

The effect of porcine IgG found in spray dried plasma on canine immune cells

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Abstract

Chronic enteropathy is a common occurrence in dogs, as a result of damage to the intestinal wall caused by e.g. canine parvo-virus. Treatments of chronic enteropathy include dietary changes, antibiotics or immunosuppressants. Since these treatments are not always effective and even carry negative side effects like antibiotic resistance or increased susceptibility to infections, alternative treatments are investigated. Spray-dried porcine plasma (SDPP) and more specifically immunoglobulins in it have been found to have positive immunomodulatory effects and support gut health in multiple species which is the reason it is investigated as a treatment alternative. However, there is little knowledge of the effects of SDPP and its immunoglobulins on canine immune cells. For this reason ELISA was used to investigate if canine serum would interfere with pIgG concentration measurements as well as if the extrusion process affects functional pIgG present in dog kibble. An attempt at characterizing FcγRs- and determining their interactions with pIgG on various canine cells was made by using flow cytometry. The activation of the canine immune system by pIgG was studied by looking at T-cell proliferation upon CPV and VP2 stimulation by means of a BrdU assay. The research done for this thesis showed that canine serum does not affect results of pIgG concentration measurements. No functional pIgG could be observed once the dog kibble underwent extrusion. It was also shown that pIgG has an inhibiting effect on T-cell proliferation when they are stimulated with CPV or VP2. In conclusion no supportive effect by pIgG on the immune system in response to CPV could be observed.

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1. Introduction

1.1 Domestic dogs and chronic enteropathy

The domestic dog, also known as *Canis familiaris*, is the most popular pet worldwide [1]. They provide companionship, act as medical service dogs as well as assist the police and military, making them an integral part of our society [2-5].

Like all animals, dogs are susceptible to health issues. They can develop severe gastrointestinal symptoms, like vomiting, loss of appetite and weight, and diarrhea. When these symptoms persist for more than three weeks, and other causes have been ruled out (like hepatic disease, parasitic disease or intestinal blockage by a foreign body or tumors) chronic enteropathy (CE) is diagnosed [6]. CE can be classified as food-responsive (FRE), antibiotic-responsive (ARE), immunosuppressant-responsive (IRE), or non-responsive (NRE) [7]. It is difficult to find reliable information on the prevalence of CE in dogs as most studies focus on the occurrence of gastrointestinal issues in general [8]. Different studies from the UK, Italy, Japan and Sweden reported a range from 1-17.8% of prevalence of gastrointestinal disease in dogs [7-10]. Some of these studies also investigated the susceptibility of dog breeds to gastrointestinal disease and found that Boxer and Rottweiler were high risk amongst others [7, 9, 10]. The development of CE can have genetic causes, can be caused by the mucosal immune system, environmental factors like diet and disruption of enteric bacteria, or an interplay between the three factors (see figure 1) [11].

An example of genetic factors could be the upregulation of Toll-like receptors (TLR) 2, 4, and 9 which have been associated with the onset of CE in dogs [12, 13]. These TLRs recognize microbe-associated molecular patterns (MAMPs), like peptidoglycans, lipopolysaccharides or unmethylated CpG oligonucleotides. Upon activation they start producing proinflammatory cytokines, causing

upregulation of costimulatory molecules as well as activation of pro-inflammatory signaling pathways. Upregulation of TLRs thus causes upregulation of the inflammatory responses, which can result in chronic inflammation of the intestine [14, 15].

Another contributor to CE prevalence are parvoviral infections caused by the canine parvovirus (CPV). CPV is a small, non-enveloped single stranded DNA virus [16]. Its viral capsid consists of three peptides: VP1, -2 and -3 [17]. Out of these, VP2 is the peptide which determines the antigenicity of the virus [18]. The symptoms of a parvoviral infection include lethargy, fever, no appetite, bloody diarrhea, vomiting and abdominal pain or bloating [18, 19].

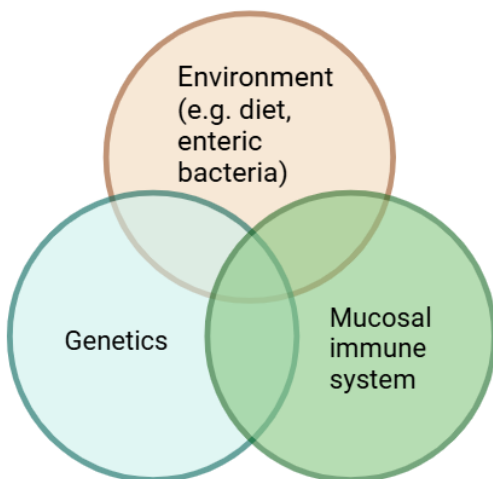


Figure 1: The three factors causing CE. Created in Biorender.com

The virus compromises the intestinal barrier by causing necrosis of the intestinal crypt epithelium, shortening or obliteration of villi, and the dilation of intestinal crypts with necrotic cellular debris which causes many of the observed symptoms [17]. For puppies a parvoviral infection can be even more harmful, as cardiac tissue can be affected. While no specific cure exists, supportive treatment, including hydration and regulating nutrition level, can raise survival rate of virally infected dogs from 9.1% to 64% [16, 19].

Treatment for CE, whether caused by CPV or not, depends on the response of the animal to the treatment. Generally, a change in the diet is first tried, followed by antibiotics and lastly immunosuppressants in case the dog does not respond to the previously tried, less invasive treatment [20]. As not every dog with CE can be helped with a change in diet, using antibiotics can cause antibiotic resistance and immunosuppressants can increase the risk of certain infections and cancers, alternative therapies are investigated, for example spray-dried porcine plasma (SDPP) [10, 21, 22].

1.2 Spray-dried plasma powder

SDPP is a byproduct of the meat industry: it is obtained from the blood of slaughtered animals which have been checked and cleared for human consumption. After addition of anticoagulants, the blood is centrifuged and the resulting plasma is concentrated using filtration or vacuum

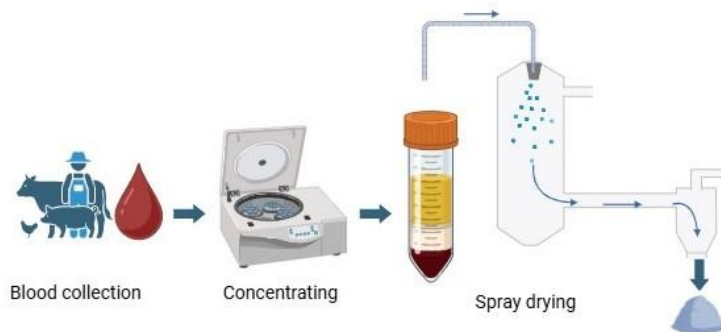


Figure 2: Schematic overview of the production process of SDPP.
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evaporation. The concentrated plasma is spray-dried, resulting in a powder containing plasma proteins (see fig. 2) [23]. Its composition is generally 50–60% albumin, 40–50% globulins and 1–3% fibrinogen. The globulins consist of a group of globular proteins which are subdivided into α , β and γ fractions, the last of which contains immunoglobulins [24].

The immunoglobulins in SDPP have been increasingly investigated because of their immunomodulatory properties. A study showing piglets fed a diet with SDPP while weaning suffered less from diarrhea than piglets offered more traditional diets, demonstrating that SDPP supports gastrointestinal health [25]. The increased gut health could be because of changes in the immune system. In rats, SDPP resulted in a decrease of pro-inflammatory- and increase of anti-inflammatory cytokines present in the mucosa of the small intestine [26]. Intestinal health is not only supported by modulating the immune system. Immunoglobulins have been found to bind a wide range of pathogenic bacteria in addition to inhibiting their growth in an *in vitro* study [27]. In addition to that, another study found that by binding of these bacteria immunoglobulins also prevent them from adhering to the intestinal wall [28]. These gut-health improving functions make SDPP and its immunoglobulins a promising option for treatment of CE.

1.3 Immunoglobulin G

Out of the different immunoglobulins, IgG is the most abundant antibody found in in SDPP [29]. It consists of two heavy chains and two light chains bound by disulfide bridges. The molecule can be split into two regions: the Fab region and the Fc region. The Fab region contains the light chain and part of the heavy chain, which both have a variable region forming the antigen-binding site. The constant Fc region is responsible for the cellular effector functions. It can activate the complement immune system by binding C1q or induce phagocytosis, degranulation, cytokine and superoxide production by various immune cells and antibody-dependent cell-mediated cytotoxicity when binding Fc receptors [30-33].

The Fc receptors IgG specifically binds to, are Fc gamma receptors (FCyR) [34]. These are a family of transmembrane receptors of which, in humans, three different classes have been found, named FcyRI (also known as CD64), FcyRII (also known as CD32) and FcyRIII (also known as CD16) (see fig. 3) [35, 36]. They are expressed by a variety of immune cells, including macrophages, B-cells, T-cells and natural killer cells (NK-cells) [37]. FcyRI is the only known high affinity receptor for IgG, meaning it can bind monomeric IgG, while FcyRII and FcyRIII only bind IgG immune complexes [36]. Binding of IgG-containing immune complexes to FcyRs generally leads to activating of the cell through signaling induced by immunoreceptor tyrosine-based activation motifs (ITAM). The exception is FcyRIIIb which is anchored to the cell by glycan phosphatidylinositol (GPI) [30, 36]. While most FcyRs have an activating role, FcyRIIb is the only known FcyR with an inhibitory function. This is because it contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) which counteracts the effects of the ITAM signaling, giving FcyRIIb a regulatory function [30]. Most of the information concerning FcyRs was found from studying human and murine FcyRs, meaning expression and function in other mammals has not been elucidated as much.

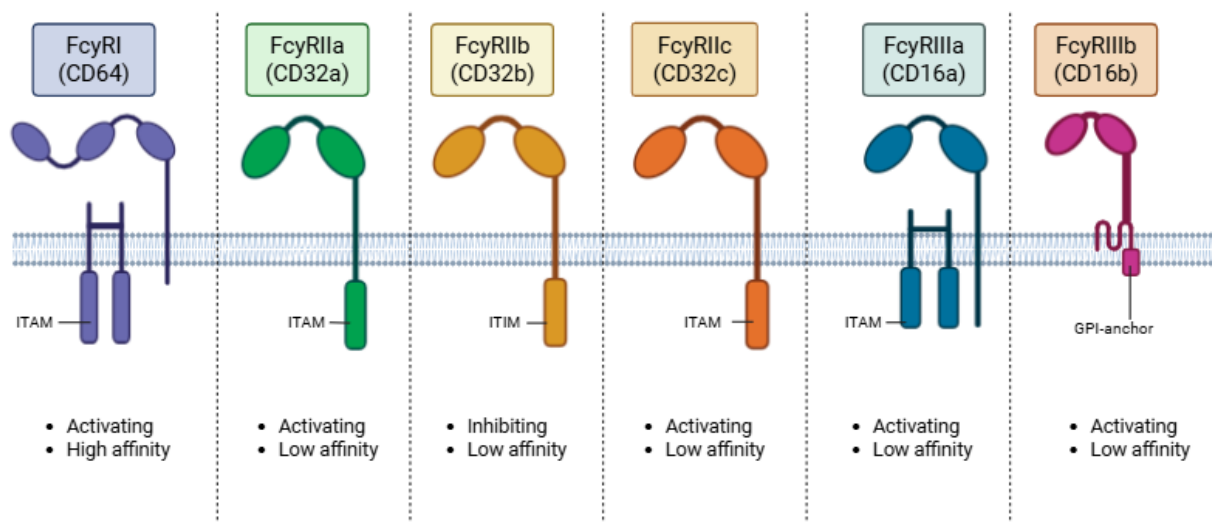


Figure 3: The different classes and their subtypes within the human FcyR family. Created with BioRender.com., adapted from Vogelpoel et al. [35].

1.4 T-cells

Like mentioned in the previous paragraph, Fcγ receptors are also expressed by T-cells. They are formed in bone marrow and then mature in the thymus into T helper cells (CD4+ T-cells) or cytotoxic T-cells (CD8+ T-cells) [38]. For naïve T-cells to be activated, an antigen presenting cell (APC) needs to present an antigen to T-cell receptors with the help of MHCII (major histocompatibility complex II) for CD4+ t-cells and MHCI for CD8+ t-cells. For activation of the T-cell a costimulatory signal is needed through interaction of CD28 receptors on t-cells with B7, a surface protein ligand present on APCs (see fig.4) [39]. Upon activation, T-cell proliferation is induced by protein tyrosine kinases. They activate the phosphatidylinositol second messenger bifurcating pathway leading to calcium/calcineurin and protein kinase C activation, the Ras pathway, and the phosphatidylinositol 3-kinase pathway leading to blast formation and the cell going from a quiescence state (G0 phase) to the G1 phase of the cell cycle. Another effect is upregulation of high affinity receptors for the pro-inflammatory cytokine IL-2 [40]. This cytokine has been found to promote T-cell proliferation as it helps T-cells to go from the G1 to the S phase of the cell cycle by up-regulating certain essential cyclins (cyclin D2, -D3, -E and E2F) and down-regulating cyclin-dependent kinase inhibitor p27kip1 [41]. Proliferation assays are a good way to investigate T-cell responses upon stimulation with a certain antigen like e.g. CPV and is an important method in this study.

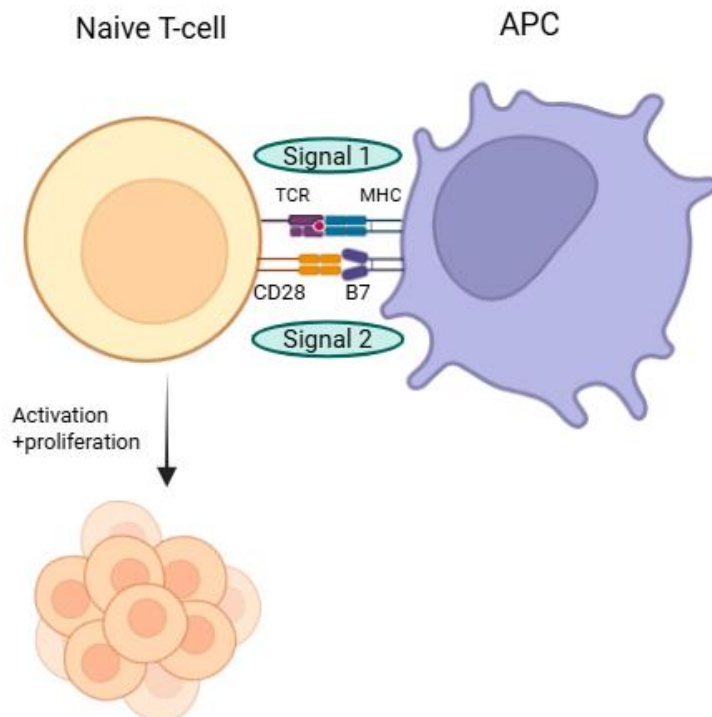


Figure 4: The activation of naïve T-cells by antigen-presenting cells requires multiple signals. Through MHC antigen is presented to the TCR (signal 1). An additionally costimulatory signal by interaction of CD28 on T-cells with B7 on APCs is needed for activation (signal 2). Upon activation proliferation of the T-cells is induced. Adapted from Lee et al. [39]. Created with BioRender.com

To summarize, dogs often suffer from CE, which is treated with medications which can have negative effects. An alternative treatment option is to use SDPP to help boost the dogs immune system. Hence this involves the use of the immune system of pigs to boost the immune system of dogs to fight infections. Therefore, the Fc region of IGG molecules of pigs must be recognizable for the immune system of dogs, a tricky issue and the crux of the study. The aim of this research is to see if adding SDPP to the diet of dogs will help improve their intestinal mucosal health and thus can be used as a treatment for CE.

1.5. Research questions

To answer the question “How does porcine IgG affect the canine immune system?” the following sub-questions were investigated:

- Does canine serum interfere with the measurement of plgG concentrations?
- How does extrusion affect functional plgG?
- Which FcyR receptors are found on which immune cell and how do they interact with plgG?
- Does plgG increase T-cell proliferation upon stimulation with CPV or VP2?

It is hypothesized that canine serum does not interfere with plgG concentration measurements. It is expected that extrusion degrades functional plgG based on the heat and pressure used during the process which degrades IgG. As literature suggests that human and canine FcyRs are analogous, it is hypothesized that in canines the different FcyRs are found on the same type of immune cells as in humans. Based on previous research done it is expected that plgG increases T-cell proliferation when they are stimulated with CPV or VP2.

2. Methods and materials

2.1 PBMC isolation

Canine blood (obtained from the University of Utrecht) was diluted 1:1 with PBS (Thermofisher, # 70011044). This dilution was gently pipetted onto the same volume of Ficoll-paque (cytiva, #17144002). The blood was then centrifuged for 35 min at 400g with the brake turned off. After centrifugation the PBMC layer was transferred by pipet into a different falcon tube and washed twice with PBS (15 min at 500g). After pouring off the supernatant the pellet was resuspended in 90% FBS+ 10% DMSO freeze medium. The vials were then stored at -80°C for at least a day after which they were transferred into liquid nitrogen.

2.2 Flow cytometry

For the flow cytometry experiments FACS buffer (1% BSA+ 0.1% NaN₃ in PBS) was used for washing as well as making dilutions. Washing was done with 200 µl FACS buffer per well for 5 minutes at 300g, unless stated otherwise.

2.2.1 Fc receptor characterization

Canine PBMCs (isolation described in PBMC isolation) were thawed and washed with PBS at 300g for 5 minutes. They were seeded in a Nunc round-bottom 96-well plate (Thermo Fisher, #267245) with 1×10^5 cells per well. Live/dead stain (Invitrogen, #L34963A) was diluted 1000x in PBS, of which 100 µl was added to each well. The plate was incubated at RT in the dark for 15 minutes. After incubation the plate was washed for 4 minutes at 400g at RT. Next the CD32- (Bio-rad, #MCA1075), CD16- and CD64 antibodies (both kindly provided by Bruche Walcheck) were diluted to concentrations of 1 µl/ml, 0.337 µl/ml and 1.03 µl/ml respectively, of which 100 µl was added to the appropriate wells. The plate was incubated at 4°C for 20 minutes in the dark. Cells were spun down for 5 minutes at 300g after which the supernatant was flicked off. As secondary antibody anti-mouse IgG Dylight 680 conjugated antibody (Invitrogen, #35518) was diluted to a concentration of 5 µl/ml, and 100 µl was added per well. Cells in wells without secondary antibody were resuspended with 100 µl FACS buffer. To the wells for the anti-porcine IgG FITC single stain 5 µl purified porcine IgG (pIgG) was added. After incubation at 4°C for 30 minutes in the dark the cells were washed and fixated for 15 min at RT with 100 µl 0.4% Formaldehyde per well. Cells were spun down (5min. at 300g) and the formaldehyde was discarded. The cells were resuspended in 100 µl ice-cold 90% methanol while the plate was on a shaker and permeabilized for 10 min. on ice. After washing additional antibodies were added: anti-porcine IgG FITC, (Southern Biotech, #6050-02), anti-canine T-cell kit (Bio-Rad, #TC014), anti canine B-cell kit (BD Biosciences, #558704), anti-CD14 PE (Miltenyi Biotec, #130-113-147) and anti-human-CD94 iFluor700 (Sigma-aldrich, #CWA-1165-25-UL). They were diluted to a concentration of 1µl/ml (3 µl/ml for the T-cell stain) and 100 µl was added to the appropriate wells. After incubation at 4°C in the dark for 30 minutes the plate was washed twice with 200 µl ice cold FACS buffer for 4 minutes at 400g. Cells were resuspended with 200 µl FACS buffer after which the samples were measured. Two flow cytometers in the CBI facility

were used. The Beckman Coulter Cytoflex LX was used to obtain shown in figure 10 while the Cytoflex was used to obtain the data shown in figure 12. Recommended laser settings were used but during optimization the laser intensity for the PE channel was increased to 600 and for the APC-A750 channel to 1038.

2.2.2. Fc receptor blocking

The execution of this experiment was identical as is described for the Fc receptor characterization. As the secondary antibody 100 µl of pIgG (500 µg/ml) was added to the appropriate wells. To the wells for the anti-porcine IgG FITC single stain and anti-porcine IgG Dylight594 single stain 5 µl purified porcine IgG (pIgG) was added. In addition to the additional antibodies mentioned in the previous chapter, 1 µl/ml Dylight 594 (Bethyl, #A100-105D4) was added to the appropriate wells. Samples were measured using the Cytoflex LX.

2.3 ELISA

For the Enzyme linked immunosorbent assays (ELISA) plates were coated using a 100mM bicarbonate/carbonate coating buffer (3.03g Na₂CO₃, 6.0g NaHCO₃, 1000ml distilled water, pH 9.6). The blocking was done using 200 µl blocking buffer (1.5% gelatin hydrosylates;Roche, #11112589001) per well. Blocking buffer was used for dilutions unless stated otherwise. Between addition of reagents, plates were washed three times with 200 µl washing buffer (0.05% v/v Tween in 1x PBS) per well.

2.3.1 Porcine IgG detection in SDPP dissolved in canine serum

A 96-wells plate (greiner bio-one, cat. Nr. 655001) was coated using 100 µl 10 µg/ml anti-porcine IgG (Bio-rad, cat. Nr. AHP865). Positive control wells were coated with 100 µl 10 µg/ml pIgG, while the negative control wells were not coated. The plate was then incubated at 4°C overnight. To prepare the samples, canine serum (obtained during PBMC isolation described previously) was thawed and its protein concentration was measured using a NanoPhotometer N60. Next 60mg of 70P SDPP (provided by Darling II) was dissolved in 10 ml of the canine serum. For the standard, 60mg of 70P SDPP was dissolved in MilliQ as well. The samples and standard were placed on a roller-bench for 20-30 minutes to mix properly after which they were incubated overnight at 4°C.

After incubation, blocking buffer was added to appropriate wells, after which the plate was incubated at 37°C for one hour. Again, the protein concentrations of the samples and the standard were measured. These were then diluted to 100- and 50ng/ml for the samples and a range of 6,25 ng/ml-100 ng/ml was prepared for the standard. After blocking 100 µl of sample and standard was added to the appropriate wells. The samples were then incubated at RT for one hour. Next 100 µl of the secondary antibody (goat anti-pig conjugated with HRP, Bio-rad, #AHP865P) was added to each well diluted 1:80000. The plate was incubated for 45 minutes in the dark at RT. For colour development 100 µl TMB (Neogen, #308177) was added to each well, which was left to incubate for 3-5 minutes (until color clearly developed in the standard range). To stop the reaction 50 µl of stopping buffer (2M H₂SO₄) was added to all wells, after which the plate was read using a

SpectraMax iD3 spectrophotometer at 450nm and at 580nm. For analysis the 580nm values were subtracted from the 450nm values.

2.3.2 Comparing IgG concentrations in SDPP from the Netherlands, US and China

For sample preparation 60mg of 70P SDPP from Netherlands, US and China (provided by Darling II) was dissolved in 10 ml MilliQ. They were placed on a roller bench for 20 min. after which they were incubated overnight at 4°C. For the standard curve, the protein concentration of purified pIgG was measured and diluted to a range of 6,25- 100 ng/ml. In addition to that, a titration to determine sensitivity of the ELISA was carried out, using a range of 0,01-10 ng/mL pIgG. The ELISA was carried out similar as described in the previous chapter.

2.3.3 Determining functional IgG pre- and post- extrusion

A Plate was coated with LPS (Sigma-aldrich, #L2630) and samples were made by dissolving 60 mg kibble mix with and without SDPP in MilliQ, as well as one unit of the extruded kibble with and without SDPP. Otherwise the ELISA was carried out as described in the “Porcine IgG detection in SDPP dissolved in canine serum” chapter.

2.3.4 Bovine IgG concentration

The sample was prepared by dissolving 60 mg bovine spray dried plasma (provided by Darling II) in 10 ml MilliQ. For the experiment the Bovine IgG ELISA kit from abcam (cat. Nr. ab205078) was used and the experiment was carried out according to the manufacturers provided protocol.

2.4. T-cell proliferation assay

Canine PBMCs were thawed and washed with culture medium (RPMI 1640 medium+ GlutaMax, gibco, #72400-021, and 1% penicillin/streptomycin) for 5 min at 300g. They were seeded in black 96-wells plates with clear bottom (revvity, #6005225) with 1×10^5 cells in each well. In addition to the cells, 100 μ l of stimulant, either CPV (strain 2A, obtained via R.J.Corbee, veterinarian) or VP2 (abbexa, #abx160043), was added in concentrations ranging from 0,156-5 μ g/ml with and without pIgG (100 μ l). The plate was then incubated at 37°C for 7 days. After 6 days 50 μ l of supernatant was removed with a pipet and stored for further experiments. After one more day of incubation the BrdU assay was done using the Cell Proliferation ELISA kit (Roche, #11669915001). The protocol of the manufacturer was followed. To measure fluorescence the plate was read using the LUM setting of the SpectraMax iD3 spectrophotometer.

2.5. Data analysis

The flow cytometry data obtained was analysed using FlowJo (ver. 10.10.0), where a compensation matrix was obtained and applied to samples. Gating strategies seen in supplementary figure 1,2,3 and 5 were used. Excel was used in combination with Graphpad prism (ver.10.4.1) to analyse data obtained from ELISAs as well as performing linear regression for experiments described in chapters 2.3.2- 2.3.4. Results from experiments described in chapters 2.3.2 and 2.3.4 were combined by multiplying the values of the samples diluted to 50 ng/ml by two and calculating the mean with results of the 100 ng/ml diluted samples. To determine the limit of detection the formula $3.3 \left(\frac{Sy}{S} \right)$

was used where S_y is the standard deviation of the response and S is the slope of the calibration curve. S_y and S were determined with the LINEST function in Excel. The limit of detection was confirmed by performing 4PL analysis. Experiments with multiple repeats are shown as mean \pm SD. Significant differences were determined using one-way ANOVA tests where P-values with $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Porcine IgG ELISA - detection limit

To determine the detection limit of an in-house ELISA used to study porcine IgG levels, titrations were carried out. A plate was coated with anti-porcine IgG antibodies, and purified porcine IgG was added at different concentrations in the range of 0,01-10 ng/ml (see fig. 5). Plotting the data and carrying out 4PL analysis helped determine a detection limit of 0,044 ng/ml while using the formula given in chapter x to calculate the detection limit a value of 0,035 ng/ml was found. When taking the average of the values found a detection limit of 0,0395 ng/ml can be assumed.

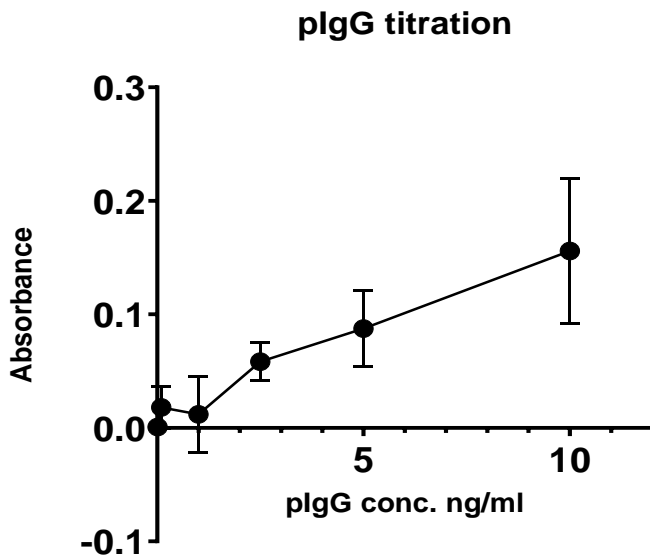


Figure 5: Absorption values of a titration curve to examine the limit of detection. A plate was coated with anti-porcine IgG while purified porcine IgG was added in a range of known concentrations (0.01-, 1-, 2.5-, 5- and 10 ng/ml). $n \geq 2$

3.2. SDPP in canine plasma

To determine if canine serum interferes with the measurement of porcine IgG, ELISAs were performed. SDPP was dissolved in canine serum from three dogs, which were then diluted to a total protein concentration of 100- and 50 ng/ml. These were then compared to a standard curve made by dissolving SDPP in MilliQ. The obtained absorption values are presented in figure 6.

The absorption values of the samples (with canine serum) lie below the standard curve. It was also shown that the measurement of just canine serum without SDPP yielded low absorption values.

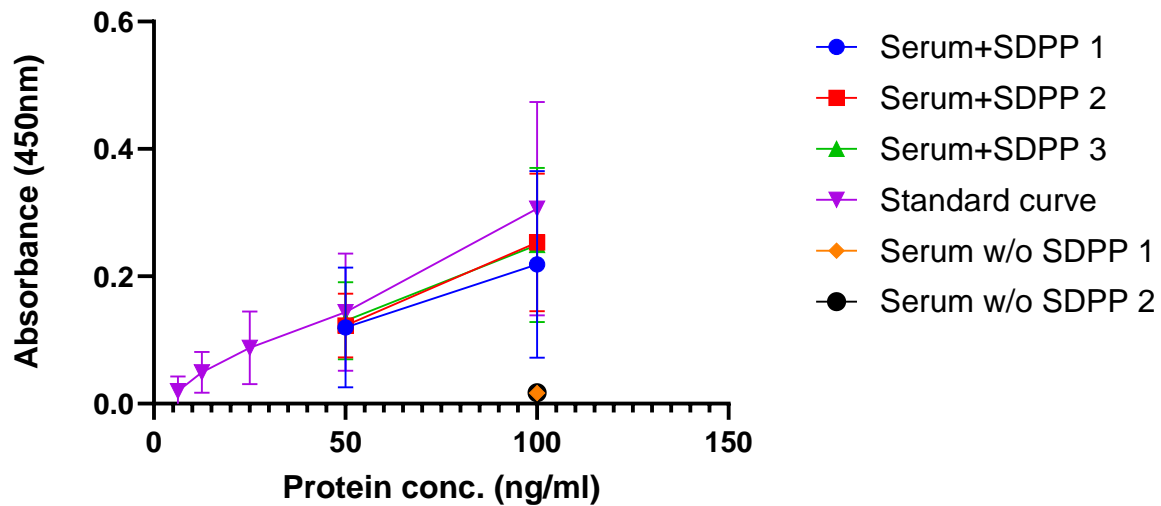


Figure 6: The absorption values of an ELISA to measure porcine IgG detection. The samples were made from serum of different dogs in which SDPP was dissolved to observe if the canine serum would interfere with the porcine IgG detection in the blood of the potential test animals. For the standard curve, SDPP dissolved in MilliQ was used and diluted to a series of known protein concentrations (100-,50-,25-,12.5- and 6.25 ng/ml.). As an extra control, canine serum without SDPP dissolved in it was measured at a protein concentration of 100 ng/ml. n=3

3.3. IgG conc in SDPP from the Netherlands, US and China

There are several factors which can influence the amount of IgG in the SDPP like the environment the pigs are held in, the type of food they eat and diseases they have. For this reason, IgG concentrations in SDPP obtained from pigs from the Netherlands, US and China were measured and compared using ELISA.

A difference in IgG percentage in the SDPP between the countries could be observed, with the SDPP from the US showing the highest percentage with an average of 47.8, followed by the Netherlands with a percentage of 47.1 (see fig. 7). The lowest concentrations could be seen for the SDPP from China with a pIgG percentage of 4.6. However, the results also do show a high standard deviation between the repeats.

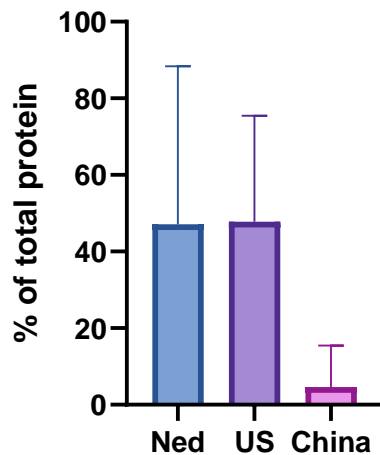


Figure 7: The measured percentage of IgG present in SDPP from pigs from the Netherlands (blue), US (purple) and China (pink). All samples were diluted to a total protein concentration of 50- and 100 ng/ml. A standard curve was used with known concentrations of purified pIgG (6.25-, 12.5-, 25-, 50- and 100 ng/ml) to obtain the percentage of pIgG in total protein with linear regression. No significant differences between samples could be found. n=4

3.4. Functional IgG pre- and post- extrusion

To get SDPP into the canine diet, multiple methods can be used. One is extrusion: here, a dough is made of the ingredients, after which the dough is formed under high heat and pressure. Therefore, an ELISA was set up to determine whether functional porcine IgG remained after the extrusion process. Dog kibble mix (the powder from which kibble is made by extrusion) with and without SDPP was dissolved in MilliQ, as well as one unit of dog kibble with and without SDPP obtained from the mixes after extrusion. The samples were then diluted to total protein concentrations of 10-, 33- and 100 µg/ml. To determine functionality, ELISA plates were coated with LPS as a model for bacteria binding.

For the dog kibble mix with SDPP functional IgG was found to be present in a concentration dependent manner. For all other samples no functional IgG could be detected (see fig. 8).

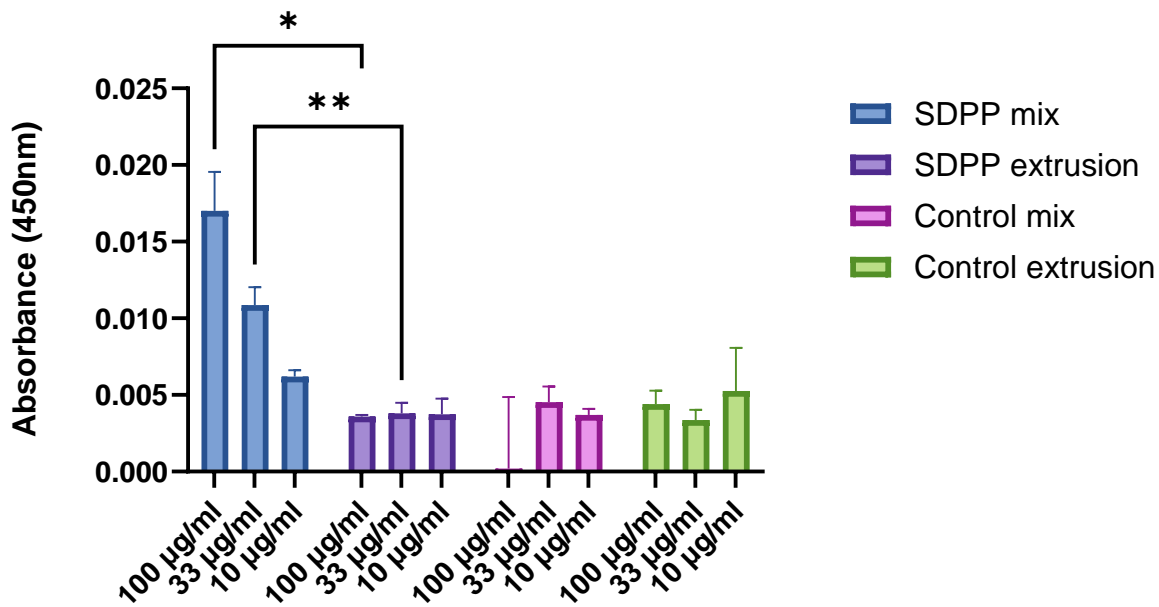


Figure 8: The measured absorption values of an ELISA to measure functional IgG. Dog kibble mix with SDPP (blue), without SDPP (pink) and units of dog kibble with SDPP (purple) and without SDPP (green) were dissolved in MilliQ and diluted to total protein concentrations of 10-, 33- and 100 µg/ml. The plate was coated with LPS as a bacterial binding model. Significant differences between samples are indicated as * p<0.05 and ** p<0.01. n=1

3.5. Bovine IgG conc

To determine the IgG concentration in bovine spray dried plasma an ELISA was performed. The sample, obtained from Darling Ingredients, was dissolved in MilliQ and diluted to 100- and 50 ng/ml total protein.

For the bovine SDPP a mean IgG percentage of total protein of 18.6 was found (see fig. 9).

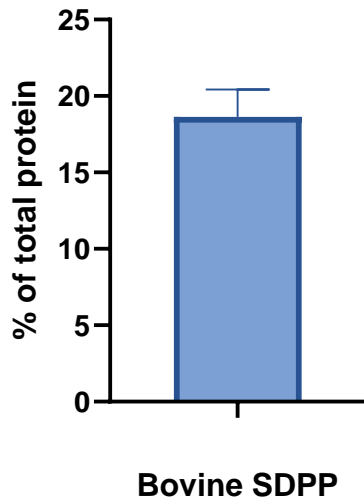
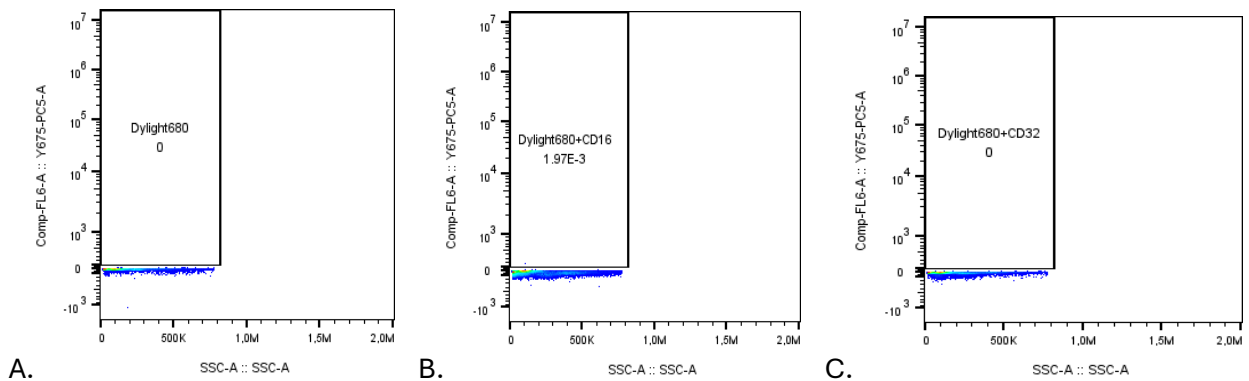


Figure 9: IgG percentage of total protein of bovine SDPP obtained through an ELISA. Bovine SDPP was dissolved in MilliQ and diluted to a total protein concentration of 50- and 100 ng/ml. A standard curve was used with known concentrations (3.125-, 6.25-, 12.5-, 25-, 50- and 100 ng/ml) of purified bovine IgG to obtain the percentage of plgG in total protein with linear regression. n=2

3.6. Fc receptor characterization optimization

To study which FcγRs are present on which canine cells, flow cytometry was used. For canine immune cells, NK-cells, macrophages, B-cells and T-cells were investigated. Unlabeled antibodies against CD16 (FcγRIII), -32 (FcγRII) and -64 (FcγRI) were used in combination with anti-mouse IgG labeled with DyLight680 to detect the expression of the three receptors on the previously mentioned canine PBMCs.

It proved to be difficult for some of the conjugated antibodies, especially the anti-mouse-IgG-DyLight680 conjugate and at times the anti-CD14-PE conjugate, to get a signal for the first experiments (see fig. 10). Because of this optimization experiments were carried out.



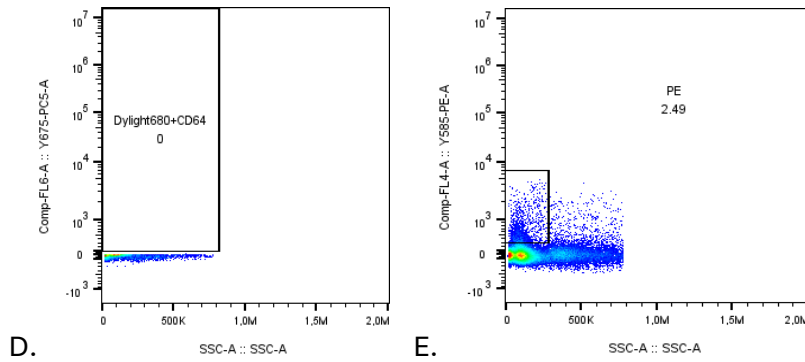


Figure 10: Dot-plots of canine PBMCs stained with anti-mouse-IgG-DyLight680 conjugate without primary antibody (A), - with anti-CD16 (B), - anti-CD32 (C) and – anti-CD64 (D) as well as PBMCs stained with anti-CD14-PE conjugate (E). For gating strategy which was used to obtain plots in this figure see supplementary figure 1.

First, a titration of the DyLight680-labeled secondary antibody was done using the anti-CD14 antibody. The DyLight680-labeled antibody binds mouse antibodies, therefore could also bind the anti-human-mouse derived anti-CD14 antibody. For the titration the DyLight680-labeled antibody was diluted to 0.1-, 1-, 2.5 and 5 µl/ml.

The results shown in figure 12 indicate that the 1 µl/ml dilution already shows a shift in intensity in the APCA-750 channel, with the intensity being even higher for the 5 µl/ml concentration (see fig. 11). Based on the titration, a concentration of 2.5 µl was chosen for further experiments.

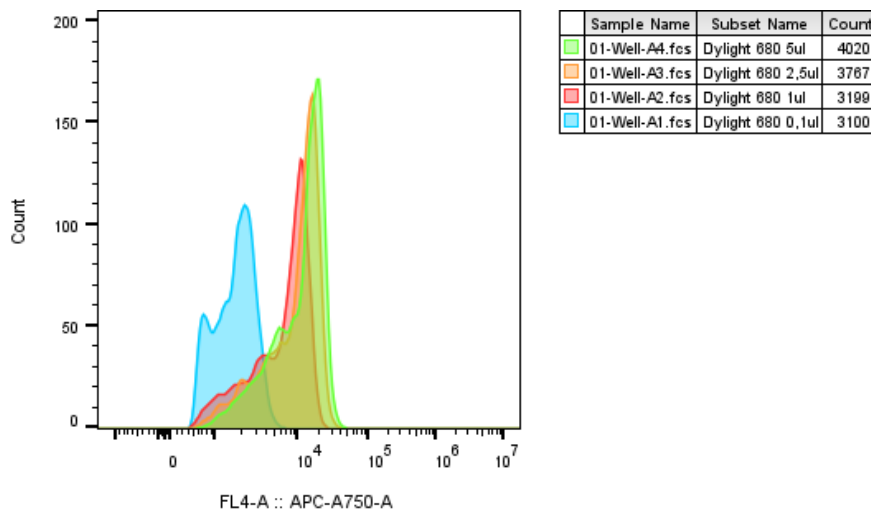


Figure 11: Histogram of Dylight680-labeled antibody titration using mouse derived anti-CD14 antibody. Dylight680 antibody was diluted to 0.1- (blue), 1- (red), 2.5- (orange) and 5 (green) µl/ml. For gating strategy which was used to obtain histograms in this figure see supplementary figure 2.

After adjusting the intensity of the lasers used for visualizing anti-mouse-IgG-DyLight680 conjugate and anti-CD14-PE conjugate as well as increasing the DyLight680 labeled antibody concentration improvements for the stains were observed (Figure 12).

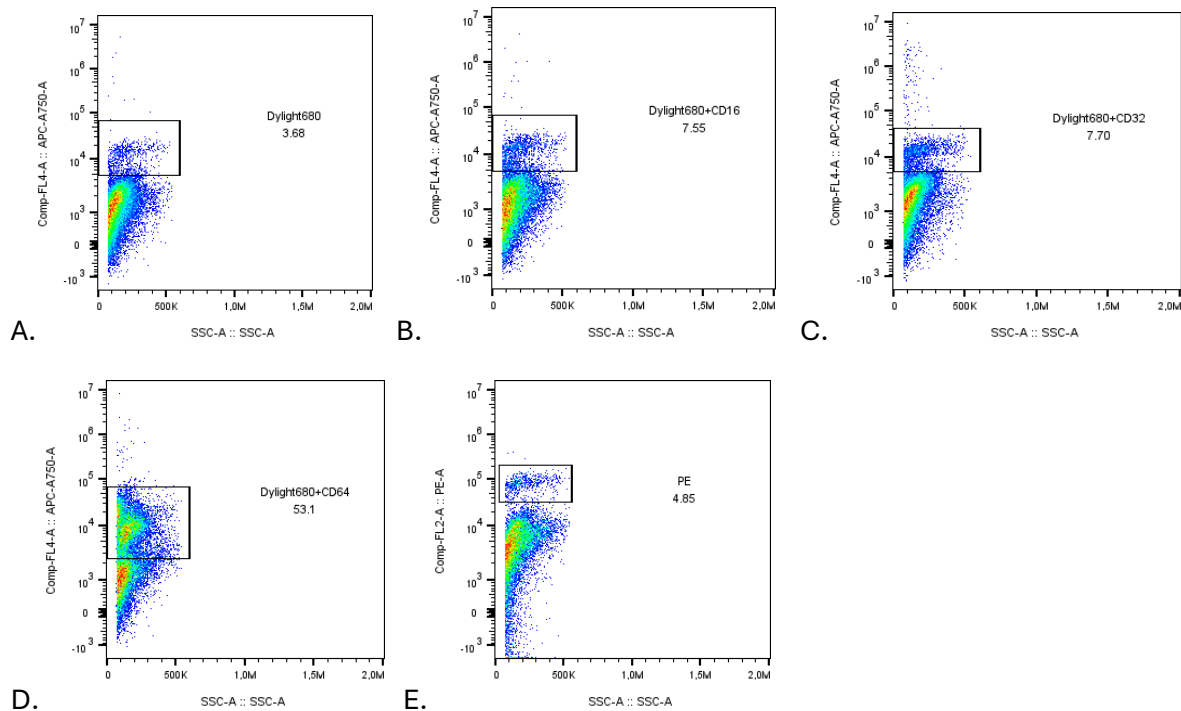


Figure 12: Dot-plots of canine PBMCs stained with anti-mouse-IgG-DyLight680 conjugate without primary antibody (A), - with anti-CD16 (B), - anti-CD32 (C) and – anti-CD64 (D) as well as PBMCs stained with anti-CD14-PE conjugate (E). For gating strategy which was used to obtain plots in this figure see supplementary figure 3.

Due to time constraints only optimization could be done and no real conclusions about Fc receptor characterization could be drawn based on the data.

3.7. Fc receptor blocking

To investigate which FcγR pIgG interacts with on different canine immune cells, flowcytometry was performed. Anti-CD16 -32 and -64 were added (on its own or in combination) to block specific FcγRs after which pIgG was added as well as anti-porcine IgG labeled with DyLight594.

First an experiment was conducted to make sure the DyLight594-labeled antibody does not bind to CD16, -32 and -64. The results seen in figure 13 confirm this, meaning that when the experiment is conducted the signal seen for DyLight594 is only from antibodies bound to pIgG.

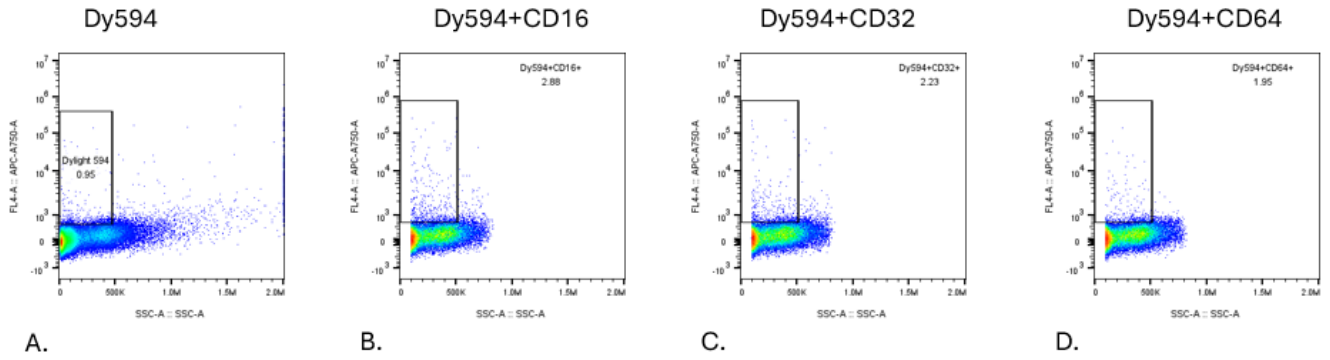
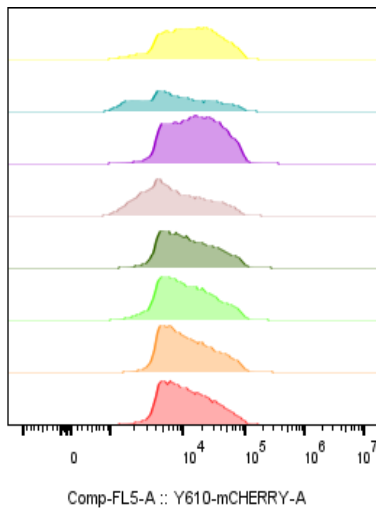


Figure 13: Dot-plots of canine PBMCs stained with DyLight594 without primary antibody (A), -DyLight680 with CD16 (B), - CD32 (C) and -CD64 (D). For gating strategy which was used to obtain plots in this figure see supplementary figure 3.

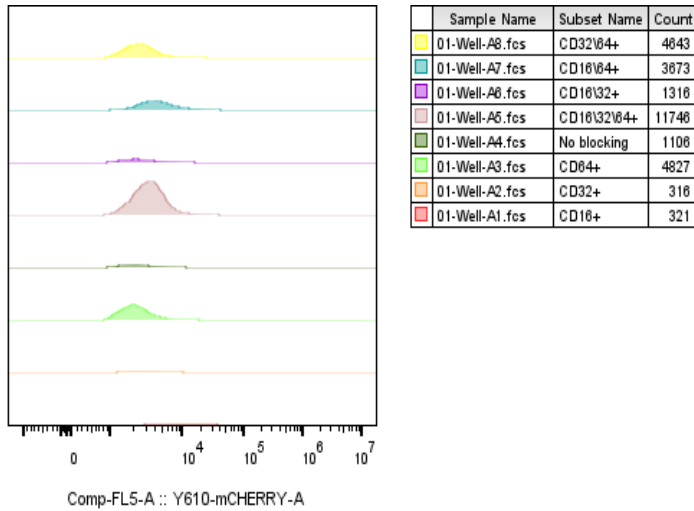
For the previous described blocking experiment the macrophages, CD4+ T-cells and B-cells were stained with antibodies conjugated with PE, however, this stain did not seem to work for all experiments (see supplementary figure 4). Because of this only NK-cells and CD8+ T-cells could be investigated. For CD4+ T-cells all CD3+ cells which were not positive for CD8 were looked at.

For CD4+ T-cells no difference could be observed between the different conditions (see figure 14 A), except for one of the repeats which showed a slight increase in intensity for the cells blocked with all three antibodies and cells blocked with the CD16+CD64 combination (see figure 14 B).



Sample Name	Subset Name	Count
01-Well-A8.fcs	CD32 ^{lo} 4+	22914
01-Well-A7.fcs	CD16 ^{lo} 4+	13958
01-Well-A6.fcs	CD16 ^{lo} 32+	33105
01-Well-A5.fcs	CD16 ^{lo} 32 ^{lo} 4+	22079
01-Well-A4.fcs	No blocking	21864
01-Well-A3.fcs	CD64+	26293
01-Well-A2.fcs	CD32+	25476
01-Well-A1.fcs	CD16+	24831

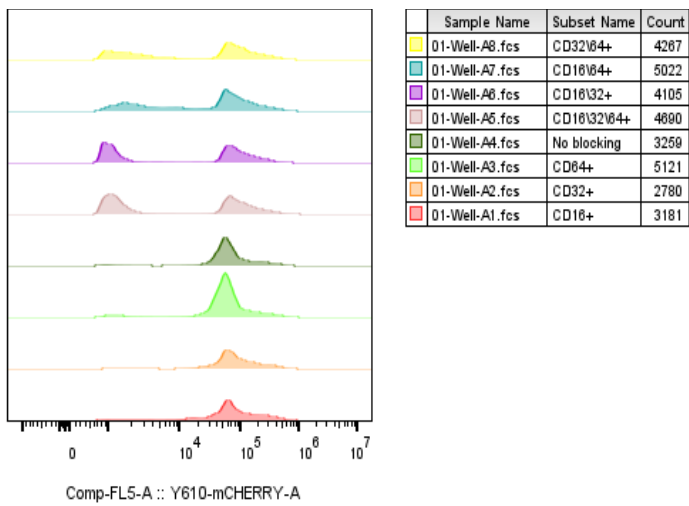
A.



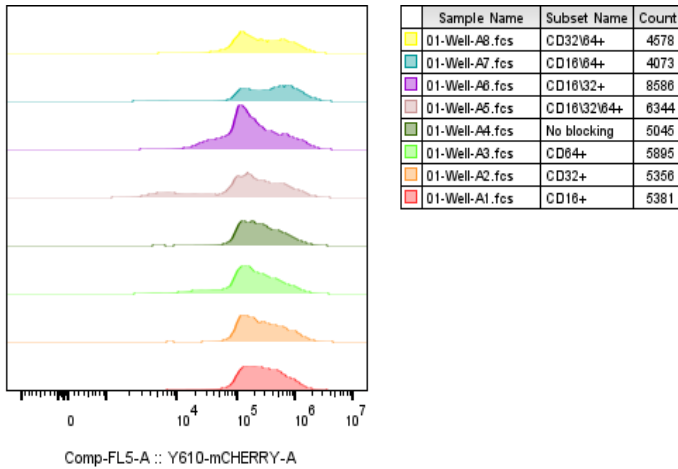
B.

Figure 14: Histograms showing binding of pIgG to CD4⁺ T-cells. FcγRs on the cells were blocked using anti-CD16, -32, -64 or any combination of the antibodies. The experiment consists of n=4 biological repeats where A is a representation of one repeat and B shows the histogram of an outlier within those repeats. For gating strategy which was used to obtain histograms in this figure see supplementary figure 3.

The CD8⁺ T-cells show two populations for the cells which were blocked with multiple antibodies for one of the repeats (see figure 16 A) while this is only the case for the cells blocked with CD16/32/64⁺ in another repeat (see figure 16 B). For the other experiments no difference in intensity between conditions was observed.



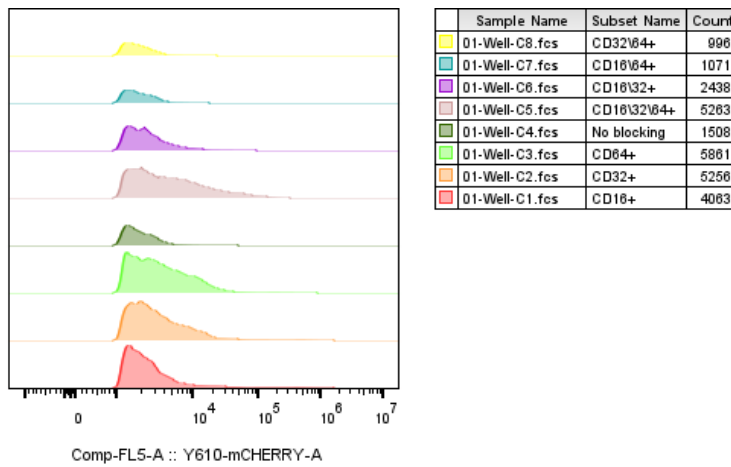
A.



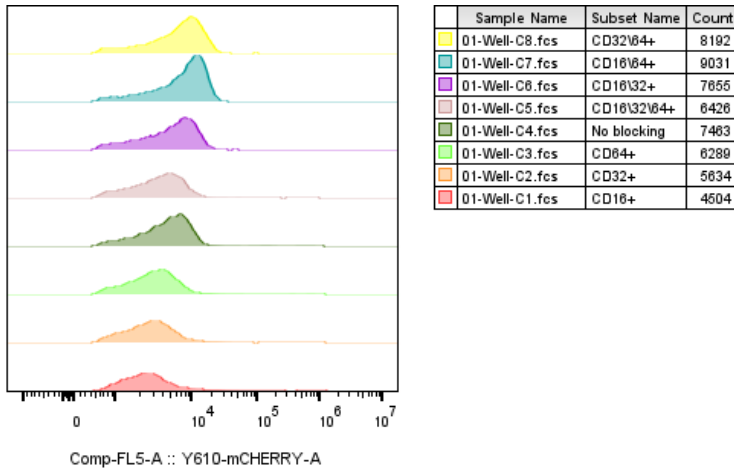
B.

Figure 15: Histograms showing binding of pIgG to CD8+ T-cells. FcγRs on the cells were blocked using anti-CD16, -32, -64 or any combination of the antibodies. The experiment consists of n=4 biological repeats where A and B both show different repeats which are outliers. For gating strategy which was used to obtain histograms in this figure see supplementary figure 3.

The NK-cells did not show a difference in intensity between conditions (see figure 16 A) except for one of the repeats, which showed a shift for the cells which were not blocked or the cells which were blocked with multiple antibodies except for the cells blocked with all three simultaneously (see figure 16 B)



A.

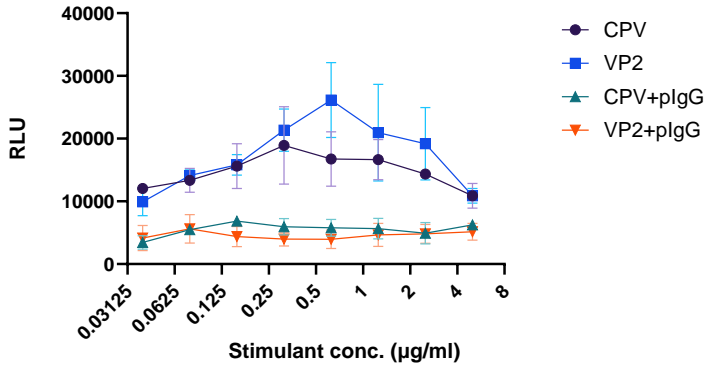


B.

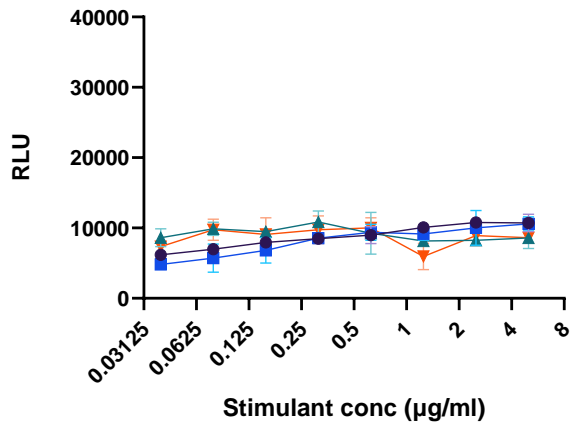
Figure 16: Histograms showing binding of pIgG to NK-cells. FcγRs on the cells were blocked using anti-CD16, -32, -64 or any combination of the antibodies. The experiment consists of n=4 biological repeats where A is a representation of one repeat and B shows the histogram of an outlier within those repeats. For gating strategy which was used to obtain histograms in this figure see supplementary figure 3.

3.8. BrdU assay

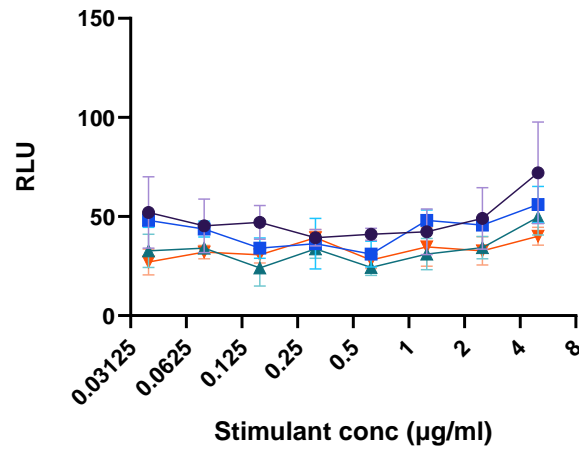
A BrdU assay for cell proliferation was performed to determine canine T-cell activation/proliferation with and without pIgG present. For this, canine PBMCs were stimulated with CPV and VP2 diluted 0-64x starting from 5 µg/ml for 7 days with and without pIgG present. As can be seen in figure 17, the addition of pIgG resulted in a decrease in fluorescence compared to when pIgG was not present, indicating a decrease in cell proliferation. For one of the repeats, however, there was an increase in fluorescence observed when pIgG was added at stimuli concentrations of 0.625 µg/ml or lower (fig. 17 B). For one of the repeats a concentration dependent increase of fluorescence was observed for the PBMCs stimulated without pIgG (fig. 17 B), however, this was not the case for other conditions and repeats.



A.



B.



C.

Figure 17: T-cell proliferation measured with BrdU assay. Fluorescence was measured after PBMCs were stimulated with CPV (dark purple), VP2 (blue), CPV with plgG (green) and VP2 with plgG (orange). On the y-axis the obtained relative light units (RLU) are plotted. Stimulant was diluted 0-64x starting from 5 µg/ml of which concentrations are plotted on the x-axis. Figure A, B and C depict the results of one of the repeats each. n=3

4. Discussion and conclusion

The aim of this thesis was to investigate the effect of porcine IgG found in SDPP on canine PBMCs, taking into account that ingestion of porcine IgG would be oral. To achieve this aim, flow cytometry experiments to analyze canine FcγR expression and study binding of porcine IgG to these receptors were optimized. Experiments confirmed specificity of the in-house porcine IgG ELISA, even when dissolved in canine serum. ELISAs further helped determine the concentration of porcine IgG in SDPP samples of various origins and showed that processing of SDPP into the diet can greatly affect the functionality of its IgG. It appears CPV-specific T-cell proliferation is reduced by the addition of porcine IgG.

4.1. Porcine IgG concentration from SDPP in canine serum

Firstly, the interference of canine serum on pIgG concentration measurements was studied. This was done as preparation for future safety studies where pIgG concentrations will be measured in canine blood samples. For this, cross-reactivity of the anti-porcine-IgG capture antibody with components from canine serum (mainly canine IgG) present in these blood samples needed to be ruled out. Literature shows that while porcine- and canine IgG share some basic similarities they do differ. Four subclasses of canine IgG have been identified, where porcine IgG is comprised of at least 6 subclasses which are divided into further subtypes [42]. For porcine IgG the CH1 domain is most conserved between subclasses, while the CH2- and CH3 domain (which comprise the lower hinge and Fc region) are highly variable [42]. For human IgG the CH2-3 domains are not as variable between different IgG subclasses [43]. This is not known for canine IgG, however, Bergeron et al. imply that it is analogous to human IgG, so a similar homology is expected [44]. Another difference between canine and porcine IgG is the glycosylation patterns in their FC region. This difference lies in their sialic acids where pigs have high levels of *N*-glycolylneuraminic acid (Neu5Gc), which is absent in dogs [45]. The variability in the hinge and FC region as well as different glycosylation patterns make the different IgGs specific as it affects their binding and functions. This leads to the hypothesis that no binding of the anti-porcine-IgG to canine IgG takes place. Results show SDPP dissolved in canine serum showed detection of porcine IgG close to the standard curve, while canine serum without SDPP did not give a clear signal. This shows that canine IgG is not bound by anti-porcine IgG, supporting the previous stated hypothesis.

4.2. Functional IgG ELISA

The study this thesis contributed to focuses on the use of porcine IgG to support canine gut health. As dog food comes in different options there are several possibilities to get porcine IgG into the canine diet. Due to its long shelf-life and convenience kibble commonly used [46]. However, it is produced through extrusion, where a dough is pressed through a die under heat and pressure. This was shown to have a negative effect on the nutritional value of dog food as it degrades heat-labile proteins and vitamins [47]. Coating the kibble after extrusion with SDPP could circumvent this issue. Fat needs to be melted as a “carrier” for the SDPP, however, this can be done at lower temperatures (up to 45°C) as described by Quigley et al [48]. Another option would be using wet dog food instead of dog kibble. This would present the same issue as the cans are sterilized at high heat (121°C–140°C) [49].

To find a suitable option to get pIgG into dog food the effect of the extrusion process specifically on pIgG in dog kibble was studied. LPS was used as a model for functionality. It was expected to only find functional pIgG in the SDPP kibble mix which hadn't gone through the extrusion process. The high temperatures used during extrusion (in this specific case at 140°C) cause IgG to be degraded. Literature shows that for IgG this happens at 70°C [50]. For the first experiments no functional IgG was found for any of the samples. However, they were left outside of the fridge to incubate on a roller bench for 3 nights, which could have degraded the functional IgG. Once new samples with a one day roller bench incubation were made, the measurements improved. Now functional IgG could be observed for the kibble mix with SDPP as we expected. The measurements yielded low values with samples diluted to 10-100 µg/ml so for future repeats it would be interesting to try less diluted samples (10-100 mg/ml e.g.) to see if this improves the results. More investigation needs to be done into how to get functional IgG into dog food. It has been shown by the results that mixing SDPP into dog kibble which undergoes extrusion is not an option as the pIgG gets degraded. For future research the other options previously mentioned need to be investigated. Based on the results it is expected that using wet dog food is also not an option as the heat treatment will degrade the functional pIgG. It appears that coating the already processed dog kibble with SDPP is the best option when it is done carefully at lower temperatures. It is not the preferred method as the SDPP is not as protected from the dogs digestive system compared to when it is mixed into the kibble, but it is an option worth exploring, nonetheless.

4.3. BrdU assay

If SDPP is able to help treat CE in dogs it is expected that pIgG can interact with and stimulate the canine immune system. To study this, T-cell proliferation was looked at. According to literature they are one of few cells able to undergo clonal proliferation which happens as a result of stimulation by a pathogen, making it a good indicator of cell activation [51]. Literature also shows that the majority of PBMCs are lymphocytes (70-90%) of which CD3+ T-cells make up 70-85% [52]. This means when looking at clonal proliferation in PBMCs most of the proliferation observed is expected to be by T-cells. For this thesis a BrdU (bromodeoxyuridine) assay was used to observe cell proliferation. BrdU is a thymidine analog, which is incorporated into newly synthesized dna and can be stained by Anti BrdU-POD conjugated with fluorescent dye [53]. The cell proliferation can then be observed by measuring the fluorescence, which should increase with more new DNA synthesized. For this experiment, an increase in cell proliferation was expected with an increase of stimulant concentration. The co-incubation with pIgG was also expected to increase cell proliferation and previous research done at CBI showed that pIgG forms immune complexes with CPV and these complexes interact with lymphocytes, granulocytes and monocytes (esp. CD14+) [54]. The dogs of which PBMCs were isolated from were vaccinated against CPV, so memory cells should be present to interact with and give response to the CPV-IgG IC. The results, however, were not as expected. For two of the dogs the PBMCs show less fluorescence with pIgG, indicating an inhibitory effect of pIgG on the cell proliferation. Literature shows that IgG can act as an antagonist for FcγRs [55]. This means it binds to a part of the receptor without activating it and blocking it in the process (in this case for canine IgG), resulting in a decrease in cell activation and thus proliferation. There is

evidence of IgG functioning as a neutralizing antibody, where it binds to a pathogen rendering it non-infectious [56-58]. This might have been the case here, where pIgG binds the CPV so effectively that it prevents canine immune cells from interacting with it, preventing activation and proliferation. One dog showed higher proliferation for cells with pIgG, however, this was only at lower stimuli concentration. This might indicate that pIgG has an effect at low pathogen concentrations, hinting at a potential for it not being a cure for CE but a prevention measure. It is evident that the results do not allow for a clear conclusion and more research needs to be done. More repeats with PBMCs of different dogs could be done to see if a certain trend in cell proliferation can be discovered with a bigger sample size. Furthermore, the activation of canine FcγRs by pIgG needs to be investigated to get a better understanding of its effect on canine immune cells. The activation of the canine immune cells by pIgG could also be studied in other ways e.g. by measuring how pIgG affects granzyme B or IFN-γ release of the cells.

4.4. FcγR characterization

As the previous paragraph shows, for IgG to have effect its interaction with FcγRs on canine immune cells important. To get a better understanding of this, an attempt to characterize the different FcγRs present on different canine immune cells was made. The results, however, were inconclusive so optimization needed to be done. After adjusting laser settings for the Dylight680 and PE channel and increasing the concentrations of the Dylight680 antibody and T-cell kit the controls improved as expected. For further research, a characterization with the optimized protocol would be helpful, which due to time constraints was not possible for me to do. Literature shows that in humans FcγRI is found on monocytes, FcγRII on neutrophils, monocytes, eosinophils, platelets and B-cells and FcγRIII on neutrophils, large granular lymphocytes, and macrophages [36]. It is assumed to be the same for canine FcγRs as Bergeron et al. suggests that they are analogous to human FcγRs [44].

5. Conclusion

Based on the results found it can be concluded that canine serum does not interfere with the measurement of pIgG in blood samples. The extrusion process degrades the functional pIgG present in the SDPP in dog kibble. pIgG also seems to have a negative effect on the activation of the canine immune system, but this cannot surely be confirmed by the obtained results. Further experiments are also needed to confirm expression of FcγRs on canine immune cells and their binding by pIgG.

6. References

1. *Domestic dog* [cited 2024 22/10]; Available from: <https://www.nationalgeographic.com/animals/mammals/facts/domestic-dog>.
2. Tahir Tak, M.D.H.G. *No bones about it: Dogs are good for your health*. 2023 [cited 2024 22/10]; Available from: <https://www.mayoclinichealthsystem.org/hometown-health/speaking-of-health/dogs-are-good-for-your-health>.
3. A., S. *Types of service dogs and how they help their humans*. . 2024 June 13 [cited 2024 23/10]; Available from: <https://www.petmd.com/dog/general-health/types-of-service-dogs>.
4. Anderson, K., & Anderson, K., *What are medical alert service dogs and what they do*, in *CertaPet® - Emotional Support Animal Letters*. 2024.
5. *Dogs in the Police Force & Military: Types & Roles* 2020 November 25 [cited 2024 23/10]; Available from: <https://www.purina.co.uk/find-a-pet/articles/dog-types/breed-guides/police-military-dogs>.
6. Dandrieux, J.R., *Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same?* *J Small Anim Pract*, 2016. **57**(11): p. 589-599.
7. Holmberg, J., et al., *Chronic Enteropathy in Dogs-Epidemiologic Aspects and Clinical Characteristics of Dogs Presenting at Two Swedish Animal Hospitals*. *Animals (Basel)*, 2022. **12**(12).
8. Dandrieux, J.R.S. and C.S. Mansfield, *Chronic Enteropathy In Canines: Prevalence, Impact And Management Strategies*. *Vet Med (Auckl)*, 2019. **10**: p. 203-214.
9. Kathrani, A., Werling, D., & Allenspach, K., *Canine breeds at high risk of developing inflammatory bowel disease in the south-eastern UK*. *Veterinary record*, 2011. **169**(24): p. 635.
10. Maria Chiara Marchesi, C.C.T., Sara Busechian, Camillo Pieramati and Fabrizio Rueca, *The role of diet in managing inflammatory bowel disease affected dogs: a retrospective cohort study on 76 cases*. *Veterinaria italiana*, 2017. **53**(4): p. 297-302.
11. Allenspach, K., *Clinical Immunology and Immunopathology of the Canine and Feline Intestine*. *Veterinary Clinics of North America: Small Animal Practice*, 2011. **41**(2): p. 345-360.
12. McMahon, L.A., et al., *Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease*. *Veterinary Immunology and Immunopathology*, 2010. **135**(1): p. 158-163.
13. Allenspach, K., et al., *Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies*. *Veterinary Microbiology*, 2010. **146**(3): p. 326-335.
14. I.A. Burgener, A.K., K. Allenspach, S.N. Sauter, J. Boisclair, M.G. Doherr, T.W. Jungi, *Upregulation of Toll-Like Receptors in Chronic Enteropathies in Dogs*. *Journal of Veterinary Internal Medicine*, 2008. **22**(3): p. 553-560.
15. Aarti Kathrani, A.H., Brian Catchpole, Lorena Alvarez, Kenneth Simpson, Dirk Werling, Karin Allenspach, *TLR5 Risk-Associated Haplotype for Canine Inflammatory Bowel Disease Confers Hyper-Responsiveness to Flagellin*. *PLoS ONE*, 2012. **7**(1).
16. Goddard, A. and A.L. Leisewitz, *Canine Parvovirus*. *Veterinary Clinics of North America: Small Animal Practice*, 2010. **40**(6): p. 1041-1053.

17. Kilian, E., et al., *Long-term effects of canine parvovirus infection in dogs*. PLoS One, 2018. **13**(3): p. e0192198.
18. Adly, M.M., et al., *Molecular characterization of full-length VP2 gene of canine parvovirus type 2 strains circulating in Egypt 2019-2021*. Comp Immunol Microbiol Infect Dis, 2024. **110**: p. 102190.
19. *Canine parvovirus*. American Veterinary Medical Association [cited 2025 13/03]; Available from: <https://www.avma.org/resources-tools/pet-owners/petcare/canine-parvovirus>.
20. Makielski, K., et al., *Narrative review of therapies for chronic enteropathies in dogs and cats*. J Vet Intern Med, 2019. **33**(1): p. 11-22.
21. Llor, C. and L. Bjerrum, *Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem*. Ther Adv Drug Saf, 2014. **5**(6): p. 229-41.
22. *Risk factors: immunosuppression*. 2015, April 29 [cited 2024 28/10]; Available from: <https://www.cancer.gov/about-cancer/causes-prevention/risk/immunosuppression>.
23. Blázquez, E., et al., *Biosafety steps in the manufacturing process of spray-dried plasma: a review with emphasis on the use of ultraviolet irradiation as a redundant biosafety procedure*. Porcine Health Management, 2020. **6**(1): p. 16.
24. Dávila, E., et al., *Heat-induced gelation of porcine blood plasma proteins as affected by pH*. Meat Science, 2007. **76**(2): p. 216-225.
25. Pérez-Bosque, A., J. Polo, and D. Torrallardona, *Spray dried plasma as an alternative to antibiotics in piglet feeds, mode of action and biosafety*. Porcine Health Management, 2016. **2**(1): p. 16.
26. Pérez-Bosque, A., et al., *Dietary Plasma Protein Supplements Prevent the Release of Mucosal Proinflammatory Mediators in Intestinal Inflammation in Rats*. The Journal of Nutrition, 2010. **140**(1): p. 25-30.
27. Jung, T.H., et al., *Purification and Anti-pathogenic Properties of Immunoglobulin Concentrates from Porcine Blood*. Korean J Food Sci Anim Resour, 2017. **37**(5): p. 743-751.
28. Hedegaard, C.J., et al., *Natural Pig Plasma Immunoglobulins Have Anti-Bacterial Effects: Potential for Use as Feed Supplement for Treatment of Intestinal Infections in Pigs*. PLoS One, 2016. **11**(1): p. e0147373.
29. Kazimierska, K. and W. Biel, *Chemical Composition and Functional Properties of Spray-Dried Animal Plasma and Its Contributions to Livestock and Pet Health: A Review*. Animals (Basel), 2023. **13**(15).
30. Andrew Getahun, J.C.C., *Of ITIMs, ITAMs, and ITAMis: revisiting immunoglobulin Fc receptor signaling*. Immunological reviews, 2015. **268**(1): p. 66-73.
31. Solana, R., et al., *Innate immunosenescence: Effect of aging on cells and receptors of the innate immune system in humans*. Seminars in Immunology, 2012. **24**(5): p. 331-341.
32. Pahl, J.H.W., et al., *CD16A Activation of NK Cells Promotes NK Cell Proliferation and Memory-Like Cytotoxicity against Cancer Cells*. Cancer Immunol Res, 2018. **6**(5): p. 517-527.
33. Daha, N.A., et al., *Complement activation by (auto-) antibodies*. Mol Immunol, 2011. **48**(14): p. 1656-65.
34. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
35. Vogelpoel, L.T., et al., *Control of cytokine production by human Fc gamma receptors: implications for pathogen defense and autoimmunity*. Frontiers in Immunology, 2015. **6**.
36. Selvaraj, P., et al., *The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria*. Nature, 1988. **333**(6173): p. 565-567.
37. Mancardi, D. and M. Daëron, *Fc Receptors in Immune Responses*, in *Reference Module in Biomedical Sciences*. 2014, Elsevier.

38. Sauls RS, M.C., Taylor BN. . *Histology, T-Cell Lymphocyte*. . May 2023 [cited Mar. 2025; Available from: <https://www.ncbi.nlm.nih.gov/books/NBK535433/>.
39. Lee, H.-G., M.-J. Cho, and J.-M. Choi, *Bystander CD4+ T cells: crossroads between innate and adaptive immunity*. *Experimental & Molecular Medicine*, 2020. **52**(8): p. 1255-1263.
40. Levine, B.L., J.J. Mond, and C.H. June, *Proliferation, Lymphocyte*, in *Encyclopedia of Immunology (Second Edition)*, P.J. Delves, Editor. 1998, Elsevier: Oxford. p. 2017-2023.
41. Shi, M., et al., *Cell cycle progression following naive T cell activation is independent of Jak3/common gamma-chain cytokine signals*. *J Immunol*, 2009. **183**(7): p. 4493-501.
42. Butler, J.E., et al., *Porcine IgG: structure, genetics, and evolution*. *Immunogenetics*, 2009. **61**(3): p. 209-30.
43. Jefferis, R., J. Lund, and M. Goodall, *Recognition sites on human IgG for Fcγ receptors: the role of glycosylation*. *Immunology Letters*, 1995. **44**(2): p. 111-117.
44. Bergeron, L.M., et al., *Comparative functional characterization of canine IgG subclasses*. *Veterinary Immunology and Immunopathology*, 2014. **157**(1): p. 31-41.
45. Nemanichvili, N., et al., *Wild and domestic animals variably display Neu5Ac and Neu5Gc sialic acids*. *Glycobiology*, 2022. **32**(9): p. 791-802.
46. Montegiove, N., et al., *The Hard Choice about Dry Pet Food: Comparison of Protein and Lipid Nutritional Qualities and Digestibility of Three Different Chicken-Based Formulations*. *Animals (Basel)*, 2022. **12**(12).
47. Quang D Tran, W.H.H., Antonius FB van der Poel, *Effects of extrusion processing on nutrients in dry pet food*. *Science of Food and Agriculture*, 2008. **88**(9): p. 1487-1493.
48. Quigley, J.D., 3rd, et al., *Effects of spray-dried animal plasma on intake and apparent digestibility in dogs*. *J Anim Sci*, 2004. **82**(6): p. 1685-92.
49. Bubelova, Z., et al., *The effect of different heat sterilization regimes on the quality of canned processed cheese*. *Journal of Food Process Engineering*, 2010. **34**: p. 1860-1878.
50. Vermeer, A.W. and W. Norde, *The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein*. *Biophys J*, 2000. **78**(1): p. 394-404.
51. *Clonal expansion*, in *Rheumatology and Immunology Therapy*, J.D. Abbott, et al., Editors. 2004, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 226-227.
52. Kleiveland, C.R., *Peripheral Blood Mononuclear Cells*, in *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, K. Verhoeckx, et al., Editors. 2015, Springer

Copyright 2015, The Author(s). Cham (CH). p. 161-7.

53. Mead, T.J. and V. Lefebvre, *Proliferation assays (BrdU and EdU) on skeletal tissue sections*. *Methods Mol Biol*, 2014. **1130**: p. 233-243.
54. Stroop, J., *Identifying immunoglobulins in spray-dried porcine*

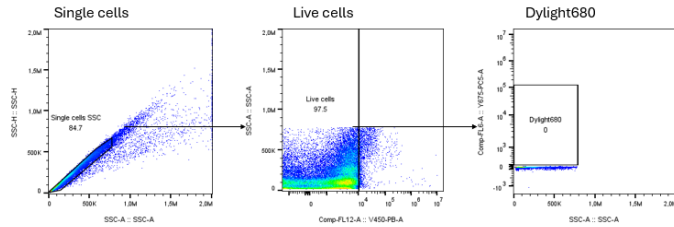
plasma and their binding capacity to canine PBMCs, in *Cell Biology and Immunology*. 2024, Wageningen University and Research.

55. van Mirre, E., et al., *Monomeric IgG in intravenous Ig preparations is a functional antagonist of FcγRII and FcγRIIIb*. *J Immunol*, 2004. **173**(1): p. 332-9.
56. Adjobimey T, M.J., Sollberg L, Bawolt M, Berens C, Kovačević P, Trudić A, Parcina M and Hoerauf A, *Comparison of IgA, IgG, and Neutralizing Antibody Responses Following Immunization With Moderna, BioNTech, AstraZeneca, Sputnik-V, Johnson and Johnson, and Sinopharm's COVID-19 Vaccines*. *Front. Immunol.*, 2022. **13**.
57. Chen, X., et al., *Human monoclonal antibodies block the binding of SARS-CoV-2 spike protein to angiotensin converting enzyme 2 receptor*. *Cellular & Molecular Immunology*, 2020. **17**(6): p. 647-649.

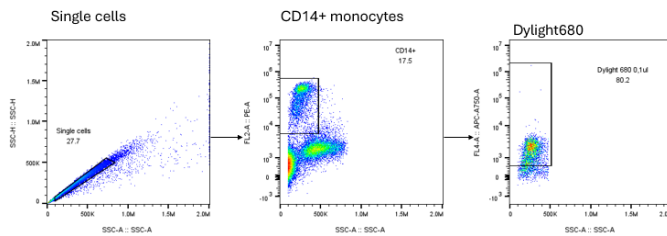
58. Burton, D.R., *Antiviral neutralizing antibodies: from in vitro to in vivo activity*. Nat Rev Immunol, 2023. **23**(11): p. 720-734.

7. Appendix

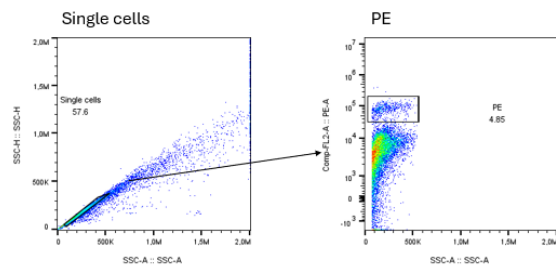
7.1 Supplementary figures + Data



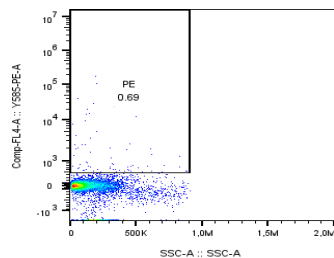
Sup. figure 1: Representative gating strategy for single stain controls pre-optimization. Single stains are identified after which live cells are gated. Lastly the specific single stain positive cells are gated.



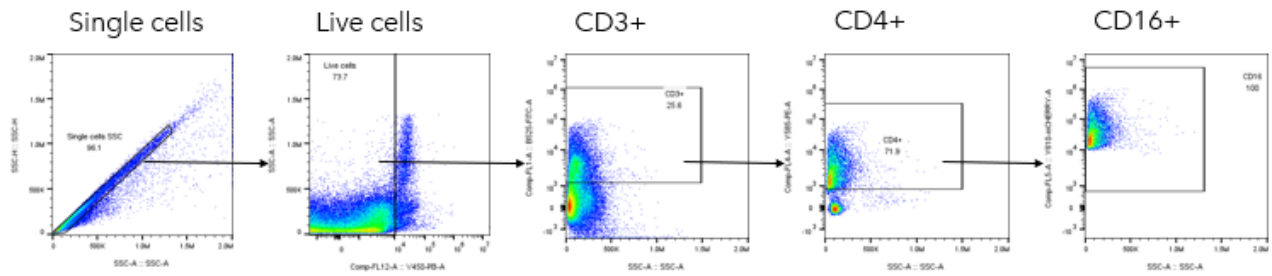
Sup. figure 2: Representative gating strategy for Dylight680 conjugated antibody titration. Single cells were gated first. Next CD14+ are gated for. Lastly the Dylight680 positive cells are gated.



Sup. figure 3: Representative gating strategy for single stain controls pre-optimization. Single stains are identified first. Lastly the specific single stain positive cells are gated.



Sup. figure 4: Representative dot plot of the PE control of one of the repeats of the Fc receptor blocking experiments.



Sup. figure 5: Representative gating strategy for identifying bound pIgG to canine immune cells. Single stains are identified after which live cells were gated. For T-cells CD3+ populations were gated first, after which the specific T-cell population was gated. Lastly the Dylight680 positive cells were gated.