Divergent effects of Western and Mediterranean diets on behavior and monocyte polarization

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Summary

Monocytes and macrophages—important mediators of innate immunity, health, and disease—are sensitive to diet and stress. Western diets promote inflammation and disease, while Mediterranean diets reduce inflammation and promote health, although the mechanisms through which diet alters immune function are unknown. Here, we conducted the first randomized, long-term diet trial in which macaques were fed either a Western- or Mediterranean-like diet to determine how diet and behavior interact to influence monocyte polarization–a precursor to subsequent health outcomes. Monocyte gene expression profiles differed markedly between the two diets, with over 40% of expressed genes being differentially expressed (FDR<0.05). The Western diet induced a more proinflammatory monocyte phenotype with changes in monocyte polarization-associated genes. Diet also shifted the co-expression of gene pairs, including small RNAs and transcription factors that are associated with metabolism and adiposity in humans. We also found that diet altered a suite of affiliative and anxiety-associated behaviors, and these behavioral changes mediated ~20% of the effect of diet on monocyte gene expression. Together, these findings suggest that diet-induced effects on monocyte polarization are due, in part, to both direct and behaviorally-mediated effects on monocyte gene expression.

Keywords

Immunogenomics, diet, behavior, monocytes, polarization

Introduction

Modern human diets vary across geography, cultures, and socioeconomic strata, and profoundly impact our health and survival. For example, Western diets–characterized by protein and fat from primarily animal sources, simple sugars, and saturated and n-6 fatty acids–are associated with increased risk for metabolic syndrome, type II diabetes, cardiovascular disease, autoimmune disorders, and depression (Jacka et al., 2010; Manzel et al., 2014), as well as increased mortality (Cordain et al., 2005; Drake et al., 2018; Smil, 1989; Smyth and Heron, 2006). Conversely, Mediterranean diets–characterized by protein and fat from primarily vegetable sources, raw fruits and vegetables, and monounsaturated and n-3 fatty acids–lower the incidence of chronic disease and increase longevity (Farchi et al., 1994; Osler and Schroll, 1997; Romagnolo and Selmin, 2017; Trichopoulou et al., 1995). Recent findings implicate inflammation as a key factor in diet-driven chronic disease, as components of the Western diet can arouse the sympathetic nervous system, increase oxidative stress, and elevate levels of inflammatory markers (Giugliano et al., 2006; Lopez-Garcia et al., 2004). In contrast, components of Mediterranean diets such as n-3 fatty acids and antioxidants promote an anti-inflammatory phenotype (O'Keefe et al., 2008). Together, these findings suggest that diets modulate inflammation, which may underlie many of the subsequent health consequences.

Mounting evidence suggests that dietary effects on inflammatory pathologies are mediated by changes in immune cell regulation (Devêvre et al., 2015; Drescher et al., 2019; Holt et al., 2009; Nanri et al., 2007; Nettleton et al., 2006). For instance, components of Western diets increase circulating inflammatory cytokine concentrations in mouse models (Drescher et al., 2019) and in humans (Holt et al., 2009; Nanri et al., 2007; Nettleton et al., 2006). The majority of these

inflammatory cytokines are secreted by monocytes, an innate immune cell. Monocytes and monocyte–derived macrophages are phenotypically variable along a spectrum. In the circulation, monocytes are generally defined as classical, intermediate, and non-classical, which upon activation may become polarized along this spectrum which ranges broadly from proinflammatory (M1-like) to regulatory/reparative (M2-like) phenotypes. An appropriate balance of monocyte phenotypes is essential for a healthy immune system. Classically-activated "M1" monocytes respond to proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ by becoming macrophages which propagate the inflammatory response to infection (Mosser and Edwards, 2008). In contrast, M2 activated monocytes mobilize tissue repair processes and release anti-inflammatory cytokines in response to interleukin (IL)-4, IL-13, and transforming growth factor (TGF)-β (Mosser and Edwards, 2008). Thus, diet may alter disease propensity by reprogramming the balance between these proinflammatory and anti-inflammatory monocyte subsets (Devêvre et al., 2015).

Diet is only one aspect of the environment that impacts health and survival by altering immune function. Components of the social environment, such as social status and social integration, may also affect these fitness outcomes by activating inflammatory programs in primary white blood cells (Cole, 2013; Snyder-Mackler and Lea, 2018; Tung and Gilad, 2013). For instance, socially subordinate rhesus macaques exhibit increased activation of the inflammatory response through MyD88-dependent Th1-mediated inflammatory activation in response to a bacterial infection (Snyder-Mackler et al., 2016). Given the similar molecular pathways through which diet and the social environment impact health, these two environmental stimuli could compound or mitigate one another. Moreover, given that changes in food intake can directly alter social behaviors

themselves (Kaplan et al., 1991; Warden and Fisler, 2008), dietary effects could plausibly act through (or interact with) social environmental effects.

Earlier investigations of diet effects on immune function primarily focused on the effects of single nutrient modifications, which precluded potentially important synergistic effects of multiple nutrients in a given diet (Hu, 2002). Indeed, whole-diet modifications are more effective at lowering heart rate than single-nutrient supplementation (Whelton et al., 1992), and dietary supplement trials have shown limited efficacy (Kimmig and Karalis, 2013; Ohlow et al., 2017; Steinhubl, 2008), emphasizing the need to study the effects of the entire diet rather than single nutrients. Clinical studies of long-term diet effects and social influences on health are challenging in humans as diet is difficult to control, and social influences are difficult to document; thus both are limited by their reliance on self-report (Stice and Durant, 2014; Suchanek et al., 2011). Further, no studies of whole diet manipulation in primates have probed the molecular mechanisms through which diet can alter immune function–data that are critical to identifying targets of future therapies and interventions. To address these gaps, we conducted a long-term diet intervention study in nonhuman primates by feeding them either Mediterranean- or Western-like diets. After 15 months on the diet, we assessed monocyte polarization and gene regulation by measuring genome-wide gene expression. As expected, proinflammatory genes were more highly expressed in animals fed a Western diet relative to a Mediterranean diet, indicative of shifts in monocyte phenotypes. Diet also affected gene co-expression patterns, altering large modules of co-regulated genes, and influenced monkey behavioral phenotypes. Western-fed monkeys became more socially isolated and exhibited more anxiety-associated behaviors. Interestingly, these behavioral changes mediated some of the effects of diet on

monocyte gene expression. Together, these results suggest both direct and behaviorally-mediated effects of diet on monocyte polarization that may contribute to chronic inflammatory diseases.

Results

Dietary intervention alters behavior

We fed adult female cynomolgus macaques either a Western-like (hereafter, "Western") or a Mediterranean-like (hereafter, "Mediterranean") diet for 15 months (the equivalent of ~4 years in a human lifespan; Fig. 1A) prior to blood collection and processing. The experimental diets were nutritionally matched with respect to caloric content of macronutrients and formulated to model human diet patterns, as previously described (Shively et al., 2019). Protein and fat were derived primarily from animal sources in the Western diet and plant sources in the Mediterranean diet. Consequently, the two diets differed in their composition of key micronutrients, including fatty acids, polyunsaturated fatty acid ratios, fiber, and sodium (Fig. 1B; see methods and SI Table 1 for a detailed comparison). As expected, the Western diet resulted in significantly increased body weight, caloric intake, body fat, insulin resistance, and hepatosteatosis relative to the Mediterranean diet (Fig. 1C; (Shively et al., 2019)).

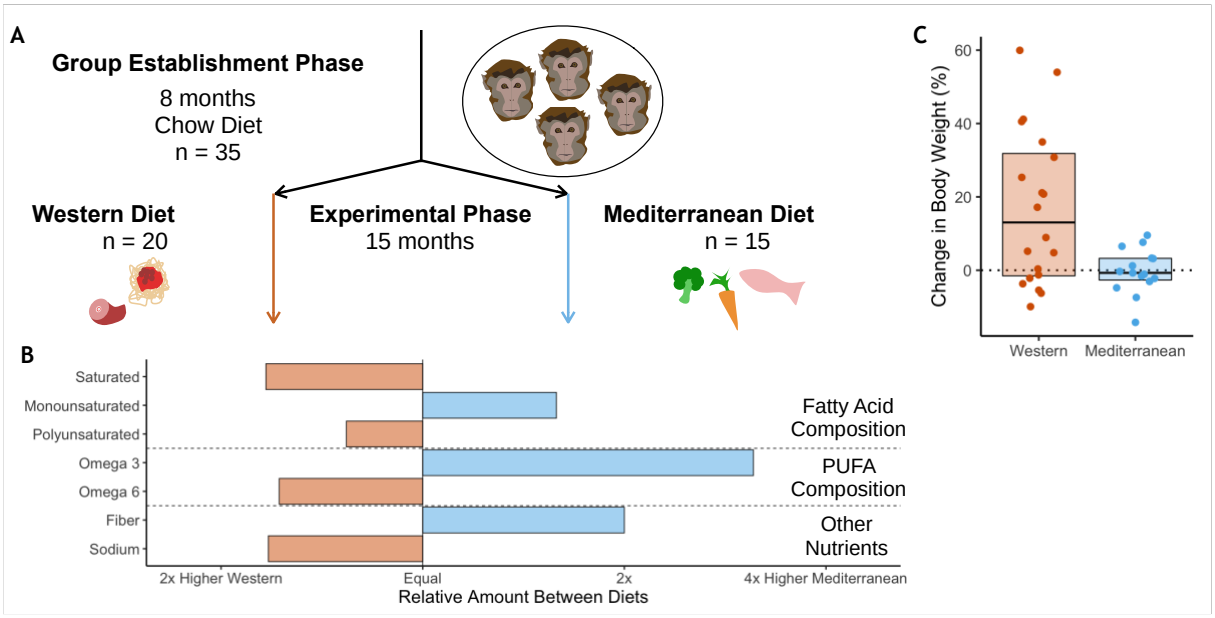

Figure 1. Experimental design and diet effects on body weight. **A)** Monkeys were housed in groups of 3-4 animals (n = 35 monkeys) and fed standard monkey chow diet for 8 months before being fed experimental diets. Behavioral data were collected during the last 6 weeks of the baseline phase and the first twelve months of the experimental phase. Body weight was measured 5 months prior to, and 14 months after the start of the experimental phase. Monocytes were isolated from blood collected 15 months after the start of the experimental phase. **B)** Experimental diets were isocaloric with respect to macronutrients, but differed in food sources and relative amounts of micronutrients. Orange bars indicate nutrients with higher concentration in the Western diet formulation, while blue bars indicate higher levels of a given nutrient in the Mediterranean diet. See SI Table 1 for a more detailed comparison of the two diets. **C)** Percent change in body weight from baseline after 14 months on the diet ($t_{(23.0)}$ = -3.02, p = 0.0023).

Monkeys fed the Western and Mediterranean diets differed in a suite of behaviors. Monkeys fed the Mediterranean diet spent significantly more time in body contact (Mann-Whitney U = 280, p_{BH} = 5.3 x 10^{-5}) and resting (U = 267, p_{BH} = 0.0016), whereas those fed the Western diet spent significantly more time alone (U = 48, p_{BH} = 0.0042; Fig. 2A). There was no significant difference in behavior between the two diet groups in all other measured behaviors (SI Fig. 1, SI Table 2), or in any behaviors during the baseline phase (SI Table 2).

We next leveraged the fact that some behaviors were correlated with one another (SI Fig. 2), and conducted a principal component analysis to reduce the dimensionality to a few behavioral factors (Benito et al., 2018; Seltmann et al., 2018). Behaviors associated with dominance interactions—including aggression, submission, and agonisms—all loaded heavily onto the first principal component, which explained 26.8% of the overall variance in behavior and did not differ between diets (Welch-Satterthwaite $t_{(29.2)}$= 0.0362, p = 0.97; SI Fig. 3, SI Note 1).

155 The second principal component, which explained 15.2% of the variance in behavior, differed
156 significantly between the two diets, and thus represented a composite of diet-altered behaviors
157 (hereafter, DAB). Mediterranean-fed monkeys had significantly higher DAB phenotype scores
158 than their Western-fed counterparts ($t_{(32.6)}$= -4.63, p = 5.6 x 10^{-5}; Fig. 2B). No other principal
159 component was significantly correlated with diet and thus DAB captures the variance in behavior
160 altered by diet (Fig. 4B, SI Table 3). DAB captured a number of anxiety- and socially-relevant
161 behaviors (SI Fig. 5, SI Table 4). Behaviors associated with social integration (e.g., percent of
162 time in body contact, rate of initiating friendly interactions) were positively correlated with
163 DAB, while behaviors related to social isolation and anxiety (e.g., percent of time alone, percent
164 of time grooming self) were associated with lower DAB values (Fig. 2C). Thus, DAB captured a
165 measure of social integration associated with consuming a Mediterranean-like diet, and social
166 isolation and anxiety associated with consuming a Western-like diet.

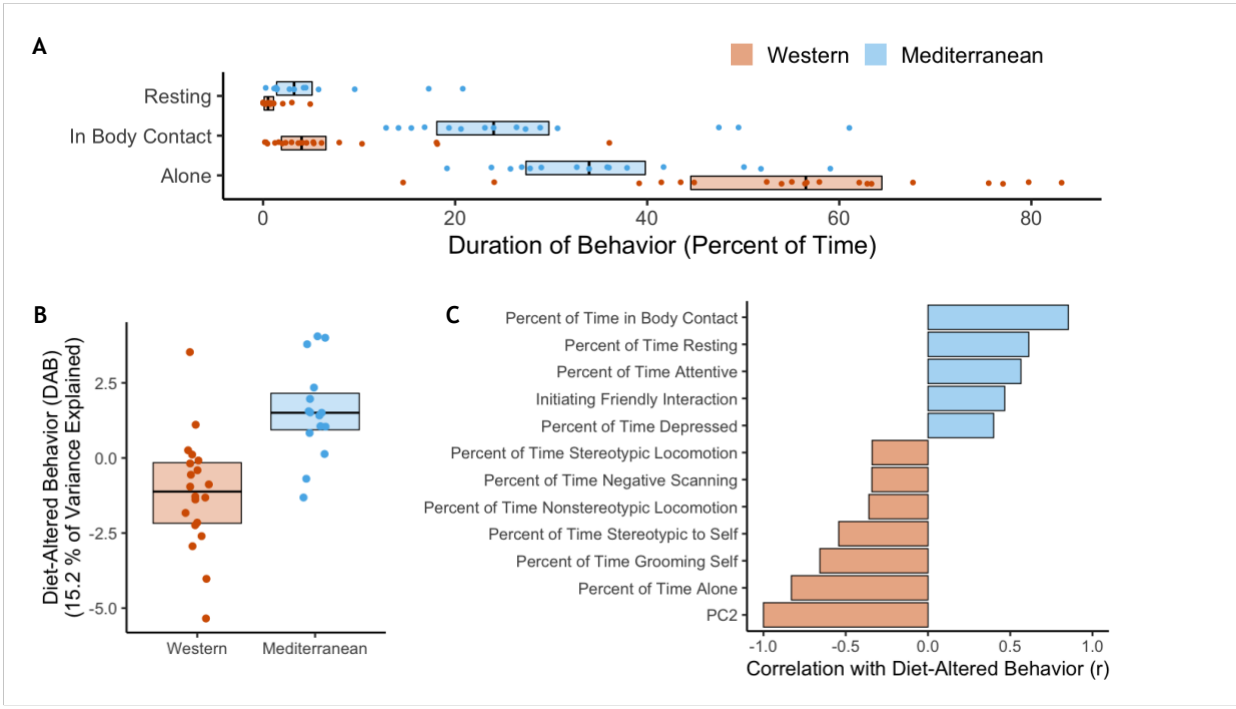

Figure 2. Diet alters behavioral phenotype. A) Three behaviors were significantly different between the two

diet groups. Monkeys fed the Mediterranean diet spent more time in body contact (p_{BH} = 5.3 x 10^{-5}) and resting (p_{BH} = 1.6 x 10^{-3}) than Western-fed monkeys. Monkeys eating the Western diet spent more time alone than Mediterranean-fed monkeys (p_{BH} 4.2 x 10^{-3}). **B)** Composite measures of diet-altered behavior (DAB) were significantly higher in Mediterranean diet compared to Western diet animals ($t_{(32.0)}$= 5.30, p = 8.2 x 10^{-6}). **C)** Eleven of the 32 behaviors observed are significantly correlated with DAB score ($p < 0.05$). Here, significant correlations with DAB in which behaviors are more frequent in Mediterranean diet or Western diet monkeys are indicated with blue or orange bars, respectively.

Diet alters monocyte gene expression and regulation

To test how diet affected circulating monocytes, we used RNA sequencing to measure genome-wide gene expression of purified CD14+ monocytes after 15 months on the experimental diets. The first principal component of genome-wide gene expression, which explained 59.2% variance, was significantly associated with diet ($t_{(25.1)}$ = -4.41, p = 1.7 x 10^{-4}; Fig. 3A), and 40% of the 12,240 expressed genes (SI Table 5A) were significantly differentially expressed between the two diets (n = 4,900 genes, FDR < 0.05; SI Table 5B).

The number of diet-responsive genes was roughly balanced between those that were more highly expressed in monkeys fed the Mediterranean diet (hereafter "Mediterranean genes"; n = 2,664) and those that were more highly expressed in monkeys fed the Western diet (hereafter "Western genes"; n = 2,236). While balanced in direction, the effects of the two diets differed significantly in magnitude ($t_{(3124.9)}$ = -26.0, p = 4.9 x 10^{-135}). The effect size of diet on Western genes was, on average, 1.6-fold larger than on Mediterranean genes (Fig. 3B). Thus, the Western diet induced stronger perturbations in monocyte gene expression than the Mediterranean diet.

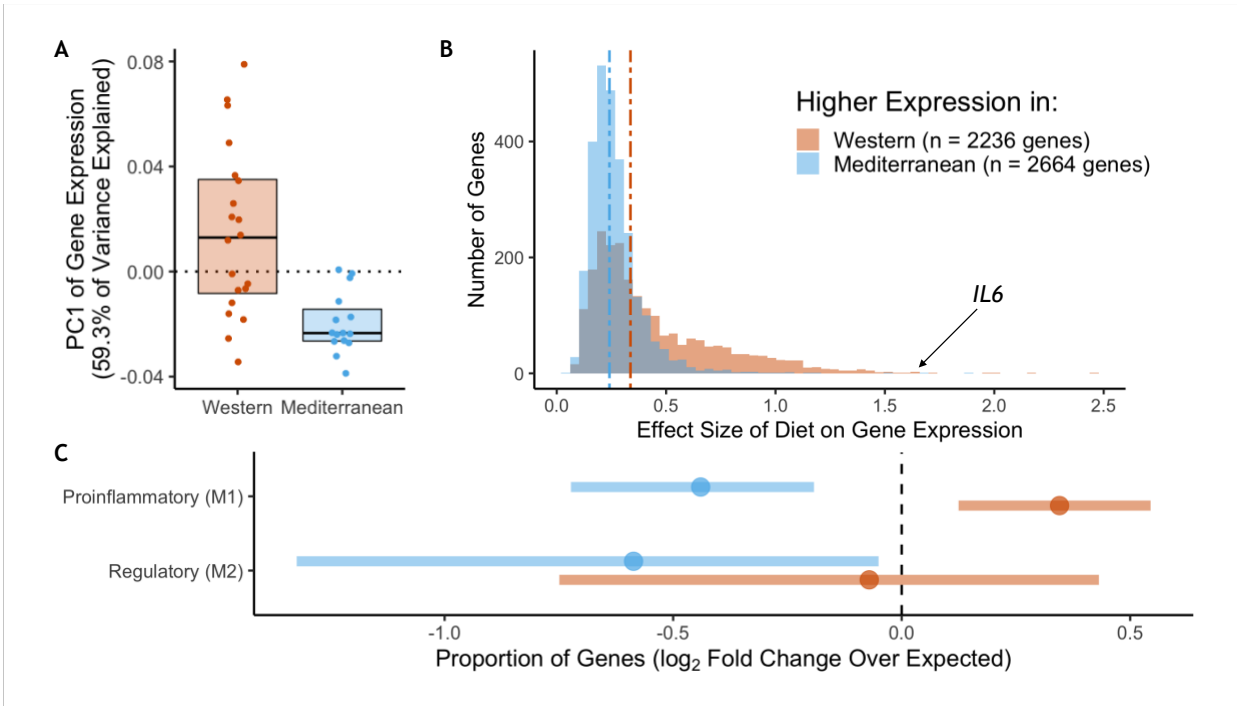

Figure 3. Diet affects monocyte gene expression. A) Diet significantly predicts the first principal component of gene expression (59.3% variance explained, $t_{(25.0)} = -4.41$, $p = 1.72 \times 10^{-4}$). **B)** The effect of diet on genes that were more highly expressed in Western-fed monkeys ("Western genes") was, on average, 1.6-fold larger than "Mediterranean genes" ($t_{(3124.9)} = -26.0$, $p = 4.9 \times 10^{-135}$; mean effect sizes indicated by dashed lines). The effect sizes for differentially expressed genes are shown (FDR < 0.05, n = 4,900 genes). **C)** The proportion of Western genes (orange) and Mediterranean genes (blue) in the subsets of proinflammatory (top) and regulatory (bottom) genes are presented as $\log_2$ fold change from the null expectation (proportion of all genes that are proinflammatory or regulatory). Error bars were calculated through 10,000 permutations of the data. Western genes were enriched for proinflammatory (M1-like) genes (fold-enrichment = 1.27, *95% CI* = 1.09, 1.46), whereas in the Mediterranean genes the same proinflammatory M1-like gene set was under-represented (fold-enrichment = 0.74, *95% CI* = 0.61, 0.88). Regulatory (M2-like) genes were also under-represented in Mediterranean genes (fold-enrichment = 0.67, *95% CI* = 0.40, 0.97), but not in Western genes (fold-enrichment = 0.95, *95% CI* = 0.60, 1.35).

The most strongly affected Western genes included well-known inflammatory-related genes, such as including interleukin-6 ($\beta_{diet} = -1.66$, FDR = 8.9×10^{-3}; Fig. 3B), interleukin-1α ($\beta_{diet} = -$

1.22, FDR = 0.03; SI Table 5B), and two subunits of the NF-κB protein (*NFKB1* β_{diet} = -0.30, FDR = 0.017; *NFKB2* β_{diet} = -0.42, FDR = 0.012). Western genes were enriched for biological processes critical to anabolic regulation of biomass content and replication, including response to growth factor (GO:0070848, $p = 4.6 \times 10^{-3}$) and response to insulin (GO:0032868, $p = 1.4 \times 10^{-3}$; SI Table 6A), suggesting that the Western diet reprogrammed proinflammatory immune, and oxidative metabolic aspects of monocyte gene regulation. Meanwhile, Mediterranean diet promoted antioxidation reductive processes (GO:0055114, $p = 4.3 \times 10^{-3}$; SI Table 6B), a critical function in muting proinflammatory monocytes.

Next, to identify putative upstream gene regulatory mechanisms, we examined if diet-induced changes in gene expression were accompanied by specific transcription factor binding sites. We identified 34 distinct transcription factor-binding motifs enriched within 2 kilobases of the transcription start sites of Mediterranean genes and one that was enriched near the transcription start sites of Western genes (FDR < 0.05; SI Table 7). Among the transcription factors with motifs enriched in Mediterranean gene promoters were key transcription factors—such as PU.1, SP1, and MEF2C—that drive monocyte fate and functional differentiation in response to extracellular signals (Schuler et al., 2008; Scott et al., 1994; Zhang et al., 1994). The binding motifs of three interferon-regulatory factors (IRF) that reprogram monocyte differentiation and polarization were enriched near transcription start sites of Mediterranean genes (Chistiakov et al., 2018). In addition to enrichment of their binding motifs, IRF3, which promotes M2 macrophage polarization and anti-viral response to TLR4 stimulation, and IRF8, which supports monocyte maturation (Günthner and Anders, 2013), also had higher gene expression in Mediterranean-fed monkeys. The sole transcription factor with binding sites enriched in Western diet-associated

genes, ATF2, is a key mediator of inflammatory pathways and diseases, including response to bacterial endotoxin, atherosclerosis, and obesity (Fledderus et al., 2007; Miyata et al., 2012; Reimold et al., 2001). Interestingly, Western genes were enriched for activation of the MAPKK pathway, which lies upstream of ATF2 transcription factor activity (GO:0000186, $p = 4.7 \times 10^{-3}$, SI Table 6A) (Herlaar and Brown, 1999). Thus, some of the diet-associated changes in monocyte gene regulation may be mediated by changes in the activity and binding of key transcription factors.

We next conducted a more targeted analysis of monocyte polarization, by focusing on genes that were previously reported to be differentially expressed between induced proinflammatory (M1) and regulatory (M2) monocyte polarization as defined in (Schmidl et al., 2014) (see SI Table 5A,B for polarization categories). Western genes were enriched for M1-associated genes (*n* = 162 genes, 27.1% more than expected, *95% CI* = +9.0%, +45.9%; Fig. 3C), but not M2-associated genes (*n* = 24 genes, 4.8% fewer than expected, *95% CI* = -59.5%, +34.9%). Conversely, the Mediterranean gene set was depleted for both M1-associated genes (*n* = 112 genes, 26.2% fewer than expected, *95% CI* = -38.9%, -14.4%) and M2-associated genes (*n* = 20 genes, 33.3% fewer than expected, *95% CI* = -60.0%, -3.3%). Together, these observations indicate that a Western diet induces a more proinflammatory (M1-like) phenotype thus providing a target for mechanistic follow-up studies.

Finally, we explored the potential for diet to alter the magnitude or direction of pairwise gene expression correlations among the most strongly diet-affected genes. If so, this could reveal gene regulatory networks that are altered by diet (de la Fuente, 2010; Gaiteri et al., 2014). Many gene

pairs were identified that were significantly associated with each other in both diets, both positively (n = 714) and negatively (n = 332, $p < 0.05$; SI Table 8A). Drawing on a newly developed approach, correlation by individual level product (CILP; (Lea et al., 2019)), we identified 445 gene pairs that were differentially correlated in Mediterranean- versus Western-fed monkeys at a relaxed FDR < 20% (SI Table 8B). The majority (97%) of these gene pairs exhibited positive associations in one diet and negative associations in the other, as we have the most power to detect differential correlation in gene pairs in which the direction of correlation is opposite in the two diets. Some of these gene pairs were significantly correlated in reversed directions (n = 23 gene pairs; nominal p-value < 0.05), suggesting that diet can reverse the co-expression relationship between two genes (Figure 4A). Importantly, we identified 16 "hub" genes that exhibited differential correlations with partner genes more so than expected by chance (Fig. 4B, SI Table 8C). These hub genes were enriched for genes encoding transcription factors (Fisher's Exact Test, FET, OR = 7.40, $p = 7.0 \times 10^{-3}$), including SOX4 (essential for normal insulin secretion and glucose tolerance) and NR4A2 (involved in lipid, carbohydrate, and energy metabolism; (Goldsworthy et al., 2008; Pearen and Muscat, 2010)), thereby implicating immune and metabolic reprogramming by the Western and Mediterranean diet. Interestingly, the hub gene involved in the greatest number of differentially-correlated gene pairs was *RF00283*–a non-coding RNA that has been associated withed BMI and HDL cholesterol in human genome-wide association studies ((Davis et al., 2017; Kanai et al., 2018; Tachmazidou et al., 2017); Fig. 4B-D).

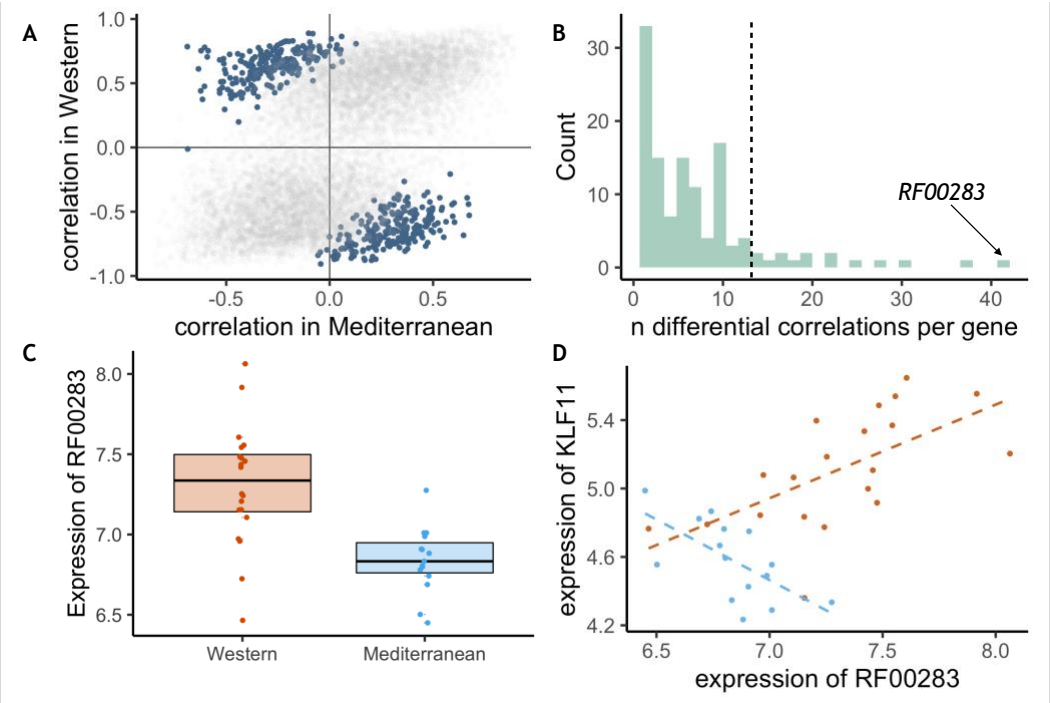

Figure 4. Diet affects monocyte gene co-expression. A) The Pearson correlation between a given pair of genes within each of the experimental diets are plotted against each other. Gene pairs that are significantly differently correlated between diets are highlighted in blue (n = 445 significant pairs, FDR < 20%). **B)** Of the genes involved in significant pairs, some were paired with more genes than expected by chance (n = 16 "hub" genes; dotted black line is the maximum number of significant pairs expected by chance). The strongest hub gene was the non-coding RNA *RF00283*. **C)** Residual normalized expression of *RF00283* is significantly greater in Western- than Mediterranean-fed monkeys (β_{diet} = -0.507, FDR = 2.3 x 10^{-6}). **D)** Example of a differential correlation involving *RF00283*. Residual normalized expression of *RF00283* is plotted against expression of *KLF11*, a differentially-expressed transcription factor that regulates insulin and has been associated with type II diabetes in humans (Neve et al., 2005). The two genes are positively correlated in Western-fed monkeys (r = 0.61, p<0.005) and negatively correlated in Mediterranean-fed monkeys (r = -0.63, p<0.01).

Diet-altered behaviors mediate some changes in monocyte gene expression

The strong effects of diet on both behavior and monocyte gene expression led us to examine if the effect of diet on gene expression was mediated by diet-induced changes in behavior. Of the

4,900 diet-affected genes, 34% were also significantly associated with DAB in a univariate model (n = 1,679, $p < 0.05$). Of these, DAB significantly mediated the effect of diet on the expression of 872 genes (18% of all diet-associated genes, bootstrapped $p < 0.05$; Fig. 5A). These DAB-mediated genes were significantly more likely to be Western genes (n = 482, 55%, two-sided binomial test $p = 0.0020$), and were enriched in biological processes such as regulation of leukocyte migration (FET $p_{BH} = 0.0042$; SI Table 9A-C). We also tested the possibility that diet effects on behavior were mediated through changes in monocyte gene expression but found no genes that significantly mediated the relationship between diet and DAB (bootstrapped $p <$ 0.05; Fig. 5B).

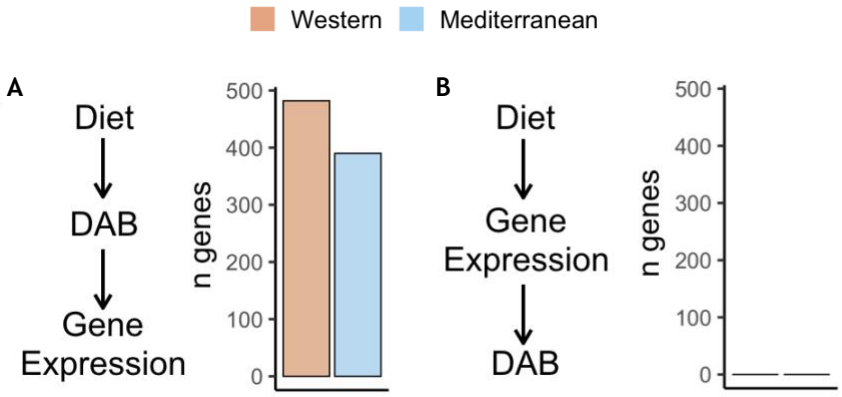

Figure 5. Behavior partially mediates the effect of diet on gene expression for 20% of diet-associated genes. **A)** Behavior significantly mediated the effect of diet on gene expression in 872 genes (n = 482 higher in Western, 390 higher in Mediterranean). **B)** Conversely, gene expression did not significantly mediate the effect of diet on DAB in any genes.

Discussion

Here, we showed for the first time that a controlled, whole-diet manipulation exerts profound effects on monocyte gene regulation and behavior in a primate. Forty percent of the expressed genes were differentially expressed between monkeys fed Western or Mediterranean diets, indicating that diet dramatically altered monocyte programming. Beyond differential gene expression, we identified differences in gene co-expression and enrichment of transcription factor binding motifs, suggesting that diet alters gene regulatory networks. More specifically, we found that the Western diet promoted a more proinflammatory monocyte phenotype, while the Mediterranean diet had a more benign effect suggesting better balance in monocyte polarization.

We identified the enrichment of binding motifs for numerous transcription factors that appear to be involved in diet-regulated gene expression. Of note, members of the E26 transformation-specific (ETS), specificity protein (Sp)/Krüppel-like family (KLF), myocyte-specific enhancer factor (MEF), and interferon-regulatory factor (IRF) families of transcription factors have all been linked to myeloid differentiation (Chistiakov et al., 2018; Schuler et al., 2008; Scott et al., 1994; Zhang et al., 1994) and were overrepresented in regulatory regions of genes with higher expression in monkeys fed the Mediterranean diet in this study. Broadly, this suggests that transcriptional networks involved in monocyte differentiation were differentially affected by our diet manipulation. IRF-1 and IRF-8 are linked to M1 monocyte polarization, while IRF-3 is associated with M2 polarization, and all three had binding motifs enriched in genes more highly expressed in monkeys fed the Mediterranean diet (i.e., more lowly expressed in monkeys fed a Western diet).

The Western diet promoted a more proinflammatory monocyte polarization relative to Mediterranean diet, which is consistent with that seen in brain myeloid cells of mice fed a Western diet (Yang et al., 2019). Conversely, Mediterranean diet could reduce proinflammatory gene expression, as has been suggested in at least one study in humans (Camargo et al., 2012). Future comparisons of each diet to the current standard diet for captive primates will help to identify which of these two non-mutually exclusive hypotheses is most likely. It is also worth noting that the M1/M2 paradigm of monocyte polarization is a simplification of the heterogeneity that exists within organisms (Martinez and Gordon, 2014; Nahrendorf and Swirski, 2016) and the patterns of gene expression following the diet manipulation described here may indicate alternative phenotypes that fall outside of the simplified M1/M2 binary.

In addition to altering monocyte gene expression, this diet manipulation also led to differences in a suite of important behaviors. Monkeys consuming the Western diet exhibited more anxiety and social isolation, a phenotype remarkably similar to that observed in juvenile Japanese macaques born to mothers consuming a high-fat Western diet (Thompson et al., 2018). In that study, offspring behavior was associated with maternal levels of macrophage-derived chemokine (MDC), which showed higher expression in Western-diet fed animals in our study (β_{diet} = -0.243, FDR = 0.059). Our findings extend the behavioral effects of a Western diet into adulthood. Future work will investigate if the direct diet effects on adult behavior are mechanistically similar to the transgenerational diet effects identified in Japanese macaques born to high-fat-fed mothers.

For a subset (18%) of genes, the diet-altered behavior (DAB) phenotype mediated the effect of

diet on monocyte gene expression. Notably, the Western diet induced both a proinflammatory monocyte phenotype, as well as social isolation and anxiety behaviors. It is, therefore, possible that a Western diet contributes to inflammation by producing a more socially isolated or anxious animal. Indeed, monocytes have been shown to be responsive to social stimuli, including social isolation (Cole, 2019) and anxiety (Cole et al., 2015), suggesting that part of the effect of diet on monocyte function may be due to diet-induced changes in socially relevant behaviors. In support of this hypothesis, animals fed a Western diet exhibited significantly higher expression of pro-inflammatory genes involved in the "conserved transcriptional response to adversity" (CTRA; Wilcox T-test $p = 0.016$; (Cole et al., 2015), and lower expression of antiviral- and antibody-related CTRA genes (Wilcox T-test $p = 0.023$; SI Fig. 6).

The mechanistic pathways through which diet affects behavior are still unclear. It is possible that diet induces changes in the central nervous system in myriad ways: including infiltration of cytokines from the periphery (Raison et al., 2006), stimulation of resident immune cells in the brain (Yang et al., 2019), alteration of membrane lipids (Du et al., 2016), and direct anti-inflammatory action of n-3 fatty acids in the brain (Layé et al., 2018). We previously showed that diet had a strong effect on the microbiome (Nagpal et al., 2018), which is a key component of the gut-brain axis (Sharon et al., 2016), representing another possible route through which diet could alter behavior. Diet could also alter behavior through other physiological systems, including those that monitor satiety and thermoregulation (Aydin and Gordon, 2013; Diekmann et al., 2019). Future work on the neurobiological responses to diet manipulations could help clarify these putative mechanisms.

Interestingly, Western diet induced substantial variation in multiple phenotypes, including body weight, gene expression, and behavior. This heterogeneity in response to diet is consistent with previous studies demonstrating that some individuals may be more resistant (or susceptible) to the effects of a Western diet (Shively et al., 2009), presumably due to genetic variation or past environmental experiences. However, we were unable to identify any consistencies in individual responsiveness across the phenotypes (SI Fig. 7). For instance, monkeys that exhibited a strong gene regulatory response to the Western diet did not also exhibit the largest increase in body weight. Change in body weight was significantly correlated with diet-altered behavior (DAB) within Western diet fed monkeys ($r_{Western}$ = -0.584, p = 0.0069; SI Fig. 7A), but change in body weight did not significantly predict the expression of any genes at an FDR < 20%. Western diet fed individuals thus exhibited a mosaic response to diet across multiple phenotypes, of which the underlying mechanisms remain unknown but presumably involve interactions between diet, environment, and the genome.

In summary, we found that diet significantly alters behavior and monocyte polarization. The Western diet increased the proinflammatory phenotype relative to a diet, which supports the role of monocyte polarization in diet-associated chronic inflammatory diseases. Thus, avoiding a Western-style diet and/or consuming a Mediterranean-style diet could be beneficial in preventing or treating chronic inflammation and disease. The majority of the effects of diet are presumably mediated through direct or combined actions of saturated/polyunsaturated fats, n-6:n-3 ratios, pro- and anti-antioxidant characteristics, and other unique features of the protein and carbohydrate constituents in the two diets. Monocyte reprogramming was also partially mediated by the diet-induced changes in behavior, although the mechanisms by which this occurred are

unknown. Ongoing and future work will address interactions between social behavior (e.g., social status) and diet to further understand how environmental stressors may impact inflammation in the periphery and in the central nervous system.

Methods

Subjects

At study initiation, subjects were 43 adult (age: mean = 9.0, range = 8.2-10.4 years, estimated by dentition), female cynomolgus macaques (*Macaca fascicularis*), obtained from Shin Nippon Biomedical Laboratories (SNBL USA SRC, Alice, TX) and housed at the Wake Forest School of Medicine Primate Center (Winston-Salem, NC) as described previously (Shively et al., 2019). Briefly, monkeys were socially housed in groups of 3-4 and consumed standard monkey chow (SI Table 1) during an eight-month baseline phase, after which they were fed either the Western (5 groups, $n = 21$) or Mediterranean (6 groups, $n = 22$) diet, balanced on pretreatment characteristics that reflected overall health, including body weight, body mass index, and plasma triglyceride concentrations ((Shively et al., 2019); Fig. 1A). Two monkeys did not tolerate the experimental diet, and were switched to standard monkey chow, three animals died during the course of the study, and a further three were identified as outliers (see "Removal of Batch Effects" below), resulting in a sample size of 35 (Western $n = 20$, Mediterranean $n = 15$). All animal manipulations were performed according to the guidelines of state and federal laws, the US Department of Health and Human Services, and the Animal Care and Use Committee of Wake Forest School of Medicine.

Experimental Diets

Experimental diets were formulated to be isocaloric with respect to protein, fat, and carbohydrate macronutrients, and identical in cholesterol content (~ 320mg / 2000 kilocalories (Cals)/day) as described previously (Shively et al., 2019). The Western diet was formulated to be similar to that consumed by American women age 40-49 as reported by the US Dept. Agriculture, with protein

and fat derived mainly from animal sources. The Western diet was relatively high in saturated fat and sodium, and low in monounsaturated fat and n-3 fatty acids. The Mediterranean diet was formulated to mimic key aspects of the traditional Mediterranean diet, with an n-6:n-3 fatty acid ratio similar to a traditional hunter-gatherer type diet (Bédard et al., 2012; Cordain et al., 2005; Kafatos et al., 2000). Protein and fats were derived mainly from plant sources, fish and dairy, and monounsaturated fatty acids were relatively high. Mediterranean diet contained more complex carbohydrates and fiber, and less sodium and refined sugars than Western diet. Key ingredients included English walnut powder and extra-virgin olive oil which were the primary components provided to participants in the PREDIMED study, a landmark dietary intervention study that illustrated the role of the Mediterranean diet on cardiovascular disease prevention (Estruch et al., 2018). For details of the composition of both diets and how they relate to monkey chow, see SI Table 1.

Behavioral Characterization

Behavioral data were collected weekly during two 10-minute focal observations, randomly ordered and balanced for time of day, for 6 weeks during the baseline phase (2 total observation hours/monkey) and for 12 months during the experimental phase (15 total observation hours/monkey). Behaviors were collected as previously described (Shively, 1998), and combined into summary behaviors (e.g., “aggression” was a combination of all total, noncontact, contact aggressive events). A complete table of all behavioral data can be found in SI Table 2. In order to quantify the overall impact of diet on behavior, we conducted a principal component analysis using the R package *FactoMineR* (Lê et al., 2008).

Blood Sample Collection

We briefly removed monkeys from their social group, drew blood via venipuncture within 9 minutes of entering the building, and returned the animal to their group. Blood was collected into EDTA-containing tubes, mixed with an equal amount of PBS without calcium or magnesium, and overlaid on a 90% Ficoll-Paque Plus/10% PBS solution in LeucoSep tubes followed by centrifugation at 800 x g for 20 min. Isolated PBMCs were then immediately used for the collection of CD14+ monocytes by positive selection using a Miltenyi bead-based protocol following manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After assessing cell viability and numbers, CD14+ monocytes were stored in 85% FBS, 15% DMSO sterile freezing media at -80°C and transferred to liquid nitrogen for storage until RNA extraction.

RNA extraction and sequencing

RNA was extracted from monocytes using the AllPrep DNA/RNA Mini Kit (Qiagen, Inc., Hilden, Germany), and quantified using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technology, Inc., Santa Clara, CA). RNA libraries were prepared for sequencing by the Cancer Genomics Shared Resource (Wake Forest University, Winston-Salem, NC) using the TruSeq-stranded total RNA kit (Illumina), which includes a ribosomal depletion step. The RNA-seq libraries were then sequenced using single-end 76-bp reads on an Illumina NextSeq 500 to an average read depth of 34.5 million reads per sample (range 25.9 – 41.6 million reads). Reads were mapped to the *Macaca fascicularis* reference genome (Macaca_fascicularis_5.0, v 93, Ensembl) (Kersey et al., 2018; Kinsella et al., 2011) using HiSat2 (Kim et al., 2015) and then converted to a sample-by-gene read count matrix

using featureCounts (Liao et al., 2014) (median = 38.0%; range 24.5 - 50.4% of reads mapped to exons).

Read Count Normalization and Removal of Batch Effects

First, we removed lowly expressed genes (median reads per kilobase per million reads mapped < 1), which resulted in 12,240 genes for our downstream analyses. We normalized read counts using the *voom* function of the R package *limma* (Ritchie et al., 2015). While investigating monocyte purity, three samples differed in CD3 gene expression from the rest by several orders of magnitude. We concluded that these samples were contaminated with CD3+ cells (i.e., inefficient CD14 purification, see SI Fig. 8) and excluded them from all analyses, leaving a final sample size of 35 monkeys (n = 20 WEST, n = 15 MED). To control for batch effects related to RNA quality and monocyte purity, we calculated the residual gene expression from a model of normalized gene expression as a function of CD14 expression, CD3 expression, RNA integrity, and RNA concentration. These residual gene expression values were used for all subsequent analyses.

Modeling Effect of Diet on Gene Expression

In order to determine which genes were significantly affected by diet, we modeled the residual expression of each gene as a function of diet using a linear mixed effects model controlling for relatedness among monkeys using the R package *EMMREML* (Akdemir and Godfrey, 2015). Relatedness was estimated using the ngsRelate program (Hanghøj et al., 2019) with SNP genotypes inferred from the RNA-seq reads using bcftools mpileup (Li et al., 2009). We calculated an empirical false discovery rate (FDR) for each gene using a permutation-based

approach (Snyder-Mackler et al., 2016). Genes that passed a threshold of FDR < 0.05 were considered differentially expressed between the two diets. To examine global patterns of variation in gene expression, we conducted principal component analysis on the correlation matrix of normalized residual gene expression using the *prcomp* function in R.

Enrichment analyses

Gene ontology (GO) enrichment analyses were conducted using Fisher's Exact Tests using the *weight01* algorithm to test for enrichment implemented in the R package *topGO* (Alexa and Rahnenfuhrer, 2019). For a more targeted analysis of M1 and M2 specific genes, we identified a set of differentially expressed genes in our data set that were previously found to be involved in monocyte polarization (Schmidl et al., 2014) (638 proinflammatory and 138 regulatory), which we used to explore monocyte polarization in the current study. We calculated the proportion of genes more highly expressed in the Mediterranean- and Western-fed animals in each polarization category and tested for significance using a permutation test (n = 100,000 permutations).

Transcription Factor Binding Site Analysis

We tested for enrichment of transcription factor binding motifs within 2 kb (upstream or downstream) of the transcription start sites of differentially expressed "Western genes" or "Mediterranean genes" (FDR < 0.05) using the program HOMER (Heinz et al., 2010) and equivalent regions around the transcription start sites of all genes expressed in these data as the background set for enrichment testing. We searched for known vertebrate transcription factor binding motifs and report the TF motifs passing a threshold of FDR < 0.05.

Gene-gene co-expression analysis

In addition to testing whether diet led to mean differences in gene expression between Western and Mediterranean animals, we also tested whether diet impacted the correlation structure among expressed genes (i.e., gene co-expression). Specifically, we used 'correlation by individual level product' (CILP) (Lea et al., 2019), to test whether diet affected the magnitude or direction of pairwise gene expression correlations among the top 140 most differentially expressed genes (n = 9730 gene-gene pairs tested, equivalent to ${}_{140}C_2$). To test whether a given pair of genes was differentially co-expressed as a function of diet, we first obtained a vector of products for each gene pair by multiplying the normalized gene expression values for two genes together. Normalization was performed by scaling expression values to mean 0 and unit variance within Mediterranean and Western subsets of the data respectively, to ensure that distributional differences between sample groups did not bias our results, following previously described procedures (Lea et al., 2019). Each of these vectors of products were used as the outcome variable in a linear mixed effects model implemented in the R package *EMMREML* (Akdemir and Godfrey, 2015), which included a fixed effect of diet and a random effect to control for genetic relatedness. To assess significance, we extracted the p-value associated with the diet effect for all 9730 gene pairs. We then reran each linear mixed effects model 100 times after permuting diet, extracted the p-value associated with the diet effect, and used these values to calculate an empirical FDR distribution (Snyder-Mackler et al., 2016).

Using this approach, we identified 445 gene pairs that were significantly differentially co-expressed as a function of diet at a 20% empirical FDR. Next, we performed two follow up analyses to understand their biological import. First, we tested for the existence of 'hub genes',

defined as genes that displayed differential co-expression to their tested partner genes more so than expected by chance. To define the null distribution for identifying hub genes, we randomly sampled 445 gene pairs from the set of all 9730 tested gene pairs 1000 times and calculated the number of partners a focal gene had in each sample; we considered a gene to be a significant 'hub gene' if it fell outside the 95$_{th}$ percentile of this distribution, which was equivalent to a focal gene that displayed significant differential co-expression with 13 or more of its tested partner genes. Second, we asked whether the set of 'hub genes' we identified were enriched for transcription factors, relative to the background set of all 140 genes tested for differential co-expression. We performed this analysis because many of the proposed mechanisms to generate large scale changes in gene co-expression patterns involve changes in transcription factor function or activity (de la Fuente, 2010; Gaiteri et al., 2014). To implement the enrichment analysis, we used the TRRUST database of known mammalian transcription factors for annotation (Han et al., 2018) paired with hypergeometric tests.

Mediation

To test if DAB mediated the effect of diet on gene expression, we conducted mediation analyses. We used a bootstrapping approach where we conducted 10,000 bootstrap iterations of two models: (Model 1) the expression of each gene as a function of diet, and (Model 2) the expression of each gene as a function of diet and DAB (Preacher and Hayes, 2004). For each bootstrap iteration, we then calculated the mediation effect (i.e., the indirect effect) of DAB as the difference between the effect size of diet in Model 1 (β_{diet}) and Model 2 (β'_{diet}). We considered there to be a mediation effect when the 95% confidence interval for the indirect effect (β_{diet}-β'_{diet}) did not include zero.

A similar method was used to calculate the mediation of gene expression on DAB, testing the difference between the effect size of diet in two models: (Model 3) DAB as a function of diet, and (Model 4) DAB as a function of diet and the expression of each gene.

Data Availability

All data and code used to complete these analyses can be found at https://github.com/cscjohns/diet_behavior_immunity. The raw data can be accessed from the gene expression omnibus repository from accession # GSE144314.

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Author Contributions

Conceptualization, C.A.S. and T.C.R.; Methodology, C.A.S., T.C.R., N.S.-M., and C.S.C.J.; Formal Analysis – C.A.S., T.C.R., N.S.-M., A.J.L., and C.S.C.J; Investigation, M.E., A.N.V., N.A.V., S.C.P., and S.Y.W.; Writing – Original Draft, C.A.S., T.C.R., N.S.-M., and C.S.C.J.; Writing – Review & Editing, all authors; Visualization – N.S.-M., A.J.L., and C.S.C.J; Funding Acquisition, C.A.S. and T.C.R.; Supervision, C.A.S., T.C.R., and N.S.-M.

Declaration of Interests

The authors declare no competing interests.

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