

Interleukin-8 and interleukin-10, brain volume and microstructure, and the influence of calorie restriction in old rhesus macaques

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Abstract Higher systemic levels of the proinflammatory cytokine interleukin-6 (IL-6) were found to be associated with lower gray matter volume and tissue density in old rhesus macaques. This association between IL-6, and these brain indices were attenuated by long-term 30 % calorie restriction (CR). To extend these findings, the current analysis determined if a CR diet in 27 aged rhesus monkeys compared to 17 normally fed controls reduced circulating levels of another proinflammatory cytokine, interleukin-8 (IL-8), and raised levels of anti-inflammatory interleukin-10 (IL-10). Further, these cytokines were regressed onto imaged brain volume and microstructure using voxel-wise regression analyses. CR significantly lowered IL-8 and

raised IL-10 levels. Across the two dietary conditions, higher IL-8 predicted smaller gray matter volumes in bilateral hippocampus. Higher IL-10 was associated with more white matter volume in visual areas and tracts. Consuming a CR diet reduced the association between systemic IL-8 and hippocampal volumes. Conversely, CR strengthened associations between IL-10 and microstructural tissue density in the prefrontal cortex and other areas, particularly in a region of dorsal prefrontal cortex, which concurred with our prior findings for IL-6. Consumption of a CR diet lowered proinflammatory and increased anti-inflammatory cytokine concentrations, which lessened the statistical association between systemic inflammation and the age-

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related alterations in important brain regions, including the hippocampus.

Keywords Calorie restriction · Interleukin-8 · Interleukin-10 · Hippocampus · Prefrontal cortex · Voxel

Introduction

Increased neuroinflammation with aging contributes to neurodegeneration and cognitive impairment (Wilson et al. 2002). Although the predominant focus of this work has been on proinflammatory cytokines (PICs) such as interleukin-6 (IL-6), other cytokines including ones with chemoattractant properties (i.e., chemokines) such as IL-8 can also induce tissue damage (Dinkel et al. 2004). Aged healthy participants older than 60 years have higher circulating IL-8 relative to participants aged 30–60 years (Alvarez-Rodríguez et al. 2012). In a recent report that examined the cytokine profile of aged rhesus monkeys (Asquith et al. 2012), there was a similar but nonsignificant trend for higher IL-8 in aged versus adult macaques. IL-8 is also more highly expressed in mild cognitive impairment and Alzheimer's dementia (Galimberti et al. 2003, 2006). While IL-8 is normally associated with neutrophil activation in the bloodstream, it is a pleiotropic chemokine that is also found in the central nervous system (CNS), where it is released from and predominantly affects microglia and endothelial cells (Ehrlich et al. 1998). IL-8 facilitates leukocyte transmigration across the blood–brain barrier under both normal (Hickey 1999) and neuropathological conditions (Cross and Woodroffe 1999). These migrating leukocytes may include polymorphonuclear cells, such as, degranulated neutrophils, which are normally present at only low levels in the CNS (Baggiolini et al. 1989; Janeway et al. 2005). B lymphocytes are attracted by IL-8 via epithelial cell secretion (Alter et al. 2003) and T lymphocytes via microglial release of IL-8 (Liu et al. 2010).

IL-8 can have neuroprotective and neurotrophic effects, at least in culture conditions (Araujo and Cotman 1993; Semple et al. 2010), as well as neurotoxic actions through secondary mediators like amyloid-beta ($A\beta$; Franciosi et al. 2005; Liu et al. 2010). Tissue damage may also be induced through the release of reactive oxygen species (ROS) and

neutrophil-mediated production of proteases (Eikelenboom et al. 2002; Dinkel et al. 2004; Weiner and Frenkel 2006). Importantly, such neurotoxic effects are mediated by microglial activation and occur in the rhesus monkey, the animal studied in this report, even though it does not spontaneously develop delayed recall deficits fully reminiscent of Alzheimer's disease (Nagahara et al. 2010) or tau pathology (Nelson et al. 1996). For instance, injecting fibrillar $A\beta$ into the brains of geriatric female rhesus monkeys produced lesion volumes and ROS, which could be abrogated in a step-wise manner through co-injection of macrophage/microglia inhibitory factor (Leung et al. 2011). By extension, given the bidirectional relationship of systemic and central cytokine release (Dantzer 2004), peripheral levels of IL-8 may indirectly gauge the degree of microglial activity in brain.

Neurotoxicity may be more prominent in hippocampus because this area has high IL-8 mRNA expression relative to many other brain regions, at least in rodents (Licinio et al. 1992). Further, IL-8 application in rodent neural tissue reduced long-term potentiation (Xiong et al. 2003), a change in synaptic plasticity thought to underlie learning and memory. Smaller entorhinal cortex volumes of adults with schizophrenia have also been attributed to prenatal IL-8 exposure (Ellman et al. 2010). To validate this regional specificity and test if higher circulating IL-8 was associated with less volume or tissue microstructure in the hippocampus and other areas, we conducted voxel-wise regression analyses of brain scans acquired using magnetic resonance imaging (MRI) following the methods of Ashburner and Friston (2000).

Similarly, it was of interest to determine if higher peripheral levels of the anti-inflammatory cytokine IL-10 could be statistically associated with larger brain volumes or denser microstructural integrity. In particular, we assessed if IL-10 may partly account for why a calorie restriction (CR) diet in our aged monkey cohort reduced IL-6 levels (Willette et al. 2010), as well as significantly reduced the association between higher IL-6 and less brain volume and tissue microstructure in the prefrontal cortex and other areas. IL-10 may also moderate some of the associations between IL-8 and the aging brain. IL-10 can lower IL-8 expression in human microglia following stimulation with PICs or endotoxins (Ehrlich et al. 1998). Such feedback is not reciprocal, however, as IL-8 does not seem

to be able to interfere with IL-10 production in cultured microglia (Franciosi et al. 2005).

The statistical associations between systemic cytokine activity and both brain volume and microstructure were investigated in aged rhesus monkeys using MRI and diffusion tensor imaging (DTI), respectively. Specifically, we extended our previous analysis of IL-6 and neural structure (Willette et al. 2010) using two representative indicators of pro- and anti-inflammatory cytokine activity. IL-8 and IL-10 were chosen based on histological and cellular analysis in humans (Sokolova et al. 2009) and rodents (Licinio et al. 1992). The cytokine levels were determined non-invasively from blood, rather than from the brain tissue or cerebrospinal fluid (CSF). This strategy was necessary because the monkeys continue to be evaluated in a longitudinal project until natural mortality, in order to determine ultimate longevity. Further, demonstrating the predictive value of blood biomarkers would enable the findings to be more readily translated to the human clinical setting or for similar associational analyses in human participants.

Our cohort consists of 27 rhesus macaques that have been consuming a 30 % CR diet for the prior 13–19 years since middle age, as well as 17 normally fed controls. CR in these nonhuman primates has a number of positive metabolic, hormonal, and neural benefits (Ramsey et al. 2000; Colman et al. 2009), some of which are inter-related, including via the diverse actions of insulin and improved gluoregulation (Willette et al. 2012b). Based on previous cell culture and blood analyses from these monkeys (Kim et al. 1997; Willette et al. 2010) and the demonstration that CR reduces IL-8 gene expression in humans (Crujeiras et al. 2008), we predicted that CR would: (1) lower blood IL-8 levels and (2) increase blood IL-10 levels. Further, we anticipated this CR-induced shift in cytokine activity would be statistically associated with volume and microstructural density in the hippocampus and several other brain regions. Cytokine concentrations, brain indices, and interactions were tested using voxel-wise regression, both with linear and nonlinear relationships. Aging has been associated with progressive as well as nonlinear changes in several neural regions (Good et al. 2001; Sowell et al. 2003). Moreover, we previously found that stress reactivity was related to brain volume and microstructure in both linear and nonlinear fashions (Willette et al. 2011). Finally, the potential influence of visceral adiposity and some

spontaneous inflammatory disease conditions was also considered because both influence inflammatory physiology (Devaux et al. 1997; Bulcão et al. 2006; Miron et al. 2010; Strober and Fuss 2011).

Methods and materials

Subjects

Forty-four rhesus monkeys (*Macaca mulatta*) between 19 and 31 years of age were used from a longitudinal cohort maintained at the Wisconsin National Primate Research Center. Twenty-seven subjects had been fed 30 % fewer calories relative to their own baseline intake since they were middle-aged. The 17 control animals were fed a standard diet ad libitum for approximately 8 h each day. Details of the CR manipulation, housing, and husbandry have been described previously (Kemnitz et al. 1993; Colman et al. 2009).

Interleukin-8 and interleukin-10 assays

Blood was collected at two time points to verify the stability of the cytokine measures over time: (1) when an animal crossed the threshold criterion for aged status at 20 years of age and (2) within 6 months of the neuroimaging scan date. The elapsed time (mean \pm SD) between baseline and MRI blood collection was 4.14 ± 2.75 years. To generate reference values for the assays, additional samples were tested from eight middle-aged monkeys and two middle-aged, healthy humans. IL-8 and IL-10 were determined by human enzyme-linked immunosorbent assay kits (ELISA; R&D Systems, Minneapolis, MN). Methods are similar to those described previously (Willette et al. 2007). ELISA, rather than multikine arrays, was used to facilitate comparison with our previous work (Willette et al. 2010) and to facilitate accuracy and sensitivity of detection. The need to use a high sensitivity kit for IL-10, and this focused approach with only two cytokines, lessened the likelihood of inadvertently finding spurious associations with brain volume and microstructure. The lower detection limit for IL-8 and IL-10 assays was 1.5 and 0.03 pg/mL, respectively. The values at the two time points were highly correlated for IL-8 [$r = .68$, $p < .001$] and IL-10 [$r = .80$, $p < .001$]. Given this stability over time, the

mean concentration for each subject was used to index cytokine activity in the statistical analyses with the brain data. A similar analytic strategy was used in our previous report on IL-6 (Willette et al. 2010).

Visceral adiposity and morbidity

Age-related morbidities such as chronic heart failure (Devaux et al. 1997), inflammatory bowel disease (Strober and Fuss 2011), and neoplastic disease including adenocarcinoma (Miron et al. 2010) may increase PIC expression. Excess adipose tissue can also elevate PIC in the systemic circulation (Bulcao et al. 2006). We previously reported a small but significant correlation between IL-6 levels near time of scan and the presence of inflammatory disease, while no association was seen between IL-6 and abdominal adiposity as estimated by MRI (Willette et al. 2010). Using the same approach to characterize central fat deposition and disease state, we explored whether or not IL-8 or IL-10 levels were correlated with the presence of pathological conditions that could be inflammatory in 13 of 44 aged monkeys. These diseases included adenocarcinoma ($n=4$), gastric bloat ($n=1$), arthritis ($n=3$), peritonitis ($n=1$), endometriosis ($n=3$) and endometriosis comorbid with gastric bloat ($n=1$). Correlations were also conducted between inflammatory biomarkers and total peritoneal adiposity. Because normal aging includes both healthy individuals as well as some with non-terminal illness, all animals regardless of disease state were utilized in all other voxel- and non-voxel analyses comparable to our report on IL-6 (Willette et al. 2010).

MRI data acquisition

Scan session and image acquisition parameters have been described in detail previously (McLaren et al. 2009; Willette et al. 2010; Bendlin et al. 2011). Briefly, monkeys were anesthetized and images were acquired using a General Electric 3.0 T scanner (GE Medical Systems, Milwaukee, WI, USA). T1-weighted scans were used to determine regional gray matter and white matter volumes. Diffusion tensor imaging encoding in 12 directions was used to quantitatively estimate tissue and tract density of gray matter and white matter using minimum energy criteria (Hasan et al. 2001). Voxels for all modalities were resampled from their native resolution to $0.5 \times 0.5 \times 0.5$ mm.

Imaging artifacts

As described in previous reports (e.g., Willette et al. 2012a, b), one T1-weighted and three DTI scans were excluded due to scanner and motion artifacts. An additional six diffusion tensor imaging scans (CR=4, controls=2) were excluded due to focal (1–2 mm), lesion-like white matter abnormalities that appeared on T1 images. Such abnormalities can strongly influence DTI parameters in local and distal tissue (Iverson et al. 2011). These images were thus excluded from analysis as in previous reports (Willette et al. 2010; Bendlin et al. 2011).

MRI processing

Processing steps have been described previously (McLaren et al. 2009; Willette et al. 2010). Briefly, T1-weighted images were segmented and normalized to 112RM-SL atlas space using DARTEL in SPM5 (Ashburner 2007). Gray matter and white matter segments were smoothed with a 4-mm full width at half maximum (FWHM) Gaussian kernel. Gray matter and white matter volumes were masked using binary transforms of their respective prior probability maps. Partial volume effects were minimized by using absolute thresholding of 0.30 for gray matter and 0.25 for white matter. As described in detail elsewhere (Willette et al. 2010; Bendlin et al. 2011), the DTIFIT function in FSL was used to calculate FA, axial diffusivity, radial diffusivity, and mean diffusivity (MD) brain maps. These maps were aligned to the 112RM-SL atlas space and smoothed with a 4-mm FWHM Gaussian kernel. FA, axial diffusivity, and radial diffusivity were calculated to examine axonal and myelin alterations (Song et al. 2002). MD was used to examine microstructural integrity of parenchyma and neuropil. Increased MD, reflecting less tissue density, has been found as age increases in both cross-sectional (Benedetti et al. 2006) and longitudinal (Rosano et al. 2012) studies of normal aging, as well as frontotemporal dementia (Whitwell et al. 2010).

Statistics: MRI analyses

Voxel-wise regression analyses were conducted in SPM5 using a general linear model framework (Ashburner and Friston 2000). Briefly, this technique uses software to perform a linear regression analysis at

every voxel that partly constitutes a given brain image, which allows one to infer regional associations in brain tissue in an in vivo manner. Diffusion tensor, gray matter, or white matter images were used as the dependent variable. Separate analyses were conducted for mean IL-8 and IL-10 values derived from averaging the samples at 20 years of age and at the time of MRI scan. Age, sex, and dietary condition were covariates; total brain volume was additionally covaried in analyses of regional gray matter volume to remove the effect of brain size. Unadjusted FA and MD values in this cohort have been described elsewhere (Bendlin et al. 2011). Based on previous reports (Colman et al. 2009; Willette et al. 2010; Willette et al. 2012a, b), it was also of interest to test if CR as compared to control animals showed a shallower or steeper slope per unit increase for IL-8 or IL-10. Therefore, a Dietary Condition \times IL-8 or Dietary Condition \times IL-10 interaction term was also used as a predictor of interest in voxel-wise regression models.

The voxel and cluster level thresholds were set at $p < .005$ (uncorrected) and $p < .05$ (corrected), respectively. We have previously described correcting for multiple comparisons at the cluster level to minimize type 1 error (Willette et al. 2012a, b). Briefly, Monte Carlo simulations (Forman et al. 1995) in the Analysis of Functional NeuroImages package were used to determine the minimum cluster size necessary to constitute a significant result. To gain a better understanding of whether myelin or axon changes predominantly drove FA results (Song et al. 2002), radial and axial diffusivity were respectively examined using a binary mask of the FA result map at a $p < .05$ (uncorrected) voxel threshold. Whole brain cluster coordinates correspond to the Saleem–Logothetis atlas (Saleem et al. 2002) and are displayed on the 112RM-SL underlays (McLaren et al. 2009). Standard monkey atlases were used to identify fibers (Schmahmann and Pandya 2006) and subcortical structures (Paxinos et al. 2000).

Statistics: non-MRI analyses

Other tests were conducted with SPSS 20.0 (SPSS, Chicago). Analysis of covariance (ANCOVA) was used to test dietary effects on IL-8 and IL-10, as well as between subject characteristics and total brain volume. The independent variable of interest was dietary condition (CR vs. control diet). Covariates included age and sex. Two-tailed Pearson's correlations were

used to evaluate whether factors were significantly associated with one another. Alpha was set at 0.05. A logarithmic transformation was used to improve normality for the IL-10 values.

Results

Subject characteristics, mean IL-8, and IL-10 levels

The age (mean \pm SD) and sex composition of the two dietary groups did not differ significantly (CR: 24.3 \pm 2.8 years, 16 females (F) and 11 males (M); control: 23.7 \pm 2.8 years, ten F and seven M). IL-10 values initially had negative skew (3.60), but the distribution was normalized after the logarithmic transformation removed skew (0.12). Monkeys consuming the CR diet had significantly lower serum IL-8 [$F(1, 40)=8.80, p=0.005$] and higher serum IL-10 [$F(1, 40)=4.37, p=0.043$] as compared to control animals (Fig. 1). Specifically, IL-8 in CR subjects was 4,610 pg/mL lower (CR: mean \pm SD=7,150 \pm 680 pg/mL; controls: mean \pm SD=11,760 \pm 1,400 pg/mL), and their IL-10 was 0.72 pg/mL higher (CR: 1.25 \pm 0.33 pg/mL; controls: 0.53 \pm 0.10 pg/mL). IL-8 and IL-10 levels were not directly correlated across groups [$r=-0.18, p=0.23$], nor in CR [$r=-0.16, p=0.43$] or control monkeys [$r=0.10, p=0.71$] when analyzed separately. The presence of a potentially inflammatory disease condition in 13 of these 44 older monkeys was not significantly associated with either IL-8 [$r=-0.04, p=0.79$] or IL-10 [$r=0.04, p=0.80$]. In addition, visceral adiposity was not related to IL-8 [$r=0.191, p=0.214$] or IL-10 [$r=-0.019, p=0.903$]. To verify that these IL-8 and

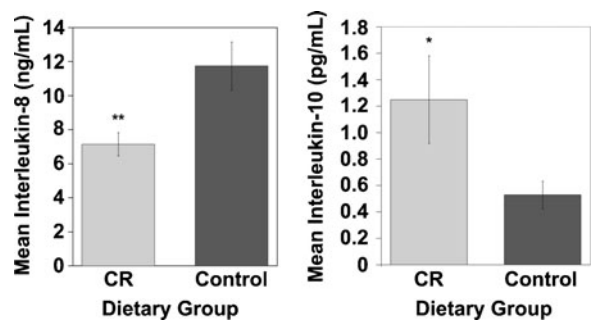


Fig. 1 The effect of calorie restriction in aged rhesus macaques ($n=27$) compared to controls ($n=17$) on interleukin-8 and interleukin-10 as measured in serum. * $p < .05$; ** $p < .01$

IL-10 values were representative for the rhesus monkey using these assay methods, comparative levels were determined for eight middle-aged rhesus monkeys and two middle-aged, healthy humans. For IL-8, middle-aged monkeys averaged 242 ± 179 pg/mL, which was significantly lower than for all of the old monkeys (data not shown). These serum IL-8 values are comparable to other recent reports of adult macaques (Asquith et al. 2012). IL-10 in the middle-aged monkeys (mean, 3.03 ± 5.06 pg/mL) did not differ from the older monkeys in either diet condition. The human cytokine data generated contemporaneously in the same assays were in the expected range, indicating that the assays were performing in accordance with the manufacturer's specifications (Boekholdt et al. 2004).

IL-8: association and interaction analyses

Voxel-wise regression analyses were used to examine if higher IL-8 was associated with regional brain volume and microstructural tissue density. No significant association or interaction relationships were found with white matter volume or DTI images (data not shown). The linear fit of IL-8 against gray matter volume was also not significant across groups. However, after adding a quadratic term, higher IL-8 predicted less gray matter bilaterally in the hippocampus. The clusters in the left hemisphere [290 voxels; peak $t=3.19$; coordinates, $-16, 8, 6$] and right hemisphere [1,041 voxels; peak $t=3.93$; coordinates at $15, 10, 8$] overlapped with the presubiculum and cornu ammonis (CA) fields, as well as dentate gyrus.

The interaction terms tested where CR monkeys showed comparatively larger gray matter volumes than controls, per picogram per liter increase in systemic IL-8, which would suggest less of an association between IL-8 and gray matter volume (Fig. 2, “hot” color). The linear estimate produced bilateral clusters restricted to the anterior hippocampus, although a quadratic term improved the fit of the model—particularly for controls. The cluster in the left hemisphere [1,310 voxels; peak $t=4.61$; coordinates, $-15, 10, 7$] included the dentate gyrus, as well as the subiculum and the CA fields. A contralateral cluster [342 voxels; $t=3.59$; coordinates, $14, 11, 7$] encompassed a similar hippocampal region. Figure 2 illustrates the interaction using a representative voxel in the left hippocampus. For controls, higher IL-8 was related to less gray matter volume [$r=-0.663$, $p=0.005$], whereas CR

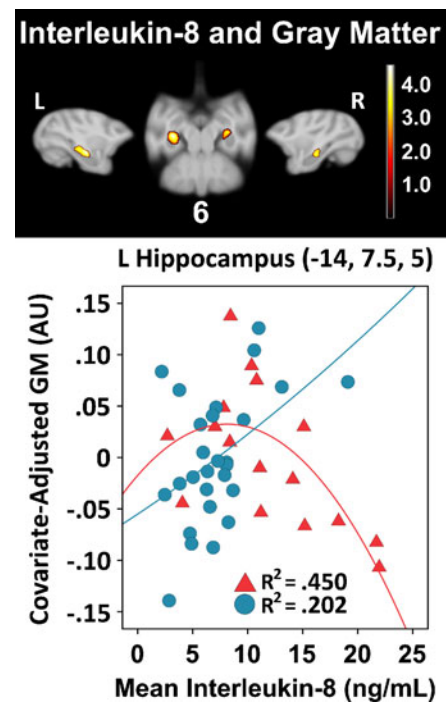


Fig. 2 The quadratic relationship between higher levels of the chemoattractant cytokine interleukin-8 and lower gray matter volume in aged control ($n=16$, red triangles) and calorie restriction monkeys ($n=27$, blue circles). One volumetric scan had been discarded from the analysis due to image artifacts. The only area that showed a significant association was the hippocampus in both the left and right hemispheres. This statistical effect was seen when looking across dietary conditions (not shown) or the Dietary Condition by IL-8 interaction (“yellow–red” color). A representative voxel in the left hippocampus illustrates this interaction. Color bars represent t values. A slice number below the axial cross-section depicts the millimeter from the origin located at ear bar zero. R^2 refers to the proportion of variance in the dependent measure explained by the predictor variable. Cross-sections from the brain are oriented in neurological space

monkeys at first showed an inverse correlation [$r=-0.45$, $p=0.024$]. However, two CR monkeys with the highest IL-8 values (13,100 and 19,100 pg/mL) drove that statistical effect. Removing these data points reduced the correlation between IL-8 and the left hippocampus to nonsignificance [$r=0.37$, $p=0.068$]. These results indicated that for controls, higher IL-8 predicts smaller gray matter volume in the hippocampus, whereas old monkeys consuming the CR diet do not show this association. However, we emphasize that a direct causal inference based on cytokine–brain associations cannot be made based on these changes in slope.

IL-10: association and interaction analyses

IL-10 and regional gray matter

We hypothesized that higher IL-10 would predict more gray matter volume, particularly in areas where CR monkeys had showed a weaker association between higher IL-6 and gray matter volume compared to controls (Willette et al. 2010). This analysis produced one significant cluster in the lunate sulcus of the right occipital lobe [2,401 voxels; peak $t=3.83$; coordinates, 14, 12, 24]. This gray matter was proximal to the sulcus, rather than actually located in a gyrus. The region of association extended ventrally through primary visual cortex and extrastriate areas V2 and V3, first medially and then subjacent to the posterior lateral ventricle. A 2-mm extent overlapped with the posterior hippocampus. The quadratic estimate produced no other clusters that reached statistical significance.

The analysis of the interaction tested whether or not and in which brain regions, unitary increases in IL-10 (picograms per milliliter) resulted in a steeper slope for the CR animal than for the controls. Such a relationship would reinforce the interpretation of a beneficial association with gray matter volume. In keeping with the between group analysis, regions that showed a relationship with higher IL-10 included primary visual cortex and extrastriate area V2, both in the right hemisphere [1,828 voxels; peak $t=3.72$; coordinates, 8, -25, 20] and the left hemisphere [1,165 voxels; peak $t=3.44$; coordinates, -10, -26, 15]. By examining the interaction using a representative voxel in primary visual cortex, CR monkeys showed a flat association [$r=0.05$, $p=0.801$], whereas higher IL-10 was related to less gray matter in controls [$r=-0.497$, $p=0.050$]. The quadratic fit model indicated no regions of association that exceeded the cluster significance threshold.

IL-10 and regional white matter

No cluster reached suprathreshold significance in the IL-10 association analysis across groups. The interaction terms examined in which brain regions, if any, did CR monkeys, per picograms per milliliter increase in IL-10, show more white matter volume than control animals. Clusters were seen in the caudal portion of the frontal forceps dorsal to the head of the caudate in the left hemisphere [cluster size=1,021 voxels; maximum $t=3.41$; coordinates, -5, 31, 11] and the right

hemisphere [cluster size=1,059 voxels; peak $t=3.38$; coordinates, 2, 32, 12]. The white matter in these voxels mostly consisted of uncinate fasciculus and remained subjacent or medial to the orbital sulcus. A representative voxel in uncinate fasciculus showed that higher IL-10 predicted lower white matter volume in controls [$r=-0.65$, $p=0.007$]. CR monkeys did not show this relationship [$r=0.02$, $p=0.932$].

IL-10 and regional fractional anisotropy of white matter

Across dietary conditions, higher IL-10 concentrations were associated with more white matter volume in regions subjacent to inferior parietal sulcus and ending caudally lateral to the lunate sulcus, both in the left hemisphere [cluster size=2,112; peak $t=5.45$; coordinates at -26, -4, 20] and right hemisphere [cluster size=5,732 voxels; peak $t=5.36$; coordinates at 25, -5, 18]. These clusters overlapped exclusively with inferior longitudinal fasciculus fibers. The interaction tested if any brain regions showed a relationship where, per unit increase in IL-10 concentration, CR monkeys had more predicted white matter density versus controls. This relationship reached significance in one cluster that spanned the splenium of the corpus callosum. Similar to the white matter and gray matter results, CR monkeys did not show a significant correlation between IL-10 and FA [$r=0.30$, $p=0.226$], whereas for controls, higher IL-10 corresponded to lower FA [$r=-0.510$, $p=0.036$]. Post hoc analyses indicated that radial diffusivity, but not axial diffusivity, maps overlapped with the FA association and interaction results, suggesting that the FA results with IL-10 were restricted to myelin fibers. Unadjusted values for FA have been described in a previous report (Bendlin et al. 2011).

IL-10 and regional mean diffusivity of gray and white matter

For IL-10, the hypothesis was that higher values would be associated with a denser tissue microstructure (i.e., lower mean diffusivity) in gray and white matter. This association was found for both dietary groups in a single cluster spanning the left posterior intraparietal sulcus [cluster size=1,597 voxels; peak $t=3.44$, coordinates at 10, -6, 24]. Next, examination of the IL-10 \times Dietary Condition interaction tested if

monkeys on the CR diet influenced tissue density per picogram per milliliter increase in IL-10. Table 1 and Fig. 3 show the clusters that reached significance. The peak voxel was located in the dorsal prefrontal cortex bordering on the agranular frontal area F2. This maximally significant cluster extended 22 mm in the sagittal cross-plane from the central sulcus, encompassing the gray matter in the primary motor cortex, the supplementary motor area (SMA) and pre-SMA, rostral premotor cortex in area F7, and the dorsal prefrontal cortex, as well as middle and anterior cingulate cortices. A representative voxel in the dorsal prefrontal cortex (Fig. 3) shows that while controls did not exhibit significant statistical associations with tissue density as IL-10 increased [$r=0.25$, $p=0.35$], the CR monkeys evinced a strong relationship between higher IL-10 and denser parenchymal microstructure, as represented by lower MD [$r=-0.63$, $p=0.007$]. It is of interest that this cluster overlapped considerably with a cluster generated from a previously observed interaction between Dietary Condition and IL-6 (Fig. 4). In that analysis, the CR monkeys had a weaker association between higher IL-6 and higher MD than did the controls (Willette et al. 2010). Additional areas where the CR diet augmented this relationship included the thalamus, striatum, and posterior cingulate cortex. For white matter tracts, the clusters overlapped with the

Table 1 Interaction effect of IL-10 and diet on mean diffusivity

Location	<i>t</i> value	x, y, z	Cluster size (voxels)
L dorsal PFC	11.25	-10, 34, 28	18,551
R pre-SMA	5.19	0, 36, 33	
R anterior cingulate cortex	3.81	2, 24, 28	
L LGN	5.19	-16, 11, 12	1,901
R putamen	4.62	8, 14, 16	5,087
R putamen	4.17	11, 24, 16	
R ventral PFC	3.8	14, 30, 22	
L retrosplenial cortex	4.62	-6, -4, 22	1,422
L caudate	3.95	-10, 21, 20	2,073

This table depicts regions where aged monkeys on CR, relative to controls, had more predicted tissue density per unit increase in IL-10. The highest *t* value for a given cluster of significant, contiguous voxels is shown. For clusters that extended over more than 15 mm, the highest *t* value in those areas is indicated. Coordinates are in Saleem–Logothetis atlas space. Brains are oriented in neurological space

L left hemisphere, PFC prefrontal cortex, R right hemisphere

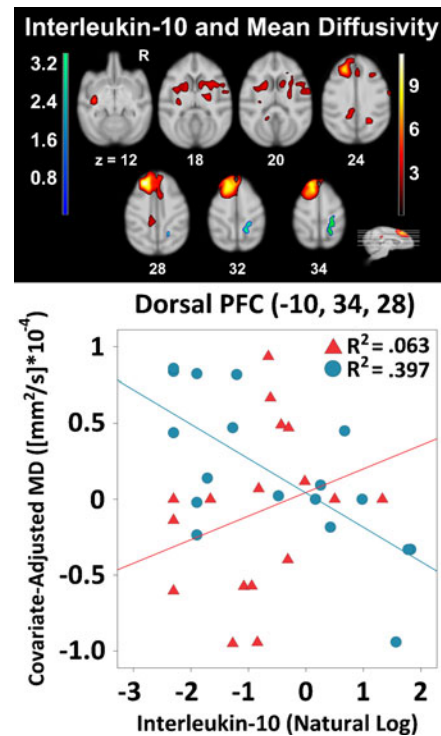


Fig. 3 The linear association between lower mean diffusivity (i.e., more tissue density) and higher concentrations of the anti-inflammatory cytokine interleukin-10. For both the CR ($n=18$, blue circles) and control ($n=17$, red triangles) groups, higher IL-10 was related to more gray matter proximal to the intraparietal sulcus (blue–green color). The Dietary Condition by IL-10 interaction showed that CR monkeys had more predicted gray matter per unit increase in IL-10 versus controls in the prefrontal cortices, thalamus, striatum, and posterior and anterior cingulate cortices. Nine DTI scans had been discarded from this analysis due to image artifacts. Color bars represent *t* values. Slice numbers below axial cross-sections depict the millimeter from the origin located at ear bar zero. R^2 refers to the proportion of variance in the dependent measure explained by the predictor variable. Brains are oriented in neurological space

superior longitudinal fasciculus I and II, extreme and external capsule, striatal bundle, dorsal-occipital bundle, fronto-occipital fasciculus, arcuate fasciculus, the cingulum, internal capsule, and prefrontal corticocortical fibers. The unadjusted values for MD have been described in a previous report (Bendlin et al. 2011).

Discussion

Consumption of a CR diet starting in middle age resulted in a significant reduction in systemic levels

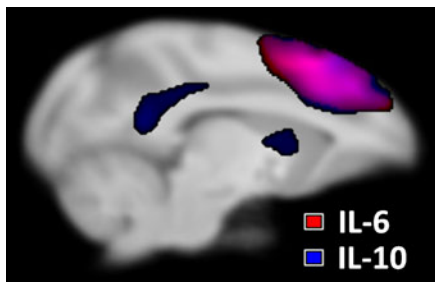


Fig. 4 The overlap between the Dietary Condition by IL-10 interaction map (in *blue*) and a Dietary Condition by IL-6 map (in *red*) produced in prior work (Willette et al. 2010). The *purple* color represents the spatial area where the two statistical effects overlap. The sagittal cross-section depicts the left hemisphere

of IL-8 in aged rhesus monkeys. The CR diet also led to higher circulating levels of the anti-inflammatory cytokine IL-10. These nutritional effects on cytokine concentrations were not due to CR-induced reductions in age-related morbidity or visceral fat (Colman et al. 2009). The occurrence of such high IL-8 values in the aged monkeys was unexpected, but the range determined for middle-aged monkeys was comparable to a recent report (Asquith et al. 2012), suggesting that the IL-8 was accurately determined.

For the brain–cytokine associations, we first discuss IL-8 and then IL-10. Higher IL-8 was associated with less gray matter volume bilaterally in the hippocampus. While IL-6 and IL-8 are both PICs, higher IL-6 in our previous report was not clearly associated with the hippocampus (Willette et al. 2010), suggesting some spatial specificity to these relationships with brain volume and microstructure. We should acknowledge that while peripheral cytokines can reflect levels in the central nervous system (Reyes and Coe 1996) and can induce cascades in brain (Dantzer 2004), there can be marked differences in concentrations across compartments and tissues. Thus, causal inferences cannot be made based on our results, and future studies are needed to determine if IL-8 is acting directly to impair hippocampal volume and whether that effect is lessened by a CR diet.

Nevertheless, based on previous cellular and neuroimaging studies, we had anticipated that higher IL-8 in the bloodstream might predict lower gray matter volume in the hippocampus and adjacent areas. Studies of rodent brains indicate, for example, that mRNA for IL-8 is higher in the hippocampus than in many other brain regions (Licinio et al. 1992). In addition, hippocampal administration of IL-8 dysregulated

hippocampal function in rats (Xiong et al. 2003). In humans, higher levels of maternal IL-8 during pregnancy was predictive of less entorhinal cortex volume in their adult offspring with schizophrenia, although no effects were found on the hippocampus in that study (Ellman et al. 2010). Sokolova et al. (2009) reported higher IL-8 and other inflammatory markers in neural tissue, as well as more A β plaques in the inferior temporal gyrus of specimens obtained postmortem from individuals with Alzheimer’s disease. This finding is important because cortical thickness in this region is significantly correlated with hippocampal atrophy in mild cognitive impairment and early Alzheimer’s disease (Desikan et al. 2010). The potential influence of IL-8 on the brain may be especially pronounced in patients with a genetic predisposition for poor vascular health (Li et al. 2009; Raz et al. 2011).

Although IL-8 likely has its own actions, it also can act in concert with other cytokines, given that they are produced and released in a cascade. These effects could occur synergistically with existing neuropathology to negatively affect gray matter. For instance, application of A β in the rat hippocampus caused increased T cell chemotaxis via expression of the PIC tumor necrosis factor- α in microglia. This effect was blocked by using a CXCR2 receptor antagonist, one of the receptors to which IL-8 binds (Liu et al. 2010). IL-8 itself can synergize with A β _{1–42} to induce PIC and production of the inflammatory enzyme cyclooxygenase-2 in human microglia (Franciosi et al. 2005). However, IL-8 concentrations in CSF are substantially higher in aged individuals with amnesic mild cognitive impairment as compared to Alzheimer’s disease, but are not correlated with disease duration, and did not change over a 3-year period in people converting from mild cognitive impairment to Alzheimer’s disease (Galimberti et al. 2006). By extension, IL-8 may facilitate neurodegeneration earlier in the Alzheimer’s disease process. Additional studies are needed in animal models, where brain tissue can be obtained, in order to determine if microglia and other cells in aged versus young brain produce more IL-8 and induce atrophy. It is also important to note that our results in monkeys cannot directly be compared to human neuropathology.

As previously highlighted, the aged monkeys on the CR diet had lower IL-8. Given the association between IL-8 and gray matter across subjects, the prediction would be that CR animals would have more

hippocampal gray matter. Furthermore, the statistical interaction between dietary condition and IL-8 was suggestive of a weaker relationship between IL-8 and gray matter in CR monkeys. This cohort of monkeys had been examined more than a decade earlier, at a much younger age, and it was demonstrated then that CR reduced IL-8 expression in peripheral blood mononuclear cells challenged oxidatively with xanthine and xanthine oxidase (Kim et al. 1997). A similar reduction of free oxygen radical production in the brain parenchyma could explain why CR monkeys, even the few with relatively high IL-8, did not evince a corresponding decrease in the hippocampal gray matter volume. Unfortunately, we cannot establish causality with these analyses because changes in the slopes of the cytokine–brain relationships are still correlational in nature. Finally, it should be noted that we did not detect a significant relationship between IL-8 and IL-10 levels in these monkeys. The literature on direct relationships between IL-8 and IL-10 is complex. For example, although IL-10 can suppress IL-8 production in human microglia cultured with PIC or endotoxin (Ehrlich et al. 1998), application of IL-8 alone to microglia did not change IL-10 expression (Franciosi et al. 2005).

Regression analyses of IL-10 revealed complex associations with brain volume and microstructure, which shared some spatial overlap with our previous analysis of IL-6 (Willette et al. 2010). Across groups, higher IL-10 was associated with more volume in primary visual areas, extrastriate cortices, and uncinate fasciculus, as well as higher FA in inferior longitudinal fasciculus and corpus callosum. Similarly, higher IL-6 levels across groups predicted lower white matter volume and FA in similar clusters, suggesting that systemic IL-6 and IL-10 may act synergistically on white matter integrity. Indeed, Henry et al. (2009) recently demonstrated that peripheral endotoxin administration in aged mice simultaneously increased PIC and IL-10 release from whole brain homogenate microglia.

The analysis of mean diffusivity indicated that higher IL-10 in CR monkeys predicted higher parenchymal tissue and tract density (i.e., lower MD), whereas in the control animals this relationship was not evident. This statistical association in the CR monkeys was most pronounced in a large cluster ranging from primary motor to prefrontal cortices along the dorsal convexity of the brain. This result was striking because the statistical cluster is nearly identical to one

highlighted before by an interaction between dietary condition and IL-6 (Willette et al. 2010). Blood levels of IL-6 and IL-10 in this cohort are not highly correlated, so the correspondence is likely not due to autocorrelation. A CR diet may thus increase anti-inflammatory activity, possibly moderating the actions of proinflammatory cytokines on brain microstructure integrity. But this type of causal inference must await future studies of direct work in brain tissue.

Our prior analyses of age-related changes in the brain of these unique, old monkeys indicated that higher age predicted less volume in the temporal and frontal cortices, which was reflected in part by decreased FA and increased MD of white matter tracts in frontal areas (Colman et al. 2009; Bendlin et al. 2011). The current IL-10 associations with microstructure concur with the previously observed age-related decreases in FA of the corpus callosum, superior longitudinal fasciculus, and other tracts (Bendlin et al. 2011). For statistical effects of CR alone, we previously found higher gray matter volume in the insula and striatum (Colman et al. 2009), which was supported by the association with IL-6, although not seen in the current IL-8 analysis. There was increased tract integrity in the CR condition, primarily in the superior longitudinal fasciculus, a tract that also emerged in the IL-10 \times Dietary Condition cluster analysis. The interaction between age and CR in prior reports implicated gray matter in the mid-cingulate cortex and small clusters in the thalamic bundle and superior longitudinal fasciculus for FA and MD. For other age-related biomarkers of interest, such as homocysteine (Willette et al. 2012a), the interaction with dietary condition revealed a shallower slope of association with gray matter in the right hippocampus. A similar association with hippocampal gray matter was found in the current analyses of IL-8. A comparable set of findings was evident for anterior cingulate cortex and MD.

Although there are many unique features of this CR model in primates, several limitations should be acknowledged. Given the value of these irreplaceable animals and the focus on natural morbidity and ultimate life span are still being evaluated, it was not possible to analyze the brain tissue. Thus, one has to speculate from values in the systemic circulation to activity within the CNS. In addition, we could not directly monitor leukocyte trafficking into the CNS

or the activation of the microglia or astroglia, which presumably are the central sources of these cytokines. Cytokines are also activated by the pathology associated with aging, both within the brain and the periphery. We did conduct separate analyses of the small subset of monkeys with overt clinical conditions and confirmed that these subjects with age-related diseases did not account for the general conclusions. There are also several methodological issues related to the MRI scans and analytic strategies applied after data acquisition. We attempted to minimize artifacts by covarying for dietary condition and age in voxel-wide models, excluding any scans with artifacts, and ensure that the scans were conducted while the monkeys were maintained in comparable plane of anaesthesia. Nonetheless, some of these factors may still have introduced bias. As rhesus monkeys do not appear to develop cognitive deficits akin to Alzheimer's disease (Nagahara et al. 2010) or tau pathology (Nelson et al. 1996), these findings should not be directly generalized to Alzheimer's disease patients. Future studies will need to establish if there is a comparable relationship between IL-8 and hippocampal gray matter in dementia.

Nevertheless, our primary hypothesis that long-term CR can shift the balance of pro- and anti-inflammatory activity was confirmed as indicated by lower IL-6 and IL-8 as well as higher IL-10. Our hypothesis that this peripheral cytokine activity would predict less gray matter volume in the hippocampus was also substantiated. Although IL-10 was not directly correlated with IL-8 levels, higher levels were associated with more white matter volume primarily in visual areas. Further, higher IL-10 was predictive of more microstructural density in the dorsal prefrontal cortex, an overlap that was similar to a result previously seen with IL-6 (Willette et al. 2010). These associational findings in the brain broadly suggest that a CR diet is effective for promoting health and perhaps slowing the natural progression of neural aging in a number of important brain regions.

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