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Blood transcript immune signatures distinguish a subset of people with elevated serum ALT from others given acetaminophen

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Abstract

The diagnosis of drug-induced liver injury (DILI) is hindered by the limited utility of clinical chemistries. We have shown that hepatotoxicants can produce peripheral blood transcriptome “signatures” (PBTS) in rodents and humans. In this study, 42 adults were treated with acetaminophen (APAP) (1 gram every 6 hours) for 7 days, followed by 3 days of placebo. 11 subjects received only placebo. After 5 days, 12 subjects (30%) had increases in serum alanine aminotransferase (ALT) levels (“responders”). PBTSs of 707 and 760 genes, respectively, could distinguish responders and non-responders from placebos. Functional analysis of the responder PBTS revealed increased expression of genes involved in TH2 mediated and innate immune responses, while the non-responders demonstrated increased gene expression consistent with a tolerogenic immune response. Taken together, these observations suggest that the clinical subjects with transient increases in serum ALT failed to maintain or intensify a hepatic tolerogenic immune response.

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CONFLICT OF INTEREST

The authors claim no conflict of interest.

AUTHOR CONTRIBUTIONS

R.F. wrote the manuscript; R.F. performed the research; P.B.W. and R.S.P. designed the research; R.F., K.G., and P.R.B. analyzed the data; S.O.S. and P.B.W. contributed new reagents/analytical tools.

Keywords

hepatotoxicity; genomics; biomarker; blood; immunology

INTRODUCTION

In the United States, drug-induced liver injury (DILI) is both the most commonly identifiable cause of acute liver failure and the major reason for regulatory actions on drugs(1, 2). Diagnosis is problematic because of the poor ability of current clinical indicators, such as increases in serum alanine amino transferase (ALT), to distinguish between benign transient and mild liver damage, allowing the patient to continue treatment safely, versus DILI that will progress to life-threatening injury if treatment is not stopped(3). Also, for patients on multi-drug therapy regimes, current clinical options cannot distinguish which particular drug is problematic. Lacking are reliable, sensitive and compound specific biomarkers, which will improve liver safety assessments in new drug trials, as well as clinical treatment.

The maturing of various “omics” technologies in the last decade has led many, including the current authors, to apply these technologies to this very end(4–9). Our impetus for pursuing peripheral blood (PB) transcriptomics in clinical studies came from in-life rat studies of 8 hepatotoxicants including APAP. This study demonstrated the feasibility of finding compound-specific PB cell transcription signatures (PBTS) that paralleled hepatotoxicity, *and* were more sensitive to toxicity than the traditional biomarker, serum ALT(10). Furthermore, by probing human PB transcriptome data from clinical overdose patients with human orthologs of the rat PBTS, we were also able to differentiate these patients from non-exposed individuals(11). Subsequently, in a clinical study, we found a PBTS that could distinguish 6 subjects receiving a single non-toxic but supratherapeutic 4 gram dose of APAP from 3 receiving placebo. The signature consisted primarily in down-regulation of genes involved in oxidative phosphorylation and other mitochondrial functions 48 hours after dosing. Notably, in this earlier study, none of the subjects demonstrated any significant rise in serum ALT, a sensitive biomarker for liver injury(4). However, we have since concluded a larger clinical study consisting of 53 healthy human volunteers enrolled in a double-blind, placebo-controlled experiment where 42 received the maximum recommended therapeutic dose of APAP (4 grams/day for 7 days), and the remaining 11 subjects received placebo. In contrast to the single dose study, 12 (29%) of the 42 dosed subjects did have significant rises in ALT after 4 days of treatment. Here we present blood transcriptome results from these subjects. The hypothesis tested in the current study was that a PBTS could be found that could distinguish between subjects with significant rises in serum ALT from those without within the first 4 days of treatment, prior to any significant ALT response. Secondarily, we wanted to determine if any such PBTS would modulate during the remaining days and if it could provide mechanistic insights into APAP induced liver injury (AILI).

RESULTS

Clinical—All subjects completed the inpatient study with no significant adverse events. Subject characteristics and a summary of ALT response are listed in Supplemental Table 1. A time-course of serum ALT levels is shown in Figure 1. No subject had statistically significant increases in any other clinical tests. Before the study began, it was determined that increases in serum ALT levels would be considered significant if, at any time during the course of the study, they exceeded 2 X the upper limit of normal (ULN) ($2 \times 30 = 60$ U/L for females; $2 \times 40 = 80$ U/L for males) and 2 X the subjects' own predose baseline values. Accordingly, of the 42 subjects receiving APAP, 12 had significantly increased ALT levels as here defined, with non-significant increases generally starting at day 4 and reaching significant peaks from days 7 to 10 (responders). 7 of the 12 subjects had increases greater than 3 X the ULN. 2 weeks after the study all subjects' ALT levels had returned to normal (data not shown.)

Gene Expression Changes—Venn diagrams of the number of differentially expressed probes (DEPs) that make up each PBTS for each time period are shown in Figure 2. For example, in the Early period (days 1 to 4), there were a total of 707 DEPs (401 unique) when non-responders were compared to placebos, reflective of the transcriptomic response of non-responders to APAP as compared with responses to placebo. Of these, 401 DEPs were unique and not shared with the responders or placebo. We found a total of 963 DEPs when responders were compared to placebos, reflective of the transcriptomic response of "responders" to APAP. Of those, 503 DEPs were unique. In addition, we found 775 DEPs when responders were compared directly to non-responders in the ANOVA, reflective of the differences in the transcriptomic responses of responders and non-responders to APAP, of which 408 DEPs were unique. Also shown are the various categories of shared DEP's. A complete list of DEPs in the PBTSs can be found in Supplemental Tables 2 through 4.

Principal Component Analysis (PCA)—PCA is a technique that allows one to visualize relationships in a complex data set in a standard graphical form. Here the PCAs are based upon the PBTSs that have been determined beforehand to be statistically significant, and are for illustrative purposes only. PCA utilizing all PBTSs from the Early period (Figure 3a) reveals clear separation for each group at each time period. Similar results were obtained for both the Middle and Late Periods (data not shown). We found there was considerable variability amongst individuals in each group at each time period. This variability is apparent in the PCA showing each individual using the unique PBTSs from the Early period for the non-responders compared to placebos, responders compared to placebos, as well as responders compared to non-responders (Figure 3b, c, d). In the figures, each shape represents one subject at a particular day while the mesh ovals represent 2 standard deviations from the center of distribution. Despite individual variability, the graphics show appreciable separation of non-responders from the placebos (Figure 3b), the responders from the placebos (Figure 3c), as well as the responders from the non-responders (Figure 3d). PCA results were obtained for the Middle and Late periods that demonstrate similar separation between groups (data not shown).

Functional Analysis of DEPs—Ingenuity Pathway Analysis® (IPA) and *Gene Set Enrichment Analysis* (GSEA) of the PBTSs showed enrichment mainly in cellular and innate immune responses across the data set for both responders and non-responders. However, the nature of the responses was quite different for the non-responders and responders. In general, for the non-responders there was an abundance of genes that have been identified with hepatic immune tolerance, while the genes for responders tended to reflect a pro-inflammatory signal.

IPA Analysis

Early—A complete listing of genes for the reported IPA biofunctions for all IPA comparisons can be found in supplemental tables 5 through 7. All comparisons for the early period are presented in Table 1. When the responders were compared to the placebos in the early period, the top rated IPA biological functions consisted of activated immune responses. Myeloid cell functions (activation, infiltration, stimulation and recruitment) and different T cell functions (activation and proliferation) were up-regulated. There were no significant down-regulated immune functions. Notable up-regulated inflammatory and immune modulatory cytokine and chemokine genes included IL-3, 4, 13, and 25 as well and FCER1A, suggesting the induction of a T_H2 cell-mediated immune response. When compared directly to the non-responders, the responders showed similar up-regulated inflammatory biofunctions, with the addition of evidence for an anti-microbial response.

As with the responders, when the non-responders were compared to the placebos during the early period top-rated IPA biofunctions involved the immune response. But unlike the responders, T cell modulatory functions (development, negative selection, quantity) were up-regulated while myeloid cell functions (activation, chemotaxis, recruitment) were down-regulated. In addition, genes involved in oxidative phosphorylation were down-regulated. Likewise, when compared directly to responders, biofunctions for T cell homing and chemotaxis as well as migration of plasmacytoid dendritic cells (DCs) were up-regulated.

Middle—All comparisons for the middle period are presented in Table 2. Comparing the responders to the placebos, IPA indicated a muted immune response in the Middle period in comparison to the results from the early period, with only significance for the response and quantity of CD8 cytotoxic T cells. Down-regulated pathways (T cell suppression, B cell generation), largely driven by lower AKT1 expression, are consistent with the survival of proinflammatory T cells. In contrast, when the responders were compared directly to the non-responders, there was abundant evidence for a continued up-regulated proinflammatory response. Top-rated up-regulated immune biofunctions involved the activation of macrophages (movement and phagocytosis), antigen presenting cells (movement), and mast cells (degranulation).

As in the early period, when the non-responders were compared to the placebos during the middle period, top-rated IPA biofunctions involved up-regulation of largely inhibitory immune responses. These included the increased activation of regulatory T-cells, lack of activation of inflammatory T and B cells, and the activation and recruitment of dendritic cells. Interestingly, IPA indicated an increased quantity of neutrophils and natural killer

cells. Also much like the Early period, down-regulated biofunctions included those for the development, binding, and chemoattraction of neutrophils and macrophages, the arrest in rolling of leukocytes, and for genes associated with liver inflammation. Comparing the non-responders directly to the responders, top-rated biofunctions included the movement, migration, and activation of leukocytes in general, but specifically for mononuclear leukocytes/T cells.

Late—All comparisons for the late period are presented in Table 3. Comparing the responders to the placebos, IPA indicated continued and increasingly significant up-regulation of biofunctions associated with neutrophil/macrophage recruitment and chemotaxis, as well as chemotaxis of T cells, function and quantity of B cells, and inflammatory liver injury. When compared directly to the non-responders, the top-rated biofunctions were very similar to those from the comparison to the placebos and as with the early period indicated a T_H2 response (mast cell degranulation).

For the non-responders compared to the placebos during the late period, the immune response seemed to be somewhat mixed and attenuated. Up-regulated biofunctions included similar anti-inflammatory categories and attendant genes of earlier periods (proliferation of dendritic and T cells). The biofunction signature for myeloid cells, interestingly, was, as in the middle period, mixed: While chemotaxis and “immune response” from these cells seemed to be increased, the classical activating chemokines/receptors and the NF- κ B genes of the responder signature were absent. At the same time, biofunctions for the infiltration, recruitment and chemotaxis of myeloid cells also were down regulated because of other genes in the dataset (see Supplemental Table 7 and the discussion for specific genes). As well, when compared directly to the responders, proliferation of dendritic cells and development of T cells were the top-rated biofunctions for the non-responders.

GSEA

Functional analysis utilizing GSEA indicated the same general trend as IPA: a generally proinflammatory response in the responders but a tolerogenic one in the non-responders. While numerous significant immune pathways were detected with most comparisons, we focus on the Leading Edge Analysis (LEA) results from GSEA(13). This function highlights the enriched genes that contribute most to the significant pathways (The pathways for each comparison can be found in supplemental tables 8 and 9). The LEA results are summarized in Figure 4, where key enriched immuno-modulatory genes are shown in a supervised heat map for the responders and non-responders to placebos comparisons. While not absolute, genes that are known to be tolerogenic/anti-inflammatory in a hepato-immunological context were more highly represented in the non-responders, while pro-inflammatory genes are more highly represented in the responders across all treatment periods. Similar results were obtained for the direct responder vs. non-responder comparisons (Supplemental Figure 1).

DISCUSSION

As hypothesized, we were able to find a PBTS that could distinguish subjects with mild, though significant rises in serum ALT from those who did not before the elevations began

on day 4. The same is true for our secondary hypothesis that this signature would modulate during the remaining days of the study and that the signature(s) could provide mechanistic insights into AILI. Specifically, the signatures we found in all post-treatment periods, including the early period before the rise in serum ALT, suggest that the responders in the early period experienced a proinflammatory T_H2 allergic response, activation of macrophages and neutrophils and anti-microbial response, including increased activation of NF- κ B and INFG pathways. While the T_H2 allergic response seemed to attenuate in the later periods, the inflammatory macrophage/neutrophil and NF- κ B/INFG responses seem to persist into the late period.

These findings are notable because we were able to use white blood cells from circulating blood as a “surrogate” tissue to detect mild liver injury. It may be that the resident liver immune cells would show a more dramatic change in gene expression than those few that find their way into the peripheral blood. In any case, that we were able to “detect” these relatively small signals of hepatic distress in peripheral blood adds weight to our earlier findings.. At the same time, we recognize that the most rigorous approach would have been to split the data into “training” and “test” sets. However, because of the subtleness of the changes in this study, we found it necessary to combine all the samples to reach acceptable statistical power. Substantive confirmation of the results would require a separate test set, and we recognize this as a limitation of this work.

Even so, recent literature on AILI has focused on immune responses as playing important roles, even though involvement of macrophages and neutrophils remain controversial(14, 15). Studies demonstrating a proinflammatory response have recently been balanced by other studies that suggest a balance between protective and pro-inflammatory functions depending upon the model(14).

Some recent APAP research has specifically focused on the ability of receptors of damage associate molecular pattern molecules (DAMPS) to detect DAMPs such as heat shock proteins and or DNA/RNA released by damaged hepatocytes. The mechanism is thought to involve innate immune processes involving TLRs, IL1B, IL18, NF- κ B and NALP3 inflammasome activation(16). In fact, a recent mouse study has shown that HSP-70 released from dying APAP treated hepatocytes contributed to a DAMP inflammatory response(17). Importantly, we did see up-regulation of TLRs, IL1B, IL18, NF- κ B, and NALP3 in the responders during the course of this study. This gives credence to the hypothesis that DAMP responses may be similarly involved in APAP DILI in humans.

Conversely, it appears that the non-responders were able to intensify or maintain a tolerogenic response that prevented any significant damage. Indeed, it is well known the liver maintains a tolerogenic immune environment under basal conditions (18). This is thought to be due to frequent exposure to innocuous antigens, such as gut-derived nutrients, antigens from aged or damaged cells, neo-antigens that arise by adduct formation of metabolic products during xenobiotic metabolism, and low levels of bacterial antigens from gut microbiota. This phenomenon is mediated by circulating DCs and resident liver Kupffer (KCs), liver sinusoidal endothelial (LSECs), and stellate cells. Importantly, in two recent publications, DC induced tolerance has been confirmed to be operative in human liver(19,

20). Even the mixed signal from myeloid cells during the middle and late periods may reflect recent reports that indicate an anti-inflammatory/regenerative role for both neutrophils and macrophages during recovery periods in acute APAP injury cases(21, 22). At the same time, these particular studies could not rule out the possibility of a pro-inflammatory effect by neutrophils and macrophages during the development of the already established injury in the cited clinical cases.

Several other recent studies support a role for tolerance in limiting AILI. It has been demonstrated that DCs protected against AILI in a mouse model by promoting a tolerogenic immune environment(23). More importantly, a mouse population-guided resequencing study of these same clinical subjects revealed that variants in CD44 contributed to the AILI in the responders(5). Subsequently, a number of studies have confirmed CD44's role in protection from hepatotoxic liver injury in mice and tolerance in human liver(24–27).

That such a tolerogenic response may be involved in maintaining the normal ALT levels of the non-responders is supported by a number of specific gene expression changes. We found evidence for the increased activity and number of dendritic cells. First, we observed up-regulation of CCR7 and several HLA-DR genes, which has been the standard for detecting circulating human dendritic cells(28). Other MHCI and MHCII genes were also upregulated, including several with SNPs that have been reported to be associated with lumiracoxib-related liver injury(29). Secondly, we found that other up-regulated genes more recently known to be indicative of DC activity were also prominent (CD11b, IRF8) as well as IL15(30, 31). Interestingly, a recent study showed that IL-15^{-/-} mice demonstrated enhanced susceptibility to AILI, though the mechanism remains elusive(32).

Other evidence for increased hepatic tolerance included a high IL-10/IL-12 ratio (increased expression of IL-10R, and concurrent decreased expression of a receptor IL-12RB(33). Up-regulation of receptors for other key cytokines important in modulating tolerance (IL4 and 6) were also observed. Of note is a recent report of IL-10/4/6^{-/-} KO mice being uniquely susceptible to AILI(34). Up-regulated iCOS expression during the Middle period may also be evidence of tolerance: Recent work has demonstrated a crucial role for iCOS in the ability of plasmacytoid DCs to differentiate CD⁺4 T-cells into an IL-10 producing tolerogenic phenotype(35). Gene expression changes in the non-responders also offered evidence for increased activity of T_{regs}, including up-regulation of IL10 receptor, FOXP3, and IL7 and its receptor(36–38). In addition, we saw evidence for the activation of the two most widely recognized inhibitory T cell signaling pathways (PDCD1 and CTLA4- Early) as well as for the more recently recognized inhibitory role for HVEM (TNFSFR14) in the presence of its ligand CD160 (Middle)(39). Other upregulated genes that provide evidence for an tolerogenic signature in the non-responders included SOCS5 (Early and Late periods - T helper 2 (T_H2) cell activity suppressor), IL11 (Late -compensatory hepatocyte proliferation) and HMOX1 (Late -modulator of inflammation)(40, 41). Finally, three significantly regulated genes, CX3CR1 (up-regulated in non-responders), LGALS3 (down in non-responders, up in responders), and PPAR γ up in non-responders, down in responders), have been shown to be involved in the anti-inflammatory activity of alternatively activated (M2) macrophages, particularly during AILI(42–44).

While the ALT responses were minimal and transient (in contrast to other clinical work that has been done largely with acute liver injury cases after the fact), our previous publication with these subjects demonstrated that the responders did have an increase in breakdown products of NAPQI(6). As well, an independent study involving therapeutic dosing of APAP, has demonstrated that elevated serum ALT is associated with elevations in serum miR122 and CK18(45). Moreover, the association of ALT elevations with CD44 noted in these subjects has also been recently observed in patients with acute liver failure due to APAP overdose where the patients did not intend to kill themselves(5, 25). These observations support the idea that the mild injury observed with therapeutic APAP ingestion may be mechanistically linked to more severe APAP liver injury.

Lastly we saw a similar decrease in oxidative phosphorylation genes for the non-responders during the early period as we had seen in our earlier study. This decrease, importantly, was not observed in the responders. Another research group recently observed a similar phenomenon in a study with 5 clinical subjects that had been given APAP, though with no increases in ALT(46). Even though the mechanistic importance of these observations is unclear, that this phenomenon has been observed in 3 independent clinical studies suggests it may be a good candidate surrogate measure of exposure to APAP that is *not* associated with liver injury. We have suggested several mechanisms that might explain this observation, including direct mitochondrial toxicity to leukocytes and/or a metabolic change that was consistent with activation of immune cells. The current study would lend more credence to the latter explanation, especially since we see modest but significant increases in expression of key genes involved in glutamate metabolism (glutaminase and glutaminase 2). Activated T- cells, tolerogenic or otherwise, are known to favor glycolysis, with glutamine as the preferred energy source, while concordantly down-regulating oxidative phosphorylation(47, 48).

In summary, the PBTS signatures suggest the ability of non-responders to intensify or maintain a protective tolerogenic response to a pharmacological APAP challenge, while that process seems largely absent in the responders, allowing for a pro-inflammatory response that most likely contributes to the rise in serum ALT. This study also provides possible insight into previous conflicting reports on the role of the immune system in AILI, and is consistent with much of the latest literature in this area of study. Importantly, these gene expression signatures were detectable before the rise in ALT in the responders, and may warrant further investigation as a biomarker in predicting which APAP intoxicated individuals are in danger of AILI. Whether this kind of immune signature is specific to APAP cannot be determined from this study. Future studies would be needed that would investigate transcriptomic profiles from blood samples from patients that experienced liver distress due to other hepatotoxicants.

METHODS

Additional details on methods with associated references are supplied in the supporting information.

Subject selection, dosing and sample collection

Written informed consent was obtained and approved by the University of North Carolina (UNC) Institutional Review Board. Subjects were in the clinic for 14 days. Data from this study has been presented in recent publications by selected authors of this publication and is referred to in the discussion(5, 6). Following an acclimation period (days -3 to 0), 42 subjects received 4 daily oral APAP doses of 1000 mgs (every six hours) for 7 days (days 1 – 7) while twelve received placebo. Both staff and the subjects were blinded to treatment assignment. Blood samples were taken at 8:00 a.m. daily prior to dosing and analyzed for complete blood count (CBC), aspartate aminotransferase (AST), ALT, total bilirubin, alkaline phosphatase, blood urea nitrogen, alpha-glutathione-S-transferase, and creatinine. Dosing began on day 0 after the 8:00 a.m. blood draw. Individual serum baseline serum ALT was determined as the mean of the values obtained prior the start of dosing. In addition, 7.5 milliliters (mls) of blood were drawn into PAXgene (PreanalytiX/Qiagen, Hilden, Germany) blood RNA collection tubes (3 tubes at 2.5 mL). For subsequent analysis, subjects that received APAP were divided into two categories according to the results of the serum ALT analysis. “Responders” were those that had a 2-fold increase in serum ALT from individual baseline values *and* were >2-fold above normal (as defined by historical data from the UNC hospital: females = 30 IU/L; males = 40 IU/L), while all others receiving APAP were classified as “Non-responders”.

RNA Isolation and Microarray Gene Expression Data Analyses were performed as previously described(4). For the analysis, study days were categorized into periods in relationship to the start of APAP or placebo dosing as follows, with rationale indicated: “Predose” (days before APAP or placebo treatment; days -3, -2, -1, and 0), “Early” period (days 1, 2, 3, and 4 after the start of dosing) before any significant rise in ALT, “Middle” period (days 5, 6, and 7), when ALT rises were significant and at or near peak, and “Late” period (days 8, 9, 10) after dosing had ceased and ALT levels had plateaued or began to decline, for a total of 14 study days. Each subject/day was normalized to their corresponding day 0 data. Pooling of days also greatly increased the study’s statistical power: Power tests indicated an 80% confidence for detecting gene expression changes of at least 1.2 fold at an alpha value of 0.05 (supplemental data). A one-way ANOVA test with post-hoc analysis was utilized within these periods to compare responders, non-responders and placebos. Statistically differentially expressed probes were identified at a false discovery q-value of 0.1 and a fold change of 1.2, for responders versus placebos, non-responder versus “placebos”, as well as “responders” versus non-responders. Similar data from the Predose period were excluded.

Biological Function Analysis

The data were analyzed for biological functionality with Ingenuity Pathway Analysis® (IPA) (Ingenuity Systems, version 14400082, build 190263, Copyright © 2012, www.ingenuity.com) as well as *Gene Set Enrichment Analysis* (GSEA, www.broadinstitute.org/gsea/)(13). IPA utilized as input the PBST’s from the ANOVA while GSEA utilized the entire data set. By utilizing the entire data set, GSEA is able to find more subtle but significant differences than IPA in biological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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STUDY HIGHLIGHTS

What is the current knowledge on the topic?

While the mechanism(s) of acetaminophen –induced liver injury are becoming better defined, questions concerning the exact role of the immune system, and more importantly, why certain individuals seem more susceptible than others still await clarification.

What question did this study address?

In an extensive clinical study, about 20% of patients given 4 grams of acetaminophen per day for 7 days showed significant transient increases in serum ALT after 4 days. We wanted to know if whole blood transcriptomics could give insight into why these particular patients had this mild ALT response (responders), and concomitantly, why the other 70% (non-responders) did not.

What this study adds to our knowledge

Our results suggest that there was an active role for pro-inflammatory mechanisms early in the responders, while the non-responders had an intensified tolerogenic/anti-inflammatory immune response and a significantly muted pro-inflammatory response.

How this might change clinical pharmacology and therapeutics

Maintenance or intensification of liver immune tolerance may be important in protecting against acetaminophen-induced liver dysfunction/toxicity. This information could help guide further studies in understanding the mechanism of variable response across patients to acetaminophen and other drugs that induce liver toxicity.

Figure 1.

*Serum ALT levels as fold change from the average of the 3 day pre-dose data (not shown). RED, responders; GREEN, non-responders; BLUE, placebos. APAP dosing began on day "0" (right after 8 a.m. blood draw) and ended on day "7": 1 gram every 6 hours for a total daily therapeutic dose of 4 grams for 7 days.

Figure 2.

Venn diagrams showing the number of differentially expressed probes (Fold change > 1.2, FDR < 0.1) for each dosing period for responders (R) vs placebos (P), non-responders (N) vs placebos, and responders vs non-responders.

(a)

(b)

(c)

(d)

Figure 3.

Scatter plots of PCA results utilized DEGs for the early dosing period. (a) Each shape represents the mean PCA location for all individuals/days in each group. (b, c and d) Shapes represent a single subject/day. Webbed elliptical shapes represent 2 standard deviations from the mean 3D PCA locale.

Intensity plot of GSEA selected leading edge (LE) genes for responders and non-responders compared to placebos. LE genes represent those that contribute most significantly to GSEA identified pathways. Rows are response type/dose period. Columns represent the genes, and shading indicates the number of times the gene appears in the significant GSEA pathways. The tolerogenic signature is largely absent in the responders, but prominent in the Non-responders, while a pro-inflammatory signature largely represents the Responders.

TABLE 1

EARLY TREATMENT PERIOD

Significant immune biofunctions according to IPA arranged according to cell type for responders and non-responders compared to placebos and to each other. P-value is singular for one biofunction category or the range for multiple categories that were associated with the same cell type.

Response Category	Comparison	Relative Expression Direction	Cell Type	Functional Annotation	p-value	
Responders	vs <i>Placebos</i>	<i>Up</i>	Myeloid cells (neutrophils, macrophages)	Activation, infiltration, stimulation, recruitment	0.000001 – 0.016000	
			T cells	Activation, proliferation	0.000064 – 0.009970	
		vs <i>Non-responder</i>	<i>Down</i>	No significant categories		
	mast cells			Activation, degranulation	0.00013 – 0.00051	
	<i>Up</i>		eosinophils	movement	0.00176	
			mononuclear leukocytes	chemotaxis	0.00410	
			antigen presenting cells	movement	0.01070	
			macrophages	movement	0.01330	
			Hematopoietic progenitor cells	differentiation	0.01450	
			lymphocyte	migration	0.01610	
			granulocytes	activation	0.00052 – 0.00357	
myeloid cells			activation, movement	0.00122 – 0.0172		
Non-responders	vs <i>Placebos</i>	<i>Up</i>	T cells	Development, negative selection, quantity	0.00003 – 0.01680	
	vs <i>Placebos</i>	<i>Down</i>	Inflammatory Dendritic cells	Chemotaxis, recruitment	0.004 to 0.008	
			Myeloid cells (neutrophils, macrophages)	Activation, chemotaxis, recruitment	0.00002 – 0.0175	
			T cell	Activation	0.0104	
			Leukocytes	Oxidative Phosphorylation	0.0033	
			T cells	homing, chemotaxis	0.00720 – 0.0187	
vs <i>Responders</i>	<i>Up</i>	Plasmacytoid dendritic cells	migration	0.00720		

TABLE 2

MIDDLE TREATMENT PERIOD

Significant immune biofunctions according to IPA arranged according to cell type for responders and non-responders compared to placebos and to each other. P-value is singular for one biofunction category or the range for multiple categories that were associated with the same cell type.

Response Category			Cell Type	Functional Annotation	p-value
Responders	vs Placebos	Up	Cytotoxic T cells	Quantity, Response	0.0183 – 0.0218
		Down	T cells	Suppression, Interaction	0.00609 – 0.00797
	vs Non- responder	Up	B cells	Generation	0.00812
			Antigen presenting cells	immune response, movement	0.000988 – .02390
			Macrophages	immune response, movement, phagocytosis	0.000986 – 0.01430
Mast cells	degranulation		0.02370		
Non-responders	vs Placebos	Up	phagocytes	immune response	0.00246
			T cells	Lack of, activation, differentiation, proliferation	0.00235 to 8.33E-16
			Regulatory T cells	Activation	0.00233
			Hematopoietic Progenitor cells	Proliferation	0.000496
			B cells	Lack of, proliferation	0.000344 – 1.01E-06
	Down		Natural Killer Cells	Quantity	0.0000665
			Dendritic cells	Recruitment, activation	0.006790 – 0.005590
			Neutrophils	Quantity	0.004270
			Bone marrow cells	Differentiation	0.0007
			Hematopoietic progenitor cells	Differentiation	0.0064
			Leukocytes	Arrest in cell rolling	0.0071
			Liver	Inflammation	0.0057
			Myeloid cells (Neutrophils, macrophages)	Myelopoiesis, chemoattraction, binding	0.0026 – 0.0066
			vs Responders	Up	Leukocytes
T lymphocytes	cell movement	0.0290			
Mononuclear Leukocytes	migration	0.0302			
blood cells	quantity	0.0102			

TABLE 3

LATE TREATMENT PERIOD

Significant immune biofunctions according to IPA arranged according to cell type for responders and non-responders compared to placebos and to each other. P-value is singular for one biofunction category or the range for multiple categories that were associated with the same cell type.

Response Category	Comparison	Relative Expression Direction	Cell Type	Functional Annotation	p-value
Responders	vs Placebos	Up	Myeloid cells (Neutrophils, Macrophages)	recruitment, chemotaxis	3.07E-7 - 0.0017
			T cells	chemotaxis	0.0101
			B cells	Quantity, Function	0.00089 – 0.00219
			No significant categories		
Non-responders	vs Non-responder	Up	granulocytes	recruitment	0.00279
			neutrophils	recruitment	0.00351
			phagocytes	recruitment	0.00542
			immune cells	proliferation	0.0066
			leukocytes	recruitment	0.0102
			mast cells	degranulation	0.0148
			lymphocytes	differentiation	0.0183
			T cells	development	0.0216
			-	inflammatory response	0.0251
			T cells	Proliferation	0.00147
			Dendritic cells	Proliferation, Quantity	0.00014 – 0.00405
			Myeloid cells (Neutrophils, Macrophages)	Immune response, chemotaxis	0.00022 – 0.00701
			Myeloid cells (Neutrophils, Macrophages)	infiltration, cell movement, chemotaxis, recruitment, adhesion	0.00002 – 0.03060
			T cells	chemotaxis	0.0101
			dendritic cells	proliferation	0.0067
			T cells	Development	0.0368
Non-responders	vs Placebos	Up	T cells	Proliferation	0.00147
			Dendritic cells	Proliferation, Quantity	0.00014 – 0.00405
Non-responders	vs Placebos	Down	Myeloid cells (Neutrophils, Macrophages)	Immune response, chemotaxis	0.00022 – 0.00701
			Myeloid cells (Neutrophils, Macrophages)	infiltration, cell movement, chemotaxis, recruitment, adhesion	0.00002 – 0.03060
Non-responders	vs Placebos	Up	T cells	chemotaxis	0.0101
			dendritic cells	proliferation	0.0067
Non-responders	vs Placebos	Down	Myeloid cells (Neutrophils, Macrophages)	Immune response, chemotaxis	0.00022 – 0.00701
			Myeloid cells (Neutrophils, Macrophages)	infiltration, cell movement, chemotaxis, recruitment, adhesion	0.00002 – 0.03060