Caffeine Synergizes with Another Coffee Component to Increase Plasma GCSF: Linkage to Cognitive Benefits in Alzheimer’s Mice

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Abstract. Retrospective and prospective epidemiologic studies suggest that enhanced coffee/caffeine intake during aging reduces risk of Alzheimer’s disease (AD). Underscoring this premise, our studies in AD transgenic mice show that long-term caffeine administration protects against cognitive impairment and reduces brain amyloid-β levels/deposition through suppression of both β- and γ-secretase. Because coffee contains many constituents in addition to caffeine that may provide cognitive benefits against AD, we examined effects of caffeinated and decaffeinated coffee on plasma cytokines, comparing their effects to caffeine alone. In both AβPPsw+PS1 transgenic mice and non-transgenic littermates, acute i.p. treatment with caffeinated coffee greatly and specifically increased plasma levels of granulocyte-colony stimulating factor (GCSF), IL-10, and IL-6. Neither caffeine solution alone (which provided high plasma caffeine levels) or decaffeinated coffee provided this effect, indicating that caffeine synergized with some as yet unidentified component of coffee to selectively elevate these three plasma cytokines. The increase in GCSF is particularly important because long-term treatment with coffee (but not decaffeinated coffee) enhanced working memory in a fashion that was associated only with increased plasma GCSF levels among all cytokines. Since we have previously reported that long-term GCSF treatment enhances cognitive performance in AD mice through three possible mechanisms (e.g., recruitment of microglia from bone marrow, synaptogenesis, and neurogenesis), the same mechanisms could be complimentary to caffeine’s established ability to suppress Aβ production. We conclude that coffee may be the best source of caffeine to protect against AD because of a component in coffee that synergizes with caffeine to enhance plasma GCSF levels, resulting in multiple therapeutic actions against AD.

Keywords: AD mice, Alzheimer’s disease, caffeine, coffee, cognitive benefits, cytokines, GCSF

INTRODUCTION

A growing number of epidemiologic studies have shown that higher levels of caffeine or coffee intake during aging is associated with cognitive protection...
and decreased risk of Alzheimer’s disease (AD) [1–8]. For example, Eskelinen and colleagues [6] found that mid-life coffee intake was associated with a large 65% decreased risk of dementia, while individuals with AD were found to have consumed less caffeine (based on questionnaires) during the two decades prior to disease diagnosis compared to disease-free individuals [7]. Though informative, such epidemiologic studies of caffeine/coffee are problematic because retrospective studies are based on recall, while prospective (longitudinal) studies over decades are impractical.

In studies involving AD models, work has focused almost exclusively on caffeine rather than coffee administration. In this context, we have demonstrated that long-term administration of a physiologic level of caffeine in drinking water protects young adult AD transgenic (AβPPsw) mice from memory impairment in older age [9], as well as reverses already-present cognitive impairment in aged AD transgenic mice [10]. These robust protective and treatment effects of caffeine across multiple cognitive domains were likely induced by caffeine’s profound ability to suppress both β- and γ-secretase, resulting in decreased Aβ production and much lower brain Aβ levels/deposition [9, 10]. We have further extended these mechanistic findings by showing that 1) caffeine administration to transgenic mice increases brain PKA levels to reduce the Raf-1/NFκB inflammatory pathway, which normally stimulates brain β-secretase, and 2) caffeine suppresses levels of GSK-3α (a key stimulator of γ-secretase) in neuronal cell cultures [10]. Underscoring caffeine’s unique suppression of both enzymes involved in brain Aβ production are acute caffeine administration studies in AD transgenic mice, wherein we have found a single i.p. or oral treatment to induce reductions in both brain and plasma levels of Aβ within several hours [11]. Other complementary and/or linked mechanisms of caffeine action that could contribute to its cognitive benefits in AD mice are: 1) caffeine’s ability to decrease hippocampal levels of pro-inflammatory cytokines (e.g., TNF-α, IL-12(p70), and IFN-γ) [11], 2) caffeine’s beneficial effects on signal transduction factors involved in neuronal plasticity and survival (Zeitlin et al., submitted for publication), and 3) caffeine’s ability to enhance brain mitochondrial function (Bradshaw et al., submitted for publication).

To what extent blockade of brain adenosine receptors is involved in these mechanisms of caffeine action is not known. Our collective findings have led us to conclude that caffeine is likely to be a multi-mechanistic, disease-modifying therapeutic against development of AD. Indeed, an entire special issue of the Journal of Alzheimer’s Disease (Supplement 1, 2010) has been devoted to caffeine as a cognitive-enhancing therapeutic against AD.

In addition to caffeine, coffee is rich in other components that may complement or synergize with caffeine’s actions to reduce risk of AD. For example, coffee is high in dietary phenols such as chlorogenic acid and caffeic acid that are antioxidants [12]. Indeed, coffee’s “per serving” content of antioxidants is higher than that of almost all other foods [13] and coffee is the primary dietary source of antioxidants in the United States [14]. Secondly, coffee has anti-inflammatory properties through as-yet unidentified compounds that are thought to be responsible for coffee’s reported risk reduction of heart disease and several cancers [15, 16]. Since brain oxidative stress/free radical damage and chronic inflammation are critical pathophysiologic processes in AD [17], coffee’s sizable content of antioxidants and anti-inflammatory compounds could counter both of these pathogenic processes and further reduce risk of AD beyond that provided by dietary caffeine alone.

To determine if the non-caffeineergic components of coffee provide cognitive benefit over and above that of caffeine, it is critical to compare the potential of caffeine to coffee (both caffeinated and decaf-feinated) for prophylactic effects against AD. The present study is the first to compare the effects of caffeine to those of coffee in an AD-relevant study. In so doing, we report a profound ability of an as-yet unidentified component of coffee to synergize with caffeine, resulting in greatly enhanced plasma levels of several beneficial cytokines. Of these, coffee-induced elevations in plasma granulocyte-colony stimulating factor (GCSF) are particularly important since exogenous GCSF treatment for several weeks to AD transgenic mice greatly improves their cognitive performance through multiple mechanisms (e.g., recruitment of bone marrow cells, enhanced synaptogenesis, increased neurogenesis) [18]. Thus, the present study is the first to provide evidence that coffee provides protective effects against AD that are not possible with caffeine or decaffeinated coffee alone.

MATERIALS AND METHODS

Acute treatment study

Animals

Mice in the acute treatment study were inbred from mating pairs originally obtained from The Jackson
Laboratories (Stock #100010; Bar Harbor, Maine). All mice had a B6C3F1/J background derived from a cross between heterozygous mice carrying the mutant AβPPK670N, M671L gene (AβPPsw) with heterozygous mutant PS1 (Tg line 6.2) mice. This resulted in offspring consisting of mutant AβPPsw+PS1, AβPPsw, PS1, and non-transgenic (NT) genotypes. After weaning and genotyping, only AβPPsw+PS1 and NT mice were selected for use in this acute study. All mice were maintained on a 12 h dark/12 h light cycle with ad libitum access to rodent chow and water. All animal procedures were performed in AAALAC-certified facilities with protocols approved by the Institutional Animal Care and Use Committee at the University of South Florida.

General protocol

At 6–8 months of age, a pre-treatment blood sample (0.1 cc) was taken via facial vein from AβPPsw+PS1 (Tg) and NT mice. Samples were collected in EDTA tubes and centrifuged at 12,000 RPM for 3 min. Plasma was then transferred into new screw cap tubes and stored at −80°C. At 10 days following the pre-treatment blood sample, 6–8 month old mice were injected intraperitoneally with 200 μl of one of the following: saline, caffeine, un-concentrated coffee, concentrated coffee, or concentrated decaffeinated coffee (see next section for treatment preparations), with 6, 7 mice per treatment group. A post-treatment blood sample (0.2 cc) was taken 3 h thereafter, with plasma separated and then stored at −80°C. Parenthetically, the pre-treatment blood sample could not be taken any closer to the post-treatment blood sample because of restrictions on frequency/volume of blood sampling in University of South Florida IACUC guidelines.

Preparation of caffeine, coffee, and decaffeinated coffee

Caffeine was purchased from Sigma Aldrich (Sigma, St. Louis MO); Maxwell House regular and decaffeinated coffees (Kraft Foods) were purchased commercially. Caffeine was dissolved in 1xPBS at 7.5 mg/ml concentration. For both caffeinated and decaffeinated coffee, 40 g of ground coffee was added to 300 ml double-distilled water. Coffee solutions were then heated to boiling, kept at boiling for 2 min, then filtered with a coffee filter into a clean container. For “un-concentrated” coffee solution, 1.5 ml aliquots (containing 1.5 mg/ml caffeine) were put into 2 ml tubes and stored at −20°C. For “concentrated” coffee and decaffeinated coffee solutions, 1.5 ml aliquots of brewed coffee were frozen at −80°C and then put into a speed vac with tube lids open for 1 h (to concentrate these solutions), after which their volume was brought to 300 μl with sterile nanopure water. These “concentrated” coffee and decaffeinated coffee solutions were then stored at −20°C. On the day of treatment, concentrated coffee and decaffeinated coffee aliquots were reconstituted to 300 μl (7.5 mg/ml caffeine for concentrated coffee) to keep all non-caffenergic components of coffee at the same concentration for both of these treatments. Unconcentrated coffee aliquots were simply taken out from freezer and thawed. For the three coffee treatment solutions containing caffeine, the amount of caffeine administered for the acute i.p. injection of 200 μl was: un-concentrated coffee (0.3 mg), concentrated coffee (1.5 mg), and concentrated decaffeinated coffee (0.06 mg). Prior to administration, the pH of each solution was measured and adjusted to pH 5.3 (the pH of un-concentrated coffee). The amount of caffeine administered for the caffeine and “concentrated” coffee treatment (1.5 mg) was equivalent to a human intake of 500 mg caffeine (e.g., five 8-oz cups of coffee).

Plasma cytokine measurement

In both pre-treatment and post-treatment plasma samples, a total 12 cytokines and chemokines were measured with Luminex assay (GCSF, GM-CSF, IFN-γ, IL-1α, IL4, IL-6, IL-10, IL-12(p40), IL-17, IP-10, TNFα, and VEGF). Cytokine expression profiles and levels were detected using the Bio-Rad Bio-Plex, with reagents being ordered from Millipore as customer kits (Millipore, CA). Samples and standards were prepared using company protocols with the initial concentration of standards ranging from 32 ng/ml to 1.95 pg/ml. Plasma samples were prepared for analysis by diluting 1 volume of the serum sample with 3 volumes of the Bio-Plex mouse sample diluent. Detail procedures were performed following the protocol provided by the manufacturer. Finally, the plates were read. Each cytokine level was calculated based on its own standard curve.

Plasma caffeine and theophylline detection

Plasma caffeine and theophylline concentrations were measured in samples collected at 3 h following treatment. ELISA Kits from Neogen (WI, USA) were utilized, following the manufacturer’s protocol. In brief, the enzyme conjugate solution was prepared by diluting the 180× enzyme conjugate stock 1 to 180 in the EIA buffer provided. Caffeine or theophylline was then diluted with EIA buffer at two fold dilutions from 200 ng/ml to 0.39 ng/ml. Then 20 μl standard of each
dilution was added into the coated plate. Plasma samples were then diluted with EIA buffer, with 20 μl of this dilution added into the coated plate. Both standard and samples were run in duplicate in the plate. Positive and negative controls of 20 μl were loaded to each plate. Then 180 μl of diluted drug-enzyme conjugate was added into each well and mixed by gently shaking the plate. Plates were covered with plastic film and incubated at room temperature for 45 min. During the incubation, a 10 x wash buffer was diluted to 1 x with DI water and mixed thoroughly. Once incubation was completed, the liquid was dumped from the wells. Plates were then taped on a clean lint-free towel to remove any remaining liquid in the wells. Then each well was washed with 300 μl of diluted wash buffer 3 times. After completing the last wash step, the bottom of the wells was wiped with a lint-free towel to remove any liquid on the outside of the wells. Then 150 μl of the K-Blue Substrate was added to each well with a multi-channel pipette. The plate was then mixed by gently shaking, followed by incubation at room temperature for 5 to 20 min. To stop the enzyme reaction, 50 μl of red stop solution was added to each well and gently mixed. The absorbance was then measured with plate reader (Synergy HT, Biotek, VT) at a wavelength of 650 nm. The absorbance was converted into concentration using Gen5 software.

**Plasma Aβ_{1-40} and Aβ_{1-42} determinations**

Aβ levels were detected by using ELISA kits (KHB3482 for 40, KHB3442 for 42, Invitrogen, CA). Standard and samples were mixed with detection antibody and loaded on the antibody pre-coated plate as the designated wells. HRP-conjugated antibody was added after wash, and substrates were added for colorimetric reaction, which was then stopped with sulfuric acid. Optical density was obtained and concentrations were calculated according a standard curve.

**Long-term treatment study**

**Animals**

Mice used in the long-term treatment study were obtained from the Florida Alzheimer’s Disease Research Center’s colony of inbred mice having a mixed background of C57/B6, SJL/SW. All mice were generated from a cross between heterozygous mice carrying the mutant AβPPK670N, M671L gene (AβPPsw) with heterozygous PS1 (Tg line 6.2) mice. After weaning and genotyping, only AβPPsw (Tg) and NT mice were selected for use in this long-term study. Lighting conditions and access to food/water were the same as for mice of the acute treatment study, with all protocols approved by the University of South Florida IACUC.

**General protocol**

At 10 months of age, AβPPsw (Tg) and NT littermates were started on twice weekly gavage treatment (100 μl) with “concentrated” regular coffee, “concentrated” decaffeinated coffee, or saline solution. Ten month old AβPPsw mice of our inbred colony have high brain Aβ levels, but do not yet have robust Aβ deposition/deposits. Thus, this study was largely “prevention-based” by involving Tg mice most associated with the prodromal/mild cognitive impairment (MCI) stage of Alzheimer’s disease. Coffee solutions were prepared as in the acute study, with both caffeinated and decaffeinated coffee solutions having the same final concentration of non-caffeine components. The final caffeinated coffee solution contained 0.75 mg caffeine/100 μl and the decaffeinated coffee solution contained 0.03 mg/100 μl. Thus, each mouse being given caffeinated coffee received the human equivalent of caffeine in 2 1/2 cups of coffee twice weekly. Each Tg treatment group consisted of 5–7 mice. At three months into bi-weekly gavage treatment and at 13 months of age, mice were tested for six successive days in the cognitive interference task (see below). Because all NT mice exhibited very good performance irrespective of treatment, they were combined into a single NT group. On the day following completion of behavioral testing, all mice were euthanized, at which time a blood sample was taken for analysis of the same 12 cytokines as analyzed in the acute study (see preceding section).

**Cognitive interference task**

We designed this task of working (short-term) memory measure-for-measure from a cognitive interference task that easily discriminates normal aged, MCI, and AD patients from one another [19]. The interference testing protocol in humans consists of four measures, only the first of which is relevant to the data being presented currently. This first measure, three-trial recall, involves the subject being presented with ten objects from “Bag A” and asked to recall the objects on three successive trials thereafter, with a brief distraction task between trials. Then, ten different objects from “Bag B” are presented to the subject, following which they are ask to name as many of those 10 objects as possible (Proactive interference; measure 2). The subject is ask to name as many of the objects from Bag A as possible (Retroactive interference; measure 3), followed
by a 20 min delay, after which naming Bag A objects once again is requested (delayed recall; measure 4).

Our analogous interference task for mice involves two radial arm water maze (RAWM) set-ups in two different rooms, each with different sets of visual cues. Animals are first tested in RAWM “A” for three successive trials, then in RAWM “B” for one trial, followed by one trial back in RAWM “A”, and a final trial in RAWM “A” after a 20 min delay. The present study only presents the first measure (three trial recall) of this complex task to exemplify the link between plasma GCSF levels and cognitive performance in long-term treated mice; the full spectrum of measures will be presented in a separate study focusing on behavior. As such, the “three trial recall” measure currently presented (utilizing only RAWM “A”) is essentially the same as the first of three successive trials in our standard RAWM task. For this three trial recall measure, an aluminum insert was placed into a 100 cm circular pool to create 6 radially distributed swim arms emanating from a central circular swim area. The number of errors prior to locating which one of the 6 swim arms contained a submerged escape platform (9 cm diameter) was determined for 3 successive trials per day, with each error (wrong arm entry) resulting in the animal being pulled back to the start arm to make another choice. The platform location was changed daily to a different arm, with a different start arm semi-randomly selected from the remaining 5 swim arms each day as well. Animals were given 60 s to find the escape platform for each trial, with the number of errors and escape latency recorded for each trial. As a distractor between trials, animals were placed in a Y-maze and allowed to explore for 60 s between successive trials of the three-trial recall task. Animals were tested for six successive days, with statistical analysis performed over the three resultant 2-day blocks and overall. We have recently demonstrated the cognitive interference task’s utility in aged AβPPsw Tg mice, which show clear impairment in the task, as well as cognitive benefit in this task from treatment with the cRaf-1 inhibitor “Sorafenib” [20] or long-term exposure to electromagnetic fields [21].

**Statistical analysis**

All data was analyzed with ANOVA and followed by post hoc pair-by-pair differences between groups (planned comparisons), which was resolved using the Fisher LSD test. Pre- versus post-treatment measures were statistically evaluated using paired Student’s t-test. In order to determine if relationships were present between or among plasma and behavioral measures, correlation analysis was performed using the Systat analytical software package. All group comparisons were considered significant at p < 0.05.

**RESULTS**

**Acute treatment study: Tg mice**

In 6–8 month old AβPPsw+PS1(Tg) mice, a single i.p. treatment with caffeine or various coffee solutions had dramatically different effects on plasma cytokine levels 3 h thereafter (Fig. 1). Evaluating the change (post-pre treatment) in plasma levels of 12 cytokines revealed that only three cytokines were significantly affected by the various treatments: GCSF, IL-10, and IL-6. Plasma levels of the remaining nine cytokines (GM-CSF, IFN-γ, IL-1α, IL-4, IL-12(p40), IL-17, IP-10, TNFα, and VEGF) were all unaffected by any treatment administered in Tg mice.

For GCSF, plasma levels were unaffected by saline, caffeine, or concentrated decaf treatment, as indexed by the change in GCSF levels for post- versus pre-treatment (Fig. 1). In sharp contrast, acute treatment with either un-concentrated or concentrated coffee resulted in a large increase in plasma GCSF levels 3 h later, resulting in post-pre changes that were highly significant compared to those for the other three treatments (Fig. 1). These results were observed whether evaluating the difference between post- and pre-treatment levels (as depicted in Fig. 1), or by evaluating post-treatment GCSF levels alone (data not presented). The elevation in plasma GCSF induced by un-concentrated coffee was even higher than that induced by concentrated coffee. Thus, caffeine and some unidentified component of coffee synergize in Tg mice to provide a large acute elevation of plasma GCSF levels, an elevation that high caffeine levels could not provide.

For both IL-10 and IL-6, plasma levels were also unaffected by saline, caffeine, or concentrated decaf treatment (Fig. 1). However, concentrated coffee treatment resulted in large post-treatment elevations in plasma levels of both IL-10 and IL-6. Un-concentrated coffee treatment induced a similarly large change in plasma IL-10 levels. However, the change in plasma IL-6 for this group was significantly less than that for concentrated coffee and failed to reach significance compared to the change in IL-6 levels for the other three treatment groups (“p” values between 0.07 and 0.10).
In 6–8 month old AβPPsw + PS1 (Tg) mice, acute treatment with coffee (concentrated or un-concentrated) substantially increased plasma levels of GCSF, IL-10, and IL-6 at 3 h post-treatment, as indexed by the difference between post- and pre-treatment levels. Caffeine solution, concentrated decaffeinated coffee treatment, or saline had no such effect on any of these cytokines. *** \( p < 0.000001 \) versus all three non-asterisk groups; ** \( p < 0.05 \) or higher level of significance versus all three non-asterisk groups; † \( p < 0.0001 \) versus concentrated coffee; # \( p < 0.02 \) or higher level of significance versus all other groups.

Analysis of plasma caffeine levels in 3-hr post-treatment blood samples revealed the following rank order, with each group being significant different in caffeine levels from all others: Caffeine solution > concentrated coffee > un-concentrated coffee > concentrated decaf (Fig. 2). The same rank order was evident for post-treatment plasma levels of the active caffeine metabolite theophylline. Thus, acute treatment with either the caffeine solution or concentrated coffee resulted in the highest plasma levels of caffeine and theophylline in Tg mice. However, only coffee solutions were able to induce large increases in GCSF, IL-10, and IL-6.

For the five treatment groups of Tg mice, blood levels of both Aβ1-40 and Aβ1-42 were not significantly different in pre-treatment or post-treatment samples (Fig. 3a, d). As well, there were no pre-treatment versus post-treatment significant differences in plasma Aβ1-40 or Aβ1-42 levels for any treatment group, although nearly significant pre- versus post-treatment reductions (\( p = 0.08 \) and 0.15, respectively) were evident for Tg mice treated with caffeine solution. These strong trends suggested to us that significant reductions in blood Aβ levels were dependent on higher blood levels of caffeine. We therefore re-analyzed the pre- versus post-treatment data, including only those animals with post-treatment caffeine levels >20,000 ng/ml irrespective of treatment group. The resultant “high caffeine level” group included all animals in the caffeine solution group, as well as all but two animals in the concentrated coffee group. For this high caffeine level group, pre- versus post-treatment plasma levels of Aβ1-40 and Aβ1-42 were both significantly reduced (Fig. 3b, e). Further underscoring the linkage between higher-caffeine containing treatments and plasma Aβ
Fig. 3. a, d) In 6–8 month old AβPPsw+PS1 (Tg) mice, plasma Aβ1-40 and Aβ1-42 levels were not affected by any of the acute treatments administered, both in comparing post-treatment levels and pre- versus post-treatment levels. b, e) If only Tg mice with post-treatment caffeine levels >20,000 ng/ml are included irrespective of treatment group, significant decreases in plasma Aβ1-40 and Aβ1-42 were evident 3 h following treatment. *p < 0.01 versus pre-treatment level. c, f) Including Tg mice from all treatment groups, significant inverse correlations were present between post-treatment plasma levels of caffeine and either Aβ1-40 (c) or Aβ1-42 (f). Higher plasma caffeine levels were associated with lower plasma Aβ levels.

Acute treatment study: NT mice

In 6–8 month old NT littermate mice, very similar patterns of plasma cytokine change and caffeine/theophylline levels were present as in Tg mice. The same nine cytokines that were unaffected by any treatment in Tg mice (GM-CSF, IFN-γ, IL-1α, IL-4, IL-12(p40), IL-17, IP-10, TNFα, and VEGF) were also unaffected in NT mice. Moreover, the same three cytokines (e.g., GCSF, IL-10, and IL-6) that were increased by un-concentrated or concentrated coffee in Tg mice were similarly elevated by these two treatments in NT mice (Fig. 4). Also as with Tg mice, none of the other three treatments induced an appreciable increase in these three cytokines. These results were observed whether evaluating the difference between post- and pre-treatment cytokine levels (as depicted in Fig. 4), or by evaluating post-treatment levels alone. Most notably, plasma GCSF levels in NT
In 6–8 month old non-transgenic (NT) mice, acute treatment with coffee (concentrated or un-concentrated) substantially increased plasma levels of GCSF, IL-10, and IL-6 at 3 h post-treatment, as indexed by the difference between post- and pre-treatment levels. Caffeine solution, concentrated decaffeinated coffee, or saline treatment had no such effect on any of these cytokines. ***p < 0.02 or higher level of significance vs all three non-asterisk groups; **p < 0.01 or higher level of significance versus all other groups; *p < 0.05 or higher level of significance versus all three non-asterisk group.

mice were greatly and similarly increased at 3 h following un-concentrated or concentrated coffee treatment (Fig. 4). All three other treatments (saline, caffeine solution, concentrated decaf) failed to induce substantive increases in plasma GCSF. Thus, the ability of coffee (un-concentrated and concentrated) to enhance plasma GCSF levels was independent of genotype. Further underscoring the universality of this effect, we have seen the same elevation in plasma GCSF (as well as IL-10 and IL-6) induced by coffee, but none of the other treatments, in several other genotypes, namely AβPPsw mice and mutant PS1 mice (data not shown).

Analysis of plasma caffeine and theophylline levels in post-treatment blood samples from NT mice (Fig. 5) revealed the same general levels and inter-group relationships as seen for Tg mice (Fig. 2). Specifically, NT mice treated with either caffeine solution or concentrated coffee had caffeine and theophylline levels that were substantially higher than those in the other three treatment groups (Fig. 5). Thus, as was the case for Tg mice, treatment with either the caffeine solution or concentrated coffee resulted in high plasma caffeine and theophylline levels. Yet only the two coffee solutions (un-concentrated and concentrated) were able to induce large increases in GCSF, IL-10, and IL-6.

Long-term treatment study

In this study, 10 month old AβPPsw (Tg) mice were given twice-weekly gavage treatment with either saline, decaffeinated coffee (concentrated), or caffeinated (concentrated) coffee. At three months into treatment, mice were tested for 3-Trial Recall in the
Fig. 6. a, c) AβPPsw (Tg) mice were cognitively evaluated at three months into bi-weekly gavage treatment with decaffeinated coffee, coffee, or saline control. In the 3-Trial Recall measure of the cognitive interference task, both Tg controls and Tg mice being treated with decaffeinated coffee (Tg+Decaf) were impaired in both errors (a) and escape latency (c) compared to NT controls. By contrast, Tg mice being given chronic coffee treatment showed performance levels in both measures that were no different from NT controls. Performance of all Tg groups for both measures was indexed and statistically evaluated as percent change from the performance of NT controls. *p < 0.05 or higher level of significance versus NT controls. b, d) Irrespective of whether decaf or coffee treatment was given to Tg mice, their errors and latency in 3-Trial Recall were both inversely correlated with plasma GCSF levels. Better cognitive performance was present in Tg mice with higher plasma GCSF levels.
DISCUSSION

A variety of epidemiologic studies have reported that either caffeine or coffee habitual intake reduces the risk of age-related cognitive impairment and AD [1–8]. Our studies in AD transgenic mice further support habitual caffeine intake as protective against AD [9]. However, the effects of caffeine have never been compared to those of coffee in a controlled AD-related study; this, to determine if non-caffeine components of coffee beneficially interact with caffeine to provide enhanced protective effects against AD. The present study is the first to address this issue in reporting that coffee (but not caffeine or decaffeinated coffee) induces large elevations in plasma GCSF, IL-10, and IL-6 levels in both AD transgenic and normal mice. The large coffee-induced increase in plasma GCSF elevations is of particular importance because only plasma GCSF levels (among all cytokines evaluated) are correlated with enhanced cognitive performance of AD transgenic mice following long-term coffee or decaf coffee treatment. Since plasma GCSF levels are substantially reduced early in AD [22] and such deficient hematopoietic support appears contributory to AD pathogenesis [23, 24], coffee-induced GCSF stimulation could be a new, disease-modifying therapeutic for reducing the risk of AD.

GCSF (filgastrim) is one of several hematopoietic growth factors (along with GM-CSF and M-CSF) that control production of circulating blood cells by the bone marrow. Both GCSF and its receptor are expressed in neurons and neural progenitor cells that reside in the hippocampal neurogenic niche [25]. Although GCSF has been used primarily as an agent to treat leukopenia, GCSF has also been studied in animal models of stroke where it has been reported to reduce brain damage and improve outcome [26–29]. Importantly, plasma levels of GCSF are decreased in early AD [22] and GCSF is one of 18 plasma signaling proteins that are collectively predictive of conversion from MCI to AD [23].

In the present study, acute administration of coffee (but not caffeine or decaffeinated coffee), induced a large increase in plasma GCSF levels 3 h thereafter. This coffee-induced increase in GCSF was independent of genotype in that it was seen not only in AβPPsw+PS1 mice, but also in non-transgenic mice, AβPPsw mice, and PS1 mice. Although caffeine treatment or concentrated coffee treatment both resulted in high plasma levels of caffeine and theophylline (a major metabolite of caffeine) in both AD transgenic and NT mice, only coffee solutions were able to induce large increases in GCSF (as well as IL-10 and IL-6); high plasma caffeine or decaffeinated coffee levels were insufficient for doing so. Moreover, these cytokine enhancements induced only by coffee solutions did not involve non-specific pH effects because all solutions had their pH measured and adjusted to pH 5.3 (the pH of coffee).

Whether or not the “acute” elevation in plasma GCSF levels with coffee administration would be seen with “chronic” coffee administration is not known. Indeed, no long-term study has yet compared oral caffeine to oral coffee to determine if coffee provides enhanced cognition relevant to AD. However, the present study did evaluate the effects of twice-weekly oral treatment with coffee for three months in 10 month old AβPPsw mice. Coffee treatment, but not decaffeinated coffee treatment, was found to enhance 3-Trial Recall in the cognitive interference task of working memory. Since treatment was given every 72 h in this study, the cognitive-enhancing ability of coffee must have involved a mechanism that out-lives the presence of coffee’s various components in plasma. We believe that mechanism likely involves long-term enhancement of plasma GCSF levels by coffee. Supportive of this premise is our finding that cognitive performance in Tg mice of this long-term treatment study was correlated with their plasma GCSF levels. This correlation with cognitive performance was not seen for any of the other 11 plasma cytokines evaluated.

Why might a coffee-induced chronic elevation in plasma GCSF levels provide cognitive protection against AD over and above protection provided by caffeine? We recently treated AβPPsw+PS1 mice (from the same inbred colony as used in the present study) with human GCSF daily for less than three weeks [18]. GCSF treatment resulted in decreased brain Aβ deposition in Tg mice, along with an ensuing improvement in cognitive performance to the level of NT controls. Key to the reduction in brain Aβ deposition was a GCSF-induced increase in brain microglial activity, which included GCSF-stimulation of blood stem cells in bone marrow to differentiate into monocytes. These recruited monocytes then traveled via blood to the brain, where they further differentiated into microglial cells to engulfed Aβ deposits along with GCSF-activated microglia already resident in the brain [18]. Parenthetically, this GCSF-induced enhancement of microglial activation did not involve an increase in pro-inflammatory cytokines within the brain. Also contributory to the cognitive improvement of GCSF-treated Tg mice was an increase in hippocampal synaptic volume and augmented hip-
pocampal neurogenesis. Thus, three complimentary mechanisms of GCSF-induced cognitive improvement were identified, and these same three mechanisms could be involved (along with caffeine’s mechanisms) in the enhanced cognitive performance induced by coffee treatment in the present study. Coffee-induced increases in plasma GCSF levels could be critically important to humans in the early stages of AD, wherein plasma GCSF levels are appreciably reduced [22]. Indeed, we have provided evidence that individuals with Rheumatoid Arthritis have a reduced risk of AD because of their innate elevation of the blood hematopoietic cytokines GCSF and GMCSF [30].

Along with GCSF, the present study found two other cytokines (IL-10 and IL-6) to be selectively increased in plasma following acute coffee administration. GCSF mobilizes/stimulates monocytes to produce IL-10 [31], so it is not surprising to find plasma levels of this key anti-inflammatory cytokine to be increased. In contrast to IL-10, IL-6 is a "pro-inflammatory" cytokine, chronically elevated in AD and thought to be contributory to the disease’s continual brain inflammation [32]. Inasmuch as IL-6 is structurally similar to GCSF and in the same tri-cytokine family, it is also not surprising that IL-6 was elevated along with GCSF following acute coffee administration. In fact, IL-6 appears to compliment the ability of GCSF to differentiate blood stem cells into monocytes by inducing migration of such monocytes into the brain [33], where they become microglia to actively ingest deposited Aβ [18]. IL-6 also plays an essential role in the final differentiation of B-cells into Ig-secreting cells, and thus stimulates Ig production. Indeed, increased plasma levels of IL-6 may have beneficial effects against AD to increase plasma levels of anti-Aβ antibodies through an immunotherapeutic action similar to that of current clinical trials with IVIG treatment [34]. Thus, the three cytokines elevated by acute coffee treatment (GCSF, IL-10, IL-6) could provide complimentary and interrelated beneficial effects against AD, if future studies show that all three remain elevated with chronic coffee treatment.

Undoubtedly, the most researched component in coffee is caffeine. Our prior work has established that long-term oral administration of caffeine protects young adult AD Tg mice from otherwise inevitable cognitive impairment later in life [9], as well as reverses established cognitive impairment in older Tg mice [10]. The multiple mechanisms responsible for these cognitive benefits in Tg mice include caffeine’s ability to: 1) suppress both β- and γ-secretase, thus decreasing brain Aβ production [9, 10], 2) decrease brain levels of pro-inflammatory cytokines [11], 3) favorably affect signal transduction factors (e.g., PKA, phospho-CREB, phospho-ERK) involved in neuronal plasticity and survival (Zeitlin et al., submitted), and 4) enhance brain mitochondrial function (Bradshaw et al., submitted). Given that the most important of these caffeine-mediated mechanisms against AD is suppression of brain Aβ production (#1), even a single caffeine treatment reduces both brain and plasma Aβ levels in AD mice within only 2-3 h [11]. Results from the present study mirror this prior findings in that Tg mice exhibiting high plasma caffeine levels (>20,000 ng/ml) at 3 h following acute treatment also had substantially decreased plasma levels of both Aβ1-40 and Aβ1-42 at that time point. Moreover, significant inverse correlations were evident post-treatment between plasma caffeine levels and plasma Aβ levels for all Tg mice collectively.

The above caffeine-mediated mechanisms against AD, in combination with multiple anti-AD mechanisms provided by other components of coffee (e.g., anti-inflammatory, antioxidant actions) endow a single dietary product (coffee) with an unparalleled array of therapeutic interventions against AD. Moreover, as the present study demonstrates for the first time, caffeine and some as-yet unidentified other component of coffee synergize to provide plasma elevations in key cytokines that can further counter AD pathogenesis. It is entirely possible that the component of coffee that synergizes with caffeine was removed during the decaffeination process, in which case that component cannot be brought back simply with the addition of caffeine to decaffeinated coffee. Thus, it will take more studies to identify the synergizing constituent.

Especially since AD pathogenesis begins several decades before the disease is diagnosed, interventions should start no later than mid-life (40 s–50 s) to be most effective. These mid-life interventions include a diet high in fruits/vegetables, as well as keeping blood pressure and LDL cholesterol normal. We believe moderate coffee intake should be added to this list of mid-life interventions to reduce risk of AD later in life. For most adults, coffee’s multiple beneficial constituents are safe at moderate consumption levels of 4-5 cups/day, a level of intake that was protective in our AD mouse studies [9] and a level that appears to be risk-reducing against a variety of age-related diseases, including AD [12].

In summary, we have discovered that caffeine synergizes with another component of coffee to increase plasma levels of several cytokines beneficial against AD (mostly notably GCSF). This synergy, in concert with the additional benefits against AD provided by...
caffeine and the non-caffenergic components of coffee separately, underscores coffee as a premiere dietary protection against AD.

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