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Investigating the *in vitro* immunomodulatory effects of cathelicidins on the porcine immune system, in the context of *Streptococcus suis*.

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ABSTRACT

Streptococcus suis (S. suis) is a bacterium that can cause severe illness in both pigs and humans. Currently, treatment of *S. suis* infection relies on the use of antibiotics. With increasing concerns regarding antibiotic resistance, there is a clear need for alternative treatments. Despite many attempts, vaccination of pigs against this pathogen has not proven effective.
Cathelicidins are peptides that are part of the innate immune system with immunomodulatory properties, making them promising vaccine adjuvants. The porcine cathelicidins PR-39 and PMAP-23 were selected for this study, in addition to a well-documented chicken cathelicidin (chCATH-2). In combination with providing more general knowledge regarding the porcine immune system, this study aimed to investigate whether cathelicidins could be of use as immune modulators of porcine dendritic cells.
Using flow cytometry and cytokine level readouts of porcine bone marrow derived dendritic cells, the effect of *S. suis* isolate S10 (both encapsulated and non-encapsulated), LPS, and host defense peptides on dendritic cell surface markers was analyzed. chCATH-2 dose-dependently lowered the immune response triggered by *S. suis*, its non-encapsulated mutant, and LPS, whereas PMAP-23 and PR-39 did not. In contrast, PR-39 increased IL-1ß concentrations in a dose-dependent manner.
Phagocytosis assays using fluorescent *S. suis* revealed that non-encapsulated *S. suis* could be taken up by dendritic cells. The capsule of *S. suis*, however, blocked phagocytosis and the investigated cathelicidins did not increase this uptake. Preliminary co-cultures, using dendritic cells and $CD4^+$ T cells, indicated that chCATH-2 boosts the activation of T helper cells. Together with their antimicrobial properties, these peptides form a promising alternative to antibiotics, either as vaccine adjuvants or immune modulatory drugs.

Keywords: *Streptococcus suis*, dendritic cells, cathelicidins, immunomodulation, porcine immune system, T helper cells

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INTRODUCTION

Antibiotic resistance has become increasingly problematic in both veterinary and human practices. *Streptococcus suis* (*S. suis*), a Gram-positive bacteria with pigs as a natural host can develop resistance against conventional antibiotics, providing a challenge to treat infected pigs (Athey *et al*., 2016; Wasteson *et al*., 1994; Yongkiettrakul *et al*., 2019). In adult pigs, carriage of *S. suis* is relatively common and most adult pigs have formed natural immunity against environmental strains (Lowe *et al*., 2011). However, some serotypes and strains are more virulent than others. Of these, serotype 2 is frequently isolated from diseased pigs and is therefore extensively studied (Haas and Grenier, 2018; Segura, 2015). Clinical signs associated with infection consist of sepsis, arthritis, pneumonia, meningitis, and sudden death (Haas and Grenier, 2018; Reams *et al*., 1994). Young piglets especially are susceptible to disease and growth retardation (MacInnes and Desrosiers, 1999).

A more modern approach than serotyping (which is based on capsule antigens), is using molecular typing based on sequencing data. Of the serotype 2 strains, the sequence type (ST)01 strain is distributed worldwide, although it is most frequently found in Europe and Asia (Athey *et al*., 2016). In Northern America, the ST25 strain, and the less virulent ST28 strain, are more common (Segura, 2015). In China, outbreaks of the highly virulent ST07 strain have been reported in both pigs (Wei *et al*., 2009) and humans (Ye *et al*., 2006). The zoonotic potential of *S. suis* emphasizes the importance of finding effective treatments. Protecting pigs against this pathogen could lead to a lower infection pressure, and an increase of health and welfare in pigs. An improvement of porcine health subsequently reduces the risk for human infection, prevents economic losses in pig industry and might also benefit food safety (Haas and Grenier, 2018).

Currently, protection of pigs primarily relies on the use of antibiotics. However, infections are often detected only when the animal has already died. In addition, the selection pressure exerted by antibiotics, has introduced and fostered the development of antibiotic resistance in numerous pathogenic bacteria, including *S. suis* (Munita and Arias, 2016; Seitz et al., 2016). Infections that were relatively easy to treat using antibiotics, can now become life-threatening (Llor and Bjerrum, 2014). Therefore, the widespread use of antibiotics is unsustainable and in particular undesired with regard to food-producing animals (European One Health action plan against AMR, 2017). For pigs, an attractive alternative would be to develop a vaccine against *S. suis*. In the field, clinical *S. suis* isolates of an affected herd can be used to develop inactivated or subunit autogenous vaccines (Lapointe *et al*., 2002). However, these autogenous vaccines are not always effective, cannot be standardized with regards to safety, and do not induce protection against *S. suis* serotypes and strains, other than the particular isolate the vaccine is derived from (Baums et al., 2009; Büttner et al., 2012; Wisselink et al., 2002). It shows there is a clear need for a broadly effective vaccine against multiple *S. suis* strains to boost protective immunity rather than to treat infected herds. But in spite of many efforts and promising results in mouse models (Li *et al*., 2011; Zhou *et al*., 2015), candidate vaccines have not proven effective in pigs (Haas and Grenier, 2018). Therefore, expanding the knowledge regarding the interaction of porcine immune cells with *S. suis* could help build a platform for further vaccine development.

In general, the mammalian immune system can be divided into the innate and the adaptive immune system. Cells of the innate immune system (e.g. dendritic cells) recognize pathogens and pathogen associated molecular patterns (PAMPs), and subsequently trigger an immune response by presenting antigen to T cells in order to raise protective immunity against these particular pathogens. Subsequently, the activated T helper cells can differentiate into various subtypes; most notably Th type-1, Th type-2, or Th type-17. This will either induce a strong IFN-y response (with viral and intracellular bacterial clearance), the activation of B-cells, or the activation of neutrophils, respectively (Kaiko et al., 2008).

The activation, proliferation, and differentiation of T helper cells is induced by interaction with dendritic cells. The induction requires recognition by the T cell receptor (TCR) of antigen presented on a MHC-II molecule. The extent of T cell activation is subsequently regulated by the interaction of dendritic cell co-stimulatory molecules - such as CD80/86 – with CD28 or CTLA-4 on the T cell surface (Gaudino and Kumar, 2019). Thereafter, as reviewed in Knosp *et al*. (2012), the local concentration of cytokines excreted by dendritic cells, such as IL-4, IL-6, IL-12, TGF-ß, and IL-21, modulates polarization of the T cells.

Porcine T-helper cells are characterized by the expression of CD3 and CD4 molecules on their cell surface (Gerner *et al*., 2009). The CD4 molecule complexes with the T cell receptor (TCR) when interacting with SLA-II. This separates them from cytotoxic T cells, being $CD3^+$, $CD4^-$, and $CD8^+$, which allows interaction with MHC-I molecules (Gerner *et al*., 2009). Contrary to humans, an additional subset of T cells ($CD4^+CD8^+$) is found in the pig (Gerner *et al*., 2015; Rodríguez-Gómez *et al*., 2016). These cells are thought to be memory T cells (Okutani *et al*., 2018) and could therefore be especially of interest in terms of vaccine development.

In vivo, the innate immune system is predominantly activated by *S. suis* through MyD88-dependent pathways, regardless of strain background (Auger *et al*., 2019). Upon activation of the MyD88 pathway, dendritic cells release a vast array of pro-inflammatory cytokines, such as TNF-α, IL-1ß, and the chemokine IL-8 (CXCL8) (Lecours *et al*., 2011; Liu *et al*., 2016). Furthermore, stimulation of dendritic cells results in the upregulation of certain cell surface markers, such as the co-stimulatory molecule CD80/86 and the antigen presenting molecule Swine Leukocyte Antigen-II (SLA-II), which is the porcine equivalent of MHC-II (Lecours *et al*., 2011).

This pro-inflammatory response is triggered by *S. suis* stimulation of dendritic cells *in vitro*, in which Lecours *et al* (2012) showed that Toll Like Receptor (TLR)2 plays an important role. TLR2 is known to recognize Gram positive-derived PAMPs, such as lipoteichoic acid (LTA). Endotoxins associated with *S. suis* can thereby activate dendritic cells. In *in vivo* mice experiments, however, only a minor role for TLR2 was found (Auger *et al*., 2019). This indicates that the immune reaction in response to *S. suis* is complex. In both models, however, the capsular polysaccharide surrounding the cell wall of *S. suis* hampers the formation of the inflammatory response (Auger *et al*., 2018; Graveline *et al*., 2007; Meijerink *et al*., 2012). This capsule shields the bacterium from recognition and subsequent phagocytosis by innate immune cells (Meijerink *et al*., 2012). Therefore, dendritic cells exposed to encapsulated *S. suis* are inadequately activated, unable to process antigen or fully upregulate their cell

surface markers, and have impaired cytokine production, all of which blocks proper T-helper cell activation.

Regarding vaccine development, proper adjuvants can help boost the immunogenicity of an inactivated or an otherwise insufficiently active subunit vaccine (Coffman *et al*., 2010). For *S. suis*, finding an adjuvant that can support dendritic cells in their recognition of a conserved *S. suis* antigen and thereby neutralize the effect of the capsular polysaccharide could be key to promote vaccine efficaciousness.

Promising adjuvants are the host defense peptides (HDPs), which are part of the innate immune system. Two major groups within the HDPs are the defensins and the cathelicidins, in which cathelicidins are of interest within this project. Cathelicidins are small, amphipathic, and positively charged peptides characterized by a conserved cathelin pro-domain (van Harten *et al*., 2018). They are antimicrobial, even against multidrug resistant bacteria (Coorens *et al*., 2017a; Veldhuizen *et al*., 2013). Many animal species are furthermore known to produce their own set of HDPs (Matsuzaki, 2019). The only human cathelicidin (LL-37) has been the most extensively studied (Coorens *et al*., 2017a; Xhindoli *et al*., 2016). In pigs, 11 cathelicidins have been found, each with its own structure and mechanism of action against pathogens (Sang and Blecha, 2009; Wessely-Szponder *et al*., 2010). Beside their antimicrobial function, cathelicidins possess immunomodulatory properties, such as the promotion of cytokine release and the enhancement of phagocytosis (Coorens *et al*., 2017a; van Harten *et al*., 2018). Thereby, HDPs contribute to the host's defense against various pathogens (Gupta *et al*., 2017). In a healthy situation, the concentration of HDPs in either blood or tissue is low (van Harten *et al*., 2018). However, upon immune activation, cells can excrete these peptides and raise the local concentration substantially. For instance, the porcine cathelicidin PR-39 is stored in neutrophils and released by degranulation (Wu *et al*., 1999). It is a proline and arginine rich cathelicidin, consisting of 39 amino acids (Holani *et al*., 2016). PR-39 shows broad antimicrobial activity through direct lysis and disruption of cellular processes of bacteria, induces *in vitro* cytokine production in macrophage cell lines, and exerts an anti-apoptotic effect (Veldhuizen *et al*., 2014). Despite its broad antimicrobial effect, PR-39 is not effective against *S. suis*, which can produce a protease capable of degrading PR-39 (LeBel *et al*., 2018).

Besides PR-39, the other 10 porcine cathelicidins consist of five protegrins (PG-1 to PG-5), 2 prophenins, and 3 porcine myeloid antimicrobial peptides (PMAP-23, PMAP-36, and PMAP-37) (Sang and Blecha, 2009; Wessely-Szponder *et al*., 2010). PMAP-36, a cathelicidin consisting of 36 amino acids, shows rapid lytic effects on bacterial membranes, but is furthermore known to bind lipopolysaccharide (LPS) and various other endotoxins (Scheenstra *et al*., 2019; Scocchi *et al*., 2005). PMAP-23 on the contrary, has no binding affinity to LPS, but nonetheless maintains its antimicrobial properties (Veldhuizen *et al*., 2017). Within the same study, Veldhuizen *et al*. (2017) showed that PMAP-23 is able to induce IL-8 production in a porcine macrophage cell line, although also observed that PMAP-23 lowered the uptake of latex beads by monocytes.

Within this project, three porcine cathelicidins (PR-39, PMAP-23, and PMAP-36) and a more extensively studied chicken cathelicidin (chCATH-2), were investigated. Similar to PMAP-36, chCATH-2 is known to bind LPS, which prevents TLR-4 activation. Moreover, the peptide binds to DNA, promoting uptake

and TLR-9 signalling (Coorens *et al*., 2017b; Scheenstra *et al*., 2019; Veldhuizen *et al*., 2017). In addition, this cathelicidin kills *S. suis* at relatively low concentrations (Scheenstra *et al.*, unpublished). The D-isomer form of chCATH-2 (DCATH-2), which cannot be proteolytically degraded, has also already shown protective effects after *in ovo* injection against pathogenic *Escherichia coli* (*E. coli*), even up to 7 days after hatching (Cuperus *et al*., 2016).

The primary objectives of this project are to provide more general knowledge on porcine *in vitro* cultures, and to investigate the interaction of *S. suis* (with and without capsule) with porcine dendritic cells and the subsequent effect on porcine T cells. The *S. suis* isolate S10 and its non-encapsulated mutant (S10-Δ*cps*), will be used for this purpose. This particular isolate with sequence type 01 shows large genomic resemblance with the well-documented P1/7 isolate, both being virulent strains (Gaiser et al., 2019; Velikova et al., 2016; Zaccaria et al., 2016).

Following initial culture and characterization of porcine dendritic cells and T helper cells, the effects of cathelicidins on these immune cells will be investigated in combination with *S. suis*. This will provide an overview on the interaction between dendritic cells, T helper cells, *S. suis*, its capsular polysaccharide, and cathelicidins as immunomodulatory agents. Addressing this matter thereby also follows the scope of the I&I faculty's aim: *"Unraveling mechanisms of infections, host defense and antimicrobial resistance*'', in which the host defense plays a central role. Read outs of the cytokine production, cell surface marker expression and phagocytotic capacity of dendritic cells, proliferation of T cells, and viability assays to assess HDP toxicity will elucidate whether cathelicidins could be of use as vaccine adjuvants. In combination with their antimicrobial properties, cathelicidins could form promising alternatives to antibiotics.

MATERIALS AND METHODS

Cells

Bone marrow cells were isolated by hip bone puncture from adult pigs and homogenized through a 70 µm cell strainer (Corning, Sigma-Aldrich). The cell suspension was pipetted onto a Ficoll density gradient of 1.077 g/ml (GE Healthcare Bio-Science AB, Sweden), followed by centrifugation at 872 x *g* for 20 minutes at room temperature with a slow start and brake. The same protocol was followed for blood cells.

The cells from the buffy coat were collected and washed with Dulbecco's phosphate buffered saline (dPBS) (GIBCO, Invitrogen). The washed cells were stored in fetal calf serum (FCS) (Bodinco B.V., Alkmaar, The Netherlands)/10% dimethyl sulfoxide (Sigma-Aldrich) at a concentration of $6 * 10^7$ cells/ml in liquid nitrogen until further use.

All animals were used and kept under the approval and guidelines of the animal ethical committee of the Utrecht University.

Dendritic cell culture

Bone marrow cells were slowly thawed and seeded into 96-well culture plates at a concentration of $0.5 * 10^6$ cells per ml in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Invitrogen), supplemented with 10% FCS and 1% penicillin/streptomycin (Thermo Fisher Scientific), for 7 days at 37 ºC, 5% CO_2. For bacterial assays, the antibiotics were left out of the dendritic cell medium.

Recombinant pig granulocyte-macrophage colony-stimulating factor (GM-CSF) (40 ng/ml or 1%) (Sanbio B.V., Uden, The Netherlands) and recombinant porcine IL-4 (40 ng/ml) (R&D systems, Minneapolis, MN, USA) were added to the wells to generate bone marrow-derived dendritic cells. Cells were fed on days 3 and 6 of culture with fresh medium containing GM-CSF and IL-4.

T cell purification

Freshly isolated peripheral blood mononuclear cells (PBMCs) were stained with 10 µl of the monoclonal antibody (mAb) CD4-PE-Cy7 *(clone 74-12-4, BD Biosciences),* diluted in 90 µl FACS buffer (PBS/0,5% BSA) (Sigma-Aldrich)) at a maximum of $2*10^7$ cells per staining. After 20 minutes of incubation (on ice) the cells were washed twice with MACS buffer (PBS/0.5% BSA/2 mM EDTA), followed by centrifugation at 500 x *g* for 5 minutes. The cells were resuspended in MACS buffer and stained with anti-Cy7 Microbeads (Miltenyi Biotec) according to manufacturer's instructions. The cells were separated using a QuadroMACS™ Separator and LS columns (Miltenyi Biotec).

Flow cytometry was used to check the cells purity (CD4 positive) and was always >90%

T cell culture

Purified cells were split into two separate groups. One group was incubated with 5 µM of carboxyfluorescein succinimidyl ester (CFSE)(Invitrogen) for 20 minutes at 37 ºC, the other group served as an unstained control. After 20 minutes, the CFSE labeling was quenched by pipetting the cells into ice-cold RPMI 1640 medium. The cells were seeded into 96-well culture plates at a concentration of $0.5 * 10^6$ cells per ml in RPMI 1640 GlutaMAX medium (GIBCO, Invitrogen), supplemented with 10% FCS, 1% penicillin/streptomycin, and 20 ng/ml IL-2 (R&D systems) and incubated at 37 ºC, 5% CO_2. Cells were stimulated with PHA (Invivogen) at a concentration of 20 µg/ml or a combination of ionomycin (Calbiochem, Merck) and PMA (Sigma-Aldrich) at a concentration of 1 µg/ml and 25 ng/ml, respectively. The

cells were fed at day 3 and day 6 with fresh medium (supplemented with mitogens for the corresponding wells).

Bacterial assays

The *S. suis* isolate S10, its non-encapsulated mutant (*S. suis* S10-Δ*cps*), and GFP-labeled *S. suis* were a gift from Dr. Jesús Arenas Busto, Utrecht University. 15 ml Todd Hewitt Broth (THB)(Oxoid Limited) medium was inoculated with *S. suis*-S10 or *S. suis*-Δ*cps* for 16 hours at 37 ºC in a small centrifuge tube (15 ml), to allow the bacteria to grow in approximate anaerobic conditions.

From this suspension, 300 µl was pipetted into 15 ml of fresh THB medium and the bacteria were grown to log-phase in 4 hours (at 37 ºC with shaking). After centrifugation (at 1200 x *g* for 10 minutes), the log-phase bacteria were resuspended in THB medium and the optical density (OD) was measured at 620 nm. With an OD of 0.1 (in 1 ml THB) corresponding to $1*10^8$ bacteria, the sample was diluted to obtain a multiplicity of infection (MOI) of 10 ($5*10^6$ bacteria per ml) and washed twice with dPBS. For several assays, log-phase bacteria were heat-killed at 80 ºC for 1 hour. The bacteria were resuspended in cell culture medium and added to the dendritic cells as described below.

As a control after each experiment, the bacterial suspension was serially diluted 10-fold up to 10^{-6}. The dilutions were spread plated onto tryptone soy agar (TSA)(Oxoid Limited) plates, supplemented with 5% sheep blood (Thermo Scientific, Oxoid Limited). The plates were incubated overnight at 37 ºC, after which the colonies were counted. This was done to certify the MOI value, the heat-killing of the bacteria and to check whether there was no contamination of the bacterial suspension and the THB medium.

Dendritic cell stimulation

After six days of culture, the supernatant was removed from the cells and discarded. Cells were stimulated with 100 ng/ml LPS Escherichia coli 0111:B4 (Invivogen) as a positive control, in the presence of various concentrations of different cathelicidins. *S. suis* was added to the cells at a MOI of 10, also in the presence of various concentrations of cathelicidins.

The following cathelicidins, chemically synthesized, were used: chιCATH-2, chɒCATH-2 (CPC scientific, Sunnyvale, CA, USA), PMAP-36, PMAP-23, and PR-39 (China Peptides, Shanghai, China), ranging in concentrations from 0.15 µM to 2.5 µM. Control groups consisted of cells that remained unstimulated (with only cell culture medium being added).

The cells were stimulated at 37°C for either 24 hours (initial LPS assays) or 2.5 hours. For the latter, the cell supernatant was collected and frozen at -20 ºC for further analysis (ELISA), after 2.5 hours. RPMI medium supplemented with FCS, GM-CSF, IL-4, and antibiotics (penicillin/streptomycin), was added to the cells for the remaining 21 hours.

Flow cytometry

After seven days of culture (24 hours after stimulation), the supernatant from the dendritic cell cultures was collected and frozen at -20 ºC. For the T cell cultures, the supernatant was collected and frozen at various timepoints (1 to 9 days after seeding). Subsequently, the cells were detached from the culture plates using a brief incubation at 37°C with PBS supplemented with 0.5 mM EDTA. The cells were detached by vigorous pipetting, washed with FACS buffer, and transferred to V-bottom plates, followed by centrifugation at 700 x *g* for 2 minutes. After washing, the cells were stained for 20 minutes (on ice) with labelled monoclonal antibodies (mAbs) for flow cytometry

(FACSCantoII, BD Biosciences, San Jose, CA, USA).
The following mAbs were used: CD14-PB *(clone TÜK4, AbD serotech, dilution 1:100),* SWC3a-PE *(clone 74-22-15, Abcam, dilution 1:1000),* Monocytes/Granulocytes mAb-PE *(clone 74-22-15, Invitrogen, dilution 1:4000),* SLA-II-FITC *(clone 2E9/13, Thermo Scientific, dilution 1:1000),* CD8*α*-AF647 *(clone 76-2-11, BD Biosciences, dilution 1:500),* CD4-PE-Cy7 *(clone 74-12-4, BD Biosciences, dilution 1:1000),* CD3ε-PerCP-Cy5.5 or CD3ε-AF647 *(clone BB23-8E6-8C8, BD Biosciences, dilution 1:1000 or 1:250, respectively),* and a soluble APC-labeled CTLA4-mouse immunoglobulin fusion protein for the detection of CD80/86 *(Ancell, dilution 1:1000*). The mAbs were diluted in FACS buffer. Control wells remained unstained to correct for background fluorescence. After staining, cells were washed and resuspended in FACS buffer and kept on ice until FACS.
Flow cytometry data was analyzed using FlowJo analysis software V10 (FlowJo, Ashland, OR, USA). To compensate for spectral overlap, BD™ CompBead Anti-Mouse compensation particles (BD biosciences) were used, according to manufacturer's instructions.

ELISA analysis
Levels of IL-1ß, IL-8, IL-10, IFN-γ and TNF-α cytokines in cell culture supernatant were measured by sandwich ELISA using ELISA Duoset kits (R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions. The cell culture supernatant was diluted - where appropriate - in PBS/1% BSA (IL-6, IL-8, IFN-γ and IL-10 assays) or TBS/0.1% BSA/0.05% Tween20 (MP Biomedicals) (TNF-α and IL-1ß assays). Absorbance was measured at 450 nm with a FLUOstar Omega microplate reader (BMG, Labtech GmbH) and corrected for absorbance at 540 nm. Results were analyzed using MARS data analysis software (BMG Labtech GmbH). All ELISA samples consisted of two technical replicates. Standard sample dilutions (two-fold) were used to create a standard curve based on the optical density for quantification of cytokine levels.

Cell viability
To assess cell viability and the toxicity of the host defense peptides, Annexin-V (BD Pharmingen) was used to stain the cells and visualized using flow cytometry, according to manufacturer's instructions. In a different assay, WST-1 (Sigma-Aldrich) was used to evaluate the mitochondrial activity of the cells. The WST reagent was diluted 10 times in cell culture medium and in every well, 100 µl of the WST solution was added after the supernatant had been removed. The cells were incubated with the WST reagent for approximately 90 minutes (until a change in color was clearly visible). The absorbance was measured at 450 nm and corrected at 650 nm using the FLUOstar Omega microplate reader.

Co-culture assays
Co-culture assays of $CD4^+$ T cells and dendritic cells took 15 days to complete. During the first week, CFSE and non-CFSE labeled $CD4^+$ T cells were kept in culture as described above, separate from the dendritic cells. On day six, the dendritic cells were stimulated with *S. suis* (S10 and S10-Δ*cps*, MOI:10) and/or 0.3125 µM of the aforementioned HDPs.
After 2.5 hours of stimulation, the medium from the dendritic cells was removed and replaced with RPMI 1640 GlutaMAX medium, supplemented with 10% FCS, 1% penicillin/streptomycin, 20 ng/ml IL-2, 40 ng/ml IL-4, and 1% GM-CSF. PBS supplemented with 0.5 mM EDTA was used to gently detach the $CD4^+$ T cells from the culture plates. The detached $CD4^+$ T

cells were added to the dendritic cells in a 1:1 ratio. The cells were fed every 3 days and flow cytometry and ELISA readouts were conducted on various timepoints (1 to 8 days after co-culture). A co-culture of dendritic cells, $CD4^+$ T cells, supplemented with PHA (20 µg/ml), served as a positive control.

Phagocytosis assays

GFP-labeled *S. suis*-S10 and GFP-labeled *S. suis* S10-Δ*cps* were used for phagocytosis assays. The bacteria and dendritic cells were cultured and stimulated as described before and flow cytometry was used to visualize phagocytosed *S. suis*. Except for the SWC3a-PE mAb *(clone 74-22-15, Abcam, dilution 1:1000)* for gating purposes, no mAbs were added to the cells. Controls consisted of wells containing only dendritic cells, GFP-labeled *S. suis*-S10, or *S. suis* S10-Δ*cps*.

In some wells, Cytochalasin D (CytoD) (Sigma-Aldrich) was added at a concentration of 1 µM prior and during BMDC stimulation. This was done to validate that the uptake of *S. suis* was caused by phagocytosis and not invasion.

Statistical analysis

All data are expressed as mean ± SEM. The datasets were analyzed for significance using the non-parametric Friedman rank sum test, due to skewed distributions of the data. A p-value < 0.05 was considered significant (*), with ** being $p < 0.01$ and *** being $p < 0.001$. Statistical analysis was performed using R studio software (RStudio Inc, Boston, USA). Experiments were repeated at least six times, unless indicated otherwise.

RESULTS

Characterization and activation of porcine dendritic cells

To investigate the effect of cathelicidins and *S. suis* on porcine dendritic cells, baseline data was collected regarding cytokine levels and cell surface markers of dendritic cells. Dendritic cells were characterized for comparison with the phenotype known in literature (Carrasco *et al*., 2001; Lecours *et al*., 2011). The cells were generated from bone marrow derived mononuclear cells harvested by Ficoll density centrifugation. After seven days of culture, the majority of the cells (>95%) were SLA-II$^+$ SWC3a$^+$ and (>80%) CD14$^+$. For CD14, however, only a shift in fluorescence intensity was observed instead of two distinct peaks to distinguish between positive and negative cells. Within the population of SLA-II$^+$SWC3a$^+$ cells, 40-50% of the cells was found to be positive for CD80/86. Once the cells were stimulated for 24 hours with *E. coli* LPS, however, >80% of the cells expressed CD80/86. The cells thereby match the phenotype known in literature corresponding to porcine bone marrow derived dendritic cells, and will hence be referred to as bone marrow derived dendritic cells (BMDCs). Extensive flow cytometry details and graphs regarding BMDC characterization can be found in Supplementary Figure 1.

Morphologically, the BMDCs started as small, round cells on day 1, but changed in size and shape as time progressed (Figure 1a-c). After approximately 3 days of culture, the cells started to form protrusions, which is typical for (porcine) dendritic cells (Carrasco *et al*., 2001; Loss *et al*., 2018). After 7 days, flow cytometry analysis of BMDCs showed that unstimulated BMDCs express high levels of SLA-II (Figure 2b). Furthermore, there was no significant change in the expression of SLA-II after *S. suis*-S10 or LPS stimulation. The expression did increase significantly after stimulation with *S. suis* S10-Δ*cps*. The same pattern was seen for the co-stimulatory molecule CD80/86 (Figure 2c). Surprisingly, and in contrast to the results obtained after 24 hours of stimulation (Supplementary Figure 1c), 2,5 hours of stimulation with LPS resulted in no upregulation of CD80/86.

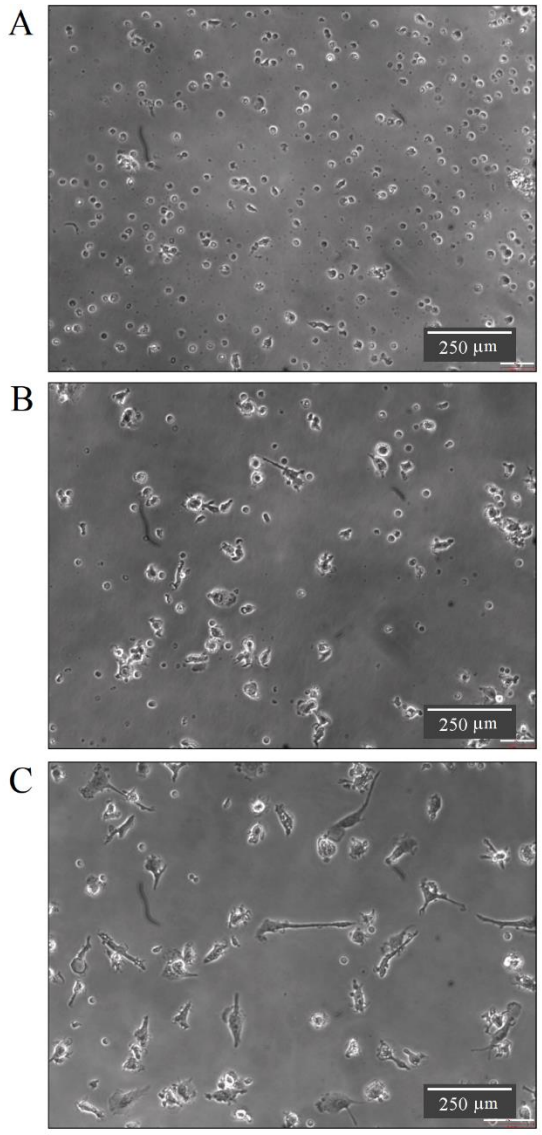

Figure 1. Morphology of porcine BMDCs. Microscopic images of BMDC morphology at various timepoints after seeding. Images are representative pictures of 12 independent experiments. **A)** Small, round cells at day 1. **B)** Growth and formation of protrusions at day 3. **C)** Full grown BMDCs at day 7.

ELISA analysis supported the notion that the cells were activated by non-encapsulated *S. suis*, but also after stimulation with *S. suis*-S10 and LPS. The production of multiple pro-inflammatory

cytokines, such as IL-8, TNF-α (Figure 2d), IL-6 and IL-1ß (Supplementary Figure 2) was induced or increased. Besides, *S. suis* S10-Δ*cps* induced significantly higher TNF-α cytokine levels, compared to its encapsulated counterpart. These findings are in accordance with studies investigating the *S. suis* P1/7 strain, were they observed the same effect for the capsule of *S. suis* on cytokine and CD80/86 levels (Auger *et al.*, 2018; Lecours *et al.*, 2011). In addition, these results provide a baseline for further experiments.

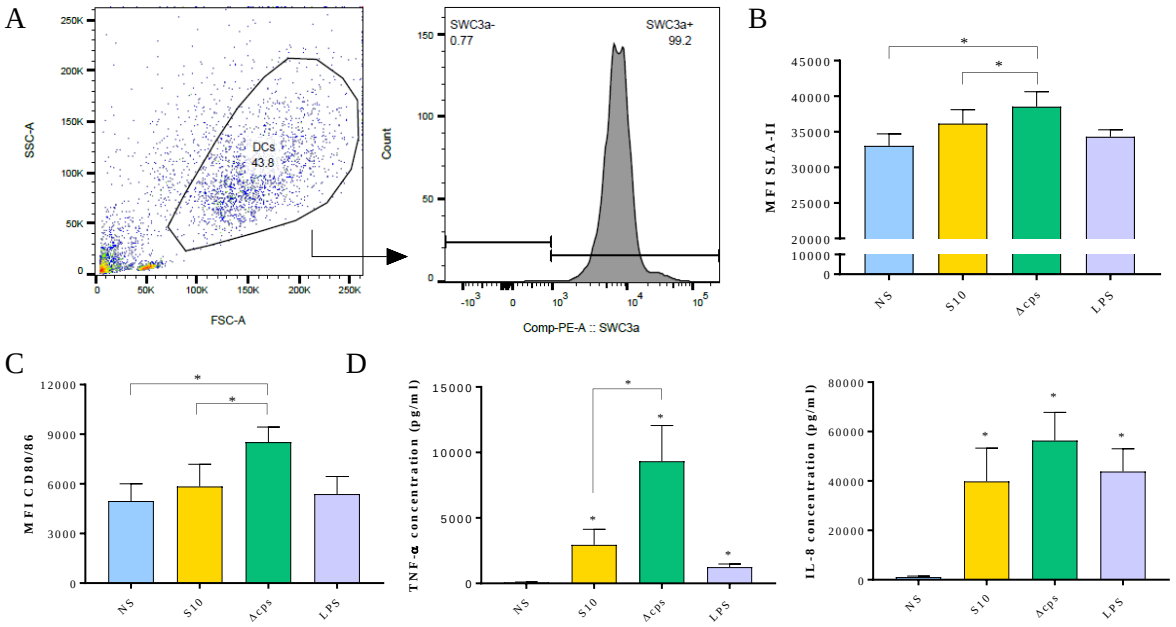

Figure 2. Activation of porcine BMDCs. Flow cytometry and ELISA read outs of dendritic cell activation markers. Cells were stimulated for 2.5 hours with LPS (100 ng/ml), *S. suis*-S10 (S10), or *S.suis* S10-Δ*cps* (Δ*cps*) (MOI:10), then rested for 21.5 hours. Non-stimulated (NS) cells served as control. The figure represents results from 6 independent experiments. **A)** Representative flow cytometry plots of a single well. The gating strategy of BMDCs was based on the forward scatter (FSC) and side scatter (SSC), followed by selection on $SWC3a^+$ cells. **B)** Summary of the median fluorescence intensity (MFI) of SLA-II labeled BMDCs. **C)** Summary of the MFI of CD80/86 labeled BMDC. **(D)** Production of TNF-α and IL-8 by BMDCs, as measured with ELISA.

Characterization and proliferation of porcine T cells

Porcine T cells have been studied to a limited extent, and optimization of cell cultures often requires numerous attempts. Preliminary attempts in which frozen T cells were thawed, purified, stained with CFSE, and subsequently cultured, resulted in relatively pure $CD4^+$ cells. However, these cells could not survive longer than 10 days in culture and were non-responsive to any of the chosen mitogens. Further optimization provided stable cell cultures, by using freshly isolated PBMCs or PBMCs that were allowed to rest for 1 hour after thawing, and the supplementation of IL-2 (20 ng/ml) in the cell culture medium. Magnetic purification of PBMCs resulted in >90% purity of $CD4^+$ cells. Within the population of purified $CD4^+$ cells, >90% was found to be $CD3^+$ (Figure 3a), thereby matching the T helper cell phenotype (Gerner *et al.*, 2015). In pigs, a $CD4^+CD8\alpha^+$ population is known (Rodríguez-Gómez *et al.*, 2016). This particular subset of T cells was also found in this study (Figure 3b). The percentage of $CD4^+CD8^+$ cells present within the $CD4^+$ T cell population varied between 15-35%.

Microscopic images revealed that the $CD4^+$ T cells were round in shape and relatively small (Figure 3c). The morphology of unstimulated T helper cells did not change

over time. Furthermore, non-stimulated T helper cells were not able to produce any detectable IFN-γ or IL-10.
T helper cells that were stimulated with mitogens (ionomycin/PMA or PHA) showed characteristics of activation. The overall cell number increased, the cells started to form clusters (Figure 4a), and the cells upregulated the production of IFN-γ and IL-10 (Figure 4b). To measure T cell proliferation, CFSE was used. A characteristic of the CFSE staining is that it diffuses into cells once it is added and binds to intracellular proteins (Tario *et al.*, 2012). When a cell divides, the cell's CFSE is assumed to divide equally between its two daughter cells. Therefore, a decrease of the CFSE fluorescent signal correlates with cell division. The fluorescence intensity of CFSE labeled cells decreased over time, in stimulated cells only (Figure 4c). From this fluorescent signal, the proliferation index can be calculated (Roederer, 2011). In accordance with the cytokine levels and microscopic images, the proliferation index of porcine T helper cells increased after stimulation with either mitogen (Figure 4d).

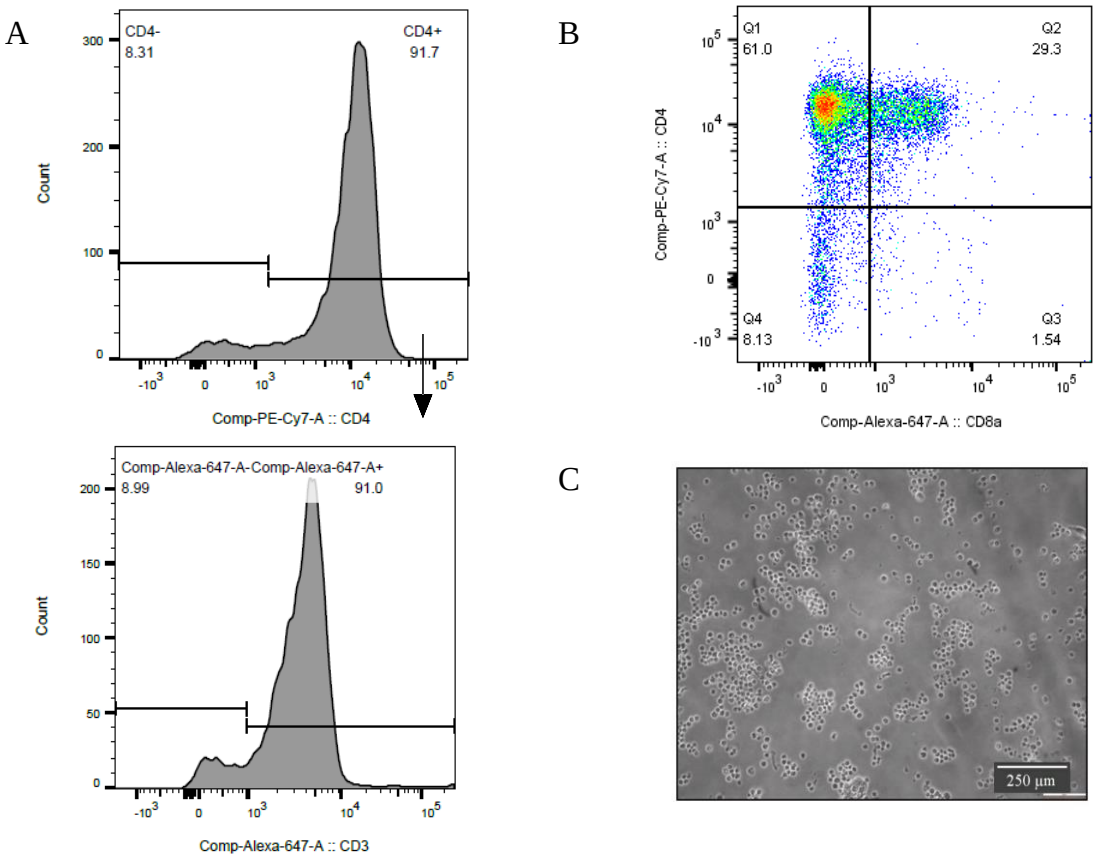

Figure 3. Characterization of porcine CD4⁺ cells. CD4⁺ T cells were cultured in 96-well plates and were harvested at various timepoints. For flow cytometry characterization, cells were stained with CD3, CD4, and/or CD8α mAbs. The figures are representative of 4 independent experiments. **A)** Representative histograms of purified CD4⁺ cells and subsequent gating strategies. **B)** Representative flow cytometry plot of CD4 and CD8α expression of unstimulated CD4⁺ T cells. **C)** Morphology of unstimulated CD4⁺ cells after 3 days of culture.

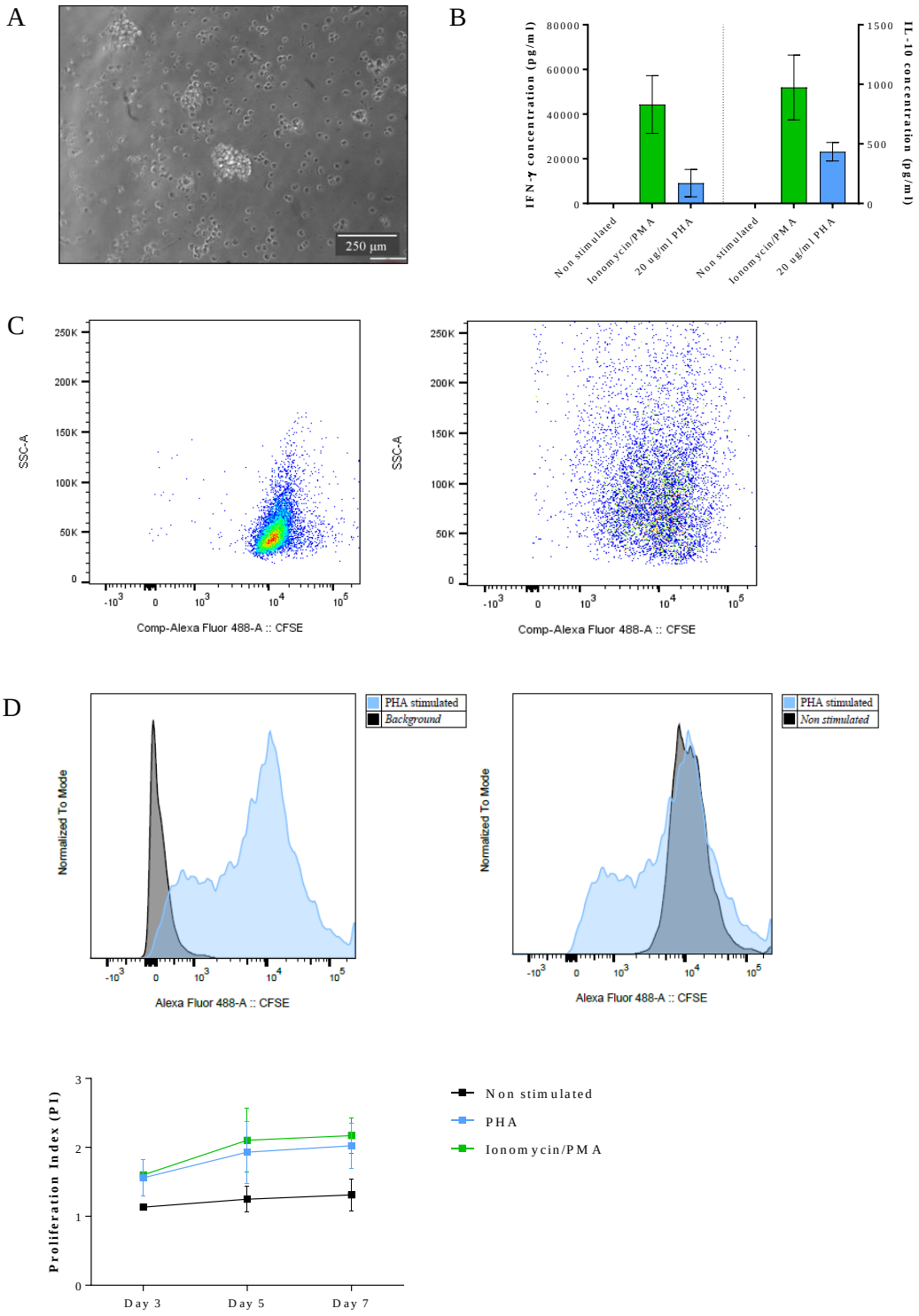

Figure 4. Proliferation characteristics of porcine CD4⁺ cells. CD4⁺ cells were cultured in 96-well plates and stimulated with either ionomycin/PMA or PHA. Images shown are representative results of 2 independent experiments. **A)** Morphology of CD4⁺ cells, stimulated with PHA for 3 days. **B)** Cytokine production (IFN-γ and IL-10) by CD4⁺ cells, 3 days after stimulation, n=2. **C)** Scatter plots displaying the CFSE fluorescence of CD4⁺ T cells, before (left) and after (right) stimulation with PHA. **D)** Representative CFSE flow cytometry histograms, 7 days after stimulation. Unstained CD4⁺ T cells (background fluorescence) and non-stimulated CD4⁺ T cells were used as controls (black histograms). The proliferation index was calculated from the CFSE fluorescence, n=2.

PMAP-36 exerts a dose-dependent effect on dendritic cell viability

Investigating the immunomodulatory properties of HDPs *in vitro* was one of the main objectives within this project. Cytotoxity, however, makes the interpretation of data challenging and for eventual *in vivo* translation, host safety must be guaranteed. For cell viability assays, dendritic cells were exposed to LPS, *S. suis*, and various concentrations of cathelicidins up to 2.5 µM. Both the WST assay and AnnexinA5 staining revealed that chCATH-2, PMAP-23, and PR-39 can be used safely up to 2.5 µM (Supplementary Figure 3). PMAP-36 on the other hand, shows a dose-dependent toxicity and kills ~50% of the dendritic cells at 2.5 µM. Due to the high toxicity of PMAP-36, this peptide was excluded from further experiments. Staining of the BMDCs with Annexin/PI showed that stimulation with either LPS or *S. suis* (MOI:10) for 2.5 hours had little to no effect on cell viability (data not shown).

ʟCATH-2 and ᴅCATH-2 lower the *S. suis* and LPS induced immune response

ʟCATH-2 and ᴅCATH-2 had already shown promising protective properties in an *in ovo* experimental setup (Cuperus *et al*., 2016) and are known to bind LPS (Scheenstra *et al*., 2019). Within this study, HDP properties were investigated by addition of the peptides to BMDCs, combined with either *S. suis* or LPS. ʟCATH-2 and ᴅCATH-2 both significantly lowered the pro-inflammatory immune response triggered by LPS in a dose-dependent manner (Figure 5a-b). This includes TNF-α, IL-8, IL-1ß and CD80/86. In contrast, no effect of LPS or the HDPs was seen on SLA-II expression (Figure 5c). In non-stimulated cells, no effects exerted by chCATH-2 were found at the concentrations tested.

In the initial setup, ʟCATH-2 and ᴅCATH-2 were added to the BMDCs simultaneously with *S. suis* at concentrations ranging from 0.625 to 2.5 µM, similar to the LPS assays. However, antimicrobial killing assays demonstrated that *S. suis*-S10 and *S. suis* S10-Δ*cps* were readily killed after the addition of 2.5 µM or 1.25 µM of peptide, respectively (data not shown). Thus, lower concentrations (0.156 µM to 0.625 µM) of ʟCATH-2 and ᴅCATH-2 were chosen for further experiments.

Even at these lower concentrations, ᴅCATH-2 showed a rapid dose-dependent reduction of TNF-α production, up to non-detectable levels at 0.625 µM (Figure 6b). This effect was seen for both *S. suis*-S10 and *S. suis* S10-Δ*cps*, indicating that this effect is not capsule dependent. For IL-8, the pattern ᴅCATH-2 exhibits resembles that of TNF-α (Figure 6a). Its effect on *S. suis* S10, however, was not found to be significant ($p = 0.1025$). For ʟCATH-2, the observed decrease on cytokine levels of *S. suis* stimulated BMDCs was less pronounced compared to ᴅCATH-2, yet still lowering the overall response. For the effect of ʟCATH-2 on TNF-a levels, the large error bars in the *S. suis* S10-Δ*cps* group can be appointed to an outlying group.

For CD80/86, as mentioned before, 2.5 hours of LPS stimulation did not suffice to upregulate CD80/86 expression (Figure 2c and 6c). *S. suis* S10-Δ*cps*, on the other hand, did enhance the expression of CD80/86. Furthermore, ᴅCATH-2 seemed to neutralize this effect at 0.625 µM, although not statistically significant. For 0.625 µM of ʟCATH-2, only a slight change in fluorescence intensity was observed in *S. suis* S10-Δ*cps* stimulated BMDCs.

To investigate the immunomodulatory role of ʟCATH-2 and ᴅCATH-2, these HDPs were added to live *S. suis* and *S. suis* S10-Δ*cps*, but also to their heat-killed

counterparts. Heat-killed *S. suis*-S10 or Δ*cps*, however, did not elicit an immune response from the BMDCs and no effect of LCATH-2 or DCATH-2 was observed (Figure 7a-d, Supplementary Figure 4).

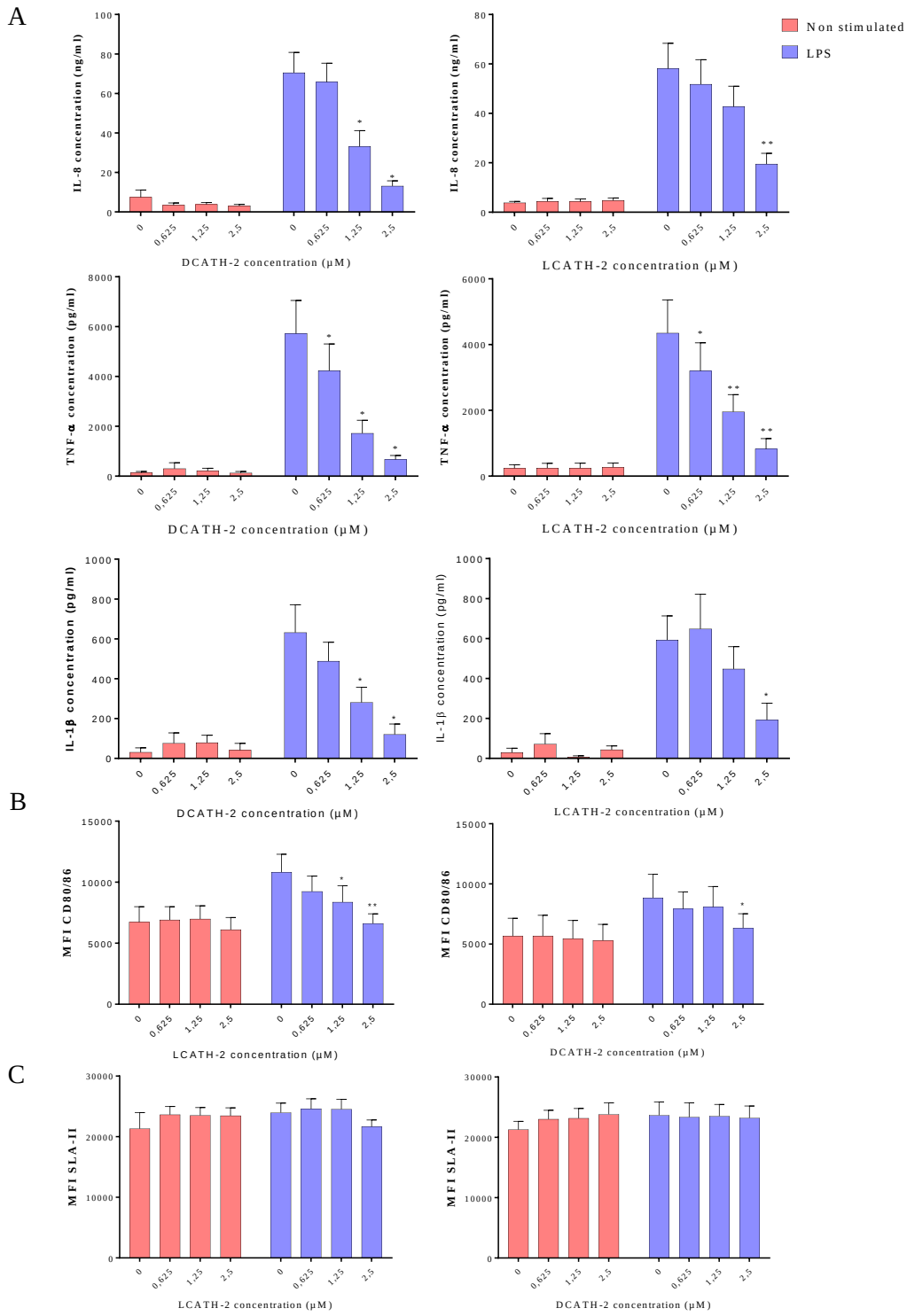

Figure 5. The LPS neutralizing properties of chCATH-2. BMDCs were cultured for 6 days, and subsequently stimulated with LPS for 24 hours. Results represent 6 independent experiments. **A)** ELISA readouts of the pro-inflammatory cytokines IL-8, IL-1ß, and TNF-α after 24 hours of stimulation, n=6. **B)** Summary of the MFI of CD80/86, measured using flow cytometry. For DCATH, n=5. For LCATH, n=6. **C)**. Summary of the MFI of SLA-II. For DCATH, n=5. For LCATH, n=6.

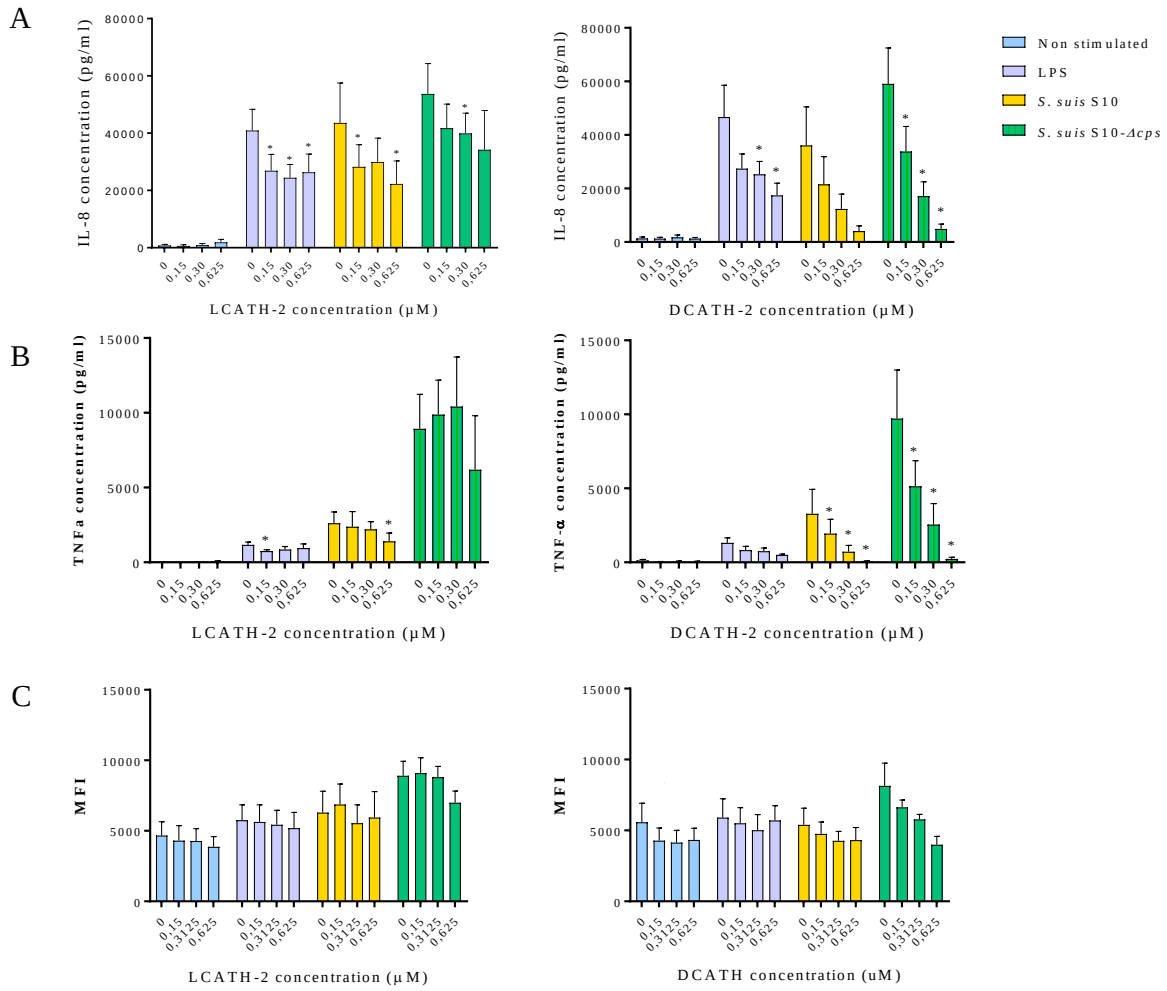

Figure 6. The effect of chCATH-2 on the CD80/86 expression and pro-inflammatory cytokine levels of *S. suis* stimulated BMDCs. After 6 days of culture, BMDCs were stimulated for 2.5 hours with either LPS, *S. suis*-S10, or *S. suis* S10-Δ*cps*, followed by antibiotic treatment to kill extracellular bacteria. Cells were allowed to rest for 24 hours, prior to the harvest of cell medium and cells. Results represent 6 independent experiments. **A)** ELISA readouts of the IL-8 production by BMDCs, n=6. **B)** The effect of LCATH-2 and DCATH-2 on TNF-α production by BMDCs, n=6. **C)** Summary of the MFI of CD80/86, n=6.

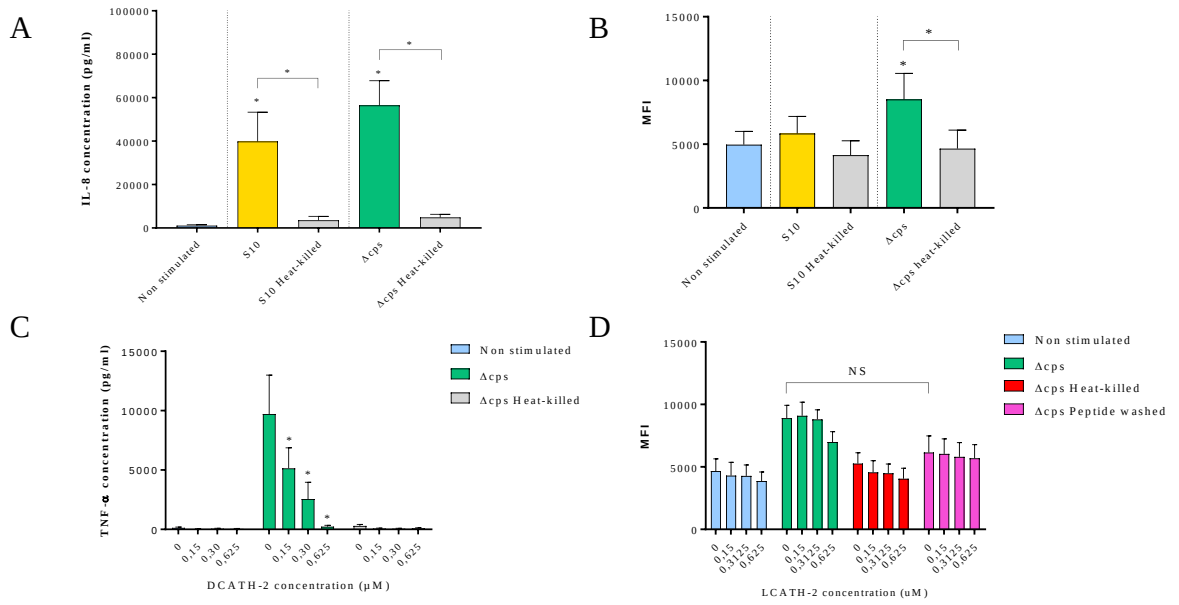

Figure 7. The immune response seen in heat-killed *S. suis* stimulated BMDCs and 'Peptide-Washed' BMDCs. BMDCs were stimulated with *S. suis* or heat-killed *S. suis* (both encapsulated and non-encapsulated). After 2.5 hours, the cells received medium containing antibiotics to kill extracellular *S. suis*. 'Peptide-washed' cells received either LCATH-2 or DCATH-2 (2.5 μM) instead of antibiotics. Results represent 6 independent experiments. **A)** ELISA readout of the IL-8 production by BMDCs, n=6. **B)** Summary of the MFI of CD80/86 expression by BMDCs, n=6. **C)** The effect of DCATH-2 on TNF-α production of S10-Δ*cps* and heat-killed S10-Δ*cps* stimulated BMDCs, n=6. **D)** Summary of the MFI of CD80/86 expression by BMDCs. 'Peptide-washed' cells received LCATH-2 instead of penicillin/streptomycin, n=5.

In addition to these experiments, cells were supplemented with 2.5 μM LCATH-2 or DCATH-2 instead of antibiotics after 2.5 hours of stimulation (labeled as 'Peptide washed'). This assay was not performed with PR-39 or PMAP-23, since these peptides do not kill *S. suis*. BMDCs that were washed with LCATH-2 instead of antibiotics, seemed to express lower levels of CD80/86, although not statistically significant (NS) (Figure 7d). Similar results were obtained for cytokine levels (i.e. IL-8 and TNF-α) and DCATH-2 washed BMDCs (Supplementary Figure 4). Comparison of cells receiving antibiotics to cells receiving chCATH-2, implies there is an immunomodulatory effect exerted by the peptides. However, none of these results were statistically significant.

Il-10 balances the pro-inflammatory immune response

The immune system is a tightly regulated system aiming to prevent pathogens from infecting the host. On the other hand, an exaggerated immune response will cause cell death and tissue damage. Therefore, pro-inflammatory responses and anti-inflammatory responses must be balanced. IL-10, the main anti-inflammatory cytokine, was found to be upregulated when BMDCs were stimulated with *S. suis* in a similar pattern as TNF-α and IL-8 (Figure 8a), presumably compensating for this pro-inflammatory response. The addition of LCATH-2 or DCATH-2 furthermore dose-dependently lowered the IL-10 cytokine levels (Figure 8b-c). Overall, a lowering of the pro-inflammatory cytokine levels was associated with a lowering of IL-10 levels and vice versa.

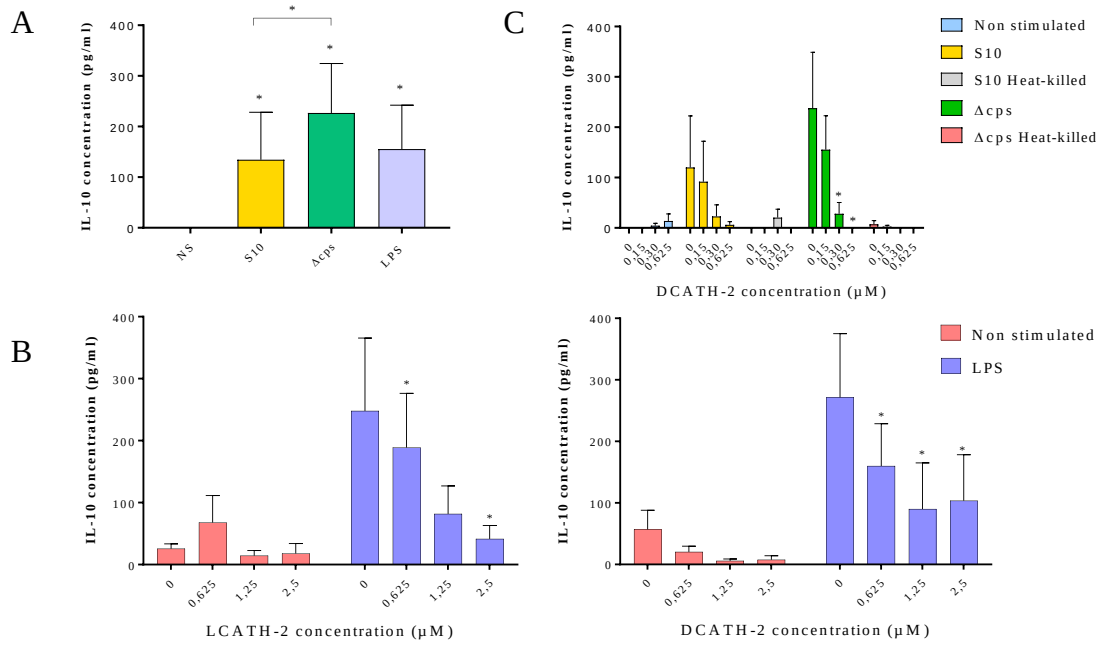

Figure 8. The influence of LPS, *S. suis*, and chCATH-2 on the anti-inflammatory response (IL-10). Dendritic cells were stimulated with LPS, *S. suis* or heat-killed *S. suis* (MOI:10). Results from 6 independent experiments of the IL-10 response are presented, as measured with ELISA. **A)** IL-10 production of BMDCs stimulated for 2.5 hours. Cells received antibiotics after 2.5 hours and were allowed to rest for another 21.5 hours, n=6. **B)** IL-10 production by BMDCs after 24 hours of stimulation with LPS and chCATH-2, n=4. **C)** The effect of ᴅCATH-2 on the IL-10 production of BMDCs after 2.5 hours of stimulation with either *S. suis*-S10 or *S. suis* S10-Δ*cps*, n=6.

Increasing T helper cell activation using ʟCATH-2

Finding an adjuvant, capable of boosting the immune response against *S. suis*, is key in vaccine development. Therefore, a co-culture assay was set-up to investigate the interaction between *S. suis*, BMDCs, T helper cells and HDPs. Due to extensive optimization time, an actual co-culture (with freshly isolated PBMCs) has only been performed once. What was observed, is that non-stimulated dendritic cells already activate T helper cells to some extent (compared to wells containing only $CD4^+$ T cells (Figure 9a-b). The proliferation index increased slightly, although no detectable IFN-γ was produced. The combination of PHA (20 µg/ml), dendritic cells, and T helper cells furthermore seemed a valid positive control. Neither *S. suis*-S10 or *S. suis* S10-Δ*cps*, on the other hand, seem to induce any response from the T helper cells. Regarding the HDPs, co-culture readouts suggested that ʟCATH-2 promotes the activation of $CD4^+$ T cells, but no statistics can confirm and replicates are needed. For PMAP-23 and PR-39, no clear effect was found (data not shown).

PMAP-23 exerts no immunomodulatory effect on porcine dendritic cells *in vitro*

Within this study, chCATH-2 exerted immunomodulatory properties on dendritic cells and T helper cells. For PMAP-23, no clear effects on the T helper cell response after *S. suis* stimulation were found. To investigate its interaction with porcine dendritic cells, similar assays to ʟCATH-2 and ᴅCATH-2 were conducted. As stated in the introduction, PMAP-23 has no binding affinity to LPS, although - at 20 µM - it does possess immunomodulatory capacities, upregulating IL-8 production in a porcine

macrophage cell line (Veldhuizen *et al.*, 2017).

For the concentrations tested in this study, PMAP-23 had no effect on the production of IL-8, TNF-α, IL-1ß, and IL-10 by dendritic cells or on expression of CD80/86 and SLA-II, in both non-stimulated and LPS stimulated groups (Figure 10a). Initial antimicrobial assays indicated that *S. suis* is resistant to PMAP-23. In addition, cell cultures with *S. suis* and PMAP-23 showed no effect of PMAP-23 on the BMDCs (data not shown). Therefore, assays studying the effect of PMAP-23 on the BMDC response after *S. suis* stimulation were not continued. For phagocytosis assays, PMAP-23 was included. Since the enhancement of phagocytosis is one of the known functions of cathelicidins, it was hypothesized that these peptides could promote the uptake of *S. suis*.

Phagocytosis of *S.* suis is blocked by its capsule (Meijerink *et al.*, 2012). In agreement with those findings, GFP-labeled *S. suis*-S10 was taken up significantly less compared to GFP-labeled *S. suis* S10-Δ*cps* (Supplementary Figure 5). However, GFP-labeled *S. suis* S10-Δ*cps* was slightly more fluorescent compared to *S. suis*-S10. Therefore, raw data was corrected as described in Supplementary Figure 5. Regarding phagocytosis, it was found that PMAP-23 had no effect enhancing or decreasing the uptake of either *S. suis*-S10 or *S. suis* S10-Δ*cps* (Figure 12a). Combining co-culture-, dendritic cell-, and phagocytosis assays, it seems that (*in vitro)* PMAP-23 exerts little to no effect on the immune response of porcine dendritic cells.

The BMDCs response to LPS is altered upon addition of PR-39

Within the group of porcine cathelicidins, PR-39 is one of the most extensively studied peptides. However, to the authors knowledge, its interactions with dendritic cells have not yet been investigated.

After simultaneous addition with LPS to BMDCs, the peptide exerts an inhibitory effect on the BMDC's cytokine production at 0.625 and 1.25 µM, but this inhibitory effect is less pronounced at a concentration of 2.5 µM (Figure 10b). This pattern was seen for IL-10, TNF-α, and IL-8. Strikingly, for IL-1ß, PR-39 dose-dependently upregulated the cytokine levels (Figure

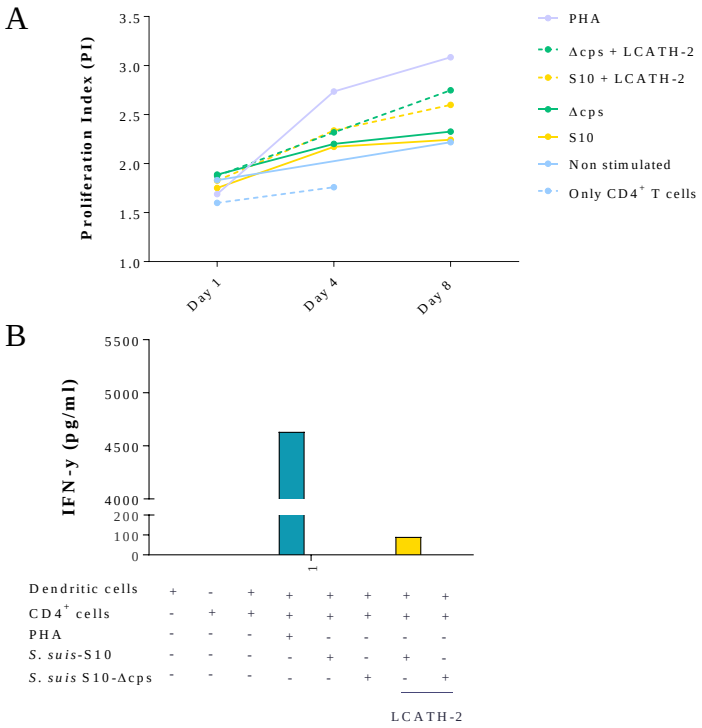

Figure 9. Readouts of the co-culture assay. 6 day old dendritic cells were stimulated with *S. suis*-S10 or *S. suis* S10-Δ*cps*. HDPs were added at a concentration of 0.3125 µM. After 2.5 hours, the bacteria were killed using penicillin/streptomycin and $CD4^+$ T cells were added to the dendritic cells in a 1:1 ratio. **A)** The proliferation index - as calculated from the CFSE fluorescence - was measured at various timepoints. For non-stimulated $CD4^+$ T cells, 1 value is missing, n=1. **B)** ELISA readout of the IFN-γ production of co-cultured $CD4^+$ T cells on day 1. Wells containing only dendritic cells did not produce any detectable IFN-γ, n=1.

10c). Within this study, it is the only cytokine found to be significantly upregulated when subjected to peptide.
For flow cytometry readouts of SLA-II and CD80/86, no changes in fluorescence intensity were observed after the addition of PR-39 to LPS and non-stimulated cells (Figure 10d).

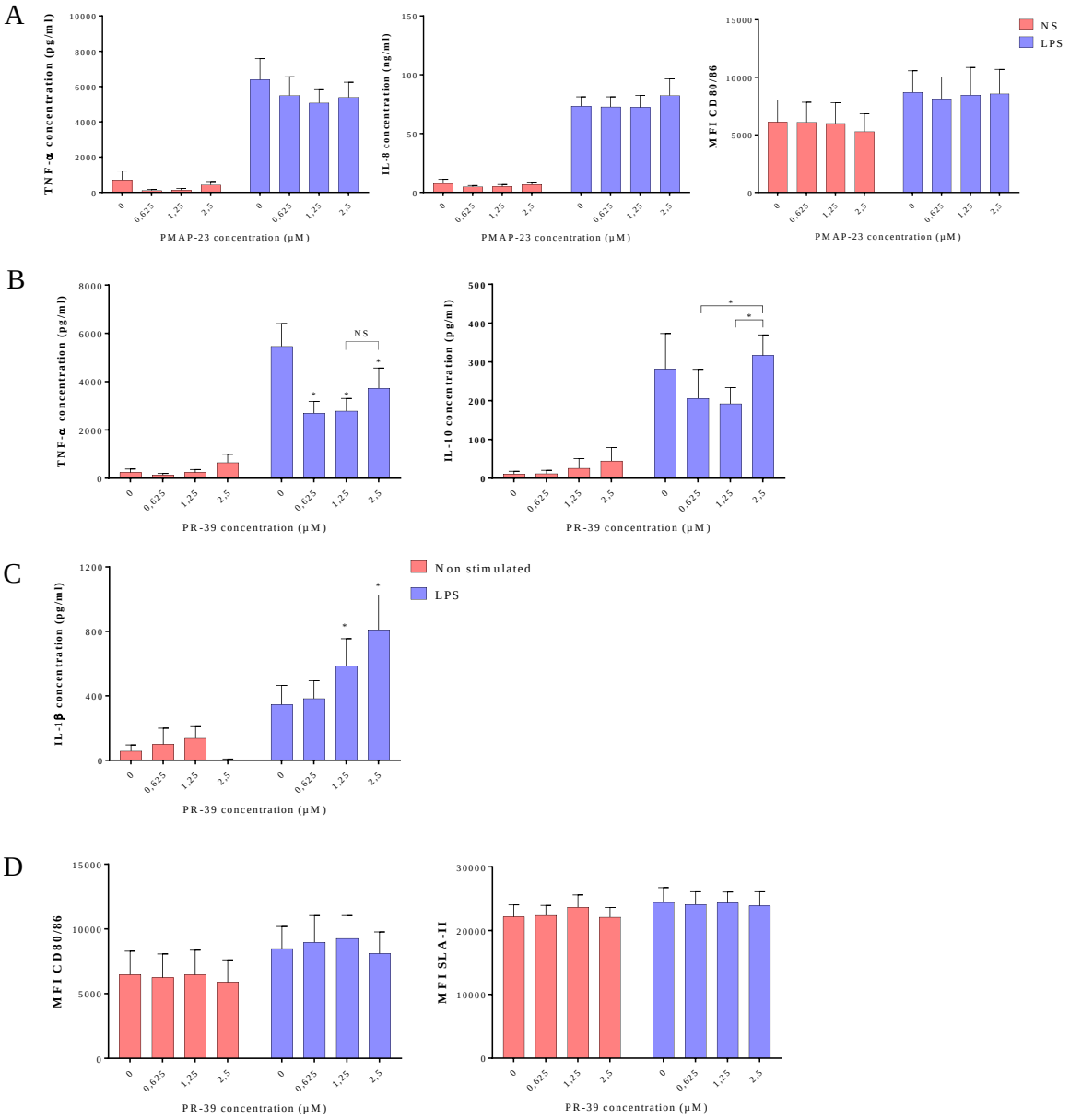

Figure 10. The immunomodulatory effects of PR-39 and PMAP-23. Readouts of the BMDC response after stimulation with LPS. Cells in 6 independent experiments were stimulated for 24 hours. **A)** TNF-α and IL-8 production by BMDCs after 24 hours of stimulation with LPS and PMAP-23 (left) and the effect of PMAP-23 on the BMDCs' CD80/86 expression (right), n=6. **B)** The effect of PR-39 on LPS stimulated and non-stimulated BMDCs. For IL-10, n=4. **C)** The effect of PR-39 on the BMDC's IL-1ß production, n=6. **D)** Summary of the MFI of CD80/86 and SLA-II expression by BMDCs after stimulation with LPS, n=5.

Unlike LCATH-2 and DCATH-2, PR-39 does not kill *S. suis*. Therefore, all assays with *S. suis* were performed with concentrations of PR-39 ranging from 0.625 to 2.5 µM. It was found that PR-39 did not influence cytokine levels or cell surface markers in both encapsulated and non-encapsulated *S. suis* stimulated BMDCs (Figure 11).

For the phagocytosis assays, no clear effect of LCATH-2 was found (Figure 12b). DCATH-2 on the other hand, lowered the phagocytosis of *S. suis* S10-Δ*cps* up to non-detectable levels (Figure 12c). Remarkably, PR-39 promoted the uptake of non-encapsulated *S. suis* in a dose-dependent manner (Figure 12d). This is in contrast to what was expected, since PR-39 is assumed to be degraded by *S. suis* (LeBel *et al.*, 2018). The effect of PR-39, however, was not seen for encapsulated *S. suis*. To conclude, whereas chCATH-2 primarily lowered the immune response (i.e. cytokine levels and phagocytosis), PR-39 showed several immune stimulating effects on porcine dendritic cells.

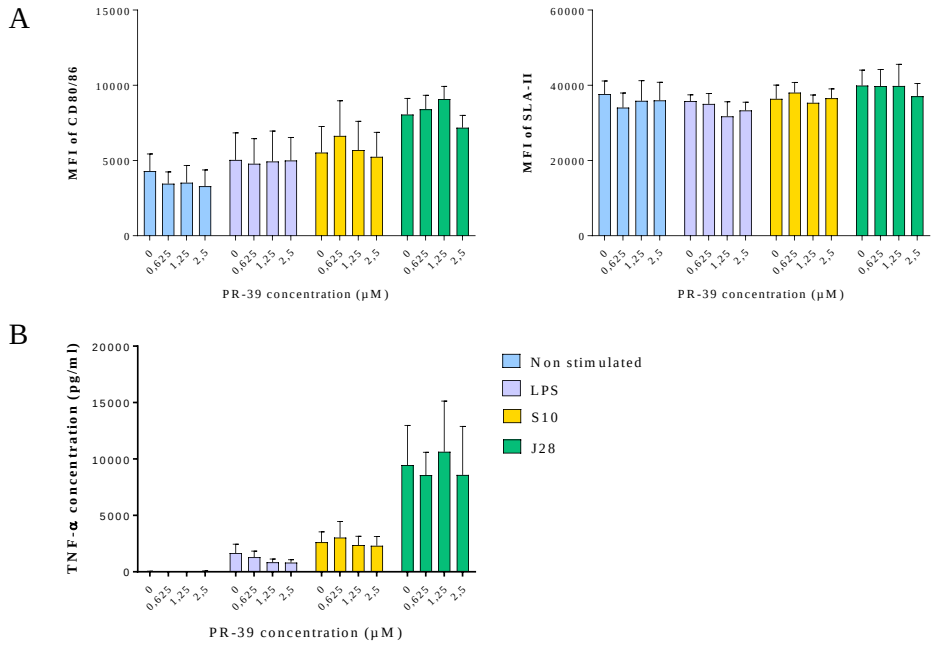

Figure 11. The influence of PR-39 on *S. suis* stimulated BMDCs. LPS, *S. suis*-S10, or *S. suis* S10-Δ*cps* stimulated BMDCs in 5 independent experiments were analyzed using flow cytometry and ELISA. **A)** Summary of the mean fluorescence intensity of CD80/86 and SLA-II expression of BMDCs, n=4. **B)** TNF-α production by BMDCs, n=5.

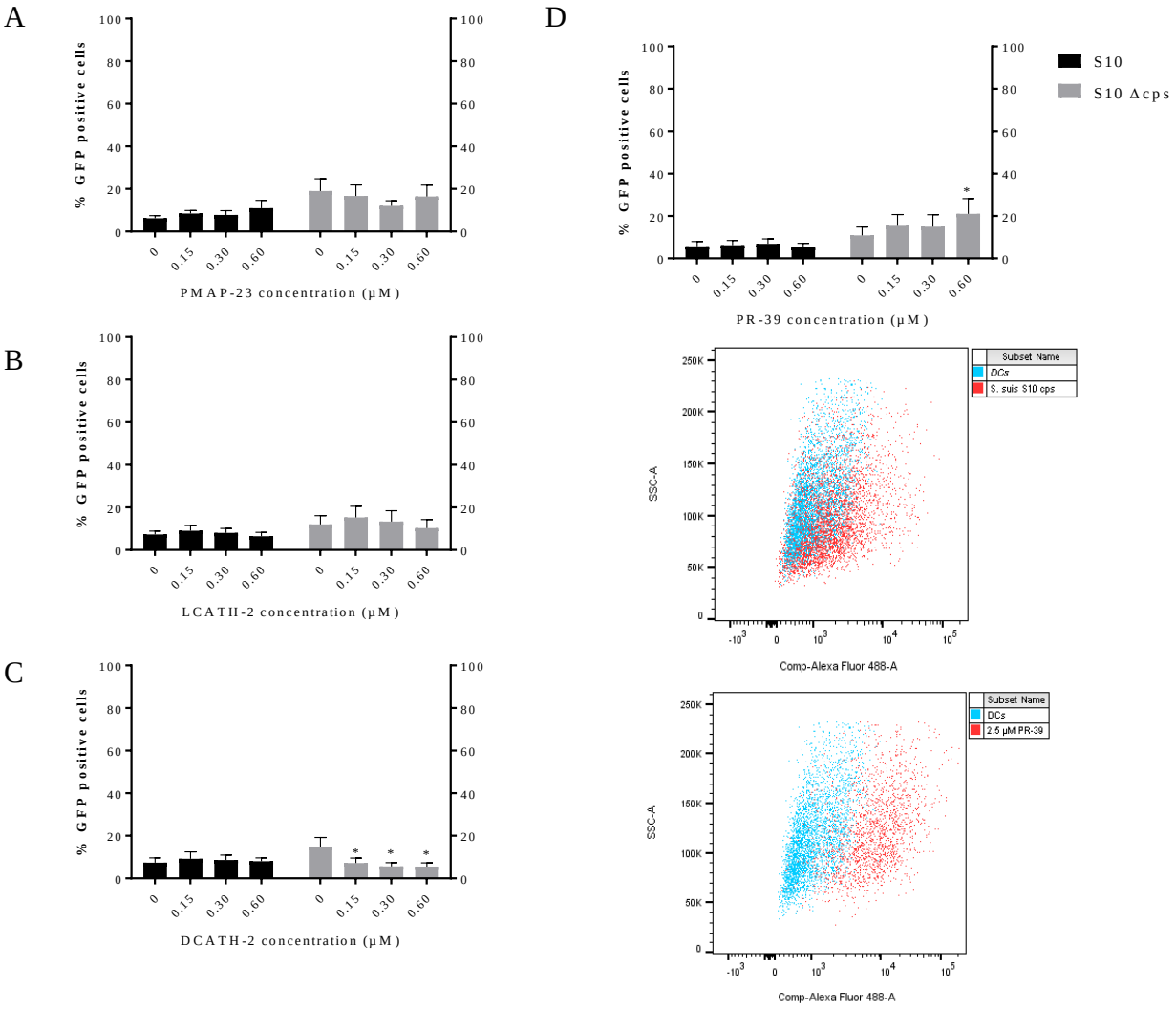

Figure 12. The influence of cathelicidins on the uptake of GFP-labeled *S. suis*. BMDCs were stimulated with GFP-labeled *S. suis*-S10 or *S. suis* S10-Δ*cps*, detectable on the Alexa Fluor 488 channel. After 2.5 hours, the cells were treated with antibiotics to kill any extracellular bacteria. After 24 hours, the cells were labeled with a SWC3a mAb for flow cytometry. Within the population of SWC3a^{+ve} cells, the GFP fluorescence was measured. **A)** The uptake of *S. suis*-S10 (black) and *S. suis* S10-Δ*cps* (grey) is presented as the percentage of GFP positive BMDCs. **B, C)** The uptake of *S. suis*-S10 and S10-Δ*cps* during concomitant addition of LCATH-2 or DCATH-2, respectively. **D)** The percentage of GFP positive BMDCs and the effect of PR-39 (top). The middle and bottom scatter plots show unstimulated dendritic cells in blue and S10-Δ*cps* stimulated cells in red, with the addition of 2.5 µM of PR-39 in the bottom graph. For all phagocytosis experiments, n = 5 independent experiments.

DISCUSSION

Dendritic cells are key players in the search for novel treatments and vaccine strategies against *S. suis,* since they form the first line of defense against pathogens. Understanding the way dendritic cells interact with *S. suis* can help develop a platform for further vaccine development. For various sequence types (i.e. ST1, -7, -25, and -28) of serotype 2, it is known that *S. suis* activates dendritic cells through the MyD88 pathway, followed by the subsequent release of several cytokines (such as IL-6, IL-12, and TNF-α) (Auger *et al*., 2019). Nonetheless, the capsular polysaccharide of *S. suis* hampers the induction of this immune response (Auger *et al*., 2018; Graveline *et al*., 2007; Lecours *et al*., 2011). In this study, the effect of the capsule on porcine BMDC activation was demonstrated using the ST01 *S. suis* isolate S10. In accordance with the aforementioned reports, the capsule of *S. suis* inhibits phagocytosis (Supplementary Figure 5) and the activation of BMDCs as measured with ELISA (Figure 2d). In addition, stimulation with *S. suis*-S10 induced significantly less expression of the cell surface markers SLA-II and CD80/86, compared to its non-encapsulated counterpart (Figure 2b-c). In order for dendritic cells to upregulate SLA-II expression and antigen presentation, the antigen is required to enter the endocytic pathway of the dendritic cell (Roche and Furuta, 2015). Thus, the ability to phagocytose bacteria is key in this process. The findings obtained from the SLA-II readouts, therefore, are in line with the observation that *S. suis* S10-Δ*cps*, but not *S. suis*-S10, is phagocytosed by BMDCs.

Since the wildtype *S. suis* is barely taken up by antigen presenting cells, studying the effect of cathelicidins on the phagocytosis of *S. suis* was one of the objectives within this project. Several HDPs are known to have immunomodulatory functions, amongst which influencing phagocytosis. For chCATH-2, Cuperus *et al*. (2018) demonstrated an increase in the uptake of pHrodo beads by PBMCs, when peptide was added. Lishko *et al*. (2016) showed the same effect for LL-37 using latex beads and human embryonic kidney cells, but furthermore observed a dose-dependent effect on live-bacteria (*E. coli* and *Staphylococcus aureus).* In contrast to these observations, several HDPs (including LL-37 and chCATH-2) were found to reduce the uptake of latex beads in a murine macrophage cell line (Coorens *et al*., 2017a). To the author's knowledge, no data is available regarding the effect of HDPs on the phagocytotic capacity of dendritic cells. From the phagocytosis assays conducted in this study, it can be stated that the investigated cathelicidins did not enhance the phagocytosis of *S. suis*-S10 (Figure 12). The phagocytosis of its non-encapsulated mutant, on the other hand, was promoted in a dose-dependent manner after addition of PR-39.

Cathelicidins are positively charged peptides (van Harten *et al*., 2018). Proposed by Lishko *et al*. (2016), is that this high positive charge allows the peptides to interact with negatively charged bacterial membranes. They found that LL-37 can serve as a ligand for the Mac-1 receptor (CD11/CD18), involved in the phagocytotic pathway. By adhering to both the Mac-1 receptor and the bacterial cell wall or cell wall components, these peptides are causing opsonization. Besides LL-37, Mac-1 binding domains have been identified in a large variety of host defense peptides (Podolnikova *et al*., 2015). Through the opsonic function of HDPs, phagocytosis could be promoted. This could explain why *S. suis* S10-Δ*cps* is taken up more efficiently after the addition of PR-39, although the same effect for the other peptides would then be expected. Generally speaking, the observed effect of PR-39 on the uptake of *S. suis* S10-Δ*cps*, was rather surprising. LeBel *et al*. (2018) demonstrated that PR-39 is degraded by a protease, dipeptidylpeptidase IV (DPPIV). This protease has been identified in 12

different *S. suis* strains (including P1/7), although the *S. suis* S10 isolate has not been tested (Jobin *et al*., 2005; Jobin and Grenier, 2003). Recently, the genome sequence of *S. suis* S10 has been published and shows >99% resemblance to the P1/7 genome (Gaiser *et al*., 2019). Furthermore, results obtained in this study, in addition to observations made by Velikova *et al*. (2016), indicate that the S10 isolate is highly comparable to the P1/7 strain. Therefore, and due to the importance of DPPIV as a virulence factor (Ge *et al*., 2009), the absence of this particular protease seems unlikely. Still, PR-39 is able to enhance the phagocytosis of *S. suis* S10-Δ*cps* and does not appear to completely lose its function. It must be mentioned though, that it has not been tested whether or not *S. suis* capsule mutants are capable of degrading PR-39.

Another interesting aspect to PR-39, is its effect on IL-1ß (Figure 10c). The cytokine IL-1ß originates from an IL-1ß precursor (proIL-1b). Upon activation of caspase-1, intracellular proIL-1ß is cleaved and can subsequently be externalized (Mortaz *et al*., 2012). Caspase-1 is downstream from the inflammasome pathway, which is induced by, amongst others, the P2X7 receptor (Elssner *et al*., 2004; Mortaz *et al*., 2012). For LL-37, Elssner *et al*. (2004) showed that this peptide could activate the P2X7 receptor and promote the subsequent release of IL-1ß. Furthermore, McHugh *et al*. (2019) provided evidence regarding the involvement of the inflammasome and the NLRP3-dependent pathway upon LL-37 stimulation. Peiró *et al*. (2018) discovered that the murine HDP 'mCRAMP' plays a pivotal role in the activation of the inflammasome and IL-1ß release in mouse alveolar macrophages. Hypothetically, PR-39 could resemble LL-37 and mCRAMP in activating the inflammasome, perhaps through the P2X7 receptor. This could provide an explanation as to why IL-1ß cytokine levels are increasing dose-dependently after PR-39 addition. However, PR-39 has never been shown to have P2X7 binding affinity. IL-1ß in its turn, has recently been shown to contribute to the clearance of bacterial burden after experimental *S. suis* infection in mice (Lavagna *et al*., 2019). Furthermore, IL-1ß has been found to promote phagocytosis of opsonized beads in microglial cells (Ferreira *et al*., 2011). Comparing Figures 10c and 12d, the IL-1ß production promoted by PR-39, might be linked to the phagocytosis of *S. suis* S10-Δ*cps*.

Regarding *S. suis*-S10, the peptides might still be able to opsonize the bacteria or promote phagocytotic pathways. Nonetheless, recognition of *S. suis* by the dendritic cells is required for the eventual phagocytosis. Thus, it seems probable that addition of HDPs will have limited effect on the phagocytosis of encapsulated *S. suis*. Since the capsule of *S. suis* is interfering with the phagocytotic capacity of dendritic cells, this hypothetically blocks T cell activation. With HDPs being potent immunomodulators, one of the objectives within this project was to evaluate their capacity to promote T cell activation. This was investigated using a culture system in which BMDCs and $CD4^+$ T cells were cultured together. For pigs, no standardized protocols for such *in vitro* culture systems are available. Additionally, the time needed to complete a culture is relatively long (15 days), causing cell viability to become an issue. For the BMDCs, the length of the culture turned out not to be a problem, in contrast to the T cells. After thawing, staining and purifying the PBMCs, the cells were no longer responding to any mitogen and died rapidly. Reducing the overall handling of the cells would presumably be beneficial to their viability. Negative selection of Th-cells, for instance, instead of positive selection on their CD4 marker, would leave cell surface receptors unaltered (Moore *et al*., 2019). However, for porcine cells, limited tools are available. As an illustration regarding negative selection, a specific mAb against natural-killer cells is required. To the author's knowledge, no such marker is known in pigs and therefore,

no mAbs against natural-killer cells are commercially available. In that perspective, porcine cell culture systems are far less developed compared to human and murine culture systems and require extensive optimization. Due to this optimization time, an actual readout was only obtained once. The effect ʟCATH-2 is exerting seems very promising, although the differences in CFSE fluorescence are rather subtle (Figure 9a). Logically, more replicates are needed to confirm the effect of ʟCATH-2 on $CD4^+$ T cells.

As reviewed in Ma *et al*. (2017), TCR activation is accompanied by various changes in membrane potentials. Intracellularly, the concentration of potassium (K^+) is relatively high and extracellularly, calcium (Ca^{2+}) and natrium (Na^+) are the predominant positive charged ions. The membrane of a T lymphocyte consists of an outer and an inner leaflet, the latter being negatively charged. Upon TCR activation (through MHC-II), K^+ diffuses out of the cell, through K^+ channels (Cahalan and Chandy, 2009). Subsequently, Ca^{2+} and Na^+ channels are activated, causing influx of these ions and a local depolarization of the T cell membrane. It is then proposed that this depolarization enables dissociation of the CD3 tails from the membrane, followed by phosphorylation of the immunotyrosine-based activation motifs (ITAMs), although the precise mechanism is still unknown.

Besides TCR activation, it has been shown that there are more pathways to cause T cell membrane depolarization. Activation of the P2X7 receptor, for instance, can open up calcium channels and thereby enable the influx of calcium (Badou *et al*., 2013). A mechanism to bypass the TCR, is to incorporate positively charged lipids into the T cell membrane (Ma *et al*., 2017b). These studies underline the importance of the electrochemical gradient in T cell signalling pathways. Therefore, the high positive charge of HDPs, or their capacity to bind the P2X7 receptor (Elssner *et al*., 2004), could play a role in the activation of T cells. Similar to PR-39, however, it is not known whether ʟCATH-2 can bind to the P2X7 receptor or not, or if these peptides interact with T cell membranes. Therefore, if ʟCATH-2 does in fact influence T cell activation, the precise mechanisms remain to be elucidated.

In addition, HDPs are known to be chemoattractants (McHugh *et al*., 2019; De Yang *et al*., 2000; Yu *et al*., 2007). LL-37, for instance, is a chemoattractant for (e.g.) neutrophils, in which G-coupled receptors (FPRL1) and the inflammasome are activated. Several reports furthermore indicate that chCATH-2 and PR-39 are also capable of attracting immune cells (Bommineni *et al*., 2014; Huang *et al*., 1997). Investigating if HDPs can affect the activation of dendritic cells and $CD4^+$ T cells through chemotaxis, would be another interesting next step. This could be done, for instance, designing a membrane migration assay with BMDCs and $CD4^+$ T cells.

Concerning the CFSE staining measuring T cell proliferation, it is assumed that the fluorescent signal of a cell is divided equally between its daughter cells upon division. The assumption of an exactly equal division of fluorescent signal in a biological system, however, rarely holds (Luzyanina *et al*., 2014). Over the past years, various reports have been published studying CFSE characteristics (Mazzocco *et al*., 2017; Roederer, 2011). As a result, some extensive statistical and mathematical models have been developed, to provide better tools for the analysis of CFSE results (Luzyanina *et al*., 2014; Miao *et al*., 2012). Since these models are extremely complex, they were not integrated within this study. The implementation of such models should be considered though, when measuring the proliferation becomes a substantial part of a project. Especially for the detection of subtle differences, using more advanced models would be advantageous.

Despite the fact that the CFSE calculations were not optimal, it became clear that PHA and ionomycin/PMA were capable of

stimulating porcine $CD4^+$ T cells (Figure 4). It was hypothesized that the capsular polysaccharide of *S. suis* would block the formation of the adaptive immune response. However, *S. suis* S10-Δ*cps* stimulated BMDCs were not able to activate T helper cells any more than *S. suis*-S10 stimulated BMDCs (Figure 9a). It has to be kept in mind, that it is uncertain whether the pigs used in this study had encountered environmental *S. suis* before, and whether a T cell with a *S. suis* specific TCR was present within the pool of $CD4^+$ cells. Furthermore, this experiment was only conducted once and any differences might be subtle. Therefore, the answer to this question remains inconclusive.

Regarding vaccine development, memory T cells are of special interest. Within the porcine immune system, $CD4^+CD8^+$ cells are thought to be memory T cells. A characteristic of the memory T cell subset, is that the proportion increases with age. As demonstrated by Rodrígez-Gómez *et al*. (2016), the proportion of $CD4^+CD8^+$ cells does in fact increase with age, reaching levels up to 80% of total $CD4^+$ cells in 4 year old pigs. The pigs used in this study were approximately 3 months old, with the proportion of double positive cells ranging from 15-35%. This matches the findings mentioned above, strengthening the idea that these cells are memory T cells. However, the effect of *S. suis* or cathelicidins on the $CD4^+CD8^+$ population of T cells still needs to be investigated.

Our initial hypothesis suggested that cathelicidins could boost the immune response of BMDCs and thereby promote T cell activation. Based on flow cytometry and ELISA data, this was not the case. LCATH-2 and DCATH-2 reduce the immune response that is induced by LPS, *S. suis*-S10, and *S. suis* S10-Δ*cps*, in a dose-dependent manner (Figures 5 and 6). A known property of various HDPs is their affinity to bind endotoxins. As stated in the introduction, chCATH-2 has a high affinity to bind LPS (Veldhuizen *et al*., 2017). The binding of HDPs to LPS and subsequent neutralization, as proposed by Coorens *et al*. (2017), blocks TLR4 activation. This would provide an explanation for the dose-dependent lowering of the immune response that was observed in LPS stimulated BMDCs (Figure 5).

For *S. suis*, what is causing the reduced activation is not known. At higher peptide concentrations, the decrease in cytokine levels and cell surface marker expression could be appointed to the bactericidal effect of the peptides. The concentrations tested in this study (0.15 to 0.625 μM), however, are too low to be bactericidal and cannot fully account for the observed decrease.

S. suis is a gram-positive bacterium and thus does not express LPS. Besides LPS, chCATH-2 is known to neutralize other endotoxins, amongst which LTA (Coorens *et al*., 2017b). If it is in fact an endotoxin of *S. suis* these peptides are binding to, then either purified LTA derived from *S. suis* (Gisch *et al*., 2018), or *S. suis* mutants lacking the endotoxin of interest (e.g. LTA or suilysin (Lavagna *et al*., 2019)), could help examine the mechanism behind the dose-dependent lowering of the *S. suis* induced immune response.

Another cause for the dose-dependent decline of the immune response could be appointed to a direct immunomodulatory effect on the BMDCs exerted by the peptides. Within this study, a reason to assume LCATH-2 and DCATH-2 possess direct immunomodulatory properties, is the pattern seen in the 'peptide washed' BMDCs (Figure 7d, Supplementary Figure 4). Although not significantly, the peptides consistently lowered the overall immune response after stimulation with *S. suis*. What was found by Mookherjee *et al.* (2006), is that LL-37 is capable of blocking the TLR4-to-NF-κB pathway, contributing to a lowering of cytokine levels (i.e. TNF-α). Choi *et al*. (2014) showed that a LL-37 derivative could suppress the production of pro-inflammatory cytokines after IL-32 stimulation in $CD14^+$ monocytes. They also observed that the peptide upregulated

MKP-1 phosphorylation and IL-1RA production, contributing to a reduction of inflammation. These studies are indicating that cathelicidins can interact directly with cells, apart from their endotoxin neutralizing capacities, dampening the immune response.

Regarding 'peptide washed' cells, a second explanation for the seemingly declined immune response, could be silent killing (Coorens *et al*., 2017b). Upon death, bacteria can release various endotoxins and other cell wall components (Lepper *et al*., 2002). After killing the bacteria, HDPs can directly bind to endotoxins that are released in the environment, altogether lowering the immune response. This implies that the killing of bacteria, using antibiotics, can trigger an additional response from dendritic cells that is absent when bacteria are killed by peptides. Therefore, treating the cells with peptide instead of antibiotics can result in a more 'natural' way of killing. This theory, however, can only hold when dead bacteria can indeed trigger an immune response. Within this project, heat-killed *S. suis* (both encapsulated and non-encapsulated) was unable to elicit a response from the BMDCs (Figure 7). This suggests that interaction between live *S. suis* and antigen-presenting cells is needed. However, these findings are in contrast to what was found by Segura *et al*. (1999). They observed an increase in TNF-α and IL-6 production after stimulation of murine macrophages with heat-killed *S. suis* strain S735 (serotype 2). These differences might be caused by differences in protocols (different cell types, *S. suis* strains, or lengths and temperatures of heath-killing). Unfortunately, no effect of the peptides on the heat-killed *S. suis* induced immune response was observed (Figure 7c).

Within this study, the cathelicidins that were investigated primarily exerted an anti-inflammatory effect rather than a stimulating effect on the immune response. Concerning adjuvant and vaccine development, dampening the immune response is usually not a desired property, although adjuvants should not trigger an exaggerated immune response that could compromise host safety (Batista-Duharte *et al*., 2018). Inactivated bacteria (bacterins) are frequently used for autogenous *S. suis* vaccines (Lapointe *et al*., 2002). It requires the attenuation or killing of the pathogen, for instance using sonification. These bacterins, however, do not seem to induce a profound immunity against strains other than the particular strain it is derived from (Baums *et al*., 2009; Büttner *et al*., 2012) Since there are many different *S. suis* strains, the development of a sub-unit vaccine containing various *S. suis* antigens derived from different strains, or a potent universal antigen (provided that such an antigen exists), would be the ideal solution. Such sub-unit vaccines, however, require profound adjuvants (Martelet *et al*., 2017). A frequently used adjuvant, aluminum hydroxide (alum), is known to trigger the release of IL-1ß (similar to PR-39), thereby boosting the immune response (Li *et al*., 2007). An advantage of PR-39 over alum could be that it was observed to be capable of lowering the IL-8 and TNF-α responses at lower concentrations (Figure 10b), restraining the formation of excessive inflammation. However, regarding *S. suis*, the influence of PR-39 was limited (Figure 11) and might therefore not be the ideal candidate for a *S. suis* specific vaccine adjuvant.

Some strains of *S. suis*, primarily associated with serotype 2 ST07, can cause a so-called streptococcal toxic shock syndrome (Lachance *et al*., 2013; Ye *et al*., 2006). This specific strain from a human outbreak in China, causes a severe IFN-γ dependent inflammatory immune response, resulting in septic shock. In this case, it might be beneficial to lower the (harmful) immune response. However, to fully elucidate whether chCATH-2 could be used as a vaccine adjuvant, further development of the *in vitro* co-culture is needed, particularly in the context of ST07.

All the results presented here, are derived from *in vitro* models. Major advantages of

such *in vitro* models consist of the relative low amounts of animals needed and the possibilities of extensive screening. *In vitro* results are extremely valuable and can give good hints towards eventual *in vivo* outcomes and help understand underlying mechanisms. The immune system, however, is a complex system in which different cell types interact with each other in complex tissue types (McComb *et al*., 2013). Looking only at dendritic cells or T cells in an artificial environment, limits the interactions of cells. This provides a challenge, translating *in vitro* results to an eventual *in vivo* model. The poor translational character of many *in vitro* studies demonstrates the need for the development of *in vitro* models that have a closer resemblance to the *in vivo* situation (Jaroch *et al*., 2018). Especially concerning the '3 Rs rule' of replacement, reduction, and refinement, optimizing translatability is crucial.

The dendritic cell culture system is a well described system, generating reproducible results. Dendritic cells are high expressers of the MHC-II molecule and are able to upregulate cytokine production and CD80/86 expression upon stimulation, mediated via the MyD88 pathway (Figure 2) (Carrasco *et al*., 2001; Lecours *et al*., 2011). *In vivo*, when cytokine levels of blood plasma or brain cytokine levels are examined after *S. suis* challenge, the same patterns are seen (Auger *et al*., 2019). Multiple cytokines, such as IL-1ß and TNF-α, are upregulated and a predominant role for the MyD88 pathway was found. BMDCs thereby closely mimic the *in vivo* situation and provide a solid baseline for further investigations. Cultures of porcine T cells, on the other hand, still need work boosting reproducibility. Co-culturing these cells, investigating the interaction between the two cell subsets, was part of an attempt to refine the *in vitro* culture. Hypothetically, an *in vitro* co-culture system containing multiple cell types (e.g. natural killer cells, T cells, B cells, dendritic cells, and/or macrophages), would be ideal to serve as a platform for vaccine development. Such systems could then elucidate what effect adjuvants are exerting on the different cell subsets and how they influence the interaction between the cells, since they mimic the complexity of the immune system more closely. Novel strategies include the development of an immune-system-on-a-chip (combined with microfluidic systems) (Polini *et al*., 2019), or involve tissue engineering combined with hydrogel scaffolds (Singh, 2017). With special regard to T cells, microfluidic systems allow better analyses of rare T cell subsets (such as T cells with a *S. suis* specific TCR). These approaches and techniques, however, have emerged only recently and require more general understanding of the immune system prior to development.

Concluding, BMDCs respond to stimulation with *S. suis*, although this response is hampered by the capsular polysaccharide. Unfortunately, PMAP-23 showed no immunomodulatory effects on BMDCs for the concentrations tested. The effects exerted by PR-39 and chCATH-2 on BMDCs, seem promising for adjuvant development, when focusing on preventing excessive inflammation. For both peptides, however, the effect on the phagocytosis of encapsulated *S. suis* stimulated BMDCs is limited. For PR-39 specifically, there is no effect on cytokine production in *S. suis* stimulated BMDCs, and might therefore not be the most logical choice of adjuvant for this particular bacterium. Overall, more work on the complexity of the porcine immune system is needed to find novel strategies targeting *S. suis* to protect both pigs and humans.

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JOURNAL OF CHOICE

This report was written as if it was designated for the journal 'Veterinary Immunology and Immunopathology'. This journal focusses on basic, comparative, and clinical immunology, including the immunology of livestock. Their instructions to authors include:

- An abstract of no more than 400 words
- Providing a maximum of 6 keywords following the abstract
- A list of abbreviations that can be used without further explaining
- References within the text should consist of the name of the first author (without initials), followed by '*et al'* when applicable, and the year of publication.

REFERENCES

Athey TBT, Teatero S, Takamatsu D, Wasserscheid J, Dewar K, Gottschalk M, et al. Population Structure and Antimicrobial Resistance Profiles of Streptococcus suis Serotype 2 Sequence Type 25 Strains. PLoS One 2016;11:e0150908. doi:10.1371/journal.pone.0150908.

Auger J-P, Benoit-Biancamano M-O, Bédard C, Segura M, Gottschalk M. Differential role of MyD88 signaling in Streptococcus suis serotype 2-induced systemic and central nervous system diseases . Int Immunol 2019:7–10. doi:10.1093/intimm/dxz033.

Auger JP, Dolbec D, Roy D, Segura M, Gottschalk M. Role of the Streptococcus suis serotype 2 capsular polysaccharide in the interactions with dendritic cells is strain-dependent but remains critical for virulence. PLoS One 2018;13:1–18. doi:10.1371/journal.pone.0200453.

Badou A, Jha MK, Matza D, Flavell RA. Emerging roles of L-type voltage-gated and other calcium channels in T lymphocytes. Front Immunol 2013;4:243. doi:10.3389/fimmu.2013.00243.

Batista-Duharte A, Martínez DT, Carlos IZ. Efficacy and safety of immunological adjuvants. Where is the cut-off? Biomed Pharmacother 2018;105:616–24. doi:10.1016/J.BIOPHA.2018.06.026.

Baums CG, Kock C, Beineke A, Bennecke K, Goethe R, Schroder C, et al. Streptococcus suis Bacterin and Subunit Vaccine Immunogenicities and Protective Efficacies against Serotypes 2 and 9. Clin Vaccine Immunol 2009;16:200–8. doi:10.1128/CVI.00371-08.

Bommineni YR, Pham GH, Sunkara LT, Achanta M, Zhang G. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. Mol Immunol 2014;59:55–63. doi:10.1016/j.molimm.2014.01.004.

Büttner N, Beineke A, de Buhr N, Lilienthal S, Merkel J, Waldmann K-H, et al. Streptococcus suis serotype 9 bacterin immunogenicity and protective efficacy. Vet Immunol Immunopathol 2012;146:191–200. doi:10.1016/j.vetimm.2012.03.012.

Cahalan MD, Chandy KG. The functional network of ion channels in T lymphocytes. Immunol Rev 2009;231:59–87. doi:10.1111/j.1600-065X.2009.00816.x.

Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, et al. Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 2001;104:175–84. doi:10.1046/j.1365-2567.2001.01299.x.

Choi K-YG, Napper S, Mookherjee N. Human cathelicidin LL-37 and its derivative IG-19 regulate interleukin-32-induced inflammation. Immunology 2014;143:68–80. doi:10.1111/imm.12291.

Coffman RL, Sher A, Seder RA. Vaccine adjuvants: Putting innate immunity to work. Immunity 2010;33:492–503. doi:10.1016/j.immuni.2010.10.002.

Coorens M, Scheenstra MR, Veldhuizen EJA, Haagsman HP. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. Sci Rep 2017a;7:1–11. doi:10.1038/srep40874.

Coorens M, Schneider VAF, de Groot AM, van Dijk A, Meijerink M, Wells JM, et al. Cathelicidins Inhibit Escherichia coli-Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner. J Immunol 2017b;199:1418–28. doi:10.4049/jimmunol.1602164.

Cuperus T, van Dijk A, Matthijs MGR, Veldhuizen EJA, Haagsman HP. Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic E. coli. Sci Rep 2016;6:26622. doi:10.1038/srep26622.

Cuperus T, Kraaij MD, Zomer AL, Van Dijk A, Haagsman HP. Immunomodulation and effects on microbiota after in ovo administration of chicken cathelicidin-2. PLoS One 2018;13:1–19. doi:10.1371/journal.pone.0198188.

Elssner A, Duncan M, Gavrilin M, Wewers MD. A Novel P2X7 Receptor Activator, the Human Cathelicidin-Derived Peptide LL37, Induces IL-1β Processing and Release. J Immunol 2004;172:4987–94. doi:10.4049/jimmunol.172.8.4987.

European One Health action plan against AMR. 2017. https://ec.europa.eu/health/amr/sites/amr/files/amr_action_plan_2017_en.pdf (accessed August 20, 2019).

Ferreira R, Santos T, Viegas M, Cortes L, Bernardino L, Vieira O V, et al. Neuropeptide Y inhibits interleukin-1β-induced phagocytosis by microglial cells. J Neuroinflammation 2011;8:169. doi:10.1186/1742-2094-8-169.

Gaiser RA, Zomer AL, Wells JM, van Baarlen P. Draft Genome Sequence of Streptococcus suis S10, a Virulent Strain Used in Experimental Pig Infections. Microbiol Resour Announc 2019;8. doi:10.1128/MRA.00227-19.

Gaudino SJ, Kumar P. Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. Front Immunol 2019;10:360. doi:10.3389/fimmu.2019.00360.

Ge J, Feng Y, Ji H, Zhang H, Zheng F, Wang C, et al. Inactivation of Dipeptidyl Peptidase IV Attenuates the Virulence of Streptococcus suis Serotype 2 that Causes Streptococcal Toxic Shock Syndrome. Curr Microbiol 2009;59:248–55. doi:10.1007/s00284-009-9425-8.

Gerner W, Käser T, Saalmüller A. Porcine T lymphocytes and NK cells - An update. Dev Comp Immunol 2009;33:310–20. doi:10.1016/j.dci.2008.06.003.

Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH, Saalmüller A. Phenotypic and functional differentiation of porcine αβ T cells: Current knowledge and available tools. Mol Immunol 2015;66:3–13. doi:10.1016/j.molimm.2014.10.025.

Gisch N, Auger J-P, Thomsen S, Roy D, Xu J, Schwudke D, et al. Structural analysis and immunostimulatory potency of lipoteichoic acids isolated from three *Streptococcus suis* serotype 2 strains. J Biol Chem 2018;293:12011–25. doi:10.1074/jbc.RA118.002174.

Graveline R, Segura M, Radzioch D, Gottschalk M. TLR2-dependent recognition of Streptococcus suis is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int Immunol 2007;19:375–89. doi:10.1093/intimm/dxm003.

Gupta S, Winglee K, Gallo R, Bishai WR. Bacterial subversion of cAMP signalling inhibits cathelicidin expression, which is required for innate resistance to *Mycobacterium tuberculosis*. J Pathol 2017;242:52–61. doi:10.1002/path.4878.

Haas B, Grenier D. Understanding the virulence of Streptococcus suis: A veterinary, medical, and economic challenge. Med Mal Infect 2018;48:159–66. doi:10.1016/j.medmal.2017.10.001.

van Harten R, van Woudenbergh E, van Dijk A, Haagsman H. Cathelicidins:

Immunomodulatory Antimicrobials. Vaccines 2018;6:63. doi:10.3390/vaccines6030063.

Holani R, Shah C, Haji Q, Inglis GD, Uwiera RRE, Cobo ER. Proline-arginine rich (PR-39) cathelicidin: Structure, expression and functional implication in intestinal health. Comp Immunol Microbiol Infect Dis 2016;49:95–101. doi:10.1016/j.cimid.2016.10.004.

Huang H-J, Ross CR, Blecha F. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. J Leukoc Biol 1997;61:624–9. doi:10.1002/jlb.61.5.624.

Jaroch K, Jaroch A, Bojko B. Cell cultures in drug discovery and development: The need of reliable in vitro-in vivo extrapolation for pharmacodynamics and pharmacokinetics assessment. J Pharm Biomed Anal 2018;147:297–312. doi:10.1016/J.JPBA.2017.07.023.

Jobin M-C, Grenier D. Identification and characterization of four proteases produced by *Streptococcus suis*. FEMS Microbiol Lett 2003;220:113–9. doi:10.1016/S0378-1097(03)00088-0.

Jobin M-C, Martinez G, Motard J, Gottschalk M, Grenier D. Cloning, purification, and enzymatic properties of dipeptidyl peptidase IV from the swine pathogen Streptococcus suis. J Bacteriol 2005;187:795–9. doi:10.1128/JB.187.2.795-799.2005.

Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: How does the immune system decide to mount a helper T-cell response? Immunology 2008;123:326–38. doi:10.1111/j.1365-2567.2007.02719.x.

Knosp CA, Johnston JA. Regulation of CD4 + T-cell polarization by suppressor of cytokine signalling proteins. Immunology 2012;135:101–11. doi:10.1111/j.1365-2567.2011.03520.x.

Lachance C, Gottschalk M, Gerber PP, Lemire P, Xu J, Segura M. Exacerbated type II interferon response drives hypervirulence and toxic shock by an emergent epidemic strain of Streptococcus suis. Infect Immun 2013;81:1928–39. doi:10.1128/IAI.01317-12.

Lapointe L, D'Allaire S, Lebrun A, Lacouture S, Gottschalk M. Antibody response to an autogenous vaccine and serologic profile for Streptococcus suis capsular type 1/2. Can J Vet Res 2002;66:8–14.

Lavagna A, Auger J-P, Dumesnil A, Roy D, Girardin SE, Gisch N, et al. Interleukin-1 signaling induced by Streptococcus suis serotype 2 is strain-dependent and contributes to bacterial clearance and inflammation during systemic disease in a mouse model of infection. Vet Res 2019;50:52. doi:10.1186/s13567-019-0670-y.

LeBel G, Vaillancourt K, Yi L, Gottschalk M, Grenier D. Dipeptidylpeptidase IV of Streptococcus suis degrades the porcine antimicrobial peptide PR-39 and neutralizes its biological properties. Microb Pathog 2018;122:200–6. doi:10.1016/j.micpath.2018.06.029.

Lecours MP, Segura M, Fittipaldi N, Rivest S, Gottschalk M. Immune Receptors Involved in Streptococcus suis Recognition by Dendritic Cells. PLoS One 2012;7. doi:10.1371/journal.pone.0044746.

Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, et al. Characterization of porcine dendritic cell response to Streptococcus suis. Vet Res 2011;42:72. doi:10.1186/1297-9716-42-72.

Lepper P, Held T, Schneider E, Bölke E, Gerlach H, Trautmann M. Clinical implications of antibiotic-induced endotoxin release in septic shock. Intensive Care Med 2002;28:824–33. doi:10.1007/s00134-002-1330-6.

Li H, Nookala S, Re F. Aluminum Hydroxide Adjuvants Activate Caspase-1 and Induce IL-1β and IL-18 Release. J Immunol 2007;178:5271–6. doi:10.4049/jimmunol.178.8.5271.

Li J, Xia J, Tan C, Zhou Y, Wang Y, Zheng C, et al. Evaluation of the immunogenicity and the protective efficacy of a novel identified immunogenic protein, SsPepO, of Streptococcus suis serotype 2. Vaccine 2011;29:6514–9. doi:10.1016/J.VACCINE.2011.07.010.

Lishko VK, Moreno B, Podolnikova NP, Ugarova TP. Identification of Human Cathelicidin Peptide LL-37 as a Ligand for Macrophage Integrin αMβ2 (Mac-1, CD11b/CD18) that Promotes Phagocytosis by Opsonizing Bacteria. Res Reports Biochem 2016;2016:39–55.

Liu J, Tian ZY, Xiao YC, Wang XL, Jin ML, Shi DS. The role of porcine monocyte derived dendritic cells (MoDC) in the inflammation storm caused by streptococcus suis serotype 2 infection. PLoS One 2016;11:1–13. doi:10.1371/journal.pone.0151256.

Llor C, Bjerrum L. Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. Ther Adv Drug Saf 2014;5:229–41. doi:10.1177/2042098614554919.

Loss H, Aschenbach JR, Ebner F, Tedin K, Lodemann U. Effects of a pathogenic ETEC strain and a probiotic Enterococcus faecium strain on the inflammasome response in porcine dendritic cells. Vet Immunol Immunopathol 2018;203:78–87. doi:10.1016/j.vetimm.2018.08.004.

Lowe BA, Marsh TL, Isaacs-Cosgrove N, Kirkwood RN, Kiupel M, Mulks MH. Microbial communities in the tonsils of healthy pigs. Vet Microbiol 2011;147:346–57. doi:10.1016/J.VETMIC.2010.06.025.

Luzyanina T, Cupovic J, Ludewig B, Bocharov G. Mathematical models for CFSE labelled lymphocyte dynamics: asymmetry and time-lag in division. J Math Biol 2014;69:1547–83. doi:10.1007/s00285-013-0741-z.

Ma Y, Poole K, Goyette J, Gaus K. Introducing Membrane Charge and Membrane Potential to T Cell Signaling. Front Immunol 2017a;8:1513. doi:10.3389/fimmu.2017.01513.

Ma Y, Yamamoto Y, Nicovich PR, Goyette J, Rossy J, Gooding JJ, et al. A FRET sensor enables quantitative measurements of membrane charges in live cells. Nat Biotechnol 2017b;35:363–70. doi:10.1038/nbt.3828.

MacInnes JI, Desrosiers R. Agents of the "'suis-ide diseases'" of swine: Actinobacillus suis, Haemophilus parasuis, and Streptococcus suis. Can J Vet Res 1999;63:83–9.

Martelet L, Lacouture S, Goyette-Desjardins G, Beauchamp G, Surprenant C, Gottschalk M, et al. Porcine Dendritic Cells as an In Vitro Model to Assess the Immunological Behaviour of Streptococcus suis Subunit Vaccine Formulations and the Polarizing Effect of Adjuvants. Pathogens 2017;6:13. doi:10.3390/pathogens6010013.

Matsuzaki K. Antimicrobial peptides : basics for clinical application. 2019.

Mazzocco P, Bernard S, Pujo-Menjouet L. Estimates and impact of lymphocyte division parameters from CFSE data using mathematical modelling. PLoS One 2017;12:e0179768. doi:10.1371/journal.pone.0179768.

McComb S, Thiriot A, Krishnan L, Stark F. Introduction to the Immune System, Humana Press, Totowa, NJ; 2013, p. 1–20. doi:10.1007/978-1-62703-589-7_1.

McHugh BJ, Wang R, Li H-N, Beaumont PE, Kells R, Stevens H, et al. Cathelicidin is a “fire alarm”, generating protective NLRP3-dependent airway epithelial cell inflammatory responses during infection with Pseudomonas aeruginosa. PLoS Pathog 2019;15:e1007694. doi:10.1371/journal.ppat.1007694.

Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE, Wells JM. Immunomodulatory effects of Streptococcus suis capsule type on human dendritic cell responses, phagocytosis and intracellular survival. PLoS One 2012;7:1–9. doi:10.1371/journal.pone.0035849.

Miao H, Jin X, Perelson AS, Wu H. Evaluation of Multitype Mathematical Models for CFSE-Labeling Experiment Data. Bull Math Biol 2012;74:300–26. doi:10.1007/s11538-011-9668-y.

Mookherjee N, Brown KL, Bowdish DME, Doria S, Falsafi R, Hokamp K, et al. Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37. J Immunol 2006;176:2455–64. doi:10.4049/jimmunol.176.4.2455.

Moore DK, Motaung B, du Plessis N, Shabangu AN, Loxton AG, SU-IRG Consortium S-I. Isolation of B-cells using Miltenyi MACS bead isolation kits. PLoS One 2019;14:e0213832. doi:10.1371/journal.pone.0213832.

Mortaz E, Adcock IM, Shafei H, Masjedi MR, Folkerts G. Role of P2X7 Receptors in Release of IL-1β: A Possible Mediator of Pulmonary Inflammation. Tanaffos 2012;11:6.

Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiol Spectr 2016;4. doi:10.1128/microbiolspec.VMBF-0016-2015.

Okutani M, Tsukahara T, Kato Y, Fukuta K, Inoue R. Gene expression profiles of CD4/CD8 double-positive T cells in porcine peripheral blood. Anim Sci J 2018;89:979–87. doi:10.1111/asj.13021.

Peiró T, Patel DF, Akthar S, Gregory LG, Pyle CJ, Harker JA, et al. Neutrophils drive alveolar macrophage IL-1β release during respiratory viral infection. Thorax 2018;73:546–56. doi:10.1136/thoraxjnl-2017-210010.

Podolnikova NP, Podolnikov A V., Haas TA, Lishko VK, Ugarova TP. Ligand Recognition Specificity of Leukocyte Integrin $\alpha_M \beta_2$ (Mac-1, CD11b/CD18) and Its Functional Consequences. Biochemistry 2015;54:1408–20. doi:10.1021/bi5013782.

Polini A, del Mercato LL, Barra A, Zhang YS, Calabi F, Gigli G. Towards the development of human immune-system-on-a-chip platforms. Drug Discov Today 2019;24:517–25. doi:10.1016/J.DRUDIS.2018.10.003.

Reams RY, Glickman LT, Harrington DD, Thacker HL, Bowersock TL. *Streptococclcs Suis* Infection in Swine: A Retrospective Study of 256 Cases. Part II. Clinical Signs, Gross and Microscopic Lesions, and Coexisting Microorganisms. J Vet Diagnostic Investig 1994;6:326–34. doi:10.1177/104063879400600308.

Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nat Rev Immunol 2015;15:203–16. doi:10.1038/nri3818.

Rodríguez-Gómez IM, Talker SC, Käser T, Stadler M, Hammer SE, Saalmüller A, et al. Expression of T-bet, Eomesodermin and GATA-3 in porcine αβ T cells. Dev Comp Immunol 2016;60:115–26. doi:10.1016/j.dci.2016.02.022.

Roederer M. Interpretation of cellular proliferation data: Avoid the panglossian. Cytom Part A 2011;79A:95–101. doi:10.1002/cyto.a.21010.

Sang Y, Blecha F. Porcine host defense peptides: Expanding repertoire and functions. Dev Comp Immunol 2009;33:334–43. doi:10.1016/j.dci.2008.05.006.

Scheenstra MR, van den Belt M, Tjeerdsma-van Bokhoven JLM, Schneider VAF, Ordonez SR, van Dijk A, et al. Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action. Sci Rep 2019;9:4780. doi:10.1038/s41598-019-41246-6.

Scocchi M, Zelezetsky I, Benincasa M, Gennaro R, Mazzoli A, Tossi A. Structural aspects and biological properties of the cathelicidin PMAP-36. FEBS J 2005;272:4398–406. doi:10.1111/j.1742-4658.2005.04852.x.

Segura M. *Streptococcus suis* vaccines: candidate antigens and progress. Expert Rev Vaccines 2015;14:1587–608. doi:10.1586/14760584.2015.1101349.

Segura M, Stankova J, Gottschalk M. Heat-killed Streptococcus suis capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun 1999;67:4646–54.

Seitz M, Valentin-Weigand P, Willenborg J. Use of Antibiotics and Antimicrobial Resistance in Veterinary Medicine as Exemplified by the Swine Pathogen Streptococcus suis, Springer, Cham; 2016, p. 103–21. doi:10.1007/82_2016_506.

Singh A. Biomaterials innovation for next generation ex vivo immune tissue engineering. Biomaterials 2017;130:104–10. doi:10.1016/J.BIOMATERIALS.2017.03.015.

Tario JD, Humphrey K, Bantly AD, Muirhead KA, Moore JS, Wallace PK, et al. Optimized staining and proliferation modeling methods for cell division monitoring using cell tracking dyes. J Vis Exp 2012:e4287. doi:10.3791/4287.

Veldhuizen EJA, Brouwer EC, Schneider VAF, Fluit AC. Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. PLoS One 2013;8. doi:10.1371/journal.pone.0061964.

Veldhuizen EJA, Scheenstra MR, Tjeerdsma-van Bokhoven JLM, Coorens M, Schneider VAF, Bikker FJ, et al. Antimicrobial and Immunomodulatory Activity of PMAP-23 Derived Peptides. Protein Pept Lett 2017;24:1–8. doi:10.2174/0929866524666170428150925.

Veldhuizen EJA, Schneider VAF, Agustiandari H, Van Dijk A, Tjeerdsma-van Bokhoven JLM, Bikker FJ, et al. Antimicrobial and immunomodulatory activities of PR-39 derived peptides. PLoS One 2014;9:1–7. doi:10.1371/journal.pone.0095939.

Velikova N, Kavanagh K, Wells JM. Evaluation of Galleria mellonella larvae for studying the virulence of Streptococcus suis. BMC Microbiol 2016;16:291. doi:10.1186/s12866-016-0905-2.

Wasteson Y, Høie S, Roberts MC. Characterization of antibiotic resistance in Streptococcus suis. Vet Microbiol 1994;41:41–9. doi:10.1016/0378-1135(94)90134-1.

Wei Z, Li R, Zhang A, He H, Hua Y, Xia J, et al. Characterization of Streptococcus suis isolates from the diseased pigs in China between 2003 and 2007. Vet Microbiol 2009;137:196–201. doi:10.1016/j.vetmic.2008.12.015.

Wessely-Szponder J, Majer-Dziedzic B, Smolira A. Analysis of antimicrobial peptides from

porcine neutrophils. J Microbiol Methods 2010;83:8–12. doi:10.1016/j.mimet.2010.07.010.

Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LA., Smith HE. Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of Streptococcus suis serotype 2. Vet Microbiol 2002;84:155–68. doi:10.1016/S0378-1135(01)00452-7.

Wu H, Zhang G, Ross CR, Blecha F. Cathelicidin gene expression in porcine tissues: roles in ontogeny and tissue specificity. Infect Immun 1999;67:439–42.

Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A. The human cathelicidin LL-37 — A pore-forming antibacterial peptide and host-cell modulator. Biochim Biophys Acta - Biomembr 2016;1858:546–66. doi:10.1016/j.bbamem.2015.11.003.

De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. Ll-37, the Neutrophil Granule–And Epithelial Cell–Derived Cathelicidin, Utilizes Formyl Peptide Receptor–Like 1 (Fprl1) as a Receptor to Chemoattract Human Peripheral Blood Neutrophils, Monocytes, and T Cells. J Exp Med 2000;192:1069–74. doi:10.1084/jem.192.7.1069.

Ye C, Zhu X, Jing H, Du H, Segura M, Zheng H, et al. Streptococcus suis sequence type 7 outbreak, Sichuan, China. Emerg Infect Dis 2006;12:1203–8. doi:10.3201/eid1208.060232.

Yongkiettrakul S, Maneerat K, Arechanajan B, Malila Y, Srimanote P, Gottschalk M, et al. Antimicrobial susceptibility of Streptococcus suis isolated from diseased pigs, asymptomatic pigs, and human patients in Thailand. BMC Vet Res 2019;15:5. doi:10.1186/s12917-018-1732-5.

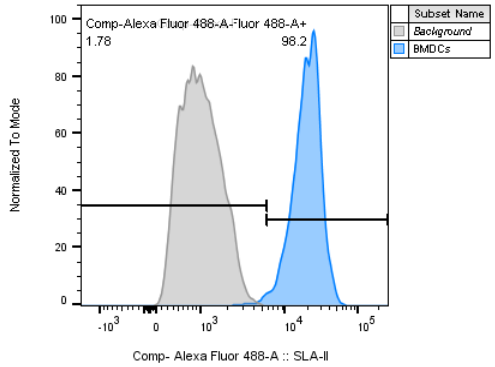
Yu J, Mookherjee N, Wee K, Bowdish DME, Pistolic J, Li Y, et al. Host Defense Peptide LL-37, in Synergy with Inflammatory Mediator IL-1β, Augments Immune Responses by Multiple Pathways. J Immunol 2007;179:7684–91. doi:10.4049/jimmunol.179.11.7684.

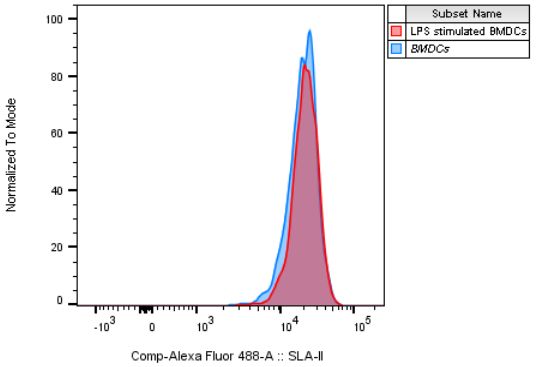
Zaccaria E, Cao R, Wells JM, van Baarlen P. A Zebrafish Larval Model to Assess Virulence of Porcine Streptococcus suis Strains. PLoS One 2016;11:e0151623. doi:10.1371/journal.pone.0151623.

Zhou Y, Wang Y, Deng L, Zheng C, Yuan F, Chen H, et al. Evaluation of the protective efficacy of four novel identified membrane associated proteins of Streptococcus suis serotype 2. Vaccine 2015;33:2254–60. doi:10.1016/J.Vaccine.2015.03.038.

SUPPLEMENTARY FIGURES

A





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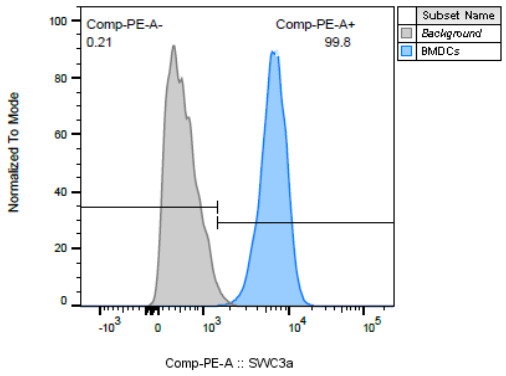

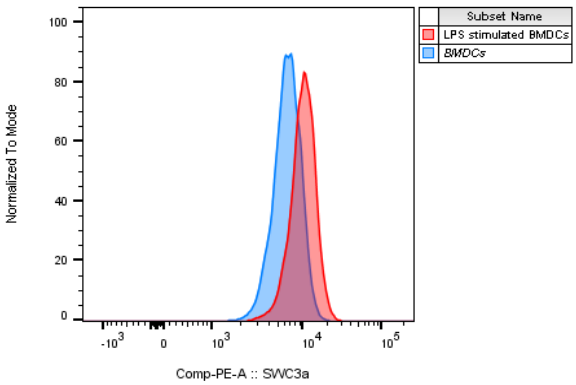

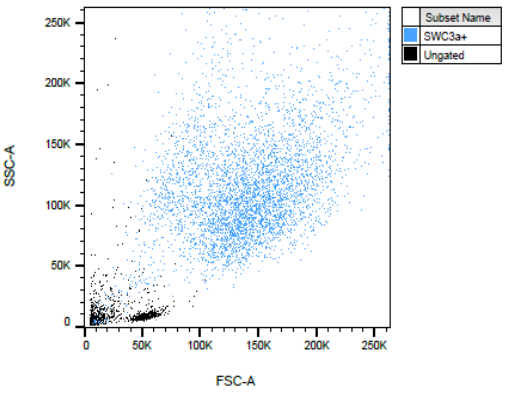

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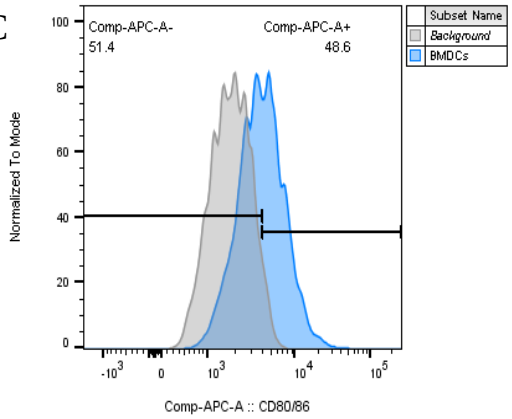

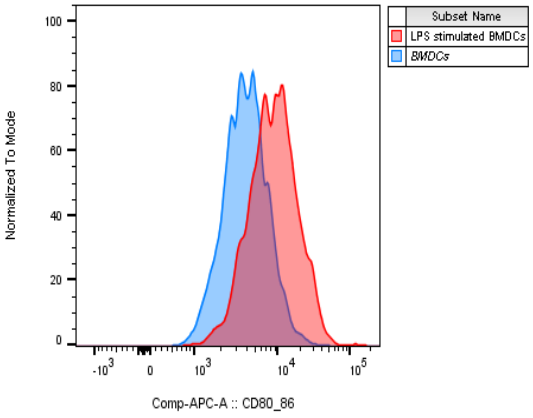

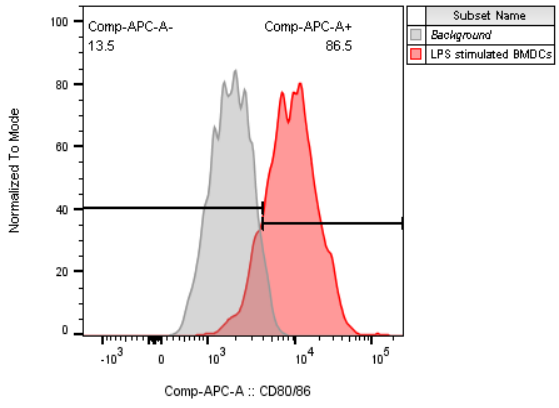

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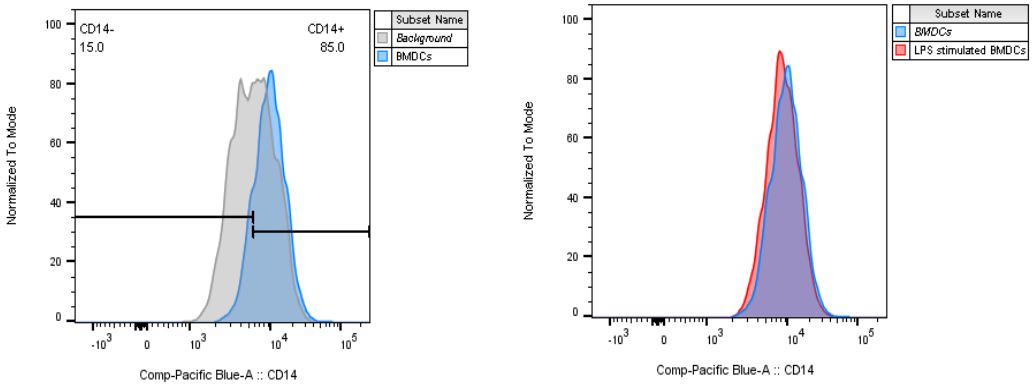

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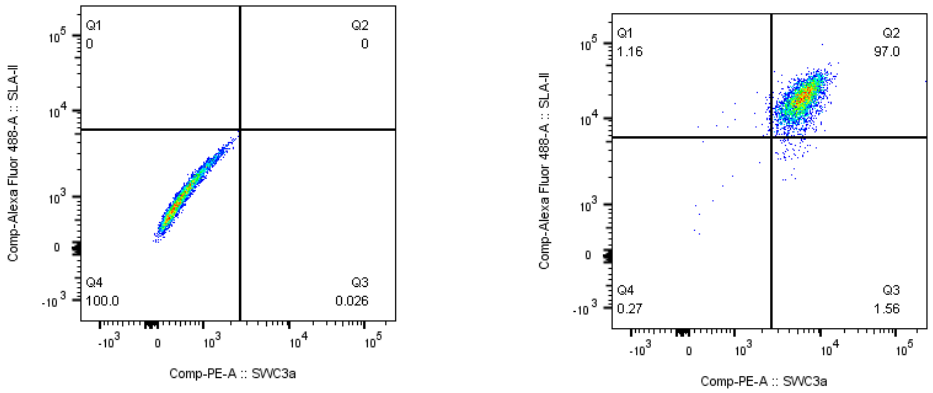
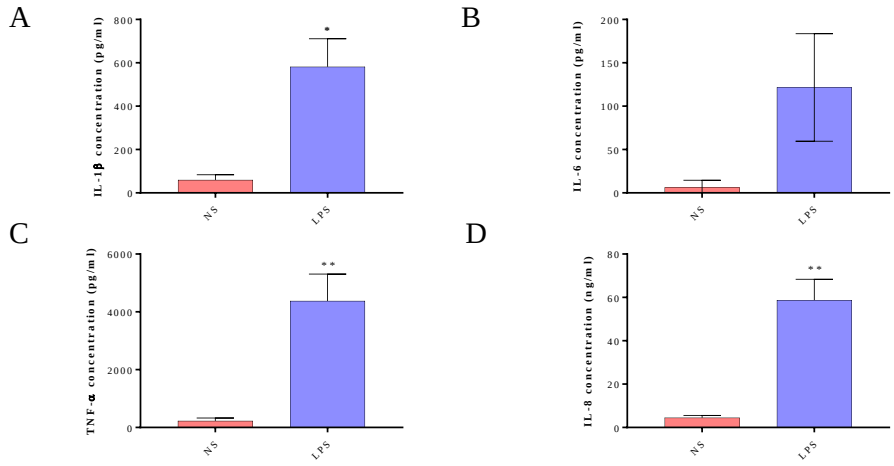
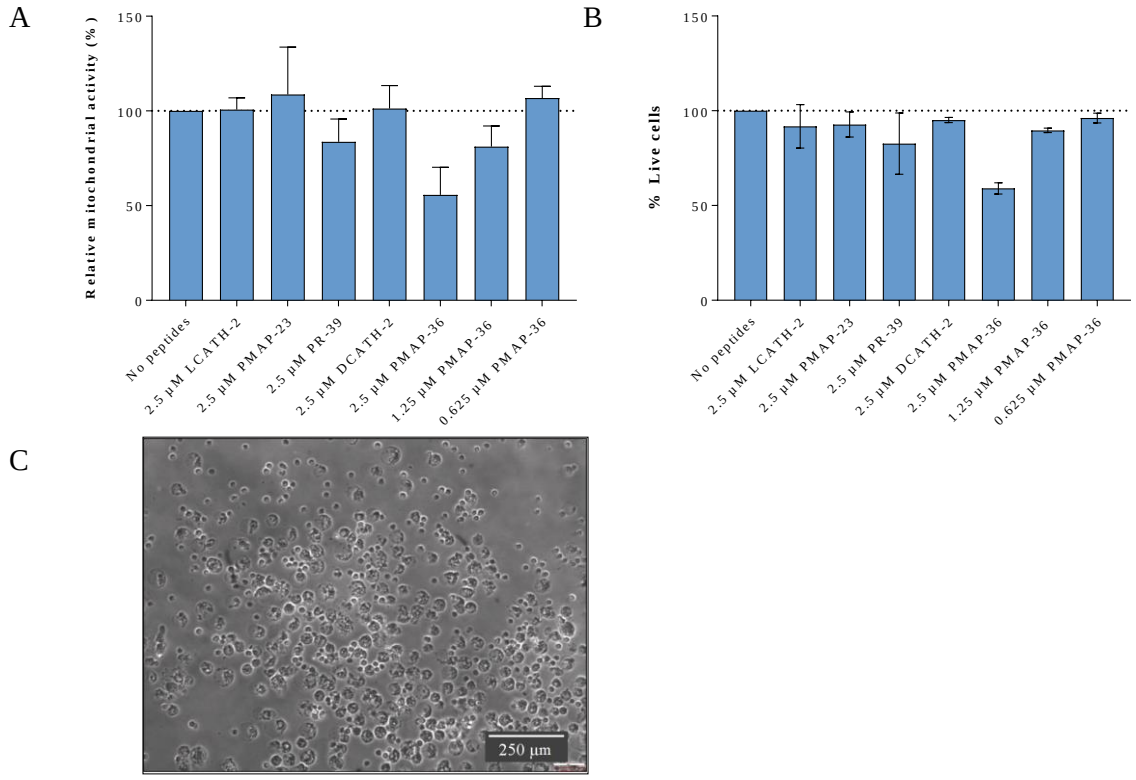


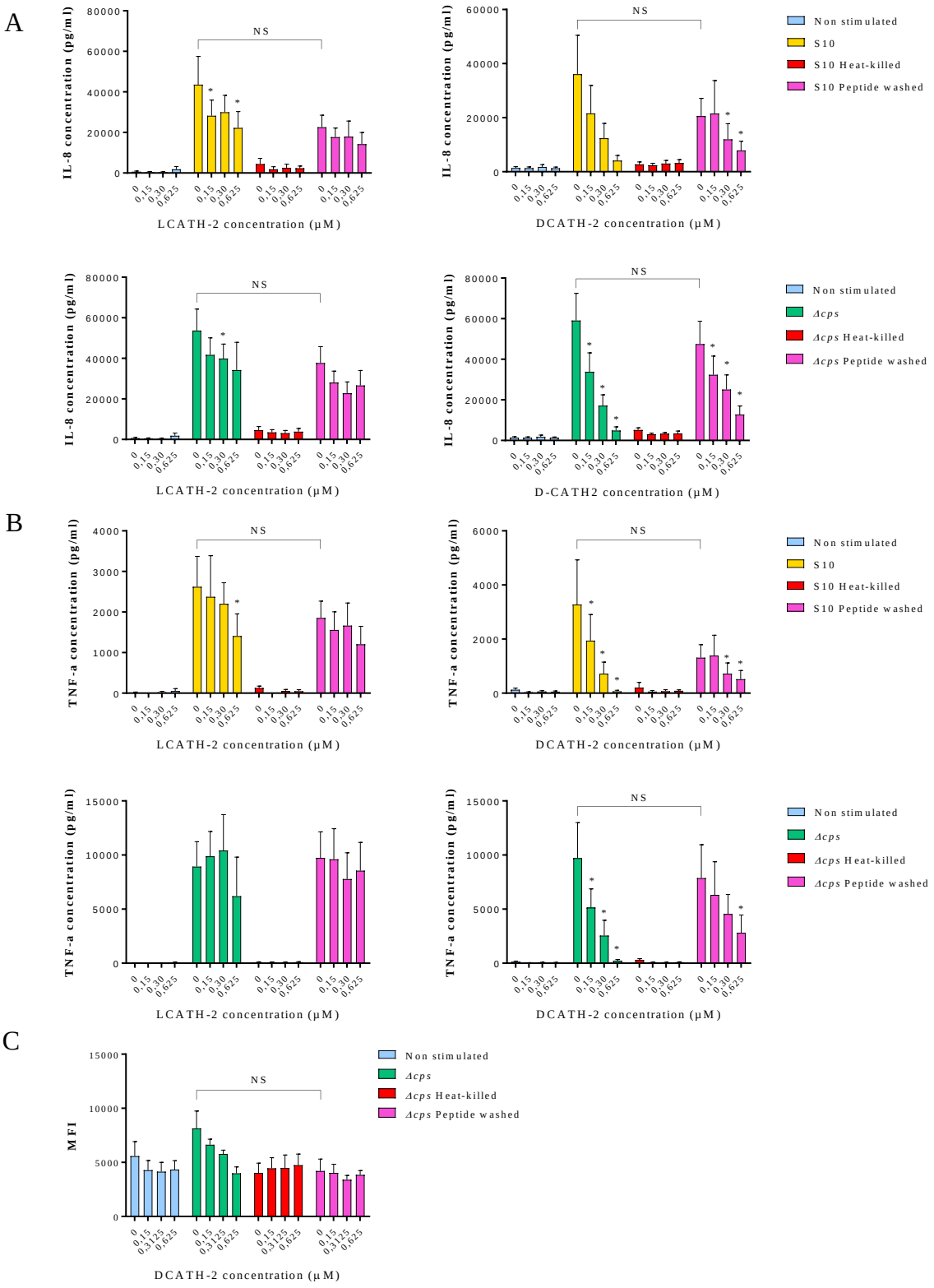
Supplementary Figure 1 - BMDC characterization by flow cytometry. BMDCs were cultured for 6 days, in the presence of GM-CSF and IL-4. After 6 days, cells were stimulated with LPS (100 ng/ml) or used as control (with only cell medium being added). After 24 hours, cells were harvested for FACS analysis. BMDCs were gated based on their FSC and SSC, as described in the results section (Figure 2a). Gated cells were examined for the expression of SLA-II, SWC3a, CD14 and CD80/86. Figures shown are representative of 6 independent experiments. **A)** Representative histogram displaying the expression of SLA-II by dendritic cells. >95% of the non-stimulated BMDCs were found to be SLA-II positive. LPS stimulation did not increase the fluorescent signal. **B)** Representative histogram of the SWC3a expression by dendritic cells. >99% of the cells were SWC3a positive and this marker was subsequently used for gating purposes. On the bottom is a scatter plot of the FSC and SSC of a representative well, displaying SWC3a positive cells in blue **C)** Representative histogram of the CD80/86 expression by non-stimulated dendritic cells (top) and LPS stimulated dendritic cells (bottom). **D)** Representative histogram of the CD14 expression by BMDCs. **E)** Scatter plots of combined SLA-II and SWC3a fluorescence, in an unlabeled (left) and labeled (right) well.



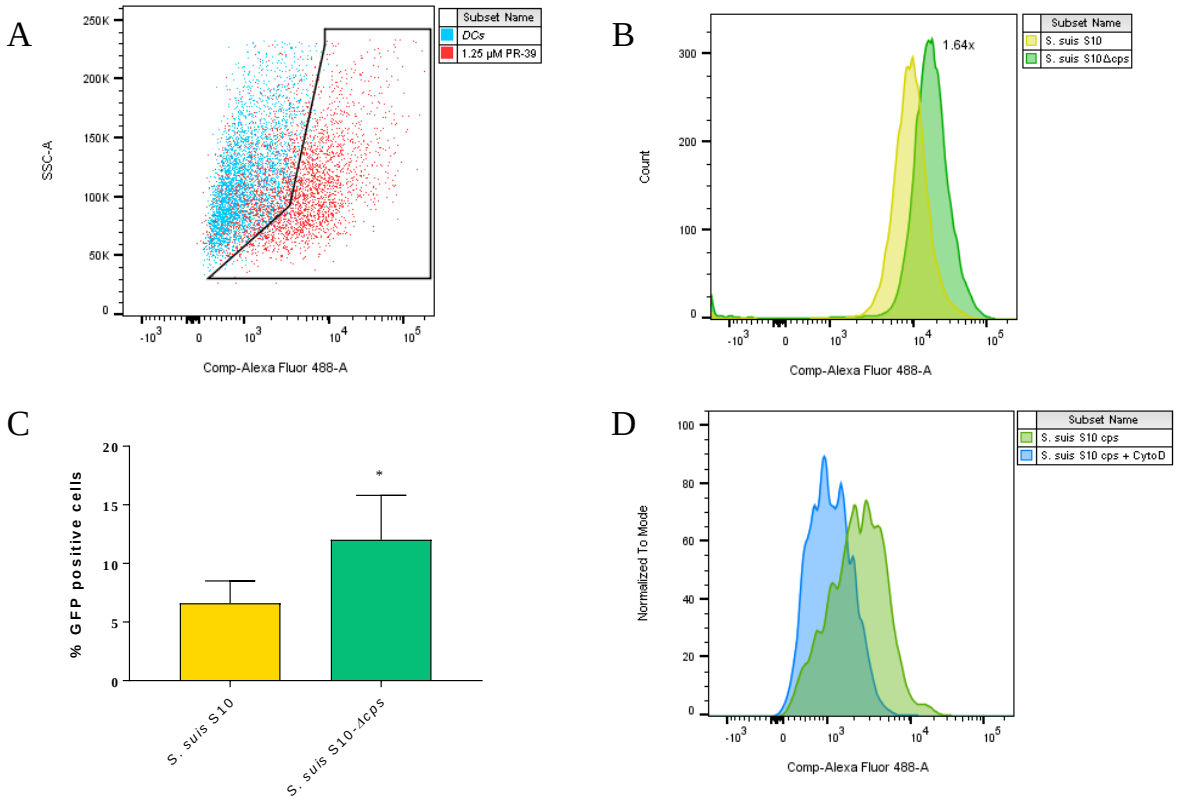
Supplementary Figure 2 - Pro-inflammatory cytokine release by BMDCs after 24 hours of LPS stimulation. The supernatant of 7 day old BMDCs, stimulated for 24 hours, was used to examine cytokine levels by ELISA. **A)** IL-1ß production, n=6. **B)** IL-6 production, n=2. **C)** IL-8 production, n=6 **D)** TNF-α production, n=6.



Supplementary Figure 3 - Peptide induced cytotoxicity by PMAP-36. To evaluate the toxicity of various cathelicidins, BMDCs were stimulated with 2.5 µM of peptide for 24 hours. For PMAP-36, lower concentrations (i.e. 1.25 and 0.625 µM of peptide) were included. After stimulation, cells were incubated with WST-1 or harvested for FACS analysis using Annexin/PI. Results are representative of 3 independent experiments. **A)** WST-1 assay readout of the relative mitochondrial activity of BMDCs. Non-stimulated cells were set at 100% as control, n=3. **B)** Percentage of live cells, as calculated from the population of Annexin negative cells. Non-stimulated cells were set at 100% as control, n=2. **C)** Morphology of apoptotic PMAP-36 (2.5 µM) stimulated BMDCs.



Supplementary Figure 4 - Overview of the response of 'peptide washed' BMDCs and heat-killed *S. suis* stimulated BMDCs. BMDCs were stimulated for 2,5 with; **(blue)** cell culture medium. **(green)** *S. suis* S10. **(yellow)** *S. suis* S10-Δ*cps*. **(red)** heat-killed *S. suis*. **(pink)** *S. suis*, followed by peptide treatment instead of antibiotics. **A)** IL-8 production by BMDCs and the effect of LCATH-2 and DCATH-2, n=6. **B)** TNF-α production by BMDCs, n=6. **C)** Summary of the CD80/86 expression of BMDCs, n=4.



Supplementary Figure 5 - Phagocytosis of *S. suis* by BMDCs – Gating strategies and controls. BMDCs were gated as previously described (based on their FSC, SSC, and SWC3a marker). Results shown are representative of 5 independent experiments *(a,c)* or 3 independent experiments *(b,d)* **A)** Subsequent gating strategy after initial gating. Non-stimulated DCs (blue) were set as control. The percentage of GFP positive cells was determined as the amount of cells within the gate as a percentage of the total. **B)** The fluorescent signal of both *S. suis* S10 and *S. suis* S10-Δ*cps.* The difference in MFI was calculated (=1.64) and all *S. suis* S10-Δ*cps* results were divided by 1.64 to correct for the observed difference. **C)** The percentage of GFP positive BMDCs after 2.5 hours of stimulation (MOI:10) and 24 hours of rest, n=5. **D)** Addition of cytochalasin D (CytoD) to the dendritic cells (blue), blocked phagocytosis of *S. suis* S10-Δ*cps* (green).

VERSLAG VAN DE GEVOLGDE CURSUSSEN

Modern Methods of Data Analysis (Universiteit Utrecht)

De cursus statistiek heb ik halverwege het onderzoeksjaar (januari) gevolgd. Het tempo van de cursus was voor mij ideaal. Eerst werd er wat stof herhaald uit de bachelor, om in te kunnen komen, waarna er vervolgens veel aandacht was voor het kiezen van het juiste type statistiek voor het onderzoek. Daardoor ben ik de verschillen tussen statistische testen beter gaan begrijpen en de voor- en nadelen die met de verschillende testen gepaard gaan.

Gedurende de cursus was er veel persoonlijke aandacht tijdens de computerpractica en kon ik met een van de docenten overleggen over mijn eigen onderzoeksopzet. Qua timing kwam de cursus ook op het juiste moment, omdat ik op dat moment het eerste deel van mijn onderzoek aan het afronden was en zo meteen kon afsluiten met statistiek. In het algemeen was ik erg tevreden over deze cursus, en dan met name de inpassing binnen het onderzoek, organisatie en tijdsduur van de cursus.

Academic Writing for Publication (Babel)

Het schrijven van verslagen is nooit mijn sterkste punt geweest, dus leek het mij ook zeker niet onverstandig om mee te doen met de cursus *Academic Writing for Publication*. In het algemeen vind ik structuur aanbrengen en het grotere geheel blijven zien het lastigste, en had ik gehoopt daar tijdens de cursus veel op te focussen. Wat dat betreft was het een kleine tegenvaller dat het grootste gedeelte van de cursus gericht was op Engels en grammatica. Uiteraard heb ik daar ook veel aan gehad en bleken vooral de ‘Error Analyses’, om onze eigen schrijffouten te leren herkennen, erg zinvol.

Doordat we in principe elke week een nieuwe deadline kregen voor een onderdeel van het verslag, is het schrijven van het verslag onbewust ook best snel opgeschoten en bleef er tussendoor nog tijd over om experimenten te doen. De timing van deze cursus (mei-juni) was dan ook ideaal en heb ik in de maanden juli en augustus de tijd gehad om met de ontvangen feedback van de andere studenten, de docent, en mijn begeleider het verslag af te ronden.

Academic English – Presenting (Babel)

Deze cursus start in september en bestaat uit een 2 uur durende sessie, met 6 sessies in totaal. De laatste les vindt 1½ week voor het symposium plaats. Op die manier blijf ik in de aanloop naar het symposium bezig met mijn onderzoek en het oefenen met presenteren. Vooral het begrijpelijk kunnen uitleggen van resultaten is iets wat ik graag uit de cursus wil proberen te halen.