**Co-operative suppression of inflammatory responses in human dendritic cells by plant proanthocyanidins and products from the parasitic nematode *Trichuris suis***

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**Running Title:**

Proanthocyanidins and helminths modulate dendritic cell activity

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Dendritic cells, proanthocyanidins, *Trichuris suis,* inflammation, parasite

**List of abbreviations**

COC – cocoa

DC – dendritic cells

ECGC – epigallocatechin gallate

F1 – fraction 1

F2 – fraction 2

GALT – gut associated lymphoid tissue

LPS – lipopolysaccharide

mDP – mean degree of polymerisation

PAC – proanthocyanidins

TsSP – *Trichuris suis* soluble products

WCF – white clover flowers

**Abstract**

Interactions between dendritic cells (DCs) and environmental, dietary and pathogen antigens play a key role in immune homeostasis and regulation of inflammation. Dietary polyphenols such as proanthocyanidins (PAC) may reduce inflammation, and we therefore hypothesised that PAC may suppress lipopolysaccharide (LPS)-induced responses in human DCs and subsequent Th1-type responses in naïve T-cells. Moreover, we proposed that, since DCs are likely to be exposed to multiple stimuli, the activity of PAC may synergise with other bioactive molecules which have anti-inflammatory activity, e.g. soluble products from the helminth parasite *Trichuris suis* (TsSP). We show that PAC are endocytosed by monocyte-derived DCs and selectively induce CD86 expression. Subsequently, PAC suppress the LPS-induced secretion of IL-6 and IL-12p70, whilst enhancing secretion of IL-10. Incubation of DCs with PAC did not affect lymphocyte proliferation, however subsequent IFN-γ production was markedly suppressed, whilst IL-4 production was unaffected. The activity of PAC was confined to oligomers (degree of polymerization ≥ 4). Co-pulsing DCs with TsSP and PAC synergistically reduced secretion of TNF-α, IL-6 and IL-12p70 whilst increasing IL-10 secretion. Moreover, both TsSP and PAC alone induced Th2-associated OX40L expression in DCs, and together synergized to up-regulate OX40L. These data suggest that PAC induce an anti-inflammatory phenotype in human DCs that selectively down-regulates Th1 response in naïve T-cells, and that they also act cooperatively with TsSP. Our results indicate a novel interaction between dietary compounds and parasite products to influence immune function, and may suggest that combinations of PAC and TsSP can have therapeutic potential for inflammatory disorders.

**Introduction**

Dendritic cells (DCs) are key players in immune surveillance and homeostasis in various organs, particularly those with large mucosal surfaces such as the gastrointestinal tract. A number of specialised populations of DCs reside in the lamina propria and the gut associated lymphoid tissue (GALT) such as the Peyer’s patches. Human intestinal DCs are not well characterised, but in mice different subsets are distinguished by their expression of CD11b, CD103, CX3CR1 and CCR7, and they play an important role through antigen sampling from the intestinal lumen and subsequent presentation of pathogen antigens to T-cells in the GALT [1](#_ENREF_1), [2](#_ENREF_2). Thus, DCs are exposed to both harmless gut flora and pathogenic intestinal microorganisms such as viruses, bacteria and parasites, as well as dietary components. They therefore play a key role in maintaining effective immune homeostasis; overt inflammatory responses by DCs such as excessive secretion of pro-inflammatory cytokines (e.g. TNF-α) may lead to the development of chronic inflammation, whilst appropriate cytokine secretion and T-cell activation is also important for effective clearance of potentially harmful pathogens [3-5](#_ENREF_3). Therefore, modulation of DC activity may be an effective strategy for ameliorating autoimmune diseases, as well as invoking desirable immune response for protection against intestinal pathogens.

The cytokine profiles secreted by DCs upon activation by microbial antigens can vary markedly according to the nature of the pathogen. The established paradigm is that pathogenic, intracellular bacteria and viruses promote a vigorous inflammatory response from DCs characterised by secretion of high levels of TNF-α, IL-12 and IL-6, and subsequent induction of Th1-type CD4+ T-cells that produce large amounts of IL-2 and IFN-γ [6](#_ENREF_6), [7](#_ENREF_7). In contrast, multicellular helminth parasites invariably invoke a Th2-type response, whereby DCs induce T-cells that secrete high amounts of IL-4, IL-5 and IL-13, and little IFN-γ. Protective immunity is thought to derive in part from an IL-4/IL-13 driven increase in gut motility and fluid secretion that removes parasites from their intestinal niche [8-11](#_ENREF_8). In addition, this Th2-driven response results in alternatively-activated macrophages with wound-healing and anti-inflammatory properties [12](#_ENREF_12). Interestingly, concurrent stimulation of DCs with helminth antigens has been shown to actively down-regulate the Th1 inflammatory response induced by TLR agonists such as lipopolysaccharide (LPS) [13](#_ENREF_13). Helminths and/or their secreted products have therefore been proposed as novel therapy for chronic inflammatory disorders such as Crohn’s disease or multiple sclerosis [14](#_ENREF_14), [15](#_ENREF_15).

Bioactive dietary compounds also have the potential to markedly influence the immunological milieu of the body, through either absorption and subsequent systemic activity or interaction with the numerous innate immune sentinel cells that reside in the gastrointestinal mucosa. Indeed, many plant compounds have been reported to have anti-inflammatory effects; the flavan-3-ol, epigallocatechin gallate (EGCG), an abundant molecule in green tea, has been shown to alleviate symptoms of autoimmune inflammation in mice [16](#_ENREF_16), [17](#_ENREF_17), and *in vitro* experiments have demonstrated that EGCG inhibits inflammatory responses in macrophages through inhibition of TLR-dependent pathways [18](#_ENREF_18). A related group of compounds are proanthocyanidins (PAC; syn. condensed tannins), which are oligomeric and polymeric forms of flavan-3-ols found in dietary components such as fruits, nuts, berries and beans.

The flavan-3-ol monomeric units that give rise to PAC are predominantly catechin or epicatechin (comprising procyanidin -type PAC) or gallocatechin or epigallocatechin (comprising prodelphinidin -type PAC, which are less numerous than procyanidins but found in large amounts in e.g. blackcurrants and other berries). The major difference between these monomeric units is an extra hydroxyl group in the B-ring of prodelphinidins (Figure 1). Large variations are also observed in molecular weight depending on the number of linked flavan-3-ol units, i.e. leading to different degrees of polymerization. These molecules have strong bioactivity as they bind readily to other macromolecules such as proteins and polysaccharides, and have been extensively studied for their antioxidant [19](#_ENREF_19), and antiviral [20](#_ENREF_20) properties. In addition, a number of studies have highlighted the anti-inflammatory properties of PAC; administration of oligomeric PAC has been shown to alleviate the symptoms of inflammatory disorders such as autoimmune arthritis [21](#_ENREF_21) or experimental autoimmune encephalomyelitis [22](#_ENREF_22) in mice. The anti-inflammatory mechanisms of PAC have not been elucidated fully, but have been suggested to involve inhibition of TLR-dependent signalling pathways and antigen-presenting capacity in macrophages [22](#_ENREF_22), [23](#_ENREF_23), as well as down-regulation of CD11b surface expression in monocytes [24](#_ENREF_24).

Despite the large recent interest in the anti-inflammatory properties of PAC, their interactions with human DCs is not yet clear. Peripheral blood monocyte-derived DCs represent a convenient and widely-used model to assess the effects of various immuno-modulatory agents on human DC activity [25-27](#_ENREF_25). Here, we prepared well-characterised PAC fractions to investigate effects on human monocyte-derived DC activity. We hypothesised that PAC would be recognised and taken up by DCs, and subsequently inhibit LPS-induced inflammatory responses. Moreover, we postulated that any anti-inflammatory activity of PAC would not act in isolation, but would interact with other modulatory substances that may be found in the same environment sampled by the DCs, which in the case of dietary compounds such as PAC, would include gastrointestinal parasites. Therefore, we also determined the effects of simultaneous DC exposure to PAC and products from the helminth *Trichuris suis,* a pathogenic pig parasite that causes large problems in swine production, but has also shown promise in treating autoimmune diseases in humans. We reasoned that the well-known Th2-polarising effects of *T. suis* [11](#_ENREF_11), [28](#_ENREF_28) may synergise with PAC to modulate DC function. We show that PAC interact directly with human DCs, and down-regulate inflammatory cytokine responses and subsequent Th1 responses in naïve T-cells. Furthermore, PAC and *T. suis* soluble products synergize to supress inflammatory responses in DCs. Our results may indicate a novel function of PAC to down-regulate DC-driven inflammatory processes, and suggest that dietary components and parasites can interact to modulate immune responses.

**Materials and Methods**

*Proanthocyanidins*

Purified PAC were prepared from either cocoa beans (COC) or white clover flowers (WCF) to obtain exclusively procyanidin- or prodelphinidin-type PAC, respectively. Two fractions were isolated from each plant extract; a first fraction (F1) that contained lower molecular weight PAC (as measured by mean degree of polymerization – mDP), and a second fraction (F2) containing higher molecular weight PAC. The extraction, purification and analysis procedures have been described before in detail [29](#_ENREF_29). Briefly, to purify PAC, plant material (50 g) was extracted with acetone/water (7:3; v/v) and PAC fractions were obtained by Sephadex LH-20 chromatography, with F1 eluted using 3:7 v/v acetone/water and F2 eluted with 1:1 v/v acetone/water. Analysis of PAC was undertaken by HPLC-MS in order to calculate purity, the molar proportions of monomeric sub-units and mean degree of polymerization (mDP). Results of these analyses have been published previously [29](#_ENREF_29), [30](#_ENREF_30), and are summarized here briefly in Table 1. In addition, 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)-tagged PAC were obtained similarly by acetone/water extraction of *Lotus corniculatus* and chromatographic purification, followed by conjugation to DTAF as previously described [31](#_ENREF_31). Untagged PAC were prepared in a similar fashion but omitting the DTAF conjugation step.

*Parasite material*

Adult *T. suis* worms were collected from the caecum and colon of experimentally-infected pigs and washed extensively in warm saline. The *T. suis* soluble products (TsSP) were prepared by homogenization and sonication of whole worms as previously described [11](#_ENREF_11).

*Isolation of monocytes and dendritic cell culture*

Buffy coats were collected (Copenhagen University Hospital, Denmark) from healthy, anonymous volunteers following written, informed consent. Peripheral blood mononuclear cells (PBMC) were isolated on histopaque (Sigma-Aldrich) and monocytes purified by anti-CD14 microbeads and magnetic separation (MACS, Miltenyi Biotech). Monocytes were cultured at 37°C and 5% CO2 in complete RPMI media (RPMI 1640 containing 10% inactivated foetal bovine serum, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin). Monocytes were differentiated into immature DC (iDC) in the presence of 12.5 ng/mL each of IL-4 and GM-CSF (R&D Systems, Abingdon, UK) and routinely used at day 4. Immature DC were then pre-treated for one hour with PAC or PBS as a control. Concentrations of fractions were adjusted to ensure an equal concentration of PAC between the different samples - thus, all concentrations refer to concentration (w/v) of PAC. After optimization, 10 µg/mL of COC PAC and 20 µg/mL of WCF PAC (w/v) were found to be optimal concentrations; higher concentrations resulted in significant cytotoxicity as judged by 7AAD staining (Figure S1). Where indicated, TsSP were added for the final 30 minutes. Lipopolysaccharide (LPS; 10 ng/mL) was then added and the cells cultured for a further 24 hours. For blocking experiments, 10 µg/mL either anti-CD11b (Clone ICRF44, BD Pharmingen, USA) or anti-67LR (Clone MLuC5, Abcam, UK), or appropriate isotype controls were added 15 minutes prior to the addition of PAC and incubated at 37°C. In some experiments, PAC fractions were pre-incubated in polyvinylpolypyrrolidone (PVPP) overnight (10:1 PVPP:PAC; w/w) at 4°C, followed by centrifugation at 3000g for ten minutes, and the supernatant retained and used to stimulate DCs. Controls consisted of media alone incubated with PVPP, and PAC with no PVPP incubated overnight in an identical fashion.

*Mixed Lymphocyte Reactions*

Immature DCs were incubated with either LPS or LPS with 20 µg/mL WCF F2 for 48 hours, then washed and counted. For preparation of responder cells, allogenic PBMC were depleted of monocytes by removal of CD14+ cells by MACS separation as described above. The responding lymphocytes were then labelled with CFSE (Sigma-Aldrich; 1 µM) and then added at a 1:10 (DC to responder cell) ratio and the cells cultured for 6 days, after which fluorescence was analysed by flow cytometry.

*Flow cytometry*

After 24 hours DCs were harvested, washed and stained with either anti CD80-PE (Clone L307.4), CD86-APC (Clone FUN-1), MHC-II-FITC (Clone Tu39), OX40L-PE (Clone Ik-1) CD11c-FITC (Clone B-ly6), CD11b-APC (Clone ICRF44), CD103-FITC (clone Ber-ACT8) or CX3CR1-PE (clone2A9-1; all from BD Pharmingen, USA). Cells were acquired on an Accuri C6 flow cytometer. Mean fluorescence intensities were calculated after gating on viable cells. Data were analysed using FCS version 5 (De Novo Software, Glendale, CA).

*ELISA*

Supernatants from DC cultures were harvested after 24 hours and the levels of TNF-α, IL-6 and IL-10 measured using the appropriate cytosets (Life Technologies, USA) according to the manufacturer’s instructions. For IL-12p70, plates were coated with anti-IL-12p70 (eBioscience, San Diego, CA) and detected with biotinylated anti-IL-12p40/70 (BD Pharmingen, USA), followed by streptavidin-horseradish peroxidase (Life Technologies, USA) and TMB substrate (Sigma-Aldrich, Schnelldorf, Germany).

*Fluorescence microscopy*

Immature DCs were stimulated for 1 or 2 hours at 37°C or 1 hour at 4°C with either media only, DTAF-tagged PAC (50 µg/mL) or an equivalent concentration of untagged PAC. Cells were then washed in PBS, fixed in 4% paraformaldehyde, settled onto poly-L-lysine coated coverslips and blocked for 1 hour at room temperature with 2% BSA in PBS. Cells were then stained for with Alexa Fluor-594 anti-human CD31 (Clone WM59, Biolegend, San Diego, CA) or permbealised with 0.1% saponin followed by staining with anti-human CD107b (Clone H4B4, Biolegend, San Diego, CA) and Alexa Fluor 594 goat-anti mouse conjugate (Thermo Fisher). Cells were mounted in Vectashield with DAPI (Vector Labs, Carlsbad, CA), and then examined microscopically at x100 magnification on a Leica HMR DC fluorescence microscope. Images were processed using ImageJ software.

*TLR4-reporter cells*

Human embryonic kidney 293 (HEK 293) cells stably expressing TLR4 [32](#_ENREF_32) were cultured in DMEM media supplemented with 10% heat inactivated bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were stimulated with 20 µg/mL WCF or COC F2 fraction and/or 10 ng/mL LPS. After 24 hours, supernatant was harvested and IL-8 production measured by ELISA.

*Th1/Th2 skewing assay*

Immature DCs were stimulated for 48 hours with either LPS alone, or in the presence of PAC and/or TsSP. Naïve, allogenic CD45RA+CD4+ T-cells were isolated using the naïve T-cell isolation kit (MACS, Miltenyi Biotech). After 48 hours DCs were extensively washed in PBS and then added to naive T-cells at a ratio of 1:10 DC:T-cells. Cells were then cultured for 12 days in complete RPMI media supplemented with 50 U/mL IL-2 (Life Technologies), with the media changed every 2-3 days for fresh media containing IL-2. Cells were then washed and stimulated for five hours with a mixture of ionomycin (Sigma-Aldrich; 1 µg/mL), phorbol 12-myristate 13-acetate (Sigma-Aldrich ; 30 ng/mL) and brefeldin A (Sigma-Aldrich; 10 µg/mL). Cells were then fixed and permeabilised using the cytofix/cytoperm kit (BD Pharmingen, USA), and intracellular cytokine staining carried out by flow cytometry using anti IL4-APC (Clone 8D4-8) and IFN-γ-FITC (Clone 4S.B3; both from BD Pharmingen, USA). Background responses from unstimulated cells were subtracted from the stimulated responses.

*Data analysis and statistics*

Where indicated, ANOVA analyses with Bonferroni post-hoc testing or paired t-tests were carried out using GraphPad Prism (v6.00, GraphPad Software, La Jolla, California, USA, www.graphpad.com). Normality of data was assessed using Shapiro-Wilk tests, and where data did not conform to a normal distribution logarithmic transformation was carried out prior to analysis. Statistical analyses was performed on either raw cytokine concentrations (ELISA) or mean fluorescent intensities/percentage of positive cells (Flow cytometry), however for ease of interpretation data is presented in most instances as a percentage of the response of cells to LPS, and means ± S.E.M. of untransformed data are presented.

**Results**

*Structurally diverse proanthocyanidins induce CD86 expression in dendritic cells*

To determine if PAC are recognized by DCs, we first asked whether DCs incubated with PAC respond by up-regulating classical cell-surface markers of DC maturation, and whether structural features of PAC may affect any such response. To this end, we used purified PAC fractions that consisted exclusively of either procyanidins (COC) or prodelphinidins (WCF). Monocyte-derived DCs were then exposed to the various PAC fractions. After 24 hours, incubation with PAC did not affect expression of CD80 or MHC-II, in contrast to the strong up-regulation induced by the TLR4 agonist LPS (Figure 2A). However, incubation with F2-fractions from both COC and WCF induced up-regulation of CD86 expression (*P*<0.01), with both PAC types inducing a similar level of up-regulation, though the expression was not as profound as that induced by LPS (Figure 2A).

Characterisation of the monocyte-derived DCs showed that majority of the DCs were CD11c+, CD11b+,and CD103-. Most of the DCs were CX3CR1-, but a small population (~5%) of the DCs were CX3CR1+ (Figure S2). Interestingly, PAC seemed to induce CX3CR1 expression with higher proportions of CX3CR1+ cells present following PAC exposure (Figure 2B). Given that PAC likely exert their activity locally in the intestinal mucosa, and in mice CX3CR1 has been shown to be important for allowing DCs to sample antigens from the intestinal lumen [33](#_ENREF_33), we examined PAC-induced CD86 up-regulation in CX3CR1- and CX3CR1+ DCs. CD86 expression induced by PAC was identical in these two populations (Figure 2C).

The effect of PAC was clearly dependent on the degree of polymerization, as F1 from COC and WCF containing an equal amount (w/w) of low mDP (≤2.3) PAC did not induce CD86 expression (*P*>0.05; Figure 2C). No interactions between LPS and PAC were evident; PAC did not inhibit LPS-induced expression of any cell-surface activation marker, nor did they additively increase the expression of CD86 (data not shown). To confirm the role of PAC, the F2 fractions were pre-incubated with PVPP to selectively neutralize PAC. CD86 expression was subsequently abolished in these PVPP-treated samples (Figure 2C). These data suggest that structurally diverse PAC are able to selectively induce CD86 expression in DCs that is dependent on their degree of polymerization.

*Proanthocyanidins inhibit LPS-induced pro-inflammatory cytokine secretion in dendritic cells*

We next investigated whether PAC induced cytokine secretion in DCs. Incubation of DCs with PAC alone did not result in any measurable cytokine production (data not shown). However, incubation of DCs with LPS and both COC or WCF F2 resulted in a reduction (*P*<0.001) in the LPS-induced secretion of the Th1-type cytokines IL-6 and IL-12p70, whilst TNF-α secretion was not affected (Figure 3A). In contrast, PAC increased the LPS-induced secretion of the Th2/regulatory type cytokine IL-10 (Figure 3A-B). The dynamic range of PAC activity was low, with concentrations of 5 µg/mL of both COC and WCF still inhibiting IL-6 and IL-12p70 secretion, but no inhibition was evident at 2.5 µg/mL (Figure S3A).

PAC from WCF appeared to more strongly inhibit IL-6 and IL-12p70 production and augment IL-10 production than COC PAC, suggesting that PAC comprised of prodelphinidins are stronger regulators of DC activity than those comprised of procyanidins. Whilst a higher concentration of WCF could be used due to lower cytotoxicity issues, we also observed that when equal concentrations of WCF and COC F2 were used, suppression of IL-12p70 secretion was greater with WCF than with COC (*P*<0.05), whereas suppression of IL-6 showed no significant difference (Figure S3B).

Similar to the CD86 expression data (Figure 2), effects on cytokine secretion were dependent on polymerization, with COC and WCF F1 proving inefficient at modulating cytokine secretion, and the activity of F2 was abolished by pre-incubation in PVPP (Figure 3B). Overall, these data suggest that oligomeric PAC induce a regulatory phenotype in DCs that may inhibit inflammatory responses

*Proanthocyanidins are internalized by dendritic cells and localise to lysosomes*

Having established that PAC modulate DC activity, we next investigated whether PAC were internalized by DCs or interacted only with the cell periphery. To accomplish this, we used PAC purified from *L. corniculatus* and tagged with the fluorophore DTAF. These PAC had a mean degree of polymerization of 9.5 (as determine by thiolysis), indicating a similar molecular weight to the biologically active F2 fractions from COC and WCF. We also confirmed that these DTAF-tagged PAC induced similar functional activity in DCs as COC and WCF PAC, as evidenced by significant inhibition of IL-6 and IL-12p70 secretion in LPS-activated DCs (*P*<0.05; Figure S4), indicating that the DTAF-tagging procedure does not alter the functional activity of PAC. We then exposed monocyte-derived DCs to either DTAF-tagged PAC or controls consisting of untagged PAC or medium only. Single-cell imaging of DCs demonstrated that the DTAF-tagged PAC were clearly visible within the cell after 1 hour of exposure (Figure 4A). No fluorescence was observed in DCs exposed to untagged PAC (Figure 4A) or medium only (data not shown). To confirm that PAC are internalized by DCs, we performed dual labelling experiments with antibodies against the DC surface protein CD31 which clearly showed that PAC were located in the interior of the cell (Figure 4B).

To determine if this was an active endocytotic process, DCs were incubated with DTAF-tagged PAC at either 37°C or 4°C, which demonstrated that internalization of PAC occurred only at 37°C, whereas at 4°C PAC bound to the cell membrane but remained on the exterior of the cell, co-localizing with CD31 (Figure 4B). These data suggest that DCs recognize and actively internalize PAC. Moreover, PAC appeared to localise to a LAMP2-bright compartment within the DC, suggesting that PAC traffic to lysosomes following internalisation (Figure 4C).

*Proanthocyanidins do not inhibit binding of LPS to TLR4 and do not signal through 67LR or CD11b*

To determine if PAC inhibited LPS-induced cytokine secretion by either binding to and neutralizing TLR4, or else binding to LPS and preventing it being bound by TLR4, we used TLR-4 reporter cells that secrete IL-8 following TLR4 ligation. As shown in Figure 5A, neither COC or WCF F2 induced IL-8 secretion in these cells, indicating that they do not interact directly with TLR4. Furthermore, IL-8 secretion was unchanged after addition of LPS in the presence of PAC, demonstrating that PAC do not inhibit the interaction of LPS with TLR4.

Given that PAC were not bound by TLR4, we asked whether there could be another cognate receptor that interacts with PAC. Previous studies have demonstrated that EGCG interacts specifically with 67LR on mouse DCs [34](#_ENREF_34), whilst oligomeric PAC have been shown to interact with CD11b on bovine monocytes [24](#_ENREF_24). We therefore tested whether blocking either or both these receptors would ablate the effects of PAC on LPS-stimulated DCs. However, anti- 67LR or anti-CD11b treatment (Figure 5B), or treatment with both mAbs (data not shown), did not reverse the inhibition of LPS-induced IL-6 and IL-2p70 secretion in PAC-treated DCs.

*Proanthocyanidin-primed dendritic cells do not inhibit proliferation but inhibit Th1 responses in naïve CD4+ T-cells*

We next assessed whether PAC-primed DCs inhibit lymphocyte proliferation and/or skew naïve CD4+ cells to a Th1 or Th2 phenotype. DCs were activated with either LPS or LPS in the presence of WCF PAC, as these had shown to be most effective at modulating cytokine secretion (Figure 2A). Proliferation of lymphocytes (monocyte-depleted PBMC) cultured with LPS-matured DCs was not inhibited by co-pulsing DCs with PAC (Figure 6A). T-cells activated by LPS-primed DCs produced both IFN-γ and IL-4, consistent with a mixed Th1/Th2 type response that is known to be induced by this antigen (Figure 6B). In contrast, T-cells activated by DCs that had been primed by LPS in the presence of PAC produced very little IFN-γ, whilst IL-4 production was unchanged (Figure 6B). Thus, the IL-4/IFN-γ ratio was far higher for T-cells activated by PAC-treated DCs (5.3 vs. 1.9; *P*<0.01 by paired t-test; Figure 6B), thus suggesting that PAC-primed DCs do not inhibit T-cell proliferation but subsequently drive a Th2 phenotype in naïve T-cells by selective down-regulation of Th1 responses.

*Proanthocyanidins synergize with* Trichuris suis *products to modulate cytokine secretion and induce OX40L expression in dendritic cells*

Given the anti-inflammatory properties of PAC, we next assessed whether PAC would interact with TsSP, which we have previously shown to markedly inhibit inflammatory responses in DCs. Consistent with our previous work [11](#_ENREF_11), [13](#_ENREF_13), we observed here significant decreases in LPS-induced TNF-α, IL-6 and IL-12p70 secretion after pre-incubation of DCs with a concentration of 40 µg/mL TsSP (mean reductions of 64, 43 and 57%, respectively; Figure 7A). Incubation of DCs with higher concentrations of TsSP (up to 160 µg/mL) failed to result in increased inhibition of secretion of TNFα [13](#_ENREF_13) and IL-6 and IL-12p70 (Fig S5), indicating a saturating effect of TsSP at this concentration. However, addition of either COC or WCF F2 further decreased secretion of TNF-α, IL-6 and IL-12p70, indicating that PAC and TsSP synergized to reduce secretion of these cytokines (mean reductions of 74, 69 and 86% respectively, for COC and 84, 79 and 97%, respectively for WCF; Figure 7A). Furthermore, an enhancement of IL-10 secretion was observed when TsSP and PAC were combined. Cells exposed to TsSP secreted more IL-10 than DCs pulsed only with LPS, and this effect was boosted by co-incubation with PAC (Figure 7A), although large variations between experiments with cells from different blood donors prevented statistically significant differences. These data suggest that reductions in Th1 cytokine secretion, and a corresponding enhancement of IL-10 secretion, are co-operatively achieved by PAC and TsSP in a combined effect that is above and beyond the effect achieved by a saturating concentration of either treatment in isolation.

Given that we have also previously observed that TsSP up-regulates OX40L expression in DCs [11](#_ENREF_11), which is important in driving Th2 responses [35](#_ENREF_35), we also assessed whether TsSP and PAC would synergize to up-regulate OX40L expression. Incubation of PAC or TsSP with immature DCs did not result in increased OX40L expression (data not shown). As expected, TsSP significantly induced OX40L expression in LPS-stimulated DCs (*P*<0.05;Figure 7B,C). Similarly, addition of both COC and WCF F2 to LPS-pulsed DCs resulted in increases in OX40L expression, although only statistically significant for WCF (Figure 7B,C). Strikingly, co-incubation of LPS-stimulated DCs with TsSP and PAC, particularly WCF F2, resulted in increased expression of OX40L compared to TsSP alone (*P*<0.001 for WCF; Figure 7B,D), indicating augmentation of the helminth-induced Th2 polarization by PAC. Thus, PAC and TsSP interact to influence both cytokine secretion and OX40L up-regulation in DCs.

Finally, we assessed whether pulsing DCs with a combination of WCF F2 and TsSP would result in a lower production of IFN-γ in naïve CD4+ T-cells, compared to that observed with WCF F2 alone (Figure 6). Similar to the data with WCF F2, T-cells activated by DCs primed by TsSP produced only low amounts of IFN-γ (Figure 8A), however IFN-γ production in T-cells activated by DCs exposed to both WCF F2 and TsSP was not further reduced from the already low levels observed with each treatment in isolation, whilst IL-4 production was again unaffected. Thus, IL-4/ IFN-γ ratios were similar (*P*=0.8) in T-cells activated by DCs treated with either WCF F2 alone, TsSP alone, or WCF and TsSP in combination (Figure 8B).

**Discussion**

The objective of this work was to test the hypothesis that PAC would modulate LPS-induced responses in human DCs, and would also interact with TsSP, which are already known to possess such inhibitory activity.

Our data clearly show that the exposure of DCs to PAC induces an anti-inflammatory phenotype, with marked reductions in secretion of inflammatory cytokines as well as a significant impairment in IFN-γ production by naïve CD4+ T-cells activated by PAC-pulsed DCs. The DC phenotype induced by PAC was remarkably similar to that induced by TsSP, as well as what has been observed previously with products from other helminths such as *Schistosoma mansoni* or *Trichinella spiralis* [11](#_ENREF_11). Notably, exposure of DCs to both PAC and TsSP resulted in an augmentation of this phenotype that was above and beyond that achieved by either single stimulus, perhaps suggesting that PAC and TsSP modulate DC activity through independent pathways that co-operatively inhibit pro-inflammatory cytokine production. Therefore, our results may shed light on a novel interaction between diet and an intestinal pathogen that may be important for regulation of inflammation and immunity.

PAC are clearly recognised by DCs, with our experiments showing that DCs actively endocytose PAC which appear to be trafficked to the lysosomes within 2 hours, consistent with the putative intracellular trafficking of EGCG and tannin-lysozyme complexes by mouse macrophages [36](#_ENREF_36), [37](#_ENREF_37). DCs then respond by selectively up-regulating CD86 (and possibly CX3CR1, to a lesser-extent), suggesting at least a partial maturation of the DC. However, no cytokine secretion was evident in the absence of TLR ligation, indicating that PAC do not fully induce functional DC activity and, instead, selectively modulate TLR4-driven responses. The presence in the lysosome raises the possibility that PAC may exert activity through a modulation of antigen processing, but, clearly, further studies will be necessary to determine the mechanistic aspects of how PAC are trafficked within DCs, and the signalling pathways that are modulated by PAC in LPS-stimulated cells. Ligation of TLR4 primarily triggers two signalling pathways: the myD88-dependent pathway, which leads to NF-κB translocation and inflammatory gene expression, and the TRIF-dependent pathway, which results in type-I interferon transcription [38](#_ENREF_38). The influence of PAC on these two pathways is clearly an area requiring investigation. In addition to a number of reports from murine models that dietary PAC may alleviate inflammatory disorders such as colitis [39](#_ENREF_39), [40](#_ENREF_40), *in vitro* studies, working mainly with model murine cell lines, have demonstrated that polymeric PAC can have specific anti-inflammatory activity such as a decrease in nitric oxide production or a reduction in NK-κβ activation and IL-1β secretion [41](#_ENREF_41), [42](#_ENREF_42). Functional consequences of this modulation of DC activity may be a marked change in naïve T-helper cell function, as evidenced by our data showing significantly impaired IFN-γ production in CD4+ T-cells activated by PAC-pulsed DCs, consistent with previous work showing that dietary PAC can selectively down-regulate Th1-type inflammatory responses in mice *in vivo* [43](#_ENREF_43). The initial contact and process(es) involved in recognition of PAC by DCs will also require further clarification. Given the strong protein-binding properties of PAC [44](#_ENREF_44), it is perhaps unlikely that PAC are bound by a cognate receptor and instead are more likely to interact with a variety of cell surface molecules, and perhaps also lipid rafts in the cell membrane, as has been shown for the interaction between cocoa PAC and human enterocytes [45](#_ENREF_45).

Similarly to bioactive dietary compounds such as PAC, helminths (or their secreted and/or somatic products) can be said to possess anti-inflammatory properties; indeed, controlled helminth infection has been shown to alleviate signs of auto-inflammatory disorders in humans [15](#_ENREF_15). We therefore hypothesised that the human DC response to helminths and PAC may share certain similarities. In support of our hypothesis, we observed a remarkably similar phenotype whereby LPS-stimulated DCs pulsed either with TsSP or PAC secreted significantly less IL-6 and IL-12p70, and more IL-10, than control cells treated only with LPS, and also resulted in a significantly increased expressed of OX40L, which has been shown to be important in driving Th2 responses in the context of helminth infections [46](#_ENREF_46). Importantly, we showed that these effects could be synergistic, in the sense that responses significantly greater than either treatment in isolation could be achieved by the combination. The effective concentrations of PAC were saturated by cytotoxicity at higher concentrations, whilst the effective range of concentrations of TsSP was also shown to be saturable at concentrations greater than 40 µg/mL [13](#_ENREF_13), perhaps due to a lack of availability of surface receptors needed to bind the active components of TsSP on DC function. However, the addition of PAC significantly enhances the effects of this saturating concentration of TsSP, suggesting that PAC operate through a mechanism distinct from that of TsSP to influence DC activity, with a similar end-result in terms of cytokine secretion and OX40L expression. Whilst we did not observe a co-operative inhibition of IFN-γ production in T-cells activated by DCs exposed to both PAC and TsSP, this can likely be attributed to the low levels of IFN-γ that were produced by each treatment in isolation, thus lowering the scope to observe synergistic effects. Overall, our data may indicate that the immune-modulating activity of *T. suis* can be augmented by the presence of PAC. Interestingly, Zhong *et al*. have recently noted a similar phenomenon in sheep leukocyte preparations exposed *in vitro* to *Haemonchus contortus* antigens, whereby enhanced IL-10 production and reduced IL-12 production was evident after co-incubation of the cells with another type of tannin, tannic acid (a mixture of hydrolysable tannins that contain glucose as a central core that is surrounded by 6 or more galloyl groups) [47](#_ENREF_47), suggesting possibly a conserved interaction between helminth antigens and oligomeric polyphenols.

This interaction between pathogens and these dietary compounds raises a number of possibilities. In the *in vivo* situation, gastrointestinal pathogens such as helminths and bioactive food compounds are likely to be found in close proximity within the gut and associated with the intestinal mucosa, suggesting that the co-operative effects we have observed *in vitro* may also be relevant *in vivo*. The concentrations of PAC used in our studies are very likely to represent physiological concentrations expected to be present in the digesta following consumption of PAC-containing foods [40](#_ENREF_40), [48](#_ENREF_48), [49](#_ENREF_49), although precise measurements of local PAC concentrations at the mucosal surface are limited by methodological difficulties[50](#_ENREF_50). Oligomeric PAC are known to be poorly absorbed by enterocytes [51](#_ENREF_51), and are thought to be largely stable through transit of the monogastric GI tract (e.g. that of humans, pigs or rats) [49](#_ENREF_49), [50](#_ENREF_50). This suggests that the immune-modulating activity of dietary PAC is likely mediated by sentinel cells in the gut mucosa, and subsequent modulation of T-cell activity in the GALT. Dendritic cells are key candidates to be the cells that mediate these effects of PAC, due to their ability to directly interact with the contents of the intestinal digesta [52](#_ENREF_52).

Intestinal DCs may acquire luminal antigens in two ways; antigens may be delivered to follicular or lamina propria-resident DCs by M-cells in the epithelium that capture antigen from the intestinal lumen, or DCs may sample the luminal contents directly, by either extending dendrites through the epithelial barrier and transporting antigens back to the sub-epithelial zone, or by actively migrating into the lumen where they interact with the intestinal digesta [52-55](#_ENREF_52). If and how DCs interact with PAC *in vivo* is a key question of interest: do DCs directly sample PAC from the intestinal lumen, or are PAC recognized by M-cells and delivered by transcytosis to DCs residing in the sub-epithelial region of the GI mucosa? In mice, intestinal DCs appear to be a heterogeneous population, with differential functions based on the expression of surface markers. In the steady state, both CD103+ and CX3CR1+ reside in the lamina propria and lymphoid tissue, and appear to have distinct functions. CX3CR1 expression has been shown to be crucial in the ability to sample luminal antigens[56](#_ENREF_56), whilst others have shown that CX3CR1+ DCs seem to be a non-migratory population within the lamina propria. Instead, CX3CR1-CD103+  cells can uptake antigen in the epithelium and are better equipped for subsequent migration to the lymph nodes and activation of T-cells, and are important in induction of T-regulatory cells and intestinal homeostasis [53](#_ENREF_53), [57-59](#_ENREF_57). In contrast, murine CD103- DCs have also been shown to have migratory and T-cell activation ability but instead induce pro-inflammatory Th1 and Th17 responses [58](#_ENREF_58), [60](#_ENREF_60). Moreover, during infection and inflammation, monocytes migrate from the blood into the mucosa and can differentiate into dendritic cells with a strong inflammatory phenotype[61](#_ENREF_61), [62](#_ENREF_62), thus probably resemble monocyte-derived DCs as utilised in our current experiments (consisting of CD103- DCs). We also observed both CX3CR1- and a small population of CX3CR1+ cells, which seemed to equally recognise PAC (as judged by CD86 expression), suggestive of a conserved response across distinct populations of DCs. However whether or not different populations of intestinal DCs respond identically to PAC is a question which warrants further attention.

It is also notable that *T. suis* infection has been observed to decrease barrier function in the intestinal mucosa in the site of the infection [63](#_ENREF_63), [64](#_ENREF_64), and Hiemstra *et al*. have shown that excretory/secretory products from *T. suis* can disrupt the tight barrier junctions in epithelial cells *in vitro*, enhancing direct contact of antigens to immune cells residing in sub-epithelial locations [65](#_ENREF_65). Thus, an intriguing possibility is that interactions between PAC and the GALT could be amplified during helminth infection due to the ‘leaky’ epithelial barriers allowing the passage of oligomeric PAC molecules that under normal circumstances may be confined to the gut lumen. The *in vivo* interactions between PAC and the gut mucosa, both alone and in the context of helminth (or other pathogen) infection clearly is an area where much work is required.

This putative interaction between a common class of bioactive dietary compounds and a gut pathogen may have far-reaching implications. PAC-rich feed supplements are becoming popular in livestock production due to their antioxidant and anti-inflammatory properties [19](#_ENREF_19), [66](#_ENREF_66), and may have beneficial effects in helminth-infected animals such as enhancement of Th2-type protective immune mechanisms that enhance worm expulsion, or control of excessive gut inflammation. Paradoxically, *T. suis* has also been investigated as a therapeutic treatment in humans suffering from autoimmune disease, in part due to the strong modulatory effects observed on DC and macrophage function [67](#_ENREF_67), [68](#_ENREF_68). However, clinical trials to investigate effects on diseases such as colitis and multiple sclerosis have not been equivocal, with some reported successes but also a number of negative results [14](#_ENREF_14), [69](#_ENREF_69), [70](#_ENREF_70). Our current data may suggest that the efficacy of helminth therapy could be augmented/enhanced by concurrent nutritional treatment with PAC, either in the form of PAC-rich dietary sources or administration of purified, concentrated PAC. Although it is possible, but expensive, to synthesize oligomeric PAC, large-scale extraction and purification procedures exist to obtain PAC from a number of inexpensive plant sources, including some agricultural waste-products such as berry pomace [71](#_ENREF_71). Moreover, as discussed above, the physiological concentrations of PAC needed to exert activity may be achievable through a PAC-rich diet rather than targeted treatment with purified PAC. Therefore, ‘combination therapy’ with helminth products and PAC may be a novel future avenue for autoimmune treatment.

In conclusion, this study provides the first evidence that oligomeric PAC can modulate human DC activity, and that the potential for synergism exists with the modulatory activity of gastrointestinal parasites which may be present within the intestine. The activity of PAC was confined to oligomers with a mean degree of polymerization of at least 4, and appeared to be more closely associated with prodelphinidins rather than procyanidins. Further investigation of the *in vivo* interactions between dietary PAC and intestinal parasites on immune function is highly warranted.

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A.R.W, I.V.D. and S.M.T. conceived and designed the study. A.R.W., E.J.K., L.C.L. and R.D. performed the dendritic cell experiments. A.R., C.F., J.D.R and I.M.H. prepared PAC samples and performed chemical analyses. H.K. and S.S. contributed essential reagents and/or materials. A.R.W wrote the manuscript, with input from all other authors.

**Conflict of Interest Statement**

The authors declare no conflict of interest.

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**Figure Legends**

**Figure 1 – Examples of procyanidin and prodelphinidin structure**

Chemical structures of epicatechin and epigallocatechin, the monomeric *cis* isomers giving rise to procyanidin- and prodelphinidin- type polymers, respectively. Note the extra hydroxyl group in the B-ring of gallocatechin.

**Figure 2 – Oligomeric proanthocyanidins induce CD86 expression in human dendritic cells**

**A)** Induction of CD80, CD86 and MHC-II expression in dendritic cells (DCs) after 24 hours incubation with COC F2 (10 µg/mL) or WCF F2 (20 µg/mL). Shown are representative FACS plots from five different experiments, each performed with cells from different donors, and comparison of expression of each DC surface molecule to that induced by LPS, which was set at 100%. Data are shown as mean ± S.E.M. \**P*<0.05; \*\* *P*<0.01 by one-way ANOVA when compared to medium alone. Grey shaded area represents isotype control.

**B)** Percentage ofCX3CR1 positive cells in unstimulated DCs or DCs incubated for 24 hours with COC F2 (10 µg/mL) or WCF F2 (20 µg/mL). Experiments were performed three times with cells from different donors, and shown is the mean and inter-donor S.E.M.

**C)** CD86 expression (mean fluorescence intensities) in DCs treated for 24 hours with WC F2 (20 µg/mL) and gated as being either CX3CR1+ or CX3CR1-. Similar results were obtained with COC F2 (10 µg/mL). The experiments were performed twice with cells from different donors, with similar results.

**D)** Induction of CD86 expression in DCs incubated for 24 hours with either LPS, 10 µg/mL COC F1, F2 or F2 pre-incubated with PVPP, or 20 µg/mL WCF F1, F2 or F2 pre-incubated with PVPP. CD86 expression was assessed by mean fluorescence intensity relative to LPS alone, which was set at 100%. Experiments were performed three times with cells from different donors, and shown is the mean and inter-donor S.E.M. \**P*<0.05; \*\* *P*<0.01 by one-way ANOVA when compared to medium alone.

**Figure 3 – Oligomeric proanthocyanidins modulate cytokine secretion in LPS-activated dendritic cells and inhibit peripheral lymphocyte proliferation**

**A)** Suppression of LPS-induced cytokine secretion in dendritic cells (DCs) by addition of 10 µg/mL COC F2 or 20 µg/mL WCF F2. Cytokine secretion in proanthocyanidin (PAC)-treated cells is expressed as a percentage of the LPS-induced secretion for each cytokine, where 100% corresponds to 32 ± 3.1 ng/mL for TNFα, 16 ± 4.4 ng/mL for IL-6, 3 ± 0.5 ng/mL for IL-12p70 and 3 ± 1.9 ng/mL for IL-10. The experiments have been performed at least five times with cells from different donors. Shown is the mean with inter-donor S.E.M. \**P*<0.05; \*\*\**P*<0.001 by one-way ANOVA compared to LPS only.

**B)** Suppression of LPS-induced cytokine secretion in DCs by addition of either COC or WCF F2, COC or WCF F1, or COC or WCF F2 pre-incubated with polyvinylpolypyrrolidone. 10 µg/mL PAC was used for COC fractions and 20 µg/mL PAC was used for WCF fractions. Cytokine secretion in PAC-treated cells is expressed as a percentage of the TLR-induced secretion for each cytokine as described above. The experiments have been performed at least three times with cells from different donors. Shown is the mean with inter-donor S.E.M. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001 by one-way ANOVA compared to LPS only.

**Figure 4 – Endocytosis of proanthocyanidins by dendritic cells**

**A)** Dendritic cells (DCs) were incubated for one hour with either DTAF-tagged proanthocyanidins (PAC) or equivalent PAC lacking the DTAF tag (‘Control PAC’). DAPI staining was used to visualize nuclei. Representative results from five different experiments with cells from different donors.

**B)** DCs were incubated with DTAF-tagged PAC at either 4°C or 37°C and stained with DAPI to visualize nuclei and Alexa-594 conjugated anti-CD31 to visualize DC surface. Representative results from three different experiments with cells from different donors.

**C)** DCs were incubated with DTAF-tagged PAC at 37°C for 2 hours and stained with DAPI to visualize nuclei and Alexa-594 conjugated anti-LAMP2 to visualize lysosomes. Representative results from three different experiments with cells from different donors. For all images scale bar indicates 5 µm.

**Figure 5 – Proanthocyanidins do not inhibit binding of LPS to TLR4 and do not bind 67LR or CD11b on dendritic cells**

**A)** TLR4 reporter cells were incubated with 20 µg/mL of either COC F2 or WCF F2 for 24 hours, and stimulated with 10 ng/mL LPS or vehicle control. TLR4 activation was assessed by measuring the production of IL-8. Results are the mean ± S.E.M. of three independent experiments.

**B)** Dendritic cells were incubated with anti-67LR, anti-CD11b or isotype controls (IC) for 15 minutes prior to the addition of COC F2 or WCF F2 and then LPS. IL-6 and IL-12p70 secretion was quantified after 24 hours. Cytokine secretion in proanthocyanidin-treated cells incubated with either isotype controls or anti-67LR or CD11b is expressed as a percentage of the secretion induced by LPS alone + isotype control, or LPS alone + anti67LR or CD11b. Mean secretion in isotype-control treated cells activated with LPS alone was 44 ± 8.6 ng/mL for IL-6 and 4 ± 1.7 ng/mL for IL-12p70. Experiments were performed three times with cells from different donors, and presented as mean ± inter-donor S.E.M. \**P*<0.05; \*\**P*<0.001 as assessed by one-way ANOVA.

**Figure 6 – Proanthocyanidin-exposed dendritic cells do not reduce lymphocyte proliferation but inhibit IFN-γ production in CD4+ T-cells**

**A)** Representative data plot from one of three experiments showing allogenic lymphocyte proliferation following stimulation with dendritic cells (DCs) matured with LPS or LPS + 20 µg/mL WCF F2.

**B)** Representative data plot from one of four experiments showing IL-4 (red, vertical axis) and IFN-γ (black, horizontal axis) production in CD4+ T-cells. Allogenic T-cells were activated by DCs previously incubated for 48 hours with either LPS or LPS + 20 µg/mL WCF F2. T-cells were cultured for 12 days and then stimulated for five hours with ionomycin and phorbol-12-myristate 13-acetate to measure cytokine production. Unstimulated cells are shown as a control.

**C)** Ratio of IL-4 positive cells to IFNγ positive cells in CD4+ T-cells activated by DCs previously incubated with LPS or LPS + WCF F2 as described above. Results are mean ± S.E.M of four independent experiments with DCs from different donors. \*\**P*<0.01 by paired t-test.

**Figure 7 – Proanthocyanidins and soluble products from *Trichuris suis* co-operatively inhibit inflammatory cytokine secretion and induce OX40L expression in LPS-activated dendritic cells**

**A)** Suppression of LPS-induced cytokine secretion in dendritic cells (DCs) by addition of 40 µg/mL *Trichuris suis*soluble products (TsSP) or TsSP combined with 10 µg/mL COC F2 or 20 µg/mL WCF F2. Cytokine secretion in treated cells is expressed as a percentage of the LPS-induced secretion for each cytokine, where 100% corresponds to 36 ± 4.2 ng/mL for TNFα, 25 ± 3.9 ng/mL for IL-6, 5 ± 0.7 ng/mL for IL-12p70 and 2 ± 1.2 ng/mL for IL-10. The experiments have been performed at least four times with cells from different donors. Shown is the mean with inter-donor S.E.M. \**P*<0.05; \*\*\**P*<0.001 by one-way ANOVA compared to TsSP only.

**B)** Representative FACS plots from one of four independent experiments showing OX40+ DCs after 24 hours incubation with media only, LPS, or LPS combined with either TsSP, COC F2 or WCF F2, and also TsSP + either COC F2 or WCF F2.

**C)** Percentages of OX40+ DCs after 24 hours incubation with LPS only or LPS combined with either TsSP, COC F2 or WCF F2. Experiments were performed four times with cells from different donors. Shown is the mean ± inter-donor S.E.M. \**P*<0.05, \*\**P*<0.01 by one-way ANOVA compared to LPS only.

**D)** Percentages of OX40+ DCs after 24 hours incubation with LPS combined with either TsSP, or TsSP + COC F2 or WCF F2. Experiments were performed four times with cells from different donors. Shown is the mean ± inter-donor S.E.M. \*\**P*<0.001 by one-way ANOVA compared to TsSP only.

**Figure 8 - IFN-γ production in CD4+ T-cells activated with dendritic cells exposed to *Trichuris suis* soluble products and/or proanthocyanidins**

**A)** Representative data plot from one of four experiments showing IL-4 (red, vertical axis) and IFN-γ (black, horizontal axis) production in CD4+ T-cells. Allogenic T-cells were activated by dendritic cells (DCs) previously incubated for 48 hours with either LPS, LPS + 40 µg/mL TsSP or LPS + 20 µg/mL WCF F2/40 µg/mL TsSP. T-cells were cultured for 12 days and then stimulated for five hours with ionomycin and phorbol-12-myristate 13-acetate to measure cytokine production.

**B)** Ratio of IL-4 positive cells to IFNγ positive cells in CD4+ T-cells activated by DCs previously incubated with LPS, LPS + WCF F2, LPS + TsSP or LPS + WCF F2/TsSP as described above. Results are mean ± S.E.M of four independent experiments with DCs from different donors.

**Table 1.** Chemical analysis of fractionated proanthocyanidins from two different plant sources. PC – procyanidin, PD – prodelphinidin , mDP – mean degree of polymerization. % purity – g PAC / 100 g fraction. COC – Cocoa beans, WCF – white clover flowers. Full details of the analyses are described elsewhere [29](#_ENREF_29), [30](#_ENREF_30).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Sample |  | % purity | mDP | % PC | % PD |
| COC | F1 | 58.5 | 2.3 | 100 | 0 |
|  | F2 | 75.5 | 5.4 | 100 | 0 |
| WCF | F1 | 11.8 | 1.8 | 1.8 | 98.2 |
|  | F2 | 100 | 8.6 | 1.3 | 98.7 |

Figure 1:

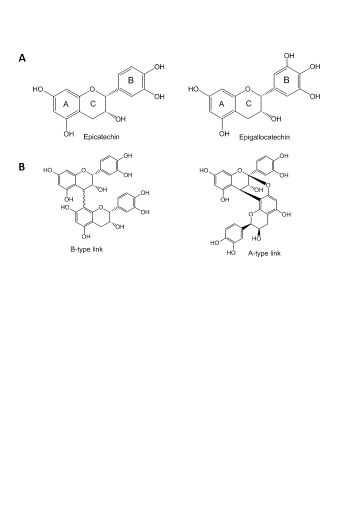


Figure 2

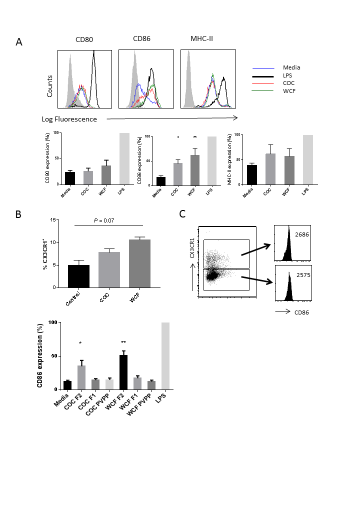


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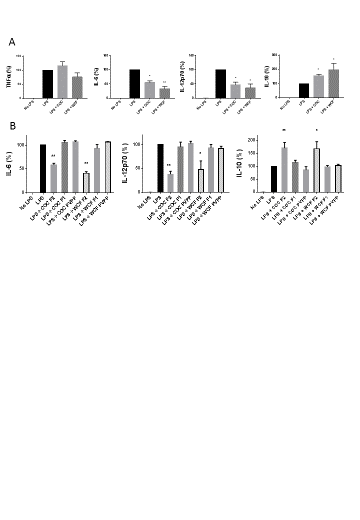


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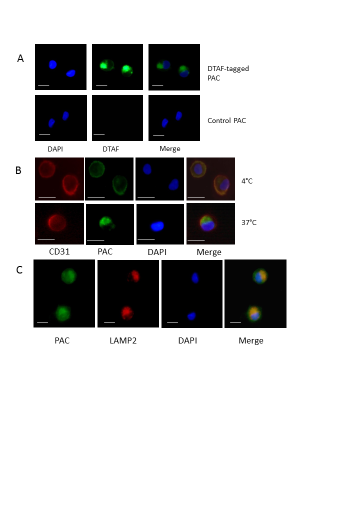


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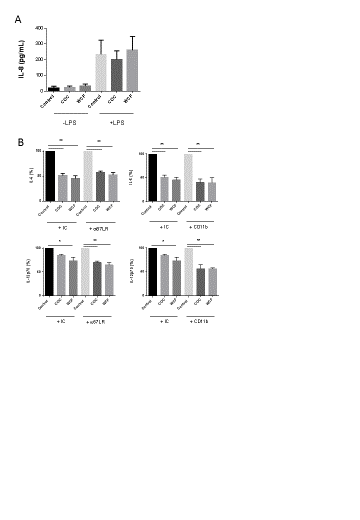


Figure 6:



Figure 7:

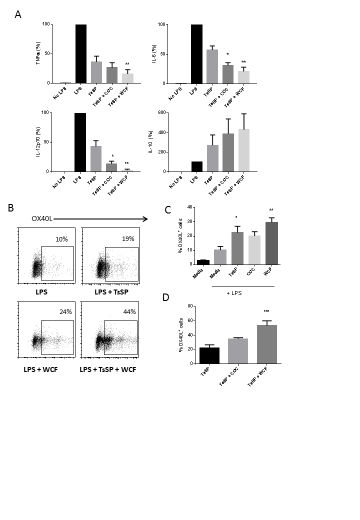


Figure 8:

