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**Specific antibodies induced by inactivated parapoxvirus ovis potently enhance oxidative burst in canine blood polymorphonuclear leukocytes and monocytes**

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25 **Abstract**

We have recently shown that inactivated parapoxvirus ovis (iPPVO) effectively stimulates canine blood phagocytes. However, a potential link between innate and adaptive immunity induced by iPPVO remained open. The objective of this study was to define the effects of repeated iPPVO treatment of dogs to evaluate (i) iPPVO-specific antibody production, and  
30 (ii) modulation of iPPVO-induced oxidative burst by anti-iPPVO antibodies. Serum analysis of dogs treated repeatedly with iPPVO (Zylexis<sup>®</sup>) showed transient production of non-neutralising iPPVO-specific IgG. There was a correlation between iPPVO-specific IgG levels and enhanced oxidative burst rates *in vitro* upon transfer of immune sera. Even four years post Zylexis<sup>®</sup> treatment considerably stronger oxidative burst rates in response to iPPVO were  
35 observed in monocytes and PMN, whereas only moderate burst rates were detected in monocytes, but not in PMN, from dogs treated with a placebo. Depletion of serum IgG by protein A-sepharose or by parapoxvirus ovis coupled to sepharose abolished the increase of oxidative burst responses and resulted in burst rates similar to blood leukocytes from control dogs. However, uptake of viral particles was found to be independent of iPPVO-specific IgG  
40 and restricted to cells with dendritic and monocytic morphology. These data demonstrate that non-neutralising iPPVO-specific IgG is produced during treatment with Zylexis<sup>®</sup>. Moreover, for the first time the interaction of iPPVO with antibodies is shown to enhance oxidative burst.

45 **Keywords:** innate immunity, oxidative burst, antibody

## Introduction

The family *Poxviridae* comprises the largest of all known human and animal DNA viruses. Poxviruses are highly immunogenic, as has been demonstrated for several attenuated virus strains such as modified vaccinia virus Ankara (MVA) (Gherardi and Esteban, 2005). The  
50 genus *Parapoxvirus* (PPV) includes parapoxvirus ovis (PPVO) as the prototype species, a pathogen that is responsible for orf (contagious pustular dermatitis), as well as parapoxvirus bovis 1 (bovine papular stomatitis) and parapoxvirus bovis 2 (pseudocowpoxvirus, milker's node) (Büttner and Rziha, 2002). Parapoxvirus ovis (PPVO) has a restricted host range for sheep and goats. Manifestation of the disease is limited to the skin and, in contrast to vaccinia  
55 virus, there is no systemic virus spread (McKeever et al., 1988). For an effective immune response, both CD4<sup>+</sup> T cells and a humoral immune response seem to be necessary, since high levels of PPVO-specific antibodies (IgG) were found to correlate with fast healing of lesions. The existence of neutralizing antibodies in sheep is reported, however these antibodies do not mediate long-lasting protection and even fail to protect newborn lambs by colostrum and milk  
60 transfer (Buddle and Pulford, 1984; Le et al., 1978). Additionally, duration of acquired PPVO-specific immunity is transient, therefore re-infection occurs frequently (Haig and Mercer, 1998; Lloyd et al., 2000; Yirrell et al., 1991). PPVO infection can be transmitted to humans also leading to a local infection (milker's nodule) and an antibody response (Yirrell et al., 1994).

65 The known effects of poxviruses on the innate immune system, the host restriction, and the transient humoral immune response make PPVO attractive for application as an immunostimulant for non-permissive hosts. The immunostimulatory capacities of PPVO have been demonstrated in various mouse models (Büttner and Mayr, 1986; Mayr et al., 1978). Effective innate immune responses generated against herpes viruses, amongst others, have  
70 been demonstrated following administration of inactivated PPVO (Castrucci et al., 2000; Weber et al., 2003; Ziebell et al., 1997). The antiviral activity of iPPVO against hepatitis B

virus and HSV1 was mediated by cytokine induction (IFN- $\gamma$ , IL-12p40, IL-18 and TNF- $\alpha$ ) as demonstrated in mice (Weber et al., 2003). In dogs, another non-permissive species for infection with PPVO, antibody production has been demonstrated after intramuscular injection followed by intra-dermal administration of an attenuated PPVO strain (Büttner et al., 1995). Multiple application of immunostimulants in short term intervals is commonly used to induce and enhance innate immune reactions in defence against a variety of infections (Mayr et al., 1978). In contrast to PPVO infection, it is unknown whether treatment of dogs with inactivated PPVO (iPPVO) is as well able to induce antibody production against parapoxviruses. Moreover, it is of interest to explore if iPPVO-specific antibodies are able to contribute to the known immunostimulatory properties of iPPVO. We have recently shown *in vitro* stimulation of canine phagocytes by iPPVO (Schütze et al., 2009). Inactivated PPVO potently enhanced phagocytosis of *Listeria* by monocytes and PMN. Moreover, oxidative burst was found to be induced by iPPVO in canine monocytes. However, a potential link between innate immune activation (e.g. oxidative burst) and adaptive immunity especially after multiple treatment (e.g. antibody production induced by iPPVO treatment of dogs) remained open.

Oxidative burst activity induced by certain parasites and *Salmonella ssp.* has been shown to be enhanced in the presence of specific antibodies (Arbo et al., 2006; Laursen et al., 2003; Uppington et al., 2006). Therefore, in this study the impact of iPPVO-specific antibodies from dogs previously treated with Zylexis<sup>®</sup> on the modulation of oxidative burst in phagocytes was analysed. Our results demonstrate a link for iPPVO-induced innate and adaptive immune responses: (i) We found that non-neutralising iPPVO-specific IgG is produced during multiple treatment with iPPVO/Zylexis<sup>®</sup>, and (ii) interestingly, iPPVO-specific IgG was found to significantly enhance oxidative burst of monocytes and induce oxidative burst in PMN which were non-responsive in the absence of immune serum.

## Materials/ Methods

### *Virus/Reagents*

100 PPVO was grown in bovine foetal kidney BK-KL-3A cells. Chemically inactivated PPVO, strain D1701 in a pharmaceutical formulation, provided as Zylexis<sup>®</sup>, with 10E7.57 TCID<sub>50</sub>/dose PPVO (resuspended in 1 ml PBS), the stabiliser (polygeline)<sup>®</sup>, chosen as a control, and filter (5 µm)-purified iPPVO, (10e7.2 TCID<sub>50</sub>/ml, without stabiliser) were supplied by Pfizer Animal Health, Louvain-la- Neuve, Belgium. Sucrose gradient-  
105 purified iPPVO prepared in Bavarian Health and Food Safety Authority, Oberschleissheim, Germany as previously described (Mayr et al., 1989) was used as immunoblot antigen and for sepharosis coating. Immunoblot results obtained using filtrated vs. sucrose gradient-purified iPPVO were similar for most viral proteins except the >250 kDa band (data not shown). For electron microscopy sucrose gradient-purified PPVO was used. Inactivation of PPVO was  
110 performed using binary ethyleneimine (BEI) or β-propiolactone (BPL), two agents commonly used for virus inactivation. Inactivation efficacy was tested by three blind passages of undiluted iPPVO in highly permissive primary bovine oesophagus cells. Lipopolysaccharide (LPS) from *Salmonella* Abortus-equi (Alexis<sup>®</sup>, Gruenberg, Germany); phorbol 12-myristate 13-acetate (PMA, Alexis<sup>®</sup>, Gruenberg, Germany) and dihydrorhodamine (Invitrogen,  
115 Karlsruhe, Germany) were purchased.

### *Animals, blood and sera*

Blood was obtained from healthy dogs by vein puncture of vena *cephalica antebrachii* into heparinised vacutainer tubes (4 µg/ml Lithium-Heparine; Kalbe-Labortechnik, Nümbrecht-  
120 Elsenroth). All dogs belong to the College of Veterinary Medicine, University of Leipzig. All procedures were performed in accordance with local and animal welfare legislations.

In the context with a previous study dealing with treatment of an experimental oral papilloma virus infection six dogs (Labrador Retriever) were treated with Zylexis<sup>®</sup> and seven with a placebo control (polygeline) at the Department of Small Animal Medicine, University of Leipzig, Leipzig, Germany (März M., 2007; Raue M., 2008). Dogs received six doses Zylexis<sup>®</sup> or placebo subcutaneously within 28 days, respectively. Serum samples were taken before treatment, and 14 days and 2 month post treatment and stored at  $-80^{\circ}\text{C}$ . Approximately four years after the last administration blood samples were taken again from three Zylexis<sup>®</sup>-treated and four placebo-treated individuals. For our study blood and serum of further untreated beagles (n=3) and a Zylexis-treated Labrador Retriever (high responder after 6 doses Zylexis; PPVO-specific ELISA: 16.1 KELA units, Western Blot analysis see Figure 3 A, C) was used. Serum of the latter dog was used for serum-transfer and antibody depletion.

#### *Oxidative burst assay*

The oxidative burst activity of the leukocytes was assessed by addition of respiratory burst sensitive dye dihydrorhodamine (DHR,  $8.9\ \mu\text{g}/\text{ml}$ ) (Rothe et al., 1988) post stimulation of leucocytes with Zylexis<sup>®</sup>, stabiliser (polygeline), purified iPPVO or PBS; LPS ( $5\ \mu\text{g}/\text{ml}$ ) and PMA ( $185\ \text{ng}/\text{ml}$ ) were used as positive controls (15 min,  $37^{\circ}\text{C}$ ). Erythrocyte lysis was performed using lysis buffer ( $150\ \text{mM}\ \text{NH}_4\text{Cl}$ ,  $8\ \text{mM}\ \text{KHCO}_3$ ,  $1\ \text{mM}\ \text{EDTA}$ ; pH 7.0) incubated for 10 min at room temperature. The lysis reaction was stopped by addition of PBS and cells were washed twice. The samples were analyzed by flow cytometry (FACSCalibur, BD) within 30 min. PMNs and monocytes were gated by FSC/SSC and analyzed separately. The burst rate equates to the percentage of oxidative burst active cells within one cell population (monocytes or PMN). The serum-transfer experiments were performed with pre-filtered ( $0.45\ \mu\text{m}$  pore) and heat-inactivated (30 min at  $56^{\circ}\text{C}$ ) sera to exclude cellular components and complement.

*PPVO-specific ELISA*

96-well maxisorb plates (Nunc, Wiesbaden, Germany) were coated with PPVO (preparation  
150 of PPVO strain Orf11) or negative control antigen (preparation of cell line, that was used for  
PPVO multiplication) in carbonate buffer (0.1 M, pH 9.6), kindly provided by Moredun  
Scientific Ltd (Midlothian, Scotland, UK). The plates were blocked with PBS/2 % skimmed  
milk powder/0.05 % Tween. Diluted sera (1:50 in PBS/2 % skimmed milk powder/0.05 %  
155 Tween) were incubated. To detect PPVO-specific IgG a goat-anti-dog IgG-HRP conjugate  
(1:4000; MP Biomedicals, Illkirch, France) which may also cross-react with IgM was used.  
Samples were developed with the substrate TMB/H<sub>2</sub>O<sub>2</sub> (KPL, purchased from Medac GmbH,  
Medel, Germany) and measured kinetically (3 times, 45 s intervals) at 450 nm with ELISA  
reader Spectra Max 340PC (Molecular Devices, Ismaning, Germany). Data were analysed  
with Softmax 5.0 software by calculating KELA units as the slope of reaction rate as  
160 previously described (Shin et al., 1993). For each serum sample the level of non-specifically  
bound Ig was measured. Finally, non-specific signals from the negative controls (see above)  
were subtracted from their respective PPVO-specific signals to determine the KELA units.

*PPVO-specific virus neutralisation test (VNT)*

165 All sera were initially diluted 1:5 in cell culture medium followed by 2-fold dilution steps  
resulting in a dilution range from 1:5 to 1:640. An equal amount of PPVO strain D1701  
(MSV +4; 110 TCID<sub>50</sub>/well as demonstrated by virus back-titration in the same assay) was  
added to each well and incubated at 37 °C for 1 h. Afterwards, 100 µl trypsinised BK-KL-3A  
cells (passage 215) were added to each well and the assay was incubated at 37 °C for 4 or 7  
170 days. Data from 4-days and 7-days readings were similar (data not shown). Data derived from  
4-days readings are presented since increasing numbers of dead cells after 7 days made the  
reading of the test more difficult. An anti-PPVO sheep antiserum with a VN<sub>50</sub>=191 was used

as a positive control in the VNT. All samples were tested in quadruplicates. The titre of the sera was calculated using the Spearman-Kärber method.

175

*Immunoblot analysis of iPPVO-specific IgG*

Purified iPPVO strain D1701 was fractionated on vertical 12 % SDS-PAGE after reduction with mercaptoethanol and heat-denaturation (2 min, 95 °C). Viral proteins were immobilized on nitrocellulose membrane (MembraPure, Bodenheim, Germany). Dog sera (dilution: 1:10 –  
180 1:100) were incubated with the membrane. Virus-protein bound serum-IgG were detected with goat-anti-canine IgG-HRP (1:1000; MP Biomedicals, Heidelberg, Germany) and developed with Chloro-2-naphtol Opti-4-CN (SIGMA-Aldrich, Taufkirchen, Germany) and H<sub>2</sub>O<sub>2</sub>. To detect PPVO F1L env protein (apparently ~37 kDa), either mAb 8D7 or 10E6 which recognize the F1L env protein (Housawi et al., 1998) followed by goat-anti-mouse  
185 IgG-HRP (1:1000; R&D Systems, Wiesbaden, Germany) were used.

*Antibody-depletion with Protein A-sepharose or iPPVO-loaded sepharose from immune serum*

0.25 g Protein A-sepharose purchased from SIGMA-Aldrich (Taufkirchen, Germany) were  
190 washed with PBS for three times. 2 ml of diluted immune-serum (1:10 in PBS) was incubated with washed protein A-sepharose for 2 h at room temperature by end-over-end rotation in a batch approach. To completely remove protein A sepharose after the 2-h incubation, the serum was centrifuged and the supernatant afterwards filtered by 0.45 µm-syringe filters (Nalgene, VWR International, Darmstadt, Germany).

195 Filter-purified iPPVO with a protein content of 4 mg protein (according to Bradford assay) was dialysed using coupling buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub>, 0.5 NaCl pH 8.3) in a ZelluTrans 6.0 dialysis tube (molecular cut-off of 8.000 - 10.000 MWCO; Roth, Karlsruhe, Germany). CnBr-activated Sepharose 4 Fast Flow (1 g, GE Healthcare, Munich, Germany) was equilibrated in

5 ml of 1 mM HCl (4 °C) and washed with 10 volumes of 1 mM HCl (4 °C) twice. The  
200 prepared sepharose was divided into 2 parts and residual HCl solution was removed. To one  
part (0.5 g sepharose) the prepared 4 mg iPPVO antigen was added. The residual CnBr-  
activated sepharose part was put into pure coupling buffer as a negative control for the  
depletion. The aliquots were mixed by end-over-end rotation at 4 °C over night. After  
removing the antigen solution the remaining active binding groups of CnBr-activated  
205 sepharose were blocked with block buffer (0.5 M Ethanolamine, 0.5 M NaCl, pH 8.3) for 2 h  
and afterwards washed with 3 cycles of alternating buffers (A: 0.1 M NaC<sub>2</sub>H<sub>3</sub>COO, pH 4.0  
containing 0.5 M NaCl and B: 0.1 M Tris HCl, pH 8.0 containing 0.5 M NaCl). The iPPVO-  
coupled or pure sepharose were divided in 3 equal parts, and 1 ml of diluted immune-serum  
(1:10 in PBS) was incubated with one part of prepared sepharoses at 4 °C over night by end-  
210 over-end rotation in batch approach. Serum was recovered by centrifugation and transferred to  
the second part, incubated 2 h at room temperature by end-over-end rotation. The same  
procedure was performed again using the third part of sepharose, respectively. After removal  
of most of the sepharose by centrifugation, the remaining sepharose particles were removed  
using a 0.45 µm-syringe filter (Nalgene, VWR International, Darmstadt).

215

#### *Electron microscopy*

Peripheral blood leukocytes (PBL) were obtained from heparinized blood samples by density  
centrifugation (400 g) in Histopaque® (d = 1.077 g/ml; Sigma-Aldrich, Taufkirchen,  
Germany). 7 x 10<sup>5</sup> cells were seeded in 24-well plates and incubated with sucrose-gradient  
220 purified iPPVO (preincubated with either immune serum or non-immune serum, final dilution  
1:100) at a MOI of 10 for 4 h at 37 °C, 5 % CO<sub>2</sub>. Subsequently the cells were harvested with a  
cell scraper (TPP, MIDSCI; St. Louis, MO, USA) and washed in PBS twice. Cell pellets were  
embedded in cold fixation buffer (3% cacodylate-buffered glutaraldehyde, pH 7.35) and  
stored at 4 °C until further processing for electron microscopy. For postfixation in cacodylate-

225 buffered 1 % osmium tetroxide, the tissues were rinsed in buffer, dehydrated in graded  
alcohols and processed into polymerized blocks of Epon resin. Sections for initial light  
microscopy to determine tissue quality and architecture suitable for ultrastructure were cut at  
1  $\mu\text{m}$  and stained with toluidene blue. For electron microscopy, thin sections were cut with a  
diamond knife, mounted on copper grids, stained with 0.2 % lead citrate and 5 % uranyl  
230 acetate, and examined using a Philips EM 208 electron microscope (Philips, Eindhoven,  
Netherlands) operating at 80 kV. Photography was performed with a Morada digital camera  
(Olympus SIS, Münster, Germany).

#### *Statistical analysis*

235 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.  
Non-parametric data were analysed by Kruskal-Wallis Test and Dunn's multiple comparison  
test was used for post test to compare groups of interest.

## Results

240 *Detection and characterization of iPPVO-specific antibodies in sera of Zylexis<sup>®</sup>-treated dogs*

To answer the question if administration of iPPVO in dogs would result in PPVO-specific antibody production the humoral response following multiple iPPVO treatment (6 doses) of dogs was analyzed. Sera from the six Zylexis<sup>®</sup> and seven placebo administrated dogs were taken from three different time points (prior to, two weeks and two month post Zylexis<sup>®</sup> or  
245 placebo administration) and analysed by PPVO-specific Western blot and ELISA.

Western Blot analysis of all sera taken before and 2 weeks after treatment demonstrates a iPPVO-specific humoral immune response in the Zylexis group in contrast to placebo-treated dogs (Figure 1A). The most abundant PPVO immuno-reactive proteins have been previously described with molecular masses of 65 kDa, 39 kDa and 22 kDa (Czerny et al., 1997). All  
250 sera of Zylexis<sup>®</sup>-treated dogs taken two weeks after the treatment show immuno-reactive bands at >250, 65 and <39, which are not detectable in sera taken prior to the Zylexis<sup>®</sup> treatment (Fig. 1A). Zylexis-treated dogs show also a signal against 22 kDa protein with different intensity. The protein close to 37 kDa could be identified with two monoclonal Abs 10E6 and 8D7 specific for the envelope protein which is also known as F1L/env protein of  
255 PPVO (data not shown) with an apparent molecular mass of 39 kDa as previously described (Housawi et al., 1998). Additionally we used a serum from a PPVO-immunized rabbit as a positive control, that show also a signal at about 37 kDa. In contrast to the >250 kDa and 65 kDa protein bands, specific serum IgG for the 39 kDa and 22 kDa bands were not detectable four years post treatment by immuno-staining. The signal against a 65 kDa protein  
260 was, surprisingly, stronger four years post treatment, but was not detectable in pre-treatment sera (data not shown).

For quantification of the iPPVO-specific antibodies sera (1:50 diluted) from these six Zylexis<sup>®</sup> and seven placebo administrated dogs were taken from three different time points (prior to, two weeks and two month post Zylexis<sup>®</sup> or placebo administration) and were

265 measured in PPVO-specific ELISA. Additionally, sera were investigated from three Zylexis<sup>®</sup>-  
and four placebo-treated dogs four years after their last treatment. A statistically significant  
increase of PPVO-specific IgG in sera of the Zylexis<sup>®</sup>-treated dogs in contrast to the placebo-  
treated individuals was observed two weeks and two month post treatment by PPVO-specific  
ELISA (Fig. 1B). However, detectable serum levels of anti-PPVO IgG could not be found by  
270 the ELISA approximately four years after the final iPPVO administration.

We were also interested to analyze whether the iPPVO-specific antibodies were neutralizing.  
Therefore, we performed a VNT with BK-KL-3A cells as target cells for PPVO. No  
neutralizing antibodies could be detected in any of the sera (<1:5) from Zylexis<sup>®</sup>-treated dogs  
taken from different time points after administration. In contrast, using an anti-PPVO sheep  
275 antiserum as positive control, neutralizing activity was detectable (VN<sub>50</sub>=191; data not  
shown). Thus, iPPVO treatment of dogs induces a humoral response similar to natural PPVO  
infection in permissive hosts and similar to viable PPVO application in dogs (Büttner et al.,  
1995), characterized by a transient production of non-neutralizing serum antibodies against  
already known immunogenic PPVO proteins.

280

*Enhanced iPPVO-induced oxidative burst rates in Zylexis-treated dogs is correlated to  
iPPVO antibody titers*

We have recently shown that iPPVO induces moderate oxidative burst in canine blood  
monocytes but not in PMN (Schütze et al., 2009). In different infection models for parasites  
285 and bacteria it was shown that the oxidative burst activity was enhanced by opsonizing  
antibodies and complement (Arbo et al., 2006; Laursen et al., 2003; Uppington et al., 2006).  
To investigate whether the PPVO-specific antibodies in serum of Zylexis<sup>®</sup> treated dogs would  
augment oxidative burst of peripheral blood leukocytes, an oxidative burst assay was  
performed by supplementing serum of Zylexis<sup>®</sup>-treated dogs (diluted 1:10) to blood cells of  
290 an untreated dog. In this experiment it was demonstrated that the iPPVO-induced oxidative

burst was enhanced by serum transfer of anti-iPPVO immune sera taken two weeks and two months following Zylexis<sup>®</sup> administration in comparison to sera sampled before treatment (\*\*P<0.001, \*P<0.05, ANOVA and Bonferroni's Multiple Comparison Post test; Fig. 1C).

In contrast, stimulation with PMA did not show any differences between sera from distinct  
295 time points. Interestingly, in the presence of immune serum not only monocytes but also PMN  
responded to iPPVO stimulation with oxidative burst, whereas samples supplemented with  
serum before treatment showed iPPVO-induced burst activity exclusively in monocytes as  
recently described (Schütze et al., 2009). Consistent with the time course of the antibody  
levels (see Fig. 1B) transfer of sera taken two months after Zylexis<sup>®</sup> treatment still enhanced  
300 the oxidative burst rate, although to a lower extent as in the presence of serum taken after two  
weeks (Fig. 1C). Furthermore, the high responder dog identified by ELISA analogously  
shows a high response in oxidative burst in monocytes after stimulation with iPPVO in  
comparison to the other Zylexis-treated individuals. Thus, the iPPVO-specific IgG in immune  
sera quantified by ELISA correlates well with the enhancement of burst activity in the serum  
305 transfer experiment performed with cells from a naive donor dog. We also investigated serum  
transfer from a placebo-treated dog to cells of an untreated dog. Here we found no  
enhancement of oxidative burst in contrast to serum of a Zylexis-treated dog (data not  
shown). Finally, we tested the serum of the cell donor itself. As expected we found a weak  
reactivity of monocytes by iPPVO stimulation in contrast to PMN (data not shown).  
310 Additionally we demonstrated by a dilution series of immune serum (derived from a high  
responder) added to blood samples of an untreated dog, that high serum concentrations  
correlate with an enhancement of iPPVO-induced burst activity (Fig. 1D).

*Long-term enhancement of iPPVO-induced oxidative burst in Zylexis<sup>®</sup>-treated dogs is  
315 mediated by a transferable serum component*

To analyze a potential long-term effect of Zylexis<sup>®</sup> blood samples from Zylexis<sup>®</sup>-treated dogs were investigated (n = 3) >four years after administration of six Zylexis<sup>®</sup> doses. Blood samples from placebo-treated dogs (n=4), administrated >four years ago, served as control group. Surprisingly, the undiluted blood samples from the Zylexis<sup>®</sup>-treated individuals showed increased burst rates after *in vitro* stimulation with iPPVO in both, monocytes and PMNs, although the treatment had been stopped four years ago (Fig. 2). In contrast, whole blood from placebo-treated dogs exhibited burst rates following iPPVO stimulation comparable to those from untreated dogs (Schütze et al., 2009), characterized by a moderate burst response to iPPVO exclusively in monocytes but not in PMNs. The enhanced burst rates observed in whole blood from Zylexis<sup>®</sup>-treated dogs were found to be specific for iPPVO stimulation. The burst responses observed for LPS or PMA did not differ between both groups of dogs. Unfortunately, due to the small group sizes (Zylexis<sup>®</sup>-treated animals, n = 3; placebo-treated dogs, n = 4), it was impossible to determine statistical significance. Nevertheless, using all separately measured values of triplicates (3 measurements for every stimulus/dog) for statistical analysis, the difference between whole blood measurement between Zylexis<sup>®</sup> and the placebo-treated group was found to be highly significant (p<0.001, Bonferroni's Multiple Comparison Test, data not shown). Thus, even when serum anti-PPVO IgG was not detectable any more in dogs four years after their last Zylexis<sup>®</sup> treatment, the oxidative burst rates were enhanced in blood samples from these dogs (see Fig 2).

In order to verify that the enhancement of iPPVO-induced oxidative burst observed in blood samples from Zylexis<sup>®</sup>-treated dogs (>4 years post administration) depends on a serum component, again serum transfer experiments were done. The undiluted serum of the Zylexis<sup>®</sup>-treated dogs (n = 2, >4 years post administration) enhanced iPPVO-induced oxidative burst in PMN and monocytes of a placebo-treated dog (data not shown). As a negative control the homologous serum of the placebo-treated dogs themselves was utilized. In contrast, cells supplemented with the different sera responded similarly upon stimulation

with PMA (data not shown). Therefore, even very low and by ELISA undetectable anti-iPPVO antibody levels appear to be sufficient for the iPPVO-induced enhancement of oxidative burst.

345

*Antibody depletion abrogates enhancement of oxidative burst by blood monocytes and PMN stimulated with iPPVO*

To prove the involvement of specific antibodies in the enhancement of iPPVO-induced oxidative burst, Ig (mainly IgG and IgM) from the immune serum of a Zylexis<sup>®</sup>-treated dog was depleted by protein A-sepharose beads as previously described (Scott et al., 1997; Peng et al., 1991). Following Ig depletion by protein A-sepharose treatment of immune serum, all specific-PPVO Ig (with only a minor reactivity against the 65 kDa band left) were depleted as demonstrated in an PPVO-specific immunoblot using protein A-depleted as compared with non-depleted sera (Fig. 3A). This Ig-depleted serum was utilized in an oxidative burst experiment in comparison to untreated immune serum. The homologous serum of the untreated dog (cell donor) was used as an additional control. After stimulation with iPPVO, the Ig-depleted immune-serum showed a strongly reduced oxidative burst activity similar to the non-immune serum, in contrast to the untreated immune serum (Fig. 3B).

355

To selectively deplete PPVO-specific antibodies from the immune serum it was treated with iPPVO-coupled sepharose beads. As a negative control, the immune serum was exposed to uncoupled sepharose. The anti-PPVO antibody level was strongly reduced as demonstrated in immunoblot analysis (Fig. 3C), albeit complete antibody depletion was not reached with the iPPVO-coupled sepharose beads. This is especially evident for the F1L/env protein at about 37 kDa. As expected, treatment with uncoupled sepharose did not change antibody levels significantly (Fig 3C). Again depleted and control-treated sera were tested in oxidative burst assay. The transfer of serum depleted of PPVO-specific antibodies to whole blood cells of a naïve dog resulted in a reduction of oxidative burst activity in iPPVO-stimulated PMN and

365

monocytes in contrast to untreated and mock-treated immune serum (Fig. 3D). This result is consistent with the decreased iPPVO immunoreactivity detected by immunoblot analysis (Fig. 370 3C).

*Uptake of iPPVO particle exclusively in cells with dendritic and monocytic morphology but not in PMN is independent of specific antibodies*

From vaccinia virus it is known that the most abundant form of poxvirus particles are intracellular mature virus (IMV) derived from cell lysates (Smith et al., 2002). Analysis of 375 short-term iPPVO-exposed canine PBL (4h) by electron microscopy provide an insight into the interaction of iPPVO with the immune cells. In the following experiment iPPVO was added to PBL in the presence vs. absence of immune serum. To allow formation of immune complexes, undiluted immune sera were incubated with iPPVO at 37 °C for 2 hours before 380 addition to cells. As control a non-immune serum was utilized. Analysis by electron microscopy of these samples demonstrated that cells with dendritic and monocytic morphology can take up viral particles (Fig. 4A,C), with the majority being found in the lysosomal compartments (Fig. 4B), but iPPVO was also found in the cytoplasm (Fig. 4 A,C,D). Dendritic cells, in particular, appeared to contain a high virus load per cell, mostly in 385 lysosomes. In contrast, PMN appeared unable to take up iPPVO (Fig. 4E,F). Comparing viral uptake using immune vs. non-immune serum samples by counting the viral particle load per cell ( $n > 15$  cells / per group) revealed no difference. This suggests that the uptake of iPPVO particles is independent of the presence of specific antibodies. However, electron microscopic analysis is limited in that it is unlikely to be fully representative or quantitative, because 390 iPPVO-positive cells were found only rarely (Fig. 4F).

## Discussion

The present study demonstrates (i) that multiple Zylexis<sup>®</sup> treatment of dogs results in the formation of PPVO-specific serum antibodies, (ii) that the moderate iPPVO-induced oxidative burst in monocytes can be enhanced, and additionally, PMN become sensitive to  
395 iPPVO-induced oxidative burst in the presence of PPVO-specific serum IgG of Zylexis<sup>®</sup>-treated dogs, (iii), that the uptake of inactivated virus particles is restricted to dendritic-like cells and monocytes, independently of the presence of PPVO-specific antibodies.

From infected hosts (man and ruminants) and immunization studies in mice it is known that non-permissive hosts generate an antibody response to infection with live PPVO (Czerny et  
400 al., 1997; Housawi et al., 1998; Yirrell et al., 1994). However, in this study animals were treated with chemically inactivated virus. The quantification of PPVO-specific antibodies demonstrates the development of specific IgG shortly after a series of six applications of Zylexis<sup>®</sup>. In the dogs accessible for this study the antibody level was found to drop below the detection limit in ELISA four years post final treatment. This result is in agreement with data  
405 from experimentally and naturally PPVO-infected sheep that also show high antibody levels which increased after productive primary infection and decreased after recovery (Lloyd et al., 2000; McKeever et al., 1987). Thus, iPPVO treatment of a non-permissive host induces a transient humoral response similar to natural PPVO infection in permissive hosts and viable PPVO administration in dogs. However, it remains open how many applications with iPPVO  
410 are necessary to induce a humoral response. In any case the humoral response seems to consist of non-neutralising antibodies with longevity and effect on oxidative burst of PMN and monocytes.

In detail, antibodies against an immuno-dominant PPVO protein previously designated as a 39 kDa protein were detected (Housawi et al., 1998). Antibody reactivity against this protein  
415 in the molecular weight range of ~40 kDa, which is enriched in viral envelope preparations, has been described previously (Balassu and Robinson, 1987; Housawi et al., 1998). The

coding gene F1L reveals an open reading frame of 1,002 bp with the potential to encode a protein with a MW of 36.7 kDa (Housawi et al., 1998). The specific band observed by immunoblot in this study was near 37 kDa, which was confirmed by different monoclonal antibodies directed against the 39 kDa protein. The 39 kDa protein is homologous to the H3L gene of vaccinia virus which has also been described as an immunogenic envelope protein (Housawi et al., 1998). Besides the 39 kDa protein other immunoreactive proteins of PPVO have been described with molecular weights of 22 and 65 kDa (Czerny et al., 1997). Like others, a strong increase of anti-65 kDa-specific IgG in the Zylexis<sup>®</sup>-treated dogs was found, that did not decline over 4 years post administration. The identity of the 65 kDa protein remains elusive. Czerny et al. suggest that the protein is a major core protein (Czerny et al., 1997). Here some cross-reactivity with this 65 kDa protein was observed in serum of dogs treated with placebo or untreated dogs. In addition, sera of all Zylexis<sup>®</sup>-treated dogs showed specific antibodies against a ~250 kDa protein and some of those also against the 22 kDa protein.

In our recent report differences between canine monocytes and PMN for the iPPVO-induced oxidative burst were found (Schütze et al., 2009). In the absence of specific antibodies, iPPVO-induced burst was restricted to canine monocytes, demonstrated in this study by blood samples of placebo- or non-treated dogs. In contrast, Förster et al. demonstrated PPVO-induced oxidative burst in human neutrophils, that was accompanied by an up-regulation of complement receptors CR1 and CR3 (Förster et al., 1994). One possible explanation for this cell specificity of oxidative burst in the absence of specific antibodies is that only monocytes express adequate pattern recognition receptors (PRR) that respond to iPPVO as a pathogen-associated molecular pattern or danger signal, whereas canine PMN might not express such receptors. Previously it was demonstrated that Toll-like receptors (TLR) mediate oxidative burst (Hoarau et al., 2007). Until now, the potential PRR used by parapoxviruses are not characterized. It is also conceivable that indirect activation of burst activity by secretion of

mediators from other cells activate oxidative burst, e.g. by G-protein coupled receptors on monocytes (Sheppard et al., 2005). The electron microscopic analysis of canine cells  
445 incubated with iPPVO additionally demonstrated differences between monocytes/dendritic cells and PMN, because only dendritic cells and monocytes were found to take up viral particles.

By serum transfer experiments this study demonstrated that immune serum mediates enhancement of the iPPVO/Zylexis<sup>®</sup>-induced oxidative burst. Interestingly the enhancement  
450 of oxidative burst was not only observed in monocytes but also in PMN, which showed no oxidative burst in naïve animals. To prove whether PPVO-specific antibodies are actually responsible for the enhanced burst activity antibody-depletion from serum containing PPVO-specific IgGs was performed. By protein-A sepharose all specific IgGs (anti-39 kDa) were depleted from immune serum, which completely abrogated the enhancement effect. A more  
455 selective strategy of depletion eliminating only specific PPVO antibodies by iPPVO-coupled sepharose did not completely abolish the presence of anti-39 kDa antibodies as demonstrated by a reduced but still visible signal for the 39 kDa protein in the immunoblot (Fig. 3C). However, the partial depletion resulted in a partial decrease of burst enhancement (Fig. 3D). This may suggest that in particular the anti-39 kDa antibodies are mediators for the  
460 enhancement of PPVO-induced oxidative burst. This finding fits well with those of other groups studying innate immunity to parasites, fungi or bacteria. Induction of oxidative burst by *Giardia lamblia* trophozoites was enhanced by opsonisation with anti-*Giardia* hyperimmune serum and complement in PMN (Arbo et al., 2006). Laursen et al. demonstrated that together with complement, lung surfactant D and mannan binding lectin the  
465 specific IgG antibodies in contrast to IgM mediate enhanced burst activity in neutrophils in response to *Pneumocystis carinii* (Laursen et al., 2003). Furthermore, the uptake of *Salmonella enterica* serovar Typhimurium and oxidative burst induction was increased by binding to high affinity IgG receptor Fc $\gamma$ RI mediated by immune serum (Uppington et al.,

2006). For our model we suggest that the presence of PPVO-specific antibodies results in  
470 formation of immune complexes that make the virus particles accessible to the low affinity  
Fc $\gamma$  receptors, which are also expressed on PMN. Suh et al. demonstrated a Fc $\gamma$ RIIA-mediated  
NADPH oxidase activation in human cells, that was considerably disrupted by  
phosphatidylinositol-3 kinase (PI3) inhibitors, whereas phagocytosis was inhibited to a lower  
extent (Suh et al., 2006).

475 Unexpectedly leukocytes of whole blood samples taken from dogs which were treated four  
years previously with Zylexis<sup>®</sup> showed enhanced iPPVO-induced oxidative burst, in contrast  
to the placebo-treated group, although PPVO-specific antibodies were no longer detectable by  
ELISA (using a pre-dilution of sera at 1:50, see Fig. 1B). This suggests that very low serum  
levels of specific IgGs are sufficient to enhance iPPVO-induced oxidative burst.

480 The electron microscopic analysis showed that iPPVO particles were taken up even in the  
presence of immune serum exclusively in dendritic cells and monocytes, but not in PMN,  
which indicates that the antibody-mediated oxidative burst activity is independent from the  
uptake of virus particles. However, further investigations are needed to confirm these  
hypotheses. In addition, it remains to be evaluated whether macropinocytosis is the  
485 mechanism for PPVO uptake in dendritic cells and monocytes as recently demonstrated for  
VACV (Mercer and Helenius, 2008). Interestingly, the 39 kDa protein of PPVO was shown to  
bind glucosamine heparane sulphate on host cells (Scagliarini et al., 2004) similar to its  
homologue, the H3L vaccinia protein that is part of the virus entry machinery (Lin et al.,  
2000).

490

### **Conclusion**

In summary, iPPVO-specific antibodies enhancing oxidative burst present an interesting  
example of collaboration between innate and adaptive immunity. In future studies the  
importance of the antibody-dependent iPPVO-induced oxidative burst activity needs to be

495 investigated *in vivo* and its relevance has to be identified in innate immunity against viral,  
bacterial and fungal infections.

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this manuscript.

**Figure 1:**

*PPVO-specific IgG in serum of Zylexis<sup>®</sup>-treated dogs results in enhanced iPPVO-induced oxidative burst activity*

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The presence of *orf virus*-specific antibodies was analysed in sera of Zylexis<sup>®</sup> or placebo-treated dogs (n = 6 and 7, respectively) from samples taken before, two weeks (2 wk), two month (2 mo) and four years (4 yr) post application. A) PPVO-specific immunoblot using vertically fractionated (12 % SDS-PAGE) and on nitrocellulose membrane immobilized iPPVO and unstained (M1) or prestained (M2) protein standards. PPVO-specific IgGs from dog sera (1:10) before (-) and 2 weeks after treatment with Zylexis (Zyl) or placebo (pl) was detected by using anti-dog IgG-HRP. As control (ctr.) a rabbit serum specific for orf virus (1:100 dilution) and anti-rabbit IgG-HRP was used. B) PPVO-specific ELISA: PPVO-antigen and control-antigen (see Mat. & Methods) were coated to 96-well plates; virus-specific IgG from the diluted sera (1:50) were detected by goat-anti-dog-HRP developed with TMB substrate. KELA units of optical density at 650 nm are shown. Statistical Analysis was performed with Kruskal-Wallis Test and Dunn's multiple comparison test (\*\*P < 0.001, \*P < 0.05); C) Sera (1:10) of 5 Zylexis<sup>®</sup>-treated dogs (2 wk and 2 mo) in comparison to sera before Zylexis<sup>®</sup>-treatment were tested in serum transfer experiments for iPPVO-induced oxidative burst activity. As control stimuli PBS and PMA (185 ng/ml) were used. All serum transfer experiments were performed with blood cells from non-Zylexis<sup>®</sup>-treated dogs (laboratory animals). Statistical analysis was performed with ANOVA and Bonferroni's multiple comparison test (\*P < 0.05, \*\*P < 0.001). D) Serial dilutions of immune sera were added to blood cells of an untreated dog in the iPPVO-induced oxidative burst assay.

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**Figure 2:***Long-lasting enhancement of oxidative burst induced by iPPVO in Zylexis<sup>®</sup>-treated dogs*

Dogs were treated on six occasions with Zylexis<sup>®</sup> (n = 3) or placebo (n = 4) respectively, four years previously. Blood samples from dogs were taken and oxidative burst activity of Zylexis<sup>®</sup> - vs placebo-treated dogs was analysed by *in vitro*-restimulation with pharmaceutical formulation of iPPVO in contrast to stabiliser control. As further control stimuli PBS (negative control), LPS (5 µg/ml) and PMA (185 ng/ml) were utilized. Measurement of oxidative burst activity in canine monocytes and PMN was performed by flow cytometry. Detection of ROIs was achieved by staining cells with DHR (8.9 µg/ml), that is oxidized by ROIs to the green fluorescent dye rhodamine. Burst rates were calculated as percentage of rhodamine-positive cells within the specified cell populations.

**Figure 3:**

*Antibody depletion in immune sera results in reduced iPPVO-induced oxidative burst activity*

545 A) Total IgG-depletion from immune serum (1:10 diluted in PBS) was performed with protein A-sepharose, demonstrated in PPVO-specific immunoblot: lane 1 – non-immune serum from the blood donor, lane 2 - Ig-depleted serum by protein A-sepharose treatment, lane 3 - untreated immune serum. The result is the same for two independent experiments.

B) Serum transfer of Ig-depleted immune serum (gray bars) in comparison to untreated immune serum (black) and non-immune serum (white) to oxidative burst assay (described above). For this application all sera were diluted 1:10 in PBS. The result is representative for two independent experiments.

C) Treatment of immune serum (lane 1-3; 1:10 diluted) with iPPVO-coated sepharose results in a strong reduction of PPVO-specific antibodies, demonstrated in immunoblot: lane 1-  
555 treatment with iPPVO-coated sepharose, lane 2 – mock-control treatment with uncoated sepharose, lane 3 - untreated immune serum. The result is representative for two independent experiments.

D) Immune sera post distinct treatments (as indicated in the legend) were transferred to oxidative burst assay, that was performed with blood from an untreated dog. As negative  
560 serum control we used the serum from the blood donor itself (white bars). The result is representative for two independent experiments.

**Figure 4:**

*Dendritic cells and monocytes but not PMN show uptake of iPPVO particles*

565 Isolated PBL were cultivated for 4h with iPPVO, incubated before with immune serum (to  
allow formation of immune complexes) or non-immune serum. Analysis by electron  
microscopy was performed as detailed in Mat & Methods. Since we could not find any  
differences between samples incubated with immune or non-immune serum, only data from  
incubation with preformed immune complexes (immune serum) are shown. Internalized  
570 iPPVO particles in: A) a dendritic cell, B) in the lysosomal compartment (inset from dendritic  
cell); or C) in monocytes D) in lysosomal compartment (white arrow) or cytoplasm (black  
arrow), see inset from monocyte. E) Granulocytes did not internalize iPPVO particles F) as  
demonstrated additionally in an overview picture in contrast to dendritic cells (black arrow).

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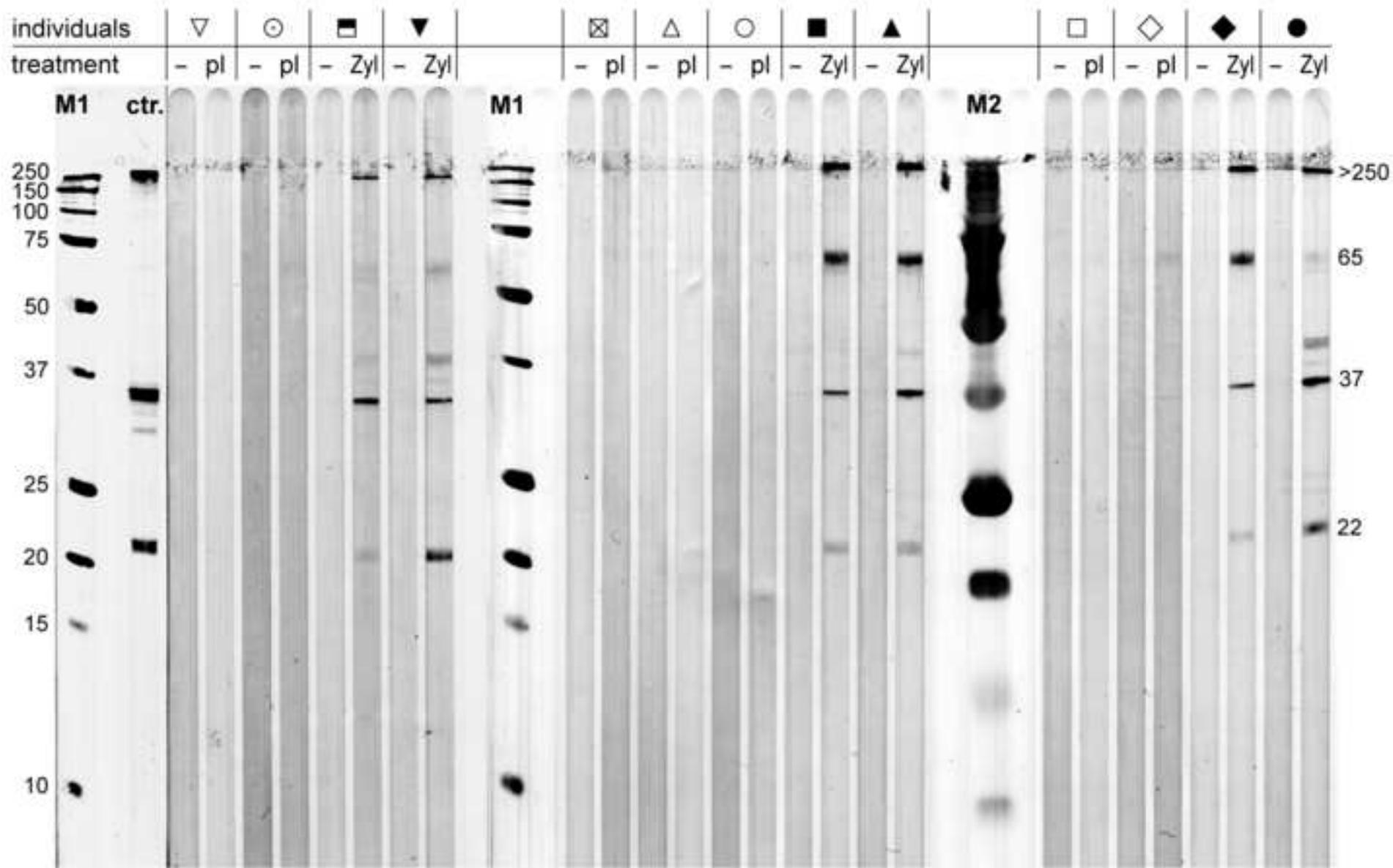
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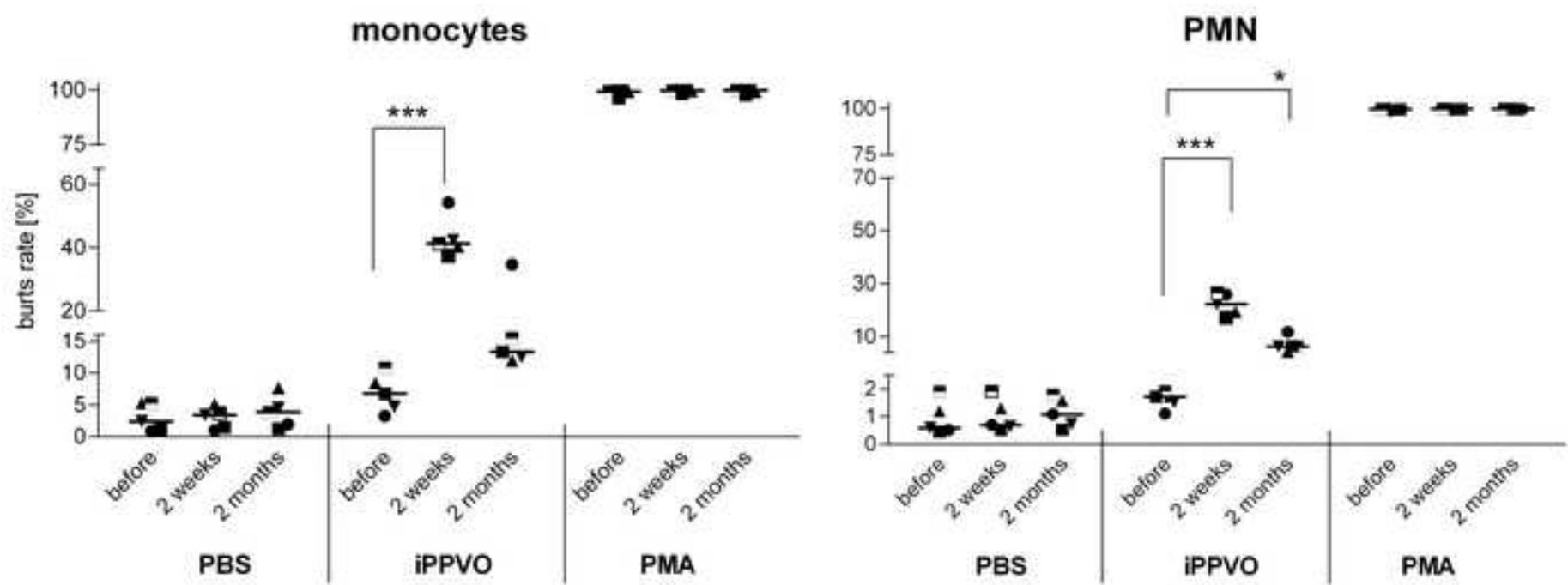
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Figure 1A





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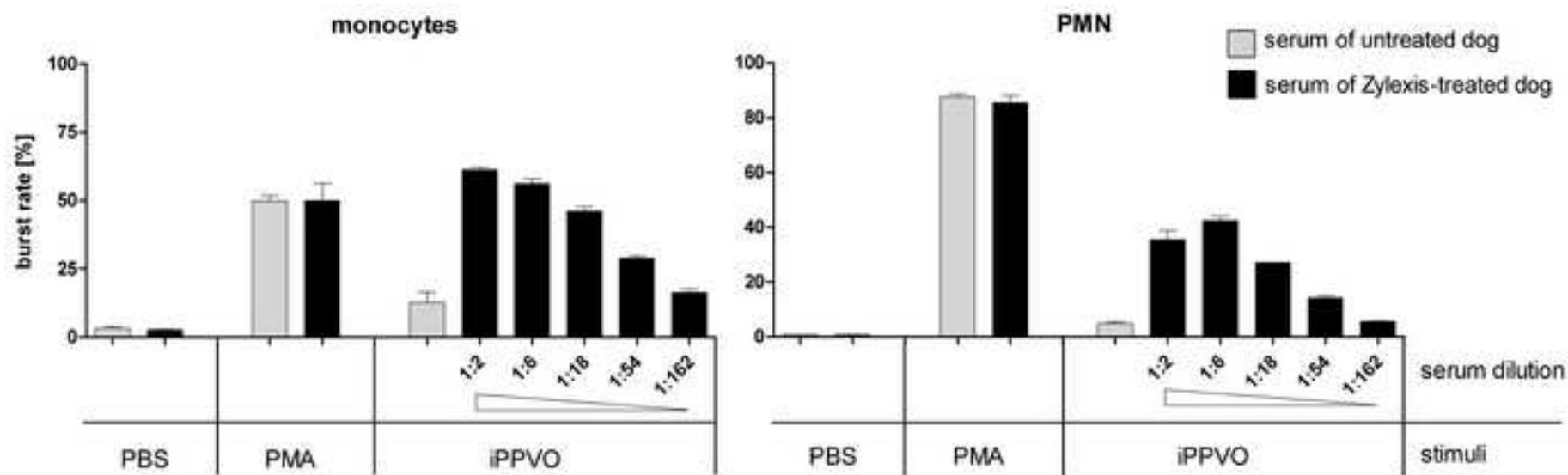
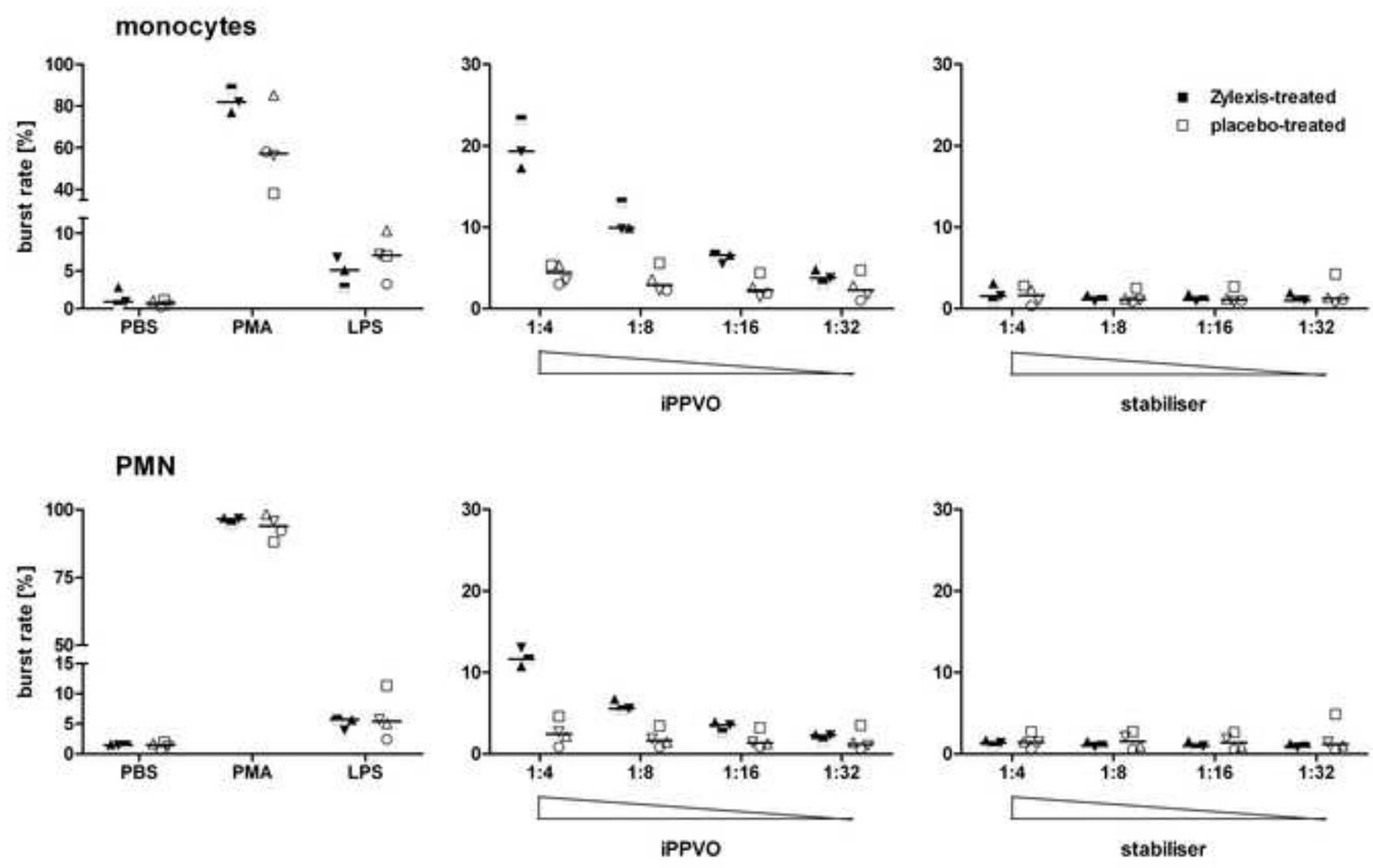
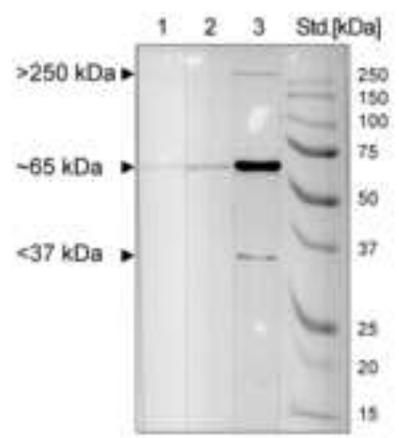


Figure 2

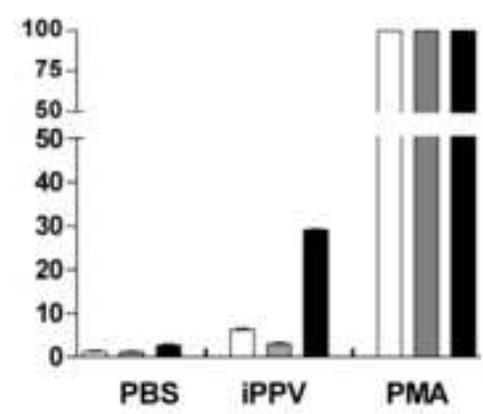
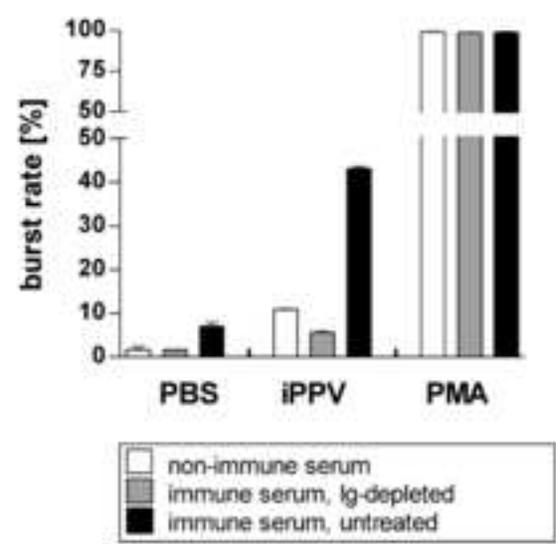


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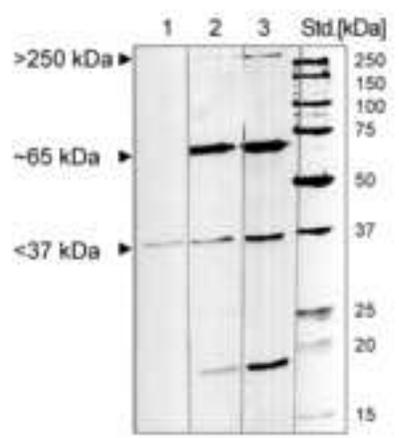
**A**



**B**



**C**



**D**

