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

Emily M. Kimmel, Maria Jerome, Jeff Holderness, Deann Snyder, Sharon Kemoli, Mark A. Jutila, and Jodi F. Hedges\*

Department of Immunology and Infectious Diseases, Montana State University, Bozeman, MT 59718

### 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 TNF $\alpha$  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.

### Keywords

procyanidins; dengue; PBMC; interferon; innate

## 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

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\*Corresponding author: Jodi F. Hedges, Department of Immunology and Infectious Diseases, Montana State University, 960 Technology Blvd., Bozeman, MT 59718, Phone: 406-994-6730, Fax: 406-994-4303, jodi.hedges@gmail.com.

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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 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<sup>®</sup>, 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; Holderness et al. 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 TNF $\alpha$  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 100,000g for 4 hours. 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 serially 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 50µl cMEM 24 and 48–72 hours after assay set up. The assay was terminated after 7 days incubation at 37° with 10% CO<sub>2</sub> by addition of 0.1% crystal violet in 20% methanol to each well. Virus was quantified (as TCID<sub>50</sub>/ml) by analyses of the pattern of cytopathic effect (CPE) adapted to sextuplet wells according to the method of Reed and Muench (Reed and Muench, 1938). Monomeric procyanidin and OPCs applied to BHK-21 cells from 1–20µg/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 hours 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 70ml of blood was collected per subject, 17ml whole blood was diluted 1:2 with Hanks buffer, and underlaid with 15ml Histopaque 1077 (Sigma) in 50 ml tubes. The tubes were centrifuged for 30 minutes at 500g. The resulting buffy layer containing PBMCs was aspirated and cells were washed once with Hanks buffer and centrifugation at 500g for 5 minutes, then RBCs were removed by water lysis, and an additional wash. 2–5×10<sup>6</sup> isolated cells were infected at 1–30 MOI (defined as BHK-21 TCID<sub>50</sub>/cell), as indicated depending on experiment in minimal volume for 2 hours 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<sup>6</sup> cells/ml. Crude extract of Applepoly®, the putative trimer or tetramer procyanidins [10µg/ml, isolated from Applepoly® by normal phase HPLC as previously described (Holderness et al. 2007)], monomeric procyanidin [catechin (Fisher) or epicatechin (Sigma), also 10µg/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 <20µg/ml were detected (Holderness et al. 2007; Holderness et al. 2008).

Cells were cultured for 48 hours 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 hour infection (n=1, a seronegative donor), samples were excluded from analyses. RNA was extracted from a

minimum of  $3 \times 10^6$  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 hours. 1000U/ml recombinant universal IFN I (PBL Interferon Source) or vehicle only was then added to cells for 20 minutes prior to collection of the cell pellets ( $5 \times 10^6$  cells per treatment) and storage at  $-80^\circ\text{C}$ . Cell pellets were lysed in 20 $\mu$ l of MPER (Pierce) with Halt Protease & Phosphatase Inhibitor (Pierce) per manufacture's suggestions on ice for 30 minutes. Lysates were centrifuged at 8000g for 2 minutes and 6 $\times$  Laemmli Sample buffer added to lysate supernatant fluids. Samples were boiled for 5 minutes, loaded onto a prepared 7.5% gel and run at 100 volts. Protein was transferred to PVDF at 100 volts for 1 hour. Blot was blocked in 3% BSA in TBST for 1 hour 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  $4^\circ\text{C}$  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 hour 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 (0.2M glycine, 3.4mM SDS, 0.01M Tween 20, pH 2.2), incubated for 10 minutes 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 (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 100U/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 hours cells were collected by gentle scraping, and stained with antibodies specific for CD11b/Mac-1 conjugated to PE (BD Pharmingen) and HLA-A,B,C conjugated to APC (clone W6/32, Biolegend) or CD86 conjugated to PE (clone IT2.2, BD Pharmingen). 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 (Figure 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<sup>®</sup> (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<sup>®</sup> extract (Holderness et al. 2007) was more effective at reduction of DENV titers than was the crude extract (Figure 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; Rodriguez-Madoz et al. 2010b), 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 hours in culture. Results in Figure 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, 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 (Figure 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 hours, then recombinant IFN I was added and cells were collected after 20 minutes 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.

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 (Figure 3A). *STAT1* expression is induced by very low concentrations of IFN I signaling and primes cells for innate responses after stimulation with IFN $\gamma$ , also critical for protection from DENV infection (Gough et al. 2010; Shrestha 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 MHC I expression pattern was unimodal, indicating the shift in expression occurred in all cells, regardless of virus infection (Figure 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.

TNF $\alpha$  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 TNF $\alpha$  production during DENV infection, we measured TNF $\alpha$  protein content in supernatant fluids from uninfected, OPC-treated, DENV-infected, and OPC17 treated/DENV-infected human PBMCs during both low and high MOI infections. The protein expression patterns were consistent with those observed in expression of *STAT1* transcripts. Namely, at very high MOI DENV infection, TNF $\alpha$  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 TNF $\alpha$  from DENV-infected cells. In contrast, at low MOI, DENV infection alone did not consistently alter detection of TNF $\alpha$  in culture supernatants, but the addition of OPCs caused a significant increase in TNF $\alpha$  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 (Figure 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 IFN $\gamma$ , and an increased expression of TNF $\alpha$  (Hober et al. 1996b; Nightingale et al. 2008; Wati et al. 2007). Our data in Figure 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 TNF $\alpha$  (Figure 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 TNF $\alpha$  are noted (Figure 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 TNF $\alpha$  which may eventually contribute to adverse pathology during DENV hemorrhagic disease (Chen et al. 2007; Hober et al. 1996b; Hober et al. 1996a). However, TNF $\alpha$  may also be beneficial for early innate immune protection. For example, treatment of DENV-infected monocytes with TNF $\alpha$  did not alter infection, but pre-infection supplementation of TNF $\alpha$  consistently decreased infection (Wati et al. 2007). Thus, OPC-induced expression of TNF $\alpha$  in bystander cells may be beneficial in limiting DENV infection. Whereas OPCs derived from cocoa have been shown to stimulate TNF $\alpha$  expression (Mao et al. 2002), addition of apple-derived trimeric OPCs to DENV-infected cells during high MOI infection decreased overt TNF $\alpha$  expression. Considering the adverse effects of excessive TNF $\alpha$  in DENV hemorrhagic disease and many other inflammatory conditions, OPC supplementation may provide benefit.

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 (Figures 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<sup>®</sup> 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 suggests that dietary supplements that contain high concentrations of OPCs, such as Applepoly<sup>®</sup>, may enhance antiviral innate immunity to benefit patients facing viral challenge.

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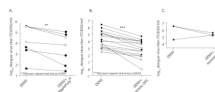
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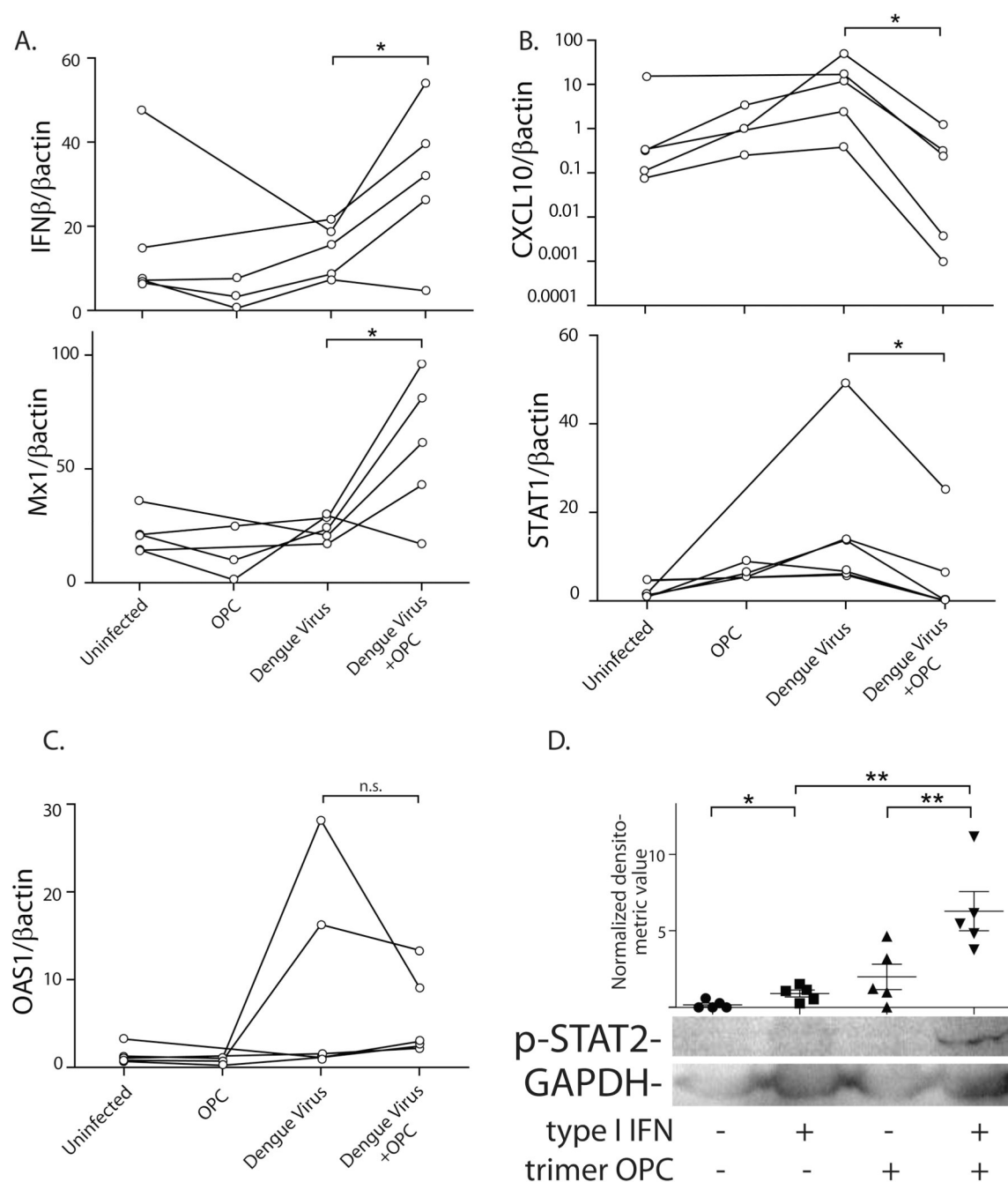
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**Figure 1.**

OPCs derived from Applepoly<sup>®</sup> 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<sup>®</sup> consistently resulted in lower titers as measured by endpoint titration. B. The putative trimeric OPCs purified from Applepoly<sup>®</sup> 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.

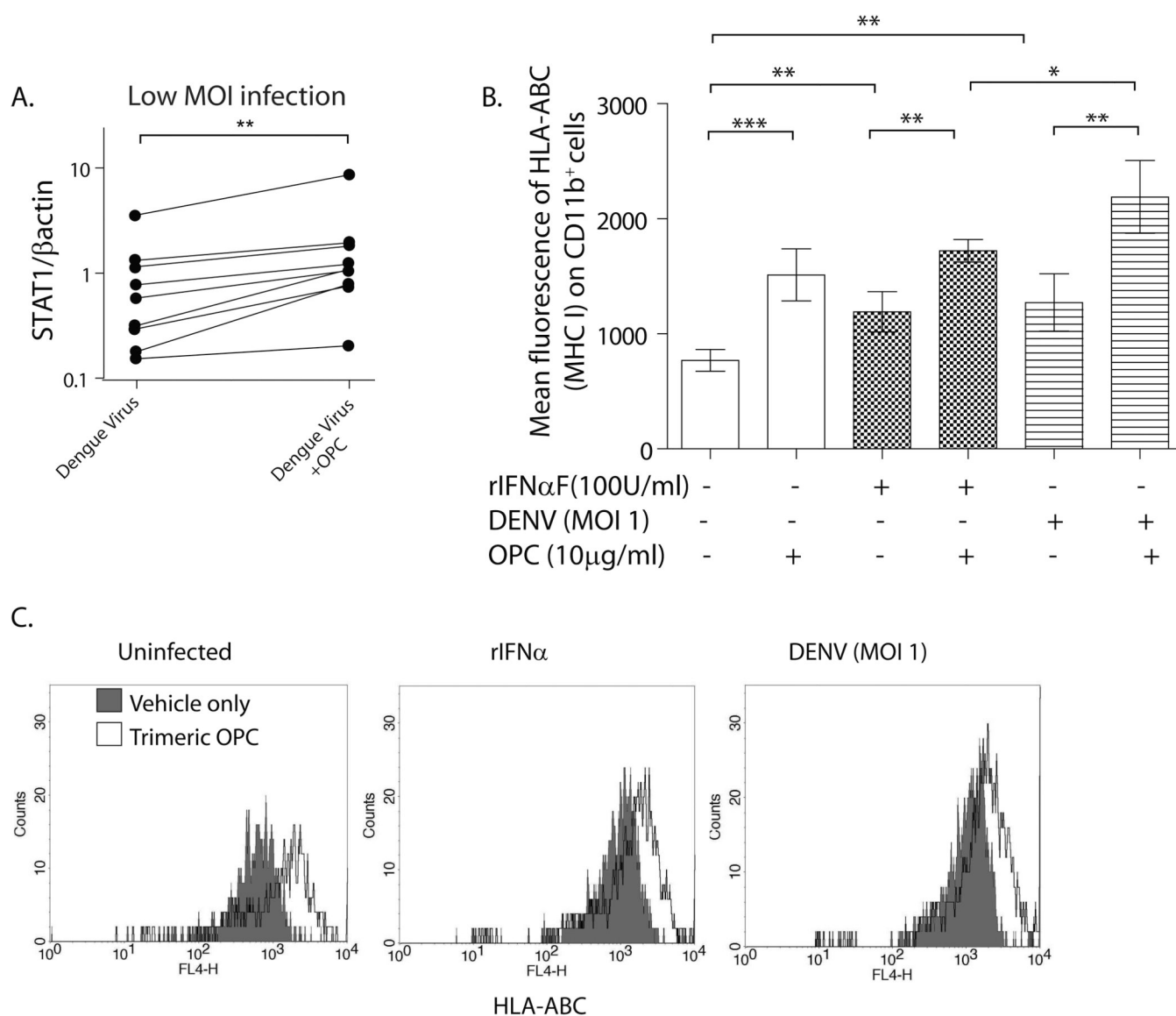


**Figure 2.**

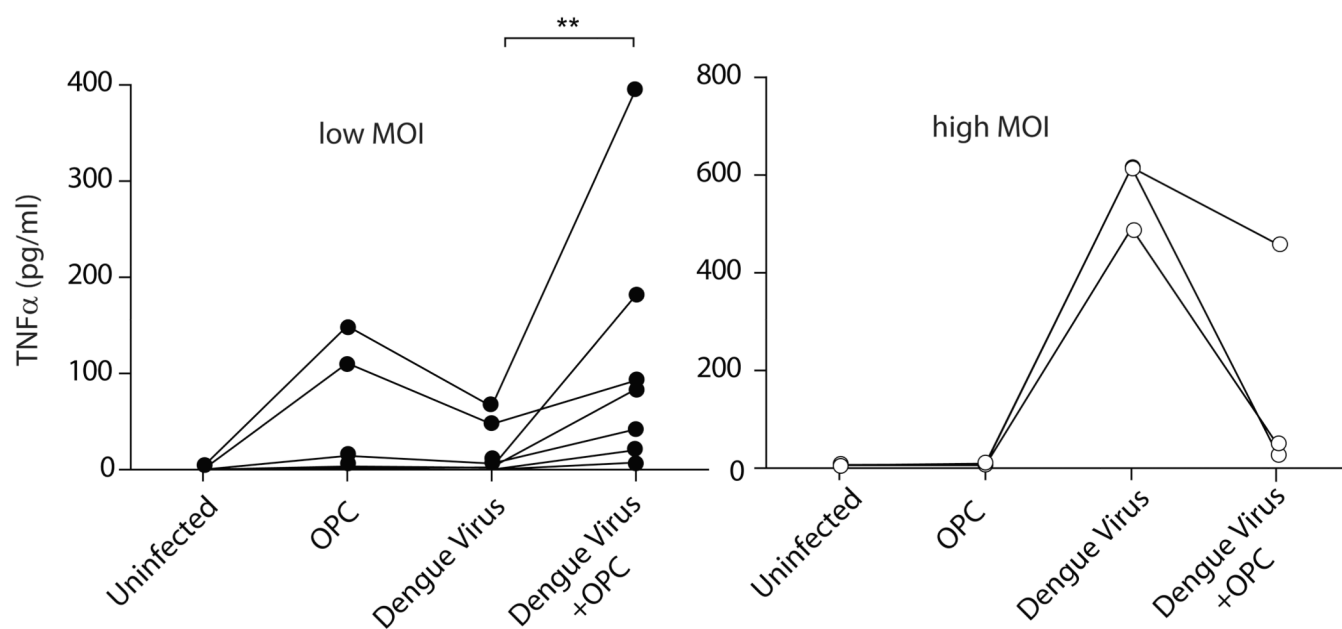
Addition of OPCs to high MOI DENV-infected human PBMC affects gene expression in the IFN I signaling pathway. A. Transcripts encoding *IFNβ* and *Mx1* remained unchanged in cells from most subjects in uninfected, OPC-treated and DENV-infected cells, but increased upon addition of OPCs to DENV-infected 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. \* $p < 0.05$ ,  $n = 5$ . D. *OAS1* transcript expression in response to DENV-infection and OPC addition is not consistent between human donors.  $n = 5$ . E. Phosphorylated STAT2 was only barely detectable 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.



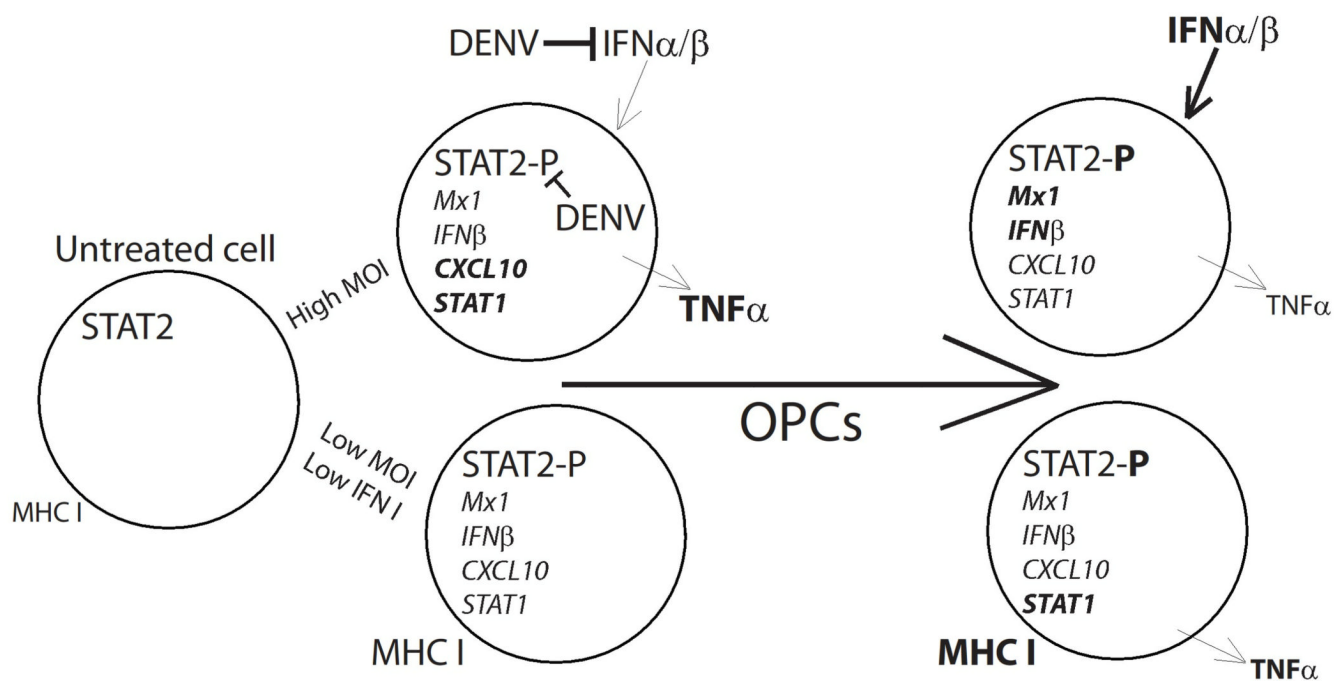
**Figure 3.**

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 (100U/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<sup>+</sup> (monocyte/macrophages) was measured by flow cytometry.  $*p < 0.05$ ,  $**p < 0.01$ . **C.** Representative histograms from one donor.



**Figure 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. \* $p=0.0156$ ,  $n=7$ . 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.



**Figure 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).