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Oligomeric procyanidins stimulate innate antiviral immunity in dengue virus infected human PBMCs

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ABSTRACT

Oligomeric procyanidins (OPCs) have been shown to have antiviral and immunostimulatory effects. OPCs isolated from non-ripe apple peel were tested for capacity to reduce dengue virus (DENV) titers. Similar to published accounts, OPCs exhibited direct antiviral activity. The possibility of enhanced innate immune protection was also tested by measuring and characterizing gene and protein expression induced by OPCs during DENV infection. Treatment of DENV-infected human PBMCs with OPCs decreased viral titers and affected the expression of critical innate antiviral immune products. OPCs enhanced expression of *MXI* and *IFNB* transcripts in high MOI DENV infected PBMC cultures, and phosphorylation of STAT2 in response to recombinant type I IFN (IFN I). During low MOI infection, addition of OPCs increased expression of *STAT1* transcripts, MHC I and TNFα protein production. Thus, OPCs exhibited innate immune stimulation of cells in DENV-infected cultures and uninfected cells treated with IFN I. While OPCs from a number of sources are known to exhibit antiviral effects, their mechanisms are not precisely defined. The capacity of OPCs to increase sensitivity to IFN I could be broadly applicable to many viral infections and two separate antiviral mechanisms suggest that OPCs may represent a novel, robust antiviral therapy.

1. Introduction

Dengue virus (DENV) is an NIAID Category A pathogen and DENV infection represents one of the most important emerging threats to human health worldwide. Over the past 30 years infection rates have dramatically increased, in part due to population urbanization. Four related viruses of different serotypes, transmitted by mosquitoes, are the cause of DENV infection. Vaccine development is complicated by difficulties in developing a small animal model relevant to human disease as well as the phenomenon of antibody-dependent enhancement (ADE). ADE involves antibodies specific for one DENV serotype that may not neutralize other DENV serotypes. Rather, non-neutralizing, cross reactive antibodies can enhance disease severity upon secondary infection with a different serotype (Halstead and O'Rourke, 1977). There are virtually no antiviral treatments for DENV and mosquito control measures have largely failed to curb DENV incidence in most parts of the world, necessitating novel approaches for protection.

The primary cellular target for DENV infection in humans is the monocyte/macrophage (Chunhakan et al., 2009; Kou et al., 2008). Although details of DENV infection are not completely understood, the early innate response appears to be critical in directing the subsequent infection to either a self-limiting dengue fever, or a severe and potentially lethal dengue hemorrhagic fever (Chen et al., 2007; Shresta et al., 2004). As with many viral infections, type I IFN (IFN I) responses are critical to DENV protection, exemplified by a commonly used mouse model, which utilizes IFN receptor-deficient mice (Shresta et al., 2004).

Polyphenols are a large and diverse group of aromatic-rich plant metabolites that have been identified in a number of food sources and dietary supplements such as cranberry juice, grape seeds, pomegranate and unripe apple peels (Holderness et al., 2008; Terra et al., 2007). Large, oligomeric procyanidins (OPCs) bind to and precipitate basic proline-rich proteins found in saliva (Charlton et al., 2002; Holderness et al., 2008). Antiviral effects of many different types of polyphenols, including monomeric and hydrolysable tannins are described (Haidari et al., 2009). Greater evidence suggests that the robust anti-viral activity is contained in larger order or oligomeric fractions [OPCs; also referred to as condensed tannins or oligomeric proanthocyanidins (Feng et al., 2008; Hauber et al., 2009; Su et al., 2010; Takeshita et al., 2009; Zhuang et al., 2009)]. For example, large-sized OPCs from blueberry leaves block the replication of hepatitis C virus (HCV) RNA in a subgenomic

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replicon expression system (Takeshita et al., 2009). Additionally, a procyanidin-rich extract from French Maritime Pine blocks HIV-1 intracellular replication and binding to host cells, although the precise mechanisms were not discovered (Feng et al., 2008). Although direct antiviral interaction between OPCs and virus is demonstrated for a variety of viral agents, the descriptions of precise effects are largely limited to blocking early steps in viral entry in cell lines, which is not necessarily relevant to their natural infection *in vivo*.

We recently identified OPCs derived from unripe apple peels, found in the dietary supplement Applepoly®, as stimulatory to innate lymphocytes. This response is specific to the oligomeric and not monomeric procyanidins, indicating a unique, non-antioxidant response (Holderness et al., 2007, 2008). We hypothesized that OPCs would also demonstrate antiviral effects. This potential was tested during DENV infection of human PBMCs. Addition of OPCs to DENV-infected cells consistently decreased viral titers *in vitro*. As expected, this effect was due, in part, to reduction of infectious titers by direct interaction of OPCs with DENV. The potential for enhanced innate responses to virus infection in the presence of OPCs was also characterized. Results indicated that OPCs enhanced innate immune responses during DENV infection in part by altering expression of genes and proteins in the type I IFN (IFN I) pathway. During high MOI infections, addition of OPCs appeared to circumvent DENV-specific mechanisms that block these pathways. OPCs also enhanced phosphorylation of STAT2 in response to recombinant IFN I and affected cells in low MOI infected cultures, by increasing expression of STAT1 transcripts, MHC I and TNFα protein. Thus, OPCs induced innate antiviral responses that may be broadly applicable to other viral infections. To our knowledge, this study is the first to identify stimulation of critical antiviral innate responses by OPCs in target cells that are highly relevant to viral infection *in vivo*.

2. Materials and methods

2.1. Virus quantification

The DENV2 strain 16681 was obtained from Dr. Robert Tesh at the World Reference Center for Emerging Viruses and Arboviruses at UTMB (WRCEVA) and was grown to high titers in C6/36 cells (ATCC). Virus was concentrated by ultracentrifugation at 100,000 × *g* for 4 h. DENV was quantified using an adapted endpoint titration assay on BHK-21 cells (ATCC) in sextuplet wells of a 96-well plate. Briefly, DENV-infected C6/36, infected human PBMC culture supernatants with monomeric or oligomeric procyanidins or vehicle only added, or concentrated virus stocks were 10-fold serial diluted in MEM in sextuplet in a 96 well plate. Low-passage BHK-21 cells were added to each well in complete MEM with 10% serum, and penicillin/streptomycin (cMEM). Wells were supplemented with 50 μl cMEM 24 and 48–72 h after assay set up. The assay was terminated after 7 days incubation at 37 °C with 10% CO_2 by addition of 0.1% crystal violet in 20% methanol to each well. Virus was quantified (as $TCID_{50}$/ml) by analyses of the pattern of cytopathic effect (CPE) adapted to sextuplet wells according to the method of Reed and Muench (1938). Monomeric procyanidin and OPCs applied to BHK-21 cells from 1 to 20 μg/ml had no effect on cell viability.

Direct interaction of OPCs with DENV was assessed by incubating DENV in MEM with varying concentrations of monomeric procyanidins or OPCs for 24 h at 37 °C. The mixtures were then applied to the endpoint titration assay.

2.2. Analyses of human cells

Human subjects were volunteers in our department. While not all volunteers were serologically tested for dengue antibodies, more than 5 were tested and all were found to be negative for dengue antibodies. More importantly, in these studies the PBMCs were separated from the sera, thus, while it would be of eventual interest, ADE was not tested in our assays. Human PBMCs were collected as previously described (Holderness et al., 2007). Approximately 70 ml of blood was collected per subject, 17 ml whole blood was diluted 1:2 with Hanks buffer, and underlaid with 15 ml Histopaque 1077 (Sigma) in 50 ml tubes. The tubes were centrifuged for 30 min at 500 × *g*. The resulting buffy layer containing PBMCs was aspirated and cells were washed once with Hanks buffer and centrifugation at 500 × *g* for 5 min, then RBCs were removed by water lysis, and an additional wash. 2–5 × 10^6 isolated cells were infected at 1–30 MOI (defined as BHK-21 $TCID_{50}$/cell), as indicated depending on experiment in minimal volume for 2 h at 37 °C. Unattached virus was washed by adding media then centrifugation. XVIVO™-15 (Lonza Walkersville, Inc.) serum-free media was then added to cells resulting in 2 × 10^6 cells/ml. Crude extract of Applepoly®, the putative trimer or tetramer procyanidins [10 μg/ml, isolated from Applepoly® by normal phase HPLC as previously described (Holderness et al., 2007)], monomeric procyanidin [catechin (Fisher) or epicatechin (Sigma), also 10 μg/ml] or vehicle only was then added to the tissue culture media. The potential for toxicity and contamination with microbial patterns in crude apple peel extracts and OPC fractions has been addressed previously (Holderness et al., 2007). Neither contamination with LPS or other innate agonists nor adverse effects to human cells at concentrations <20 μg/ml were detected (Holderness et al., 2007, 2008).

Cells were cultured for 48 h before supernatant fluid and cells were collected. The supernatant fluids were used to measure DENV titers in endpoint titration assays as well as TNFα by ELISA (Biolegend). When DENV was not detected after 48 h infection ($n = 1$, a seronegative donor), samples were excluded from analyses. RNA was extracted from a minimum of 3 × 10^6 adherent cells, and gene expression patterns were analyzed by qPCR as previously described (Hedges et al., 2005).

2.3. Western blotting

To assess STAT2 phosphorylation in human PBMCs in response to OPCs and IFN I, human PBMCs were collected and stimulated with OPCs or vehicle only for 24 h. 1000 U/ml recombinant universal IFN I (PBL Interferon Source) or vehicle only was then added to cells for 20 min prior to collection of the cell pellets (5 × 10^6 cells per treatment) and storage at −80 °C. Cell pellets were lysed in 20 μl of MPER (Pierce) with Halt Protease & Phosphatase Inhibitor (Pierce) per manufacture's suggestions on ice for 30 min. Lysates were centrifuged at 8000 × *g* for 2 min and 6× Laemmli Sample buffer added to lysate supernatant fluids. Samples were boiled for 5 min, loaded onto a prepared 7.5% gel and run at 100 V. Protein was transferred to PVDF at 100 V for 1 h. Blot was blocked in 3% BSA in TBST for 1 h at RT. Primary antibody, phospho-Stat2 (Santa Cruz Biotech sc-21689-R) was diluted 1:400 in 3% BSA in TBST and incubated overnight at 4 °C rocking. The blot was washed and secondary antibody goat anti-rabbit IgG-HRP was diluted 1:4000 in 3% BSA TBST was added and incubated for 1 h at RT rocking. The blot was again washed before adding Novex ECL (Invitrogen) and then exposed to radiography film and processed. Immediately after, the blot was stripped using a mild stripping buffer (0.2 M glycine, 3.4 mM SDS, 0.01 M Tween 20, pH 2.2), incubated for 10 min two times, and then washed twice with PBS and twice with TBST. To confirm uniform protein loading the same number of cells per pellet were always used and the blot was blocked and then incubated overnight with anti-GAPDH antibody (Santa Cruz Biotech sc-25778) diluted 1:500 in 3% BSA in TBST. The blot was then processed and developed as described above. Densitometry was calculated using ImageJ

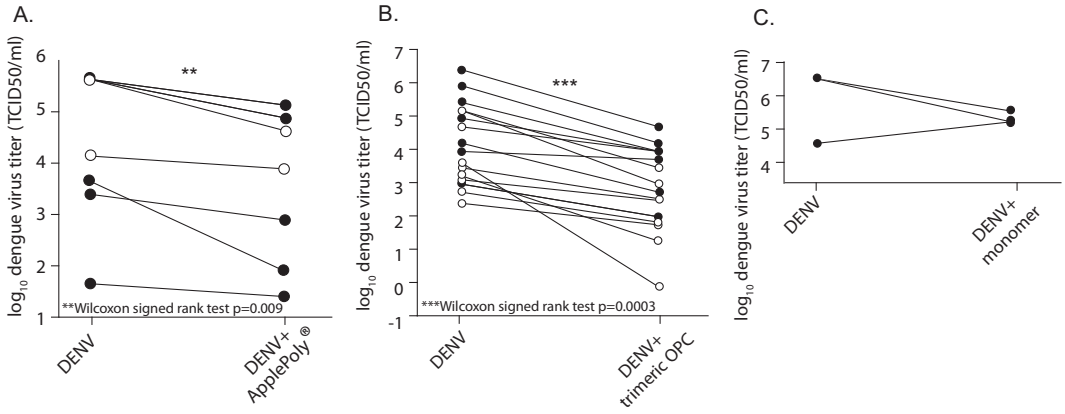

Fig. 1. OPCs derived from Applepoly® consistently decreased DENV titers upon addition to infected human PBMCs. (A) In DENV-infected human PBMCs from different human subjects (each represented by a different line, open circle denotes MOI > 10, closed circle denotes MOI < 5), addition of a crude extract from Applepoly® consistently resulted in lower titers as measured by endpoint titration. (B) The putative trimeric OPCs purified from Applepoly® were slightly more effective and consistent than crude extract at reducing titers in DENV-infected human PBMCs. (C) As shown in other viral systems, monomeric procyanidins (catechin or epicatechin) had a minimal and inconsistent effects on viral titers in the same assays.

(NIH) software and band regions were normalized to lane background.

2.4. Flow cytometry

Human PBMCs from 4 donors were collected and were either uninfected, treated with 100 U/ml recombinant universal IFN I (PBL InterferonSource), or infected with DENV at MOI 1, and each of these was treated with either trimeric OPCs or vehicle only. After 36 h cells were collected by gentle scraping, and stained with antibodies specific for CD11b/Mac-1 conjugated to PE (BD Phamingen) and HLA-A,B,C conjugated to APC (clone W6/32, Biolegend) or CD86 conjugated to PE (clone IT2.2, BD Phamingen). Cells were washed with PBS, 2% horse serum and read on a BD FACSCalibur flow cytometer.

2.5. Statistical analyses

Statistical significance was calculated for comparison of data derived using human PBMCs with the Wilcoxon signed rank test for paired data in Prism 5 (GraphPad, Inc.), unless otherwise noted. Otherwise significance was calculated using the Student's paired *t*-test.

3. Results

OPCs with innate immunomodulatory activity (Graff and Jutila, 2007; Holderness et al., 2007) were tested for their ability to reduce DENV titers from infected human PBMCs *in vitro*. During low and high MOI infections, a crude extract of OPCs derived from unripe apple peels consistently decreased DENV titers (Fig. 1A). The capacity of DENV to replicate varied between human subjects, even when infected at the same MOI. The average reduction in DENV titers with the crude OPC extract was 10-fold. We had previously identified the oligomeric, but not monomeric, OPCs as the active fraction of this extract from Applepoly® (Holderness et al., 2007). Similarly, commercial monomeric forms of OPCs such as epicatechin and catechin did not consistently reduce DENV titers (Fig. 1C), but the same concentration of the putative trimeric OPCs purified from the Applepoly® extract (Holderness et al., 2007) was more effective at reduction of DENV titers than was the crude extract (Fig. 1B). In this case the average reduction of DENV titers upon addition of OPCs was 35.9-fold, highly significant, and observed in infected cells from all human subjects tested, despite substantial genetic and experiential variation between subjects, suggesting OPCs may represent a robust antiviral treatment.

Because OPCs bind to proteins (Charlton et al., 2002), and direct interaction of OPCs and virus appears to be a common mechanism for reducing virus titers (Feng et al., 2008; Hauber et al., 2009; Su et al., 2010; Zhuang et al., 2009), the possible importance of this interaction was tested. As found in other systems, oligomeric forms (trimers and tetramers) of procyanidins directly blocked DENV infectivity resulting in reduced titers, but similar concentrations of the monomeric procyanidin (catechin) had little effect (data not shown).

Experiments were then conducted to determine if the reduction in viral titers conferred by OPCs could also be the result of innate immune enhancement in infected human monocyte/macrophages. DENV is known to interfere with, and be sensitive to IFN I pathways (Ashour et al., 2009; Mazzon et al., 2009; Rodriguez-Madoz et al., 2010a,b), thus, gene transcription relevant to this pathway was measured in human PBMCs. Cells were infected with DENV at a high MOI (>10). Uninfected cells, cells treated with OPCs only, DENV-infected, and OPC-treated/DENV-infected cells were compared after 48 h in culture. Results in Fig. 2A–C suggested that while OPCs alone had very little effect on transcript expression, addition of OPCs during DENV infection altered mRNA contents. *IFNB* expression is induced by IRF3/7 and NFκB after cell sensing of viral RNA and can be blocked by DENV infection (Rodriguez-Madoz et al., 2010b). In human cells, DENV establishes infection, at least in part, by also attenuating IFN I signaling via inhibition of STAT1/2 phosphorylation (Ashour et al., 2009; Mazzon et al., 2009). MX1 is a key antiviral product induced by phosphorylated STAT1/2 phosphorylation following signaling by IFN I. Since DENV blocks STAT2 phosphorylation, *MX1* expression is unchanged in DENV-infected cells (Mazzon et al., 2009). Consistent with these accounts, no significant changes in *IFNB* and *MX1* transcript levels were detected whether the cells were uninfected, treated with OPCs, or DENV-infected. In contrast, both *IFNB* and *MX1* were significantly increased in OPC-treated/DENV-infected cells, by an average of 2- and 2.5-fold respectively, over cells infected with DENV (Fig. 2A). The induction of both of these genes in response to OPCs is likely related, since IFNβ can in turn induce *MX1* expression. These data suggest that addition of OPCs can stimulate antiviral innate immune responses and appear to reverse viral inhibition of transcription of genes in the IFN I pathway.

Other genes related to the IFN I pathway were also measured. Surprisingly, transcripts encoding CXCL10 [IP-10, induced by IFNγ,

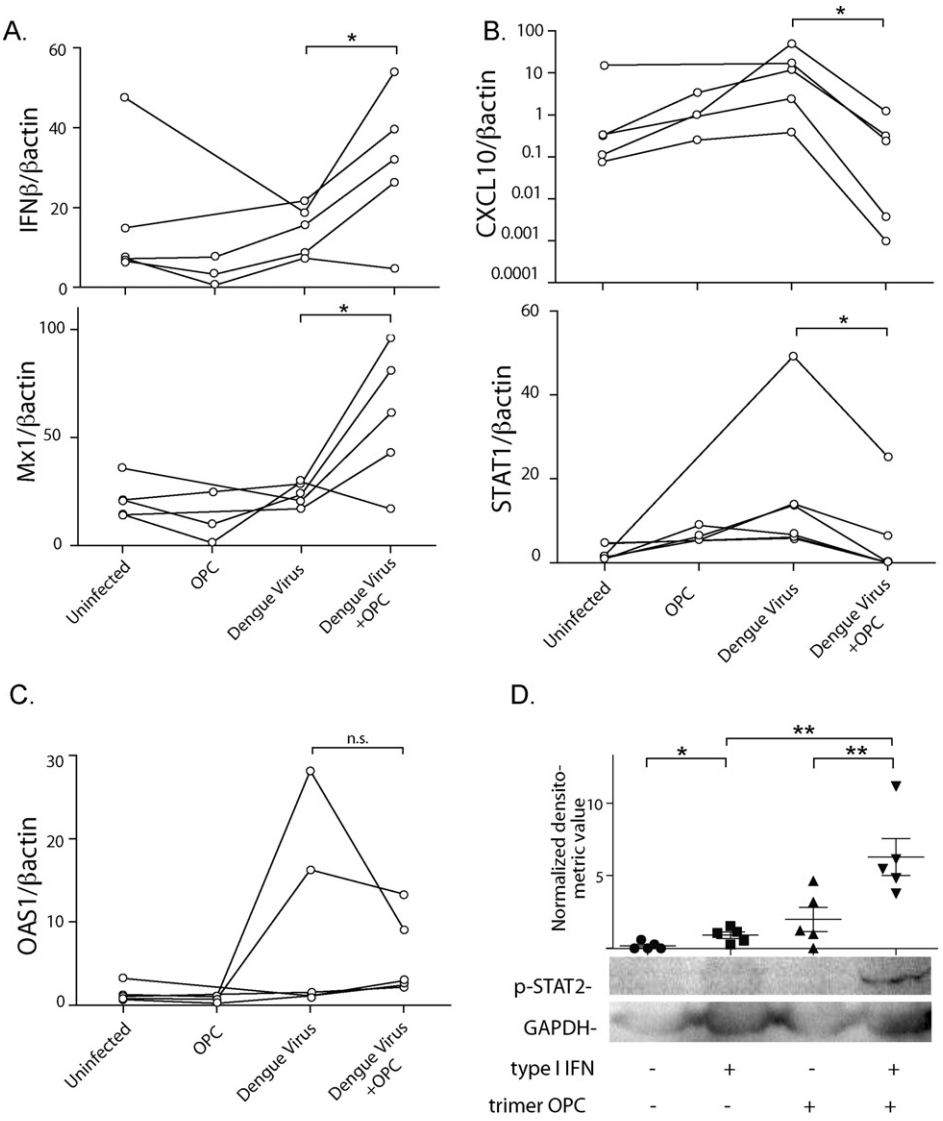

Fig. 2. Addition of OPCs to high MOI DENV-infected human PBMC affects gene expression in the IFN I signaling pathway. (A) Transcripts encoding *IFNB* and *MX1* remained unchanged in cells from most subjects in uninfected, OPC-treated and DENV-infected cells, but increased upon addition of OPCs to DENV-infected cultures. Significance was calculated using the Student's paired *t*-test, $^{*}p < 0.05$, $n = 5$. (B) The expression of *CXCL10* and *STAT1* were consistently reduced in the presence of OPC during DENV infection. $^{*}p < 0.05$, $n = 5$. (C) *OAS1* transcript expression in response to DENV-infection and OPC addition is not consistent between human donors. $n = 5$. (D) Phosphorylated STAT2 was only barely detectible in IFN only-treated cells and consistently clearly detected in OPC- and IFN I-treated human PBMCs. Normalized densitometric values for each blot ($n = 5$) and a representative blot is shown. Significance was calculated using the Student's paired *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$.

IFNβ, p38 and DENV infection (Hsieh et al., 2006; Ip and Liao, 2010)] and STAT1 (induced by low amounts of IFN I) had patterns of expression opposite of *MX1* and *IFNB* in response to OPCs. DENV infection increased the expression of CXCL10 and STAT1 and the addition of OPCs to the infected cells dramatically reduced their expression by averages of 235-fold and 168-fold respectively, compared to DENV infection alone (Fig. 2B). The gene expression changes induced by OPCs during DENV infection were statistically significant despite the substantial genetic and experiential differences between human donor subjects evident in the figures. These substantial differences were clearly demonstrated in transcription of *OAS1*. This gene followed expression patterns similar to *MX1* in response to DENV NS5 expression (Mazzon et al., 2009). In contrast, no clear pattern for *OAS1* expression in response to OPC treatment in human PBMCs was noted, rather expression appeared to be specific to donor subject. Interestingly, a specific mutation in OAS1 in humans accounts for increased susceptibility to West Nile virus infection (Lim et al., 2009). Despite such differences between subjects, addition of OPCs to DENV-infected human PBMCs had an opposing effect on gene expression compared to DENV infection alone for several different genes. The results suggest a partial rescue of DENV-mediated alterations of gene expression by OPC treatment.

To discover the impact of OPCs on the IFN signaling pathway in the absence of viral interference, we performed experiments to determine if OPCs enhanced responses to IFN I. Human PBMCs were incubated with OPCs, or vehicle only, for 24 h, then recombinant IFN I was added and cells were collected after 20 min for analysis of STAT2 phosphorylation by Western blot. Under the conditions used, phosphorylation of STAT2 with IFN I alone was only very faintly detected. However, with cells from every donor ($n = 5$ donors), samples treated with both OPCs and IFN had distinct bands indicating phosphorylation of STAT2 in response to IFN was stronger in OPC-treated cells (Fig. 2D). Such induction of enhanced responses to IFN I by OPCs may be relevant to many viral challenges. These data suggested that addition of OPCs might induce changes in gene expression in both DENV-infected and in bystander cells in infected cultures.

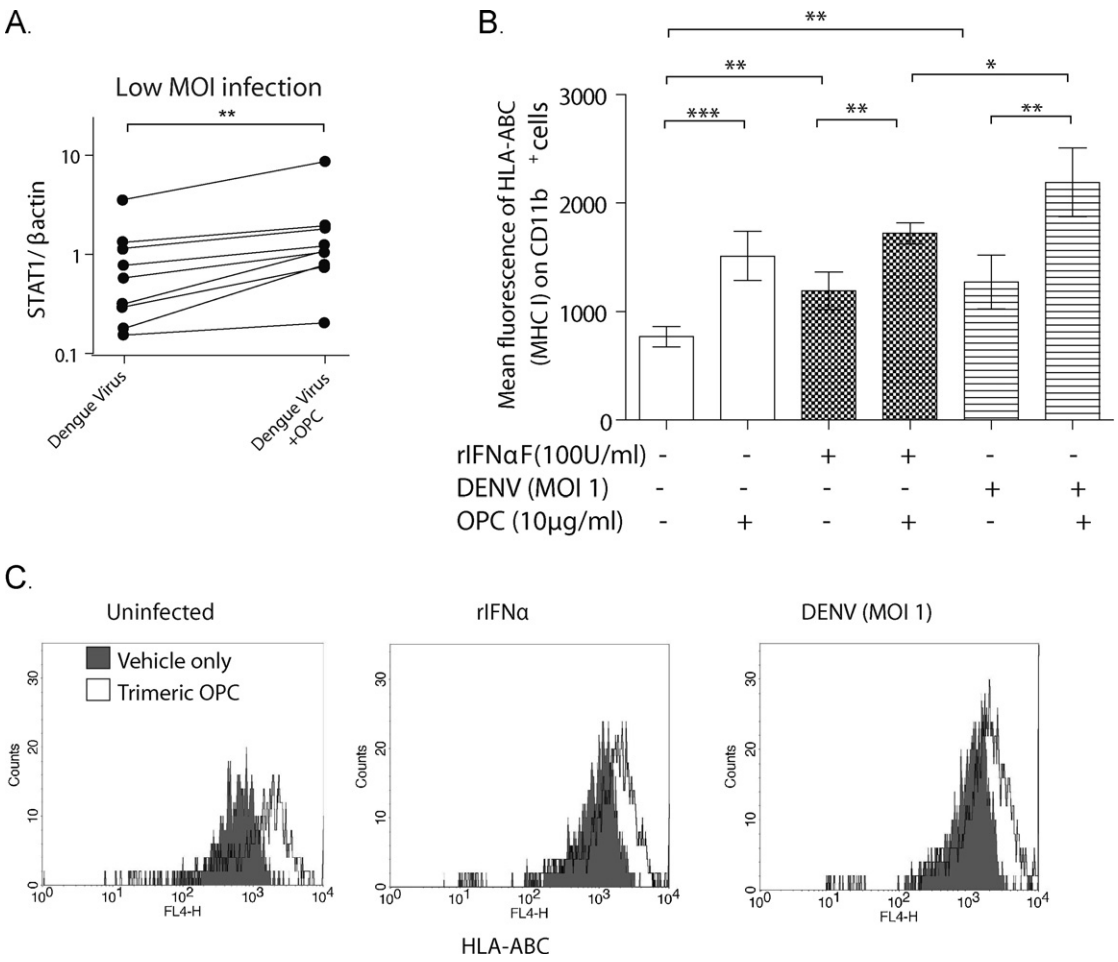

Fig. 3. OPCs altered gene and protein expression during low MOI DENV infection and stimulation with IFN I. (A) When OPCs were added to low MOI DENV infected cells, expression of *STAT1* was increased compared to vehicle only controls $^{**}p < 0.01$. (B) Human cells (from $n = 4$ donors) were untreated, treated with low rIFN I (100 U/ml), or infected with DENV at MOI = 1, then stimulated with OPCs or vehicle only and expression of MHC I (HLA-A, B, C) on $CD11b^+$ (monocyte/macrophages) was measured by flow cytometry. $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Representative histograms from one donor.

Since OPCs improved the IFN signaling response, we sought to measure the IFN response in bystander cells during low MOI DENV infection. This approach allows neighboring, uninfected cells to respond to IFN and other cytokines produced by the infected cells and is therefore more predictive of *in vivo* infection than a high MOI-infected culture. Alteration of gene expression by OPCs in uninfected bystander cells present in low MOI (0.5–1) DENV-infected cultures was tested. At low MOI, there were very low expression levels and no consistent changes in transcription of *MX1, IFNB* and *CXCL10* (data not shown) suggesting transcription of these genes only consistently changed in OPC-treated, high MOI DENV-infected cultures. However, whereas in high MOI-infected, OPC-treated, cultures *STAT1* expression decreased, uninfected cells represented in low MOI infections consistently increased *STAT1* transcript expression with OPC treatment with an average increase of 2.3-fold (Fig. 3A). STAT1 expression is induced by very low concentrations of IFN I signaling and primes cells for innate responses after stimulation with IFNγ, also critical for protection from DENV infection (Gough et al., 2010; Shresta et al., 2004). These results indicate OPCs improve the IFN signaling capacity of infected cells and/or the IFN response of bystander cells during DENV infection.

Potential downstream consequences of increased sensitivity to IFN and STAT1 signaling were then tested. Changes in expression of MHC I and CD86 were measured in uninfected, recombinant IFN I treated, or low MOI DENV-infected cells treated with trimeric OPCs. Mean fluorescence intensity (MFI) of MHC I significantly increased by treatment with IFN I and DENV infection alone compared to untreated cells (Fig. 3B). In each of these treatments, addition of OPCs further increased MHC I expression. Whereas there was no difference between OPC-treated uninfected and IFN-treated cells, OPC-treated/DENV-infected cultures had a larger increase in MHC I expression, suggesting an increased response to DENV induced cytokines other than IFN I. In all cultures the MHCI expression pattern was unimodal, indicating the shift in expression occurred in all cells, regardless of virus infection (Fig. 3B). Expression of CD86 followed the same general trends, but variation between donor subjects precluded significant differences (data not shown). OPC-induced increases in MHC I, which presents peptide antigen to $CD8^+$ T cells, and the costimulatory molecule CD86, on monocyte/macrophages suggest that OPCs may also enhance downstream adaptive immune responses. These results suggest that in the presence of OPCs, bystander cells may better respond to DENV infection and have initiated antiviral protocols to better protect themselves from further infection.

TNFα is an important inflammatory cytokine in DENV pathogenesis and progression to the severe hemorrhagic form of the disease (Chen et al., 2007; Devignot et al., 2010). To determine the effects of OPCs on TNFα production during DENV infection, we measured TNFα protein content in supernatant fluids from uninfected, OPC-treated, DENV-infected, and OPC-treated/DENV-infected human PBMCs during both low and high MOI infections (Fig. 4). The protein expression patterns were consistent with those observed in expres-

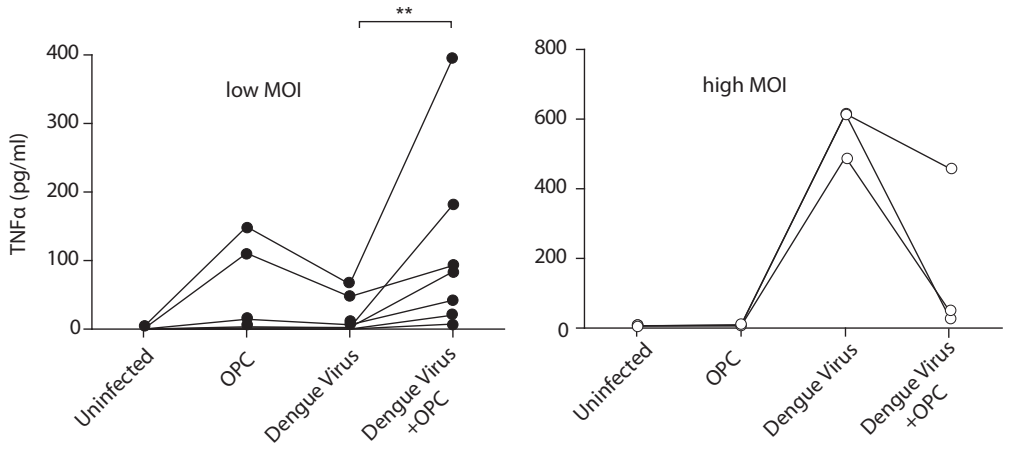

Fig. 4. OPCs altered production of TNFα during DENV infection *in vitro*. (A) At low MOI (closed circles) infection, addition of OPCs significantly increased production of TNFα in supernatant fluids from infected human PBMCs. $^{*}p = 0.0156$, $n = 7$. (B) At high MOI (open circles), DENV infection alone appeared to increase production of TNFα and the addition of OPCs decreased it. $n = 3$, insufficient for statistical significance.

sion of *STAT1* transcripts. Namely, at very high MOI DENV infection, TNFα expression was highly induced, consistent with published reports (Chen et al., 2007; Nightingale et al., 2008) and addition of OPCs appeared to decrease expression of TNFα from DENV-infected cells. In contrast, at low MOI, DENV infection alone did not consistently alter detection of TNFα in culture supernatants, but the addition of OPCs caused a significant increase in TNFα expression by presumably largely bystander cells. These data suggest that addition of OPCs can affect several aspects of innate immune responses to viral infection.

4. Discussion

Our data suggest that in addition to a direct antiviral effect, OPCs derived from Applepoly® enhance antiviral innate immunity (Fig. 5). During high-MOI DENV infection viral mechanisms block production of and responses to IFN I in infected cells (Ashour et al., 2009; Mazzon et al., 2009; Rodriguez-Madoz et al., 2010b). In DENV infection, there is an increase in *CXCL10* (Chen et al., 2006), presumably through expression of IFNγ, and an increased expression of TNFα (Hober et al., 1996b; Nightingale et al., 2008; Wati et al., 2007). Our data in Fig. 2 are consistent with these responses and also suggest a slight increase in *STAT1* expression in response to very low IFN I in DENV infected cells (Gough et al., 2010). Addition of OPCs during high-MOI infection appeared to reverse these viral mechanisms resulting in increased transcription of *IFNB* and *MXI*, decreases in *CXCL10* and *STAT1* and reduction of TNFα (Fig. 5, top panel). In the low-MOI infection a smaller minority of the cells is infected with DENV and responses of uninfected bystander cells predominate. This state is likely most relevant to initial infection *in vivo* during which cytokines expressed by infected cells promote local antiviral responses and can, in part, be modeled by low IFN I supplementation. Upon addition of OPCs in low MOI infection, increases in *STAT1*, MHC I and TNFα are noted (Fig. 5, lower panel). Our data suggests that addition of OPCs can result in an intensified innate antiviral state that warrants further study *in vivo*.

DENV-infected cells increase production of TNFα which may eventually contribute to adverse pathology during DENV hemorrhagic disease (Chen et al., 2007; Hober et al., 1996a,b). However, TNFα may also be beneficial for early innate immune protection. For example, treatment of DENV-infected monocytes with TNFα did not alter infection, but pre-infection supplementation of TNFα consistently decreased infection (Wati et al., 2007). Thus, OPC-induced expression of TNFα in bystander cells may be beneficial in limiting DENV infection. Whereas OPCs derived from cocoa have been shown to stimulate TNFα expression (Mao et al., 2002), addition of apple-derived trimeric OPCs to DENV-infected cells during high MOI infection decreased overt TNFα expression. Considering the adverse effects of excessive TNFα in DENV hemorrhagic disease and many other inflammatory conditions, OPC supplementation may provide benefit.

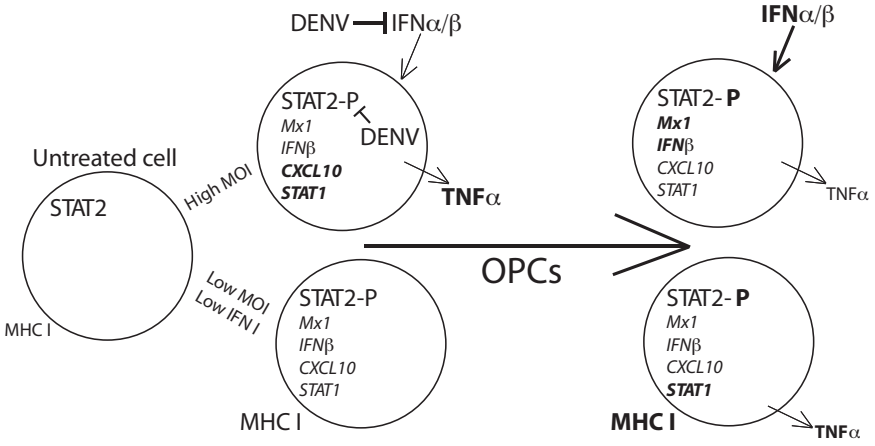

Fig. 5. Model for responses of cells to OPCs. Circles represent cells. Larger font and boldface type denotes intensified expression of genes (in cells) or proteins (on or by cells).

Considering the global challenges of DENV infection, and multiple viral threats with few to no treatment options, this dietary supplement could have substantial benefit. For example, recombinant IFN I is used as a therapy for HCV infection. Since Applepoly® is safe to ingest and can reach relevant concentration in the serum after ingestion (Holderness et al., 2007; Shoji et al., 2006), OPCs block HCV replication (Takeshita et al., 2009), and may render human cells more responsive to IFN I (Figs. 2 and 3), its use as a dietary supplement in viral infections or in combination with IFN treatment in HCV patients may be warranted.

OPCs from Applepoly® demonstrated direct viral interaction to block infection and enhancement of several different aspects of innate antiviral immunity. Antiviral treatments with single defined mechanisms of action for single-stranded RNA viruses should be used as part of a combination therapy, because of the high likelihood of such viruses rapidly evolving resistance (Valinotto et al., 2010). A viral therapeutic with two distinct mechanisms of antiviral action, such as OPCs, is likely to reduce the risks of evasion by highly mutable viruses. Our data suggest that dietary supplements that contain high concentrations of OPCs, such as Applepoly®, may enhance antiviral innate immunity to benefit patients facing viral challenge.

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References

Ashour, J., Laurent-Rolle, M., Shi, P.Y., Garcia-Sastre, A., 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. 83, 5408–5418.
Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J., Haslam, E., Davies, A.P., Williamson, M.P., 2002. Polyphenol/peptide binding and precipitation. J Agric. Food Chem. 50, 1593–1601.
Chen, H.C., Hofman, F.M., Kung, J.T., Lin, Y.D., Wu-Hsieh, B.A., 2007. Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J. Virol. 81, 5518–5526.
Chen, J.P., Lu, H.L., Lai, S.L., Campanella, G.S., Sung, J.M., Lu, M.Y., Wu-Hsieh, B.A., Lin, Y.L., Lane, T.E., Luster, A.D., Liao, F., 2006. Dengue virus induces expression of CXC chemokine ligand 10/IFN-γ-inducible protein 10, which competitively inhibits viral binding to cell surface heparan sulfate. J. Immunol. 177, 3185–3192.
Chunhakan, S., Butthep, P., Yoksan, S., Tangnararatchakit, K., Chuansumrit, A., 2009. Early diagnosis of dengue virus infection by detection of dengue viral antigen in peripheral blood mononuclear cell. Pediatr. Infect. Dis. J. 28, 1085–1088.
Devignot, S., Sapet, C., Duong, V., Bergon, A., Rihet, P., Ong, S., Lorn, P.T., Chroeung, N., Ngeav, S., Tolou, H.J., Buchy, P., Couissinier-Paris, P., 2010. Genome-wide expression profiling deciphers host responses altered during dengue shock syndrome and reveals the role of innate immunity in severe dengue. PLoS One 5, e11671.
Feng, W.Y., Tanaka, R., Inagaki, Y., Saitoh, Y., Chang, M.O., Amet, T., Yamamoto, N., Yamaoka, S., Yoshinaka, Y., 2008. Pycnogenol, a procyanidin-rich extract from French maritime pine, inhibits intracellular replication of HIV-1 as well as its binding to host cells. Jpn. J. Infect. Dis. 61, 279–285.
Gough, D.J., Messina, N.L., Hii, L., Gould, J.A., Sabapathy, K., Robertson, A.P., Trapani, J.A., Levy, D.E., Hertzog, P.J., Clarke, C.J., Johnstone, R.W., 2010. Functional crosstalk between type I and II interferon through the regulated expression of STAT1. PLoS Biol. 8, e1000361.
Graff, J.C., Jutila, M.A., 2007. Differential regulation of CD11b on γδ T cells and monocytes in response to unripe apple tannins. J. Leukoc. Biol. 82, 603–607.
Haidari, M., Ali, M., Ward Casscells III, S., Madjid, M., 2009. Pomegranate (Punica granatum) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. Phytomedicine 16, 1127–1136.
Halstead, S.B., O'Rourke, E.J., 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146, 201–217.
Hauber, I., Hohenberg, H., Holstermann, B., Hunstein, W., Hauber, J., 2009. The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. Proc. Natl. Acad. Sci. U.S.A. 106, 9033–9038.
Hedges, J.F., Lubick, K.J., Jutila, M.A., 2005. γδ T cells respond directly to pathogen associated molecular patterns. J. Immunol. 174, 6045–6053.
Hober, D., Delannoy, A.S., Benyoucef, S., De Groote, D., Wattre, P., 1996a. High levels of sTNFR p75 and TNF alpha in dengue-infected patients. Microbiol. Immunol. 40, 569–573.
Hober, D., Shen, L., Benyoucef, S., De Groote, D., Deubel, V., Wattre, P., 1996b. Enhanced TNF alpha production by monocytic-like cells exposed to dengue virus antigens. Immunol. Lett. 53, 115–120.
Holderness, J., Hedges, J.F., Daughenbaugh, K.F., Kimmel, E., Graff, J.C., Freedman, B., Jutila, M.A., 2008. Response of γδ T cells to plant-derived tannins. Crit. Rev. Immunol. 28, 377–402.
Holderness, J., Jackiw, L., Kimmel, E., Kerns, H.M.M., Radke, M., Hedges, J.F., Petrie, C., McCurley, P., Glee, P.M., Palecanda, A., Jutila, M.A., 2007. Select plant tannins induce IL-2Rα up-regulation and augment cell division in γδ T cells. J. Immunol. 179, 6468–6478.
Hsieh, M.F., Lai, S.L., Chen, J.P., Sung, J.M., Lin, Y.L., Wu-Hsieh, B.A., Gerard, C., Luster, A., Liao, F., 2006. Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. J. Immunol. 177, 1855–1863.
Ip, P.P., Liao, F., 2010. Resistance to dengue virus infection in mice is potentiated by CXCL10 and is independent of CXCL10-mediated leukocyte recruitment. J. Immunol. 184, 5705–5714.
Kou, Z., Quinn, M., Chen, H., Rodrigo, W.W.S., Rose, R.C., Schlesinger, J.J., Jin, X., 2008. Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. J. Med. Virol. 80, 134–146.
Lim, J.K., Lisco, A., McDermott, D.H., Huynh, L., Ward, J.M., Johnson, B., Johnson, H., Pape, J., Foster, G.A., Krysztof, D., Follmann, D., Stramer, S.L., Margolis, L.B., Murphy, P.M., 2009. Genetic variation in OAS1 is a risk factor for initial infection with West Nile virus in man. PLoS Pathol. 5, e1000321.
Mao, T.K., van de, W.J., Keen, C.L., Schmitz, H.H., Gershwin, M.E., 2002. Modulation of TNF-alpha secretion in peripheral blood mononuclear cells by cocoa flavanols and procyanidins. Dev. Immunol. 9, 135–141.
Mazzon, M., Jones, M., Davidson, A., Chain, B., Jacobs, M., 2009. Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J. Infect. Dis. 200, 1261–1270.
Nightingale, Z.D., Patkar, C., Rothman, A.L., 2008. Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. J. Leukoc. Biol. 84, 1028–1038.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hygiene 27, 493–497.
Rodriguez-Madoz, J.R., Bernal-Rubio, D., Kaminski, D., Boyd, K., Fernandez-Sesma, A., 2010a. Dengue virus inhibits the production of type I interferon in primary human dendritic cells. J. Virol. 84, 4845–4850.
Rodriguez-Madoz, J.R., Belicha-Villanueva, A., Bernal-Rubio, D., Ashour, J., Ayllon, J., Fernandez-Sesma, A., 2010b. Inhibition of the type I interferon response in human dendritic cells by dengue virus infection requires a catalytically active NS2B3 complex. J. Virol. 84, 9760–9774.
Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., Goda, Y., 2006. Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. J. Agric. Food Chem. 54, 884–892.
Shresta, S., Kyle, J.L., Snider, H.M., Basavapatna, M., Beatty, P.R., Harris, E., 2004. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J. Virol. 78, 2701–2710.
Su, X., Howell, A.B., D'Souza, D.H., 2010. The effect of cranberry juice and cranberry proanthocyanidins on the infectivity of human enteric viral surrogates. Food Microbiol. 27, 535–540.
Takeshita, M., Ishida, Y.i., Akamatsu, E., Ohmori, Y., Sudoh, M., Uto, H., Tsubouchi, H., Kataoka, H., 2009. Proanthocyanidin from Blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. J. Biol. Chem. 284, 21165–21176.
Terra, X., Valls, J., Vitrac, X., Merrillon, J.M., Arola, L., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., Blay, M., 2007. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J. Agric. Food Chem. 55, 4357–4365.
Valinotto, L.E., Diez, R.A., Barrero, P.R., Farias, J.A., Lopez, E.L., Mistchenko, A.S., 2010. Emergence of intratreatment resistance to oseltamivir in pandemic influenza A H1N1 2009 virus. Antiviral Ther. 15, 923–927.
Wati, S., Li, P., Burrell, C.J., Carr, J.M., 2007. Dengue virus (DV) replication in monocyte-derived macrophages is not affected by tumor necrosis factor alpha (TNF-{alpha}), and DV Infection induces altered responsiveness to TNF-{alpha} stimulation. J. Virol. 81, 10161–10171.
Zhuang, M., Jiang, H., Suzuki, Y., Li, X., Xiao, P., Tanaka, T., Ling, H., Yang, B., Saitoh, H., Zhang, L., Qin, C., Sugamura, K., Hattori, T., 2009. Procyanidins and butanol extract of Cinnamomi Cortex inhibit SARS-CoV infection. Antiviral Res. 82, 73–81.