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1 **Polymerization-dependent activation of porcine $\gamma\delta$ T-cells by proanthocyanidins**

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9

10 **Key words:** $\gamma\delta$ T-cells, activation, proanthocyanidins, innate immunity

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Abstract

Plant-derived proanthocyanidins (PAC) have been promoted as a natural method of improving health and immune function in livestock. It has previously been shown that PAC are effective agonists for activating ruminant $\gamma\delta$ T-cells *in vitro*, however effects on other livestock species are not yet clear. Moreover, the fine structural characteristics of the PAC which contribute to this stimulatory effect have not been elucidated. Here, we demonstrate activation of porcine $\gamma\delta$ T-cells by PAC via up-regulation of CD25 (IL-2R α) and show that 1) activation is dependent on degree of polymerization (DP), with PAC fractions containing polymers with mean DP >6 significantly more effective than fractions with mean DP <6, whilst flavan-3-ol monomers (the constituent monomeric units of PAC) did not induce CD25 expression and 2) both procyanidin and prodelphinidin-type PAC are effective agonists. Furthermore, we show that this effect of PAC is restricted to the $\gamma\delta$ T-cell population within porcine peripheral mononuclear cells as significant CD25 up-regulation was not observed in non $\gamma\delta$ T-cells, and no activation (via CD80/86 up-regulation) was evident in monocytes. Our results show that dietary PAC may contribute to enhancement of innate immunity in swine via activation of $\gamma\delta$ T-cells.

1 **Introduction**

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3 There is currently intense interest in increasing the efficiency and output of modern livestock production, in order to
4 ensure food security for a rapidly growing population (Wu et al., 2014). At the same time, consumer demand for
5 healthy animal produce and rising antibiotic resistance due to prophylactic inclusion in animal feed, means that
6 routine use of antimicrobial drugs to control pathogens and ensure high quality end products is unsustainable
7 (Marshall and Levy, 2011; Thornton, 2010). An alternative approach is to identify natural dietary compounds that
8 promote healthy and robust animal performance, through modulatory and/or stimulatory effects on the animal's
9 immune system.

10

11 The gastrointestinal (GI) tract is one of the most immunologically active tissues in the body, and is exposed to a variety
12 of bacterial and parasitic pathogens that can result in acute and chronic inflammation, diarrhoea and weight loss (Hale
13 et al., 1985; Nagy and Fekete, 2005) . Therefore, maintenance of healthy gut function is essential for robust
14 performance. The GI mucosa is home to a variety of innate immune system effectors including defensins, natural killer
15 cells and $\gamma\delta$ T-cells (Bevins et al., 1999; Smith and Garrett, 2011). $\gamma\delta$ T-cells are increasingly recognised as crucial
16 players in responses to GI pathogens. A key distinguishing feature from $\alpha\beta$ T-cells is that $\gamma\delta$ T-cells are not clonal for a
17 specific antigen, and do not require antigens to be presented in the context of major histocompatibility complex
18 (MHC) molecules, but rather have the ability to recognise conserved pathogen pattern receptors such as
19 phosphoantigens (Gu et al., 2015). In contrast to humans and mice, where $\gamma\delta$ T-cells make up only a small proportion
20 of the peripheral lymphocyte population (Kalyan and Kabelitz, 2013), mammalian livestock (particularly young
21 animals) have large numbers of circulating $\gamma\delta$ T-cells – in some cases up to 50% of the lymphocyte pool, depending on
22 age – and this is true of both ruminants (Holderness et al., 2007; Tibe et al., 2012) and swine (Gerner et al., 2009).
23 Despite this large population of $\gamma\delta$ T-cells in livestock, how these cells function during GI infections is not fully
24 understood (Gerner et al., 2009). Therefore, much of our knowledge on the role of $\gamma\delta$ T-cells in GI infections derives
25 from studies in mice, where it is known that they are able to secrete both pro- and anti-inflammatory cytokines, help
26 to recruit neutrophils and B-cells, and maintain homeostasis and repair of mucosal barriers (Witherden and Havran,
27 2013). Moreover, their importance has been demonstrated in a number of host-pathogen systems. Mice deficient in
28 $\gamma\delta$ T-cell receptor expression have impaired immunity to the GI helminth *Nippostrongylus brasiliensis*, resulting in

1 higher parasite burdens as well as increased lesions and pathology in the intestine compared to wild-type mice
2 (Inagaki-Ohara et al., 2011), and are also more susceptible to infection with the mucosal bacterium *Salmonella*
3 *typhimurium* (Ismail et al., 2011). Furthermore, $\gamma\delta$ T-cells have been shown to play important roles in control of viral
4 (MCMV) and fungal (*Candida albicans*) infections (Conti et al., 2014; Khairallah et al., 2015), indicating key functions
5 against a variety of pathogens.

6
7 It has become apparent that in addition to activation by pathogen ligands, $\gamma\delta$ T-cells may also be activated by non-
8 pathogen related molecules such as bioactive dietary compounds. For example, proanthocyanidins (PAC) are a group
9 of plant secondary metabolites that are widely found in common plant sources such as berries, grapes and nuts. They
10 consist of polymers of flavan-3-ol monomers, and their structure can vary widely depending on the degree of
11 polymerization and the nature of the monomeric sub-units, most of which are either catechin and its *cis* isomer
12 epicatechin (which give rise to procyanidin-type PAC – PC) or gallo catechin and its *cis*-isomer epigallocatechin (which
13 give rise to prodelphinidin-type PAC – PD). The major difference between these groups is an extra hydroxyl group in
14 the B-ring of PD-type PAC (Figure 1).

15
16 PAC have been widely investigated for both their anti-oxidant activities and their putative positive effects on gut
17 health due to both anti-microbial and immuno-stimulatory properties (Martinez-Micaelo et al., 2012). It has been
18 shown that PAC can effectively activate both human and ruminant $\gamma\delta$ T cells *in vitro* by inducing cell proliferation and
19 expression of CD25 and CD69 (Holderness et al., 2007; Tibe et al., 2012). In addition, increased numbers of peripheral
20 $\gamma\delta$ T-cells have been observed in sheep fed PAC-containing willow fodder (Ramírez-Restrepo et al., 2010) and
21 peripheral $\gamma\delta$ T-cell proliferation is increased in humans ingesting PAC-rich cranberry juice (Nantz et al., 2013).
22 However, the structural characteristics of PAC that contribute to these stimulatory effects on $\gamma\delta$ T-cells have not been
23 fully elucidated. Moreover, there is no information on whether porcine $\gamma\delta$ T-cells can be activated by PAC in a similar
24 fashion to ruminants. PAC-containing feedstuffs have recently been investigated as a cost-effective feed supplement
25 for pigs to promote gut health without reliance on antimicrobials (Feisel et al., 2014; Sehm et al., 2007) and thus an
26 exploration of the effects of PAC on porcine $\gamma\delta$ T-cell function is warranted.

1 Here, we used well characterised PAC fractions containing distinct PC and PD profiles and mean degree of
2 polymerization (mDP) to determine whether porcine $\gamma\delta$ T cells could be effectively activated by PAC via increased
3 CD25 expression, and the contribution of PC/PD profile and mDP to this effect.

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1 **Methods:**

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3 *Materials*

4 Skins from hazelnut (*Corella avellana*; hereafter referred to as HN) were provided by Dr Hervé Hoste (INRA, Toulouse,
5 France). Leaves from blackcurrant (*Ribes nigrum*; hereafter referred to as BC) were sourced from Goring-upon-
6 Thames, UK. Catechin, epicatechin, concavalin A (con A) and lipopolysaccharide (LPS) were obtained from Sigma-
7 Aldrich (Schnelldorf, Germany).

8

9 *Extraction, fractionation and analysis of proanthocyanidins*

10 Detailed procedures for the extraction and purification of PAC are described elsewhere (Williams et al., 2014). Briefly,
11 plant material was extracted with acetone/water, and the resulting extract was freeze-dried, suspended in water and
12 then loaded onto Sephadex LH-20 columns. Low-molecular weight PAC were eluted with acetone water (3:7) to yield
13 fraction 1 (F1), and high-molecular weight PAC eluted with acetone/water (1:1) to yield fraction 2 (F2). Fractions were
14 analysed using thiolytic degradation and HPLC-MS (Gea et al., 2011) which yielded information on the purity of PAC,
15 mDP and PC/PD ratio.

16

17 *Blood samples and isolation of PBMC*

18 The use of animals was approved by the Experimental Animal Unit, University of Copenhagen, and procedures carried
19 out according to the guidelines of the Danish Animal Experimentation Inspectorate (Licence number 2010/561-1914).
20 Blood samples were drawn by jugular venipuncture from 9-12 week old healthy female pigs (Danish
21 Landrace/Yorkshire/Duroc) into vacutainers containing sodium heparin. PBMCs were isolated on histopaque 1.077,
22 washed in PBS and then suspended in complete culture media (RPMI 1640 supplemented with 10% inactivated foetal
23 bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin). Viability of PBMC was
24 assessed by trypan blue staining and was routinely >98%.

25

26 *Cell culture*

27 For whole PBMC culture, cells were seeded in duplicate at 10^6 /mL in 48-well plates. Purified PAC fractions were
28 dissolved in PBS and added at concentrations of 20 or 10 µg/mL – preliminary experiments showed that higher

concentrations significantly reduced cell viability (data not shown). Con A (5 µg/mL) and PBS-stimulated cells were included in all experiments as positive and negative controls, respectively. Cells were incubated for 48 hours at 37°C and 5% CO₂ before being harvested for analysis. For monocyte culture, monocytes were purified (>90% purity) from PBMC with anti-human CD14 microbeads (Auray et al., 2013) and magnetic separation (MACS, Miltenyl Biotech). Purified monocytes were suspended in RPMI 1640 media supplemented with 10% inactivated foetal bovine serum, 1% MEM non-essential amino acids, 10 mM HEPES, 50 µM β-mercaptoethanol, 100 U/mL of penicillin and 100 µg /mL of streptomycin. The cells were rested for 24 hours, and then stimulated in duplicate for a further 24 hours with either LPS (500 ng/mL) or PAC fractions at 20 or 10 µg/mL. For PBMC, four separate experiments were performed with cells from different pigs. and for monocytes, three separate experiments were performed with cells from different pigs.

Flow cytometry

For PBMC, cells were harvested and stained with anti-porcine γδ TCR (Clone PGBL22A; Monoclonal Antibody Centre, Washington State University, Pullman, USA) and anti-porcine CD25 (Clone K231.3B2; AbD serotec, Kidlington, UK). CD25 (IL-2Rα) was used as a common and well-studied marker of peripheral T-cell activation (Caruso et al., 1997; Jouen-Beades et al., 1997). The mAbs were conjugated to either FITC or APC fluorophores using Zenon antibody labelling kits (Invitrogen), according to the manufacturer's instructions. For monocytes, cells were stained for CD80/86 expression using human CTLA-4/mouse IgG-FITC fusion protein (Ansell, Bayport, USA). Appropriate isotype controls (BD biosciences) were included in every experiment. Cells were acquired on an Accuri C6 flow cytometer. Gating for lymphocytes or monocytes was carried out based on forward/side scatter and at least 10000 events were acquired. Data were analysed using FCS express version 5 (De Novo Software, Glendale, CA).

ELISA

TNF-α in monocyte culture supernatants was quantified using the swine TNF-α ELISA kit (Life Technologies) according to the manufacturer's instructions.

Statistical Analysis

Differences in CD25 or CD80/86 expression were determined by ANOVA with Bonferroni post-hoc testing (Graphpad Prism v6.00, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

1 **Results and Discussion**

2

3 Extraction and purification of two fractions each from two different plant sources (HN and BC) yielded PAC with
4 contrasting PC/PD profiles. Full details of the chemical analysis have been presented previously (Williams et al., 2014),
5 and are summarised briefly in Table 1. Proanthocyanidins purified from HN consisted of around 80% PC units, whereas
6 those from BC were PD-rich, with more than 90% of the PAC consisting of PD units. For both plant sources, F1
7 contained lower molecular weight polymers with mDP < 6, and F2 contained high-molecular weight PAC with mDP >6.

8

9 We first tested the PC-rich PAC fractions from HN for their ability to induce CD25 (IL-2R α) expression in $\gamma\delta$ T-cells. The
10 F2 fraction (20 μ g/mL) induced significant up-regulation of CD25, with around 50% of $\gamma\delta$ TCR⁺ cells positive for CD25
11 expression compared to <5% in unstimulated cells (P <0.01) although the effect was not as strong as that induced by
12 the positive control con A (Figure 2B,C). This effect was dose-dependent, as a concentration of 10 μ g/mL induced
13 lower, but still statistically significant up-regulation (data not shown). When the F1 fraction was tested at an
14 equivalent concentration of PAC, activation was still observed but significantly less CD25 positive cells were present
15 compared to the F2 fraction (P <0.05; Figure 2B,C), showing clearly that this effect is dependent on degree of
16 polymerization. Consistent with this, we observed that the flavan-3-ol monomers catechin and epicatechin, which are
17 the dominant monomeric units of HN PAC polymers, did not induce CD25 expression (Figure 2C). In addition, this
18 effect appeared to be largely confined to the $\gamma\delta$ T-cell population; whilst there was an increase in the number of
19 CD25⁺ cells in the non- $\gamma\delta$ lymphocyte population after exposure to the F2 fraction, this was not statistically significant
20 (Figure 2D).

21

22 We next tested whether the predominantly PD polymers purified from BC could also induce CD25 expression. Similar
23 to the results with the HN PAC, we found that incubation of $\gamma\delta$ T-cells with the F2 BC fraction induced marked up-
24 regulation of CD25 (P <0.05; Figure 3A), although the effect tended to be less strong than that observed with HN PAC.
25 This effect was still present, but not significantly (P >0.05), with an equivalent concentration of the F1 fraction (Figure
26 3B). Thus, both PC and PD type PAC are able to induce CD25 up-regulation on porcine $\gamma\delta$ T-cells, and the degree of
27 polymerization of the molecule appears to play a major role in the degree of activation.

28

As the activity of PAC seemed to be confined to $\gamma\delta$ T-cells within the lymphocyte population, we investigated whether other mononuclear cells such as monocytes could be activated by PAC or whether $\gamma\delta$ T-cells were particularly amenable to this effect. Monocytes were purified from PBMC and exposed to either the TLR agonist LPS, or the F2 fractions from HN and BC, and up-regulation of CD80/86 and TNF- α secretion was quantified. We detected a small, but statistically significant up-regulation of CD80/86 in monocytes exposed to LPS. Moreover, LPS induced a marked secretion of TNF- α , indicating a significant activation of the monocytes ($P<0.01$; Figure 4A,B). In contrast, no increased expression of CD80/86 or TNF- α secretion was induced by either of the PAC fractions (Figure 4A, B). Thus, within the porcine mononuclear population, $\gamma\delta$ T-cells appear to be specifically responsive to PAC.

The present work has demonstrated that PAC are effective agonists for porcine $\gamma\delta$ T-cells *in vitro*. This is consistent with previous work showing this effect in human, bovine and caprine $\gamma\delta$ T-cells (Holderness et al., 2007; Tibe et al., 2012). Thus, this appears to be a highly conserved process across different animal species. Whilst for practical reasons our experiments were performed from PBMC-derived $\gamma\delta$ T-cells, rather than GI-resident cells, this apparently widely conserved process suggests that all $\gamma\delta^+$ cells may respond in a similar fashion to PAC - as evidenced by Holderness *et al.* (2007) who reported similar responses to PAC from the two major primary subsets ($CD8^+$ or $CD8^-$) of bovine $\gamma\delta$ T-cells (with the $CD8^+$ cells being primarily a tissue-associated subset), as well as observing similar responses between V δ 1 and V δ 2 human T-cells.

The ability of PAC to effectively activate these cells may be reflected in functional activity in animals fed a PAC-rich diet, such as enhanced innate immunity to GI pathogens such as helminths, protozoa and bacteria, if $\gamma\delta$ T-cells primed by dietary PAC respond faster and more robustly to the presence of pathogens. The role of $\gamma\delta$ T-cells in immunity to porcine pathogens has yet to be fully elucidated, however they are known to produce IL-17 and IFN- γ after stimulation with mitogens or bacterial antigens (Lee et al., 2004; Stepanova et al., 2012), and increased numbers of $\gamma\delta$ T-cells are present shortly after infection with parasites such as *Cystoisospora suis* (Gabner et al., 2014), suggesting that these cells likely play a functional role in immunity to GI pathogens, as has been reported in mice (Smith and Hayday, 2000).

Interestingly, Nantz et al. (2013) noted that, unlike $\gamma\delta$ T-cells, $\alpha\beta$ T-cell and B-cell proliferation was not enhanced by dietary PAC in humans. Similarly, Holderness *et al.* (2007) reported that whereas bovine $\gamma\delta$ T-cells were activated by

PAC, $\alpha\beta$ T-cells were not, however in this study a proportion of human $\alpha\beta$ T-cells were also responsive to PAC. Our current results with porcine cells demonstrated that whilst some activation of $\alpha\beta$ T-cells was evident, this was non-significant and clearly not as pronounced as the robust response of the $\gamma\delta$ T-cell population. Thus, it appears whilst there may be some conserved lymphocyte responses to PAC, the invariant nature of the $\gamma\delta$ TCR seems to be uniquely suited to ligation by PAC.

We also observed that the ability of PAC to induce CD25 expression was clearly dependent on the degree of polymerization, whilst monomeric flavan-3-ols had no effect. This is consistent with a number of studies demonstrating that the biological effects of PAC are related to polymer size. Our group and others have shown that *in vitro* anti-parasitic effects are also enhanced by increasing degree of polymerization (Quijada et al., 2015; Williams et al., 2014). In addition, other immune-modulating effects of PAC, such as the phosphorylation of STAT1 in human CD11b⁺ cells, are instigated only by oligomeric PAC and not by monomeric sub-units such as catechin (Snyder et al., 2014). It is also apparent that differences in biological activity of PAC can be related to the ratio of PC to PD within PAC (Novobilský et al., 2013; Scalbert, 1991). Increased biological activity is often associated with PAC molecules that have a high percentage of PD, perhaps due to the increased possibility of hydrogen-bonding with proteins resulting from the extra hydroxyl group in the B-rings. Our current data demonstrated that both PC and PD rich-PAC were able to induce CD25 expression, though the PC-type PAC from HN appeared to be more potent. However, this difference between HN and BC cannot be ascribed only to PC/PD ratio as mDP values were higher in the HN fractions (Table 1), which makes direct comparisons problematic. Further studies using larger panels of well-defined PAC will be necessary to determine the exact contribution of these monomeric subunits to the observed activity. For now, our data indicate that both PC and PD-type PAC are capable of priming porcine $\gamma\delta$ T-cells, and that the size of the PAC molecule appears to be a critical factor in determining the potency of the effect.

In conclusion, we have shown for the first time that PAC from two different plants are able to activate porcine $\gamma\delta$ T-cells through CD25 up-regulation. The degree of polymerization of the PAC polymers appeared to be the most critical structural characteristic for effective activation. Further *in vivo* experiments will aim to determine the functional importance of this relationship.

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1 **Table 1**

2 Chemical analysis of fractionated proanthocyanidins from two different plant sources. PC – procyanidin, PD –
 3 prodelphinidin , mDP – mean degree of polymerization. % purity – g PAC / 100 g fraction.
 4 Also reported by Williams *et al.* (2014).

Sample	Fraction	PC:PD ratio	mDP	% purity
Hazelnut skin	F1	81.7 : 18.3	4.6	51.3
	F2	79.1 : 20.9	9.1	70.3
Blackcurrant leaves	F1	6.3 : 93.7	2.5	59.9
	F2	5.5 : 94.5	6.5	100

Figure Legends

Figure 1 – Structure of the *trans* flavan-3-ols catechin (gives rise to procyanidin-type PAC) and gallocatechin (gives rise to prodelphinidin-type PAC). Note the extra hydroxyl group in the B-ring of gallocatechin.

Figure 2 - Proanthocyanidins (PAC) from hazelnut skin (HN) induce CD25 expression in porcine $\gamma\delta$ T-cells.

A) Gating strategy and staining controls. Lymphocytes were gated based on forward/side scatter. Staining controls consisted of APC and FITC isotype controls.

B) Representative plots from one experiment of CD25 expression in $\gamma\delta$ T-cells. The percentage of CD25 positive cells in the $\gamma\delta$ T-cell population was quantified after exposure to 20 $\mu\text{g/mL}$ HN PAC (Fraction 1 or 2) or 5 $\mu\text{g/mL}$ con A (positive control).

C) Mean results of 4 independent experiments using cells from different pigs. Results are presented as mean \pm SEM.

'F2' refers to Fraction 2 and 'F1' to refers to Fraction 1 (see materials and methods). 'C' – catechin. 'EC' – epicatechin.

** $P < 0.01$, * $P < 0.05$, ns – non-significant.

D) PAC from fraction 2 of HN do not significantly increase percentage of CD25+ cells in the $\gamma\delta$ - lymphocyte population from porcine PBMC. 'ns' – non-significant. Results are mean \pm SEM from four experiments with cells from different pigs.

Figure 3 - Proanthocyanidins (PAC) from blackcurrant leaves (BC) induce CD25 expression in porcine $\gamma\delta$ T-cells

A) Representative plots from one experiment of CD25 expression in $\gamma\delta$ T-cells. The percentage of CD25 positive cells in the $\gamma\delta$ T-cell population was quantified after exposure to 20 $\mu\text{g/mL}$ BC PAC (Fraction 1 or 2) or 5 $\mu\text{g/mL}$ con A (positive control).

B) Mean results of 4 independent experiments using cells from different pigs. Results are presented as mean \pm SEM.

'F2' refers to Fraction 2 and 'F1' to refers to Fraction 1 (see materials and methods). ** $P < 0.01$. * $P < 0.05$.

Figure 4 – LPS, but not proanthocyanidins (PAC), induce CD80/86 expression and TNF- α secretion in porcine monocytes.

A) Representative histograms showing CD80/86 expression in porcine monocytes exposed to LPS, or 20 μ g/mL PAC from fraction 2 of hazelnut skins (HN) or blackcurrant leaves (BC).

B) Mean fluorescence intensity (MFI) of CD80/86 and TNF- α secretion from monocytes. Mean results of 3 independent experiments using cells from different pigs. Results are presented as mean \pm SEM. *** P <0.001, ** P <0.01.

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