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Temporal changes in postprandial blood transcriptomes reveal subject-specific pattern of expression of innate immunity genes after a high-fat meal☆

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Abstract

White blood cells are among the first responders to dietary components and their metabolites absorbed from the gut. The objective of this study was to determine the whole blood transcriptome response to high-fat challenge meals. A total of 45 fasting and postprandial (3-h and 6-h) whole blood transcriptomes from 5 subjects in a crossover intervention trial of a high-fat meal supplemented with placebo, blueberry powder or docosahexaenoic acid (DHA) were analyzed using RNA sequencing. Select target genes were validated by quantitative reverse-transcription polymerase chain reaction in 180 samples from 20 subjects. The largest contributor to variance was the subject (13,856 genes differentially expressed), followed by the subject on a specific day (2276 genes), followed by the subject's postprandial response (651 genes). After determining the nonsignificance of individual dietary treatments (blueberry, DHA, placebo), treatments were used as replicates to examine postprandial responses to a high-fat meal. The universal postprandial response (95 genes) was associated with lipid utilization, fatty acid beta-oxidation and circadian rhythms. Subject-specific postprandial responses were enriched for genes involved in the innate immune response, particularly those of pattern recognition receptors and their downstream signaling components. Genes involved in innate immune responses are differentially expressed in a subject-specific and time-dependent manner in response to the high-fat meals. These genes can serve as biomarkers to assess individual responsiveness to a high-fat diet in inducing postprandial inflammation. Furthermore, the dynamic temporal change in gene expression in postprandial blood suggests that monitoring these genes at multiple time points is necessary to reveal responders to dietary intervention.
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Keywords: Postprandial; Inflammation; Blood transcriptome; RNA-Seq; High-fat; Biomarkers

1. Introduction

Hormonal, metabolic and immunological changes in response to dietary components digested and absorbed after a meal are manifested in postprandial blood. Blood granulocytes and mononuclear cells are innate immune effector cells that can respond to dietary components and their metabolites absorbed from the gut. Blood monocytes express pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain-like receptors (NLRs) that play a central role in inducing innate immune responses in response to invading pathogens and various endogenous molecules. PRRs recognize pathogen-associated molecular patterns or certain endogenous molecules and induce infection-induced proinflammatory responses or sterile inflammation, respectively.

Saturated fatty acids, such as palmitic acid, have been shown to activate TLR2 and TLR4-mediated signaling pathways in monocytes and macrophages [1–8]. It has also been implicated that high-fat diets can induce the absorption of endotoxin from the gut [9,10]. Thus, there are at least two possible mechanisms by which a high-fat meal may induce postprandial inflammation.

Despite mechanistic evidence and the general acceptance that a high-fat meal causes inflammation during the postprandial period, the evidence from human interventions is mixed. In a review of 57 studies

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of high-fat-meal-induced acute postprandial inflammation, most plasma-borne markers of inflammation were not significantly increased after a high-fat meal [11]. In another review of studies for which inflammatory markers were assessed post high-fat meal in healthy adults, only IL-6 was consistently changed [12]. However, 30 of the 32 reviewed studies in which increased IL-6 was reported had used cannulation, which has been shown to increase IL-6 even without a high-fat meal [13,14]. Discordant results from these studies may reflect differences in postprandial sampling times in view of the fact that concentrations of many inflammatory markers can vary in a time-dependent manner. For example, one study showed that the concentrations of proinflammatory cytokines (IL-6, IL-8 and TNFα) decreased in the postprandial plasma at 3 h [15].

It is possible that the immediate postprandial period may not allow enough time for the production of some cytokine proteins affected by dietary treatment. Gene expression precedes protein synthesis. Therefore, mRNA may provide earlier and more sensitive markers of postprandial inflammation than circulating proteins. However, the whole blood transcriptional response to a high-fat meal is unknown. In the present study, we analyzed whole blood RNA by RNA sequencing from subjects at fasting and at 3 and 6 h after a high-fat meal with 36% kcal from fat which is similar to the fat content (mean 34% kcal) of the typical American diet [16]. The present study was a substudy of a parent trial. The purpose of the parent trial was to examine whether a high-fat meal causes monocyte activation leading to increased inflammation and whether concomitant intake of DHA or blueberries rich in anti-inflammatory polyphenols suppresses the high-fat-diet-induced postprandial inflammation. In the present study, all 45 transcriptomes from 5 participants in the parent study were sequenced to investigate the contributors to variance in whole blood responses to individual challenge meals. Upon discovering that there was no effect of treatment arm (blueberry, DHA or placebo), samples from the same time point (fasting, 3 h, 6 h) on different study days from each subject were subsequently used as replicates to examine subject-specific postprandial responses to a moderately high-fat meal.

2. Material and methods

2.1. Ethics statement and recruitment

This study conforms to the principles outlined in the Declaration of Helsinki, was approved by the Institutional Review Board for Human Subjects at the University of California Davis (IRB 537073) and was registered at Clinicaltrials.gov as NCT02472171. Written informed consent was obtained from all subjects who were recruited from the greater Sacramento, California, area. The health screening and study visits were conducted at the Western Human Nutrition Research Center (WHNRC) in Davis, California.

2.2. Subjects

Inclusion criteria included (1) age between 18 and 60 years and (2) a normal BMI (in kg/m^2, 18–24.9). Exclusion criteria included (1) total blood cholesterol >240 mg/dl, (2) TGs >300 mg/dl, (3) hemoglobin <11.5 mg/dl, (4) blood pressure >140/90 mm Hg, (5) abnormal results in clinical chemistry and hematology panels, (6) inflammatory or metabolic diseases, (7) use of nonsteroidal anti-inflammatory drugs including asthma and allergy medications, (8) unwillingness to discontinue use of dietary supplements before and during the study period and (9) vegetarianism. Sixty-two subjects (36 female and 26 male) completed the parent study. For the present study, five subjects were randomly selected for the RNA-Seq study. This included three male and two female subjects. An additional 15 subjects (a total of 20 subjects) were randomly selected for the reverse-transcription polymerase chain reaction (RT-PCR) study. These 20 subjects included 11 females and 9 males, with a mean BMI of 22.85 (range 19.3–25) and a mean age of 27.4 y (range 19–57).

2.3. Study design

This investigation was a double blinded, placebo-controlled and randomized crossover study. Subjects were fed a test breakfast on the 3 test days in a random order with at least 4 weeks of washout period between each test day. Subjects and study coordinators were blinded with respect to the treatments received with the test breakfast. Researchers handling samples and data did not know what treatments subjects received on all test days. The study diets were calculated by a registered dietitian using the Nutrition Data System for Research 2014 (Nutrition Coordinating Center, University of Minnesota) and produced using ProNutra (Viocare, Inc.) software programs. The registered dietitian coded the different test breakfasts, obtained a randomized list from a statistician and assigned subjects sequentially as subject numbers were assigned. If a subject dropped out after being randomized but before initiating feeding, the next subject was assigned to the treatment schedule of the dropped subject. If a subject dropped out after being randomized and after initiating feeding, the next subject was assigned to the next available slot. Subjects were instructed by a registered dietitian or study coordinator to follow a low-polyphenol and low-omega-3 FA diet and limit consumption of fruits; vegetables; soy; fatty fish (*e.g.*, salmon); nonfatty fish, crustaceans and mollusks (*e.g.*, cod, lobster, clams, respectively); whole grains; flaxseed, walnut, canola and other vegetable oils; nuts and seeds; coffee; tea; alcohol-based beverages; herbs, spices and condiments; and chocolate starting 3 days before each test day. Participants were also allowed to take one study team-approved multivitamin per day that did not contain any added antioxidants (*e.g.*, green tea extract).

2.4. Pretest meal

Between 6:00 p.m. and 8:00 p.m. on the night before each test day, subjects were instructed to eat a standardized dinner provided by the metabolic kitchen that included a sandwich (bagel, butter, egg patty, beef sausage, cheddar cheese), vanilla wafer cookies, chocolate-hazelnut spread, yogurt (yogurt, heavy cream, raspberry-flavored syrup) and lemonade. Subjects were instructed to refrain from eating or drinking anything after 8:00 p.m., except plain water; they were instructed to document the time they ate the meal and to document any deviations on the meal checklists provided with the meal. The calculated nutrient composition of this meal is given in Supplemental Table 1. The pretest meal minimizes masking effects of previous diet and variations in the fasting levels of endpoints caused by the different dinners consumed by the subjects [17].

2.5. Study day and test breakfast

On each test day, subjects arrived at the WHNRC after a 12-h overnight fast. Subjects had their body temperature, blood pressure and weight measured and had fasting blood withdrawn by venipuncture. Subjects were then fed a breakfast meal with a test smoothie supplemented with either placebo powder or blueberry powder and either placebo control oil or DHA oil. The breakfast meal included a sandwich (bagel, butter, egg patty, beef sausage, cheddar cheese) and vanilla wafer cookies. The test smoothie base used on all treatments contained nonfat milk, water, blackberry-flavored Italian soda syrup, heavy whipping cream and xanthan gum. The test smoothie was supplemented with either placebo control powder (sucrose, wheat dextrin, artificial blueberry flavor, whey protein, high-oleic sunflower oil, maltodextrin, artificial red and blue food coloring, dextrose, ascorbic acid, citric acid and silicon dioxide), placebo control oil (high-oleic sunflower oil), blueberry powder [freeze-dried 50/50 blend of Tifblue/Rubel (*Vaccinium virgatum (ashei)*/*Vaccinium corymbosum*) blueberries] or DHA oil (algal vegetable oil from *Schizochytrium* sp.). The total calculated energy of the meal and smoothie was 849 +/− 2.6 kcal with approximately 36.2% kcal from fat by calculation. This test meal is referred to as a high-fat meal in this study. More specifically, it is a moderately high-fat, high-saturated-fat meal. The meals with the placebo, blueberry powder or DHA smoothies contained 15.4 g, 15.4 g or 14.9 g of saturated fat, respectively, primarily as palmitic acid and stearic acid. The complete calculated nutrient composition is provided in Supplemental Table 1.

The test meal food components were weighed and prepared in advance as "kits," stored at −20°C, thawed overnight at 4°C and weighed again as the test meal was assembled to double-check the weights. The DHA oil was stored at 21°C in capsules that were impermeable to light and air. The blueberry powder was received from one lot in sealed 454-g cans with oxygen absorbers and were stored at −20°C until needed. Before opening each can, it was kept at 21°C for a maximum of 24 h to prevent condensation of ambient moisture on the powder. The powder was weighed into food-grade 4.0-mil mylar bags with added oxygen and moisture absorbers, blown with food-grade nitrogen gas to expel ambient air and then stored at −20°C until needed. On the day before each test day, the powder was transferred to storage at 21°C before opening and weighing into the test smoothie immediately prior to feeding on test days. The DHA capsules were cut open and added to the test smoothies just prior to service.

Subjects were given 20 min to consume the entire breakfast, after which they were provided water *ad libitum* and remained in the Metabolic Unit in the WHNRC until they completed the test day. Postprandial blood draws were conducted at 1, 3 and 6 h after consumption of the test meal. Postprandial peaks of the plasma concentration of TGs occurred on average 3.5 h after the consumption of a high-fat breakfast [17]. Following the last postprandial blood draw, subjects were allowed to return to their normal dietary habits until 3 days before their next test day.

2.6. RNA extraction, RNA-Seq library preparation and sequencing

Three milliliters of venous whole blood was drawn into a Tempus Blood RNA tube (Life Technologies, Foster City, CA, USA), shaken vigorously and then frozen at −80°C until use. Total RNA was purified with the Tempus Spin RNA Isolation Kit (Life

Technologies) with minor modifications to the manufacturer's protocol. To remove residual genomic DNA, RNA samples were treated on-column with RNase-Free DNase (Qiagen, Hilden, Germany) per manufacturer's instructions. RNA quantity, quality and integrity were assessed with NanoDrop 1000 (NanoDrop, Wilmington, DE, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All isolated RNAs had A260/A280 ratios greater than 2 and RNA integrity numbers higher than 7.3. RNA-Seq libraries were constructed at the DNA Technologies and Expression Core (University of California, Davis) using the Ovation Human Blood RNA-Seq Library System (NuGEN Technologies, San Carlos, CA, USA) which utilizes "Insert Dependent Adaptor Cleavage" technology to enrich for non-rRNA and nonglobin sequences. Sequencing was performed in a 2×100-bp format with 45 samples multiplexed on 3 lanes on an Illumina HiSeq 4000. The sequencing data and metadata have been uploaded to NCBI's Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with study accession number GSE127530 and series record GSE127530.

2.7. RNA-Seq data processing

Fastq files were demultiplexed to assign reads to the originating sample. Read pairs were processed using the expHTS tool [18], which trims low-quality 3′-ends of read pairs using a modified version of Sickle [19] and then merges overlapping read pairs using a modified version of FLASH [20], which trims off Illumina adapters as a by-product of read merging. Both sets of single-ended reads (merged by FLASH2) and paired-end reads (unmerged) were aligned to UCSC annotations from the hg19 iGenomes database for *Homo sapiens* using STAR [21] with the default parameters. Gene counts from single- and paired-reads were summed within each sample.

2.8. RNA-Seq analyses

The R package, DESeq2 [22], was used for analysis of the count data. To determine the largest contributors to whole-transcriptome variance, the count data were transformed to stabilize the variance using a regularized log transform, and the principal components were computed and plotted using the DESeq2 package.

To determine differentially expressed genes, the raw count data were used to build a DESeqDataSet object using the DESeq2 package. Transcripts that did not have more than one count in the experiment were removed from further consideration. Transcripts differentially expressed as a function of the main effect of each experimental factor — Subject, Time, Arm, Day — were independently assessed using Wald tests. Differences were considered statistically significant if $P<.05$ after adjustment for multiple hypothesis testing using the method of Benjamini and Hochberg (method = "BH") [23]. Next, to determine transcripts differentially expressed as a function of interaction terms (*e.g.*, Time:Arm), models with and without the interaction term were compared using a likelihood ratio test. The likelihood ratio test determines if the increased likelihood of the data in the full model, which include all terms, is more than expected if the terms excluded in the reduced model are really zero. Transcripts with small *P* values from this test are those which show a response to the interaction term excluded in the reduced model. Again, transcripts were considered significantly differentially expressed if the multiple hypothesis BH-adjusted $P<.05$. Finally, to identify differentially expressed genes due to the main effect of time within a subject, study arms were treated as replicates, Wald tests were conducted for individual subjects using the *DESeq* function, and contrasts between each pair of time points were extracted using the *results* function with BH-adjusted $P<.05$ considered to be significant. The counts data file, metadata file and R code for this analysis are provided in the public GitHub repository: https://github.com/dglemay/whole_blood_RNASeq .

The R package variancePartition [24] was used to determine the contributors to variance in the transcriptome data of the study. All variables were jointly considered in a fixed effect model. See https://github.com/dglemay/whole_blood_RNASeq for R code.

To conduct clustering analyses, the DESeq2 [22] and Mfuzz [25] packages in R were used. Count data were variance stabilized using a regularized log transform. The expression values of genes were then standardized to have a mean value of 0 and a standard deviation of 1 so that vectors with similar changes were close in Euclidian space. Gene expression profiles were then clustered using mfuzz, a "soft" clustering algorithm which allows genes to be assigned to more than one cluster and quantifies the degree to which the cluster represents the gene's expression profile.

2.9. RNA-Seq functional analyses

Functional enrichment analyses were conducted for gene lists of interest using the Database for Annotation, Visualization and Integrated Discovery version 6.8 [26] with the default options and the background list of *Homo sapiens*. For each gene list of interest, annotations with a Benjamini and Hochberg adjusted $P<.05$ were taken to be significant.

Pathway enrichment analyses were conducted using Ingenuity Pathways Analysis (IPA) [27]. An IPA Core analysis was run on each list of genes that were significantly regulated at the 3- or 6-h time points relative to fasting in each subject. An IPA Comparison Analysis was then run on these 10 (5 subjects, 2 time points relative to fasting) Core Analyses. Disease-specific pathways were disabled, and only pathways with a BH multiple testing correction $P<.05$ are reported, unless otherwise stated. An IPA Upstream Analysis was conducted to identify transcriptional regulators.

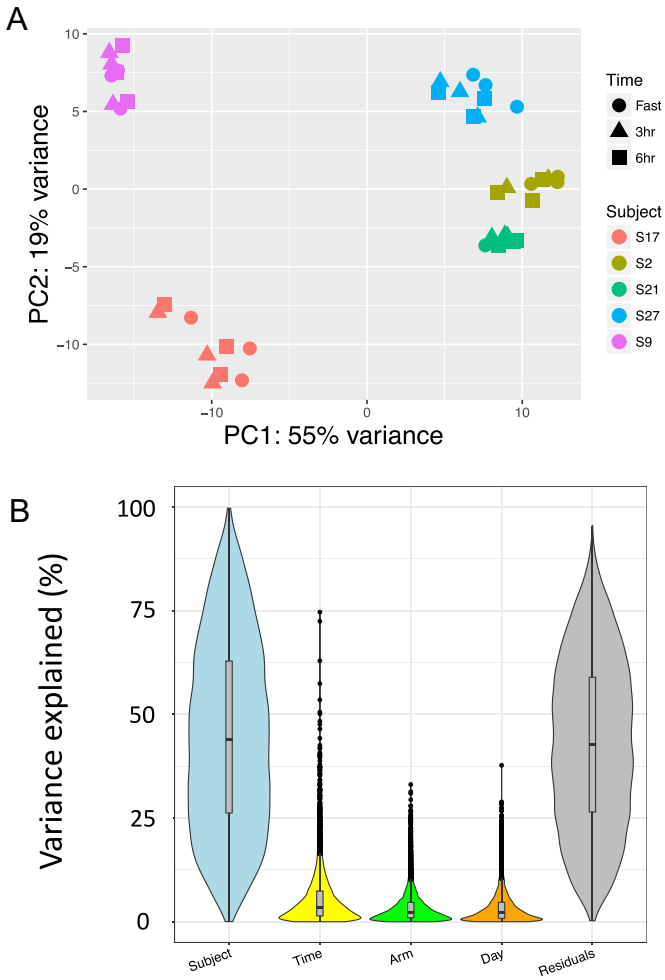

Fig. 1. (A) Principal components analysis (PCA) and (B) variance partitioning demonstrate that the largest contributor to variance is "Subject." (A) In this PCA plot, the *x*-axis shows the first principal component, which explains 55% of the variance. The *y*-axis shows the second principal component, which explains 19% of the variance. Each point in the plot represents an entire transcriptome. The shape indicates the time point (circle, fasting; triangle, 3 h; square, 6 h), and the color indicates the subject (salmon, S17; olive green, S2; bright green, S21; blue, S27; pink, S9). (B) This violin plot shows the median (circle within the dark bar), interquartile range (dark bar), 95% confidence interval (light bar) and probability density (light blue, yellow, green, orange) of the percent of the variance explained (*y*-axis) for each variable (*x*-axis). The gray plot corresponds to the residuals or unexplained portion of variance.

2.10. Quantitative RT-PCR

One microgram of total RNA was converted into cDNAs using an iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). For quantitative PCR analysis, synthesized cDNAs were diluted 10-fold with UltraPure DNase/RNase-free double-distilled water (Thermo Fisher Scientific), and 2 μl was used with a TaqMan Fast Advanced Master Mix and a TaqMan target probe set or a TaqMan housekeeping probe set. All TaqMan probe sets used in this study were purchased from ThermoFisher Scientific (Supplemental Table 2). Quantitative PCR was run in triplicate, and the average crossing-point value was used for relative expression calculations ($-2^{\Delta\Delta CT}$ method) [28]. The expression of the target genes was normalized to the expression of *ACTB* or *YWHAZ*. Quantitative PCR was performed on a QuantStudio 7 Flex Real-Time PCR System.

3. Results

3.1. Contributors to variance in whole blood transcriptome response

Whole blood transcriptomes encompass all genes being expressed by all cells in the whole blood sample. RNA sequencing of 45 fasting

and postprandial whole blood samples collected from human participants revealed 21,028 expressed transcripts. In the current experiment, whole blood was collected from human participants at fasting, 3 h and 6 h after three test meals (coded R, S or T; now unblinded, R = blueberry + sunflower oil, S = placebo + sunflower oil, T = placebo + DHA). The main variables in our model are therefore "Subject," "Time" (fasting, 3, or 6 h) and "Arm" (R, S, or T). To understand the biggest contributors to variance in our experiment, we conducted a principal components analysis of the whole blood transcriptomes. Transcriptomes strongly cluster by subject (Fig. 1A), suggesting that whole blood transcriptomes differ more among individuals than they do by time or by high-fat challenge meal.

To better quantify the contribution of the main variables to whole blood transcriptomes in the meal challenge experiments, we determined the number of differentially expressed genes associated with the main effects of Subject, Time and Arm (see Methods). There were 13,856 genes that significantly differed between Subjects, 95 genes that significantly differed by Time and 0 gene differed by Arm. The effect of a postprandial meal was captured by the Time:Arm interaction term — the effect of Arm relative to the fasting time point. Conducting a likelihood ratio test to compare the full study design inclusive of the Time:Arm interaction term and a reduced model without this term, there were no differentially regulated genes. This suggests that there may be no postprandial effect of the meal that differs between the study arms. The Time:Subject interaction term was significant for 651 genes, suggesting that there is postprandial effect of a high-fat meal challenge that is subject-specific. The Subject:Arm interaction term was significant for 2175 genes, suggesting that there could be an effect of Arm that is subject-specific. However, was this due to the meal, which does not affect fasting samples, or due to the fact that the test was conducted on a different day? Given that participants were randomized for the meal challenge sequence (*e.g.*, subjects did not all have the same test meal on Day 1), it is also possible to consider the test day as another variable, "Day," to estimate the amount of variation due to the day of the test. Dropping "Arm" from the model and replacing with "Day" (essentially randomizing the meal given), all statistical tests were repeated. There were two genes significantly different when testing the main effect of Day (compared to 0 gene by Arm). Just as in the Time:Arm interaction, the Time:Day interaction was also significant for 0 gene. The Subject:Day interaction

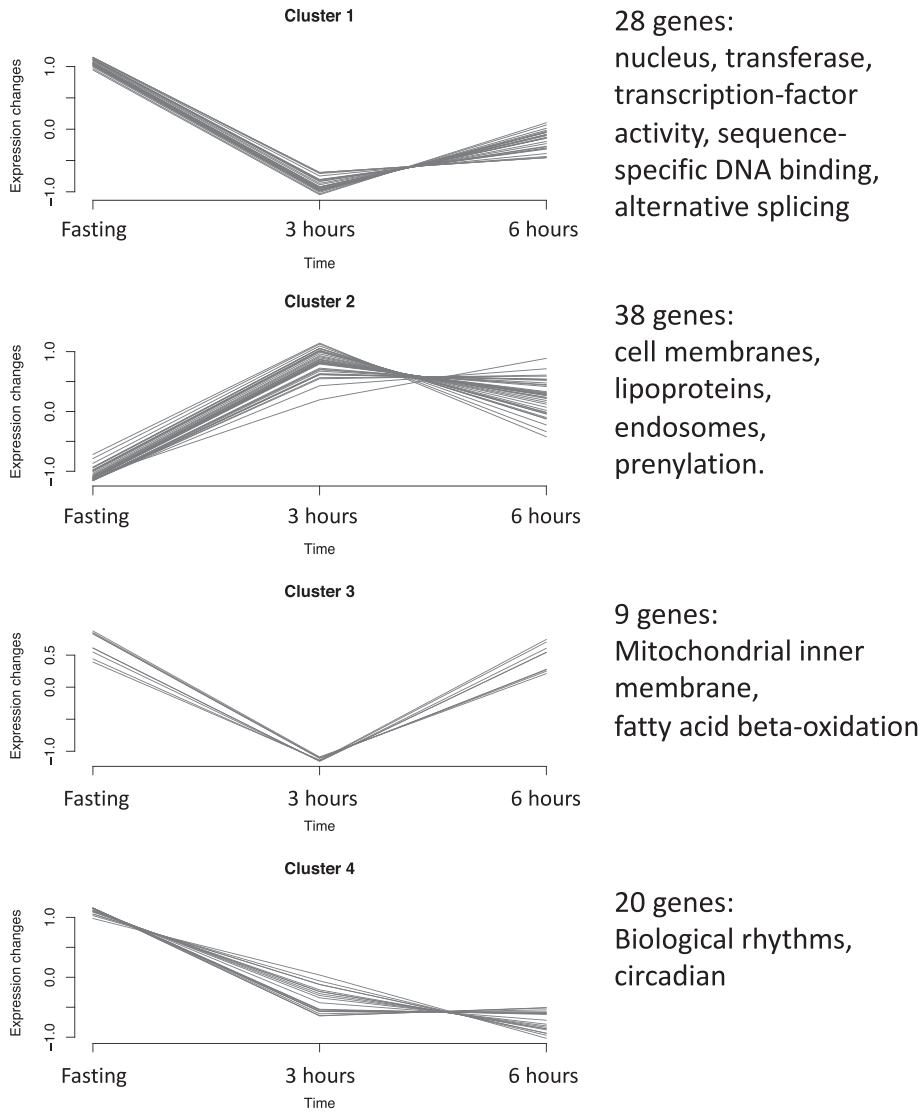

Fig. 2. Trajectory clusters and functional enrichment of genes clustered by temporal gene expression patterns. Each *x*-axis indicates the time point (fasting, 3 h, and 6 h); *y*-axis shows gene expression changes. Four clusters (1–4) represent the universal postprandial gene expression patterns. To the right of each cluster, the number of genes represented by the cluster is indicated, as well as significantly enriched functional annotations associated with the cluster.

term was significant for 2276 genes, comparable to the Subject:Arm interaction (2175 genes), suggesting that the day is what contributes the variance, not the particular version of high-fat meal presented on that day. In summary, the largest contributor to variance was the subject (13,856 genes), followed by the subject on a specific day (Subject:Day, 2276 genes), followed by the subject's postprandial response (Time:Subject, 651 genes) to the high-fat meal.

As an alternative method to determine the contributors to variance, we fit a linear mixed model that jointly considers the contribution of all specified variables on the expression of each gene. Visualization of the contribution of each variable (Fig. 1B) confirms that the subject was the largest contributor to variance with a mean contribution of 45%. The time after the meal challenge (0, 3 or 6 h) contributed a mean of 5.3% of the variance. The Day and Arm contributed means of 3.8% and 3.1%, respectively. A considerable proportion of the variance (mean 42.9%) was unexplained. This analysis confirms that the subject was the largest source of variance (45%) but that the postprandial response may be the second highest contributor (5.3%).

3.2. Cell marker transcripts in response to the test meals

The proportions of cell types in whole blood, which collectively contribute to the whole blood transcriptome, may be variable between subjects and between days within the same subject. To estimate the quantities of immune cells in each sample, we used EPIC [29] which deconvolutes transcriptome data using 65 marker genes derived from gene expression studies of 6 blood-circulating immune cell types. The 45 whole blood transcriptomes contained a mean of 25.3% neutrophils, 3.3% monocytes, 16.5% CD4+ T cells, 17.3% CD8+ T cells, 4.8% B cells, 0.03% NK cells and 32.9% unclassifiable cells. Overall, the cell populations appeared stable across the 45 transcriptomes (**Supplemental Fig. 1**). Linear mixed models with the random effect of Subject were used to determine the effects of Time, Arm and their interaction. Only Time was significant for any cell type. The fraction of neutrophils was significantly higher at 3 and 6 h relative to fasting, $P=.0017$ and $P=.0028$, respectively (**Supplemental Fig. 2A**). The fraction of CD4+ T cells was lower at 3 and 6 h relative to fasting ($P=.0159$ and $P=.0049$, respectively), and the fraction of CD8+ T cells was lower at 3 h ($P=.003$) (**Supplemental Fig. 2B–C**). However, the magnitude of the changes was quite small (<0.05%), suggesting that cell population changes were unlikely to be the primary reason for whole blood transcriptome changes.

3.3. Universal postprandial response to the test meals

Despite the extreme amount of variability between subjects and even within subjects on different days, there were still 95 genes with a significant effect of time. Using soft clustering, the 95 genes have 4 types of expression patterns (Fig. 2), 1 of which has the lowest expression at fasting (cluster 2) and the other 3 have the highest expression at fasting, which either decreases by 3 h postprandial without recovery by 6 h (cluster 1) or does recover by 6 h (cluster 3) or decreases more gradually throughout the day (cluster 4).

The cluster of genes with expression that is lowest at fasting and increases in response to the high-fat challenge meal (cluster 2, Fig. 2) includes 38 genes. These genes are associated with cell membranes (15 genes, $P<.023$), lipoproteins (8 genes, $P<.019$), endosomes (7 genes, $P<.022$) and prenylation (5 genes, $P<.013$). Movement and utilization of lipids are expected postprandial responses to a high-fat challenge meal.

The cluster of genes with expression that is highest at fasting and lowest at 3 h without recovery by 6 h (cluster 1, Fig. 2) includes 28 genes. This gene list is not statistically enriched for any annotations after multiple testing correction. However, marginally enriched annotations clusters include nucleus, transferase, transcription-factor activity, sequence-specific DNA binding and alternative splicing.

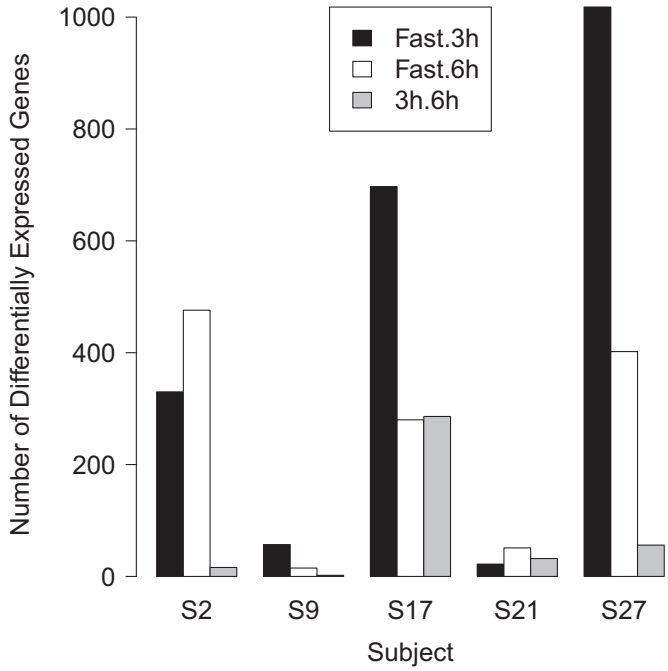

Fig. 3. Barplot of the number of transcripts differentially expressed between time points in each subject. Time point comparisons include 3 h relative to fasting (Fast.3 h, black bars), 6 h relative to fasting (Fast.6 h, white bars) and 6 h relative to 3 h (3 h.6 h, gray bars).

There is a small set of just nine genes that have lowest expression at the 3-h time point, but recover by 6 h postprandial (cluster 3, Fig. 2). These genes are associated with the mitochondrial inner membrane (five genes, $P<.00028$). The list is also marginally enriched for genes associated with fatty acid beta-oxidation (two genes, $P<.018$): acetyl-CoA acyltransferase 2 and carnitine palmitoyltransferase 1A. It is expected that catabolism of free fatty acids would decrease soon after a meal.

The last cluster comprises 20 genes that are at their highest expression at the fasting time point and steadily decrease throughout the day (cluster 4, Fig. 2). These genes are associated with biological rhythms (five genes, $P<.00038$), particularly circadian (Period circadian-like, C-terminal, three genes, $P<.00021$). In summary, despite substantial inter- and intrasubject variance, trends such as lipid utilization at 3 h postprandial, fatty acid beta-oxidation at 6 h postprandial and circadian rhythms reflect a universal postprandial response to a high-fat meal challenge.

3.4. Subject-specific postprandial response to the test meals

Given that the subjects contribute most of the variance, study arms were then treated as replicates to identify statistically significant changes due to the main effect of time within subject. The responses to the test meals are quite different between subjects. The total number of genes differentially expressed across time by subjects S2, S9, S17, S21 and S27 are 618, 66, 861, 90 and 1136, respectively. Subjects also differ in whether the greatest difference from fasting occurs at 3 or 6 h (Fig. 3). For subjects S9, S17 and S27, the greatest difference in the transcriptional response occurs between fasting and 3 h. For subjects S2 and S21, the greatest difference occurs between fasting and 6 h. This suggests that the transcriptional response to the meal varies by individual.

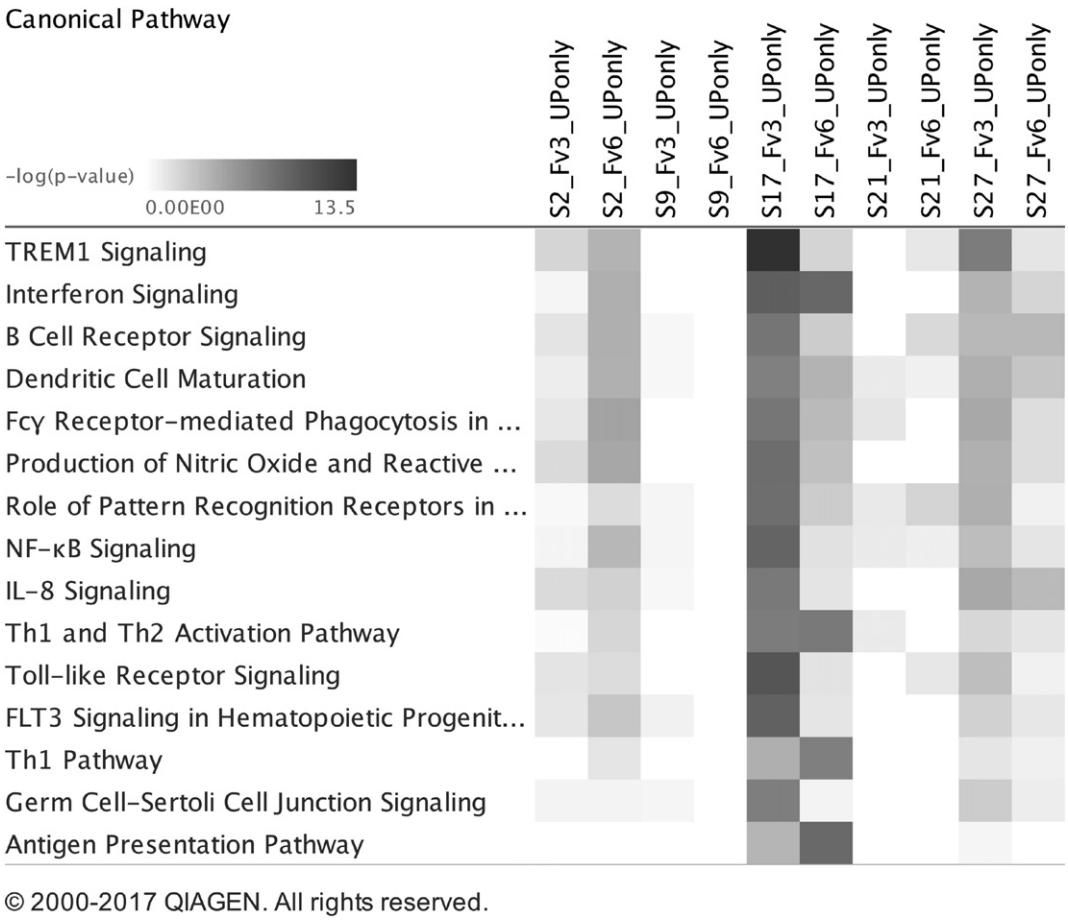

Fig. 4. Top pathways significantly enriched with genes up-regulated after fasting. This heatmap shows all pathways that are the most significantly enriched ($-\log(P) = 7$; $P=10^{-7}$ Fisher's Exact Test), ranked by significance. Each column represents an up-regulated gene list from one of the subjects at either the 3- or 6-h time point relative to fasting. The darker squares denote increased significance.

To determine functional differences between subject's postprandial responses, gene lists were analyzed for enrichment within biological pathways. More than 100 pathways are enriched with genes up-regulated by 3 h or 6 h relative to fasting (**Supplemental Fig. 3**). The top pathways are involved in the inflammatory response, with TREM1 signaling as the most significantly enriched pathway (Fig. 4). Across all of the significantly enriched inflammatory pathways, the enrichment of genes differentially expressed relative to fasting appears to be the strongest at the 3-h time point, compared to the 6-h time point, in Subjects S17 and S27, while the 6-h time point is more prominent for Subjects S2 and S21 (Fig. 4). Inflammatory pathways may be stimulated earlier in some subjects than others.

A hierarchically clustered heatmap of differentially expressed genes in the TREM1 signaling pathway further demonstrates differential postprandial transcriptional responses to the test meals among subjects (**Supplemental Fig. 4**). Subjects S17 and S27 at the 3-h time point cluster most closely together and show highly up-regulated TREM1 signaling relative to fasting. Meanwhile, the 6-h time point of Subject S2 clusters more closely with the 3-h time point of Subjects S17 and S27 than does the 3-h time point of Subject S2. While the pathway is predicted to be activated for most subjects and time points, TLR2 is down-regulated at the 6-h time point in three of the subjects, and the pathway is predicted to be inhibited in Subject S21 by 6 h. This suggests that not only might some subjects be quicker to stimulate inflammatory pathways postprandially, some subjects might also be quicker to down-regulate these pathways.

Some molecules, such as transcription factors, microRNAs and kinases, act as transcriptional regulators and affect the expression of their downstream target genes. Given known relationships of transcriptional regulators and their target genes, it is possible to predict, based on the expression of target genes, which upstream transcriptional regulators are activated or inhibited. To identify key transcriptional regulators and how they might differ between subjects and time points, an IPA Upstream Regulators Analysis was conducted for genes that are differentially regulated at 3 or 6 h relative to fasting. Even with an extreme P value cutoff of 10^{-10}, there are approximately 100 predicted transcriptional regulators. The top 20 regulators, when sorted by P value, are shown in **Supplemental Fig. 5**. For upstream regulators that are predicted to be activating, activation by these central regulators generally appears to be stronger at the 3-h time point in Subjects S17 and S27 but at the 6-h time point in Subjects S2 and S21.

Transcriptional regulators do not have to be differentially expressed to activate their targets. Therefore, when transcriptional regulators are differentially expressed, they can have an outsized role on downstream expression. Of the top 20 regulators (Supplemental Fig. 4), 3 are differentially expressed in more than 1 subject: IRF1 (2 subjects), TNFSF10 (3 subjects) and IL1B (4 subjects). IL1B is significantly different by the 3-h time point in subjects S17 and S27 but not until the 6-h time point in subjects S2 and S21 (**Supplemental Fig. 6**). This differential timing of the transcriptional activation of a central regulator IL1B may in part explain subject-specific differences in immune activation.

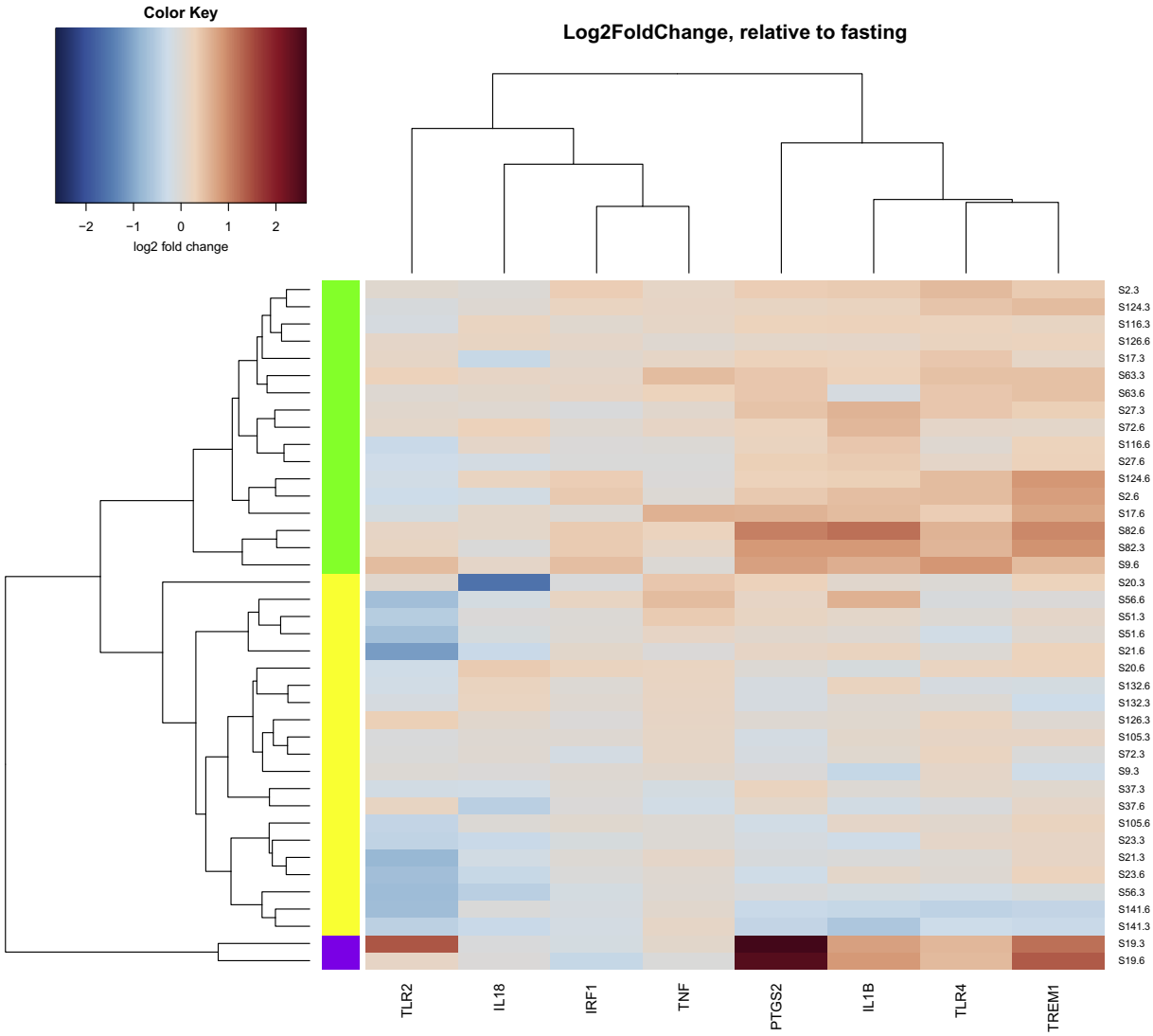

Fig. 5. Heatmap of transcript abundances measured by quantitative RT-PCR. Transcript abundances are plotted as log2 fold change relative to fasting. Orange indicates an increase relative to fasting; blue indicates a decrease relative to fasting. The *x*-axis indicates the probed gene, and the *y*-axis indicates the subject and time point with the format "subject.time": ".3" = 3 h, ".6" = 6 h. The data are clustered by both genes and subject/time. The dendograms across the top and left show the distances between samples, with longer bars indicating a greater difference. The three main sample clusters are annotated as green (upregulation of TREM1, TLR4, IL1B, PTGS2), yellow (unchanged TREM1, TLR4, IL1B, PTGS2) and purple (upregulation of TREM1, TLR4, IL1B, PTGS2 as well as TLR2).

3.5. Validation by quantitative RT-PCR

To determine whether the expression of innate immune genes in the postprandial period after a high-fat meal is subject-specific, we evaluated the abundances of mRNA of specific target genes in 180 samples from 20 subjects, including those previously sequenced. The specific target genes chosen included four genes from the TREM1 signaling pathway — TREM1, TLR2, TLR4 and IL1B — and one TLR-signaling target gene (PTGS2, also known as COX-2). In addition to IL1B, we probed two other transcriptional regulators, IRF1 and TNFα, and a proinflammatory cytokine, IL-18.

When the RT-PCR data are clustered by mRNA log2 fold change relative to fasting, TREM1, TLR4 and IL1B have the most similar expression profiles across subjects (Fig. 5). TLR2 is the most down-regulated, consistent with the RNA-Seq data (Supplemental Fig. 4). IL-18, IRF1 and TNFα are not differentially regulated. The *y*-axis is clustered by subject, annotated as green, yellow and purple clusters (Fig. 5). Subjects in the green and purple clusters are characterized by the up-regulation of TREM1, TLR4, IL1B and PTGS2, while these genes are not up-regulated in subjects annotated by the yellow cluster. Subjects in the green and purple clusters could be considered "responders," while those in the yellow cluster could be considered "nonresponders." Three of the 20 subjects have expression profiles that fall in different clusters at different times. In all three cases, the expression of innate immune genes is low/unchanged at 3 h (yellow cluster) and higher at 6 h (green cluster). These subjects may be "late responders." In short, the quantitative RT-PCR data support the observation that expression of innate immune genes in the postprandial period is variable between subjects.

4. Discussion

Previous studies with high-fat meals have yielded conflicting results on the status of postprandial inflammation (reviewed in [11,12]). In the current study, we monitored the whole blood transcriptome in a crossover study design with the subjects fed

high-fat meals supplemented with placebo, blueberry powder or DHA and identified the sources of variance. The largest contributor to variance was the subject [13,856 genes differentially expressed (DE)], followed by the test day (2276 genes DE), followed by the subject's postprandial response (651 genes DE) to the test meal. Using linear mixed models to determine each variable's contribution to the variance after correcting for other variables, the mean contributions to variance of the subject, the postprandial time, the study day and the study arm (diet) were 45%, 5.3%, 3.8% and 3.1%, respectively. The high intersubject variability possibly explains the conflicting results in previous studies of postprandial responses to high-fat meals. This finding underscores the need to utilize crossover study designs with outcome measurements at multiple postprandial time points for dietary intervention studies.

While each dietary intervention contained a supplement, every dietary intervention on all study arms was a moderately high-fat breakfast meal, so we chose to focus the analysis on the postprandial transcriptional response to that high-fat meal by using the study arms as replicates. In doing so, we were able to identify innate immune signaling genes up-regulated in the whole blood during the postprandial state. Deconvolution of the transcriptome data to determine the cell populations in each sample demonstrated that relative changes in postprandial whole blood cell populations were small but significant. Only one immune cell type — neutrophils — increased in the postprandial state, suggesting that it is possible that increases in the expression of innate immune signaling genes were due to an increase in circulating neutrophils. It is therefore not clear how much of the increase in innate immune signaling was due to the slight increase in neutrophil fraction and how much was due to up-regulation of innate immune signaling genes. However, the magnitude of the neutrophil population changes was quite small (mean change <0.05%), suggesting that cell population changes were unlikely to be the primary reason for whole blood transcriptome differences.

When probing innate immune signaling genes by quantitative RT-PCR in the fasting and postprandial blood of 20 subjects fed the test meals on the 3 different days, some subjects appeared to be more responsive than others (Fig. 5). Even random data can appear to show responders and nonresponders, but when data points are clustered in a biologically coherent manner (*e.g.*, elements of the same signaling pathway up-regulated together), the notion of true responders and nonresponders is more valid. It could be argued that the 11 subjects who have coregulated elements of the TREM1 signaling pathway and downstream targets (TREM1, TLR4, IL1B, PTGS2) are "responders" to the test meal, while the 9 subjects who did not show an up-regulated pattern of these genes at either time point would be considered "nonresponders." Further, one might consider the three subjects with an up-regulated pattern only at the late time point to be "late responders." In a whole blood transcriptome study of seven obese and seven nonobese individuals given high-fat meals of increasing caloric doses, some of the obese individuals had a response that diverged from nonobese individuals, indicative of a loss of metabolic flexibility [30]. Our data are consistent with their observation that the whole blood transcriptome can be used to differentiate subclinical differences in study subjects. We further hypothesize that responsiveness to a high-fat meal challenge indicates an elevated systemic inflammatory tone which may be associated with increased risk of chronic disease.

Surprisingly, there have been only a few studies of the whole blood transcriptome in response to an acute meal challenge. There are two studies of the postprandial response to yogurt or acidified milk, both of which show a down-regulation of inflammatory response genes by 6 h postprandial [31,32]. A study of the whole blood transcriptome postprandial response to a high-protein or high-carbohydrate breakfast showed down-regulation of inflammatory response genes, but acetaminophen, which is known to suppress inflammation [33], was included with both breakfasts [34]. In a study of the obese and nonobese subjects fed high-fat meals of increasing caloric dose, the caloric dose–response gene set was statistically enriched for "oxidative phosphorylation" but not for other biological pathways [30]. In the current study, we found that the postprandial response of the whole blood transcriptome was enriched for dozens of signaling pathways of the innate and adaptive immune system. This is likely due to the fact that subjects in the present study were given the same caloric dose (approximately 849 kcal) of a high-fat meal (approximately 36.2% kcal from fat), containing saturated fats known to stimulate innate immune signaling [1–6], on all 3 study days. These saturated fats were primarily 16:0 (7.4–7.6 g/meal) and 18:0 (3.2–3.4 g/meal). Additionally, RNA sequencing is known to have a large dynamic range and is more sensitive than microarrays (Fig. 1 in [35]) and may have enabled the detection of more modest changes in gene expression. In short, our study is the first to report strong up-regulation of innate immune signaling pathways during the postprandial period after a high-fat meal, likely due to the choice of fatty acids in the meal and the sensitive technology (RNA sequencing) used to monitor the transcriptome.

In a study of the postprandial blood transcriptome response to different dairy products, the authors reported that the kinetics differ between subjects [28]. This is consistent with our data. Our RNA-Seq data showed peak responses in inflammatory pathways at 3 h in two subjects and 6 h in two other subjects. In our RT-PCR data, 3 of 20 subjects had unchanged innate immune gene signaling at 3 h which increased by 6 h. These observations confirm the importance of postprandial kinetics and the need to include multiple time points in studies of postprandial response.

One of the clusters of genes that are low at fasting and high at the 3-h time point includes PRR genes, notably TLR1, TLR4 and TLR6; an inflammasome component, NLRP1; and their downstream signaling molecule, MAP3K5 (Supplemental Fig. 3). Some of these PRRs can be activated by microbial products or endogenous molecules leading to production of proinflammatory effector molecules including proinflammatory cytokines [36]. Thus, these results imply that if corresponding protein expression is correlated, postprandial inflammatory responses could be at least in part mediated by the activation of these receptors.

TLR2, unlike TLR4, was down-regulated during the postprandial period among most individuals, and this trend was validated in more subjects using RT-PCR (Fig. 5). Both TLR2 and TLR4 can be activated by saturated fatty acids; however, TLR4 is the specific receptor for endotoxin which is the outer cell membrane component of all gram-negative bacteria. Endotoxin is perhaps the most ubiquitously contaminating bacterial product in the environment. The fact that TLR4, but not TLR2, is up-regulated in the postprandial period suggests that postprandial blood may be more sensitive than fasting blood to gram-negative bacteria or endotoxin after consuming a high-fat diet. The possibility that postprandial blood may become more susceptible than fasting blood to endotoxin, which is implicated to be absorbed from the gut during dysbiosis, needs to be determined in the future.

Petrov et al. promoted the concept of whole blood RNA as a source of biomarkers in nutrition and metabolic health studies [37]. In a review of 57 high-fat meal challenge experiments, Herieka and Erridge found that leukocyte and mRNA markers, unlike plasma markers of inflammation, were elevated in the few studies in which they were measured [11]. Our results confirm that mRNA markers are highly sensitive to the high-fat meal. We used RNA sequencing to identify a panel of markers of immune genes such as TREM1, TLR4, IL1B and PTGS2 that were then verified by RT-PCR. These specific mRNA markers could be used to phenotype subjects to determine whether they have elevated systemic inflammatory tone. Further, our study identifies a universal postprandial transcriptional response such as changes in expression of circadian rhythm genes and genes involved in

the movement and utilization of lipids. Such mRNA markers could conceivably be used to verify subject compliance.

Although the meals were isocaloric with nearly identical percentage of kcal from fat, one of the diets included 2 g of DHA and another diet included blueberry powder. Both DHA and blueberries would be expected to decrease inflammation due to, at least in part, the suppression of toll-like receptor signaling by DHA [3–5,35,38] and by polyphenols [39]. There was no main effect of dietary supplements (blueberry powder or DHA capsules) for any genes, suggesting that the differentially expressed genes (95 genes) in postprandial time period across all subjects are mainly affected by the meal itself. This result suggests that the potential anti-inflammatory effects of DHA or blueberry powder may not be manifested after ingestion of single meal or that the sample size was not enough to give statistical power. The strengths of the study included the repeated measures of high-fat diet challenges in the same subjects, the standardized dinner preceding each day of the test meal and the use of venipuncture to reduce local inflammation.

In conclusion, the results show that the genes involved in innate immune responses, particularly germline-encoded pattern recognition receptors and their downstream signaling components, are differentially expressed in the postprandial time period after a high-fat meal compared to the fasting period in a subject-specific manner. These results suggest that the activity of these innate immune receptors which are constitutively expressed in innate immune cells can be regulated not only by agonist concentrations but also at the transcriptional step during the postprandial time period in response to a meal. The variability between subjects and time-dependent expression of the genes likely explain prior inconsistent reports on the postprandial response to high-fat meals. Future studies to assess the postprandial responses to diets could use a panel of mRNA biomarkers — IL1B, TLR4, TREM1, PTGS2 — to distinguish responders and nonresponders.

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Appendix A. Supplementary data

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