



HAL
open science

Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes

Nicole Schütze, Rüdiger Raue, Mathias Büttner, Gottfried Alber

► **To cite this version:**

Nicole Schütze, Rüdiger Raue, Mathias Büttner, Gottfried Alber. Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes. *Veterinary Microbiology*, 2009, 137 (3-4), pp.260. 10.1016/j.vetmic.2009.01.035 . hal-00485542

HAL Id: hal-00485542

<https://hal.science/hal-00485542>

Submitted on 21 May 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes

Authors: Nicole Schütze, Rüdiger Raue, Mathias Büttner, Gottfried Alber



PII: S0378-1135(09)00056-X
DOI: doi:10.1016/j.vetmic.2009.01.035
Reference: VETMIC 4350

To appear in: *VETMIC*

Received date: 7-10-2008
Revised date: 14-1-2009
Accepted date: 21-1-2009

Please cite this article as: Schütze, N., Raue, R., Büttner, M., Alber, G., Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.01.035

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes

Nicole Schütze^{1,2}, Rüdiger Raue³, Mathias Büttner⁴, and Gottfried Alber¹

5

¹Institute of Immunology, College of Veterinary Medicine, University of Leipzig, Germany

²present address: Helmholtz University Young Investigators Group LIPAD, Centre for Environmental Research, Leipzig and Department of Dermatology, Venerology and Allergology, Leipzig University Medical Center, Leipzig, Germany

10 ³Pfizer Animal Health, VMR&D, Biologicals Development, Sandwich, Kent, U.K.

⁴Bavarian Health and Food Safety Authority, Oberschleissheim, Germany

Address correspondence and reprint requests to Dr. Gottfried Alber, Institute of

15 Immunology, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 11, 04103 Leipzig, Germany.

E-mail address: alber@rz.uni-leipzig.de

phone number: (+49) 341-9738328, fax number: (+49) 341-9738147

20

Abstract

Inactivated parapoxvirus ovis (iPPVO) shows strong immunomodulatory activities in several species and is used in veterinary medicine as an immunostimulatory biological for the prevention and/or treatment of infectious diseases. In this study the immunostimulatory capacity of iPPVO on the innate immune system was investigated *in vitro* by the evaluation of induction of the oxidative burst and modulation of phagocytosis by canine blood leukocytes (polymorphonuclear cells and monocytes) of dogs. In addition, the activation of canine T lymphocytes was also studied. After stimulation with iPPVO the phagocytosis of FITC-labeled *Listeria monocytogenes* was increased in canine blood monocytes and neutrophils. Enhanced burst rates by canine monocytes stimulated with iPPVO were observed and the MHC-II expression on canine CD14⁺ monocytes was elevated following stimulation with iPPVO compared to the stabiliser control. Canine CD4⁺ T cells were activated for oligoclonal proliferation in response to iPPVO. This study shows that iPPVO is able to stimulate both phagocytotic and T-cell-dependent immune mechanisms in canine blood leukocytes.

35

keywords: cell activation, phagocytosis, oxidative burst, monocyte/macrophages, virus

40 Introduction

The family of Poxviridae contains the largest known DNA viruses. Due to their complex genetic structure poxviruses possess various mechanisms for immune evasion by utilizing a variety of genes encoding virulence proteins that counteract the host immune response.

45 Otherwise attenuated poxviruses have immunogenic properties and induce strong innate host immune response (Burgers et al., 2008; Gherardi and Esteban, 2005), probably due to facilitated cellular uptake by macropinocytosis (Mercer and Helenius, 2008). Parapoxvirus ovis (PPVO) shows a restricted host range for sheep and goats with a tropism to the skin and mucous membranes and lack of systemic virus spread (Büttner and Rziha, 2002; McKeever et

50 al., 1988). The virus provides proteins that are viral variants of soluble host immune factors such as an inhibitor of ovine GM-CSF and IL-2 (GIF), viral IL-10, that is very similar to ovine IL-10 and others such as viral vascular endothelial growth factor (VEGF) (Deane et al., 2000; Imlach et al., 2002; Lateef et al., 2003; Scagliarini et al., 2006). The stringent host specificity of parapoxvirus ovis results from capture of host-derived protective

55 immunomodulatory factors (Haig and Fleming, 1999; Haig and McInnes, 2002). For an effective immune response against PPVO, CD4⁺ T-cells and a humoral immune response seem to be relevant. However, the duration of acquired PPVO-specific immunity is transient commonly resulting in re-infection of the host (Haig and Mercer, 1998; Lloyd et al., 2000).

The known immunostimulating properties on the innate immune system combined with a

60 weak and transient immune response to the virus makes PPVO an optimal candidate for an immunoactivator. These characteristics are even preserved after inactivation. The immunostimulant Zylexis[®] is based on the chemically inactivated PPVO (iPPVO) strain D1701 originally isolated from a diseased lamb (Mayr et al., 1981). Before its use as an immunostimulant the strain D1701 had been attenuated by serial cell culture passages which

65 led to substantial genomic changes (Cottone et al., 1998). The attenuated and genetically

stable strain D1701 was successfully utilized for the aid in the prevention and treatment of infectious diseases in non-permissive species, especially in several herpes virus infection models (Castrucci et al., 2000; Weber et al., 2003; Ziebell et al., 1997). A major anti-viral effect may result from the strong interferon (IFN)-inducing capacity of PPVO (Büttner et al., 70 1995).

In this study the immunomodulatory effects of iPPVO were investigated on selected mechanisms of the innate immune response by canine leukocytes. The internalisation and degradation of pathogens in phagocytotic cells form a first barrier of the immune system against pathogens (Serbina et al., 2008; Egan et al., 2008). The engulfment of pathogens by 75 phagocytosis as well as the production of bactericidal active reactive oxygen intermediates (ROI) is primarily achieved by monocytes/macrophages and neutrophils. The latter process is described as oxidative burst because the responsible enzyme complex NADPH oxidase assembles in activated phagocytes and increases consumption of oxygen (Roberts and Camacho, 1967). The impact of iPPVO during early immune responses such as phagocytosis 80 and oxidative burst has not completely been characterized particularly in dogs.

Following uptake and destruction of pathogens by antigen-presenting cells (APC) the pathogens are processed to antigenic peptides and bound to major histocompatibility complex (MHC) molecules for presentation to T cells. In the process the MHC molecules are up-regulated. For MHC-II molecules the class II transactivator (CIITA) is the master regulator of 85 MHC-II transcription that modulates the expression of MHC-II genes (Drozina et al., 2005). In this context it was reported that in PPVO-infected sheep an accumulation of MHC-II+ dendritic cells in skin lesions was found (Haig et al., 1997).

Consequently, the activation of T-cells by iPPVO was a further topic of this investigation. In general, naive T cells that recognize pathogen-derived antigenic peptides/MCH complexes 90 with their T cell receptor become activated and start to proliferate. Following infection with PPVO in sheep, lymphoproliferation in draining lymph nodes and also in efferent lymph was

found some days later (Yirrell et al., 1991). In a non-permissive host system such as swine *in vitro* T cell proliferation following stimulation with iPPVO was also observed. In this system the predominant proliferation of T helper cells was induced by iPPVO functioning in a superantigen-like manner independent of antigen processing by APC, demonstrated by a
95 general inhibition of proliferation by an inhibitory anti-MHC II antibody (Fachinger et al., 2000a).

In this report we demonstrate by several *in vitro* assays for the first time that iPPVO enhances phagocytosis and oxidative burst in canine neutrophils and/or monocytes as well as induces
100 the up-regulation of MHC-II and the proliferation of T helper cells.

Materials/ Methods

105 Reagents

Lipopolysaccharide (LPS) from *Salmonella* Abortus-equi (Alexis[®], Gruenberg, Germany); phorbol 12-myristate 13-acetate (PMA, Alexis[®], Gruenberg, Germany); dihydrorhodamine 123 (Invitrogen, Karlsruhe, Germany), fluorescein isothiocyanate (FITC) and cytochalasin D (SIGMA-Aldrich, Taufkirchen, Germany) were purchased. Bacteria *Listeria monocytogenes* strain EGD were heat-inactivated (hi) at 60 °C for 1 h and 5×10^8 colony forming units (cfu),
110 incubated with 0.5 mg/ml FITC for 1 h at 37 °C for labelling and afterwards washed 3 times with PBS.

Virus

115 PPVO was chemical inactivated (iPPVO). Pharmaceutical formulation of commercial available iPPVO (Zylexis[®]) for dogs and cats with at least 230 IFN units of iPPVO strain D1701 per dose, the stabilizer control (polygeline) as well as purified iPPVO (pu iPPVO;

without stabilizer) were supplied by Pfizer Animal Health, UK. One dose of pharmaceutical formulation (in the following declared as iPPVO) contains 1×10^7 TCID₅₀ of PPVO (pre-
120 inactivation) and was resuspended in 1 ml of PBS or medium. For most experiments a 2-fold dilution series of one dosis was used starting from 1:4 up to 1:32 (2.5×10^6 , 1.25×10^6 , 0.63×10^6 and 0.31×10^6 TCID₅₀/ml).

Animals/blood

125 Blood was obtained from healthy dogs (breeds: beagles, n = 8; hybride, n = 1) by venepuncture of the anterior cephalic vein into heparinised vacutainer tubes (4 µg/ml lithium-heparin; Kalbe-Labortechnik, Nümbrecht-Elsenroth). Dogs belonged to the College of Veterinary Medicine, University of Leipzig. All procedures were performed in accordance with local legislations and animal welfare legislations.

130

Phagocytosis assay

Heparinized blood (150 µl/sample) was stimulated with iPPVO or stabiliser control, LPS (5 µg/ml), or PBS for 20 min at 37 °C in a waterbath. For specific inhibition of phagocytosis Cytochalasin D (5 µg/ml) was co-incubated. 100 µl of FITC-labelled *L. monocytogenes* was
135 added at a multiplicity of infection (MOI) of 10 relative to leukocytes (microscopically counted in Tuerk's reagent in a Neubauer counting chamber) and incubated for a further 15 min at 37 °C. Phagocytosis was stopped by chilling samples on ice for 5 min. For lysis of erythrocytes, blood samples were incubated with lysis buffer (150 mM NH₄Cl, 8 mM KHCO₃, 1 mM EDTA; pH 7.0) for 10 min. The lysis was stopped by adding PBS (4 °C), and
140 cells were washed twice (4 °C). Cells were fixed with 1 % v/v formaldehyde in PBS and measured within 60 min by flow cytometry (FACS Calibur, Beckton-Dickinson, Heidelberg, Germany). Extracellular FITC-labeled bacteria were quenched by the addition of 1 mg/ml trypan blue before measurement. Polymorphonuclear cells (PMN) and monocytes were gated

by forward/sideward scatter (FSC/SSC) and analysed separately. The phagocytosis rate is the
145 percentage of phagocytic active cells within one cell population (PMN or monocytes). Since
phagocytosis rates of PBS controls differed between experiments, net phagocytosis rates were
used to normalize all experiments ($\Delta PR = PR_{\text{treatment}} - PR_{\text{PBS}}$).

Oxidative burst

150 Blood samples were stimulated with diluted iPPVO or stabiliser, LPS (5 $\mu\text{g/ml}$) or PMA
(185 ng/ml) for 15 min at 37 °C in a water bath. The oxidative burst activity of the leucocytes
was assessed by adding the respiratory burst-sensitive dye dihydrorhodamine 123 (DHR,
8.9 $\mu\text{g/ml}$) and incubating for 10 min at 37 °C (Rothe et al., 1988). Subsequently lysis of
erythrocytes was carried out and cells were washed with PBS twice at 4 °C. The samples were
155 measured by flow cytometry within 30 min. PMN and monocytes were gated by FSC/SSC
and analysed separately. The burst rate equates to the percentage of oxidative burst active
cells within one cell population (PMN or monocytes).

MHC-II expression

160 Peripheral blood leucocytes (PBL) were purified from heparinised blood by density
centrifugation (1.077 g/ml ; Histopaque, SIGMA-Aldrich). Cells (7×10^5 per well) were
seeded out in 24 well, flat bottom plates (TTP, Midsci; St. Louis, MO, USA) and stimulated
for 48 h with iPPVO (1×10^6 TCID₅₀/ml) or pu iPPVO in RPMI medium supplemented with
10 % FCS, 2 μM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin. In an analogous
165 manner, cells were stimulated with the same dilutions of stabiliser control or cultivated in
medium. After 48 h cells were harvested with a cell scraper (TPP, Midsci; St. Louis, MO,
USA). Before staining for FACS analysis cells were treated with a mixture of rat/mouse/dog
normal serum (5 % of each in PBS) to block Fc receptors. To define the monocyte population,
cells were stained with anti-human-CD14 Alexa Fluor[®] 647 antibody (found to be cross-

170 reactive with dog CD14 by the manufactures; Serotec, Düsseldorf, Germany). The cells were
counterstained with anti-canine-MHC-II FITC, clone YKIX-334-2 (Cobbold and Metcalfe,
1994) or rat-IgG2a FITC isotype control antibody (Serotec, Düsseldorf, Germany).

CFSE proliferation assay

175 PBL were purified from heparinized blood by density centrifugation using Histopaque®
(Sigma Aldrich). Cells (1×10^7 /ml 0.1 % BSA in PBS) were stained with 5 μ M carboxy
fluorescein diacetate succinimidyl ester (CFSE) at 37 °C in darkness for 10 min. Staining was
stopped by adding the same volume of FCS for 3 min. The cells were washed three times with
RPMI. Stained cells (1×10^5 per well) were seeded into a 96-well round-bottom plate (TTP,
180 Midsci; St. Louis, MO, USA) and stimulated with diluted iPPVO or stabiliser control. As
further controls medium and concanavalin A (ConA, 5 μ g/ml) were used. Post cultivation for
7 d at 37 °C and 5 % CO₂, cells were harvested and stained with anti-canine-CD4 PE and
-CD8 Fluor® 647 antibodies or rat-IgG2a PE and rat-IgG1 Fluor® 647 antibodies as isotype
controls, respectively. Cells were measured by FACS (Calibur®, BD, Heidelberg, Germany).
185 All FACS analyses were performed with the CellquestPro software.

Statistical analysis

Statistical analysis of parametric data (Gaussian distribution) was performed by ANOVA.
Bonferroni's multiple comparison test was used for post test to compare groups of interest. To
190 analyse separate dilutions of iPPVO in oxidative burst assay paired t-tests was used for post
test.

Results

IPPVO enhances the phagocytotic activity of canine monocytes and PMN

We investigated modulation of phagocytotic activity in canine leucocytes by iPPVO. Canine
200 peripheral blood leucocytes, both monocytes and PMN showed a noticeable increase of
phagocytotic activity after stimulation with iPPVO (Fig. 1A). As demonstrated by a dilution
series of iPPVO, there is a dose-dependent increase of the phagocytosis rate. In comparison to
LPS, iPPVO raised phagocytosis rate significantly (** $P < 0.01$; *** $P < 0.001$).

Cytochalasin D, a potent inhibitor of actin polymerisation, which results in inhibition of
205 phagocytosis, was used as an additional control. IPPVO-stimulated phagocytotic activity of
canine leukocytes was found to be strongly reduced in the presence of cytochalasin D, to
levels equivalent to those of the PBS control (Fig. 1B).

iPPVO induces oxidative burst in canine monocytes

210 To study the ability of iPPVO to induce oxidative burst in canine leukocytes, we stimulated
blood samples from several dogs with iPPVO in comparison to stabiliser and PBS control. For
detection of ROIs we utilized the oxidative burst sensitive substrate dihydrorhodamine123.
Canine monocytes showed dose-dependent moderate, but statistically significant (** $P < 0.01$,
*** $P < 0.001$, * $P < 0.05$) increase of burst rates following stimulation with iPPVO in comparison to
215 accordant stabiliser control (Fig. 2). In contrast, PMN were not activated for oxidative burst by
iPPVO, whereas positive controls, LPS and PMA, clearly induced the oxidative burst in both
cell populations, monocytes and PMN.

IPPVO induces MHC-II up-regulation on canine monocytes

220 MHC-II is up-regulated during activation of antigen presenting cells. In order to investigate
the change of MHC-II molecules expression level in response to iPPVO canine monocytes

were stimulated with pu iPPVO or the pharmaceutical formulation of iPPVO and compared to medium or stabiliser control. The MHC-II expression increased on iPPVO-stimulated cells in comparison to stabiliser control, as demonstrated in the histogram (Fig. 3A). The same result
225 was found for pu iPPVO (without the stabiliser polygeline) stimulated cells compared to medium cultivated cells. Analyses of geometric means of fluorescence intensity (MFI) for MHC-II expression demonstrates a statistically significant difference between stimuli pu iPPVO vs. medium and iPPVO vs. stabiliser control (** $P < 0.001$, Fig. 3B).

230 *iPPVO enhances proliferation of canine CD4+ T cells*

Since T cell proliferation was described in permissive and non-permissive hosts (Yirrell et al., 1991; Fachinger et al., 2000a) we were interested in the ability of iPPVO to stimulate innate lymphocyte response and addressed this question for canine T cells by a proliferation assay with CFSE-labeled PBL. Profound T cell proliferative activity following stimulation with
235 iPPVO was found in the CD4+ T cell fraction. This activity was substantially increased compared to stabiliser or medium control and showed dose dependency (ANOVA $P < 0.001$ and Bonferroni's multiple comparison test, ** $P < 0.001$, * $P < 0.05$; Fig. 4A). Surprisingly CD4/CD8 double positive (CD4+/CD8+) T cells were detectable in this proliferation assay. This cell population was found in medium, stabiliser control, and following stimulation with
240 ConA or iPPVO. The iPPVO-induced proliferation rate of CD4+/CD8+ T cells was higher than in CD4+ T cells (Figure 4A). In contrast, most cytotoxic CD8+ T cells (CD8 single positive) did not proliferate in response to iPPVO. Only PBL from two dogs, that were found also to be highly responsive in the CD4+ and CD4+/CD8+ populations, proliferated by iPPVO stimulation, but the differences to the stabiliser control were not statistically
245 significant. The analysis with CFSE demonstrated that only a portion of the total T cells divided. Most of these proliferating cells formed a cluster, i.e. most cells went through the same number of cell divisions (Fig 4B). Moreover, we also tested iPPVO for its ability to

induce proliferation of canine B cells. CD19⁺ B cells did not show any proliferation after 7 d cultivation in the presence of iPPVO (data not shown). From this results we conclude that in contrast to ConA, which induces a polyclonal expansion, iPPVO stimulates an oligoclonal proliferation of canine CD4⁺ single and CD4⁺/CD8⁺ double positive T cells, which were synchronized in their cell divisions.

255 **Discussion**

In this study it was shown that after iPPVO stimulation the phagocytotic activity was enhanced in canine monocytes and PMN. However, the induction of oxidative burst showed only a significant increase in the monocyte population. In human neutrophils enhancement of both phagocytosis and oxidative burst were demonstrated, whereas monocytes were not investigated (Förster et al., 1994). However, Fachinger *et al.* (2000b) could not find any modifying capacity by iPPVO stimulation on phagocytotic activity and oxidative burst in porcine PMN and monocytes. It has to be noted that these investigations were performed using two commercial test kits for human whole blood which were not validated for porcine blood.

The enhanced phagocytic activity of monocytes and PMN does not mean an obligatory recognition of iPPVO by these cell types, since cytokines (TNF- α , GM-CSF) or mediators (like leucotrien B₄) released by these cell types or other cell populations can enhance phagocytosis (Cross et al., 1997; Peres et al., 2007). Friebe *et al.* (2004) showed that stimulation of human peripheral mononuclear cells (PBMC) and whole blood with iPPVO and ConA induced secretion of the proinflammatory cytokines TNF- α and IL-6. However, further investigations are necessary to clarify the mechanisms of the iPPVO actions particularly in several species.

Innate and adaptive immunity are connected via antigen processing and presentation, which
275 results in the presentation of antigenic peptides to T cells on the MHC molecules. MHC
class II (MHC-II) determinants present antigens to CD4⁺ T helper cells, which are the main
regulators of the adaptive immune response. In PPOV infected sheep a dense accumulation of
MHC-II⁺ dendritic cells in the skin lesions was observed (Haig et al., 1997). Here the up-
regulation of the MHC-II expression on canine monocytes was shown by iPPVO *in vitro*.
280 IFN- γ induces MHC-II transcription via CIITA (Drozina et al., 2005). An iPPVO-induced
release of IFN- γ was shown by several groups (Fachinger et al., 2000b; Friebe et al., 2004;
Voigt et al., 2007; Weber et al., 2003). It seems possible that the observed MHC-II up-
regulation on canine monocytes is mediated by IFN- γ production induced by iPPVO. Nitric
oxide (NO) is a downstream mediator from IFN- γ (Blanchette et al., 2003). However, NO
285 release from cultivated PBL following stimulation with iPPVO was not observed (data not
shown). In this context it needs to be noted that using CD14 as a marker to define the
monocyte population does not definitely exclude dendritic cells (DC) from this cell
population. It is unclear, whether canine blood derived DC express CD14 *in vivo* as
demonstrated for *in vitro* DC differentiation. The expression of CD14 on monocyte-derived
290 DC appears to depend on differentiation factors such as Flt-3L (fms-like tyrosine kinase 3
ligand) (Ibisch et al., 2005; Wang et al., 2007; Mielcarek et al., 2007).

From depletion experiments in infected lambs it is known that CD4⁺ T-helper cells are
important for the immune response against PPVO in sheep, whereas the role of CD8⁺
cytotoxic T cells is not completely understood (Lloyd et al., 2000). We tested the
295 proliferation of PBL isolated from dogs never treated with iPPVO before to exclude a
memory response resulting from previous priming. A dose-dependent proliferation of CD4⁺
T cells but mostly not of CD8⁺ T cells was observed. This is in agreement with results from a
proliferation study with porcine PBL. In this study it was shown that T-helper cells
(CD4^{high}), expressing activation marker CD25 and MHC-II are predominant after cultivation

300 in the presence of iPPVO (Fachinger et al., 2000b). In a subsequent study this group demonstrated that iPPVO functions as a superantigen, i.e. iPPVO was not processed by APC but activated T cells by binding directly to MHC molecules (Fachinger et al., 2000a). Surprisingly we could identify a further T-cell subpopulation in 7d-cultivated PBL, i.e. CD4⁺/CD8⁺ double positive T cells. This CD4⁺/CD8⁺ T cell subpopulation, that could not
305 only be stimulated by iPPVO, but also by ConA, showed an increased proliferation activity in comparison to single CD4⁺ or CD8⁺ cells. Canine CD4⁺/CD8⁺ double positive T cells were found previously in a similar PBL cultivation system consisting of ConA together with recombinant human IL-2 (Kato et al., 2007). Human peripheral CD4⁺/CD8⁺ T cells were characterized as anti-viral effector memory cells (Nascimbeni et al., 2004). From this we
310 suggest that CD4⁺/CD8⁺ T cells are activated T cells. Use of CFSE to assess proliferation of CD4⁺ T cells allows tracing of cell divisions (Lyons and Parish, 1994) contrary to previous work, which utilised ³H-thymidine incorporation (Fachinger et al., 2000b). By our method an oligoclonal proliferation was revealed, that is characterised by synchronised cell divisions, since proliferating CD4⁺ T cells cluster at the same CFSE intensity (see Fig. 4B). Since
315 oligoclonal activation is one of the key features of superantigens (Irwin et al., 1992), it is tempting to speculate that iPPVO activates canine T cells in a superantigen-like manner. Further, another group demonstrated by stimulation with the superantigen staphylococcus enterotoxin B that reactive T cells (V β 8⁺) undergo a discrete number of cell divisions before cells go into apoptosis (Renno et al., 1999). The exact mechanism of iPPVO-induced
320 proliferation of canine CD4⁺ T cells as well as the proliferating cell population need further characterisation.

In summary, the induction of several early innate immune mechanisms as well as proliferation of CD4⁺ and CD4⁺CD8⁺ T cells by iPPVO was shown in canine cells for the first time. This broad activation profile promotes iPPVO as a potent immunoadactivator.

325 **Conclusion:** In this report we show nonspecific immunostimulatory activity of iPPVO for
 canine leukocytes, that contributes to evidence of effectiveness of iPPVO in the non-
 permissive host dog.

Acknowledgement

330 We thank J. Richter for technical assistance and I. Hochheim from the Institute of
 Pharmacology for professional blood sampling. These studies were funded by Pfizer Animal
 Health.

335 Reference List

1. Blanchette, J., Jaramillo, M., Olivier, M., 2003. Signalling events involved in interferon-
 gamma-inducible macrophage nitric oxide generation. *Immunology* 108, 513-522.
2. Burgers, W.A., Shephard, E., Monroe, J.E., Greenhalgh, T., Binder, A., Hurter, E., Van
 340 Harmelen, J.H., Williamson, C., Williamson, A.L., 2008. Construction, characterization,
 and immunogenicity of a multigene modified vaccinia Ankara (MVA) vaccine based on
 HIV type 1 subtype C. *AIDS Res. Hum. Retroviruses* 24, 195-206.
3. Büttner, M., Czerny, C.P., Lehner, K.H., Wertz, K., 1995. Interferon induction in
 peripheral blood mononuclear leukocytes of man and farm animals by poxvirus vector
 345 candidates and some poxvirus constructs. *Vet. Immunol. Immunopathol.* 46, 237-250.
4. Büttner, M. and Rziha, H.J., 2002. Parapoxviruses: from the lesion to the viral genome.
J. Vet. Med. B Infect. Dis. Vet. Public Health 49, 7-16.
5. Castrucci, G., Osburn, B.I., Frigeri, F., Ferrari, M., Salvatori, D., Lo, D.M., Barreca, F.,
 2000. The use of immunomodulators in the control of infectious bovine rhinotracheitis.
 350 *Comp Immunol. Microbiol. Infect. Dis.* 23, 163-173.
6. Cobbold, S. and Metcalfe, S., 1994. Monoclonal antibodies that define canine
 homologues of human CD antigens: summary of the First International Canine
 Leukocyte Antigen Workshop (CLAW). *Tissue Antigens* 43, 137-154.
7. Cottone, R., Büttner, M., Bauer, B., Henkel, M., Hettich, E., Rziha, H.J., 1998. Analysis
 355 of genomic rearrangement and subsequent gene deletion of the attenuated Orf virus
 strain D1701. *Virus Res.* 56, 53-67.
8. Cross, C.E., Collins, H.L., Bancroft, G.J., 1997. CR3-dependent phagocytosis by murine
 macrophages: different cytokines regulate ingestion of a defined CR3 ligand and
 complement-opsonized *Cryptococcus neoformans*. *Immunology* 91, 289-296.

- 360 9. Deane, D., McInnes, C.J., Percival, A., Wood, A., Thomson, J., Lear, A., Gilray, J.,
Fleming, S., Mercer, A., Haig, D., 2000. Orf virus encodes a novel secreted protein
inhibitor of granulocyte-macrophage colony-stimulating factor and interleukin-2. *J.*
Virol. 74, 1313-1320.
10. Drozina, G., Kohoutek, J., Jabrane-Ferrat, N., Peterlin, B.M., 2005. Expression of MHC
365 II genes. *Curr. Top. Microbiol. Immunol.* 290, 147-170.
11. Egan, C.E., Sukhumavasi, W., Bierly, A.L., Denkers, E.Y., 2008. Understanding the
multiple functions of Gr-1(+) cell subpopulations during microbial infection. *Immunol.*
Res. 40, 35-48.
12. Fachinger, V., Schlapp, T., Saalmüller, A., 2000a. Evidence for a parapox ovis virus-
370 associated superantigen. *Eur. J. Immunol.* 30, 2962-2971.
13. Fachinger, V., Schlapp, T., Strube, W., Schmeer, N., Saalmüller, A., 2000b. Poxvirus-
induced immunostimulating effects on porcine leukocytes. *J. Virol.* 74, 7943-7951.
14. Förster, R., Wolf, G., Mayr, A., 1994. Highly attenuated poxviruses induce functional
priming of neutrophils in vitro. *Arch. Virol.* 136, 219-226.
- 375 15. Friebe, A., Siegling, A., Friederichs, S., Volk, H.D., Weber, O., 2004.
Immunomodulatory effects of inactivated parapoxvirus ovis (ORF virus) on human
peripheral immune cells: induction of cytokine secretion in monocytes and Th1-like
cells. *J. Virol.* 78, 9400-9411.
16. Gherardi, M.M. and Esteban, M., 2005. Recombinant poxviruses as mucosal vaccine
380 vectors. *J. Gen. Virol.* 86, 2925-2936.
17. Haig, D.M. and Fleming, S., 1999. Immunomodulation by virulence proteins of the
parapoxvirus orf virus. *Vet. Immunol. Immunopathol.* 72, 81-86.
18. Haig, D.M., McInnes, C., Deane, D., Reid, H., Mercer, A., 1997. The immune and
inflammatory response to orf virus. *Comp Immunol. Microbiol. Infect. Dis.* 20, 197-
385 204.
19. Haig, D.M. and McInnes, C.J., 2002. Immunity and counter-immunity during infection
with the parapoxvirus orf virus. *Virus Res.* 88, 3-16.
20. Haig, D.M. and Mercer, A.A., 1998. Ovine diseases. *Orf. Vet. Res.* 29, 311-326.
21. Ibisch, C., Pradal, G., Bach, J.M., Lieubeau, B., 2005. Functional canine dendritic cells
390 can be generated in vitro from peripheral blood mononuclear cells and contain a
cytoplasmic ultrastructural marker. *J. Immunol. Methods* 298, 175-182.
22. Imlach, W., McCaughan, C.A., Mercer, A.A., Haig, D., Fleming, S.B., 2002. Orf virus-
encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits
cytokine synthesis in murine peritoneal macrophages. *J. Gen. Virol.* 83, 1049-1058.
- 395 23. Irwin, M.J., Hudson, K.R., Fraser, J.D., Gascoigne, N.R., 1992. Enterotoxin residues
determining T-cell receptor V beta binding specificity. *Nature* 359, 841-843.

24. Kato, M., Watarai, S., Nishikawa, S., Iwasaki, T., Kodama, H., 2007. A novel culture method of canine peripheral blood lymphocytes with concanavalin a and recombinant human interleukin-2 for adoptive immunotherapy. *J Vet Med Sci.* 69, 481-486.
- 400 25. Lateef, Z., Fleming, S., Halliday, G., Faulkner, L., Mercer, A., Baird, M., 2003. Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. *J. Gen. Virol.* 84, 1101-1109.
- 405 26. Lloyd, J.B., Gill, H.S., Haig, D.M., Husband, A.J., 2000. In vivo T-cell subset depletion suggests that CD4+ T-cells and a humoral immune response are important for the elimination of orf virus from the skin of sheep. *Vet. Immunol. Immunopathol.* 74, 249-262.
27. Lyons, A.B. and Parish, C.R., 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171, 131-137.
- 410 28. Mayr, A., Herlyn, M., Mahnel, H., Danco, A., Zach, A., Bostedt, H., 1981. Control of contagious ecthyma in sheep by means of a new paranteral cell culture derived live vaccine. *J. Med. Vet. B.* 535-549.
29. McKeever, D.J., Jenkinson, D.M., Hutchison, G., Reid, H.W., 1988. Studies of the pathogenesis of orf virus infection in sheep. *J. Comp Pathol.* 99, 317-328.
- 415 30. Mercer, J. and Helenius, A., 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320, 531-535.
31. Mielcarek, M., Kucera, K.A., Nash, R., Torok-Storb, B., McKenna, H.J., 2007. Identification and characterization of canine dendritic cells generated in vivo. *Biol. Blood Marrow Transplant.* 13, 1286-1293.
- 420 32. Moss, B. and Shisler, J.L., 2001. Immunology 101 at poxvirus U: immune evasion genes. *Semin. Immunol.* 13, 59-66.
33. Nascimbeni, M., Shin, E.C., Chiriboga, L., Kleiner, D.E., Rehmann, B., 2004. Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions. *Blood* 104, 478-486.
- 425 34. Peres, C.M., Aronoff, D.M., Serezani, C.H., Flamand, N., Faccioli, L.H., Peters-Golden, M., 2007. Specific leukotriene receptors couple to distinct G proteins to effect stimulation of alveolar macrophage host defense functions. *J. Immunol.* 179, 5454-5461.
35. Renno, T., Attinger, A., Locatelli, S., Bakker, T., Vacheron, S., MacDonald, H.R., 1999. Cutting edge: apoptosis of superantigen-activated T cells occurs preferentially after a discrete number of cell divisions in vivo. *J. Immunol.* 162, 6312-6315.
- 430 36. Roberts, J. and Camacho, Z., 1967. Oxidation of NADPH by Polymorphnuclear Leucocytes during phagocytosis. *Nature* 216, 606-607.
37. Rothe, G., Oser, A., Valet, G., 1988. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* 75, 354-355.

- 435 38. Scagliarini, A., Dal, P.F., Gallina, L., Guercio, A., Vaccari, F., Battilani, M., Ciulli, S., Prosperi, S., 2006. In vitro activity of VEGF-E produced by orf virus strains isolated from classical and severe persistent contagious ecthyma. *Vet. Microbiol.* 114, 142-147.
39. Serbina, N.V., Jia, T., Hohl, T.M., Pamer, E.G., 2008. Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26, 421-452.
- 440 40. Voigt, H., Merant, C., Wienhold, D., Braun, A., Hutet, E., Le Potier, M.F., Saalmüller, A., Pfaff, E., Büttner, M., 2007. Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). *Vaccine* 25, 5915-5926.
- 445 41. Wang, Y.S., Chi, K.H., Liao, K.W., Liu, C.C., Cheng, C.L., Lin, Y.C., Cheng, C.H., Chu, R.M., 2007. Characterization of canine monocyte-derived dendritic cells with phenotypic and functional differentiation. *Can. J. Vet. Res.* 71, 165-174.
42. Weber, O., Siegling, A., Friebe, A., Limmer, A., Schlapp, T., Knolle, P., Mercer, A., Schaller, H., Volk, H.D., 2003. Inactivated parapoxvirus ovis (Orf virus) has antiviral activity against hepatitis B virus and herpes simplex virus. *J. Gen. Virol.* 84, 1843-1852.
- 450 43. Yirrell, D.L., Reid, H.W., Norval, M., Entrican, G., Miller, H.R., 1991. Response of efferent lymph and popliteal lymph node to epidermal infection of sheep with orf virus. *Vet. Immunol. Immunopathol.* 28, 219-235.
- 455 44. Ziebell, K.L., Steinmann, H., Kretzdorn, D., Schlapp, T., Failing, K., Schmeer, N., 1997. The use of Baypamun N in crowding associated infectious respiratory disease: efficacy of Baypamun N (freeze dried product) in 4-10 month old horses. *Zentralbl Veterinarmed B* 44, 529-536.

460 **Figure captions**

Figure 1: *iPPVO enhances the phagocytotic activity of canine monocytes and PMN.*

A) Net phagocytosis rates ($\Delta PR = PR_{\text{treatment}} - PR_{\text{PBS}}$) of canine leucocytes were calculated as
 465 a difference of phagocytosis rates from stimulated cells (diluted iPPVO or stabiliser, LPS) and
 PBS control. Each data point represents the mean of three separate measurements. Six
 independent experiments are shown using four different dogs. Statistical analysis was
 performed by ANOVA ($P < 0.001$) and Bonferroni's multiple comparison test ($*** P < 0.001$,
 $** P < 0.01$). B) Inhibition of phagocytosis by Cytochalasin D (5 $\mu\text{g/ml}$) of iPPVO treated cells
 470 demonstrated by diminished phagocytosis rate.

Figure 2: *Induction of oxidative burst by iPPVO in canine monocytes*

Oxidative burst in canine leucocytes post stimulation with iPPVO, stabiliser, PMA
 (185 ng/ml), or LPS (5 $\mu\text{g/ml}$) was detected with ROI sensitive DHR (8.9 $\mu\text{g/ml}$) by FACS.
 475 Burst rate represents percentage of rhodamine-positive cells in the indicated cell population.
 Data are derived from 6 independent experiments using three dogs. Statistical analysis was
 performed with ANOVA ($P < 0.001$) and paired t-tests for the separate dilutions ($*** P < 0.001$,
 $** P < 0.01$, $* P < 0.05$).

480 **Figure 3:** *MHC-II up-regulation on canine monocytes in the presence of iPPVO*

PBL purified from peripheral blood were cultivated for 48 h and stimulated with purified
 (pu) iPPVO (1×10^6 TCID₅₀/ml) or the pharmaceutical formulation of iPPVO (diluted 1:4).
 As controls cells were cultivated in the presence of LPS (1 $\mu\text{g/ml}$), in medium or stabiliser
 (1:4). All CD14⁺ cells were analysed for expression level of MHC-II on the cell membrane.
 485 Cells were counterstained with anti-canine-MHC-II mAb or isotype control before. The
 fluorescence intensity was measured by flow cytometry. A) Histogram for electronically

selected CD14⁺ monocytes shows the fluorescence intensity of bound anti-MHC-II-FITC and isotype control (rat IgG2a-FITC). B) Mean + SD of the geometric means of fluorescence intensity of the MHC II fluorescence channel from all tested dogs (n = 8). Statistical analysis
490 was performed with ANOVA (P<0.001) and Bonferroni's multiple comparison test (***) P<0.001).

Figure 4: Proliferation of canine CD4⁺ T-cells in the presence of iPPVO

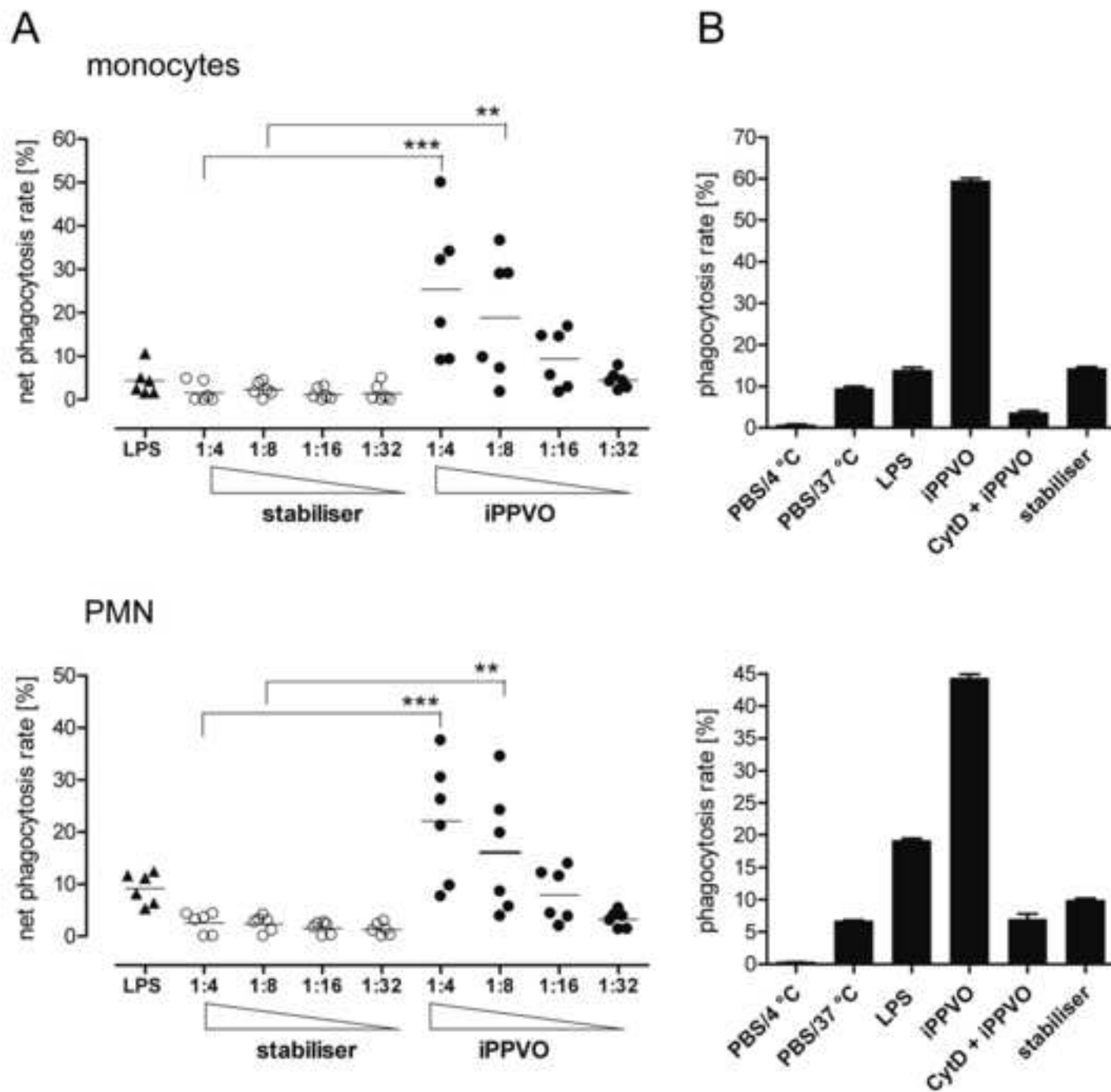
Freshly isolated PBL were cultivated for 7 d post CFSE-staining in the presence of iPPVO or
495 stabiliser control. Concanavalin A (ConA; 5 µg/ml) and medium were used as controls.

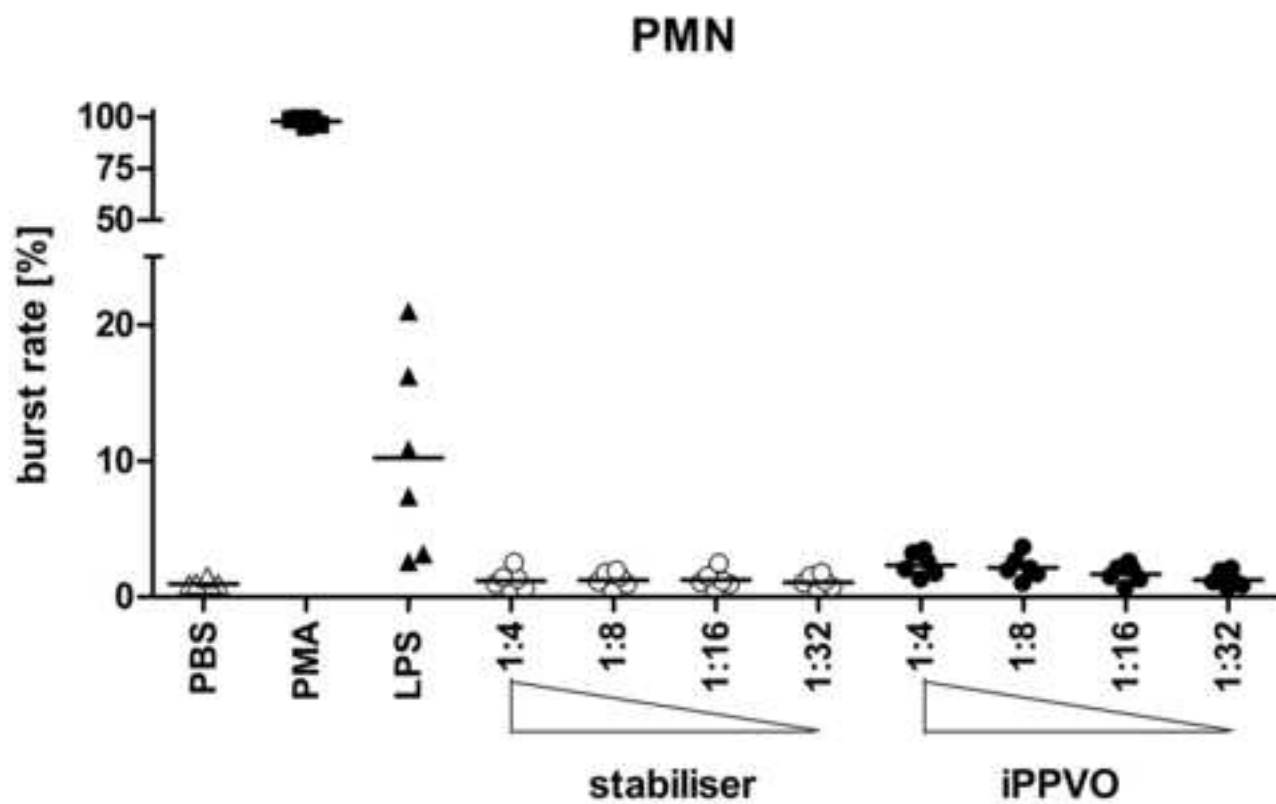
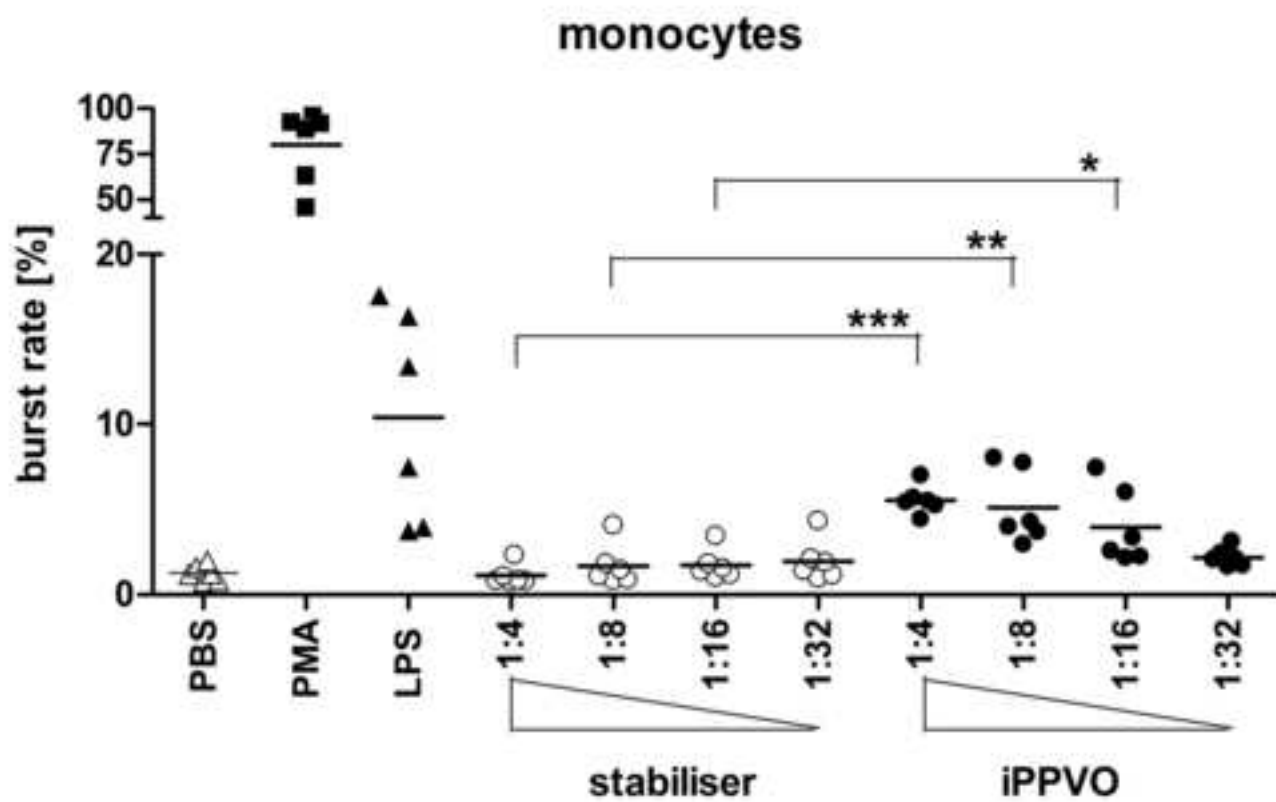
A) Proliferation rates derived from blood samples of 6 individual dogs are shown. Proliferation rates are defined as percentage of CFSE-diminished (dim) cells within the CD4⁺, the CD4⁺/CD8⁺ or the CD8⁺ T cell population. Statistics was performed with ANOVA (P<0.001) and Bonferroni's multiple comparison test (***) P<0.001, *P<0.05).

500 B) Dot plots with gated CD4⁺ or CD4⁺/CD8⁺ cells were utilized to determine portion of proliferating cells in the specified cell fraction.

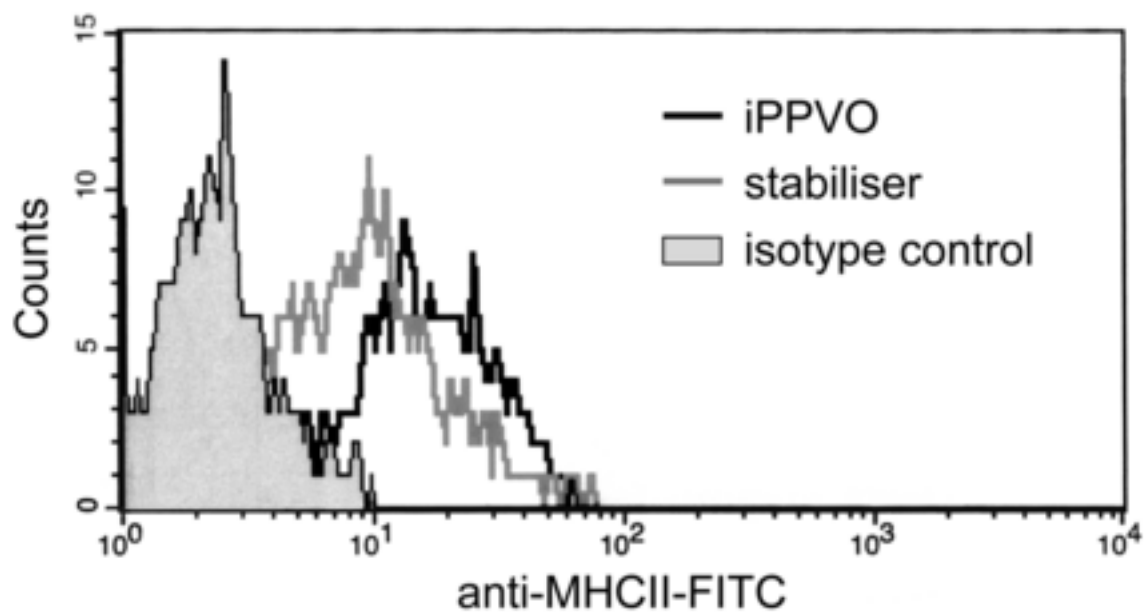
505

Figure 1





A



B

