Immune Effects of Cocoa Procyanidin Oligomers on Peripheral Blood Mononuclear Cells

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There has been considerable work on the relationships between nutrition and the immune response, particularly on studies that have focused on adaptive responses. There is increasing recognition of the importance of innate immunity in host protection and initiation of cytokine networks. In this study, we examined the effect of select cocoa flavanols and procyanidins on innate responses *in vitro*. Peripheral blood mononuclear cells (PBMCs), as well as purified monocytes and CD4 and CD8 T cells, were isolated from healthy volunteers and cultured in the presence of cocoa flavanol fractions that differ from another by the degree of flavanol polymerization: short-chain flavanol fraction (SCFF), monomers to pentamers; and long-chain flavanol fraction (LCFF), hexamers to decamers. Parallel investigations were also done with highly purified flavanol monomers and procyanidin dimers. The isolated cells were then challenged with lipopolysaccharide (LPS) with quantitation of activation using CD69 and CD83 expression and analysis of secreted tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, and granulocyte macrophage colony-stimulating factor (GM-CSF). The chain length of flavanol fractions had a significant effect on cytokine release from both unstimulated and LPS-stimulated PBMCs. For example, there was a striking increase of LPS-induced synthesis of IL-1β, IL-6, IL-10, and TNF-α in the presence of LCFF. LCFF and SCFF, in the absence of LPS, stimulated the production of GM-CSF. In addition, LCFF and SCFF increased expression of the B cell markers CD69 and CD83. There were also unique differential responses in the mononuclear cell populations studied. We conclude that the oligomers are potent stimulators of both the innate immune system and early events in adaptive immunity.
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Introduction

The innate immune system constitutes the first line of defense against pathogens. In addition to providing physical and chemical barriers, innate immunity consists of several specific immune cells, including monocyte/macrophages, natural killer (NK) cells, and polymorphonuclear cells. Target recognition by these cells is pathogen-nonspecific and dependent on highly conserved macromolecules found on microorganisms (1, 2). These structures, known as pathogen-associated molecular patterns, include surface molecules such as lipopolysaccharide (LPS) and lipotechoic acid found in gram-negative and gram-positive bacteria.

The innate immune system also plays a critical role in initiating the adaptive immune response (3, 4). These effects are partly mediated by cytokines, the pattern of which largely depends on the types and combinations of toll-like receptors that are engaged by a specific type of pathogen (5). These cytokine profiles, along with other factors such as efficient antigen presentation, may promote an early and vigorous response by the CD4 and CD8 T cells of the adaptive immune system (6). Also, the early cytokine profile will regulate the determination of a Th1 or Th2 cellular immune response (7). Thus, study of innate immunity is an important bridge between several disciplines, including cardiovascular disease, cancer, and autoimmunity (8, 9). Previously overlooked, the idea is becoming increasingly hypothesized that specific dietary components, in particular flavanols, and their oligomeric forms, the procyanidins, can have a beneficial influence on a variety of early immune responses.

Cocoa is a flavanol-rich food that contains monomeric flavanols, such as epicatechin and catechin, and their oligomeric derivatives known as procyanidins (these oligomers can be larger than 10 subunits; Refs. 10–12). Cocoa extracts enriched in these flavanols and procyanidins have been shown to modulate immune cells (peripheral blood mononuclear cells [PBMCs] and in macrophages) and to alter cytokine and chemokine production (13–17). To further characterize cocoa's direct influence on immune cells, we directly quantified the activation of PBMCs using

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the surrogate markers CD69 and CD83, and also quantitated the production of specific cytokines in the presence or absence of LPS with defined concentrations of short or long oligomers of cocoa flavonoids (18, 19). Based on this data, we then studied the direct effect of cocoa flavanols and procyanidins on cytokine production on purified CD4 and CD8 populations. We report herein that immune cells have a differential response to the various cocoa flavanols and procyanidin oligomers. Moreover, flavanol-induced cytokine secretion differs depending on size and structure (hydroxyl modification).

Materials and Methods

Human Subjects. Forty milliliters of peripheral blood was drawn after overnight fasting from 10 healthy male volunteers ranging in age from 22 to 28 years old. Samples were drawn into citrate-containing tubes and mixed 1:1 with Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) without addition of calcium and magnesium. Subjects were asked to refrain from the use of dietary supplements and multivitamins for a minimum of 30 days prior to this study. Blood samples were layered over Histopaque-1077 gradient and centrifuged at 500 *g* for 30 mins at room temperature. PBMCs were then harvested, washed twice in HBSS, and counted using a hemocytometer and an estimation of viability by a trypan blue exclusion assay. Viability was consistently greater than 95%. Cells were finally resuspended in serum-free X-VIVO 15 (BioWhittaker, Walkersville, MD) and numbers were adjusted to 2×10^6 cells/ml.

Isolation of Monocytes, CD4 T Cells, and CD8 T Cells. CD4+ and CD8+ cells were isolated by positive selection. Briefly, 10^7 PBMCs in 90 μl were incubated with 10 μl of either anti-CD4+ or CD8+ magnetic reagent and incubated at 4°C for a half hour. The cells were then separated by magnetic cell-sorting using a Miltenyi Biotec (Auburn, CA) system and were counted and diluted to a concentration of 2×10^6 cells/ml with BioWhittaker X-VIVO 15 (Walkersville, MD). Autologous plasma from the subject was added to a concentration of 1% by volume. A 96-well plate was treated with anti-human CD3 in phosphate-buffered saline (PBS; BD Biosciences, San Diego, CA) at a concentration of 0.5 μg/ml for 4 hrs. After the 4-hr incubation, the plate was washed 4 times with PBS. Purified CD4+ or CD8+ cells were added to each well along with various concentrations of the individual cocoa compounds or controls. The cultures were then incubated at 37°C for 1 day, and supernatants were harvested and stored at −80°C.

For isolation of monocytes from PBMCS, we used the monocyte isolation kit (Miltenyi Biotec), which involved an indirect magnetic labeling system *via* magnetic depletion of T cells, NK cells, B cells, DCs, and basophils. The purity of negatively-selected monocytes was assessed by flow cytometry and found to be 95% pure. Cells were resuspended at 2×10^6 cells/ml in X VIVO 15 medium (Cambrex Bio Science) supplemented with penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were then dispensed at 2×10^5 cells/well into individual wells of 96-well flat-bottom microtiter plates (Corning, Acton, MA). Equal volumes of media containing the appropriate compound and controls was added to triplicate wells of monocyte cultures. Cultures were incubated at 37°C for 72 hrs, pulsed with 1.0 μCi of tritiated thymidine for 12 hrs, harvested, and counted using a Wallac 1205 Betaplate LCS counter (LKB/Wallac, Gaithersburg, MD).

Purification of Cocoa Flavanols by Preparative Normal-Phase HPLC. Approximately 0.7 g of semi-purified cocoa extract was dissolved in 7 ml of acetone/water/acetic acid in volume ratios of 70:29.5:0.5, respectively using previously described methods (11). Separations were performed at room temperature using a 5-μm Supelcosil LC-Si 100 Å HPLC column (Sigma-Aldrich, St. Louis, MO) and the flavanols eluted by linear gradient solvent system. The separation of monomers and oligomers was monitored by UV absorbance at 280 nm and fractions collected at intervals between peaks (corresponding to oligomers). Fractions with equal retention times from several preparative separations were combined, evaporated under partial vacuum, and freeze-dried. Each fraction was resuspended in sterile PBS at a final concentration of 5 mg/ml. All collected fractions representing the monomeric flavanols and the procyanidin oligomers with up to 5 monomeric subunits (i.e., dimers, trimer, tetramers, and pentamers) were combined and designated short-chain flavanol fraction (SCFF). All fractions containing procyanidin oligomers that contained from 6 to 10 monomeric subunits (heptamers to decamers) were combined and designated long-chain flavanol fraction (LCFF).

Additional highly purified flavanols and procyanidins, including (−)epicatechin, (+)catechin, and procyanidin dimer B2 and procyanidin B5 were isolated from cocoa to a purity of >95%. In addition, 3′-*O*-methyl-(−)epicatechin and 4′-*O*-methyl-(−)epicatechin were synthesized and purified to a purity <99%. The procyanidin dimers A1 and A2 were isolated from peanut skins and purified to a level >96%. All the fractions and purified materials were provided by Mars Inc. (Hackettstown, NJ).

Culture Conditions. PBMCS or purified T cells were placed in either 96-well round bottom culture plates (2×10^5 cells/well) or 48-well culture plates (Corning, Corning, NY) at a final concentration of 1×10^6 cells/well. Cells were cultured in triplicate with the addition of serial dilutions (20, 2.0, or 0.2 μg/ml) of each cocoa flavanol fraction or a saline control for 16 hrs. LPS (100 μg/ml) was then added. Cells for fluorescent-activated cytometric scan (FACS; BD Biosciences, San Jose, CA) analysis were harvested 2 hrs post–LPS challenge. Supernatant fractions for cytokine analysis were harvested after 24 hrs of LPS challenge.

Cytokines and FACS Analysis. Cytokine profiles were determined using a BD Cytometric Bead Array Human

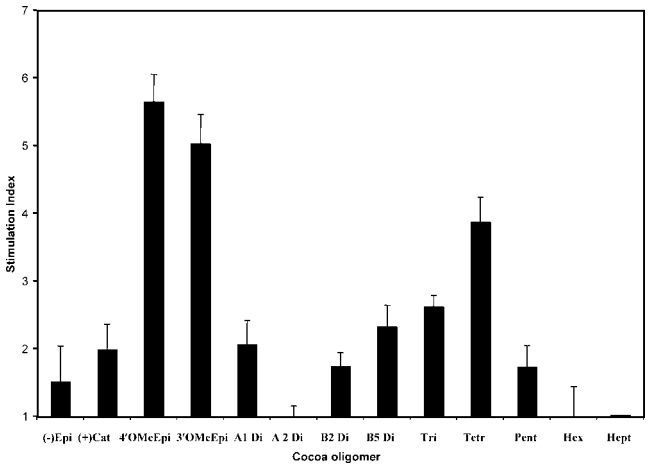

Figure 1. 72-hr proliferation data of purified monocyte cultures from individuals treated with 2.0 μ*M* of (–)epicatechin, (+)catechin, 4′OMe-epicatechin, 3OMe-epicatechin, A1 dimer, A2 dimer, B2 dimer, B5 dimer, trimer, tetramer, pentamer, hexamer, and heptamer in triplicate wells. Results are reported as a stimulation index (treatment value by media control value). $N = 5$.

Inflammation Kit (BD Biosciences). Briefly, 50 μl of culture supernates and standards was added to 100 μl of capture beads and detection reagent. Tubes were incubated for 3 hrs at room temperature, washed with 1 ml of wash buffer, and centrifuged prior to collection of the supernate. Wash buffer (300 μl) was added and samples immediately analyzed on the flow cytometer after vortexing. Known positive and negative samples and a standard curve were included with each assay and all samples analyzed in triplicate. In addition, cultured cells (1×10^6) were washed in PBS with 0.1 % bovine serum albumin (PBS/BSA), resuspended in 100 μl PBS/BSA, and incubated with anti-human CD3, CD19, CD69, and CD83 for 1 hr at 4°C. Cells were resuspended in 1.0 ml PBS/BSA and analyzed on the flow cytometer. Isotype controls as well as known positive and negative samples are included in each assay.

Statistical Analysis. Analysis of variance (ANOVA) models were used to compare the induced cytokine levels in each of the 6 treatment conditions (with the purified compounds, there are more than 6 treatments; SCFF, LCFF, or control treatment, each with or without LPS challenge). Values not following a normal distribution were transformed using the following methods: square root, logarithmic, nonparametric ranking, and 1/4 power transformations when appropriate. Values were then back-transformed. If the results of the overall F test in the analysis of variance were significant, pairwise comparisons were made in order to identify which group differed from the other. All analyses were two-tailed, and P values <0.05 were considered statistically significant.

Results

Proliferation of Monocytes and Cytokine Profiles of PBMCs. Purified monocyte cultures were treated with 13 different flavanols and procyanidins. Both 3′-*O*-methyl- and 4′-*O*-methyl-(–)epicatechin–treated monocytes generated approximately 3-fold the proliferation levels of unmodified epicatechin (Fig. 1). As the size of the oligomer increased, from dimers to tetramers, there was a corresponding increase in stimulation (the exception appears to be procyanidin A2 dimers). The larger oligomers, pentamers through heptamers, demonstrated no significant stimulation.

Supernates from PBMCs cultured with different concentrations of SCFF, LCFF, and a medium control, with or without LPS challenge, were analyzed and concentrations of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, and GM-CSF measured as described above (Table 1). Treatment of PBMCs with 20 μg/ml of LCFF or SCFF was shown here because of its most significant result compared with other concentrations. LCFF or SCFF alone did not significantly affect the synthesis of TNF-α compared to incubation with medium alone. Both LCFF and SCFF significantly increased the LPS-induced production of TNF-α (432.3 ± 122.2) to 820.8 ± 100.5 and 587.9 ± 136.8 pg/ml, respectively ($P = 0.0001$ and $P = 0.0004$, respectively). After incubation with 20 μg/ml of LCFF, IL-1β levels were increased significantly by 36.5 ± 11.8 pg/ml compared with the medium control value of 11.5 ± 4.2 pg/ml, and, when activated by the addition of LPS, were 83.8 ± 19.1 pg/ml higher than with LPS alone (28.4 ± 8.1 pg/ml). The SCFF plus LPS treatment had a smaller but still significant increase over LPS media control. In the absence of LPS, levels of IL-6 after treatment of PBMCs with LCFF or SCFF were low at all concentrations, and were not significantly different from those observed in the medium control. In contrast, following LPS challenge, LCFF treatment was associated with IL-6 levels of 1338 ± 224 pg/ml versus LPS control values of 720 ± 147 pg/ml ($P < 0.001$). Minimal concentrations of IL-10 were detected in the culture supernates of PBMCs treated with 20 μg/ml of LCFF, SCFF, or medium alone. When combined with LPS, however, treatment with 20 μg/ml LCFF and SCFF resulted in the release of 47.2 ± 24.6 pg/ml and 76.6 ± 53.5 pg/ml of IL-10 over medium control, representing a 71% and 116% increase, respectively, compared with treatment with LPS alone (66.2 ± 27.7 pg/ml; $P = 0.013$ and 0.033, respectively). We note that IL-10 was the only cytokine for which SCFF was a stronger stimulus than LCFF; however, the difference between the two treatments did not reach statistical significance. We note the significant standard error following LPS stimulation. In the absence of LPS, treatment of PBMCs with both SCFF and LCFF resulted in significant induction of GM-CSF compared with medium alone (65.2 ± 25.4 pg/ml and 268.2 ± 134.4 pg/ml, respectively, vs. 13.6 ± 2.0 pg/ml). The increases induced by LCFF and SCFF were significantly higher than those seen with the media control. The addition of LPS to treatments of specific oligomeric fractions from cocoa resulted in 524 ± 204.1 and 280 ± 331 pg/ml, respectively, in GM-CSF levels compared with the value obtained for the control LPS media (85.6 ± 24.6 pg/ml). The concentration

Table 1. Cytokine Secretion (pg/ml) in the 20 μg/ml Treatment of LCFF and SCFF in Unchallenged and LPS-Challenged PBMCs[a]

		Mean ± SD			
Cytokine	Oligo	Media	Cocoa	Media + LPS	Cocoa + LPS
TNF-α	LCFF	Nd[b]	Nd	432 ± 122	820.8 ± 100.5***
	SCFF	Nd	Nd	432 ± 122	587.9 ± 136.8***
IL-1β	LCFF	11.5 ± 4.2	36.5 ± 11.8**	28.4 ± 8.1	83.8 ± 19.1**
	SCFF	11.5 ± 4.2	23.5 ± 7.5**	28.4 ± 8.1	37.4 ± 6.9**
IL-10	LCFF	8.9 ± 2.6	11.5 ± 2.4	66.2 ± 27.7	113.4 ± 16.5**
	SCFF	8.9 ± 2.6	14.7 ± 5.3	66.2 ± 27.7	142 ± 62.3**
IL-6	LCFF	14.7 ± 5.8	22.9 ± 10.2	720 ± 147	1338 ± 224***
	SCFF	14.7 ± 5.8	28.5 ± 5.3**	720 ± 147	795 ± 144.7
GM-CSF	LCFF	13.6 ± 2.0	268.2 ± 134.4**	85.6 ± 24.6	524.1 ± 204.1**
	SCFF	13.6 ± 2.0	65.2 ± 25.4**	85.6 ± 24.6	280.6 ± 331

[a] *P* values were achieved by using Student's *t* test to compare the media versus the cocoa or media + LPS versus cocoa + LPS treatments for a given cytokine.
[b] Nd, values were below, or equal to, the detection limit.
** $P < 0.01$; *** $P < 0.001$.

of GM-CSF seen after incubation with LCFF combined with LPS was significantly increased compared with treatment with LPS alone ($P = 0.004$), whereas the difference between LPS alone and LPS combined with SCFF did not reach statistical significance. Again, the induction of GM-CSF seen in LCFF-treated cells was higher than with SCFF, but the difference did not reach statistical significance.

Interestingly, we observed dose-dependent increases in the secretion of TNF-α, IL-1β, and IL-6 after treatment with LCFF combined with LPS, and in the release of TNF-α and IL-10 after incubation with SCFF plus LPS (data not shown) for the three concentrations studied (0.2, 2.0, and 20 μg/ml). However, significant differences in the results obtained with LPS alone were observed only at the 20 μg/ml concentrations of LCFF and SCFF.

Cytokine Profiles of Treated CD4 and CD8 T Cells. Isolated CD4 and CD8 T cells activated with anti-CD3 were treated with (−)epicatechin, 3′-*O*-methyl-(−)epicatechin, B5 dimer, and hexamers and assayed for cytokine production. Activated CD4 T cells treated with the different cocoa compounds reflected increased levels of cytokine production over media control. 3′-*O*-methyl-(−)epicatechin–treated CD4 T cells demonstrated significantly higher levels of interferon (IFN)-γ, TNF-α, and IL-10 over media control and (−)epicatechin-treated cells. CD8 T cells in response to (−)epicatechin and 3′-*O*-methyl-(−)epicatechin produced significantly higher amounts of IFN-γ over the media controls (data not shown).

Activation of Cell Surface Markers. Data on cell surface markers are reported as mean fluorescence intensity (MFI), which is regarded as a measure of receptor density, and percentage of cells expressing the respective activation markers. A significant staining for CD69 after incubation with the cocoa fractions was observed on CD19+ B cells. A dose-response analysis was completed for both cocoa treatments (SCFF and LCFF) in LPS-challenged and unchallenged cells. LPS treatment did not affect expression of CD 69 (LPS data not shown). Treatment with LCFF was associated with a dose-dependent increase in CD69 expression on B cells, with the concentration of 20 μg/ml giving the highest MFI (Fig. 2A). There was also a greater than 2-fold increase in the percentage of CD69+ B cells following LCFF treatment compared with incubation with medium only (57% and 23%, respectively; Fig. 2B). The MFI and percentage of B cells expressing CD69 was consistently lower after SCFF than after LCFF treatment (Fig. 2A and B). No statistically significant changes in the intensity of staining or the number of CD69-expressing cells were observed for either of the cocoa treatments after LPS challenge (data not shown). B cells were the only PBMC subpopulation to demonstrate significant surface expression of CD83 following cocoa treatment. Similarly to CD69 expression, CD83 expression was enhanced by LCFF treatment in a dose-dependent manner, with the highest concentration tested (20 μg/ml) resulting in the greatest induction (Fig. 3). Compared with the medium control, the percentage of CD83+ B cells was significantly elevated in PBMCs treated with the highest concentration of LCFF ($P = 0.049$), whereas the increase after incubation with SCFF did not reach statistical significance (52% in control cells, 80% with LCFF, and 60% with SCFF; Fig. 3B). Again, when PBMCs were challenged with LPS, neither of the cocoa procyanidin treatments caused a significant change in staining profiles.

Discussion

The consumption of foods rich in flavonoids have been purported to decrease certain risk factors for cardiovascular disease (20). Although the cardioprotective mechanism(s) of flavonoid-rich foods remains to be determined, studies support that flavonoids, and that cocoa flavanols and procyanidins in particular, may confer vascular benefits

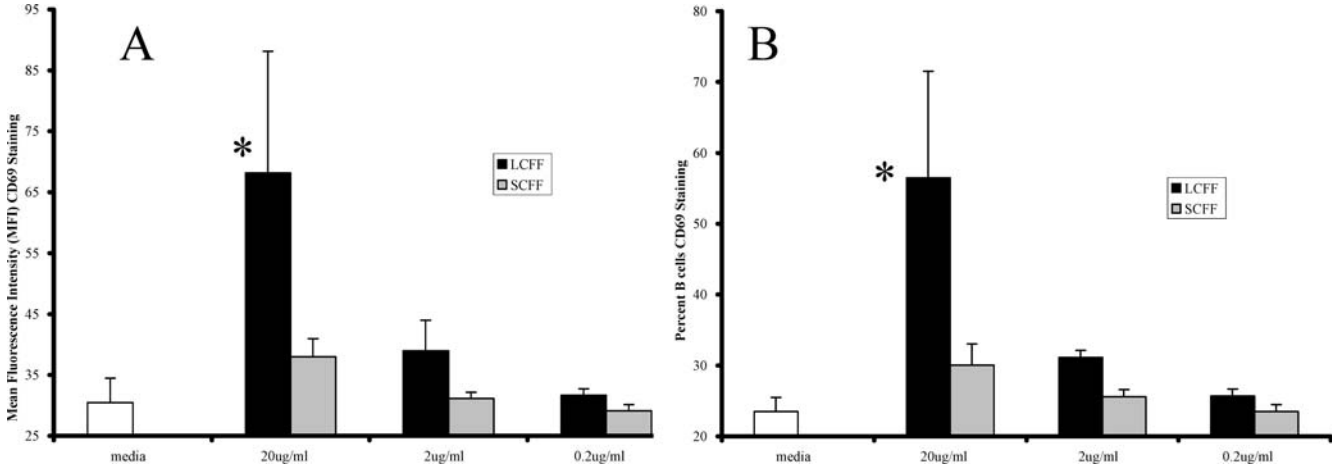

Figure 2. (A) MFI of CD 19–stained B cells expressing CD 69 after 16-hr treatment with LCFF (black bars) or SCFF (gray bars). *Significant differences ($P < 0.05$) between cocoa treatment and media control (white bar). (B) Percentage of CD 19–stained B cells expressing CD 69 after 16-hr treatment with LCFF (black bars) or SCFF (gray bars). *Significant differences ($P < 0.05$) between cocoa treatment and media control (white bar).

via mechanisms such as improving endothelium-dependent relaxation (21, 22), increasing the bioavailable pool of nitric oxide (21, 22), reducing platelet aggregation (23, 24), modulating the immune system through changes in cytokine and eicosanoid levels involved in the inflammatory response (13, 25, 26), and reducing low-density lipoprotein oxidation (27).

Raw cocoa contains monomeric flavanols (epicatechin and catechin) and oligomeric procyanidins. The oligomerization and interflavan linkages are features of procyanidin structure and chemistry that may contribute to the array of biological (cardiovascular or immunologic) effects reported for these compounds. The antioxidant capacity of flavanols is thought to be the mechanism that is immediately involved in many of these processes. For example, in Jurkat T cells and RAW 264.7 macrophages, transcription factor NF-kB activation is sensitive to oxidant stimuli; the smaller flavanols have been found to inhibit production of oxidative products in these cells, resulting in decreased NF-kB activation (28, 29). The larger flavanol oligomers have shown reduced bioavailability; however, their pharmacologic and immune-modulating properties may still be relevant (30). A major and unresolved issue is the likelihood that individual oligomers in mixtures may exhibit synergistic or antagonistic effects on the immune parameters studied herein. This is an issue that will require further study.

Previous work has suggested that individual monomers and oligomers (dimers through decamers) of cocoa flavanols can modulate PBMC production of TNF-α, IL-1β, IL-2, IL-4, IL-5, and transforming growth factor (TGF)-β in the absence or presence of the T-cell mitogen phytohemagglutinin (PHA; Refs. 13–17, 31, 32). The nature and extent of this modulation is strongly dependent on degree of

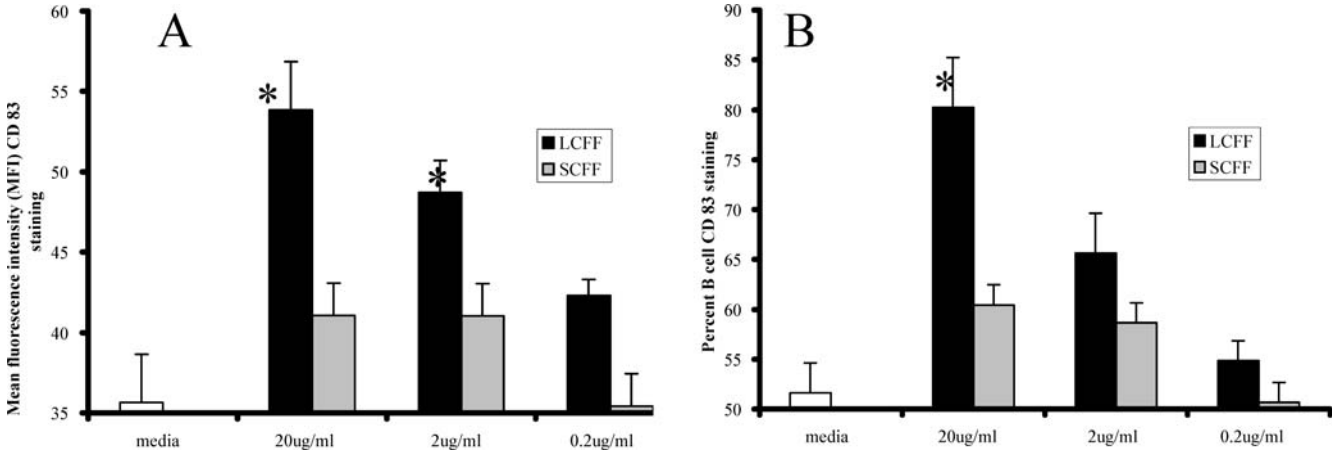

Figure 3. (A) MFI of CD 19–stained B cells expressing CD 83 after 16-hr treatment with LCFF (black bars) or SCFF (gray bars). *Significant differences ($P < 0.05$) between cocoa treatment and media control (white bar). (B) Percentage of CD 19–stained B cells expressing CD 83 after 16-hr treatment with LCFF (black bars) or SCFF (gray bars). *Significant differences ($P < 0.05$) between cocoa treatment and media control (white bar).

polymerization of the procyanidins. Cocoa procyanidins, like other flavonoids, can be powerful antioxidants *in vitro,* but there are mixed reports of clear antioxidant effects *in vivo* (33). A number of redox-sensitive signal transduction pathways are involved in triggering a variety of innate immune responses (34). Although the existing research suggests that cocoa flavanols and procyanidins might modulate innate immunity, only limited studies have addressed this issue (32, 35, 36). To address this issue, we first looked at the capability of the cocoa compounds to stimulate purified monocyte cultures. Second, we determined the direct effect of the cocoa compounds on activated CD4 and CD 8 T cells. Lastly, we used LPS to simulate bacterial activation of the innate immune system and assessed the *in vitro* effects of cocoa flavanols and procyanidins on cytokine production.

An important finding herein is our observation that smaller oligomer cocoa compounds, and especially the 3′- and 4′-*O*-methylated (–)epicatechins, *in vivo* metabolites of epicatechin, induce monocyte/macrophage cultures to proliferate. The finding that specific factors augment macrophage longevity is significant because macrophages are integral in the early signaling in many innate immune scenarios. Macrophage-derived IL-1, IL-6, IL-8, IL-12, and TNF-α are the main factors that initiate local tissue inflammation. Moreover, along with facilitating antigen presentation, these cytokines also activate T cells. Consequently, macrophages constitute a bridge between innate resistance and specific immunity (37). Secondly, we find that (–)epicatechins and 3′-*O*-methyl epicatechin can directly induce increase IFN-γ, TNF-α, and IL-10 cytokines in activated CD4 cells and IFN-γ in activated CD8 T cells. These data demonstrate that these compounds can directly act on T cells independent of the influences of macrophage signaling, and may promote differentiation (Th1/Th2) and extend the recognition phase of the immune response depending on the cytokine microenvironment.

The results presented here indicate that SCFF does not significantly affect, whereas LCFF enhances, the IL-1β synthesis of unstimulated PBMCs. These findings are similar to the results of previous studies from our laboratory, in which PMBCs were incubated with individual cocoa monomers and procyanidins for 72 hrs (13). Incubation of PBMCs with 25 μg/ml of isolated cocoa flavanol monomers, dimers, trimers, and tetramers had little effect on IL-1β protein release in some subjects, but slightly reduced it in a majority of them. The larger flavanol oligomers (hexamer through decamer), however, increased IL-1β synthesis, although the degree of induction varied considerably between subjects (13, 14). Whereas we report here that both SCFF and LCFF augmented the LPS-induced release of IL-1β, previous studies from our laboratory showed that the monomers through tetramers inhibited IL-1β protein production of PHA-stimulated PBMCs, whereas the larger oligomers enhanced it (13, 14). Whereas LPS preferentially stimulates B cells and macrophages, PHA is a T-cell mitogen. Hence, the observation that the small oligomer fractions have different effects on IL-1β synthesis depending on the mitogen used suggests that their activity is cell type–specific.

LCFF and SCFF by themselves had little effect on TNF-α secretion by PBMCs. Similarly, others reported that a 24-hr incubation of RAW 264.7 macrophages with epicatechin, catechin, or procyanidin dimers B1 or B2 at a concentration of 100 μg/ml (344 μ*M* and 173 μ*M*, respectively) did not significantly affect the secretion of TNF-α (32). In contrast, 100 μg/ml (115 μ*M*) of the procyanidin trimer C2 significantly enhanced the RAW 264.7 macrophage synthesis of TNF-α. A previous study from our laboratory found that individual cocoa monomers and oligomers enhanced TNF-α secretion in unstimulated PBMCs, with tetramers through octamers being the most active (15). Together, these results suggest that when they are used singly, monomers and oligomers have different effects on TNF-α secretion than when they are combined into the LCFF and SCFF fractions. It is also possible, however, that the different stimulation protocols used in the three studies accounted, at least partly, for the observed differences.

The present findings indicate that LCFF and SCFF can augment the release of TNF-α induced by LPS, that is, from macrophages and T cells. The catechin monomer, at concentrations of 31 and 63 μ*M*, slightly but significantly inhibited the synthesis of TNF-α by RAW 264.7 macrophages incubated with both IFN-γ and LPS, but had no effect at higher concentrations (35). Others, using only IFN-γ for stimulation of TNF-α production by RAW 264.7 macrophages, have reported that 100 μg/ml (344 μ*M*) of epicatechin or catechin almost completely inhibited the production of TNF-α (32). In the same study, 100 μg/ml (173 μ*M*) of the procyanidin dimers B1 and B2 (from apples) only slightly reduced TNF-α synthesis in response to IFN-γ stimulation, whereas the trimer C2 markedly reduced it. Although not directly comparable with our results because of the use of individual procyanidins and the use of different stimulation protocols, these findings could suggest that the procyanidins larger than dimers, or even trimers, are primarily responsible for the enhanced TNF-α synthesis that was observed with the SCFF fraction. Of note, we previously reported a similar pattern for PBMC secretion of TNF-α in response to PHA (i.e., by T cells), in that it was slightly inhibited by the cocoa monomers and dimers, but significantly enhanced by tetramers through octamers (15).

Cells of the innate immune system, including macrophages, NK cells, and neutrophils, play a role in the differentiation and activation of immune components largely through the production of cytokines and chemokines. These steps are important in determining the nature not only of the innate but also of the adaptive immune responses (3, 4). LPS-stimulated upregulation of CD69 on B cells requires the presence of macrophages or supernatants from LPS-stimulated monocytes/macrophages (38). This

suggested that soluble factors, possibly cytokines, mediate the induction of CD69 on B cells. IL-1, as well as TNF-α and IL-6, is very rapidly induced dependent on the stimulus; peak levels are typically observed within 6–24 hrs (18, 39).

CD69 and CD83 are cell surface markers expressed early during activation on human T and B cells as well as NK T cells in response to inflammatory stimuli such as bacteria. LCFF enhances CD69 and CD83 expression on human B cells independently of the presence of a proinflammatory stimulus such as LPS. The limited data available on the function of CD83 suggest that it enhances the ability of B cells to present antigen and to stimulate T-cell proliferative and cytolytic responses (19, 40). Based on these findings, we hypothesize that if select cocoa flavanols and procyanidins could augment the percentage of B cells expressing this activation marker and the density of its expression *in vivo*, their consumption would be associated with a more efficient and rapid response to immune challenges. In contrast, findings in CD69-null mice suggest that the enhanced expression of CD69 could modulate immune function by stimulating the synthesis of TGF-β (41–43), a regulatory cytokine known to inhibit the release of a variety of proinflammatory mediators. *In vivo* studies will allow determination of what (if any) ability cocoa procyanidins have in modulating the expression of CD83 and CD69 on B cells. There are a number of implications of these data for humans, including, for example, the potential of nutritional therapy for cytokine manipulation.

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