Mediterranean Diet Reduces Monocyte Inflammatory Gene Expression and Influences Social Behavior in Nonhuman Primates

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Abstract: Western diet consumption is associated with inflammation, cardiometabolic disease, and mortality in humans, while Mediterranean diet consumption confers protective effects. One likely pathway for this association is through environmentally induced changes in monocyte function, yet the underlying mechanisms remain elusive. We conducted the first randomized, long-term diet manipulation in a non-human primate model to determine whether Western- or Mediterranean-like diets alter monocyte polarization and health. Monocyte gene expression profiles differed markedly between the two diet groups, with significant differences in over 40% of expressed genes. The Western diet induced a more proinflammatory monocyte phenotype overall and upregulated specific monocyte polarization genes. Diet also disrupted the coexpression of numerous gene pairs, including small RNAs and transcription factors associated with metabolism and adiposity in humans. Diet altered affiliative and anxiety-associated behaviors and mediation analysis showed that the diet-altered behaviors contributed significantly (~50% of the effect of diet on gene expression) to 25% of the differentially expressed genes, suggesting that diet effects on central mechanisms also modulate monocyte gene expression. Together, these results identify both behavioral and molecular mechanisms underlying the health benefits of a Mediterranean diet regimen.

Significance Statement: Some of our largest public health burdens are driven by dietary changes associated with industrialization, but we still know very little about the molecular mechanisms underlying this link. Characteristic "Western diets" have been associated with increased risk for diseases related to chronic inflammation, while Mediterranean diets have anti-inflammatory benefits. Here, we identify causal effects of diet on inflammatory gene expression where consumption of the Mediterranean diet reduced inflammatory gene expression in monocytes. Additionally, our diet manipulation induced behavioral changes associated with anxiety and social integration, where Mediterranean-fed animals exhibited more positive affiliative behaviors and reduced anxiety. These behaviors were associated with 25% of the diet-affected genes, suggesting an important behavioral route through which diet can impact immune function.

61 **[Main Text]**

62 **Introduction**

63 Modern human diets profoundly impact our health and survival, and vary across geography,
64 cultures, and socioeconomic strata. In general, the Western diet derives most of its protein and
65 fat from animal sources, and is high in simple sugars and saturated and n-6 fatty acids. These
66 constituents can arouse the sympathetic nervous system, increase oxidative stress, and elevate
67 levels of inflammatory markers[1–6], and are thus associated with increased risk for metabolic
68 syndrome[7], type II diabetes[8], cardiovascular disease[7,9], autoimmune disorders[10], depression[11],
69 and increased mortality[12]. By contrast, Mediterranean diets are richer in protein and fat from
70 vegetable sources, raw fruits and vegetables, and are higher in monounsaturated and n-3 fatty
71 acids. These latter components have been associated with an anti-inflammatory phenotype[13],
72 reduced incidence of chronic disease, and increased longevity[14–17]. Despite these associations,
73 the casual nature of these links and mechanisms through which these diets induce their effects
74 remain largely unknown.
75
76 To date, attempts to understand how Western versus Mediterranean diets affect health through
77 changes in immune phenotypes have relied on (i) correlational analyses of self-reported diet in
78 humans, (ii) limited and short-term dietary interventions in humans, or (iii) experimental
79 manipulations of single nutrients in animal models[18–22]. Approaches (i) and (ii) are limited in
80 their ability to address causality[23,24], and approach (iii) cannot address the potentially important
81 synergistic effects of multiple nutrients. Further, few studies have probed the molecular
82 mechanisms through which diet can alter immune function–data that are critical for
83 understanding the immunological consequences of diet and identifying targets of future therapies

and interventions.

Circulating monocytes are likely to play a key role in modulating the effects of diet and other factors on health[3–6,25]. Monocytes are important mediators of inflammation, sensitive to local and systemic factors such as diet and stress, and may provide a key nexus for understanding stress effects as well as novel targets for therapies. Monocytes and monocyte-derived macrophages are innate immune cells that vary phenotypically along a spectrum which ranges broadly from proinflammatory (M1-like) to regulatory/reparative (M2-like). 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[26]. 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[26]. Diet may alter disease propensity by reprogramming the balance between these proinflammatory and anti-inflammatory monocyte subsets, but this hypothesis remains to be tested[25].

In addition to altering the regulation of immune cells directly, diet may affect inflammatory phenotypes indirectly by altering social behaviors, which are known to shape gene expression programs in immune cells. In particular, multiple sources of social adversity, such as low social status and poor social integration, have been shown to increase the expression of inflammatory genes in primary white blood cells in humans and other animals[27–32]. Given that some food contituents can directly alter social behaviors themselves[33–37], it is therefore possible that diet

effects on immune cell regulation may, to some degree, be mediated by changes in social environmental conditions. However, because no detailed studies of diet, social behavior, and immune cell phenotypes have been conducted, it remains unclear how these factors are linked and how they ultimately scale up to affect health.

To address these gaps, we conducted a whole-diet manipulation to directly compare the effects of Mediterranean and Western diets on behavior, monocyte gene expression, and physiological outcomes related to metabolic health in nonhuman primates. By implementing a randomized preclinical trial design, we were able to identify causal effects of realistic complex diet patterns. After 15 months of dietary manipulation, cardio-metabolic phenotypes were significantly worse and proinflammatory gene expression was significantly higher in animals fed a Western diet relative to a Mediterranean diet. Diet also affected monocyte polarization, altered gene co-expression patterns, and influenced behavior. Western-fed monkeys became more socially isolated and exhibited more anxiety-associated behaviors, and these behavioral changes mediated some of the effects of diet on monocyte gene expression. These behavioral effects imply that the diet altered monocyte gene expression in part via the central nervous system. Together, these results suggest both direct and behaviorally-mediated effects of diet on monocyte polarization may contribute to chronic inflammatory diseases, and identify potential mechanisms by which Mediterranean-like diets may lead to health benefits.

Results

Diet induced alterations in health indices

Adult female cynomolgus macaques were fed either a Western-like (hereafter, “Western”) or a

130 Mediterranean-like (hereafter, "Mediterranean") diet for 15 months (the equivalent of ~4 years in
131 a human lifespan; Fig. 1A). The experimental diets were nutritionally matched with respect to
132 caloric content of macronutrients and formulated to model human diet patterns, as previously
133 described[38]. Protein and fat were derived primarily from animal sources in the Western diet and
134 plant sources in the Mediterranean diet. Consequently, the two diets differed in their composition
135 of key micronutrients, including fatty acids, polyunsaturated fatty acid ratios, fiber, and sodium
136 (Fig. 1B; see methods and Table S1 for a detailed comparison). As previously reported, Western
137 diet significantly increased body weight, caloric intake, body fat, insulin resistance, and
138 hepatosteatosis relative to the Mediterranean diet[38] (Fig. 1C).
139

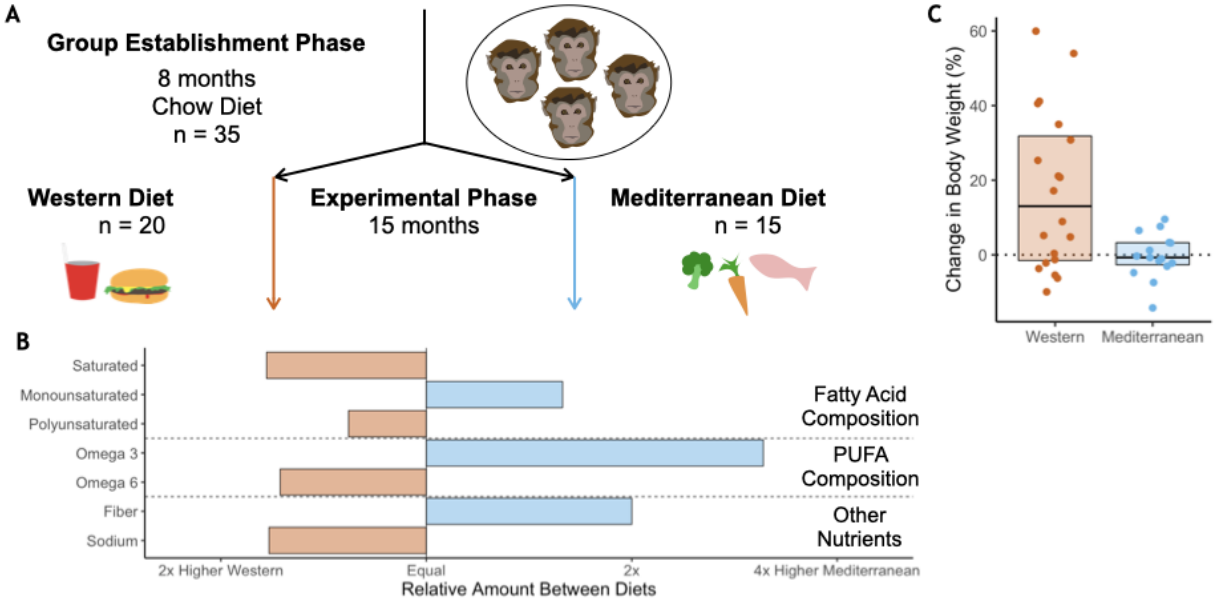

Figure 1. Experimental design and diet effects on body weight. **A)** Monkeys were housed in groups of 3-4 animals (*n* = 35 monkeys) and fed standard monkey chow diet for 8 months before being fed experimental diets. Behavioral data were collected during the last 6 weeks of the baseline phase and the during months 1-14 of the experimental phase. Body weight measurements reported are from 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 Table S1 for diet compositions. **C)** Percent change in body weight from baseline after 14 months on the diet ($t_{(23.0)} = 3.02$, $p = 0.0023$).

Diet induced major shifts in monocyte gene expression

To test how diet affected the phenotypes of circulating monocytes, we used RNA sequencing to measure genome-wide gene expression of purified CD14+ monocytes after 15 months on the experimental diets. Diet had a strong effect on monocyte gene expression: the first principal component of gene expression, which explained 59.2% variance, was significantly associated with diet ($t_{(25.1)} = 4.41$, $p = 1.7 \times 10^{-4}$; Fig. 2A), and 40% of the 12,240 expressed genes (Table S2A) were significantly differentially expressed between the two diets ($n = 4{,}900$ genes, FDR < 0.05; Table S2B). The number of diet-responsive genes was roughly balanced between those that were more highly expressed in monkeys fed the Mediterranean diet ($n = 2{,}664$; hereafter "Mediterranean genes") and those that were more highly expressed in monkeys fed the Western diet ($n = 2{,}236$; hereafter "Western genes"). While balanced in direction, the distributions of effect sizes in these two sets of genes differed significantly (one sided Kolmogorov-Smirnov test, $D = 0.33$, $p = 5.2 \times 10^{-112}$) and the effect size of diet on Western genes was, on average, 1.6-fold larger than on Mediterranean genes (Mann-Whitney $U = 4.1 \times 10^{6}$, $p = 6.1 \times 10^{-117}$; Fig. 2B). Thus, the strongest effects are seen in genes that are either activated by a Western diet or suppressed by a Mediterranean diet.

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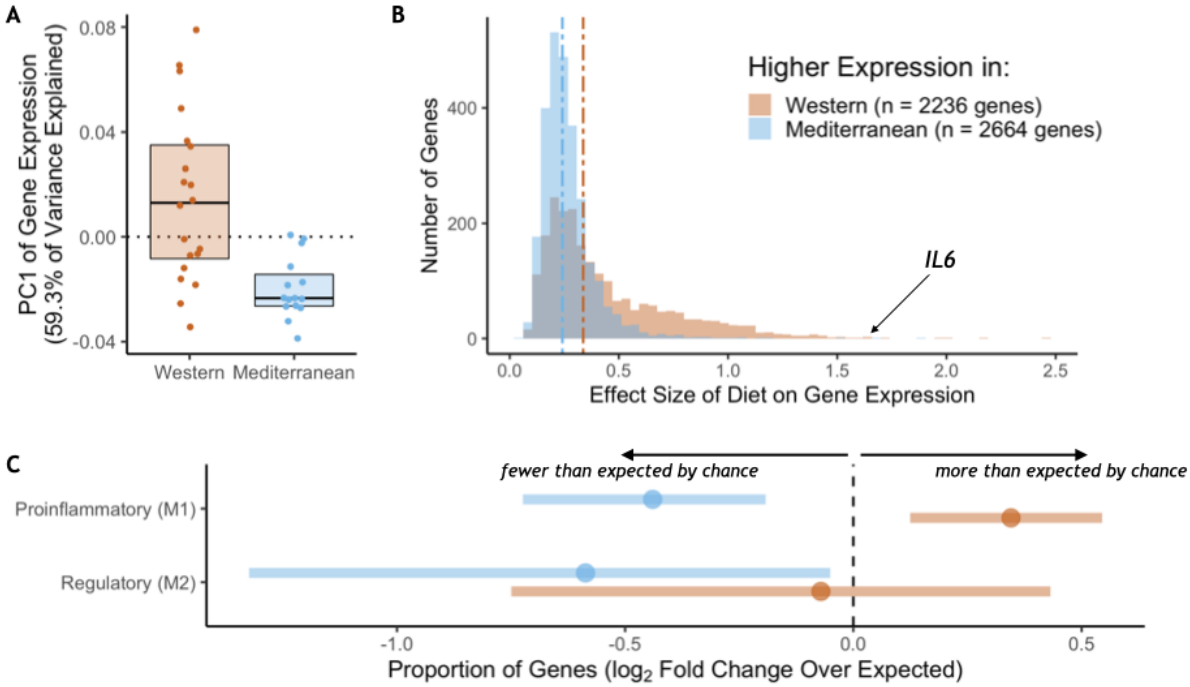

Figure 2. Diet effects on monocyte gene expression. A) Diet significantly predicts the first principal component of gene expression (59.3% variance explained, $t_{(25.0)} = 4.41$, $p = 1.72 \times 10^{-4}$). **B)** The average effect size of diet on Western genes was 1.6-fold larger than the effect size of diet on Mediterranean genes (Mann-Whitney $U = 4.1 \times 10^6$, $p = 6.1 \times 10^{-117}$). **C)** Log_2 fold enrichment of proinflammatory (top) and regulatory (bottom) genes in Western genes (orange) and Mediterranean genes (blue). Western genes contained more M1 genes than expected by chance, indicating that the Western diet induced a shift towards a proinflammatory monocyte phenotype. Western genes were enriched for proinflammatory (M1-like) genes (fold-enrichment = 1.27, *95% CI* = 1.09, 1.46), while Mediterranean genes were depleted of these same M1-like genes (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).

159

160 Monocytes in animals fed the Western diet had higher expression of a number of well-known
161 inflammatory-related genes, including interleukin-6 ($\beta_{diet} = 1.66$, FDR = 8.9×10^{-3}; Fig. 2B),
162 interleukin-1α ($\beta_{diet} = 1.22$, FDR = 0.03; Table S2B), and two subunits of the NF-κB protein

(*NFKB1* β_{diet} = 0.30, FDR = 0.017; *NFKB2* β_{diet} = 0.42, FDR = 0.012; Table S2B). Western genes were significantly more likely to be involved in replication and metabolic cellular processes, including response to growth factor (GO:0070848, weighted Fisher's Exact Test (FET) p = $4.6x10^{-3}$) and response to insulin (GO:0032868, weighted FET p = $4.0\ x10^{-4}$; Table S3A), suggesting that the Western diet also reprogrammed oxidative metabolic aspects of monocyte gene regulation. Conversely, Mediterranean diet monocyte expression patterns indicated enhanced oxidation-reduction processes (GO:0055114, weighted FET p = $6.0x10^{-3}$; Table S3B), a critical function in muting proinflammatory monocytes.

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[39] (see Table S2C for polarization categories). Western genes were enriched in M1-associated genes (n = 162 genes, fold-enrichment = 1.27, *95% CI* = 1.09 - 1.46; Fig. 2C), but not M2-associated genes (n = 24 genes, fold-enrichment = 0.95, *95% CI* = 0.60 - 1.35). Conversely, both M1-associated genes (n = 112 genes, fold-enrichment = 0.74, *95% CI* = 0.61 - 0.88) and M2-associated genes (n = 20 genes, fold-enrichment = 0.67, *95% CI* = 0.40 - 0.97) were underrepresented among Mediterranean genes. Together, these observations indicate that a Western diet induces a more proinflammatory (M1-like) phenotype.

Next, to identify putative upstream gene regulatory mechanisms, we examined whether diet-induced changes in gene expression were associated with *cis*-regulatory 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; Fig. 3, Table S4). Diet significantly altered expression of the genes encoding for seven of these 35 transcription factors, including IRF3, IRF8, MEF2C, and SP1, which drive monocyte fate and polarization in response to extracellular signals[40–44]. Thus, some of the diet-associated changes in monocyte gene regulation may be mediated by changes in the expression and *cis*-regulatory binding of key transcription factors.

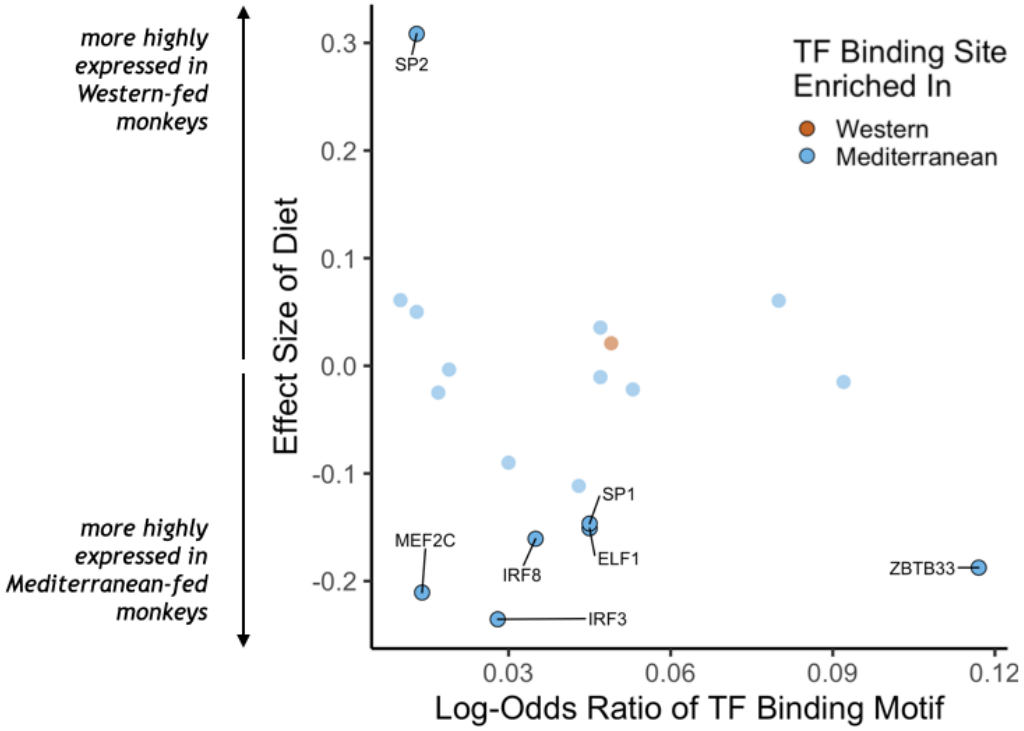

Figure 3. Transcription factor (TF) binding motifs correlated with diet effects on gene expression. The log-odds ratio of TF binding motif enrichment in Western genes (orange) or Mediterranean genes (blue) are depicted on the x-axis. The y-axis shows the effect size of diet on the expression of the gene that encodes for the TF. Only TFs with binding motifs significantly enriched in either gene set and that were detectably expressed in our samples are shown, with those significantly effected by diet outlined and labeled.

194

195 *Diet alters gene co-expression patterns*

196 Next we asked whether diet altered the magnitude or direction of pairwise gene expression
197 correlations among the most strongly diet-affected genes, as such effects could reveal key gene
198 regulatory networks that are altered by diet, that may themselves be regulated by key upstream
199 targets[45,46]. Drawing on a newly developed approach, "correlation by individual level product"
200 (CILP)[47], we identified 445 gene pairs that exhibited significant changes (FDR < 20%) in their
201 correlation between the Mediterranean- and Western-fed monkeys (Table S5A; Fig. 4A). The
202 majority (97%) of these gene pairs exhibited positive associations in one diet and negative
203 associations in the other, suggesting that diet can completely reverse the co-expression
204 relationship between two genes (Figure 4A). We further identified 16 "hub" genes that exhibited
205 differential correlations with partner genes more so than expected by chance (Fig. 4B, Table
206 S5B). These hub genes were enriched for genes encoding transcription factors (OR = 7.40, FET
207 $p = 7.0 \times 10^{-3}$), including SOX4 (essential for normal insulin secretion and glucose tolerance)
208 and NR4A2 (involved in lipid, carbohydrate, and energy metabolism[48,49]), suggesting immune
209 and metabolic reprogramming by the diet manipulation. Interestingly, the hub gene involved in
210 the greatest number of differentially-correlated gene pairs was *RF00283, aka SCARNA18,* a non-
211 coding RNA that has been associated with BMI, HDL cholesterol, and aging in human genome-
212 wide association studies[50–53] (Fig. 4B-D), identifying it as a key regulatory RNA that is altered
213 by diet and has a cascading effect on other genes and pathways.

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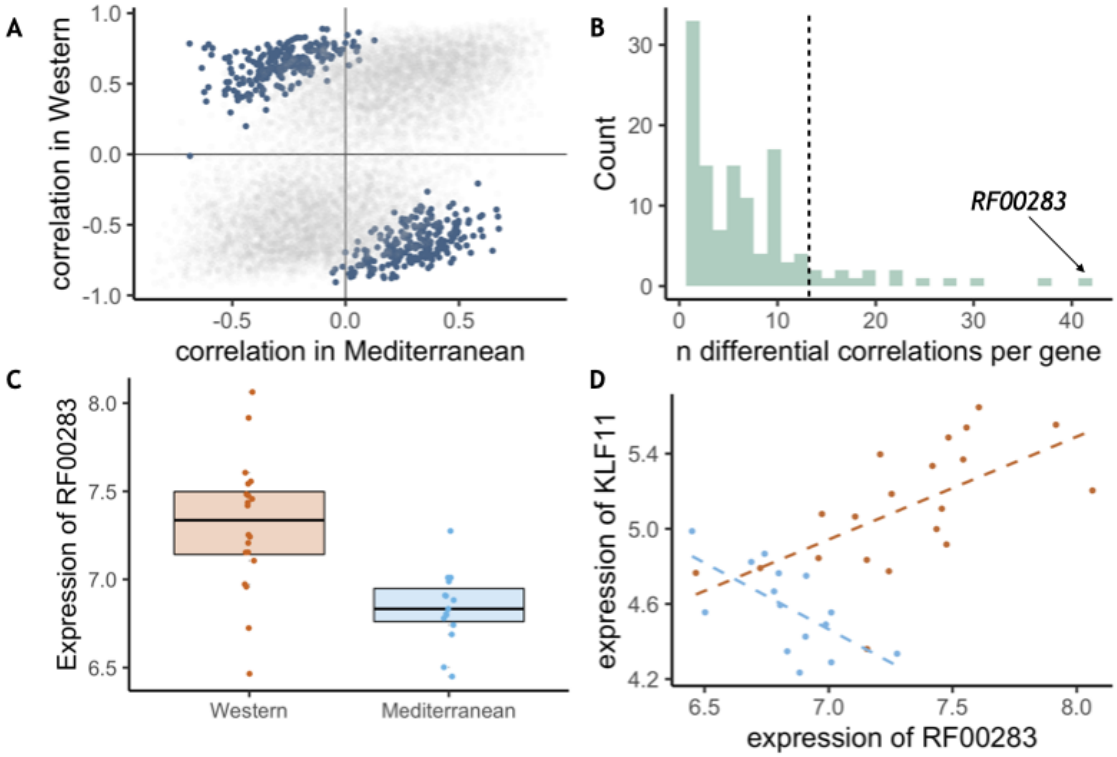

Figure 4. Diet affects monocyte gene co-expression. A) The Pearson correlation between each pair of genes within each of the experimental diets. Gene pairs that are significantly differently correlated between diets are highlighted in blue (n = 445 significant pairs, FDR < 20%). **B)** Of the genes involved 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[54]. The two genes were more highly expressed in Western monocytes, and were positively correlated with one another in Western-fed monkeys (r = 0.61, p < 0.005) and negatively correlated in Mediterranean-fed monkeys (r = -0.63, p < 0.01).

215

216 *Diet altered social behavior*

There were no differences in behavior during the baseline phase (all $p > 0.1$; Fig. S1A, B). While on the experimental diets, monkeys fed the Mediterranean diet spent significantly more time in body contact (Mann-Whitney $U = 280$, Holm-Bonferroni adjusted p (p_{HB}) = 1.2 x 10^{-5}) and resting ($U = 267$, p_{HB} = 1.6 x 10^{-3}), while those fed the Western diet spent significantly more time alone ($U = 48$, p_{HB} = 4.7 x 10^{-3}; Fig. 5A). All other measured behaviors did not pass our stringent p-value threshold after multiple hypothesis testing correction (Fig. S1C,D), although two additional behaviors differed at an uncorrected p-value < 0.05 (percent of time attentive and rate grooming self). Therefore, to increase our ability to identify diet-affected suites of behaviors, we leveraged the fact that many behaviors co-occurred (Fig. S2) by conducting a principal component analysis[55,56]. Behaviors associated with dominance interactions—including aggression, submission, and being groomed—all loaded heavily onto the first principal component, which explained 32.2% of the overall variance in behavior and did not differ between diets (Welch-Satterthwaite $t_{(30.3)}$= 0.323, $p = 0.75$; Fig. S3, Table S6A). The first principal component was significantly correlated with dominance rank (Fig. S4, Note S1).

The second principal component, which explained 18.8% of the variance in behavior, differed significantly between the two diets ($t_{(26.8)}$= -4.02, p = 4.2 x 10^{-4}; Fig. 5B), and thus represented a composite of diet-altered behaviors (hereafter, DAB). No other principal component was significantly correlated with diet and thus PC2 captures the primary behavioral component causally affected by diet (Fig. 5C, Table S6B). PC2 captured a number of anxiety and social behaviors (Fig. S5, Table S6A). Specifically, body contact is indicative of social integration and was positively correlated with PC2 loading (hereafter, DAB score), which was higher in Mediterranean fed animals. Conversely, behaviors related to social isolation and anxiety[57–62]

240 (e.g., percent of time alone, rate of grooming self, rate of scratching) were associated with lower
241 DAB scores, and hence more prevalent in animals fed the Western diet (Fig. 5C). Thus, PC2
242 captured a measure of social integration associated with consuming a Mediterranean-like diet,
243 and social isolation and anxiety associated with consuming a Western-like diet.
244

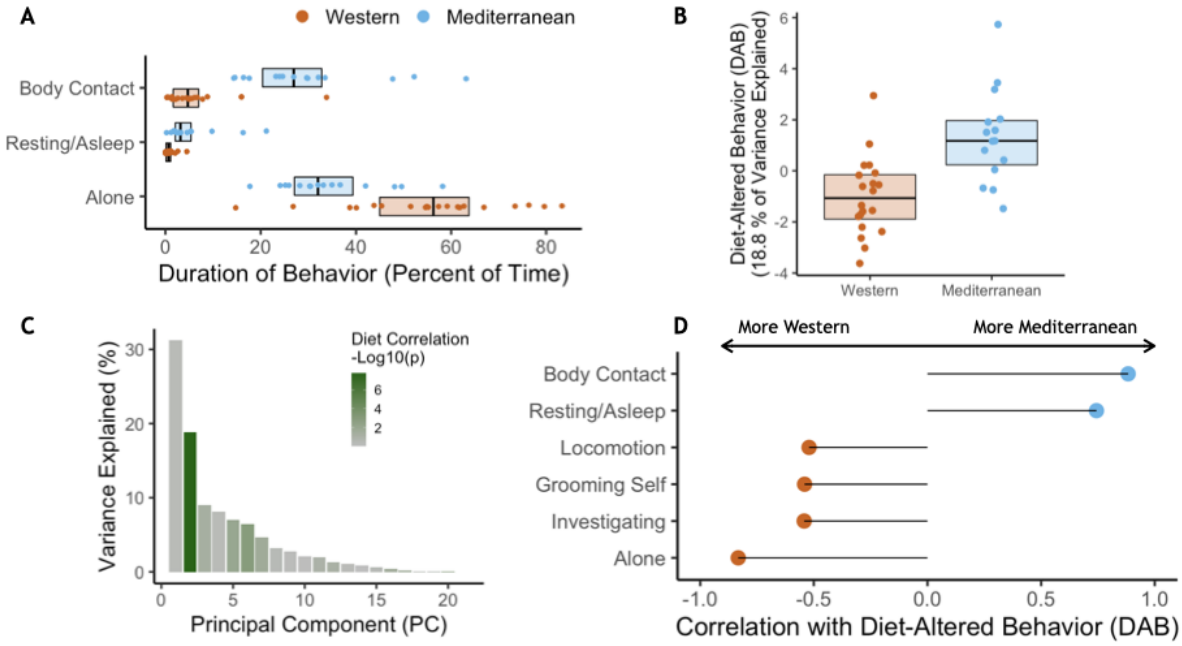

Figure 5. Diet alters behavioral phenotype. A) Three behaviors were significantly different between the two diet groups. Monkeys fed the Mediterranean diet spent more time in body contact (p_{HB} = 1.2 x 10^{-5}) and resting (p_{HB} = 1.6 x 10^{-3}) than Western-fed monkeys. Monkeys eating the Western diet spent more time alone than Mediterranean-fed monkeys (p_{HB} = 4.7 x 10^{-3}). **B)** Composite measures of diet-altered behavior (DAB scores) were significantly higher in Mediterranean diet compared to Western diet animals ($t_{(32.0)}$= 5.30, p = 8.2 x 10^{-6}). **C)** Principal component 2 (PC2) explained 18.8% of the variance in behavior and was the only PC significantly correlated with diet (see Table S6B for correlation between diet and other PCs). **D)** Six of the 21 behaviors observed are significantly correlated with DAB score (Benjamini-Hochberg adjusted $p < 0.05$). Here, significant correlations with DAB score in which behaviors are more frequent in Mediterranean diet or Western diet

monkeys are indicated with blue or orange points, respectively.

Diet-altered behaviors mediate expression of 25% of differentially expressed monocyte genes

Given the strong effects of diet on both behavior and monocyte gene expression, we tested if the effect of diet on monocyte gene expression was mediated by the diet-induced changes in behavior. Of the 4,900 diet-affected genes, 29% were also significantly associated with DAB score in a univariate model (n = 1,418, FDR < 0.05). Of these, DAB score significantly mediated the effect of diet on the expression of 1220 genes (25% of all diet-associated genes, $p < 0.05$; Fig. 6A). DAB score mediation accounted for significantly more of the effect of diet in DAB-mediated Western genes (μ = 51.1 %, δ = 12.4%), than DAB-mediated Mediterranean genes (μ = 44.2 %, δ = 10.0%; Mann-Whitney $U = 2.4 \times 10^5$, $p = 7.5 \times 10^{-23}$; Fig. 6B). These DAB-mediated genes were also significantly more likely to be Western genes than Mediterranean genes (n = 741 Western genes, 61%, two-sided binomial test $p = 6.3 \times 10^{-14}$), and were enriched in regulation of inflammatory response (GO:0050727, weighted FET $p = 2.9 \times 10^{-3}$; Table S7A-C). Together, this shows that the effect of diet on monocyte gene regulation may partially be due to diet-induced changes in key social behaviors.

In support of this mediation effect, we compared expression of a well-studied set of social adversity-responsive genes known as the "conserved transcriptional response to adversity" (CTRA)[28] in the Western- and Mediterranean-fed animals in our study. Animals fed a Western diet exhibited significantly higher expression of pro-inflammatory genes included in the CTRA (Mann-Whitney U = 222, p = 0.016) and lower expression of antiviral- and antibody-related

267 CTRA genes (Mann-Whitney $U = 82$, $p = 0.023$; Table S2C, Fig. S6).

268

269 We also tested the hypothesis that diet could alter behavior through its changes on peripheral
270 immune cell gene expression. We tested this in the 28% of genes for which monocyte gene
271 expression significant predicted DAB in a univariate model ($n = 1{,}353$, FDR < 0.05), and found
272 that gene expression significantly mediated the effect of diet on DAB score in 940 genes (19% of
273 all diet-associated genes, $p < 0.05$; Fig. 6A). Almost all of these genes (99.5%; 936/940) were
274 significantly mediated by diet-induced changes in DAB. As with DAB score mediating gene
275 expression, the genes that mediated the effect of diet on DAB score were more likely to be
276 Western genes than Mediterranean genes ($n = 558$ Western genes, 59%, two-sided binomial test
277 $p = 1.0 \times 10^{-8}$). Unlike DAB score mediating the effect of diet on gene expression, the portion of
278 the effect of diet that was accounted for by gene expression did not vary between Western ($\mu =$
279 27.5 %, $\delta = 5.4\%$) and Mediterranean genes ($\mu = 27.3$ %, $\delta = 4.6\%$; Mann-Whitney $U = 1.1 \times$
280 10^5, $p = 0.75$; Fig. 6B).

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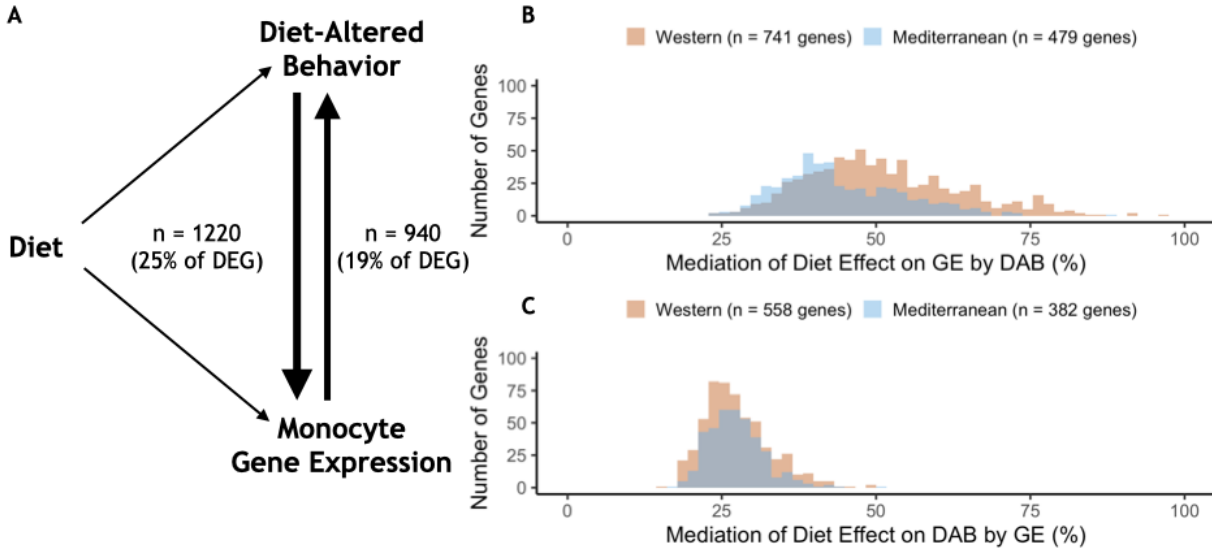

Figure 6. Behavior partially mediates the effect of diet on gene expression for 25% of diet-associated genes. **A)** Diet-altered behavior (DAB) mediated the effect of diet on gene expression for 25% (n = 1220) of genes for which diet had an effect (DEG). For 19% of differentially expressed genes (DEG), gene expression mediated the effect of diet on DAB score. **B)** DAB score mediated 24-97% of the effect of diet on gene expression in 1220 genes (n = 741 Western genes, orange; n = 479 Mediterranean genes, blue). DAB score mediated a greater number of Western genes than Mediterranean genes ($p = 6.3 \times 10^{-14}$) and accounted for a greater portion of the effect size of diet ($p = 7.5 \times 10^{-23}$) in Western genes. **C)** In gene-by-gene models of DAB score as a function of diet + gene expression, gene expression mediated 15-51% of the effect of diet on DAB in 940 genes (n = 558 Western genes; n = 382 Mediterranean genes). Gene expression mediated a greater number of Western genes than Mediterranean genes ($p = 1.0 \times 10^{-8}$), although expression of these genes did not account for more of the effect of diet on DAB score than Mediterranean genes (Mann-Whitney $U = 1.1 \times 10^{5}$, $p = 0.75$).

Western diet induces mosaic response

Western diet induced substantial variation in multiple phenotypes, including body weight, gene expression, and behavior; consistent with previous studies demonstrating that some individuals may be more resistant (or susceptible) to the effects of a Western diet[63], presumably due to genetic variation or past environmental exposures. However, we were unable to identify any

consistencies in individual responsiveness across the phenotypes (Fig. S7). For instance, monkeys that exhibited a strong gene regulatory response to the Western diet did not exhibit a large increase in body weight or a strong negative DAB score (all $p > 0.2$). Furthermore, 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, presumably involving interactions between diet, environment, and the genome.

Discussion

This study shows, for the first time, that a whole-diet manipulation exerted profound effects on monocyte function and social behavior in a primate. Forty percent of expressed genes were differentially expressed between monkeys fed Western or Mediterranean diets, indicating that diet dramatically altered monocyte programming. Relative to Western group monocytes, Mediterranean group monocytes exhibited reduced proinflammatory gene expression and regulatory gene expression. Our findings recapitulate and extend previous studies, such as a randomized human cross-over trial that demonstrated that peripheral blood monocytes from elderly individuals consuming a Mediterranean like diet enriched in olive oil had reduced proinflammatory gene expression relative to diets more enriched in saturated fat (butter)[64]. Beyond mean differences in gene expression levels, we also identified differences in gene co-expression and enrichment of transcription factor binding motifs, suggesting that diet exerts a strong effect on gene regulatory networks.

We identified 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

311 (ETS), specificity protein (Sp)/Krüppel-like family (KLF), myocyte-specific enhancer factor
312 (MEF), and interferon-regulatory factor (IRF) families of transcription factors, which have all
313 been linked to myeloid differentiation[40–43], were overrepresented in regulatory regions of genes
314 with higher expression in the Mediterranean diet group ("Mediterranean genes). IRF-1 and IRF-8
315 are linked to M1 monocyte polarization, while IRF-3 is associated with M2 polarization, and all
316 three transcription factors had binding motifs enriched in Mediterranean genes. The sole
317 transcription factor with binding sites enriched in Western diet-associated genes, ATF2, is a key
318 mediator of inflammatory pathways and diseases, including response to bacterial endotoxin,
319 atherosclerosis, and obesity[65–67]. Interestingly, Western genes were enriched for activation of the
320 MAPKK pathway, which lies upstream of ATF2[68], supporting its putative role in monocyte gene
321 regulation. Transcription factors were also overrepresented in the pairs of differentially co-
322 expressed genes, indicating that diet may be altering the networks through which inflammatory
323 genes are regulated. Broadly, this suggests that the two experimental diets differentially affect
324 transcriptional networks involved in monocyte differentiation and polarization. It is also worth
325 noting that the M1/M2 paradigm of monocyte polarization is a simplification of the more
326 complex heterogeneity of monocytes.[69,70] For example, there are at least 3 classes of monocytes
327 in the circulation, classical, intermediate, and non-classical, which individually have different
328 phenotypes. We did not assess proportions of these or try to isolate individual monocyte subsets
329 in the current study, thus the patterns of gene expression observed could represent altered
330 proportions of these subsets and well as shifts in monocyte polarization within subsets[71,72].
331
332 Diet induced changes in behavior, as monkeys consuming the Western diet exhibited more
333 behaviors related to anxiety and social isolation, a phenotype remarkably similar to that observed

in juvenile Japanese macaques born to mothers consuming a high-fat Western diet[73]. 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 suggest that a Western diet may also exert similar behavioral effects in adulthood.

We observed that for a subset (25%) of genes, the diet-altered behavior (DAB) score mediated the effect of diet on monocyte gene expression. This observation suggest involvement of mechanistic pathways in which diet first impacts the brain, which in turn impacts monocyte function. Monocytes have been shown to be responsive to social isolation[29] and anxiety[28]. Social isolation and anxiety, produced by Western diet consumption, may be accompanied by increased sympathetic outflow and increased hypothalmic-pituitary adrenal production of cortisol, both of which modulate monocyte intracellular processes governing inflammatory molecule production[74–76]. Supporting the involvement of these systems, we previously reported that the Western diet group had increased sympathetic activity, and increased cortisol concentrations[77]. Therefore, it is possible that a Western diet contributes to inflammation by producing a more socially isolated or anxious animal with increased sympathetic and hypothalamic pituitary adrenal activity, which in turn alters monocyte function. Higher expression of genes in the conserved transcriptional response to adversity support this pathway[28,29].

There are numerous pathways through which diet may affect behavior. Diet may induce changes in the central nervous system by altering gut microbiota which alters vagal input to the brain[78]. We previously showed in these NHPs that diet had a strong effect on the gut microbiome[79], and

that compared to the Mediterranean group, Western diet NHPs had lower parasympathetic (vagal) activity at the time the monocyte transcriptome was assessed[77]. Taken together these observations suggest that diet-induced changes in vagal tone in the gut-brain axis may be one pathway through which diet impacted brain function, potentially affecting behavior.

We also observed that for some genes (19%), diet-induced changes in monocyte gene expression significantly mediated the effect of diet on behavior (DAB). This observation suggests underlying mechanisms which first impact peripheral monocyte function, which in turn impacts brain function. Western diet may disrupt the blood-brain barrier, increasing infiltration of Western-diet induced cytokines, chemokines, and myeloid cells from the periphery[80,81]. Once in the brain these molecules can alter BDNF production, neurotransmitter systems, and hypothalamic-pituitary-adrenal function[80]. Western diet induced inflammatory molecules also may effect the brain through direct effects on the afferent vagus nerve[82], activation of glial cells[83], and alter neuronal membrane lipid composition affecting neurotransmission[84], whereas a Mediterranean diet may have direct anti-inflammatory actions by increasing n-3 fatty acids in the brain[85]. These results support both mediation pathways, suggesting that multiple mechanistic pathways contributed to these observations.

The behavioral analysis also showed that the first principal component described dominance-related behaviors. While the dominance component accounted for the largest proportion of the variance in behavior, it was notably unaffected by the diet manipulation. From a socio-biological perspective this suggests that dominance-related behavior is resistant to perturbation, which is consistent with known stability of dominance hierarchies in female cynomolgus monkeys[33]. The

second principal component that captured affiliation, anxiety, and social isolation was significantly affected by the two diets, suggesting that these behaviors are susceptible to dietary interventions.

In summary, we found that diet significantly alters behavior and monocyte polarization. The Western diet promoted a proinflammatory monocyte phenotype relative to a Mediterranean 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, carbohydrate, and fat 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.

Materials and Methods

Subjects

Forty-three adult (age: mean = 9.0, range = 8.2-10.4 years, estimated by dentition), female cynomolgus macaques (*Macaca fascicularis*), were obtained (Shin Nippon Biomedical Laboratories, USA SRC, Alice, TX) and housed at the Wake Forest School of Medicine Primate

Center (Winston-Salem, NC) as previously described [38]. Briefly, the monkeys were socially housed in groups of 3-4 and consumed standard monkey chow (Table S1) during an eight-month baseline phase, after which pens were assigned to receive 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 ([38]; 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 three samples were removed for insufficient CD14 purification (see "Removal of Batch Effects" below), resulting in a final sample size of 35 animals (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 (Table S1) were formulated to be isocaloric with respect to protein, fat, and carbohydrates, and identical in cholesterol content (~ 320mg / 2000 kilocalories (Cals)/day) as previously described[38]. 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[12,86,87]. 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 in cardiovascular disease prevention[88].

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 hours/monkey total) and for 14 months during the experimental phase (17.7 hours/monkey total). Behaviors were collected as previously described[89], and combined into summary behaviors (e.g., "aggression" was a combination of all total, noncontact, contact aggressive events). No significant differences in behavioral variables were observed between the diet groups which consuming the baseline standard monkey chow diet. In order to quantify the overall impact of diet on behavior, we conducted a principal component analysis using the R package *FactoMineR* [90].

Blood Sample Collection

The monkeys were trained to run out of their social groups on voice command. Blood was drawn via venipuncture within 9 minutes of entering the building,. 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 School of Medicine, 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)[91,92] using HiSat2[93] and then converted to a sample-by-gene read count matrix using featureCounts[94] (median = 38.0%; range 24.5 - 50.4% of reads mapped to exons).

Read Count Normalization and Removal of Batch Effects

First, we removed genes with low expression (median reads per kilobase per million reads mapped < 1), which resulted in 12,240 genes for downstream analyses. We normalized read counts using the *voom* function of the R package *limma*[95]. 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 Fig. S8) and excluded them from all analyses, leaving a final sample size of 35 monkeys (n = 20 fed the Western diet, n = 15 Mediterranean diet). 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*[96]. Relatedness was estimated using the ngsRelate program[97] with SNP genotypes inferred from the RNA-seq reads using bcftools mpileup[98]. We calculated an empirical false discovery rate (FDR) for each gene using a permutation-based approach[30]. 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 and the *weight01* algorithm to test for enrichment implemented in the R package *topGO*[99]. 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[39] (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[100] 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)[47], 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[47]. Each of these vectors of products were used as the outcome variable in a linear mixed effects model implemented in the R package *EMMREML*[96], 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 repeated 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[30].

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 95th 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[45,46]. To implement the enrichment analysis, we used the TRRUST database of known mammalian transcription factors for annotation [101] paired with hypergeometric tests.

Mediation

To explore relationships between DAB score and differential gene expression, we conducted mediation analyses using a bootstrapping approach involving 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 score[102]. For each bootstrap iteration, we then calculated the mediation effect (i.e., the indirect effect) of DAB score 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 90% 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.

Supplementary Materials

Fig. S1. Diet manipulation altered behavior.

Fig. S2. Behaviors exhibit significant correlations with one another.

Fig. S3. Correlation of observed behaviors with PC1.

Fig. S4. The first PC of all behavioral data captures dominance rank.

564 Fig. S5. Correlation of observed behaviors with diet-altered behavior measure (DAB; PC2).

565 Fig. S6. Expression of genes in the conserved transcriptional response to adversity (CTRA[28])

566 indicate inflammatory effects of a Western diet that parallel the effects of social adversity.

567 Fig. S7. Greater phenotypic variability in Western diet fed monkeys does not show consistency

568 in individual responsiveness across phenotypes.

569 Fig. S8. Quality control of cell purity by CD14 and CD3 expression levels: three samples were

570 excluded due to lower CD14 and high CD3 – possible T cell contamination.

571 Fig. S9. RNA Integrity was correlated with both uncorrected gene expression and relative rank.

572 Table S1. Nutritional Contents of Human and Nonhuman Primate Diets

573 Table S2. Effects of Diet on Gene Expression

574 Table S3A. Biological Processes Enriched in Western Genes Compared to Other Measured

575 Genes

576 Table S3B. Biological Processes Enriched in Mediterranean Genes Compared to Other Measured

577 Genes

578 Table S4. Transcription Factor Binding Site Motif Enrichment

579 Table S5A. Gene Pair Correlations Across and Within Diet Groups

580 Table S5B. Differentially Correlated Genes

581 Table S6A. Behavior Loadings onto Principal Components 1 and 2 and Correlation with Diet

582 and Rank

583 Table S6B. Behavior Principal Components and Correlation with Diet

584 Table S7A. Biological Processes Enriched in Behavior-Mediated Differentially-Expressed Genes

585 (DEG)

586 Table S7B. Biological Processes Enriched in Behavior-Mediated Western Genes

587 Table S7C. Biological Processes Enriched in Behavior-Mediated Mediterranean Genes

588 Note S1. Regarding rank and RNA integrity (RIN).

589

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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.