de crescimento endotelial e vascular (VEGF), via do complemento e na via de sinalização do fator de transcrição nuclear-kappa-β (LYTTLE et al., 1994; FLEMING et al., 1997; McINNES et al., 1998; WESTPHAL et al., 2007; DEANE et al., 2009; DIEL, 2009; 2011). Assim, pode-se vislumbrar que a estratégia de infecção do PPVO baseia-se no balanço entre um conjunto complexo de eventos imunoestimulatórios e mecanismos de escape da resposta imune do hospedeiro (WEBER et al., 2003).

Alguns trabalhos sugerem o importante papel das células dendríticas no reconhecimento e apresentação de antígenos do PPVO, demonstrando que a imunidade local e, aparentemente, de curta duração em infecções pelo PPVO, pode estar relacionada a um mecanismo viral capaz de mobilizar as DCs no local da infecção, impedindo assim, a recirculação deste tipo celular até os linfonodos regionais para que ocorra a apresentação do antígeno de forma eficiente às células T (LATEEF et al., 2010).

As propriedades moduladoras e imunoestimulantes da imunidade inata dos poxvírus aliadas à fraca e transiente resposta imune às infecções despertaram o interesse científico para sua utilização como candidato a imunoativador (SCHÜTZE et al., 2009). Assim, MAYR & MAYR (1999) demonstraram que o PPVO inativado quimicamente (iPPVO) apresentava

características imunomoduladoras no hospedeiro, efeito posteriormente denominado *paraimunidade*.

Durante décadas, a comunidade científica vem estudando as características de uma nova categoria de substâncias biológicas, chamadas de imunoestimulantes. Estes produtos podem ter origem tanto natural como sintética. Os imunoestimulantes seriam responsáveis por reforçar a resistência natural do organismo, estimulando diferentes componentes da resposta imunológica: fagocitose, sistema do complemento, IFNs, citocinas e atividade de células do sistema imune (linfócitos T, linfócitos B, NK, DC) (PETRUNOV et al., 2007).

A atividade imunoestimulante do iPPVO tem sido demonstrada em diferentes enfermidades animais ao longo dos anos, tanto em caráter experimental quanto na prática clínica (MAYR & MAYR, 1999). Um produto utilizado comercialmente durante muitos anos contém uma preparação inativada da cepa D1701 do PPVO. O D1701 após múltiplas passagens em cultivo celular sofreu atenuação devido a deleções, duplicações e rearranjos no genoma (COTTONE et al., 1999). O imunomodulador chegou a ser lançado no mercado com diferentes formulações comerciais, tanto na Europa quanto nas Américas com os nomes PIND-ORF®, Baypamune®, Baypamum®, sendo hoje comercializado na América no Norte com o nome de Zylexs® (COTONE et al., 1998; WEBER et al., 2007). O iPPVO chegou a ser lançado no Brasil com o nome de Baypamun®, no entanto foi retirado do mercado no ano seguinte, provavelmente pelo desconhecimento científico a respeito das suas propriedades imunológicas e/ou pelo custo elevado.

Segundo Mayr & Mayr (1999), a paraimunização induzida pelo iPPVO poderia ser utilizada na forma profilática e/ou terapêutica, na prevenção e regulação das enfermidades com curso clínico crônico e/ou que apresentam certo grau de resistência ao hospedeiro. Ao contrário da imunização (vacinação), o imunomodulador iPPVO atuaria principalmente sobre a resposta imune inata, ativando a via sinalizadora dos Th-1 e, em um segundo momento a via

Th-2, evitando assim efeitos colaterais indesejáveis, como as doenças auto-imunes (MAYR & MAYR, 1999, WEBER et al., 2012; PALLIOT et al., 2013).

Neste sentido, nas últimas décadas vários trabalhos foram realizados na tentativa de demonstrar a eficácia do iPPVO como imunomodulador. Em bovinos, trabalhos realizados por Castrucci et al. (2000) demonstraram que animais tratados com o iPPVO apresentaram sinais clínicos de menor intensidade e por período de tempo inferior ao grupo controle, além de conferir proteção dos animais tratados quando desafiados com o BoHV-1. No trabalho relacionado a doenças do complexo respiratório bovino, Ziebell e colaboradores (ZIEBELL et al., 1997 a; b) avaliaram a frequência do tratamento com antibióticos, manifestações clínicas, excreção viral e resposta sorológica. Os animais que receberam o iPPVO apresentaram uma redução significativa na frequência de tratamento com antimicrobianos, e principalmente nas manifestações clínicas relacionadas à infecção. TURK et al. (2005) descreveram a utilização do iPPVO associado à vacina autógena na antecipação da regressão de dois casos de papilomatose generalizada em bovinos.

Em suínos, o iPPVO foi avaliado em experimentos *in vivo* sob o desempenho reprodutivo de leitoas, no controle da doença de Aujeszky, circovirose suína, pneumonia enzoótica, complexo MMA e na morbidade neonatal (POTOCNJAK et al., 2006; BIUK-RUDAM et al., 2004; KRYAKIS et al., 1998; 2002). Em equinos, o imunomodulador foi testado no tratamento de doenças respiratórias, estresse induzido pelo transporte e na imunomodulação de secreção de citocinas *in vivo* (ZIEBELL et al., 1997a; b; HOROHOV et al., 2008). Houve tentativas em demonstrar a utilização do iPPVO como alternativa para o tratamento de infecções por *Rodococcus equi*, com o propósito de reduzir as elevadas taxas de mortalidade por pneumonia em potros, além de minimizar a utilização excessiva de antimicrobianos. Os trabalhos demonstraram que não foi possível reduzir significativamente as taxas de pneumonias em potros, comparando-se com o grupo placebo. Entretanto, foi possível detectar

um aumento na quantidade de células secretoras de INF-γ, além de uma elevação na capacidade fagocítica dos neutrófilos (RYAN et al., 2010; STURGILL et al., 2011). Em pequenos animais, o iPPVO foi utilizado em felinos no tratamento da leucemia, peritonite infecciosa e rinotraqueíte infecciosa felina (MAYR & MAYR, 1999; HARTMANN et al., 1998). Em caninos, o imunoestimulante foi testado no controle de infecções de herpesvírus canino e na regressão de tumores mamários (SCHÜTZE et al., 2009; 2010; MAYR & MAYR, 1999).

O imunomodulador foi também avaliado em estudos *in vitro* utilizando linfócitos periféricos (PBMC) em uma variedade espécies, incluindo humanos, caninos, felinos, ovinos, suínos, equinos e murinos. Nestes trabalhos foram observados os efeitos imunoestimulantes do iPPVO sobre a produção de diversas citocinas, índice de fagocitose, ativação de neutrófilos, estimulação de células NK e sobre outros aspectos da imunidade inata (FÖRSTER et al., 1994; AHNE & MAYR, 1996; FACKINGER et al., 2000; WINNICKA et al., 2000; HOROHOV et al., 2008; SIEGEMUND et al., 2009).

Os resultados obtidos, tanto nas avaliações experimentais quanto em estudos clínicos, demonstram que o ORFVi apresenta propriedades imunomoduladoras sobre a imunidade inata do hospedeiro. Os principais efeitos consistem de uma forte modulação na secreção de citocinas que, em um primeiro momento atuariam no sentido pró-inflamatório e, mais tardiamente no sentido anti-inflamatório, controlando assim a intensidade da resposta e evitando os indesejáveis efeitos colaterais (WEBER et al., 2007).

Um dos grandes entraves que dificultou o estabelecimento da real eficácia dos efeitos induzidos pelo iPPVO, que gera ainda muita controvérsia, é o seu mecanismo de interação com o sistema imunológico do hospedeiro, pois o produto consiste unicamente de suspensão de partículas víricas inativadas quimicamente. Alguns autores sugerem que o tamanho e a estrutura complexa do PPVO seriam responsáveis pela interação com as células do sistema

imunológico, desencadeando os efeitos imunomodulatórios observados (FRIEBE et al., 2004; WEBER et al., 2012). Fiebig et al. (2011) caracterizaram alguns destes componentes do iPPVO por meio da produção de uma biblioteca de genes do vírus, que posteriormente foram expressos isoladamente ou em combinações em um vetor. Neste trabalho, foi possível observar que as propriedades imunomodulatórias parecem estar relacionadas com o abundante conteúdo proteico das partículas víricas, pois o efeito modulador é abolido por tratamento proteolítico prévio. Com os avanços da biologia molecular alguns autores por sequenciamento do genoma do PPVO e da geração de uma biblioteca genômica, identificaram um forte candidato a imunomodulador. Os autores observaram que o produto do gene B2L (proteína B2) demonstrou atividade imunomoduladora quando utilizado em modelos murinos com uma boa estimulação do sistema imune (FRIEBE et al., 2011; McGUIRE et al., 2012).

Fiebig (2011) e Rintoul et al. (2012) investigaram as propriedades terapêuticas do iPPVO como alternativa para o tratamento de neoplasias. Neste trabalho, os autores observaram que, camundongos desafiados com as células cancerígenas pela via intravenosa (iv) e que, em seguida, receberam a administrações do iPPVO, apresentaram uma redução no índice de mortalidade e na formação de metástases pulmonares.

Nos trabalhos realizados até o presente, seja pelas diferentes condições experimentais e/ou pela complexidade do sistema imunológico, não foi possível demonstrar a cinética de atuação do iPPVO sobre o sistema imune. O que se conhece até o momento, são os principais efeitos desta imunomodulação, como a síntese de citocinas e ativação de vários tipos celulares. Apesar do grande número de experimentos com a atividade imunomodulatória do iPPVO, os resultados obtidos não convergem para um mesmo sentido. Uma possível explicação é que, nos diferentes trabalhos, foram utilizadas espécies animais e metodologias distintas. Grande parte dos trabalhos, apesar da demonstração de resultados que indicam efeitos imunoestimulantes do iPPVO, apresentam detalhes metodológicos questionáveis, seja pela

baixa sensibilidade das técnicas empregadas e/ou, seja pelo caráter experimental em que se propõem utilizar o produto (MAYR & HÖRBER, 1992; ZIEBELL et al., 1997; CASTRUCCI et al., 1995; 1996; 1998; 2000; TURK et al., 2005; STURGILL et al., 2011).

Assim, o presente trabalho teve como objetivo investigar os efeitos do iPPVO sobre imune inata celular e humoral de camundongos. Como diferencial da maioria dos trabalhos anteriores, após estimulação *in vivo*, grande parte dos efeitos também foram avaliados *in vivo*.

2. CAPÍTULO 1

Effects of inactivated *Parapoxvirus ovis* on selected events of the innate immune response in mice

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Effects of inactivated *parapoxvirus ovis* on selected 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. This study investigated the effects of intraperitoneal inoculation of iPPVO (10^7 $TCID_{50}$) on selected events of the innate immune response in mice. Sera collected from iPPVO treated mice at 6h and 12h post-inoculation (pi) displayed marked IFN-I activity as ascertained by plaque reduction and inhibition of cytopathic effect by murine encephalomyocarditis virus. A significant increase ($p<0.01$) in neutrophil activity was observed by NBT reduction test in blood collected from iPPVO treated mice at 6hpi. Peritoneal macrophages collected from iPPVO treated mice displayed increased phagocytic activity ($p<0.01$) on *Candida albicans in vivo* (from 12 to 96 hpi) and *in vitro* (24 to 72 hpi). Bactericidal activity in sera of iPPVO treated mice against *Escherichia coli* was also increased ($p<0.05$) at 24 h and 72 hpi. On the other hand, no significant effect was observed on lysozyme activity and complement activation in iPPVO treated mice. Taken together, these results demonstrate that iPPVO administration leads to a transient stimulation of some innate

immune mechanisms, likely contributing for the immunostimulant effects observed against viral and bacterial infections *in vivo*.

Key words: contagious ecthyma virus, orf virus, immunostimulant, innate immunity.

1. Introduction

Parapoxvirus ovis (PPVO), also known as *orf* virus (OV or OrfV) is one of the four members of the genus *Parapoxvirus* (PPV) of the family *Poxviridae,* along with bovine parapoxvirus 1 (BPV-1 or bovine papular stomatitis virus, BPSV), BPV-2 (or pseudocowpoxvirus, PCPV) and New Zealand red deer PPV (Robinson and Mercer, 1995). In the natural hosts, sheep and goats, PPVO infection produces a debilitating mucocutaneous disease known as contagious ecthyma or contagious pustular dermatitis. The disease is characterized by inflammatory, proliferative and scabby lesions in the mucocutaneous junction of lips, labial commissure, muzzle and, less frequently, in the udder and coronary bands. The virus may be occasionally transmitted to human by contact resulting in self-limiting vesicular and pustular lesions in the hands and fingers (Haig and Mercer, 1998). Contagious ecthyma is endemic in most sheep and goat raising countries and leads to important economic losses (Haig and Mercer, 1998). In several countries, vaccination has been used with relative success to reduce the losses associated with the disease (Haig, 2006).

The virus is able to repeatedly infect its hosts in spite of a strong immune response (Haig and McInnes, 2002). Neutralizing antibodies are barely – if so – detected and seem not to confer protection to reinfection (Haig, 2006). During infection, PPVO strongly stimulates various events of the innate immunity including phagocytosis, NK cell activity, and production of several cytokines (IFN-α, β; TNF-α, GM-CSF) (Weber et al., 2003). On the other hand, a number of immune escape mechanisms has been demonstrated or proposed for this virus (Haig and Mercer, 1999, Haig and McInnes, 2002, Haig, 2006). The 138kb PPVO genome encodes a series of factors interfering with the host innate response, including an interferon resistance gene (McInnes et al., 1998), an inhibitor of GM-CSF and IL-2 (Deane et al., 2000, Deane et al., 2009), a gene encoding a Bcl-2-like inhibitor of apoptosis (Westphal et al., 2007), an IL-10 homologue (Fleming et al., 1997), a vascular endothelial growth factor

(VEGF) (Lyttle et al., 1994) and inhibitors of the NF-κβ signaling pathway (Diel et al., 2011). The survival strategy of PPVO, therefore, seems to rely upon a balance between immune stimulatory and escape mechanisms (Weber et al., 2003).

In addition to the complex interactions with the host immune system during infection, inactivated PPVO (iPPVO) has long been recognized to stimulate the innate immune response. Administration of inactivated PPVO (and fowl poxvirus as well) was first demonstrated to reduce the mortality of mice challenged with *Pseudomonas aeruginosa* (Mayr, 1978). Subsequently, a number of studies investigated, with some promising results, the effects of iPPVO as immunostimulator in different pathological conditions.

The promising effects of iPPVO as immunostimulant led to the development and use of a commercial immunostimulant called Baypamun (Mayr et al., 1991). Subsequently, a number of studies have demonstrated its immunostimulatory effects in infectious diseases and stress-mediated diseases in several animal species (Biuk-Rudan et al., 2004). Castrucci (2000) and Ziebell (1997a, 1997b) demonstrated the effect of comercial product against bovine respiratory distress, reducing viral shedding and the severity of clinical signs. iPPVO has also been used associated with autogenous vaccine against bovine papilomatosis, improving the regression of warts (Turk et al., 2005). iPPVO was tested in swine in viral infections and to reduce losses associated with stress and reproductive performance (Kyriakis et al., 1998, 2002, Biuk-Rudan et al., 2004, Potocnajak et al., 2006). In horses, comercial preparations of iPPVO have been used for stress and prevention and treatment of infectious and/or stress-induced diseases (Ziebell et al., 1997a, and 1997b, Horohov et al., 2008, Ryan et al., 2010, Sturgill et al., 2011). The Immunomodulator effect of iPPVO was also tested in cat and dogs against viral diseases, mammary tumors and improvement of immunological status (Hartmann et al., 1998, Mayr and Mayr, 1999, Schutze et al., 2009, 2010).

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 release (Förster et al., 1994, Winnicka et al., 2000, Schutze et al., 2009, Siegemund et al., 2009). Stimulation of PBMC, notably the proliferation and resulting IL-2, IFN-α and IFN-γ increase, were observed in swine blood cells (Fachinger et al., 2000). iPPVO was also shown to exert a strong influence on cytokine secretion by human immune cells, leading to release of inflammatory and Th-1 cytokines (IFN-γ, IL-6, IL-2, IL-8, IL-18) as well as anti-inflammatory and Th-2 cytoquines (IL-4, IL-10) (Friebe et al., 2004). Tumor necrosis factor-alfa (TNF-α), IL-2 and GM-CSF were also shown to be up regulated upon *in vitro* iPPVO stimulation of immune cells (Ahne and Mayr, 1996, Weber et al., 2003). Siegemund (2009) demonstrated that plasmacytoid dendritic (pDC) secreted IFN-α and IFN-β in response to iPPVO stimulation *in vitro*.

In spite of the promising results from early clinical studies and the identification of some putative molecular and biological mechanisms *in vitro*, the mechanisms mediating the immunomodulatory effects of iPPVO remain largely unknown. A number of *in vivo* findings have been questioned and, eventually, some results are difficult to reproduce (Hartmann et al., 1998, Biuk-Rudan et al., 2004, Sturgill et al., 2011). Likewise, some *in vitro* findings are controversial, discrepant and difficult to reconcile with observations *in vivo* (Fachinger et al., 2000, Horohov et al., 2008, Ryan et al., 2010 and Sturgill et al., 2011). Thus, it has become progressively clear that findings from *in vitro* assays should 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.

Thus, the present article reports an investigation of the effects of iPPVO inoculation in selected mechanisms of the innate immune response in mice.

2. Materials and Methods

2.1. Experimental design

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

2.2. Animals

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 Ética e Bem-estar Animal, UFSM, approval # 069/2011).

2.3. Viruses

Viruses: PPVO IA-82 (passage # 5) was kindly provided by Dr Daniel Rock (University of Illinois at Urbana/Champaign). Bovine herpesvirus 1 (BoHV-1 Cooper strain) Brazilian VACV isolate Pelotas 1 (P1V) and murine encephalomyocarditis virus (EMCV) were from our lab collection (Brum et al., 2010).

2.4. Cells

Ovine fetal turbinate cells (OFTu) were used to amplify ORFV IA-82; MDBK cells were used to multiplication of BoHV-1 and Vero cells were used to amplify VACV. L_{929} murine fibroblast cells line was used to EMCV production and interferon type I (IFN-I) assays. Cells were grown in MEM eagle (minimum essential medium), supplemented with

10% fetal calf serum (Nutricell, Brazil), 100 U/ml of penicillin and 100 µg/ml of streptomycin and maintained at 37 ºC and 5% CO_2.

2.5. Yeast and bacteria

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 x 10^8 cells/ml. *Escherichia coli*/ATCC-25922 and *Micrococcus luteus*/ATCC-7468 were kindly provided by Dr. Agueda C. Vargas (DMVP, UFSM, Santa Maria, RS, Brazil) and Dr. Luiz Carlos Kreutz (UPF, Passo Fundo, RS, Brazil), respectively. Bacteria were cultivated in BHI broth at 37 °C for 18 h, centrifuged and washed three times with PBS (pH 7.4). The bacterial suspension was adjusted to a final concentration of 0.4 OD_{600nm} in PBS (*E. coli*) and at 0.2 OD_{550nm} in sodium phosphate buffer 0.05 M, pH 6.2 (*M. luteus)*.

2.6. Interferon

To provide internal reference standard, recombinant murine interferon-β (IFN-$\beta^{r)}$ was kindly provided by Dr. Paulo Cezar Pelegrino Ferreira, Universidade Federal de Minas Gerais - UFMG. The standard IFN was used in a titer equivalent to 400.

2.7. Preparation of iPPVO

PPVO strain IA82, passage # 10 was propagated in OFTu cells. When cells were 80-90% confluent, the virus was inoculated and the cultures were incubated at 37 °C for 3 to 5 days in a 5% CO_2 incubator. The inoculum was harvested when the cytopathic effect (CPE) reached about 90% of the monolayer. The supernatant was collected and submitted to three freeze-thaw cycles followed by centrifugation at 220 x g for 10 min at 4 °C to remove cell debris. Then, the supernatant was harvested and submitted to virus quantitation by limiting dilution. Virus titers were calculated according to Reed & Muench (1938) and expressed as

log_{10} median tissue culture infectious doses per milliliter ($TCID_{50}$/ml). The viral suspension was inactivated with binary ethilenimine (BEI) for 18-24 h at 37 °C. BEI 0.1 mol/l was added to the virus suspension at final concentration of 0.1% and residual BEI were hydrolyzed by the addition of 1 mol/l of sterile Na-thiosulfate solution at final concentration of 1%. Viral particles in the supernatant were concentrated by ultracentrifugation at 68.000 x g for 2 h at 4 °C and stored at -80 °C.

2.8. Type I interferon (IFN-I) assays

Serum samples obtained at different times post-inoculation (6, 12, 24 hpi) from mice inoculated with iPPVO or control (iBoHV-1 or iVACV) were assayed for IFN-I activity against murine encephalomyocarditis virus (EMCV) according to Vogel (2001). An interferon standard (IFN-β^r) was included in all assays to monitor cell condition and assay reproducibility. Briefly, confluent L_{929} cells grown in 96-well plates were incubated with two-fold dilutions of mouse sera for 6 h. Then, plates were drained, washed three times with MEM and each well was inoculated with 100 TCID of EMCV and incubated for 2 h at 37 ºC. The inoculum was removed, cells were washed three times and incubated with culture medium containing 2% FCS during 48 h when cell monolayers were monitored for EMCV-induced CPE. The IFN activity was expressed as the reciprocal of the serum dilution that produced an inhibition of CPE in 50% of the cell monolayers. Results were expressed as log 2 geometric mean titers.

IFN-I activity in mouse serum was also determined by plaque reduction assay (Green et al., 1980) comparing both groups (iPPVO treated x control). Briefly, L_{929} cells were seeded at 6-wells plate at a density of $1x10^6$ per well at the day before the experiment. On the following day, the medium was removed, cells were washed three times with medium and incubated with duplicates of mouse serum diluted 1:10 (MEM) during 6 h. In addition to mock-controls, monolayers incubated with sera of mice inoculated with iBoHV-1 and iVACV

were used as controls. Then, cells were drained, washed three times and inoculated with 100 TCID of EMCV. After 2 h of adsorption at 37 ºC, cultures were washed and overlaid with MEM supplemented with 2% of FCS and incubated for 48 h at 37 ºC and 5% CO_2. After that, monolayers were fixed with formalin 10 % and stained with crystal violet (0.3 %) for 2 h. Viral plaques were counted for each replicate and results were expressed as the percentage of plaque reduction and showed as mean ± SEM.

2.9. Nitroblue tetrazolium test

Phagocyte respiratory burst was measured in blood samples using the Nitroblue Tetrazolium (NBT) dye as described by Panigrahi (2004). Heparinized blood (100 μl) collected at early moments of the immune response (6, 12 and 24 hpi) from iPPVO and control groups were tested. Blood samples were added in triplicates at 96 microplates and mixed with equal amount of 1mg/ml of NBT (Sigma). Negative control (unstimulated cells) and positive control for each sample tested (stimulated cells - Zimozam 5 mg/ml; Sigma), were added. After an incubation of 1 h at 37 °C, an aliquot of 50 μl of the NBT-blood cell suspension was taken and added to a microtube containing 1 ml of N, N Dimethylformamide (Sigma). Samples were then centrifuged for 5 min at 2600 x g and supernatants were added to a cuvette for absorbance reading at 540_{nm}. The results were shown as the mean ± SEM and submitted to statistical analysis by student *t* test.

2.10. Assays for phagocytosis in vitro and in vivo

For phagocytosis assays, peritoneal phagocytes were collected at different intervals after iPPVO inoculation. For collection, animals were previously anesthetized with isoflurane by inhalation followed by cervical dislocation. Then, the abdomens were sprayed with 70% ethanol and the peritoneal cavity was injected with 3 ml of cold PBS (Ca^{++} and Mg^{++} free) and massaged for approximately 30 sec. After, PBS containing peritoneal cells was collected and

centrifuged at 226 x g for 10 min. Cells were suspended in MEM supplemented with 3% FCS, counted in a hematocytometer and adjusted to a final concentration of 10^6 cells.

2.10.1. Phagocytosis in vitro

Peritoneal phagocytes were first assayed for phagocytic activity *in vitro*. Peritoneal cells collected at 12, 24, 48, 72, 96, 120 and 144 hpi were assayed for the ability to phagocytize *C. albicans.* For the assay, 10 μl of yeast (10^8/ml) was mixed with 100 μl of peritoneal macrophages (MΦ/10^6 cells) in MEM supplemented with 3% of FCS. The *C.albicans*/MΦ mixture was layered over a multispot microscope slides in duplicates (40 μl/well) from each mice and incubated for 30 min at 37 °C. After that, slides were washed three times with warm PBS to remove non-adherent cells. The multispot slides were stained with fast Panoptic method and the phagocytic activity (PA%) was determined by counting 100 cells in 10 different fields using an epifluorescent microscope (1000 x). The number of macrophages containing phagocytized *C. albicans* was registered for each mouse and the results were expressed as percentage of PA by the mean ± SEM (standard error of the mean) from both groups assayed. The results were than submitted to statistical analysis by *t* student test.

2.10.2. Phagocytosis in vivo

Based on the results obtained on the *in vitro* assay, we investigated the ability of peritoneal phagocytes to internalize *C.albicans in vivo.* Mice were inoculated i.p. with iPPVO or with MEM (control group). At different intervals (24, 48, 72 and 96 h post iPPVO treatment), mice were inoculated with inactivated yeast cells suspended in 100 μl of PBS (10^7 yeast cells/mouse). After 1 h, peritoneal cells were collected as described above and each sample (40 μl/well) was placed in duplicates on glass multispot slides, followed by incubation for 1 h at 37 °C, washed three times with PBS and staining by the fast Panoptic method. In each replicate, 100 cells were counted in a total of 10 microscope fields. The mean number of

cells engulfing *C.albicans* was registered for each mouse. Results were expressed as mean±SEM for iPPVO and control groups (n = 5 animals/group). Results were submitted to statistical analysis by *t* student test.

2.11. Serum bactericidal activity

The direct antimicrobial killing was assayed by exposing mouse serum with a suspension of *E. coli* to quantify the reduction of CFU/ml. Briefly, following an overnight incubation (18h), *Escherichia coli* (ATCC 25922) was then peaked in BHI and grown at 37 °C in an orbital shaker for approximately 3h to reach the early log phase (OD_{600nm} of 0.4). The bacterial cells were harvested, washed three times, pelleted and suspended in HANK'S solution (Mg^{++}, 10 mM EGTA, 6 mM HEPES). The suspension was adjusted to 0.1 (OD_{600nm}) and kept in an ice bath until use. Aliquots of serum (18 μl, complement free) were added in a microtube (in duplicate) and mixed with a suspension of 2 μl of *E. coli* (diluted 10^4) and incubated during 1 h at 37 °C. At the end of incubation, 80 μl of HANK'S solution were added in each tube pour-plated in BHI agar, and incubated 18 h at 37 °C. The number of bacteria was determined by counting the colonies that grew in the plates. The results were expressed as mean ± SEM of CFU/ml comparing both groups (iPPVO x controls) and data were analyzed statistically by student *t* test ($p \leq 0.05$).

2.12. Other assays

The lytic activity of lysozyme was measured using a turbidimetric assay against *Micrococcus luteus*. The results were expressed in units/ml, as one unit defined as a decrease of 0.001 on OD_{450nm}U/min. Results obtained from each group were analyzed by the means±SEM and submitted to statistical *t* test. Complement hemolytic activity as assayed an results calculated according to Ekdahl et al. (2007).

3. Results

3.1.Type I interferon induction

To investigate IFN-I induction/activity, mouse L_{929} cells were incubated with two-fold dilutions of sera collected from mice inoculated with iPPVO, followed by inoculation with EMCV, whose inhibition of replication is an indicator of IFN activity. Controls included sera of mock-inoculated mice, mice inoculated with iBoHV-1 and iVACV, and assay using recombinant interferon. By using these assays, 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 h pi (Table 1). At 24 h pi, sera from 2 of 6 animals exhibited antiviral effect. No antiviral activity was detected in sera collected from mice inoculated with iBoHV-1 or iVACV at 6 and 12 hpi. Thus, the inhibitory effect of EMCV replication was likely derived from IFN-I activity induced by iPPVO stimulation.

To better characterize the inhibitory IFN activity on EMCV replication, a plaque reduction assay (PRA) was performed. Again, a marked inhibitory effect on EMCV replication was observed in sera collected at 6hpi from mice inoculated with iPPVO (Table 1). The > 94 % plaque reduction observed at 6h decreased to 28% at 12 h and was no longer observed at 24 h. No plaque reduction was observed in sera of mice inoculated with iBoHV-1 or iVACV indicating that it was an iPPVO-specific effect. Our intra-assay and inter-assay control standard interferon showed an 85% plaque reduction when tested in a 1:100 dilution These results indicate that iPPVO inoculation leads to a significant and transient increase in IFN-I production – as measured by IFN-I inhibitory effect on EMCV replication - which seems to be iPPVO - specific rather than a general response to virus inoculation.

3.2. Nitroblue tetrazolium test

During innate immune response, stimulation of neutrophil membrane triggers the production of microbicidal oxygen free radicals or reactive oxygen species such superoxide anion (Förster et al., 1994). Animals treated with iPPVO presented a transient increase in

respiratory burst by neutrophils compared to mock-treated animals. The effect was detected only at 6hpi ($p < 0.01$) (Fig. 1). No differences were observed at other time points, indicating an early and transient effect, as expected, considering the kinetic response of neutrophils during inflammatory events. Thus, iPPVO inoculation leads to a transient increase in respiratory burst by neutrophils as determined by NBT test.

3.3. Phagocytosis in vitro

The effect of iPPVO stimulation in the phagocytic activity of peritoneal macrophages *in vitro* was investigated at different time-points post-inoculation (p.i.), comparing the iPPVO and placebo/control groups. The results of counting macrophages engulfing *C. albicans in vitro* are shown in Table 2. Fig. 2 shows the increase of phagocytic activity for each time point. A significant increase in phagocytic activity was observed between 12 and 96 hpi, with the highest response detected at 72 h (ran increase of 40%; $p \leq 0.01$). No significant increase in phagocytic activity was observed after 96 h p.i. (120 and 144 h). Thus, iPPVO inoculation in mice resulted in an improvement of phagocytic activity by peritoneal macrophages as measured by *in vitro assays*.

3.4. Phagocytosis in vivo

An *in vivo* phagocytosis assay was performed to investigate whether iPPVO inoculation would increase phagocytic activity by peritoneal macrophages. As shown in Table 3 and Fig. 3, peritoneal macrophages of mice inoculated with iPPVO presented an increased phagocytic activity compared to control group. The stimulatory effect was observed at 24, 48 and 72 hpi, with the highest increase at 48 hpi. Thus, iPPVO inoculation also led to a boost on phagocytic activity by peritoneal macrophages *in vivo*. Comparing the time points tested, increased phagocytosis *in vitro* was detected longer (up to 96 h) than *in vivo* (up to 72 h). The different profile of response between both assays could be explained by the strong challenge promoted by *C. albicans* inoculation. Regardless, these results showed that phagocytosis by

peritoneal macrophages of mice is transiently increased following inoculation of iPPVO, an effect that was observed up to 72 h (*in vivo*) or 96 h (*in vitro*).

3.5. Bactericidal activity

The bactericidal activity of sera of iPPVO treated mice was assayed at different time points after inoculation. Compared with mock-infected group, the iPPVO treated group exhibited a significant increase in bactericidal activity at 24 hpi and 72 hpi ($p \leq 0.05$ and $p \leq 0.01$, respectively; fig. 4). An increase, yet not statistically significant, was also observed at 48 h. No difference was observed in sera collected at 12 and 96 hpi. Thus, iPPVO inoculation in mice led to an increase in bactericidal activity of serum against *E.coli.*

3.6. Other assays of innate immunity

Lysozyme activity in sera of iPPVO treated mice showed a slight increase in all time-points compared with control groups, yet the differences were not statistically significant ($p > 0.05$). No increase in complement activity was observed in sera of mice inoculated with iPPVO compared to controls, regardless the time point examined. These results may either reflect the lack of effect of iPPVO on this events or a low sensitivity of our assays, or both.

4. Discussion

Our results confirm and extend previous findings that iPPVO exerts stimulatory effects on cellular and humoral events of the innate immune response (Mayr and Mayr, 1999, Weber et al., 2003, Weber, 2013) The effects demonstrated herein seem to be iPPVO-specific – since they were not observed upon inoculation of other inactivated viruses (iVACV, iBoHV-1) - and are probably derived from the stimulation of host immune cells by structural components of viral particles, as described previously (Fachinger et al., 2000, Kruse et al., 2001, Weber et al., 2003, Fiebig et al., 2011). Regardless the nature, the effects of iPPVO stimulation on innate mechanisms appear to be transient. These effects, if acting solely, would

probably not suffice to explain the enhanced resistance to certain pathogens as described in the literature (Mayr & Mayr 1999, Castrucci et al., 2000, Weber et al., 2003, Biuk-Rudan et al., 2004). Rather, these events probably represent the first defense line, acting promptly, preceding and paving the way for subsequent innate and/or adaptive events that would, eventually, confer resistance to microorganisms. Extending previous studies, our results demonstrate effects against a range of microorganisms, e.g. viruses (e.g. EMCV), gram-negative (*E. coli*) and gram-positive bacteria (*M. luteus*) and yeast (*C. albicans*). Taken together, our findings may help to explain the immunostimulatory effects of iPPVO demonstrated in diverse disease models and animal species over the years (Castrucci et al., 1995, Mayr & Mayr, 1999, Weber et al., 2003, Biuk-Rudan et al., 2004. Nevertheless, the biological and molecular mechanisms underlying these effects remain far from being completely understood and will demand continuous and more in depth studies.

The immunomodulatory properties of inactivated parapoxviruses (iPPVO) on the innate immunity have led to a high scientific interest for their use as immunostimulants (Schutze et al., 2010). Mayr & Mayr (1999) first demonstrated that chemically inactivated PPVO (iPPVO) exerted immunomodulatory properties in the host, an effect termed *paraimmunity*. The immunostimulatory activity of iPPVO has been demonstrated in different animal species against different pathogens over the years, both experimentally and in clinical practice (Ziebell et al., 1997, Kyriakis et al., 1998, Mayr & Mayr, 1999, Castrucci et al., 2000) According to current data, these effects could be exploited as prophylactic and/or therapeutic tools against diseases with chronic course, situations with a degree of resistance, persistence or even in cancer-related events (Mayr et al., 1991, Castrucci et al., 1995, Kyriakis et al., 2002, Fiebig et al., 2011).

The main effects of iPPVO on the innate immunity seem to rely upon a stimulation of immune cells and secretion of cytokines. iPPVO particles would activate antigen-presenting

cells, mainly dendritic cells (DC), mediating their immunostimulatory effects by TLR-independent an TLR-dependent pathways promoted by certain viral components (Siegemund et al., 2009). The activation of immune cells are followed by the release of various cytokines, including type I interferons. IFN-I upregulation (IFN-α and IFN-β) in response to iPPVO has been demonstrated mainly *in vitro,* by exposing peripheral blood mononuclear cells (PBMC) or dendritic cells to iPPVO and measuring the cytokines in culture medium by ELISA or their mRNA by real time PCR (Horohov et al., 2008, Siegemund et al., 2009). This response has been demonstrated for PBMC of a variety of animal species including sheep, cattle, pigs, mice and humans (Fachinger et al., 2000, Weber et al., 2003, Friebe et al., 2004). In this sense, our study presented major differences: i. iPPVO stimulation was performed *in vivo*, by mouse inoculation; ii. IFN-I production/activity was measured in sera collected from inoculated animals; iii. The presence/activity of IFN-I was measured by biological assays, e.g. inhibition of ECMV replication. Regardless the approach, our results confirmed previous findings and demonstrated a prompt and transient IFN-I response upon iPPVO inoculation. The peak IFN antiviral effect was observed at 6 h pi and remained up to 24 h pi (table 1). Previous studies have already demonstrated that PBMC and DCs stimulated *in vitro* with iPPVO produce IFN-I between 6 and 24 h pi (Ahne and Mayr, 1996, Horohov et al., 2008, Siegemund et al., 2009). Also confirming earlier *in vitro* studies (Fachinger et al., 2000, Siegemund et al., 2009), 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 and activity may trigger downstream mechanisms involved in microbial resistance and clearance by the immune system. It is plausible that ligand-receptor interactions, represented in this study by iPPVO, could trigger the Jack-Stat downstream pathway that is followed by transcriptional regulation of many effector genes that may contribute for the pleotropic response by IFNs (Takaoka and Yanai, 2006). It is likely that the cascade of antiviral genes

activated by the IFN-I system participate in the broad responses observed in this study. Here, a bioassay demonstrated that iPPVO stimulates the interferon pathway, which is an important mechanism for host defense in early stages of viral infections. In this line, a number of clinical studies in different species have demonstrated decrease in virus shedding and reduction in clinical signs following iPPVO stimulation (Castrucci et al., 1995, 1996, 1998, 2000, Ziebell et al., 1997, Weber et al., 2003, Biuk-Rudan et al., 2004, Fiebig et al., 2011).

The present study also demonstrated a significant neutrophil stimulation upon iPPVO inoculation, confirming previous results (Förster et al., 1994, Winnicka et al., 2000). As granulocytes play an important role in the inflammatory response, the intracellular generation of superoxide anion by NBT reduction test (respiratory burst) was assayed after administration of iPPVO. An increase in respiratory products by neutrophils was detected as soon as at 6 hpi (Fig. 1) and, as demonstrated by (Winnicka et al., 2000), NBT reduction values decreased over the experimental period (6 to 24 hpi). Circulating neutrophils are quickly recruited to sites of infection and inflammation by host-and/or pathogen-derived components, priming these host cells for enhanced microbicidal activity (Kobayashi et al., 2005). Based on our results, we demonstrate that iPPVO can promote prompt neutrophil activation, increasing respiratory burst, which could be essential for bacterial and fungal killing in early stages of infection. This effect could have also been demonstrated *in vivo* in horses treated with iPPVO, in which an increased response to challenge with *Rodococcus equi* was observed (Ryan et al., 2010, Sturgill et al., 2011). Neutrophil and macrophages work in concert in the antimicrobial immunity. This association may include neutrophils as inducer and effector of adaptative immunity, cooperating with macrophages by producing important inflammatory cytokines and chemokines that attract and activate macrophages (Silva et al., 2010).

The effects of iPPVO on phagocytic activity of peritoneal phagocytes – mainly

macrophages –, was another interesting finding of the present study. Intraperitoneal inoculation of mice with iPPVO resulted in a strong and significant increase in phagocytosis, as measured *in vivo* and *in vitro*. Both assays showed a similar pattern of response, however with some differences in magnitude and duration. Increased in PA *in vitro* was observed between 12 to 96 hpi, with a significant burst on the phagocytic function at 72 h ($p \leq 0.01$). Upon microscopic examination, it was possible to identify most of the phagocytic cells as large peritoneal macrophages (LPM). Assay for phagocytic activity *in vivo* yielded similar results, yet with enhanced PA being observed for a short period of time (24-72 hpi). Herein, small peritoneal macrophages (SPM) predominated over LPM as phagocytic cells. It could be considered that immunization and inflammation induces a influx of SPM into the peritoneal cavity, which are considered the primary phagocytic cells in the peritoneal cavity capable to induce an immune response (Ghosn et al., 2010, Hussain et al., 2012).

Enhanced phagocytic activity has already been proposed as one of the effects of iPPVO stimulation of the innate immune system. Nevertheless, most studies demonstrated this effect by *in vitro* stimulation of PBMCs (Förster et al., 1994, Fachinger et al., 2000, Winnicka et al., 2000, Ryan et al., 2010) and the assays probably detected phagocytosis by other cell populations. In contrast, the present study performed an *in loco* and *in vivo* stimulus (intraperitoneal iPPVO inoculation). In addition, the cells obtained by peritoneal lavage were composed predominantly by macrophages (> 90%) (Ghosn et al., 2010). Thus, *in vivo* stimulation of peritoneal macrophages by iPPVO led to increased phagocytic activity measurable both *in vitro* and *in vivo*

Finally, we performed a serum bactericidal assay to investigate a possible association between iPPVO and this humoral branch of innate immune response. To test the hypothesis that iPPVO could improve serum bactericidal activity, we first inactivated serum complement, and used a gram-negative bacteria to exclude lysozyme activity. The term host

defense peptides refer to a large number of peptides as the cathelecidins and defensins characterized by their antibiotic and antifungical activities (Auvynet et al., 2009). We found that iPPVO was able to increase serum bactericidal activity at 24 and 72 h pi. Compared with mock-infected group, iPPVO treated group reduced statistically ($p \leq 0.01$) the number of *E. coli* colony former units (CFU/ml). This results could explain clinical effects observed in others studies where iPPVO improve host resistance against microbial infections (Weber et al., 2003, Ziebell et al., 1997a, 1997b, Ryan et al., 2010, Sturgill et al., 2011).

In summary, our results contribute to explain the immunostimulatory effects of iPPVO on the innate immune response. Taken individually, none of the observed effects (IFN-I induction, neutrophil activation, increased phagocytosis and bactericidal activity) would likely suffice to explain the enhanced microbe resistance observed in iPPVO-treated animals. Rather, these effects would probably act in concert with other yet unidentified or poorly characterized mechanisms as to increase the ability of the immune system to control such infections. Properly identifying and manipulating these mechanisms would constitute a notable challenge towards using iPPVO as an immunostimulant in clinical practice. In this context, the enhancement of host immunity by iPPVO would be one of the most efficient strategies to control and prevent certain infectious diseases of domestic animals.

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Table 1. Antiviral activity against EMCV in sera of mice inoculated with iPPVO. Antiviral activity was measured by reduction of cytopathic effect and plaque formation by EMCV in L_{929} cells.

Bioassay of interferon type I							
Group		Inhibition of cytopathic effect (GMT Log 2)[a]			Plaque reduction assay (% Reduction)		
	n	6hpi	12hpi	24hpi	6hpi	12hpi	24hpi
iPPVO	6	4.3 ± 0.9*	4.4±0.5*	4.3±0.2[b]	94.3*±2.8	28.9±5*	-
iBoHV-1	5	-	-	-	-	-	-
iVACV	5	-	-	-	-	-	-
MEM	5	-	-	-		-	-

[a] Interferon inhibition of cytophatic effect was expressed as log 2 geometric mean titer; n indicates the number of replicates; iPPVO: inactivated parapoxovis virus; iBoHV-1: inactivated bovine herpesvirus 1; iVACV: inactivated vaccinia virus; [b] inhibition of cytopathic effect in 2 of 6 animals;* $p \leq 0.05$.

Table 2. Phagocytic activity *in vitro* by peritoneal macrophages collected from mice inoculated with iPPVO, at different time points after inoculation, compared to control/placebo groups.

Phagocytosis *in vitro*

	Control	**iPPVO**	
hpi	**PA % (mean ±SEM)**	**PA % (mean ±SEM)**	**p value**
12	30.45 (± 0.08)	34.29 (± 0.02)	p≤0.01
24	43.36 (± 2.3)	61.77 (± 4.0)	p≤0.01
48	58.57 (± 4.7)	76.23 (± 2.2)	p≤0.01
72	36.55 (± 7.0)	77.69 (± 1.9)	p≤0.01
96	49.99 (±4.0)	70.96 (± 2.3)	p≤0.01
120	49.79 (± 6.5)	50.3 (± 2.4)	p>0.05
144	43.34 (± 2.6)	45.74 (± 5.6)	p>0.05

Control group was inoculated with MEM; PA (%) phagocytic activity; Fold increase relative to control (Mean ± SEM); SEM, standard error of the mean; P value calculated with 95% confidence.

Table 3. Phagocytic activity *in vivo* by peritoneal macrophage followed iPPVO inoculation.

	Phagocytosys *in vivo*		
	Control	**iPPVO**	
hpi	**PA %**	**PA %**	**p value**
24	40.09 (± 2.4)	67.25 (± 8.6)	$p \leq 0.01$
48	41.82 (± 1.7)	68.61 (± 4.3)	$p \leq 0.01$
72	47.76 (± 2.1)	63.8 (± 1.8)	$p \leq 0.01$
96	54.08 (±2.3)	57.82 (± 5.5)	$p > 0.05$

Results are shown as Phagocytic activity (PA %) expressed by mean ± SEM. Fold increase relative to control (Mean ± SEM); Control group was inoculated with MEM; SEM, Standard error of the mean; P value calculated with 95% confidence.

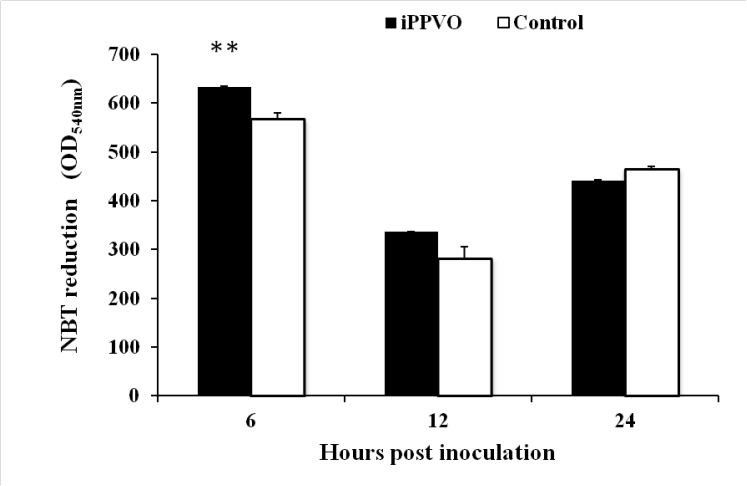

Fig. 1. NBT reduction test. The oxidative burst of neutrophil was measured in whole blood of mice inoculated ip with iPPVO and the results were compared with control group, inoculated with MEM. Results are show as mean ± SEM. **p≤0.01

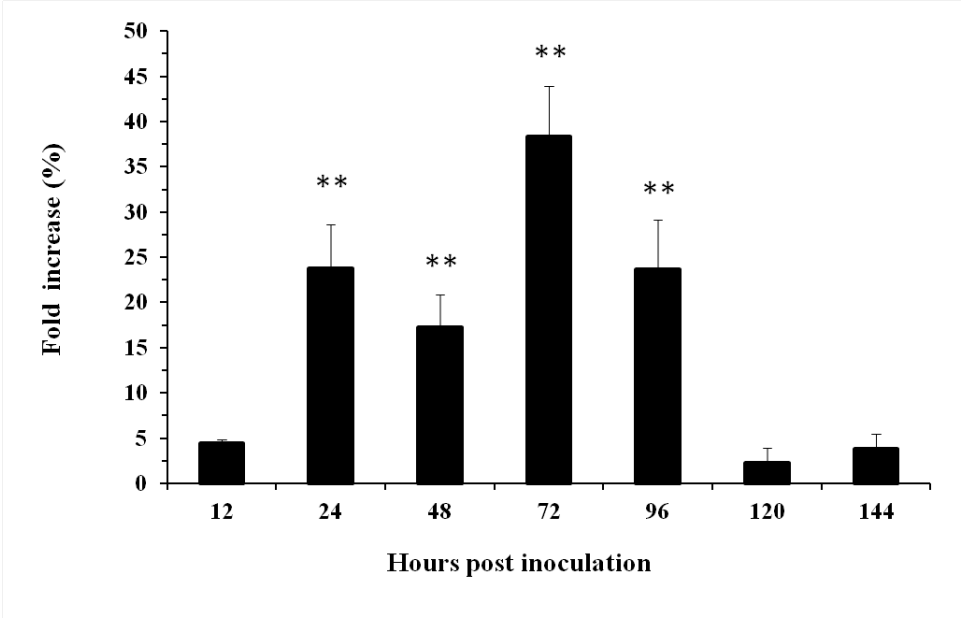

Fig. 2. Increase in phagocytic activity *in vitro* by peritoneal macrophages collected from mice inoculated with iPPVO. Values are shown as –fold increase comparing iPPVO-treated and control groups (**$p \leq 0.01$).

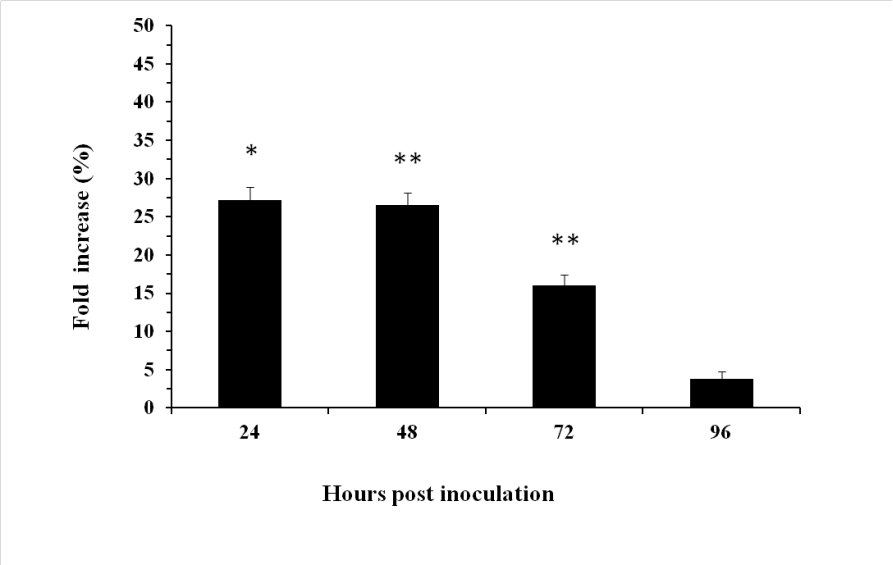

Fig. 3. Phagocytic activity *in vivo* by peritoneal macrophages following inoculation of iPPVO. Values are shown as –fold increase comparing iPPVO-treated and control groups **p≤0.01; * p≤0.05.

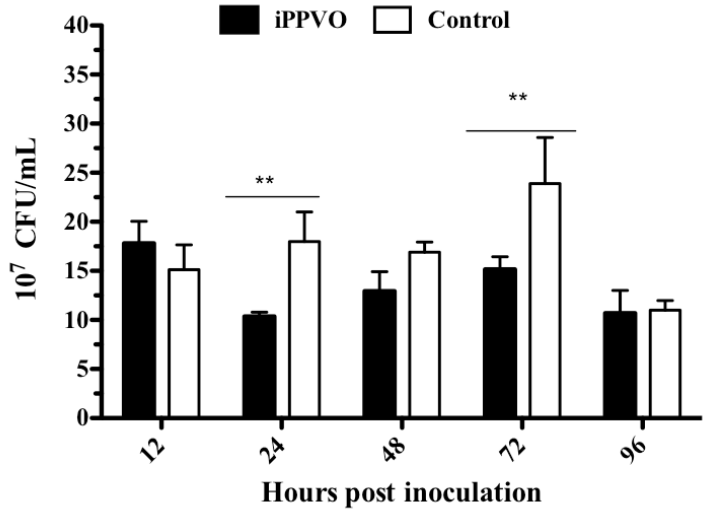

Fig. 4. Bactericidal activity in sera of mice inoculated with iPPVO. Serum samples were inactivated for complement and incubated with a suspension of *E. coli* to quantify the reduction in colony-former unity (CFU/ml). Results are show as mean±SEM and submitted to statistical evaluation by student *t* test.**p≤0.01; * p≤0.05.

3. CAPÍTULO 2

Inactivated parapoxvirus ovis induces a transient increase in the expression of proinflammatory, Th-1-related and autoregulatory cytokines in mice

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Inactivated parapoxvirus ovis induces a transient increase in the expression of proinflammatory, Th-1-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 in 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 qPCR, serum protein concentration by ELISA and IFN-α/β activity by a bioassay. The spleen of iPPVO treated animals showed a significant increased in mRNA expression of all cytokines assayed, with different kinetics and magnitude. Proinflammatory cytokines IL-1β, TNF-α and IL-8 mRNA peaked at 24 hours post-inoculation (hpi, 5.4-fold increase), 48hpi (3-fold and10-fold), respectively. A 15-fold increase in IFN-γ and 6-fold IL-12 mRNA increase were detected at 48 and 24hpi, 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 increase, respectively. Type I interferon (IFN-I) antiviral activity against encephalomyocarditisvirus (EMCV) 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-1, with a similar kinetics of that observed by qPCR, especially for IL-12 and IFN-γ. These data demonstrate that iPPVO induces a transient and complex cytokine response, initially represented by Th1-related cytokines followed by an auto regulatory and Th-2 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 produces vesiculo-papular lesions in the hands and fingers (3). The 138kb double stranded DNA PPOV 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-1 homologue; granulocyte macrophage colony-stimulating factor), an IL-2 inhibiting protein, the vaccinia E3L gene encoding an interferon-resistance product (7) and several gene products interfering with the NF-kappa β pathway (8-10). Nevertheless, the immune evasion by PPVO seems to be rather complex to be explained by a single factor/mechanism individually (6).

The immunomodulatory effects of PPVO have long been recognized and raised a significant interest in veterinary research in the last decades. Inactivated PPVO (iPPVO) retains many immunomodulatory properties of live virus suggesting that these effects should rely on the structural components of the viral particle. The effects of iPPVO in 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 auto-regulated loop of initial upregulation of inflammatory and Th1-related cytokines, followed by regulatory and Th2-related cytokines (11). Early pro-inflammatory cytokine secretion include IL-6, IL-8 TNF-alpha by monocytes and/or by antigen presenting cells (APCs), IL-2 IFN-alpha/beta and Th1 cytokines (IL-12, IL-

16, IFN-gamma) by T-helper lymphocytes. IFN-gamma secretion by T lymphocytes or 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 in the innate immune response and the knowledge derived thereof were performed *in vitro* (13,15,18-21).

In this 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 burst, serum bactericidal activity and IFN-α/β production in treated mice (Anziliero A, submitted). In the present article we describe a detailed investigation on the cytokine profile following iPPVO administration in mice, by qPCR, ELISA and IFN-I biological assay.

Material and Methods

Experimental design

Mice were inoculated with iPPVO ($10^7 TCID_{50}$) by the intraperitoneal route (i.p.). Spleen and blood samples were collected at different times post-inoculation (hpi) (12, 24, 48, 72, 96, 120) and assayed for cytokine expression. Cytokine expression in spleen (mRNA) was quantitated 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 six to eight-weeks-old, female Swiss mice (*Mus musculus*), weighing 23 - 30g 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*. Ten mice per group were used in all experiments. The study was approved by the Institutional Ethics and Animal Welfare Committee (Comitê de Ética e Bem-estar Animal, UFSM, approval # 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, USA). Bovine herpesvirus 1 (BoHV-1 Cooper strain) and the Brazilian VACV isolate Pelotas 1 (P1V) (22) and murine encephalomiocarditis virus (EMCV) kindly provided by Dr Erna G. Kroon (UFMG).

Cells: primary ovine fetal turbinate cells (OFTu) were used to amplify ORFV IA-82; MDBK cells were used for the amplification of BoHV-1; Vero cells were used to amplify VACV. Cells were grown in MEM eagle (Minimum Essential Medium), supplemented with 10% fetal bovine serum (Nutricell, Brazil), 100 U/mL of penicillin and 100 µg/mL of streptomycin and maintained at 37 ºC and 5% CO_2.

Preparation of iPPVO

The iPPVO inoculum was prepared as described previously (Anziliero A, submitted). Briefly, ORFV strain IA - 82, passage #10, was propagated in primary ovine fetal turbinate cells (OFTu) and harvested when the cytopathic effect (CPE) 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 expressed as $\log_{10}$ median tissue culture infectious doses per milliliter ($TCID_{50}$/mL). The

viral suspension was inactivated with binary ethilenimine (BEI) for 18 – 24 h at 37 °C. BEI 0.1 mol/L was added to the virus suspension to a final concentration of 0.1% and residual BEI was hydrolyzed by the addition of 1mol/L of sterile Na-thiosulfate solution at final concentration of 1%. Viral particles in the suspension were then concentrated by ultracentrifugation at 116.939 x 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 (10^7TCID$_{50}$) by the intraperitoneal route (i.p.) in a volume of approximately 100 μL. At different times post-inoculation (6, 12, 24, 48, 72, 96, 120h, 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 i.p. ultracentrifuged supernatant of OFTu cells (5-7 mice/group). In experiments assayed for type I interferon (IFN-I), control groups included mice inoculated i.p. with inactivated bovine herpesvirus 1 (iBoHV-1) and vaccinia virus (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 (50mg/animal). Specimens from individual animals were then placed in RNAlater stabilization reagent (Qiagen) and stored at -80 ºC until RNA extraction. Blood was collected from cardiac chamber and left to clot overnight at 4 ºC. The blood was then centrifuged for 20 min at 160 x 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 (QIAGEN) according to the manufacturer's instructions. The RNA concentration and purity was determined by the absorbance ratio at 280_{nm} and 260_{nm}. RNA integrity was accessed by denaturing gel electrophoresis in 1% agarose gel stained with ethidium bromide. All samples were treated with amplification-grade DNase I (Invitrogen) to remove traces of genomic DNA contamination (24). According to RNA concentrations, treated RNA concentration was adjusted for 1μg of RNA/sample.

RNA samples were reverse transcribed (RT) using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer instructions. Briefly, 1μg of DNase treated RNA was mixed with 1μL of oligo$(dT)_{20}$ primer (50 μM) and 1μ**L** dNTP mix (10 mM) and completed up to 10μL with DNase/RNase free water. Samples were heated at 65ºC for 5 min and subsequently cooled on ice for 1min. After that, 10μL of 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 50ºC for 50 min followed by heating at 85ºC during 5min and chilled on ice. To remove RNA template from cDNA 2μL RNase H was added to the mixture given a final volume o 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 ºC until qPCR assays.

Quantification of cytokine mRNA expression by qPCR

The relative gene expression was assayed by real time PCR (qPCR) using the iCycler iQ5 RT-PCR system (Bio-Rad) using standard conditions. All samples were analyzed in

triplicates. Each reaction contained 12.5 µL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), 8 µL of H_2O, 1 µL of forward primer (20 µM), 1 µL of reverse primer (20 µM) and 2,5 µL of cDNA. Custom-made specific primers and internal controls for all targets were designed using the online program Primer3Imput (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 genes) 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 ºC to 95 ºC with a 0.1ºC/s ramp rate to verify product specificity. Changes in cytokine gene expression were calculated by relative quantitation using the $\Delta\Delta C_T$ method (26), where $\Delta\Delta C_T$ = iPPVO (cytokine gene$^{\Delta CT}$ - housekeeping gene$^{\Delta CT}$) – Control (cytokine gene$^{\Delta CT}$ - housekeeping gene$^{\Delta C}$). Treatment-induced changes in cytokine gene expression for each individual sample were calculated by $2^{-\Delta\Delta CT}$. Results are expressed as the mean ± Standard error of the mean (SEM) fold-change in cytokine gene expression from iPPVO group over control group. To determine the efficiency of qPCR assays, 2-fold serial dilutions of cDNA samples were used, with the threshold cycle (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+$10^{(-1/\text{slope})}$). Mean ± SEM was calculated for each group and results were expressed 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. For ensure specificity of iPPVO effect, control groups included mice inoculated i.p. with iBoHV, iVACV and ultracentrifuged supernatant of mock-infected OFTu cells. The cytokines related to innate immune response (IL-1β, IL-12, TNF-α, IFN-γ and IL-10) were assayed using a mouse cytokine enzyme immunoassay kit (BD Biosciences, San Diego, CA). Briefly, 100 µL of 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; q.s. to 1L; pH to 6.5), coated at ELISA plates (Nunc, USA), incubated overnight at 4 ºC and then washed 3 times (PBS with 0.05% Tween-20, freshly prepared). Plates were blocked with 200 µL/well (PBS with 10% FBS, pH 7.0) and incubated at room temperature (RT) 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 µL of serum sample and standard curve dilutions for each cytokine were added in duplicates to plates and incubated at RT for 2 h. Plates were washed 5 times, blotted to remove any residual content and 100 µL of working detector solution (detection antibody + streptavidin-HRP reagent) was added to the wells and incubated for 1 h at RT. Wells were aspirated, plates were washed again (7 x) and added of 100 µL of substrate solution (tetramethylbenzidine [TMB] and hydrogen peroxide) incubated for 30 min at room temperature in a dark chamber. Finally, the reaction was stopped adding 50 µL of stop solution and the absorbance was measured at 450nm. Absorbance was transformed to cytokine concentrations (pg/mL) using the detection limits assessed from standard curve for each cytokine. Results are expressed as pg/ml represented by group means values (mean ± SEM). Results were submitted to statistical analysis by Shapiro–Wilk test to verify normality, followed by Student's *t* test to compare mean values between groups. The statistical significance

was accepted as p value < 0.05.

Type I interferon (IFN-I) assays

Serum samples obtained at different times post-inoculation (6, 12, 24 hpi) from mice inoculated with iPPVO or control (inactivated bovine herpesvirus 1 /iBoHV-1 and vaccinia virus/iVACV) was assayed for IFN-I activity against murine encephalomyocarditis virus (EMCV) according to previous study (27). An interferon standard (IFN-β^r) was included in all assays to monitor cell condition and reproducibility

Confluent L_{929} cells (2 x10^6 cell/mL) grown in 96 well plates were incubated with two-fold dilutions of mouse sera for 6 h. Then, plates were drained, washed three times with MEM, inoculated with 100 TCID of EMCV and incubated for 2 h at 37 ºC. The inoculum was removed, cells were washed three times and incubated with culture medium containing 2% FCS during 48 h and monitored for EMCV cytopathic effect. The interferon activity was expressed as the reciprocal of the serum dilution that produced an inhibition of cytopathic effect in 50% of the cell monolayers. Results were expressed as $\log_2$ geometric mean titers.

The antiviral activity of IFN-I was also determined by plaque reduction assay (28) comparing both groups (iPPVO x control). Briefly, L_{929} cells were seeded at 6-wells plates at a density of 1x10^6 per well at the day before the experiment. On the following day, the medium was removed, cells were washed three times with medium and incubated with duplicates of mouse serum diluted 1:10 (MEM) during 6 h. In addition to mock-controls, monolayers were inoculated with sera of mice inoculated with iBoHV-1 and iVACV. Then, cells were drained, washed three times and inoculated with 100 TCID of EMCV. After 2 h of adsorption at 37 ºC, cultures were washed and overlaid with MEM supplemented with 2% of FCS and incubated for 48 h at 37 ºC and 5% CO_2. After that, monolayers were fixed with formalin 10% and stained with crystal violet (0.3%) for 2 h. The percentage of 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 were expressed as the percentage of plaque reduction and showed as mean ± SEM.

Results

Standardization of PCR conditions by conventional PCR

Initially, a conventional PCR was performed to verify the specificity and functionality of the primers and to optimize the PCR conditions. For this purpose, iPPVO and control DNA samples were used. PCR settings were considered satisfactory when a single well-defined band was observed after electrophoresis in 1% agarose gel. Based on the efficiency of amplification and variability results, GAPDH was selected as the housekeeping, control gene for qPCR. During the whole 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 *R2* value of ≥0.98 (not shown).

Cytokine mRNA expression upon iPPVO stimulation

The standardized qPCR was then used to measure the expression of cytokine mRNAs in total RNA extracted from spleen of mice inoculated with iPPVO. qPCR was performed in total RNA extracted from spleens collected from mice at different times post-inoculation. For this purpose, cytokines from the pro-inflammatory route (IL-1β, 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 group of cytokines (IL-1β, 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 supernatant of iPPVO ultracentrifugation tested at the same intervals.

Increased expression of pro-inflammatory cytokines (mRNA) was first detected for IL-8 at 12 hpi, with a four-fold increase over the controls (Figure 1A). At this time,

expression of TNF-α and IL-1β mRNA remained unaltered. At 24 hpi, all three mRNAs were increased (>five-fold increase for Il-1β, 2-fold for TNF-α and 4-fold for IL-8, Figure 1A). High IL-1β expression was detected up to 96hpi, with a progressive reduction in magnitude of expression observed over time (> five-fold at 24hpi to <three-fold at 96 hpi). Expression of TNF-α remained within the range of 2 to 3-fold increase over the period. IL-8 expression remained high along 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 and IFN-γ and IL-12 mRNA was first detected at 24 hpi, with three - and <six-fold increase, respectively. IL-12 expression showed a slight reduction by 48 hpi, but remained above the control values (two to four-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.

The levels of mRNA of regulatory, Th-2 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 95 hpi (4.5-fold). The two mRNAs were below detection limits at 120 hpi.

Taken together, these results show that iPPVO stimulation results in a time-dependent, transient and auto-regulatory 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 pro-inflammatory and Th1 cytokines showed a peak in expression at 24 and 48 hpi, whereas Th-2 regulatory cytokines peaked at 72 h and 96hpi. Expression of all tested cytokines returned to stead state at 120 hpi.

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 by the mean absorbance for each set of duplicates standards subtracted the mean zero standard absorbance. The standard curve OD values were plotted on a log-log graph to determine cytokines concentrations over samples absorbance. Figures 1B, 2B and 3B present the levels of the respective groups of cytokines in sera at different intervals after iPPVO inoculation. A marked increase in cytokine levels were detected in iPPVO treated animals compared to control/placebo groups for all cytokines assayed, with the profile varying according to the respective cytokine. Among pro-inflammatory cytokines, levels of IL-1β were high from 24 to 72 hpi, presenting a progressive reduction towards near basal levels at 96 hpi. TNF-α levels also remained increased, with peaks at 24 and 96 hpi, respectively. Among Th1-related cytokines, a peak in 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 all over the 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, with a late peak (96 hpi). Consistent with mRNA findings, measurement of IL-1β, 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 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.

Interferon type I induction

The production of type I IFN by iPPVO-treated mice was assayed by investigating IFN-I activity in a biological assay against EMCV (28-29). Inhibition of EMCV replication

was detected in sera collected 6 to 24 h after iPPVO inoculation, with a peak on antiviral activity observed between 6 and 12 hpi. At 24 hpi, sera from 2 of 6 animals exhibited antiviral effect. Murine internal laboratory standard interferon (IFN^{r}) provided an intra-assay and inter-assay control during the experiment. No antiviral activity was detected in sera collected from mice inoculated with MEM, supernatant from iPPVO ultracentrifugation, iBoHV-1 or iVACV at any time assayed. Thus, the inhibitory effect of EMCV replication seems to be specific of iPPVO stimulation. To better characterize the inhibitory IFN activity on EMCV replication, a plaque reduction assay (PRA) 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 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 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 demonstrate 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 pro-inflammatory and Th1 cytokines were 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 extent previous results from *in vitro*

studies, demonstrating a broad stimulatory effect on pro-inflammatory, Th1-and Th2-related cytokines. These effects would likely contribute for 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 towards 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 pro-inflammatory, Th1- and Th-2 related cytokines (30). This complex cytokine response is also associated with activation of several cell populations, including monocytes and Th1-like cells, human neutrophils, canine monocytes, murine bone-marrow derived DCs, among others (13,15-18,30-31). 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 burst, serum bactericidal activity and IFN-α/β production (Anziliero A, submitted). 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, submitted) and demonstrated a prompt and transient IFN-I response, peaking at 6 hpi and remaining up to 24 hpi. Previous studies have demonstrated that PBMC and DCs exposed to iPPVO *in vitro* produced IFN-I in the range of 6 and 24 hpi (17,19,31). Also confirming earlier *in vitro* studies (13,18, Anziliero A, submitted), 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 pro-inflammatory cytokines was a consistent finding in the present study (Figure 1). Increased IL-8 mRNA expression was detected as early as at 12 hpi, followed by increased expression of IL-1β and TNF-α. In fact, iPPVO immune-modulation *in vitro* has been associated with production of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) by monocytes or antigen presenting cells (APCs) (30). Mouse bone-marrow derived plasmacytoid cells (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 (30). Thus, an early induction of pro-inflammatory cytokines in immune cells appears to be a common effect of iPPVO stimulation both *in vivo* and *in vitro*.

Cytokines TNF-α, IL-1β and IL-8 mediate the initial response of innate immune system to a challenge, infection or injury (32-33). TNF and IL-1β activate endothelial cells attracting polymorphonuclear cells and monocytes to the site of inflammation enhancing their movement throw the blood. IL-8 is a chemokine, acting as a monocyte chemoattractant, and later is responsible for IL-10 synthesis (32-33). Antigen presenting cells secrete IL-1β 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 this pro-inflammatory 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). By 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 Th-1 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 Th-1-type immune response by iPPVO has been detected in different species and has been suggested that this response is elicited by the viral particles themselves (11,15,31). These effects have also been demonstrated *in vivo*, as 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 h post-induction (19). Taken together, these results suggest that IFN-γ synthesis by T lymphocytes and/or NK cells, thus directing a Th-1 type response, plays a pivotal role in iPPVO immunostimulatory activity (30).

The inflammatory and Th-1-response observed at early times after iPPVO stimulation are usually limited by subsequent up regulation of regulatory and Th-2 cytokines, namely IL-1RA and IL-10, followed by IL-4 (18,29). The late up regulation of IL-1R (natural antagonist of IL-1beta), IL-10 (regulatory cytokine) and IL-4 (Th2 cytokine, Th-1 cytokine antagonist) in peripheral blood mononuclear cells (PBMC) 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 Th-2 and regulatory cytokines (IL-10, IL-4) were detected at late times, noticeably at 48-72 hpi (IL-10) and 72 hpi (IL-4) (Figure 3).

In summary, our results confirm and extend previous findings that iPPVO exerts a strong effect on cytokine expression by immune cells, leading to an initial induction of pro-inflammatory and Th1-related cytokines followed by a Th2 and regulatory cytokine response. Understanding the regulatory mechanisms and effects are pivotal towards the manipulation of the immune response and correct use of iPPVO as an immunostimulant/therapeutic in

difficult-to-treat human and animal infectious diseases. In addition, the present study illustrates the usefulness of using a combination of molecular, immunoassays and biological assays to measure cytokine response.

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

Target	Primer sequence	Product size (bp)
GAPDH	F: CAGCCTCGTCCCGTAGACAA	178
	R: ACCCCGTCTCCGGAGTCCATCACAAT	
B-Actin	F: AGGCCAACCGTGAAAAGATGACCCAGAT	139
	R: GTAGACCATGTAGTTGAGGTCAATGAA	
IL-1β	F : TGGCCTTCAAAGGAAAGAATCTATACCTGTCC	179
	R: GTTGGGGAACTCTGCAGACTCAAACTCCAC	
IL8	F: GACGCCCCCAGGACCCCACTG	162
	R: AGCCCAGGCTCCTCCTTTCCAGGTC	
TNF-α	F: CAGGCCTTCCTACCTTCAGACCTTTCCAGAT	166
	R: ACACCCCGCCCTTCCAAATAAATACATTCAT	
IL-12_{p40}	F: TGCCCCCACAGAAGACGTCTTTGATGAT	158
	R: GATGGCCACCAGCATGCCCTTGTC	
INF-γ	F: GCCAAGACTGTGATTGCGGGGTTGTATCT	178
	R: TAAAGCGCTGGCCCGGAGTGTAGACA	
IL-10	F: AGCTGCAGGGCCCTTTGCTATGGTG	180
	R: GATGAAGCGGCTGGGGGATGACAGTA	
IL-4	F: CACTGAGAATGAAAGGCCCCAAAGTCTTGA	168
	R: AGCCGGGAGGACAGATCTCTGGTGAAG	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; β-Actin: Beta-actin; IL-1: interleikin-10; IL-10: interleukin 10; IL-12p40: interleukin 12, subunit p40; IL-8 interleukin 8, IL-4: interleukin 4; IFN-γ**:** interferon Gama; TNF-α: Tumor Necrosis Factor-Alpha; F and R stand for forward and reverse primer, respectively, 5'-

3’orientation; bp: base pairs

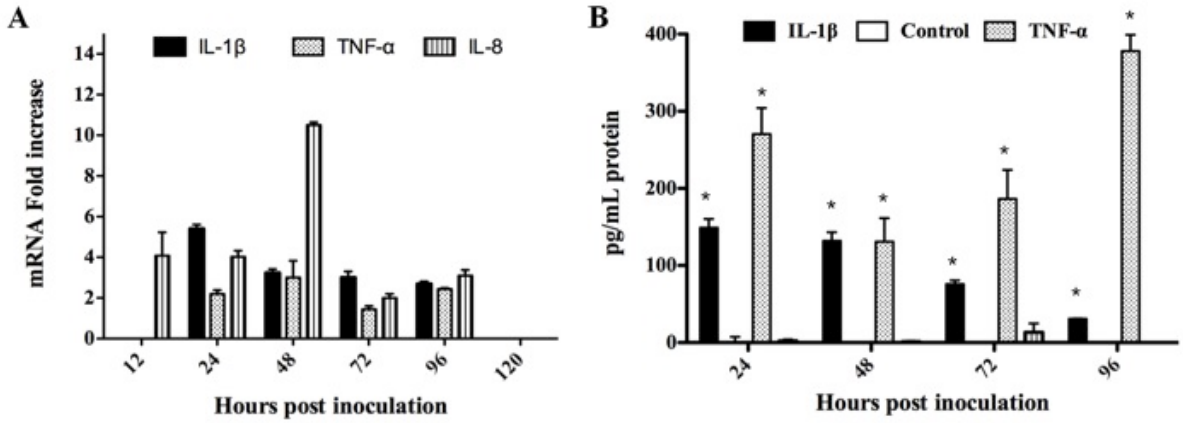

Fig.1. Pro-inflammatory cytokines measured by qPCR in mice spleen (A) and by ELISA in serum samples (B) at different time points after iPPVO inoculation. Results are expressed by mean ± SEM as times-fold increase over the control group (qPCR) and as pg/mL of serum protein in ELISA; ** P≤0.01. IL-1β mRNA was measured up to 120 hpi.

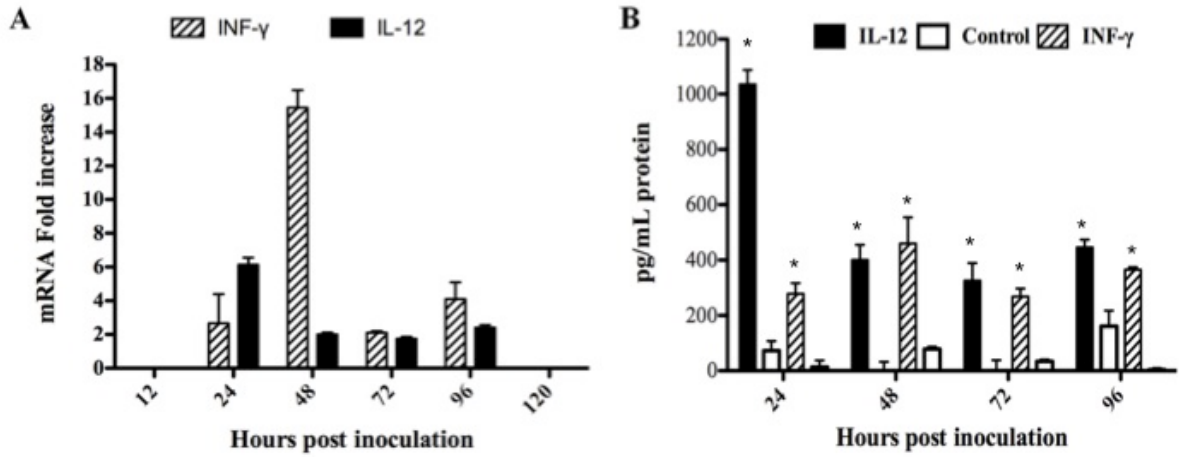

Fig.2. Th1 related cytokines in the spleen of mice measured by qPCR (A) and serum levels of protein assayed by ELISA (B) at different time points after iPPVO treatment. Results are expressed by mean ± SEM as times-fold increase over the control group (qPCR) and as pg/mL of serum protein in ELISA; ** $P \leq 0.01$.

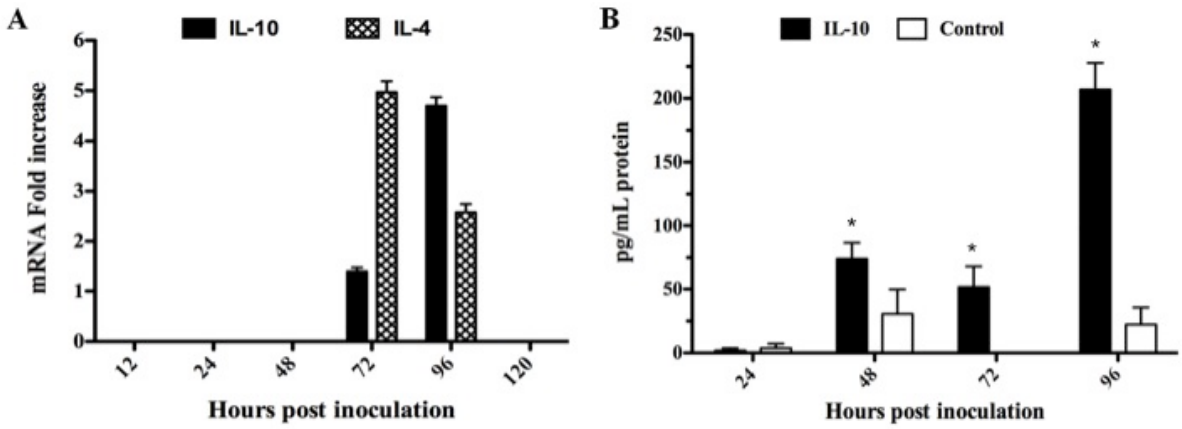

Fig.3. Quantification of mRNA Th2 cytokines in spleen of mice inoculated with iPPVO at different intervals. Results are expressed by mean ± SEM as times-fold increase over the control group (A); IL-10 mRNA was measured up to 120 hpi. Panel B demonstrates the levels of IL-10 in the sera of mice after inoculation of iPPVO, measured by ELISA. Results are expressed by mean ± SEM as pg/mL of serum protein; ** $P \leq 0.01$.

CONCLUSÕES

Embora substâncias ou formulações imunoestimulantes venham sendo estudadas há décadas, a utilização de vírus como imunomoduladores representa uma exceção na Medicina Veterinária. Neste aspecto, desde os estudos iniciais o iPPVO demonstrou propriedades imunomoduladoras em diferentes modelos animais, com resultados promissores tanto *in vitro* quanto *in vivo*. No entanto, o otimismo e as perspectivas de seu uso na prática clínica foram gradativamente caindo em descrédito, sobretudo pela escassez de estudos científicos bem delineados que comprovassem seus efeitos imunomoduladores. Nesse sentido, os resultados obtidos contribuem para o entendimento dos efeitos imunoestimulantes promovidos pelo iPPVO sobre diferentes eventos da resposta imune inata. Se considerados individualmente, provavelmente esses efeitos não são suficientes para explicar o aumento da magnitude da resposta imune frente a diferentes patógenos observada em estudos clínicos.

A estimulação tanto celular quanto humoral promovida pelo iPPVO parece atuar de forma conjunta e orquestrada. No presente trabalho, foi possível observar que o iPPVO está envolvido nas etapas iniciais da resposta imune inata, relacionadas a ativação de macrófagos, incrementando a capacidade fagocítica dos fagócitos tanto *in vitro* quanto *in vivo*. Observou-se também uma marcada ativação de fagócitos circulantes, basicamente neutrófilos, por meio da atividade microbicida, representada pela geração superóxidos reativos ao oxigênio.

Com relação a resposta humoral inespecífica, foi possível observar que o iPPVO ativa os mecanismos responsáveis pela atividade bactericida do soro, o que poderia explicar parte dos resultados obtidos *in vivo* por meio de estudos clínicos de resistência a diferentes agentes bacterianos.

O papel exercido pelo iPPVO sob o perfil cinético das citocinas envolvidas da imunoestimulação foi determinado pela mensuração da expressão gênica e dos níveis séricos. Os resultados desses ensaios demonstram que o iPPVO atua como modulador do sistema imunológico do hospedeiro, atuando incialmente na potencialização da resposta inflamatória por meio da indução de um conjunto de citocinas pró-inflamatórias (IL-1β, TNF-α e IL-8). O presente estudo, corrobora trabalhos prévios que sugerem que o tipo de resposta induzida pelo iPPVO está predominantemente relacionado resposta do tipo Th1. Neste sentido, a magnitude da resposta observada na indução de INF-γ e IL-12 confirma esta hipótese e, provavelmente esteja relacionada com a potencialização da resposta imune celular observada pela ativação de células apresentadoras de antígenos (APC), na fagocitose e secreção de outras citocinas. Também foi possível observar que apesar da forte resposta inflamatória, observou-se que, após a imunoestimulação, inicia-se um processo de auto-regulação, representado pela produção de citocinas anti-inflamatórias do tipo Th2 como a IL-10 e IL-4, em resposta tardia ao tratamento. Por fim, foi possível demonstrar que o iPPVO está envolvido em mecanismos de resistência antiviral, observado pela indução de interferon tipo I poucas horas após a estimulação.

Em resumo, os resultados deste trabalho demonstram que o iPPVO é capaz de ativar um complexo mecanismo de atuação na resposta imune inata do hospedeiro, que provavelmente está envolvido no aumento de resistência a patógenos. A determinação exata dos mecanismos e como manipulá-los se constituem em desafios notáveis no sentido de utilizar o iPPVO como imunoestimulante na prática clínica.

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