Structure-function analysis of purified proanthocyanidins reveals a role for polymer size in suppressing inflammatory responses

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Abstract

Proanthocyanidins (PAC) are dietary compounds that have been extensively studied for beneficial health effects due to their anti-inflammatory properties. However, the structure-function relationships of PAC and their mode-of-action remain obscure. Here, we isolated a wide range of diverse PAC polymer mixtures of unprecedented purity from plant material. Polymer size was a key factor in determining the ability of PAC to regulate inflammatory cytokine responses in murine macrophages. PAC polymers with a medium (9.1) mean degree of polymerization (mDP) induced substantial transcriptomic changes, whereas PAC with either low (2.6) or high (12.3) mDP were significantly less active. Short-term oral treatment of mice with PAC modulated gene pathways connected to nutrient metabolism and inflammation in ileal tissue in a polymerization-dependent manner. Mechanistically, the bioactive PAC polymers modulated autophagic flux and directly inhibited lipopolysaccharide-induced autophagy. Collectively, our results highlight the importance of defined structural features in the health-promoting effects of PAC-rich foods.

Keywords

Proanthocyanidins; V-ATPase; Autophagy; Inflammation; polymerization; macrophage

Introduction

There is currently intense interest in elucidating how dietary components may influence health and disease, in part due to increasing prevalence of chronic inflammation in humans throughout the world^{1,2}. Plant bioactive components found in the diet that may modulate inflammation include carotenoids, plant sterols, glucosinolates, and polyphenols³. Polyphenols represent one of the major groups of natural antioxidants with more than 8000 polyphenolic compounds identified in various plant species⁴. They are specialized plant metabolites and are commonly found in the human diet, especially in fruits and vegetables^{5,6}. Polyphenols act as the active component in many commercialized plant preparations, and have been associated with beneficial effects on health^{3,6}. Epidemiological and experimental studies have thus investigated how polyphenols may have protective properties against cardiovascular diseases, cancer, diabetes, neurodegenerative disease and microbial infections, among other diseases^{4,7,8}.

Proanthocyanidins (PAC), also known as condensed tannins, are among the most common dietary polyphenols⁹. They are characterized by their high molecular weight, and consist of oligomers and polymers of flavan-3-ol monomeric sub-units^{6,10}. Flavan-3-ols are formed by two phenyl rings, A and B, and one heterocyclic ring C, with different hydroxylation patterns of rings A and B^{11,12}. They may also display gallic acid residues attached by an ester bond to the C-ring hydroxyl, as seen in catechin gallates¹³. The four most common flavan-3-ols are (+)-catechin and its *cis* isomer (-)-epicatechin, and (+)-gallocatechin and its *cis* isomer epigallocatechin¹⁰, which can be joined by inter-flavanol bonds to form oligomers or polymers. When part of an oligomeric or polymeric PAC structure, (epi)catechins are known as procyanidin (PC) units and (epi)gallocatechins are known as prodelphinidin (PD) units (**Figure 1A**). Depending on the nature of their linked flavan-3-ol subunits, PAC are thus referred to as either procyanidin (PC)-type PAC, which are the most common type of

77 PAC, prodelphinidin (PD)-type PAC, or as PC/PD mixtures^{10,12}. PAC are further characterized based
 78 on their galloylation and polymerization. The percentage of galloylation describes the proportion of
 79 galloylated monomers, while the number of monomeric sub-units dictates the degree of
 80 polymerization¹³. The polymer chain lengths of PAC molecules vary greatly depending on the plant
 81 sources and thus, mean degree of polymerization (mDP) is a commonly used measure for the average
 82 number of monomeric sub-units.

83

84 Inflammation is a natural response to harmful stimuli, and results in the secretion of several cell-
 85 derived mediators, including cytokines, prostaglandins and reactive oxygen species (ROS) to protect
 86 cells and tissues. The detrimental effects of inflammation arise when it perseveres and causes
 87 permanent damage of the tissues due to homeostatic imbalance of the immune regulatory functions
 88 ⁵. Numerous studies have shown that PAC can have beneficial effects on inflammatory and metabolic
 89 disorders, such as obesity and inflammatory bowel disease^{14,15}. Furthermore, epidemiological studies
 90 recorded lower incidences of cancer, atherosclerosis and metabolic syndrome in human populations
 91 consuming procyanidin-rich foods, although conclusive evidence is still lacking to support beneficial
 92 effects on human health^{8,16}. The mechanisms underlying the anti-inflammatory effects of PAC are
 93 unclear, but are speculated to include direct interactions between PAC molecules and immune cell
 94 functions and pathways, as well as a potential prebiotic effect on the gut microbiota. There is a general
 95 consensus that PAC molecules remain stable in the stomach, and that only monomers are absorbed
 96 in the small intestine^{17,18}. In this way, polymeric PAC remain at the lumen-mucosal interface where
 97 they may directly interact with epithelial or immune cells. Several studies have shown that PAC can
 98 suppress several inflammatory markers in macrophages and intestinal epithelial cells *in vitro*, such as
 99 ROS and tumor necrosis factor- α (TNF- α), as well as the expression of inducible nitric oxide synthase
 100 (iNOS) and cyclooxygenase-2 proteins, and thereby suppressing nitric oxide (NO) and prostaglandin

101 E₂.. This may be due to the downregulation of the p38 mitogen-activated protein kinase (MAPK) and
 102 Akt signaling pathways, leading to the inhibition of the transcription factor NF-κB, which is important
 103 for the regulation of genes involved with inflammation^{19–22}. The direct effects of PAC on other cell
 104 lines, including lymphoid cell lines and peripheral blood mononuclear cells, has also been
 105 demonstrated by their ability to down-regulate transcription and secretion of pro-inflammatory
 106 cytokines and to up-regulate the secretion of anti-inflammatory cytokines^{12,20,23,24}.

107

108 Despite these documented effects of PAC on inflammatory activity, mechanistic and structure-
 109 function activity studies are limited. Investigation of the bioactivity of PAC is challenging due to the
 110 complexity of the PAC molecules and associated difficulties with characterization and purification.
 111 The isolation of PAC with high polymerization remains a challenge due to the difficulties related to
 112 separating polymeric molecules of different sizes from each other. Thus, structure-function
 113 relationship studies with PAC have been limited to relatively crude fractions consisting of mixtures
 114 of low or high mDP. Studies utilizing such fractions have suggested that the degree of polymerization
 115 of PAC may be an important parameter for the outcome observed *in vitro* and *in vivo*, with higher
 116 mDP often correlating with higher biological activity²⁰. For example, γδ T-cells are more effectively
 117 activated by PAC fractions with an mDP higher than six²⁵, and inhibition of lipopolysaccharide
 118 (LPS)-induced cytokine production in macrophages also appears to correlate with increasing mDP²⁰.
 119 In addition, the PC/PD ratio is thought to play a role for the activity of PAC, potentially due to the
 120 ability of PD-type polymers to form greater numbers of hydrogen bonds with proteins because they
 121 have more hydroxyl groups than PCs²⁶.

122

123 Recently, we developed a new method that splits the characteristic PAC polymeric mixtures into
 124 precise fractions by Sephadex LH-20 fractionation followed by semi-preparative liquid

chromatography (LC) ²⁷. This resulted in chemically well-characterized PAC samples with higher purity, differing mDPs and PC/PD ratios, and thus allowed a better comprehension of the relationship between the structure and bioactivity of PAC. Here, we investigated how highly purified PAC with differing structural characteristics may affect inflammatory responses. We utilized interleukin 6 (IL-6) production from LPS-activated RAW 264.7 macrophages as a screening tool to assess structure-function relationships of PAC isolated from two plant sources in unprecedented detail. Combined with *in vitro* and *in vivo* transcriptomics after exposure to different PAC polymers, we identified novel relationships between PAC structure and bioactivity that may have broad implications for the development of novel, PAC-based dietary supplements.

Results

Purification of PAC and analysis of structural features

As a first step in elucidating the structural characteristics that govern the bioactivity of PAC, we selected two plant sources to serve as source material for isolation of purified fractions. In order to assess the relative importance of PC or PD units, we selected plants that contain either PC rich PC/PD mixtures (hereafter PC-type PAC) or PD rich PC/PD mixture (hereafter PD-type PAC). Grape (*Vitis vinifera*) pomace, hereafter GP, is a by-product of wine production that is rich in PC-type PAC. In contrast, alpine currant (*Ribes alpinum*), hereafter AC, is a temperate herb found in Northern Europe that primarily contains PD-type PAC. Thus, material from these two distinct plant sources allowed us to derive pure PAC samples mainly consisting of either PC- or PD-type polymers, which we further sought to fractionate into samples of a precisely defined mDP. PAC were extracted with acetone/water (80/20, v/v) and fractionated by Sephadex LH-20 gel chromatography. Semi-preparative LC was subsequently used to derive 24 and 64 highly-purified samples from AC and GP, respectively²⁷. UPLC-MS/MS analysis of each of the isolated PAC samples was used to assess structural features²⁸. The samples derived from AC were 81-99% PD-type PAC with mDP values ranging between 3.2 and 12.1. In contrast, samples derived from GP were 78-96% PC-type PAC with mDP values ranging between 2.4 and 13.2 (**Figure 1B and C**). Thus, our technique successfully isolated highly purified PAC covering a wide range of mDP.

Reduction of IL-6 secretion in LPS-activated macrophages stimulated with PAC

To assess the relative ability of the isolated PAC to modulate inflammatory responses, murine RAW 264.7 macrophages were stimulated with LPS alone, or LPS combined with purified PAC derived from GP or AC. Samples were initially screened at concentrations of 3-50 µg/ml that resulted in no toxicity to the cells (data not shown), and was comparable to previous studies, which have demonstrated anti-inflammatory activity of PAC-rich extracts in cells at similar or higher

concentrations^{19,21}. PAC samples (15 µg/mL) derived by semi-preparative liquid chromatography significantly reduced LPS-induced IL-6 secretion. For GP, samples with low mDP (<4) inhibited IL-6 secretion inefficiently, with around 30% inhibition. Greater inhibition (up to 60%) was observed in samples with higher mDP, with a consistently high inhibition evident in a group of samples with mDPs between 6-10 (**Figure 2A**). However, increasing mDP above 10 did not enhance activity but resulted in a plateauing of the positive correlation between mDP and IL-6 inhibition. Indeed, the samples with the highest mDP had lower activity than those with an mDP in the 6-10 range (**Figure 2B**). As the different samples were tested at equal w/v amounts, the results suggest that the mDP was an important contributor to the bioactivity. Results obtained from samples with differing mDP tested at equimolar concentrations of 7.8 µM (**Figure S1A**), showed similar results, with medium mDP samples being most effective, confirming that the polymerization determined the activity. Overall, these results demonstrate that increasing the mDP of GP PAC enhanced anti-inflammatory activity up to a threshold, where after mDP no longer was associated with high activity.

Evaluation of the AC samples, comprised almost exclusively of prodelphinidin-type PAC, revealed a generally lower suppression of IL-6 secretion than that achieved by GP PAC across a similar range of mDP (**Figure 2C**). Moreover, the relationship between mDP and inhibition of IL-6 secretion was also less apparent, although again the most active samples tended to fall in the middle of the mDP range of around 7-10 (**Figure 2D**). This was also true for AC samples with differing mDP tested at equimolar concentrations of 7.8 µM (**Figure S1B**).

Thus, these data demonstrate a clear relationship between mDP and anti-inflammatory activity, with a positive correlation that appears to plateau at high mDP.

184 Grape pomace PAC modulate macrophage transcriptional responses in a polymerization- 185 dependent manner

186 To investigate the mechanistic basis by which PAC regulated macrophage function, three samples
187 derived from GP by semi-preparative LC were selected for transcriptomic experiments. Cells were
188 stimulated with samples with mDP 2.6 (low), 9.1 (medium) and 12.3 (high), respectively, and global
189 transcriptomic profiles were determined 6 hours later. Principal component analysis demonstrated a
190 strong influence of LPS (**Figure 3A**). Moreover, a clear effect of PAC was observed that correlated
191 closely with mDP. Consistent with the IL-6 inhibition data, the largest transcriptional changes were
192 observed in cells exposed to medium mDP PAC, whereas PAC with high or low mDP were less
193 effective at modulating the cellular responses both in resting cells and those activated with LPS
194 (**Figure 3**). Low mDP PAC were particularly ineffective at inducing transcriptional changes,
195 indicating that an $mDP \geq 3$ is necessary for optimal regulation of inflammatory activity. Moreover,
196 cells stimulated with the high mDP PAC were also not as differentiated from their respective controls
197 as the cells stimulated with medium mDP PAC, indicating that increasing mDP beyond a certain
198 threshold appears to decrease bioactivity.

199 *Transcriptional responses in resting macrophages*

200 In resting cells (no LPS), high mDP PAC treatment induced the expression of genes involved with
201 inflammatory responses, such as cytokines from the interleukin 1 family (e.g. *Il1a*, and *Il1b*), as well
202 as a number of inflammatory chemokines (*Cxcl2*, *Ccl22*, *Ccl3*) (**Figure 3B**). This was supported by
203 pathway analysis suggesting that the interferon-signaling and inflammatory pathways were
204 upregulated. Similarly, resting cells stimulated with medium mDP PAC also showed an upregulation
205 of chemokines (*Cxcl2*, *Cxcr4*) and interleukins. Consistent with this, the most upregulated pathway
206 in resting cells stimulated with medium or high mDP PAC was the Erythrocyte Differentiation
207 Pathway, which includes genes encoding colony stimulating factor (*Csf2* and *Csf3*), interleukins

(*Il1a*, *Il3*, *Il6*, *Il9*, and *Il11*) and transforming growth factors (*Tgfb1*, *Tgfb2*, *Tgfb3*). Furthermore, both samples decreased the REACTOME cholesterol biosynthesis pathway (**Figure 4A and 4B**), and upregulated the activating transcription factor 3 (*Atf3*). In contrast, treatment with low mDP PAC induced minimal transcriptional changes. Thus, in the absence of inflammatory stimuli, PAC with a medium-high mDP induced semi-maturation of resting macrophages, whereas PAC with low mDP lacked this stimulatory capacity.

Transcriptional responses in lipopolysaccharide-activated macrophages

To explore the mechanisms underlying the anti-inflammatory activity during LPS activation, we next investigated transcriptional responses in LPS-stimulated cells. Medium mDP PAC had the most prominent impact on gene expression levels and the number of significantly regulated gene pathways, compared to low and high mDP PAC (**Figure 3C**). However, investigation of the top 15 regulated genes in LPS-activated macrophages showed that 25 % of the genes were regulated by at least two of the three samples (**Figure 3D**). Despite the activation of some inflammatory pathways by PAC in resting cells, in LPS-stimulated cells we observed a strong suppression of multiple inflammatory pathways including JAK-STAT signaling, which was significantly downregulated in LPS-activated cells stimulated with medium mDP PAC (**Figure 4C**).

Another characteristic of the medium mDP PAC was its ability to upregulate pathways connected to lysosome function and transferrin endocytosis. The transferrin endocytosis pathway was upregulated regardless of LPS-activation. Genes in this pathway encode for numerous vacuolar-type H⁺ATPases (V-ATPases) responsible for the acidification of phagolysosomes, allowing for endosome trafficking and neutralization of microorganisms²⁹⁻³¹. Most of the upregulated V-ATPases belonged to the V1 Domain responsible for ATP hydrolysis, whereas fewer genes belonged to the V0 domain responsible for proton translocation. Of note, *Atp6v0d2* was the most upregulated V-ATPase

gene in LPS-activated macrophages stimulated with low, medium, and high mDP PAC, and the expression level was also significantly increased in resting macrophages stimulated with medium mDP PAC (**Figure 3B and 3C**).

Both medium and high mDP PAC significantly downregulated the cytokine inducible SH2-containing protein (*Cish*), which negatively regulates cytokine signaling of the JAK-STAT pathway^{32,33}, and colony stimulating factor 2 (*Csf2*), which regulates function and differentiation of macrophages. Interestingly, all three samples significantly upregulated *Slamf8*, which has shown to negatively regulate inflammatory response, including *Nox2* activity in macrophages³⁴.

Collectively, these data show that the anti-inflammatory profile induced by all three samples was qualitatively similar, with a suppression of genes encoding inflammatory cytokine and signaling-related genes, and an up-regulation of genes involved in lysosome function. However, mDP had a substantial impact, with low mDP PAC being ineffective at regulating transcription, and medium mDP PAC clearly being the most efficient at regulating LPS-induced changes in gene expression.

Grape proanthocyanidins directly induce transcriptional changes *in vivo* in a polymerization-dependent manner

Having established that polymerization was an important factor for direct modulation of cellular responses by PAC, we next assessed whether *in vivo* administration of purified grape-derived PAC Sephadex fractions of differing mDP would also bring about direct transcriptional changes. To this end, mice were dosed repeatedly over a short time-period of ten days to assess responses in the intestine by RNA-sequencing. In mice dosed with low mDP PAC, only 2 significantly altered genes were identified in ileal tissue compared to the control group. In contrast, a total of 27 genes were significantly modulated in tissue from mice dosed with medium mDP PAC that had been shown to be highly active *in vitro* (**Figure 5A**). Principal component analysis also demonstrated a clear effect

of medium mDP PAC, whereas low mDP PAC were not substantially differentiated from controls (Figure 5B). Notably, in both groups, the most upregulated gene was *S100g*, encoding the S100 calcium binding protein G, which transports Ca^{2+} from the apical to the basolateral membrane of the enterocytes and sustains the essential intracellular concentration of calcium³⁵. Other notable genes upregulated by medium mDP PAC included *Rspo1*, encoding R-spondin-1, a growth factor-like protein that induces crypt cell proliferation and promotes intestinal epithelial healing³⁶, and which has shown to be protective against intestinal damage and colitis. Downregulated genes were largely related to both inflammation and lipid metabolism, consistent with reports that acute PAC intake modulates bile acid metabolism and lipid homeostasis³⁷. Notable genes included the Myd88-responsive anti-microbial peptide gene *Reg3g*, as well as the reactive free radical nitric oxide 2 (*Nos2*) and *Gata4*, a gene known to be involved in bile acid homeostasis³⁸. This was supported by gene-set enrichment analysis which demonstrated that gene pathways including lipid metabolism and pro-inflammatory pathways (JAK-STAT signaling) were significantly suppressed (Figure 5C; Supplementary File 1). qPCR analysis confirmed the regulation of these genes in mice dosed with medium mDP PAC, whereas responses in mice dosed with low mDP PAC were generally less differentiated from control mice (Figure S2). Thus, direct changes in immunological and metabolic-related transcription can be brought about *in vivo* by orally delivered PAC with a defined mDP profile.

Bafilomycin A1 antagonizes the effect of grape proanthocyanidins *in vitro*

We next sought to establish the mechanistic basis of the activity of the most active PAC sample with medium mDP. We noted that our *in vitro* transcriptomic data showed a suppression of TLR signaling pathways and an up-regulation of genes involved in V-ATPase activity and lysosome function. Therefore, we first assessed the expression of *Tlr4*, *Rab7b* and *Atp6v0d2* in both resting and LPS-activated macrophages by qPCR. Interestingly, we saw a significant increase in the expression of

277 *Rab7b* in resting cells after adding GP PAC, but not in LPS-activated cells. In contrast, *Atp6v0d2*
 278 expression was upregulated in both resting and LPS-treated cells, whilst, the expression of *Tlr4* was
 279 attenuated by PAC compared to controls (**Figure 6A**). However, we did not observe a decrease in
 280 either surface or intracellular expression of TLR4 protein following PAC exposure (data not shown).
 281 Microscopically, we also observed large numbers of structures resembling autophagosomes in PAC-
 282 stimulated cells (**Figure 6B**). This may suggest either a marked increase in phagosome formation, or
 283 an accumulation of autophagosomes due to altered lysosomal flux and impaired formation of
 284 autophagolysosomes. Together with the increased expression of *Atp6v0d2*, this prompted us to
 285 examine the role of V-ATPase activity in the observed effects of PAC. We co-stimulated cells with
 286 PAC and Bafilomycin A1 (Baf), a macrolide antibiotic isolated from *Streptomyces gresius*, and a
 287 potent V-ATPase inhibitor, to explore whether inhibiting V-ATPase activity would reverse the effects
 288 of PAC. Notably, we observed that co-stimulation with Baf prevented the PAC-induced accumulation
 289 of the autophagosome-like structures in the cells, suggesting that PAC may inhibit inflammatory
 290 responses by modulating V-ATPase activity (**Figure 6B**).

291 Interestingly, we observed that Baf treatment alone inhibited IL-6 secretion (**Figure 6C**) and also
 292 downregulated *Tlr4* expression in LPS-activated cells (**Figure 6D**). Surprisingly, whilst Baf is known
 293 to inhibit the activity of the V-ATPase, expression of *Atp6v0d2* was markedly increased in Baf-treated
 294 cells, similar to that observed with PAC. Thus, Baf and PAC in isolation each induced similar changes
 295 in cellular activity, suggesting that they may act through similar mechanisms of action, and that PAC
 296 may therefore function as a V-ATPase inhibitor. However, consistent with the observed inhibitory
 297 effect of Baf on PAC-induced autophagosome accumulation, during co-incubation we observed that
 298 Baf tended to antagonize the effect of PAC. Thus, PAC-induced changes in the expression of *Tlr4*
 299 and *Atp6v0d2* were attenuated by co-stimulation with Baf (**Figure 6D**). Collectively, these data show

that PAC induces transcriptional and biochemical changes indicative of a modulatory role in V-ATPase, and hence phagosomal, activity.

Proanthocyanidins suppress lipopolysaccharide-induced autophagy

Given the putative association between GP PAC and V-ATPase activity, we next asked whether GP PAC would regulate autophagy, as Baf is a well-known autophagy inhibitor and other natural compounds with anti-inflammatory activity have also been shown to suppress autophagy in macrophages³⁹. We found that GP PAC significantly suppressed autophagy in LPS-activated cells, to a similar extent as that seen in Baf-treated cells (**Figure 6E**). These observations were further validated using mLC3 autophagy-reporter cells. LPS-activated cells showed the characteristic appearance of autophagy with a marked abundance of red punctate spots, indicating increased autophagolysosome formation. In contrast, LPS-activated cells co-stimulated with either PAC or Baf showed punctate spots and co-localization of green and red fluorescence, indicating accumulation of autophagosomes and inhibition of the full autophagy pathway (**Figure 6F**). Overall, these data suggest a novel role for PAC in modulating inflammatory activity in macrophages by suppressing LPS-induced autophagy, in a manner analogous to the V-ATPase inhibitor Baf.

Discussion

The beneficial effects of PAC are numerous, and especially their ability to suppress inflammatory markers has been demonstrated in several mouse models and *in vitro* studies. Direct interaction with immune cells, immune cell-derived mediators, and gut microbiota may cause the prevention and alleviation of gut-related inflammation⁴⁰. Thus, the anti-inflammatory properties of PAC have been attributed to their interaction with protein receptors and transcription factors of the NF- κ B and MAPK signaling pathways, and by modulating the arachidonic acid pathway and inhibiting enzymatic activity of eicosanoid generating enzymes^{12,20,41,42}. Despite the large body of research investigating the bioactivities of PAC, their mechanism of action is still not fully understood but their anti-oxidant and anti-inflammatory activities are believed to be dependent to their varying chemical structures⁵. Thus, the bioavailability, stability and activity of polyphenolic compounds may be correlated with the amount of hydroxyl groups, mean polymerization, and/or bond type and position between the monomers^{26,43–46}. Our aim was thus to investigate how differing structural characteristics of PAC would affect bioactivity, and to explore which cellular mechanisms lead to this activity.

We initially observed a pronounced suppression of IL-6 secretion in LPS-activated murine macrophages stimulated with Sephadex fractions (data not shown) and PAC samples derived from semi-preparative LC. The higher potency of samples derived from semi-preparative LC versus Sephadex LH-20, suggests that Sephadex LH-20 separation followed by semi-preparative LC is a powerful tool to isolate highly purified bioactive PAC with differing mDP. Also, our data showed that the PC-rich GP samples appeared to be more efficient at suppressing inflammatory responses in RAW 264.7 macrophages compared to PD-type AC PAC. Moreover, particularly in PC-type PAC, a non-linear relationship exists between mDP and anti-inflammatory activity, with the most potent inhibition of inflammatory cytokine secretion found in polymers with a medium (7-10) mDP. From

346 a chemical point of view, it is worth considering the stability of compounds in i.e. cell cultures where
347 PD-type PAC might be prone to oxidation, which could explain the lack in bioactivity.

348 The stimulation of macrophages with GP PAC upregulated the expression of genes related to
349 inflammatory responses, such as *Atf3*, which suggests that PAC are recognized by and have some
350 activating activity in resting macrophages. This is consistent with data showing that immature human
351 dendritic cells adopt a semi-mature state after exposure to PAC ⁴⁷. *Atf3* is induced by a variety of
352 stimuli, such as stress, and plays an important role in immune regulation, including downregulation
353 of TLR4, which consequently reduces the expression of several inflammatory genes, such as *Il6*,
354 *Il12b* and *Tnf* ^{48,49}. Whilst medium and high mDP PAC tended to induce transcription of similar
355 genes, the small number of genes that were induced by low mDP PAC were markedly different,
356 indicating a qualitative difference in the interaction of different sized PAC polymers with
357 macrophages.

358 Our data also suggests that GP PAC modulate macrophage transcriptional responses in a
359 polymerization-dependent manner. We demonstrated that PAC with differing mDPs modulate similar
360 genes but with varying impact on expression levels. This suggests, that there is no inherent difference
361 in the mechanism of how different size PAC affect the cell activity, but rather that the mDP may
362 influence the ability of the cells to process the PAC molecules. Thus, we here demonstrated that GP
363 PAC with an mDP of 9.1 had a more prominent impact on gene expression levels, especially in LPS-
364 activated macrophages, than samples with mDPs of 2.6 or 12.3. Overall, our data demonstrated strong
365 immunological properties of medium mDP PAC in presence of LPS in RAW 264.7 macrophages
366 with a downregulation of inflammatory mediators and an upregulation of anti-inflammatory
367 mediators. The decreased bioactivity seen in low mDP PAC could be caused by limited number of
368 structural units in PAC, thus resulting into e.g. lower affinity towards possible target proteins as
369 shown by Leppä et al. ⁵⁰. On the other hand, PAC with high mDP might exceed the saturation of

370 contact points with the cells, and complicate efficient structural interactions and/or endocytosis, and
371 thereby explain the reduced activity seen in cells stimulated with high mDP PAC.

372 What was also noteworthy was the prominent upregulation of JAK-STAT and inflammation
373 pathways in LPS-activated macrophages, which were consequently down-regulated by co-stimulation
374 with medium mDP PAC. Furthermore, *Slamf8*, which is a negative regulator of inflammatory
375 responses, was significantly upregulated by all three samples in LPS-activated macrophages. A
376 number of genes related to the suppression of inflammatory responses were thus regulated by PAC,
377 including *Cish* and *Csf2*. Interestingly, our data suggests that PAC seemed to elicit an inflammatory
378 response in resting cells by upregulating interleukins as well as chemokines, however, PAC acted as
379 an anti-inflammatory agent in LPS-activated cells.

380 GP Sephadex LH-20 fractions were tested *in vivo*, and the effects of medium mDP PAC on ileum
381 transcriptomics were substantial in a healthy mouse model compared to low mDP PAC. Here, we
382 gavaged mice every other day for 10 days with 200 mg/kg BW of highly purified GP Sephadex
383 fractions with either low or medium mDP PAC. A significant increase in the number of regulated
384 genes was observed in mice dosed with medium mDP PAC compared to mice dosed with low mDP
385 PAC. Notably, the downregulation of immune-related genes, such as *Asah2* and *Enpp7*, and the
386 upregulation of *Rspo1*, which promotes cell proliferation of crypt cells. Taken together, our *in vivo*
387 data suggests that dietary PAC, when comprised of medium mDP polymers, are able to regulate
388 cellular functions related to nutrient metabolism and immune function in the intestinal mucosa.

389 The question remains as to how and where PAC interacts with immune cells. There are some
390 controversies concerning whether or not larger PAC molecules can be taken up by macrophages like
391 monomers and dimers, or whether they mainly interact with the proteins and lipids on the cell
392 membrane^{51,52}. However, we have previously shown internalization of PAC with an mDP of 9.5 in

dendritic cells ⁴⁷. Based on our microarray data, we hypothesized that PAC may decrease TLR4 receptors on the cell surface due to increased degradation into RAB7B promoted lysosomes ⁵³. However, our hypothesis was not supported by flow cytometry investigations (data not shown). We observed that the KEGG Lysosome and transferrin pathway was significantly upregulated by medium mDP GP PAC, moreover the vacuolar gene *Atp6v0d2* reached high expression levels. *Atp6v0d2* plays a key role for lysosome acidification and autophagosome-lysosome fusion and thereby ensures the appropriate turnover of intracellular organelles and infective agents ⁵⁴. The observed upregulation of V-ATPase is in coherence with previous studies suggesting localization of PAC to lysosomes following endocytosis in human DCs ^{47,55}. The upregulation of the KEGG lysosomal pathway, and thereby genes encoding subunits of V-ATPase, in LPS-activated macrophages was also observed in DCs stimulated with PAC isolated from cocoa ⁵⁵. Inhibitors of V-ATPase have shown to increase NF-κB activation ⁵⁶, and thus a higher expression level of V-ATPase may explain the suppression of inflammatory markers observed in our study. Based on our findings with Baf added to LPS-activated cells stimulated with PAC, our data supports the notion that the intracellular activity of PAC is intimately connected to V-ATPase activity. The inhibitory effect of Baf on the expression of *Atp6v0d2* in LPS-activated cells stimulated with PAC, suggests that PAC has an important role in the lysosomal pathway. However, our mechanistic experiments investigating the role of PAC during autophagy, suggested a decrease in autophagy levels. This was also indicated in our studies with mLC3 cells, where we observed fewer autophagolysosomes in LPS-activated cells stimulated with PAC or Baf, compared to cells stimulated with LPS only. Taken together, these findings suggest that PAC in fact inhibits the autophagy pathway in terms of autophagolysosome formation, similarly to Baf. The enhanced expression of *Atp6v0d2* could however be explained by an accumulation of lysosomes or autophagosomes in the cytoplasm, in response to the inhibition of the autophagolysosome formation. Another explanation could be that an inhibition of the ATP6V0D2

protein by PAC and Bafilomycin in isolation, leads to a positive feedback-loop in the expression of the *Atp6v0d2* gene.

A large body of research suggests that a combination of phytochemicals lead to more pronounced beneficial health effects, rather than singled out molecules^{8,23}. Synergistic effects of selected PAC samples derived by semi-preparative LC were not investigated in this study, however, we clearly demonstrated a variation in cell stimulation between PAC samples derived from the same Sephadex fraction. These findings strongly suggest an impact of PAC structure on bioactivity, and supports the notion of how polymeric mixtures observed on chromatograms in fact depict numerous PAC molecules with distinctive chemical and biological properties.

Conclusion

We showed that pronounced transcriptional changes induced by PAC occur in a polymerization-dependent manner both *in vitro* and *in vivo*, which suggests that the chemical structure affects the bioactivity of PAC. Thus, there is an indication that medium and high mDP PAC modulate similar pathways but the level of gene expression is dependent on the mDP. Furthermore, we propose that PAC may have an important impact on V-ATPases, lysosome function and the autophagy pathway. Our results highlight the importance of considering structural features of PAC-rich food sources when examining their bioactivity in different models of inflammation. Future research should focus on the intracellular signaling pathways modulated by PAC, as well as how defined structural features may modulate the efficacy of PAC-based supplements as a novel tool for control of health and disease.

441 **Material and Methods**

442 **Samples and materials**

443 The samples and materials used for this study are depicted in **Table S1**

444 **Purification of Proanthocyanidins**

445 Extraction of PAC from plant material was performed in accordance with the protocol from Leppä et
 446 al.²⁷. In brief, we conducted a series of extraction with acetone, followed by Sephadex LH-20
 447 separation, and semi-preparative LC. For this study, the method was applied for the isolation of PAC
 448 from alpine currant (AC) and grape pomace (GP), and an overview of the purification steps is
 449 described in **Table S2**.

450 **Extraction**

451 The solid phases from AC and GP were stored at 4 °C in 80 % analytical acetone with shaking. The
 452 samples were filtered through a Whatman filter paper in a Büchner funnel, and the liquid phase was
 453 transferred to a container and placed for evaporation overnight (O/N). Following evaporation, the
 454 extracts were filtered again 1-3 times through a Whatman filter paper in a Büchner funnel and the
 455 water phase was stored at -20 °C.

456 The remaining plant material was immersed in 1 L of 80/20 acetone/water (v/v) and stored at 4°C
 457 with shaking O/N for further extractions. A total of five extractions from the plant materials were
 458 performed and each sample was analyzed by UPLC-MS/MS²⁸. The chromatograms of the GP
 459 samples depicted high similarities between the samples, which were thus pooled together. Likewise,
 460 the samples derived from AC were pooled and liquid-liquid extraction with 300 ml ethyl acetate
 461 followed by liquid-liquid extraction with 300 ml butanol was performed. Finally, the solvents were
 462 removed from the samples by rotary evaporation.

463 **Sephadex fractionation**

464 The samples were mixed with Sephadex LH-20 material by magnetic stirring O/N. Following O/N
465 mixing, the solution was poured into a Büchner funnel and all liquid material was filtered through a
466 Whatman filter paper. A series of filtrations through the Sephadex material was performed for each
467 sample, with 5 fractions of 200 ml milliQ water, 5 fractions of 200 ml methanol and 5 fractions of
468 200 ml of 80/20 acetone/water (v/v). A final fraction of 400 ml acetone was also collected to wash
469 the Sephadex material for eventual remaining compounds. In order to remove the solvents from the
470 samples prior to freeze-drying, O/N evaporation and rotary evaporation was applied to the samples
471 containing acetone and methanol, respectively. After the Sephadex LH-20 fractionation, a
472 comprehensive analytical characterization was performed by UPLC-MS/MS, and the samples were
473 subsequently freeze-dried and weighed. Eventually, the methanol and acetone fractions were
474 selectively pooled based on the similarity of their respective chromatograms. This resulted in 3
475 Sephadex AC fractions and 8 Sephadex GP fractions with weights varying between 400-1500 mg.

476 **Semi-preparative liquid chromatography**

477 The 11 Sephadex fractions were further purified by semi-preparative LC utilizing similar
478 fractionation procedure and instrumentation as described by Leppä et al.²⁷. In short, approximately
479 100-120 mg of each Sephadex fraction was fractionated via gradient elution utilizing 0,1/99,9 formic
480 acid/water (v/v) and acetonitrile as eluents. The gradient elution started with isocratic 5% acetonitrile
481 following a gradient from 8% to 55% acetonitrile at 4–32 mins and from 55% to 80% acetonitrile at
482 32–35 mins and lastly finishing with column wash (80% acetonitrile) and stabilization. The flow state
483 was set to 12 mL/min and the column used was a 150 × 21.20 mm, Gemini®10 µm,C-18, 110 Å,
484 Axia packed, Phenomenex. The samples were collected from 5.0 to 33.0 mins into 2 mL Eppendorf
485 tubes resulting in total of 168 individual tubes. The chromatographic PAC mixture (at 280 nm) was
486 integrated from 5 to 33 mins via TargetLynx software (V4.1 SCN876 SCN 917© 2012 Waters Inc)

and the total integrated area was divided into 8 equal parts (**Figure S3**). The 2 mL Eppendorf tubes were then pooled accordingly resulting in total of 8 semi-preparative samples per Sephadex fraction. All 88 semi-preparative samples (24 AC and 64 GP semi-preparative samples) were analyzed via UPLC-MS/MS²⁸ for their PC/PD composition and mDP. Semi-preparative samples were brought to water phase and freeze-dried. The weights of the samples derived by semi-preparative LC varied between 2-17 mg.

UPLC-MS/MS analyses

UPLC-MS/MS analyses were carried out with similar instrumentation and methodology as described by Engström et al.²⁸. The quantitation of the PC and PD units as well as the calculation of mDP were performed with the Engström method²⁸ as described by Malisch et al.⁵⁷. Similar standards were used for the quantitation of PC and PD as well as calibration curve for the calculation of the mDP as described by Leppä et al.²⁷. The stability of the mass analyzer response was monitored with frequent injections of 1 µg/mL catechin⁵⁷.

Cell culture

RAW 264.7 murine macrophages (ATCC TIB-71) and RAW-DifluoTM mLC3 cells (mLC3, InVivoGen) were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Low passage numbers (<20) were used for all experiments. When culturing mLC3 cells, the media was supplemented with Normocin (100 µg/ml), and Zeocin (200 µg/ml, every 2nd passage). For experimental purposes, neither Normocin or Zeocin was added to the media.

Cytotoxicity testing

Cytotoxicity of the isolated PAC used for the stimulation of murine macrophages was assessed using neutral red assays (Sigma-Aldrich) according to the manufacturer's instructions.

***In vitro* screening of anti-inflammatory activity of proanthocyanidins**

Macrophages were plated out on 96-well plates at a concentration of 2.5×10^5 cells/ml. Cells were allowed to adhere for 2 hours before LPS stimulation (500 ng/mL). Where appropriate, cells were pre-incubated with AC or GP PAC or vehicle control (PBS) 30 minutes prior to LPS addition. After 24 hours of incubation, the supernatant was collected and frozen at -20°C , and IL-6 secretion was assessed by ELISA (R&D Systems). Each semi-prep sample (15 $\mu\text{g/ml}$) was tested in triplicates, and at least two independent experiments were performed. For equimolar tests, the molarity of samples was calculated based on PC/PD ratios and mDP.

Stimulation with Bafilomycin A1

As described above, murine macrophages were plated out on 24-well plates at a concentration of 2.5×10^5 cells/ml. Cells were allowed to adhere for 2 hours before they were treated in the following order with Baf (10 nM), PAC (15 $\mu\text{g/ml}$), and LPS (500 ng/mL) with 30 min incubation time between each treatment. Stimulated cells and appropriate controls were left for incubation for 6 hours before RNA extraction.

Assessment of autophagy by flow cytometry

Autophagy in RAW 264.7 macrophages was assessed by measuring autophagic vacuoles with the Autophagy Assay Kit (Abcam ab139484) according to the manufacturer's protocol. In brief, cells were plated out in a 96-well plate and stimulated with either Baf (10 nM) or GP PAC (15 $\mu\text{g/ml}$) followed LPS stimulation (500 ng/mL) where appropriate. After 20h of incubation, the cells were trypsinized, washed by centrifugation and the pellet was re-suspended in indicator free cell culture medium containing 5% FBS. The cells were then incubated with staining solution for 30 minutes at RT in the dark, followed by a series of washing steps, before analysis by flow cytometry.

532 **Preparation of mLC3 cells for fluorescence microscopy**

533 mLC3 cells were plated out on 6-well plates containing cover slips, at a concentration of 2.5×10^5
 534 cells/ml. Cells were allowed to adhere for 2 hours before LPS stimulation (500 ng/mL). Where
 535 appropriate, cells were pre-incubated with either Baf (10 nM) or GP PAC (15 µg/ml) 30 minutes prior
 536 to LPS addition. After 18 hours of incubation, the cells were washed twice with PBS and fixed for 15
 537 min with 4% formaldehyde, followed by another washing step with PBS. Finally, the coverslips were
 538 recovered and placed onto a slide for microscopy using a Leica DM 5000B Fluorescence Microscope.
 539 Images were prepared using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

540 **Transmission Electron Microscopy**

541 Murine macrophages were plated out on 24-well plates at a concentration of 5×10^5 cells/ml. Cells
 542 were allowed to adhere for 2 hours before they were treated as appropriate in the following order with
 543 Baf (10 nM), and/or GP PAC (15 µg/ml), and/or LPS (500 ng/mL) with 30 min incubation time
 544 between each treatment. The cells were incubated for 24h before fixation and preparation for
 545 transmission electron microscopy (TEM). Cells were fixed with 3% glutaraldehyde (Merck,
 546 1042390250) in 0.1 M mNa-phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in 0.1 M
 547 Na phosphate buffer, dehydrated stepwise in a graded ethanol series, intermediate solution propylene
 548 oxide followed by pure epon O/N and next day, embedded in Epon (TAAB, T031). Semi-thin (2 µm)
 549 sections were cut with glass knives (KnifeMaster II, LKB Bromma 7800) on an ultramicrotome (Leica
 550 Ultracut, Leica Microsystems, Wetzlar, Germany), stained firstly with 1% toluidine blue (VWR
 551 34187.185) in 0.1% Borex (VWR 27727.231). Ultra-thin (50 nm to 60 nm) sections were sectioned
 552 with a diamond knife (Jumdi, 2 mm) on an ultramicrotome (Leica Ultracut), contrasted with 2%
 553 uranyl acetate (Polyscience, 21447) and lead citrate(Reynold, 1963), and examined using a Philips
 554 CM100 transmission electron microscope operating at 60 kV. Photographs were taken using Olympus
 555 Morada 11 megapixel camera and iTEM software (Olympus).

RNA extraction from macrophages and transcriptomic analysis

LPS-activated and non-activated RAW 264.7 macrophages were cultured with appropriate treatments as described above, and harvested for RNA extraction after 6 hours of incubation using RNeasy kits (Qiagen). cDNA was synthesized from 500 ng of RNA using Quantitect Reverse Transcriptase kits (Qiagen). Transcriptomic analysis was performed using the GeneChip WT PLUS Reagent kit (Thermo Fisher Scientific, CA, USA) and Affymetrix mouse Clariom S HT 24-array plate pipeline (Eurofins AROS, Denmark), with array plate processing carried out on a GeneTitan Instrument (Thermo Fisher Scientific). Transcriptome Analysis Console (TAC) software (Thermo Fisher Scientific) was used to analyze microarray data, and additional pathway analysis performed using Gene Set Enrichment Analysis software (GSEA; Broad Institute, MA, USA), using values of FDR q-value <0.2 and nominal p-value <0.02 to assess significance. Microarray data is available at GEO, under the accession number GSE167063. qPCR was performed using perfeCTa SYBR green fastmix (Quanta Bioscience) using the following program: 95°C for 2 minutes followed by 40 cycles of 15 seconds at 95°C and 20 seconds at 60°C. Primer sequences are listed in **Table S3**.

Treatment of mice with PAC fractions

6-week old female C57BL/6JOLA-Hsd mice (Envigo, the Netherlands) were distributed into 3 groups of 5 mice. The mice were allowed 1-week of acclimatization, and were fed with a purified control diet (13 kJ% fat; ssniff Spezialdiäten GmbH, Germany) throughout the entire study. Each group was orally gavaged every second day for 10 days with either low mDP GP PAC, medium mDP GP PAC (200 mg/kg BW) sephadex fractions dissolved in distilled water, or water alone. Mice were euthanized by cervical dislocation, and tissue collected from the ileum was preserved in RNAlater for RNA-sequencing and qPCR. Primer sequences are listed in **Table S3**.

578 **RNA-sequencing of mouse intestinal tissue**

579 RNA was extracted from ileal tissue as described above. Libraries were prepared and sequenced
 580 (paired-end reads of 100 bp) on a BGISEQ-500 sequencer (BGI, Copenhagen, Denmark). Quality
 581 control was performed using SOAPnuke v1.5.2 (github.com/BGI-flexlab/SOAPnuke) to remove
 582 adaptors, reads with unknown bases more than 10%, and low quality reads. Clean reads were mapped
 583 to the mouse genome ([mm10](#)) using Bowtie2 (v2.2.5). Differentially expressed genes were detected
 584 using NOIseq, using a Q-value for significance of >0.8 and a fold change of at least two⁵⁸. Pathway
 585 analysis was conducted using GSEA. Predicted genes and those with no gene symbol assigned were
 586 excluded from analysis. For qPCR validation, cDNA synthesis and PCR was performed as above. Raw
 587 RNAseq data is available at GEO under accession number GSE168138.

588 **Statistical analysis**

589 Data analysis was performed using the statistical software GraphPad Prism 7 (GraphPad Inc.) and
 590 Excel (2016). All *in vitro* data regarding IL-6 secretion in LPS-activated RAW 264.7 macrophages
 591 was analyzed to investigate the difference in percentages in IL-6 secretion, compared to an LPS-
 592 control. In GraphPad Prism, D'Agostino-Pearson and Shapiro-Wilk normality tests were used to
 593 assess data sets for normal distribution for one-way ANOVA analysis and Tukey's multiple
 594 comparisons test. If the data did not follow a Gaussian distribution, non-parametric tests were applied.

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604 **Conflict of interest**

605 The authors declare no conflicts of interests regarding this study.

606 **Ethical statement**

607 All experiments involving animals were conducted in agreement with the Danish legislation and the
608 Danish Animal Experiments Inspectorate with the license number 2015-15-0201-0076.

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618 Figures

Figure 1 - Molecular characteristics of proanthocyanidins

A) The structures of (epi)catechin and (epi)gallocatechin, which give basis to the most common proanthocyanidin (PAC) structural units, procyanidin (PC) and prodelphinidin (PD) units are displayed as single building blocks. The figure also depicts a model structure of an oligomeric PAC consisting of PD and PC extension units and a PC terminal unit (n = number of PC or PD extension units). PAC samples used in this study showed varying chemical characteristics, with samples extracted from **B)** alpine currant (*Ribes alpinum*) being rich in prodelphinidins (PD), whereas samples extracted from **C)** grape (*Vitis vinifera*) pomace were rich in procyanidins (PD). Samples were purified and extracted by Sephadex LH-20 fractionation with methanol followed by acetone. Further purification was conducted by semi-preparative liquid chromatography, resulting in 8 highly purified samples for each of the Sephadex fractions. Each sample was analyzed by UPLC-MS/MS. Samples produced from grape pomace were prepared in two batches, thus fractions labeled with “*” represent the second batch of samples. mDP: mean degree of polymerization.

Figure 2 - Suppression of IL-6 secretion in lipopolysaccharide-activated macrophages by proanthocyanidins is influenced by polymerization

A) Proanthocyanidins (PAC) derived from grape pomace (GP) reduced IL-6 secretion in lipopolysaccharide (LPS)-activated macrophages by up to 60 %. The green bars represent the samples selected for transcriptomics. **B)** The inhibition of IL-6 secretion by GP PAC was dependent on mDP. **C)** PAC derived from alpine currant (AC) showed less efficiency at inhibiting IL-6 secretion compared to GP PAC. **D)** The mDP dependency of IL-6 inhibition was less evident in LPS-activated RAW 264.7 macrophages stimulated with AC PAC. Each PAC sample (15 μ g/ml) was tested in triplicates, and at least two independent experiments were performed.

Figure 3 - Proanthocyanidin samples affected the regulation level of similar genes in macrophages in a polymerization-dependent manner

A) Principal Component Analysis plot showing a clear effect of lipopolysaccharide (LPS) and proanthocyanidins (PAC). Medium mDP PAC was the most differentiated treatment from respective controls with and without LPS. The most prominent transcriptional changes were observed in cells exposed to PAC with an mDP of 9.1, whereas PAC with a higher or lower mDP were generally less effective at modulating the cellular responses. **B)** Top 15 up- and down-regulated genes by PAC in resting cells (no LPS treatment). **C)** Top 15 up- and down-regulated genes by PAC in LPS-activated cells. **D)** The top 15 genes regulated by each PAC samples in resting or LPS-activated RAW 264.7 macrophages were investigated. Out of a total of 69 regulated genes, 25% were regulated by at least 2 of the PAC samples with differing mDPs in LPS-activated RAW 264.7 macrophages. Experiments were conducted with triplicate samples.

Figure 4 - Regulation of gene pathways in RAW 264.7 macrophages stimulated with proanthocyanidins with differing degrees of polymerization

Gene pathways regulated by **A)** medium mDP (9.1) **B)** and high mDP (12.3) PAC in resting RAW 264.7 macrophages. Gene pathways regulated by **C)** low mDP (2.6), **D)** medium mDP (9.1) and **E)** high mDP (12.3) GP PAC in lipopolysaccharide activated RAW 264.7 macrophages. Experiments were conducted with triplicate samples.

Figure 5 - Regulation of gene expression in mouse ileum tissue by proanthocyanidins

A) Significantly regulated genes (Fold change >2; q-value >0.8) obtained from RNA sequencing of mouse ileum tissue, in mice dosed with low mDP proanthocyanidins (PAC) or medium mDP PAC. Fold changes are relative to mice dosed with only water. **B)** Principal Component Analysis showed an effect of PAC mDP demarcated in two distinct clusters. ($n=5$ mice per treatment group). **C)** Gene pathways identified in the KEGG database that were downregulated (Nominal p value <0.005; q-value <0.1) by treatment with medium mDP PAC, as shown by gene-set enrichment analysis.

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Figure 6 Proanthocyanidins regulate gene expression, autophagosome formation and autophagy in RAW264.7 cells

A) Expression of *Tlr4*, *Rab7b*, and *Atp6v0d2* in RAW264.7 macrophages exposed to proanthocyanidins (PAC) in either resting conditions or following lipopolysaccharide (LPS) stimulation. Fold changes are relative to cells exposed to medium only (resting cells) or LPS only (LPS activated cells). qPCR was conducted with triplicate samples.

B) Transmission Electron Microscopy (TEM) pictures showing how pre-incubation with Bafilomycin A1 (Baf; 10 nM) inhibits the formation of autophagosome-like structures in macrophages stimulated with PAC (15 µg/ml).

C) LPS-activated cells showed a significant decrease in IL-6 secretion when pre-stimulated with Baf. ELISA experiments were conducted twice in duplicates.

D) Expression of *Tlr4* and *Atp6v0d2* in RAW264.7 cells stimulated with LPS with PAC and/or Baf. qPCR was conducted twice with triplicate samples.

E) PAC and Baf both significantly decreased autophagy flux in LPS-activated RAW 264.7 macrophages compared to cells stimulated with LPS only. The experiment was conducted with triplicate samples.

F) PAC and Baf both reduce the formation of autophagolysosomes in LPS-activated mLC3 cells. (Un-paired t-tests, Mann-Whitney's tests or one-way ANOVA were applied, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

References

1. Milani, R. V. & Lavie, C. J. Health care 2020: Reengineering health care delivery to combat chronic disease. *American Journal of Medicine* **128**, 337–343 (2015).
2. Anderson, E. & Durstine, J. L. Physical activity, exercise, and chronic diseases: A brief review. *Sport. Med. Heal. Sci.* **1**, 3–10 (2019).
3. Mathers, J. & Wolever, T. M. *Introduction to Human Nutrition. Introduction to Human Nutrition* (2009). doi:10.1016/B978-0-12-384947-2.00699-1
4. Pandey, K. B. & Rizvi, S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2**, 270–278 (2009).
5. Zhang, H. & Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **8**, 33–42 (2016).
6. Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. Polyphenols - Food Sources and Bioavailability.pdf. *Am J Clin Nutr* **79**, 727–47 (2004).
7. Hartley, L. *et al.* Increased consumption of fruit and vegetables for the primary prevention of cardiovascular diseases. *Cochrane Database Syst. Rev.* **2013**, (2013).
8. Costa, C. *et al.* Current evidence on the effect of dietary polyphenols intake on chronic diseases. *Food and Chemical Toxicology* **110**, 286–299 (2017).
9. Scalbert, A. & Williamson, G. Dietary Intake and Bioavailability of Polyphenols. *J. Med. Food* **3**, 121–125 (2000).
10. Williams, A. R. *et al.* Anthelmintic activity of trans-cinnamaldehyde and A-and B-type proanthocyanidins derived from cinnamon (*Cinnamomum verum*). *Sci. Rep.* **5**, (2015).
11. Teixeira, N., Mateus, N. & de Freitas, V. Updating the research on prodelphinidins from dietary sources. *Food Res. Int.* **85**, 170–181 (2016).
12. Martinez-Micaelo, N., González-Abuín, N., Ardèvol, A., Pinent, M. & Blay, M. T. Procyanidins and inflammation: Molecular targets and health implications. *BioFactors* **38**, 257–265 (2012).
13. Iglesias, J., Medina, I. & Pazos, M. Galloylation and Polymerization: Role of Structure to Antioxidant Activity of Polyphenols in Lipid Systems. *Polyphenols Hum. Heal. Dis.* **1**, 323–338 (2013).
14. Liu, W. *et al.* Grape seed proanthocyanidin extract ameliorates inflammation and adiposity by modulating gut microbiota in high-fat diet mice. *Mol. Nutr. Food Res.* **61**, 1601082 (2017).
15. González-Quilen, C. *et al.* Grape-seed proanthocyanidins are able to reverse intestinal dysfunction and metabolic endotoxemia induced by a cafeteria diet in wistar rats. *Nutrients* **11**, (2019).
16. Engler, M. B. & Engler, M. M. The Emerging Role of Flavonoid-Rich Cocoa and Chocolate in Cardiovascular Health and Disease. *Nutr. Rev.* **64**, 109–118 (2006).
17. Rios, L. Y. *et al.* Cocoa procyanidins are stable during gastric transit in humans. *Am. J. Clin. Nutr.* **76**, 1106–1110 (2002).
18. Tsang, C. *et al.* The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br. J. Nutr.* **94**, 170–181 (2005).

- 775 19. Bak, M. J., Truong, V. L., Kang, H. S., Jun, M. & Jeong, W. S. Anti-inflammatory effect of procyanidins
776 from wild grape (*Vitis amurensis*) seeds in LPS-induced RAW 264.7 cells. *Oxid. Med. Cell. Longev.*
777 (2013). doi:10.1155/2013/409321
- 778 20. Terra, X. *et al.* Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated
779 RAW 264.7 macrophages by inhibiting NFκB signaling pathway. *J. Agric. Food Chem.* **55**, 4357–4365
780 (2007).
- 781 21. Chu, H., Tang, Q., Huang, H., Hao, W. & Wei, X. Grape-seed proanthocyanidins inhibit the
782 lipopolysaccharide-induced inflammatory mediator expression in RAW264.7 macrophages by
783 suppressing MAPK and NF-κB signal pathways. *Environ. Toxicol. Pharmacol.* **41**, 159–166 (2016).
- 784 22. Wang, Q. Q. *et al.* Procyanidin A2, a polyphenolic compound, exerts anti-inflammatory and anti-
785 oxidative activity in lipopolysaccharide-stimulated RAW264.7 cells. *PLoS One* **15**, (2020).
- 786 23. Pallarès, V. *et al.* Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated
787 fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. *Nutrition*
788 **28**, 447–457 (2012).
- 789 24. Nallathambi, R., Poulev, A., Zuk, J. B. & Raskin, I. Proanthocyanidin-rich grape seed extract reduces
790 inflammation and oxidative stress and restores tight junction barrier function in Caco-2 colon cells.
791 *Nutrients* **12**, (2020).
- 792 25. Williams, A. R. *et al.* Polymerization-dependent activation of porcine γδ T-cells by
793 proanthocyanidins. *Research in Veterinary Science* **105**, 209–215 (2016).
- 794 26. Mueller-Harvey, I. *et al.* Benefits of condensed tannins in forage legumes fed to ruminants:
795 Importance of structure, concentration, and diet composition. *Crop Science* **59**, 861–885 (2019).
- 796 27. Leppä, M. M., Karonen, M., Tähtinen, P., Engström, M. T. & Salminen, J. P. Isolation of chemically
797 well-defined semipreparative liquid chromatography fractions from complex mixtures of
798 proanthocyanidin oligomers and polymers. *J. Chromatogr. A* **1576**, 67–79 (2018).
- 799 28. Engström, M. T. *et al.* Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and
800 polymers by UPLC-MS/MS. *J. Agric. Food Chem.* **62**, 3390–3399 (2014).
- 801 29. Forgac, M. Structure and properties of the vacuolar (H⁺)-ATPases. *Journal of Biological Chemistry*
802 **274**, 12951–12954 (1999).
- 803 30. Nishi, T. & Forgac, M. The vacuolar (H⁺)-ATPases - Nature's most versatile proton pumps. *Nature*
804 *Reviews Molecular Cell Biology* **3**, 94–103 (2002).
- 805 31. Maxson, M. E. & Grinstein, S. The vacuolar-type H⁺-ATPase at a glance - more than a proton pump.
806 *J. Cell Sci.* **127**, 4987–4993 (2014).
- 807 32. Wormald, S. & Hilton, D. J. Inhibitors of Cytokine Signal Transduction. *Journal of Biological Chemistry*
808 **279**, 821–824 (2004).
- 809 33. Liongue, C., O'Sullivan, L. A., Trengove, M. C. & Ward, A. C. Evolution of JAK-STAT pathway
810 components: Mechanisms and role in immune system development. *PLoS One* **7**, (2012).
- 811 34. Wang, G. *et al.* Cutting Edge: Slamf8 Is a Negative Regulator of Nox2 Activity in Macrophages. *J.*
812 *Immunol.* **188**, 5829–5832 (2012).
- 813 35. Diaz De Barboza, G., Guizzardi, S. & Tolosa De Talamoni, N. Molecular aspects of intestinal calcium
814 absorption. *World J. Gastroenterol.* **21**, 7142–7154 (2015).

- 815 36. Zhao, J. *et al.* R-spondin1, A Novel Intestinotrophic Mitogen, Ameliorates Experimental Colitis in
816 Mice. *Gastroenterology* **132**, 1331–1343 (2007).
- 817 37. Downing, L. E., Edgar, D., Ellison, P. A. & Ricketts, M. L. Mechanistic insight into nuclear receptor-
818 mediated regulation of bile acid metabolism and lipid homeostasis by grape seed procyanidin
819 extract (GSPE). *Cell Biochemistry and Function* **35**, 12–32 (2017).
- 820 38. Beuling, E. *et al.* Conditional Gata4 deletion in mice induces bile acid absorption in the proximal
821 small intestine. *Gut* **59**, 888–895 (2010).
- 822 39. Kuang, M. *et al.* Artesunate Attenuates Pro-Inflammatory Cytokine Release from Macrophages by
823 Inhibiting TLR4-Mediated Autophagic Activation via the TRAF6-Beclin1-PI3KC3 Pathway. *Cell. Physiol.*
824 *Biochem.* **47**, 475–488 (2018).
- 825 40. Andersen-Civil, A. I. S., Arora, P. & Williams, A. R. Regulation of Enteric Infection and Immunity by
826 Dietary Proanthocyanidins. *Frontiers in Immunology* **12**, (2021).
- 827 41. González-Quilen, C. *et al.* Health-promoting properties of proanthocyanidins for intestinal
828 dysfunction. *Nutrients* **12**, (2020).
- 829 42. Gentile, C. *et al.* Polymeric proanthocyanidins from Sicilian pistachio (*Pistacia vera* L.) nut extract
830 inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. *Eur. J. Nutr.* **51**, 353–
831 363 (2012).
- 832 43. Zumdick, S., Deters, A. & Hensel, A. In vitro intestinal transport of oligomeric procyanidins (DP 2 to
833 4) across monolayers of Caco-2 cells. *Fitoterapia* **83**, 1210–1217 (2012).
- 834 44. Karas, D., Ulrichová, J. & Valentová, K. Galloylation of polyphenols alters their biological activity.
835 *Food Chem. Toxicol.* **105**, 223–240 (2017).
- 836 45. Bitzer, Z. T. *et al.* Cocoa procyanidins with different degrees of polymerization possess distinct
837 activities in models of colonic inflammation. *J. Nutr. Biochem.* **26**, 827–831 (2015).
- 838 46. Leppä, M. M., Laitila, J. E. & Salminen, J. P. Distribution of Protein Precipitation Capacity within
839 Variable Proanthocyanidin Fingerprints. *Molecules* **25**, (2020).
- 840 47. Williams, A. R. *et al.* Co-operative suppression of inflammatory responses in human dendritic cells by
841 plant proanthocyanidins and products from the parasitic nematode *Trichuris suis*. *Immunology* **150**,
842 312–328 (2017).
- 843 48. Gilchrist, M. *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like
844 receptor 4. *Nature* **441**, 173–178 (2006).
- 845 49. Hai, T., Wolford, C. C. & Chang, Y. S. ATF3, a hub of the cellular adaptive-response network, in the
846 pathogenesis of diseases: Is modulation of inflammation a unifying component? *Gene Expression* **15**,
847 1–11 (2010).
- 848 50. Leppä, M. M., Laitila, J. E. & Salminen, J. P. Distribution of Protein Precipitation Capacity within
849 Variable Proanthocyanidin Fingerprints. *Molecules* **25**, (2020).
- 850 51. Fraga, C. G. & Oteiza, P. I. Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell
851 signaling. *Free Radical Biology and Medicine* **51**, 813–823 (2011).
- 852 52. Verstraeten, S. V., Hammerstone, J. F., Keen, C. L., Fraga, C. G. & Oteiza, P. I. Antioxidant and
853 membrane effects of procyanidin dimers and trimers isolated from peanut and cocoa. *J. Agric. Food*
854 *Chem.* **53**, 5041–5048 (2005).

- 855 53. Wang, Y. *et al.* Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in
856 macrophages by promoting lysosomal degradation of TLR4. *Blood* **110**, 962–971 (2007).
- 857 54. Xia, Y. *et al.* The macrophage-specific V-ATPase subunit ATP6V0D2 restricts inflammasome
858 activation and bacterial infection by facilitating autophagosome-lysosome fusion. *Autophagy* **15**,
859 960–975 (2019).
- 860 55. Midttun, H. L. E., Ramsay, A., Mueller-Harvey, I. & Williams, A. R. Cocoa procyanidins modulate
861 transcriptional pathways linked to inflammation and metabolism in human dendritic cells. *Food*
862 *Funct.* **9**, 2883–2890 (2018).
- 863 56. Thomas, L. *et al.* Selective upregulation of TNF α expression in classically-activated human monocyte-
864 derived macrophages (M1) through pharmacological interference with V-ATPase. *Biochem.*
865 *Pharmacol.* **130**, 71–82 (2017).
- 866 57. Malisch, C. S. *et al.* Large Variability of Proanthocyanidin Content and Composition in Sainfoin
867 (*Onobrychis viciifolia*). *J. Agric. Food Chem.* **63**, 10234–10242 (2015).
- 868 58. Tarazona, S., García-Alcalde, F., Dopazo, J., Ferrer, A. & Conesa, A. Differential expression in RNA-
869 seq: A matter of depth. *Genome Res.* **21**, 2213–2223 (2011).

870