FOOD & FUNCTION

Carica papaya increases regulatory T cells and reduces IFN-γ^+CD4$^+$ T cells in healthy human subjects

Maha Abdullah[1,2], Pei-Shin Chai[1], Chiew-Yee Loh[1], Mun-Yee Chong[1], Huai-Wei Quay[1], Sharmili Vidyadaran[1], Zainina Seman[3], Mirnalini Kandiah[4] and Heng-Fong Seow[1]

[1] Immunology Unit, Department of Pathology, Faculty of Medicine and Health Sciences (FMHS), Universiti Putra Malaysia (UPM), Malaysia
[2] MAKNA-Cancer Research Laboratory, Institute of Biosciences, UPM, Malaysia
[3] Hematology Unit, Department of Pathology, FMHS, UPM, Malaysia
[4] Department of Nutrition and Dietetics, FMHS, UPM, Malaysia

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Fruit and vegetables have therapeutic potential as they dampen inflammation, have no known side-effects and as whole foods have prospective additive and synergistic benefits. Th1 (IFN-γ^+CD4$^+$)/Th2 (IL-4$^+$CD4$^+$) T cells play a vital role in mediating inflammatory responses and may be regulated by regulatory T cells (Tregs). Effects of *Carica papaya* on cells of healthy individuals were determined using flow cytometry methods. Significant down-regulation of IFN-γ^+CD4$^+$ ($p = 0.03$, $n = 13$), up-regulation of IL-4$^+$CD4$^+$ ($p = 0.04$, $n = 13$) T cells and up-regulation of CD3$^+$CD4$^+$CD25$^+$CD127$^-$ ($p = 0.001$, $n = 15$) Tregs were observed after papaya consumption. In vitro cultures showed up-regulation of Tregs in male subjects and was significantly associated with levels of IL-1β in culture supernatants ($R^2 = 0.608$, $p = 0.04$, $n = 12$). Other inflammatory cytokines were significantly suppressed. Papaya consumption may exert an anti-inflammatory response mediated through Tregs and have potential in alleviating inflammatory conditions.

Keywords:
Carica papaya / Cytokines / Immunoregulation / Inflammation / Regulatory T cells

Fruit and vegetables produce phytochemicals such as flavonoids and antioxidants, which modify the immune system [1] and lower markers of inflammation and oxidative stress [2], reducing risk of chronic diseases such as cancer and cardiovascular disease [3]. They have potential to improve conditions in autoimmune and transplantation diseases as phytochemicals suppress T-lymphocyte proliferation in vitro and in vivo [4]. Flavonoid such as naringenin [5], green tea polyphenol extract [6], encapsulated fruit and vegetable juice powder concentrate [7] were shown to reduce levels of inflammatory cytokines. Papaya (*Carica papaya* Linn) is low in calories, high in vitamins, minerals and a rich source of enzymes [8]. Its rich content of ascorbic acid provide anti-oxidative protection [9] and its shoot exhibit high levels of flavonoids such as quercenin and kaempferol [10]. Papaya is also appreciated worldwide for its flavour, nutritional qualities and digestive properties and valued for its medicinal and pharmacological properties including immunomodulatory potentials [8].

Regulatory T cells (Tregs) maintain homeostasis of the immune system and suppress immune responses of a wide range of immune cells, including T cells, natural killer and B cells. Their dysfunction causes fatal autoimmune disease, immunopathology and allergy [11]. Deficiencies in Tregs may also be linked with infertility, miscarriage and pre-eclampsia [12]. The potential role of Tregs in the resolution of diseases in which IFN-γ Th1$^+$ cells play a pathologic function is recognized [13]. The involvement of Tregs in the immunomodulatory effect of fruits and vegetable has not been studied. We examined the effect of papaya on Th1, Th2, Tregs and inflammatory cytokines in healthy human.

Correspondence: Dr. Maha Abdullah, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, UPM 43400, Serdang, Selangor, Malaysia
E-mail: maha@medic.upm.edu.my
Fax: +603-89412787

Abbreviations: PBMC, peripheral blood mononuclear cell; **TNF,** tumor necrosis factor; **Treg,** regulatory T cell

Apparently healthy individuals with no history of chronic or acute illness, no recent invasive procedures and not on medication were recruited. Six male and nine female subjects were recruited for the in vivo study. Mean age was 24 ± 1 year (range 23–26 years). Six male and six female subjects were recruited for the in vitro study. Mean age was 25 ± 3 year (range 21–32 years). The studies were approved by the Institutional Medical Research Ethics Committee, Universiti Putra Malaysia. Informed consents were obtained.

A 5-day experiment was designed. Food intake was controlled with standard meals consisting of bread/rice, chicken/fish, vegetables and liquid. The menu for day 3 and day 4 were replicates of day 1 and day 2, respectively. A pre-exposure period of 2 days (without papaya) was followed by 2 days with 100 g of fresh papaya fruit (fruit color index 4) in the day's three major meals. A dietary recall and medical call was conducted every day. A peripheral blood sample (20 mL) was collected in the morning before meal of day 3 (0 h) and day 5 (48 h).

Reagents and protocols for surface marker staining and intracellular cytokine staining for flow cytometry analysis are detailed in Supporting Information (Supplemental Method).

Cleaned, skinned and deseeded papaya fruits were weighed, homogenized and freeze-dried. Weighed lyophilized extract was reconstituted in culture medium and centrifuged at 3000 rpm for 5 min and then the supernatant filtered with a 0.22 μm syringe filter. Fresh preparation of this extract was used for each treatment.

Methods for peripheral blood mononuclear cells (PBMC) isolation and culture and method to determine cell viability are detailed in Supporting Information (Supplemental Method). For phenotyping analysis, triplicate wells containing 1×10^6 cells/mL PBMC were treated with 125, 1000, 4000 and 16 000 μg/mL papaya extracts. Plates were incubated at 37°C in 5% CO_2.

Pro-inflammatory cytokines in cultures were detected using BD^{TM} Cytometric Bead Array (BD Bioscience) kit with anti-IL-8, anti-IL-1β, anti-IL10, anti-tumor necrosis factor-α (TNF-α) and anti-IL-12p70. Standards and the BD FCAP Array^{TM} software were used to determine cytokine concentrations.

Non-parametric Wilcoxon matched pair test was used to compare paired groups. Spearman's correlation was used to determine association between variables. Statistical analysis was performed using SPSS (version 16.0). $p < 0.05$ was considered significant.

In vivo human study showed significant suppression of IFN-γ^+CD4$^+$ ($1.48 \pm 1.19\%$ versus $3.52 \pm 3.59\%$; $p = 0.03$, $n = 13$), up-regulation of IL-4$^+$CD4$^+$ ($2.08 \pm 2.52\%$ versus $1.44 \pm 2.08\%$; $p = 0.04$, $n = 13$) T cells and up-regulation of CD3$^+$CD4$^+$CD25$^+$CD127$^-$ ($9.01 \pm 8.20\%$ versus $5.30 \pm 4.22\%$; $p = 0.001$, $n = 15$) after 48 h consumption of papaya (Fig. 1). Flow cytometry plots are shown in Supporting Information (Figs. S1 and S2). No significant changes were observed for CD8$^+$ T cells. T-cell percentages were highly variable between individuals. We were unable to detect cytokines in plasma of subjects, using the Cytometric Bead Array kit.

In vitro treatment with papaya extract maintained PBMC cultures at high viability (>80%) at 6, 24 and 48 h exposure (Supporting Information, Fig. S3). While no effect was observed on Tregs in general, a significant increase in CD4$^+$CD25$^+$CD127$^-$ Tregs was observed among male subjects at 4000 μg/mL after 48 h of treatment (Fig. 2).

IL-8, IL-6, IL-10 and TNF cytokines, but not IL-1β were significantly suppressed in PBMC supernatants after 48 h treatment with papaya (Fig. 3A). IL-1β was higher in male subjects (Fig. 3B) and percentage changes correlated

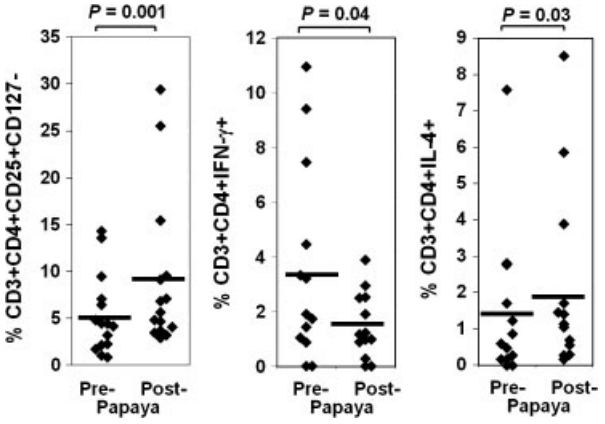

Figure 1. Percentage of CD25$^+$CD127$^-$ ($n = 15$) Tregs and CD4$^+$ T cells expressing intracellular IFN-γ^+ ($n = 13$) and IL-4$^+$ ($n = 13$) in blood samples of healthy individuals before (Pre-) and after (Post-) papaya consumption.

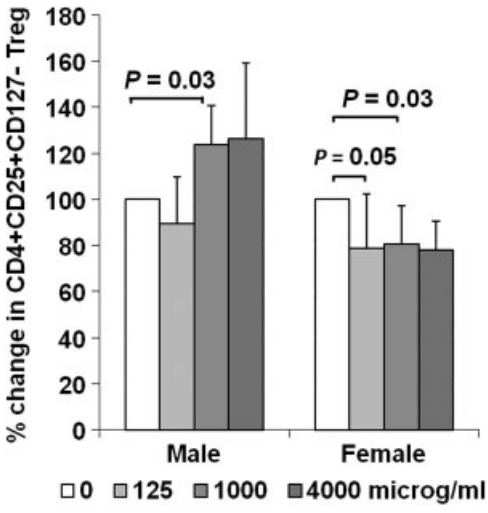

Figure 2. Percentage change in CD4$^+$CD25$^+$CD127$^-$ regulatory T cells in male ($n = 6$) and female ($n = 6$) subjects after 48 h treatment with papaya. Results are expressed in mean ± SD(%).

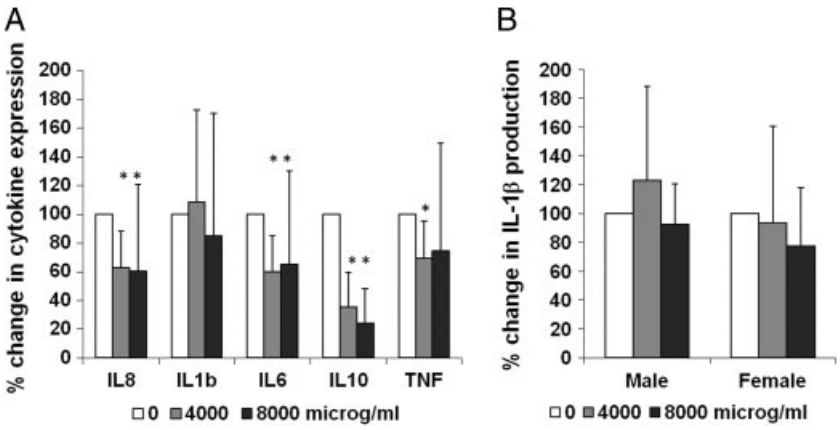

Figure 3. Percentage change in (A) cytokines production ($n = 10$) and (B) IL-1β production in male ($n = 5$) and female ($n = 5$) subjects, in supernatant of cultured PBMC after 48 h treatment with papaya. Results are expressed in mean ± SD(%).

positively with Tregs ($R^2 = 0.770$, $p = 0.009$, $n = 10$) at 48 h of treatment (results from papaya treatment at 4000 and 16 000 μg/mL). No significant correlation was observed among female subjects.

In blood of healthy volunteers, $CD25^+CD127^{lo}$ cells may comprise 7–8% [14] of $CD4^+$ T cells. In chronic hepatitis C infection, an increased percentage of $CD4^+CD25^{hi}$ Tregs (4.79% versus 2.08%) versus healthy controls may impair viral effector T-cell responses [15]. In asthma patients, increase of several percentages of $FoxP3^+CD4^+$ cells can lead to severe symptoms (4.18% versus 1.68%) [16]. Thus, Tregs are detected in low levels in the blood but small changes may induce significant damages. A study has also shown that Th2 cells are less susceptible than Th1 to the suppressive activity of $CD25^+$ Tregs [17], thus skewing toward a decreased Th1/Th2 ratio observed in allergies. This skewness was also seen in this study.

The significant suppression of IFN-γ in vivo and IL-8, IL-6, IL-10 and TNF-α in vitro by papaya correlated with the anti-inflammatory effect of fruit and vegetable shown to reduce levels of IFN-γ [5, 6, 7], IL-2 [5], IL-6 [2] and TNF-α [2, 5, 6]. $CD4^+CD25^{hi}$ Tregs co-cultured with $CD4^+CD25^-$ cells suppressed secretion of IFN-γ (and IL-13) [18].

Increased $CD4^+IL\text{-}4^+$ Th2 cells with papaya consumption is consistent with report showing enhanced levels of IL-4 (and IL-2) in serum after tomato juice consumption [19]. Papaya may enhance the role of Th2 in mediating humoral immunity and protecting against infection and parasites.

While Tregs in general, were hyporesponsive to stimulation [14, 18] intermediate concentration of papaya was observed to stimulate Tregs in standard in vitro culture system in male samples. The immune system of men and women are not equal as women are more resistant to infection, mount stronger humoral responses to vaccines and reject allografts and tumors more successfully. Various cells of the immune system express estrogen receptors and increased estrogen levels augment immune responses including the production of IL-6, IL-10 and IFN-γ [20]. Isolating PBMC from blood removes the influence of sex hormones on cells and may have influenced the different results observed in female samples implying different mechanisms in Treg induction.

Levels of IL-1β increased with percentage of Tregs in PBMC cultures of male subjects. Functional IL-1β is essential in development of a Th2-mediated immunity [21]. Another study showed expression of IL-1β receptors (IL-1R1) on activated human FoxP3 Tregs [22]. Our results support the implied role of IL-1β and Tregs in upregulating Th2.

The benefits of fruit and vegetables as whole foods have been undermined. The additive and synergistic effects of a complex mixture of phytochemicals are responsible for the potent antioxidant and anticancer properties. Furthermore, individual antioxidants studied in clinical trials do not appear to have consistent preventive effects [3]. Although synthetic drugs such as steroidal anti-inflammatory drugs and NSAIDs are currently used to treat acute inflammation, these drugs have not been successful in curing chronic inflammatory diseases. They also cause side-effects with serious consequences as exhibited by the recently withdrawn Vioxx and Celebrex [23]. Thus, the benefits from consumption of fruits such as papaya on regulation of immunity and inflammation warrants further investigation.

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